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Original Article

Canarium album extract restrains lipid excessive accumulation in hepatocarcinoma cells

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Abstract: Lipid metabolism is an important section of human body metabolism, and lipid metabolism disorder can lead to multiple diseases. *Canarium album* is a nature food, whose extract has been reported hepatoprotective, anti-inflammatory and antioxidant. In this study, we extracted polyphenol and flavonoid substances from *Canarium album* fruits, and demonstrated that they restrain lipid excessive accumulation induced by oleic acid in hepatocarcinoma cells. Moreover, polyphenol and flavonoid extracted from *Canarium album* fruits facilitated phosphorylation of adenosine monophosphate activated protein kinase (AMPK) and regulated several lipid metabolism related genes expression, including fatty acid synthase (FAS), sterol regulatory element binding protein (SREBP)-1 and peroxisame proliferator activated receptor (PPAR)-α. Therefore, for the first time, we demonstrated that *Canarium album* extract restrained lipid excessive accumulation by activating AMPK signaling pathway, downregulating SREBP-1 and FAS, upregulating PPAR-α in hepatocarcinoma cells, which may be of great significance for prevention and clinical treatment of lipid metabolism disorders.

Keywords: *Canarium album*, polyphenol, flavonoid, hepatocarcinoma cells, lipid, AMPK

Introduction

Lipid metabolism is one of the three major metabolisms of human body, a crucial component of the material and energy cycle. Lipid metabolism imbalance may lead to multiple diseases, including obesity, diabetes, fatty liver, ketosis and cardiovascular disease [1]. Liver is the dominating organ of lipid metabolism, and fatty liver is a common lipid metabolism disorder caused by unusual accumulation of lipid. A variety of factors can lead to disorders of lipid metabolism, including genetic factors, pathological factors and irrational diet. It is important to keep a balanced diet and to avoid excessive intake of lipid. It has been reported that some food plays a vital role in restraining lipid accumulation, for example, curcumin, a polyphenol derived from *Zingiber officinale* R., decreases lipid accumulation [2] and improves obesity-mediated inflammation and diabetes of mice [3]. *Aronia melanocarpa* juice, which is rich in phenolic and flavonoid, shows an antihyperlipidemic effect on rats [4]. Moreover, widdrol, being contained in various plants including *Juniperus lucayana* [5], induces lipolysis in adipocytes of mice [6, 7].

Canarium album (Lour.) *Raeusch* belongs to Burseraceae, *Canarium*, differing from *Olea europaea* L. in Mediterranean European. *Canarium album* was native to south of China, currently mainly distributed in southeast of Asia. The medical value of *Canarium album* has been long known. Four compounds, brevifolin, hypierin, ellagic acid and 3,3'-di-O-methylellagic acid, isolated from *Canarium album* fruits and leaves, were demonstrated hepatoprotective [8]. Tamai Masaharu extracted other seven hepatoprotective compounds from *Canarium album* [9]. Tannins, isolated from leaves, twigs and stem bark of *Canarium album,* were proved to be with antioxidant activity [10]. In addition, Chinese medicine study found *Canarium album* extract with effects on hangover, inflammatory and sterilization. However, it has not been reported whether *Canarium album* regulates lipid accumulation.

In this study, we exacted polyphenol and flavonoid from *Canarium album* fruits, and explored

their effect on lipid metabolism and the underlying mechanism. Since the important position of the liver, a common human hepatocarcinoma cell line HepG2 was selected to perform the experiments.

Materials and methods

Extraction of polyphenol and flavonoid from Canarium album fruits

Canarium album fruits "Changying" were collected from Hongwei farm, Minhou county, Fuzhou city, Fujian province, China. The fresh *Canarium album* fruits were squeezed to juice, and the juice was filtered and centrifuged at 14000 rpm at 4°C for 30 min. The supernatant was collected and extracted with a triple volume of ethyl acetate for 3 times and a triple volume of n-hexane for 3 times. Both organic phases were mixed and rotary evaporated in 45°C water bath to powder. The powder was dissolved with distilled water, and the polyphenol solution was gained.

The fresh *Canarium album* fruits were removed pericarp and kernel, and the mesocarp was dried at 55°C until constant weight. Thereafter, the mesocarp was smashed to powder and filtered through a 60 mesh sieve. The powder was soaked in petroleum ether (m:v = 1:8) for 1 h, heated reflux at 70°C for 1 h, filtered and dried. The gained powder was dissolved in 60% ethyl alcohol (m:v = 1:20), heated reflux at 80°C for 1.5 h. After removing ethyl alcohol, the solution was filtered and rotary evaporated in 45°C water bath to powder. The powder was dissolved with distilled water, and flavonoid solution was obtained.

Content determination of polyphenol and flavonoid

Content of polyphenol was determined using Folin-Cioealteu (FC) method [11-13]. 1 ml polyphenol solution was diluted with 30 ml distilled water. 2.5 ml Folin-Cioealteu reagent (Sigma, St. Louis, MO, USA) and 7.5 ml 20% Na_2CO_3 were added into the solution, mixed and diluted with distilled water to 50 ml. The mixture was heated at 75°C in dark for 10 min, and its absorbance at 760 nm was detected with an ultraviolet spectrophotometer (Yoke, Shanghai, China) (diluted 50 times). The standard curve was drawn with gallic acid (Sangon, Shanghai, China), and the content of polyphenol was calculated according to the standard curve.

Content of flavonoid was determined by spectrophotometric method [14]. 0.25 ml flavonoid solution was diluted with 70% ethyl alcohol to 10 ml, and the absorbance of diluted solution was detected at 360 nm with an ultraviolet spectrophotometer (Yoke) (diluted 40 times), with petroleum ether as the control. The standard curve was drawn with rutin (Sangon), and the data were calculated according to the standard curve.

Cell culture

Human hepatocarcinoma cell line HepG2 was purchased from Shanghai Cell Bank of Chinese Academy of Science. The cells were cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Beyotime, Haimen, Jiangsu, China) at 37°C in an atmosphere consisting of 5% CO_2.

MTT assay

To measure the cytotoxicity of *Canarium album* extract, MTT assay was performed. HepG2 cells were seeded in 96-well plates with a density of 3×10^3/pore. When cells adhered to the plate, oleic acid (1 mM) was added into the medium with or without drugs of different concentration. 24 h later, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) (Wanleibio, Shenyang, Liaoning, China) was added into the medium (0.5 mg/ml) and cells were continued to culture for 4 h. After centrifuging at 1000 rpm for 10 min, culture medium was removed from the plates, and dimethyl sulfoxide (DMSO) (Sigma) was added into wells to dissolve formazan particles with 200 μl per pore, and the optical density of formazan solution was detected at 490 nm with a microplate reader (Biotek, VT, USA).

Oil red O staining

To observe the lipid accumulation in cells, oil red O staining was carried out. HepG2 cells were seeded in 6-well plates. When cells adhered to plates, oleic acid (1 mM) was added into medium with or without *Canarium album* extract of different concentration. 24 h later, the cells were rinsed with PBS (Double-helix, Shanghai, China) for 3 times and fixed with 4% paraformaldehyde (Sinopharm, Beijing, China) for 30 min. After rinsing with 60% isopropanol, cells were stained with 1% oil red O reagent (Sigma) for 1 h, rinsed with distilled water for 2

Table 1. Sequence of real-time PCR primers

Name	Sequence (5'-3')
FAS Forward	5'-TTCCCATCCTCCTGACCAC-3'
FAS Reverse	5'-CTCGTAAACCGCTTCCCTC-3'
SREBP-1 Forward	5'-ACTTCATCAAGGCAGACTCGC-3'
SREBP-1 Reverse	5'-ACTCACCAGGGTCGGCAAA-3'
PPAR-α Forward	5'-TCATCACGGACACGCTTTC-3'
PPAR-α Reverse	5'-CCCGCAGATTCTACATTCG-3'
β-actin Forward	5'-CTTAGTTGCGTTACACCCTTTCTTG-3'
β-actin Reverse	5'-CTGTCACCTTCACCGTTCCAGTTT-3'

Table 2. Content of Polyphenol and Flavonoid solution extracted from *Canarium album* fruits

Extracting Solution	Concentration
Polyphenol	124.57 (μg/ml)
Flavonoid	7.94 (mg/ml)

times, and observed under an inverted microscope (Motic, Xiamen, Fujian, China).

Content measurement of triglyceride (TG) in cells

Content of TG was measured by TG testing kit (Dongou, Wenzhou, Zhejiang, China) according to the manufacturer's protocol. Standard curves were drawn using BCA protein quantitative kit (Wanleibio).

RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted with total RNA rapid extract kit (BioTeke, Beijing, China) according to the protocol. After testing the concentration, 1 μg RNA was reversely transcribed (RT) into cDNA with M-MLV reverse transcriptase (BioTeke), with oligo(dT) and random as the primers. Reagents and instruments used during RNA extraction and reverse transcription were RNase-free.

The cDNA sample was used for real-time PCR (1 μl cDNA for each reaction) with Taq PCR MasterMix (BioTeke) abd SYBR Green (Solarbio, Beijing, China) to detect levels of fatty acid synthase (FAS), sterol regulatory element binding protein (SREBP)-1and peroxisome proliferator-activated receptor (PPAR)-α, with β-actin as the internal control. Sequences of real-time PCR primers were shown in **Table 1**. The PCR reaction was set as follow: 95°C for 10 min, 40 cycles of 95°C for 10 sec, 60°C for 20 sec and 72°C for 30 sec, and finally 4°C for 5 min. The data were calculated as $2^{-\Delta\Delta Ct}$ method.

Western blot

Protein was extracted with whole cell lysis kit (Wanleibio) from HepG2 cells, denatured by boiling, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Boston, MA, USA). After blocking with 5% skim milk (YILI, Hohhot, Inner Mongolia, China) for 1 h, the PVDF membrane was incubated with follow antibodies at 4°C overnight: rabbit anti-FAS (1:1000) (Wanleibio), rabbit anti-SREBP-1 (1:1000) (Wanleibio), rabbit anti-PPAR-α (1:1000) (Wanleibio), rabbit anti-p-adenosine monophosphate activated protein kinase α1 (p-AMPKα1) (1:500) (Bioss, Beijing, China) and rabbit anti-AMPKα1 (1:1000) (Abcam, Cambridge, UK). After rinsing with TBST, the PVDF membrane was incubated with goat anti-rabbit IgG labeled with HRP (1:5000) (Wanleibio) for 45 min at 37°C, and exposure with ECL reagent (Wanleibio). After removing the antibodies with stripping buffer (Wanleibio), the PVDF membrane was incubated with rabbit anti-β-actin (1:1000) (Wanleibio) and goat anti-rabbit IgG-HRP (1:5000) (Wanleibio) to detect the internal control, β-actin.

Statistical analysis

The data in this work were present as means ± standard deviation (SD) of three individual experiments, and analyzed with one-way ANOVA test. Which $P < 0.05$ was considered statistically significant (*$*P < 0.05$, $**P < 0.01$, $***P < 0.001$, ns: no significance*).

Results

Canarium album extract has scarcely cytotoxicity on hepatocarcinoma cells

The polyphenol and flavonoid were extracted from the *Canarium album* fruits according to the previous description [15, 16] with some modification, and the concentrations of polyphenol and flavonoid solution were measured as the description in material and method. The data were shown in **Table 2**.

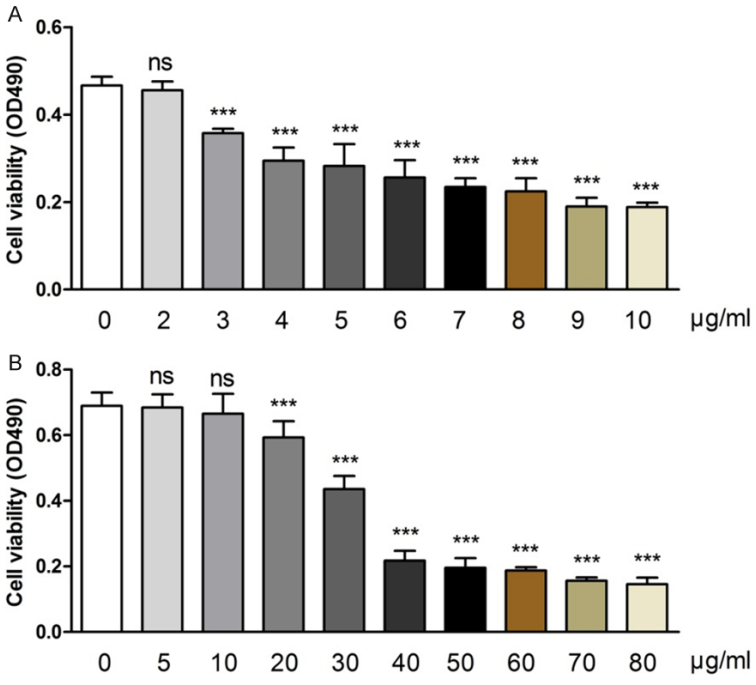

Figure 1. MTT assay was performed to detect cytotoxicity of *Canarium album* extract on HepG2 cells. A. Cell viability of HepG2 cells cultured with different concentration of polyphenol. B. Cell viability of HepG2 cells cultured with different concentration of flavonoid. The concentration of polyphenol and flavonoid solutions were shown in the figure (The data were analyzed with one-way ANOVA, compared to data in 0 μg/ml groups, ****P < 0.001, no: no significance*).

To determine whether the polyphenol and flavonoid solution were cytotoxic to hepatocarcinoma cells, the MTT assay was performed to detect the cell viability after adding polyphenol or flavonoid of distant concentration. The data showed that the cell viability was inhibited obviously when concentration of polyphenol over 3 μg/ml (**Figure 1A**), flavonoid over 20 μg/ml (**Figure 1B**), but changed seldom or never when the concentration was lower (polyphenol ≤ 3 μg/ml, flavonoid ≤ 20 μg/ml). Based on the results of MTT assay, 1 μg/ml, 2 μg/ml and 3 μg/ml were determined to be the low, medium and high concentration of polyphenol, and 5 μg/ml, 10 μg/ml and 20 μg/ml were defined to be the low, medium and high concentration of flavonoid in the subsequent experiments.

Canarium album extract restrains intracellular lipid accumulation induced by oleic acid

It has been well known that oleic acid induces the intracellular lipid accumulation. In our study, lipid accumulation model cells were established by culture with oleic acid of 1 mM. Cells in experimental groups were cultured with the same concentration of oleic acid and different concentration of polyphenol or flavonoid. Oil red O straining revealed that the lipid content in cells of model group obviously increased compared to the normal cells, while polyphenol or flavonoid both decreased lipid content significantly (**Figure 2A** and **2B**).

Intracorporal lipid contains TG, fatty acid, phospholipids, cholesterol, lipoprotein and others, among which TG is the most abundant. Hence the content of TG we examined in the HepG2 cells in each group. Similar to the oil red O straining, TG content in cells of model group was much more than cells in normal group because of the addition of oleic acid. However, polyphenol of different concentrations decreased content of TG by 44%, 82% and 74%, respectively (**Figure 2C**), and flavonoid of different concentrations decreased TG content by 52%, 43% and 45%, respectively (**Figure 2D**). These results demonstrated that polyphenol and flavonoid extracted from *Canarium album* inhibited lipid accumulation induced by oleic acid in HepG2 cells.

Canarium album extract regulates lipid metabolism related genes

To figure out the mechanism of polyphenol and flavonoid extracted from *Canarium album* inhibiting lipid accumulation, three lipid metabolism related genes, FAS, SREBP-1 and PPAR-α were tested by real-time PCR and western blot. Real-time PCR results showed that polyphenol of different concentrations decreased the FAS by 12%, 19% and 21% (**Figure 3A**), decreased SREBP-1 by 17%, 19% and 34% (**Figure 3C**), increased PPAR-α by 1.31 folds, 2.02 folds and 1.65 folds (**Figure 3E**) in mRNA level compared with cells in model group. Similarly, flavonoid decreased FAS by 26%, 47% and 53% (**Figure 3B**), decreased SREBP-1 by 15%, 22% and 17%

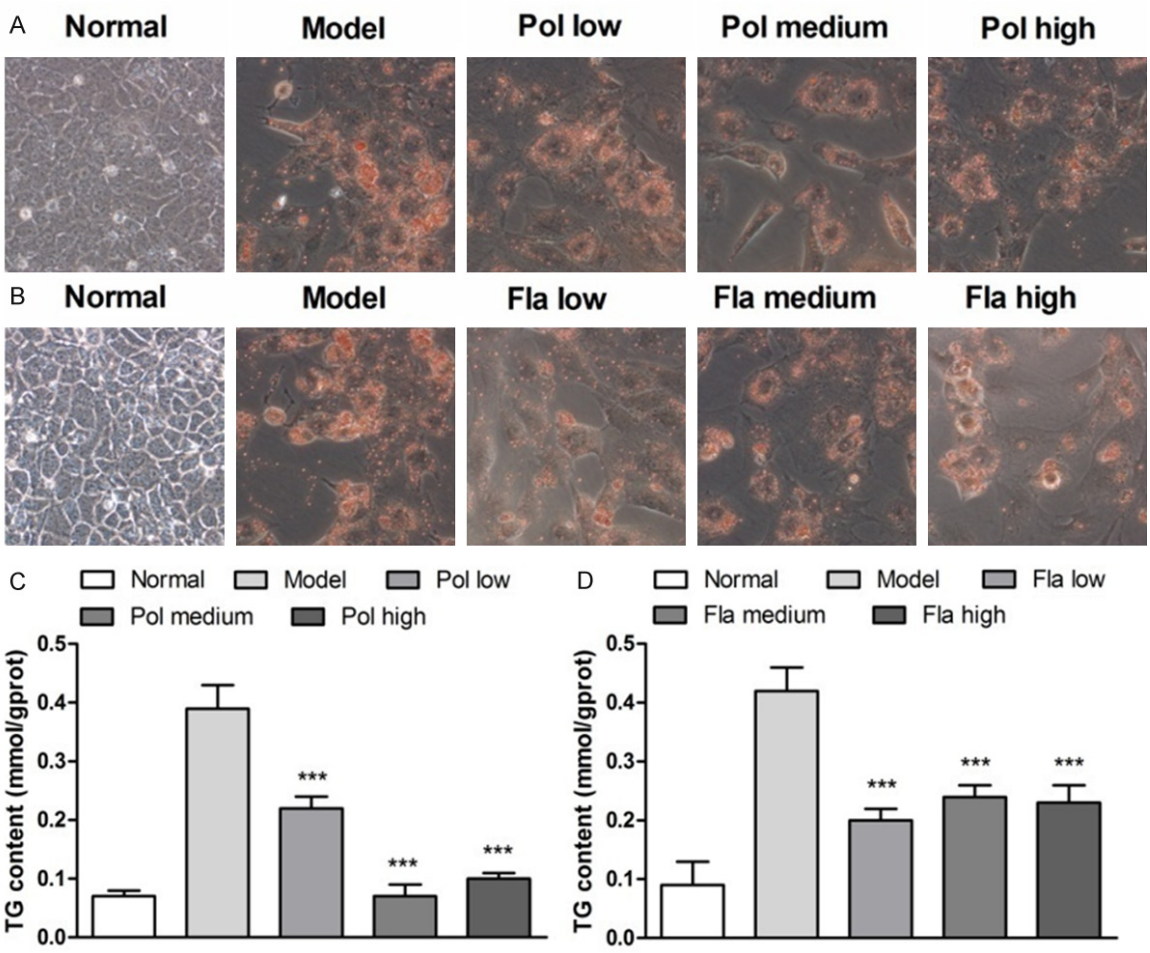

Figure 2. *Canarium album* extract restrains intracellular lipids accumulation. (A) Oil red O staining was performed to test whether polyphenol or falvonoid (B) affects lipid accumulation induced by oleic acid in HepG2 cells. (C) Polyphenol and flavonoid (D) extracted from *Canarium album* decreased content of triglyceride (TG) in HepG2 cells. (Pol: polyphenol; Fla: flavonoid) (The data were analyzed with one-way ANOVA, with cells in model groups as the controls, ****P < 0.001*).

(**Figure 3D**), increased PPAR-α by 1.4 folds, 2.33 folds and 1.86 folds in mRNA level compared with cells in model group (**Figure 3F**). Western blot revealed similar consequences in protein level (**Figure 4A-F**).

Canarium album extract promotes phosphorylation of AMPK

AMPK cascade system plays a vital role in biological energy control. It has been reported that the stress-induced AMPK phosphorylation inhibited synthesis of fatty and sterols [17]. Therefore we examined whether *Canarium album* extract affects the activity of AMPK. Since AMPK was activated via phosphorylation in α subunit, expression levels of AMPKα1 and p-AMPKα1 were detected by western blot, and calculated the relative level of p-AMPKα1/AMPKα1. The results showed that oleic acid decreased cellular p-AMPKα1/AMPKα1 level obviously, however, polyphenol of medium concentration increased p-AMPKα1/AMPKα1 level by 5.2 folds (**Figure 5A**) and flavonoid of medium concentration increased p-AMPKα1/AMPKα1 level by 3.22 folds (**Figure 5B**). So we concluded that polyphenol and flavonoid extracted from *Canarium album* activated AMPK by promoting its phosphorylation.

Discussion

In this study, we extracted polyphenol and flavonoid from *Canarium album* fruits, and demonstrated that *Canarium album* extract inhibited lipid excessive accumulation induced by oleic acid in hepatocarcinoma cells. On the

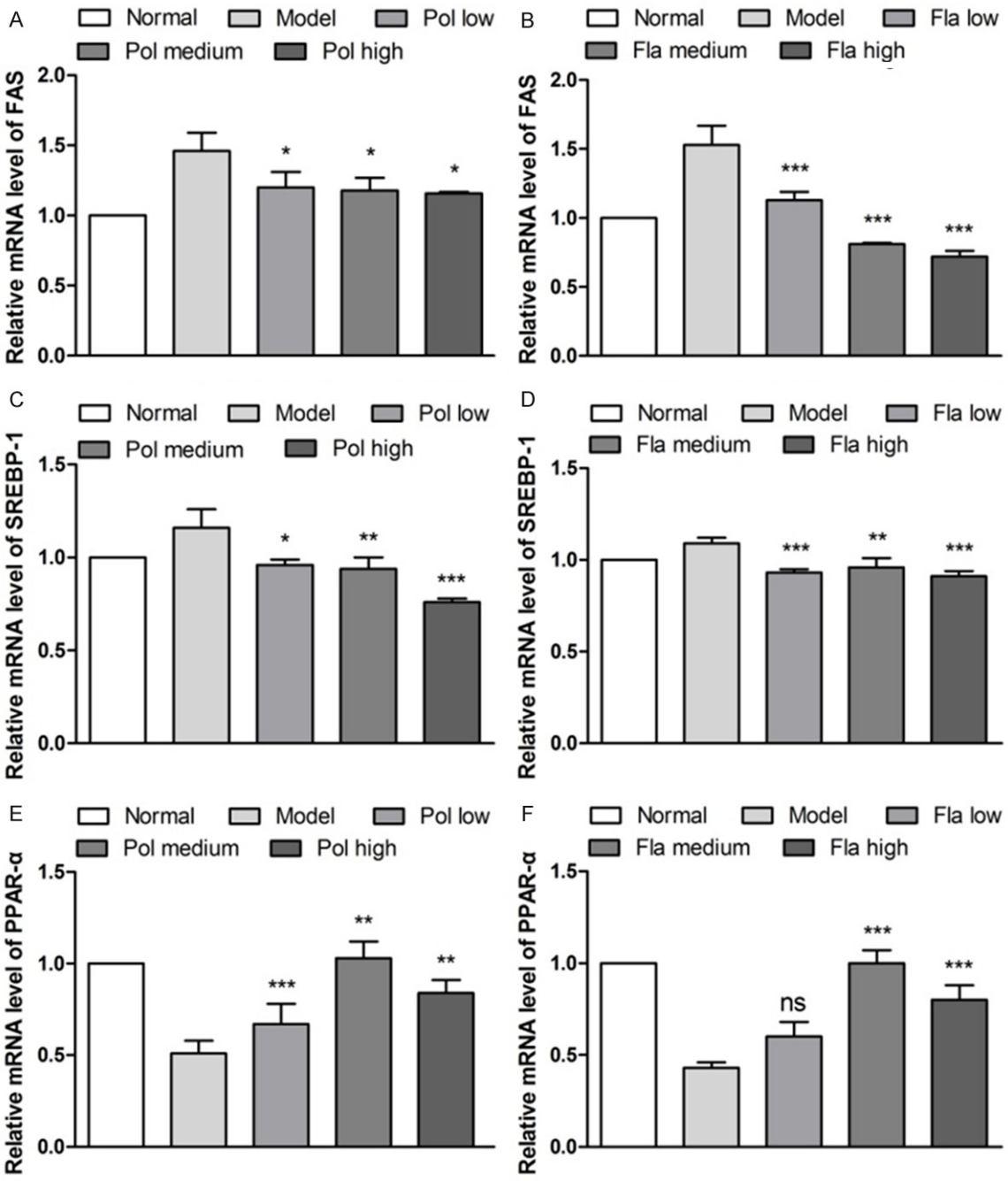

Figure 3. *Canarium album* extract regulates the mRNA levels of lipid metabolism related genes detected by real-time PCR. (A) Polyphenol and flavonoid (B) extracted from *Canarium album* decreased the mRNA level of fatty acid synthase (FAS) detected by real-time PCR. (C) Polyphenol and flavonoid (D) extracted from *Canarium album* declined the mRNA level of sterol regulatory element binding protein (SREBP)-1. (E) Polyphenol and flavonoid (F) extracted from *Canarium album* increased the mRNA level of peroxisame proliferator activated receptor (PPAR)-α (The data were analyzed with one-way ANOVA, with cells in model groups as the controls, **P < 0.05, **P < 0.01, ***P < 0.001, ns: no significance*).

other hand, lipid metabolism is regulated by multiple genes. In the study, expression of lipid metabolism related genes, FAS, SREBP-1 and PPAR-α were examined.

FAS is a vital enzyme in de novo synthesis of fatty acid [18]. FAS expression is directly controlled by the transcription factor SREBP-1. SREBPs are known to regulate multiple genes

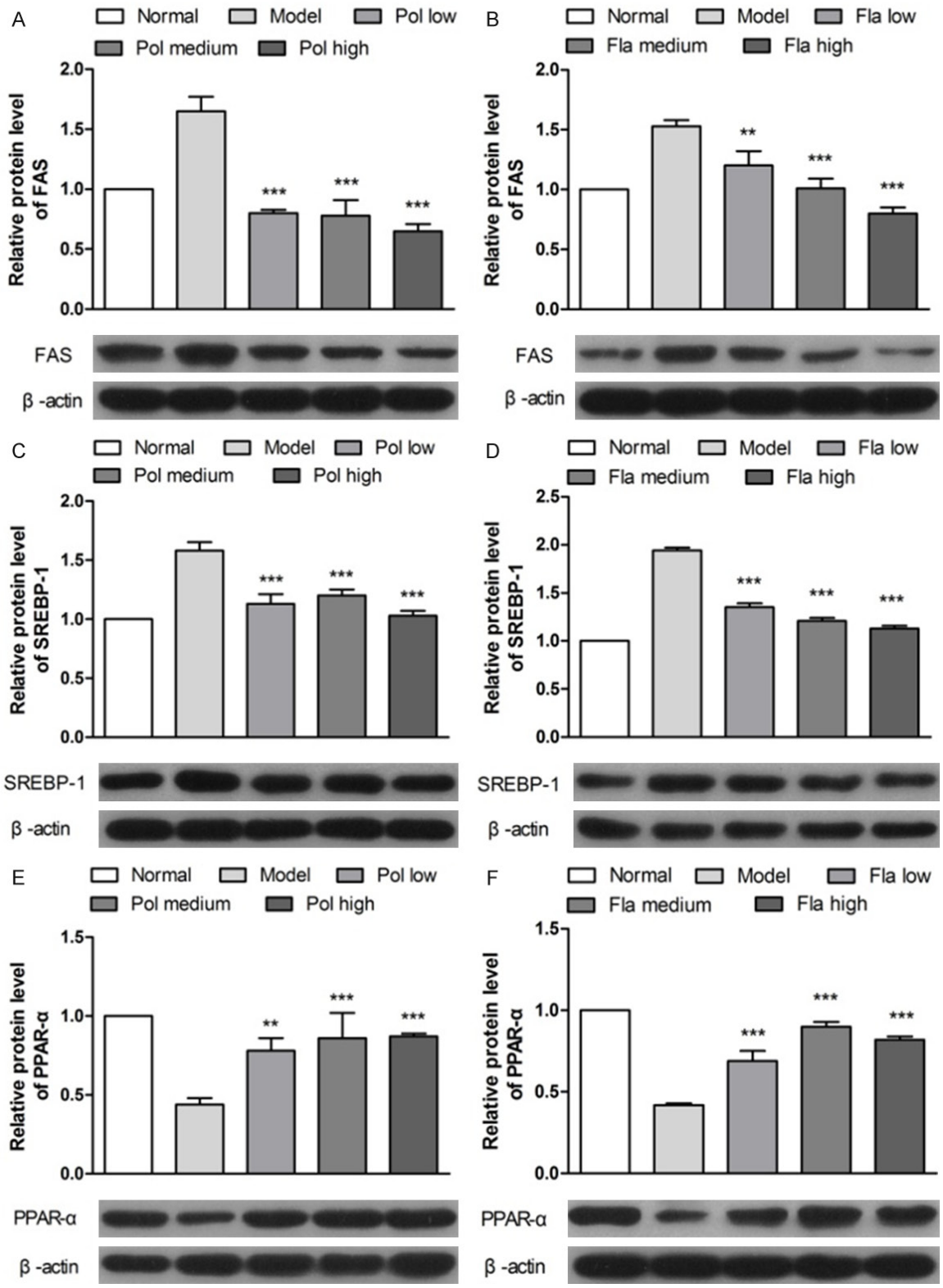

Figure 4. *Canarium album* extract regulates the protein levels of lipid metabolism related genes detected by western blot. (A) Polyphenol and flavonoid (B) extracted from *Canarium album* decreased the expression level of FAS detected by western blot. (C) Polyphenol and flavonoid (D) extracted from *Canarium album* declined the protein level of SREBP-1. (E) Polyphenol and flavonoid (F) extracted from *Canarium album* increased the protein level of PPAR-α (The data were analyzed with one-way ANOVA, with the cells in model groups as the controls, ***P < 0.01, ***P < 0.001*).

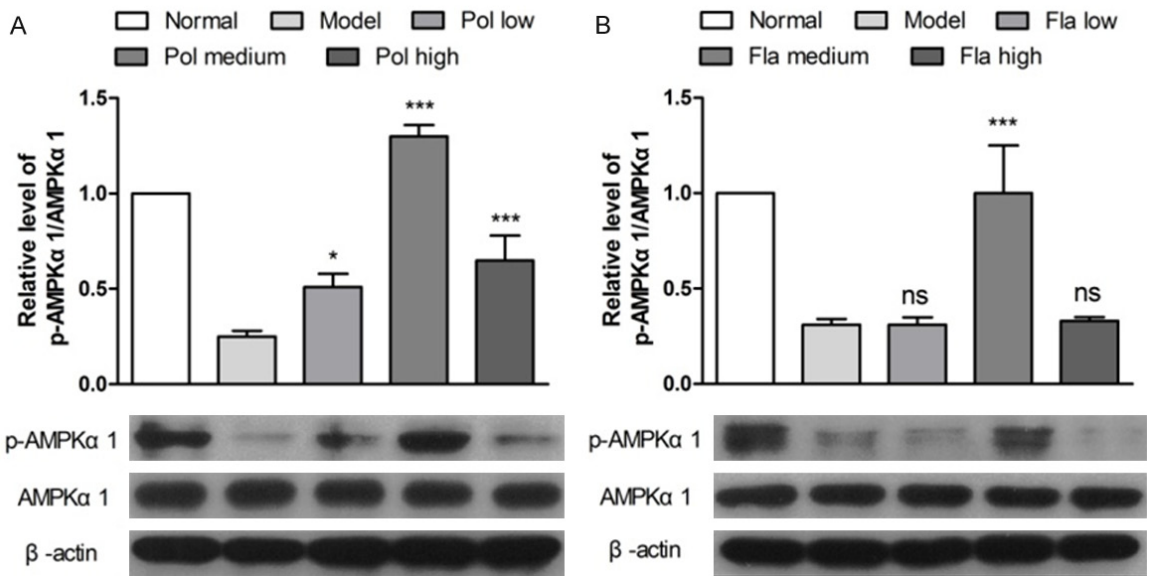

Figure 5. *Canarium album* extract promotes phosphorylation of AMPK in HepG2 cells. (A) Polyphenol and flavonoid (B) extracted from *Canarium album* increased relative level of p-AMPKα1/AMPKα1 detected by western blot (The data were analyzed with one-way ANOVA, with the cells in model groups as the controls, **P < 0.05, ***P < 0.001, ns: no significance*).

involved in cholesterol biosynthesis and uptake, including 3-hydroxy-3-methylglutary coenzyme A reductase (HMG-CoA reductase), HMG CoA synthase, farnesyldiphosphate synthase and squalene synthase [19, 20]. Moreover, SREBPs also modulates transcription of genes encoding enzymes of fatty acid synthesis and uptake, including FAS, acetyl CoA carboxylase (ACC), stearoyl CoA desaturase-1 and lipoprotein lipase [21-23]. In the liver, the regulation of SREBP-1 is in the dominant position. PPAR-α is another gene involvel in lipid metabolism. PPARs belong to the superfamily of nuclear hormone receptors, playing a crucial role in obesity-related metabolic diseases, including hyperlipidemia, insulin-resistance and coronary artery disease [1, 24, 25]. Among three isoforms, PPAR-α, PPAR-δ/β and PPAR-γ, PPAR-α is highest expressed in liver, and modulates fatty acid uptake, activation, mitochondrial β-oxidation, peroxisomal fatty acid oxidation, ketogenesis, and fatty acid elongation and desaturation [26]. In the case of fasting or a high fat diet, PPAR-α-mull mice showed a massive accumulation of lipid in their livers and a dramatic inhibition of fatty acid oxidation compared with the wild-type [27]. In this study, culture with oleic acid increased FAS and SREBP-1, and decreased PPAR-α expression in mRNA and protein levels, consistent with lipid content in hepatocarcinama cells.

Furthermore, It has also been reported that AMPK downregulates SREBP-1c expression, and then inhibits the glucose-stimulated transcription of FAS [28, 29]. AMPK is a central component of protein kinase cascades, playing a critical role in metabolism. When AMP:ATP ratio elevates, AMPK is activated through phosphorylation of Thr172 in α subunit [30]. It has been reported that activated AMPK modulates enzymes participating in lipid metabolism, including ACC, SREBP-1c, HMG-CoA reductase and biotin carboxylase. ACC phosphorylation decreases its production malonyl-CoA, which is an inhibitor of camitine palmitoyltransferase-1 (CPT-1), the transporter of fatty acid entering mitochondria [31]. These reports suggest that AMPK participates in lipid metabolism via different signaling pathways, including SREBP-1/FAS, and the overall effect of AMPK activation is to inhibit the lipogenesis and promote the lipolysis. In this study, we detected the p-AMPKα1 expression, and found that *Canarium album* extract facilitated phosphorylation of AMPKα1, and recovered the increased expression of FAS and SREBP-1 and decreased expression of PPAR-α induced by oleic acid in hepatocarcinama cells.

In summary, we demonstrated that *Canarium album* extract restrained lipid excessive accumulation induced by oleic acid by facilitating

phosphorylation of AMPK, downregulating FAS and SREBP-1, and upregulating PPAR-α in hepatocarcinoma cells. These findings may provide research foundations for the prevention and clinical treatment of lipid metabolism disorders.

Disclosure of conflict of interest

None.

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