



## Research article

# Protective effects of *Gnetum montanum* extract against oleic acid-induced lipotoxicity in hepatocytes

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### ABSTRACT

Hepatic lipid accumulation plays a key role in the early stages of fatty liver disease and can lead to liver cell dysfunction and damage. Although *Gnetum montanum* has demonstrated antioxidant and metabolic regulatory effects, its role in lipid metabolism pathways is not yet fully understood. This study aimed to investigate whether *G. montanum* extract (GME) exerts protective effects against lipid accumulation and lipotoxicity. We found that treating HepG2 cells with oleic acid (OA) induced significant intracellular lipid accumulation, as evidenced by the presence of lipid droplets, which was assessed via Oil Red O and BODIPY 493/503 staining, and an increase in intracellular triglyceride levels. OA exposure also triggered oxidative stress, cell membrane damage, and apoptosis. Pretreatment with GME at concentrations of 3 and 10 µg/mL significantly reduced OA-induced lipid droplet formation and intracellular triglyceride levels. Additionally, GME effectively attenuated malondialdehyde (MDA) levels, reduced the release of hepatic enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and inhibited caspase-3 nuclear translocation and cell apoptosis. These effects were comparable to those of silibinin (SIL), a well-established positive control. In summary, these findings suggest that GME may possess promising anti-lipid accumulation and hepatoprotective properties.

### INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), primarily characterized by excessive lipid accumulation in hepatocytes, is currently one of the most common liver

disorders worldwide. This abnormal lipid buildup results from an imbalance among fatty acid uptake, synthesis, oxidation, and triglyceride export [1]. The global prevalence of NAFLD is approximately 30%, and its

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disease burden is substantial and expected to rise further in the coming years [2-4]. Despite its prevalence, effective therapeutic options for NAFLD remain limited. Some clinical guidelines recommend drugs such as pioglitazone and vitamin E for select patient groups [5, 6], but their routine use in clinical practice is still restricted. Meanwhile, several investigational agents, including dapagliflozin, semaglutide, and aramchol, are undergoing phase III clinical trials [7]. Most recently, resmetirom became the first FDA-approved drug specifically indicated for patients with non-alcoholic steatohepatitis (NASH) with evidence of advanced fibrosis, offering a promising new therapeutic option [8].

To mimic the steatotic conditions of NAFLD *in vitro*, oleic acid (OA), a monounsaturated fatty acid, is commonly used to induce lipid accumulation in liver cells [9, 10]. Oleic acid (OA) is taken up by liver cells via membrane fatty acid transport proteins such as FATPs and CD36, leading to elevated intracellular fatty acid concentrations. These fatty acids are either oxidized in mitochondria to produce adenosine triphosphate (ATP) or esterified in the cytosol to form triglycerides. When intracellular fatty acid levels exceed the liver's capacity for  $\beta$ -oxidation and very low-density lipoprotein (VLDL) secretion, excessive triglyceride synthesis and abnormal lipid droplet accumulation occur [11]. In addition, OA stimulates *de novo* lipogenesis by activating the transcription factor SREBP-1c and its downstream targets [12-15]. Excessive lipid accumulation further promotes mitochondrial dysfunction, increases reactive oxygen species (ROS) production, and triggers oxidative stress. This oxidative burden enhances lipid peroxidation, generating cytotoxic products such as

malondialdehyde (MDA), and leads to cellular damage and apoptosis through both direct and indirect mechanisms [16].

In the search for safer and more effective hepatoprotective agents, herbal medicines have attracted significant interest due to their multitargeted actions and good tolerability. Several plant extracts and naturally derived compounds have been shown to reduce hepatic lipid accumulation, highlighting the potential of medicinal plants as sources of bioactive agents for managing hepatic steatosis and protecting hepatocytes [14, 17, 18]. *Gnetum montanum* Markgr., a lesser-studied species from the Gnetaceae family, has traditionally been used in folk medicine to treat rheumatism, musculoskeletal pain, irregular menstruation, snake bites, and various liver-related disorders. Extracts of *G. montanum* have been reported to exhibit antioxidant properties, anti-inflammatory effects, and xanthine oxidase inhibition [19-21]. Despite these promising bioactivities, its effects on hepatic lipid metabolism and lipotoxicity remain poorly understood. Our previous findings provided initial evidence for the anti-lipid accumulation and hepatoprotective effects of *G. montanum* extract (GME). In a valproic acid-induced steatosis model, GME significantly reduced intracellular lipid accumulation in HepG2 cells by lowering neutral lipid content and suppressing increases in triglyceride and total cholesterol levels [22]. A complementary study using ethanol-induced metabolic dysfunction models further supported these observations. *In vitro*, GME pretreatment inhibited xanthine oxidase activity, reduced lipid droplet formation, reversed triglyceride accumulation, and downregulated lipogenic gene expression, thereby protecting hepatocytes from acetaldehyde-induced metabolic stress. *In*

*vivo*, GME attenuated ethanol-induced hepatic lipid accumulation, oxidative stress, and liver injury markers [23].

Together, these findings suggest that GME exerts multifaceted hepatoprotective effects. However, valproic acid (VPA) and ethanol/acetaldehyde are indirect inducers of hepatocellular lipid accumulation and may not fully replicate the pathogenesis or direct lipotoxic mechanisms associated with metabolic-associated fatty liver disease (MAFLD). As noted above, oleic acid (OA) directly increases the intracellular load of extracellular fatty acids and is widely used as a model for lipid overload. To date, the ability of GME to counteract OA-induced hepatic lipid accumulation has not been investigated. Therefore, this study aims to evaluate the effects of GME on OA-induced lipid accumulation, oxidative stress, and hepatocyte apoptosis using an *in vitro* HepG2 cell model, addressing this critical knowledge gap.

## MATERIALS AND METHODS

### *Chemicals and reagents*

Dulbecco's Modified Eagle's Medium (DMEM), RPMI-1640, fetal bovine serum (FBS), and penicillin/streptomycin/amphotericin B solution were purchased from PAN-Biotech GmbH (Aidenbach, Germany). Trypsin/EDTA solution 1X, 1,1,3,3-tetramethoxypropane, Oil Red O, oleic acid, 2-thiobarbituric acid (TBA), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), isopropanol, paraformaldehyde, silibinin (SIL), and Triton™ X-100 were procured from Sigma-Aldrich (St. Louis, Missouri, USA). 4,4-Difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY™ 493/503), phosphate-buffered saline (PBS), and bovine serum albumin

(BSA) were purchased from Thermo Scientific (Rockford, Illinois, USA). Primary antibody against caspase-3, Alexa 488-conjugated anti-rabbit secondary antibody, and mounting medium with DAPI were obtained from Abcam (Cambridge, United Kingdom). Acetic acid was purchased from Xilong Scientific (Guangdong, China). Assay kits for protein and triglyceride were purchased from Beyotime (Shanghai, China). Assay kits for AST and ALT were purchased from Erba Mannheim (Mannheim, Germany). The One-step TUNEL In Situ Apoptosis Kit was bought from Elabscience (Hubei, China).

### *Collection and preparation of plant extract*

*Gnetum montanum* Markgr. lianas were collected in April 2022 from Muong Nhe, Dien Bien Province, Vietnam. Plant identification was carried out by Prof. Dr. Tran The Bach from the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology (IEBR-VAST). A voucher specimen (DL2-2023) has been deposited at the same institute for future reference. The collected plant materials were dried at 50°C and ground into a fine powder. Extraction was performed by refluxing the powdered material with 95% aqueous ethanol for 3 hours. After combination, the extracts were evaporated at 50°C under low pressure. The extraction yield was determined to be 13.4%. Chemical profile of GME was characterized in a previous study. Briefly, the total phenolic content of GME, determined by the Folin-Ciocalteu method, was 24.87 mg GAE/g. Stilbenoids in GME were profiled using a Shimadzu HPLC system equipped with an SPD-M20A DAD detector. Based on their chromatographic characteristics, seven stilbenoids were identified in the extract:  $\epsilon$ -

viniferin, gnetifolin E, gnetifolin K, shegansu B, gnetulin, isorhapontigenin, and resveratrol. The content of resveratrol was 0.81%.

A stock solution (50 mg/mL) was prepared by dissolving the extract in DMSO and stored at  $-20^{\circ}\text{C}$  until further use. Working solutions were prepared by diluting the stock solution in cell culture media before experimentation.

#### **Cell culture and treatments**

The human hepatocellular carcinoma cell line HepG2 was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in complete DMEM media (containing 10% FBS and 1X antibiotic-antimycotic solution) and incubated at  $37^{\circ}\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$ . Subculturing or subsequent experiments were conducted when cell confluence reached 70 - 80%. Cells were seeded into appropriate culture dishes and incubated overnight before being used for further experiments.

Appropriate concentrations of GME for the following experiments were referred to our previous study on its effects on cell viability of hepatocytes. As previously reported, GME at  $30\ \mu\text{g/mL}$  significantly reduced HepG2 cell viability, while concentrations of  $10\ \mu\text{g/mL}$  or lower did not induce cytotoxicity [22]. Therefore, GME concentrations of  $10\ \mu\text{g/mL}$  or lower were selected for further assays. In all experiments, cells were pretreated with the plant extract at the indicated concentrations or with silibinin ( $30\ \mu\text{M}$ , positive control) for 2 h. Cells were then treated with  $500\ \mu\text{M}$  oleic acid (OA) for 24 h in all experiments, except for the malondialdehyde (MDA) quantification assay, which was conducted after 48 h of treatment. This can be explained by the fact that oxidative stress and lipid peroxidation develop more slowly than intracellular lipid

accumulation. A longer exposure period allows sufficient time for reactive oxygen species to accumulate and for MDA, the end-product of lipid peroxidation, to become detectable.

#### **Measurement of lipid accumulation**

##### *Oil red O staining*

Cells were plated at a density of  $4 \times 10^4$  cells/well in an eight-well glass chamber slide. Cells were treated as described above. After 24 h of incubation with OA, the culture media were removed. Cells were fixed using 4% paraformaldehyde in PBS for 20 min at room temperature (RT). Subsequently, the fixed cells were stained with 0.3% Oil Red O (ORO) solution for 15 min at RT, followed by washing with 60% isopropanol. Haematoxylin was used to stain the nuclei lightly. Lipid droplets were visualized under an inverted light microscope (Nikon Eclipse Ti, Tokyo, Japan) and the area of lipid droplets was estimated by Image J software.

##### *Bodipy 493/503 staining*

To detect lipid droplets in fixed cells, cells were plated and subsequently fixed with 4% paraformaldehyde. Then, the cells were incubated with  $2\ \mu\text{M}$  Bodipy 493/503 solution in PBS to stain lipid droplets. Lipid accumulation in cells with DAPI-stained nuclei was observed using a fluorescent microscope (ZEISS Apotome 3, ZEISS, Baden-Württemberg, Germany).

To evaluate lipid storage in living cells, cells were seeded at the density of  $2 \times 10^4$  cells/well in 96-well black plates. Following the indicated treatments, the culture media were removed. A  $2\ \mu\text{M}$  BODIPY 493/503 solution was then added to each well and incubated for 15 min at  $37^{\circ}\text{C}$  in the dark. Cells were washed three times with PBS. Subsequently, PBS was added to each well, and fluorescence intensity at 493/503 nm was

measured using a Varioskan™ LUX multimode microplate reader (Thermo Scientific).

#### *Measurement of intracellular triglyceride*

HepG2 cells were plated in 6-well plates at a density of  $4 \times 10^5$  cells per well and incubated overnight. At the end of the treatment period, the cells were collected, lysed in isopropanol, and subsequently centrifuged at 14,000 rpm for 10 min at 4°C. The supernatants were collected and used for triglyceride (TG) quantification using a fluorescence-based method with a commercial kit (Beyotime, Shanghai, China), following the manufacturer's protocol. TG assay was performed in a 96-well black plate. A reaction mixture consisting of cell lysate, assay buffer, and lipase was added to each well and incubated at 37°C for 20 min. Then, a working solution containing assay buffer, enzyme mix, cofactor, and Amplex Red was added to each well, followed by incubation at 37°C for 60 min. The reaction product, resorufin (the oxidized form of Amplex Red), was assessed by measuring fluorescence intensity at Ex/Em of 560/590 nm using Varioskan™ LUX multimode microplate reader. Total protein concentrations in the lysates were determined using a commercial kit from Beyotime according to the manufacturer's instructions. The intracellular TG levels were normalized to total protein content and expressed as relative values compared to those of control cells.

#### *Determination of intracellular MDA*

Cells were plated in 6-well plates at a density of  $4 \times 10^5$  cells per well. Following the treatment period, cells were collected and lysed in 1% Triton X-100/PBS with brief sonication. The cell lysate was centrifuged at 14,000 rpm for 10 min at 4°C, and the supernatants were collected for MDA

quantification. MDA levels were estimated by measuring thiobarbituric acid reactive substances in cell lysates, as described by Wasowicz [24] with minor modifications to suit the laboratory conditions. Briefly, the reactions were carried out in capped glass tubes, containing 0.5% TBA solution prepared in 50% acetic acid, distilled water, and cell lysate. The reactions were maintained at 95 - 100°C for 1 h in a water bath. After that, the mixture was allowed to cool to RT, and the absorbance was measured at 532 nm using a 96-well plate. The intracellular MDA levels were normalized to total protein content and expressed as relative values compared to those of control cells.

#### *Determination of AST and ALT activity*

Cells were seeded at a density of  $2 \times 10^4$  cells/well in 96-well plates and incubated in a sterile incubator overnight. After the indicated treatments, the cell culture media were collected. The assay was performed in wells of a 96-well plate, containing cell culture media and assay reagent provided in AST (or ALT) kits. The absorbance was immediately monitored every 30 seconds for 3 min at 340 nm. AST and ALT activity were expressed as relative values compared to those of control cells.

#### *Immunocytochemistry (ICC)*

Immunocytochemistry (ICC) was used to detect the nuclear translocation of caspase-3. Cells were seeded and then fixed following the same procedure used in ORO staining experiments. Then, cells were permeabilized with 0.2% Triton X-100/PBS and blocked with 3% BSA/PBS in 1 h at RT. Subsequently, cells were incubated with a primary antibody against caspase-3 at 4°C overnight. Afterward, cells were incubated with an Alexa 488-conjugated anti-rabbit secondary antibody for 90 min at RT in the dark. At the

end of the procedure, the slides were mounted with the mounting medium with DAPI (Abcam). Images were captured using a fluorescent microscope and analyzed using Image J software.

#### ***TUNEL staining***

Cells were seeded and then fixed following the same procedure used in ORO staining experiments. Subsequently, cells were permeabilized with 0.2% Triton X-100/PBS for 10 min at RT. The TdT-mediated dUTP nick end labeling (TUNEL) was performed using the Elabscience® One-step TUNEL In Situ Apoptosis Kit according to the manufacturer's instructions. Finally, the slides were prepared using the mounting medium with DAPI (Abcam). Images were captured using a fluorescent microscope and analyzed using Image J software.

#### ***Statistical analysis***

All experiments were conducted three times independently. Data were presented as mean  $\pm$  standard error (SE). Statistical comparisons among groups were performed using one-way analysis of variance (ANOVA) in the GraphPad Prism 8.0.2 software (San Diego, CA, USA). *p* values below 0.05 were considered statistically significant.

## **RESULTS AND DISCUSSIONS**

### ***RESULTS***

#### ***GME reduces oleic acid induced lipid accumulation in HepG2 cells***

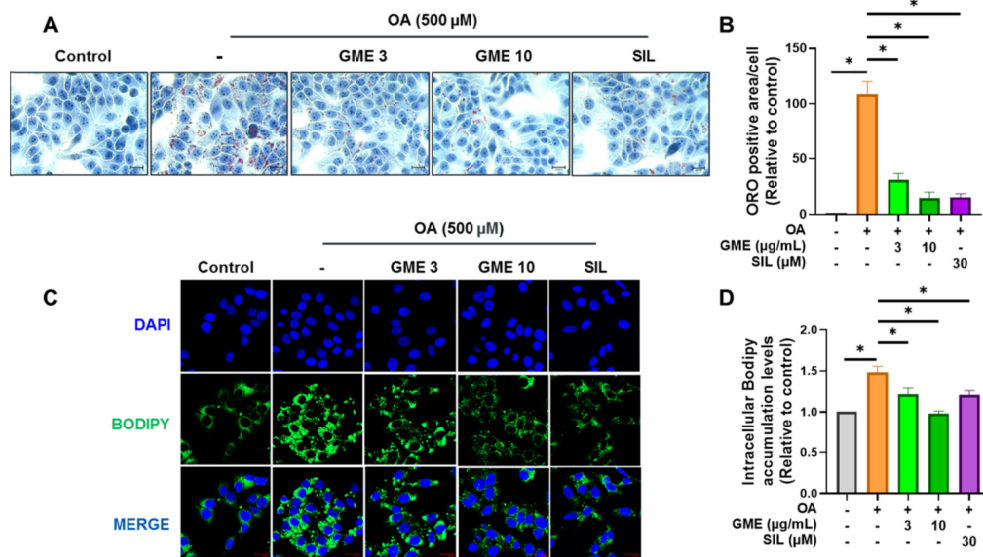
To evaluate the effect of GME on OA-induced lipid accumulation, HepG2 cells were pretreated with GME (3 and 10  $\mu\text{g}/\text{mL}$ ) or the positive control silibinin (30  $\mu\text{M}$ ), followed by incubation with 500  $\mu\text{M}$  OA. Lipid droplets were visualized using Oil Red O (ORO) and BODIPY 493/503 staining. As shown in Fig. 1A and 1C, OA significantly

increased intracellular lipid droplet formation compared with the control group, evident as red-stained structures in ORO-stained cells and green fluorescence in BODIPY-labeled cells. Pretreatment with GME significantly reduced lipid droplet density in a dose-dependent manner compared with the OA-treated group, and the effect was comparable to that observed with silibinin.

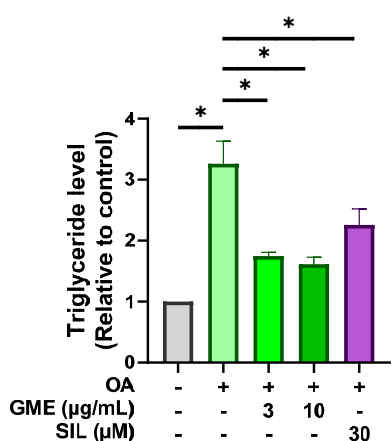
Quantitative analysis supported these findings. GME significantly decreased the mean ORO-positive area per cell (Fig. 1B) and reduced intracellular BODIPY signal intensity (Fig. 1D). Consistently, OA exposure markedly increased intracellular triglyceride levels compared to the control group. This OA-induced triglyceride accumulation was significantly attenuated by GME at both concentrations, as well as by silibinin. Specifically, GME at 3 and 10  $\mu\text{g}/\text{mL}$ , and silibinin at 30  $\mu\text{M}$ , reduced intracellular triglyceride levels by 46.4% ( $p = 0.0035$ ), 50.5% ( $p = 0.0019$ ), and 30.8% ( $p = 0.0455$ ), respectively, relative to the OA group (Fig. 2). These results indicate that GME effectively attenuates lipid storage in OA-stimulated hepatocytes.

#### ***GME attenuates oxidative stress and preserves hepatocellular membrane integrity***

Malondialdehyde (MDA), a major end-product of lipid peroxidation, is commonly used as a biomarker of oxidative stress [25]. Treatment with 500  $\mu\text{M}$  OA induced a significant increase in intracellular MDA levels relative to the control group, reflecting enhanced oxidative stress and lipid peroxidation. The presence of GME (3 and 10  $\mu\text{g}/\text{mL}$ ) abrogated this effect, effectively reducing MDA levels. The reductions in MDA content were 45.3% ( $p = 0.0402$ ) and 60.6% ( $p = 0.0069$ ), respectively, when compared with the OA group. This effect of



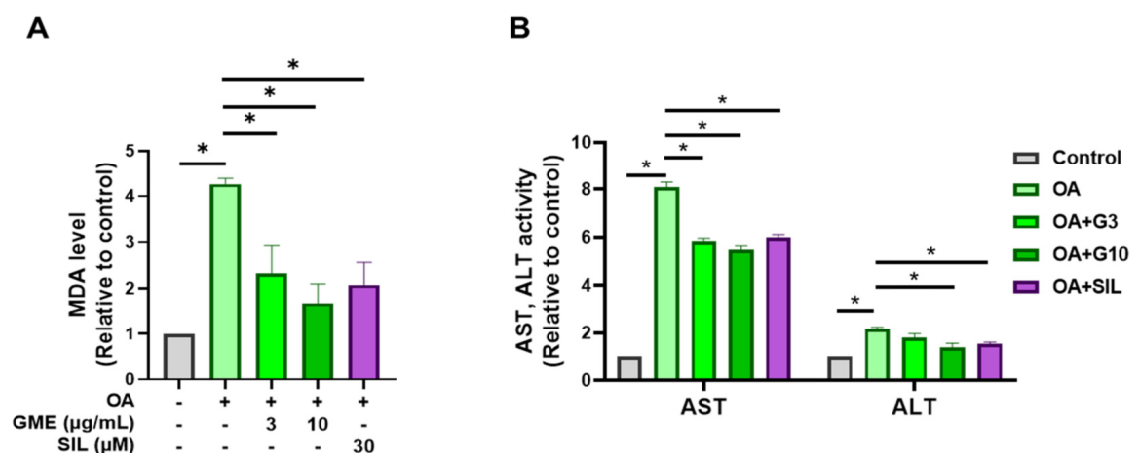
**Fig 1.** Effects of GME on lipid droplet formation stimulated by OA in HepG2 cells. Cells were pretreated with GME at concentrations of 3 μg/mL (GME3) and 10 μg/mL (GME10) for 2 h, followed by incubation with OA (500 μM) for 24 h. (A) Representative images of intracellular lipid droplets stained with ORO. (B) Quantification of mean ORO positive area per cell. (C) Representative images of intracellular lipid droplets stained with Bodipy 493/503. (D) Intracellular Bodipy accumulation levels. \* denotes  $p < 0.05$ ;  $n = 3$ .



**Fig 2.** Effects of GME on cellular triglyceride levels in OA-treated HepG2 cells. Cells were pretreated with GME at concentrations of 3 μg/mL (GME3) and 10 μg/mL (GME10) for 2 h followed by incubation with OA (500 μM) for 24 h. Triglyceride levels were determined in cell lysates using the commercial triglyceride assay kit. \* denotes  $p < 0.05$ ;  $n = 3$ .

GME was comparable to that of silibinin (30 μM) (51.3% reduction,  $p = 0.0201$ ) (Fig. 3A).

In addition, the increase in oxidative stress is closely associated with the generation of reactive groups that attack membrane lipids and proteins, destabilize the structure and damage the cell membrane [16]. Consistent with this mechanism, OA exposure disrupted cell membrane integrity and markedly elevated the release of aminotransferases (AST and ALT) into the culture medium relative to the control group. Pretreatment with GME effectively counteracted these changes in comparison with OA-treated cells. Both concentrations of GME (3 and 10 μg/mL) significantly reduced AST activity by 27.8% ( $p < 0.0001$ ) and 32.3% ( $p < 0.0001$ ), while a significant reduction in ALT activity was observed at 10 μg/mL GME (36.7% reduction,  $p = 0.0058$ ).



**Fig 3.** Effects of GME on cellular MDA levels and AST, ALT activities in OA-treated HepG2 cells. Cells were pretreated with GME at concentrations of 3  $\mu\text{g/mL}$  (GME3) and 10  $\mu\text{g/mL}$  (GME10) for 2 h followed by incubation with OA (500  $\mu\text{M}$ ). (A) MDA levels in cell lysates. (B) AST and ALT activities in culture media. \* denotes  $p < 0.05$ ;  $n = 3$ .

A similar effect was also seen with the positive control silibinin, which reduced AST and ALT activities by 26.1% ( $p < 0.0001$ ) and 29.4% ( $p = 0.0238$ ), respectively (Fig. 3B).

#### ***GME protects hepatocytes from OA-induced apoptosis***

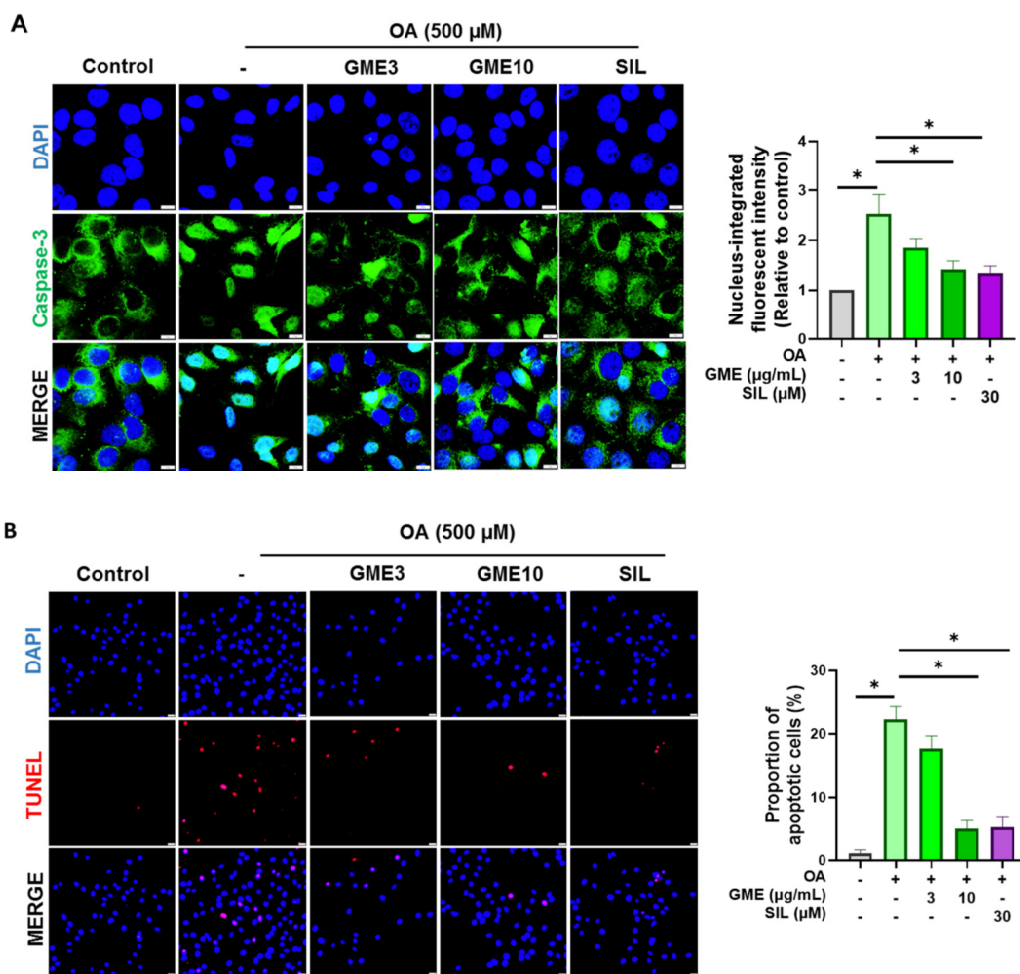
Excessive oxidative stress not only damages cell membrane but also triggers apoptotic signalling pathways [16]. To further investigate the protective role of GME, apoptosis was assessed in OA-treated HepG2 cells by monitoring caspase-3 translocation and TUNEL staining.

The results showed that caspase-3 was translocated into the nucleus of HepG2 cells upon OA stimulation. However, pretreatment with GME effectively inhibited this nuclear translocation in a dose-dependent manner compared with OA-treated cells. Statistical analyses confirmed that GME at 10  $\mu\text{g/mL}$  significantly reduced the nucleus-integrated fluorescent intensity of caspase-3 (green fluorescence) as compared to the OA group (Fig. 4A). Consistently, treatment with OA increased the number of apoptotic cells, as

indicated by TUNEL-positive staining. GME at 10  $\mu\text{g/mL}$  significantly decreased the proportion of apoptotic cells relative to OA-treated cells (Fig. 4B). These effects were comparable to those observed with the positive control, silibinin (30  $\mu\text{M}$ ).

#### ***DISCUSSION***

Intracellular lipid accumulation, especially triglycerides, is a characteristic sign of non-alcoholic fatty liver disease and other hepatic lipid-related disorders, which contributes to impaired liver cell function and promotes disease progression. In this study, we used a lipid accumulation model induced by OA in HepG2 cells to investigate whether the *Gnetum montanum* extract (GME) could exert anti-lipid accumulation effects. The results demonstrated that GME significantly reduced lipid droplet formation and intracellular TG levels. This effect was comparable to that of a well-known hepatoprotective agent - silibinin (30  $\mu\text{M}$ ), which was used as a positive control. Silibinin, a flavonoid compound and the



**Fig 4.** Effects of GME on apoptosis markers in OA-treated HepG2 cells. Cells were pretreated with GME at concentrations of 3  $\mu$ g/mL (GME3) and 10  $\mu$ g/mL (GME10) for 2 h followed by incubation with OA (500  $\mu$ M) for 24 h. (A) Representative images and quantification of caspase-3 nuclear translocation. (B) Representative images and the proportion of apoptotic cells in TUNEL staining. Nucleus-integrated fluorescent intensity and the proportion of apoptotic cells were analyzed using Image J software \* denotes  $p < 0.05$ ;  $n = 3$ .

major active constituent of silymarin (a lipophilic extract from milk thistle seeds), is widely used in *in vitro* models at concentrations of 25 - 50  $\mu$ M due to its antioxidant and lipid-regulating effects [26, 27].

Two intracellular lipid staining methods (ORO and Bodipy 493/503) were applied and gave consistent results, indicating that GME

effectively reduced lipid accumulation in OA-stimulated hepatocytes. This was shown via a marked decrease in the density of lipid droplets observed under microscopy, as well as a reduction in the mean lipid droplet area per cell and intracellular Bodipy accumulation levels. GME inhibited this accumulation in a dose-dependent manner. In addition, GME at both concentrations (3 and 10  $\mu$ g/mL)

significantly decreased intracellular TG levels, further supporting its anti-lipid accumulation activity. These findings suggest that GME can effectively counteract OA-induced lipid accumulation in hepatic cells and may help regulate intracellular lipid balance.

Previous studies have shown that high concentrations of OA in liver cells can disrupt lipid metabolism, stimulate the production of reactive oxygen species (ROS), and promote lipid peroxidation, leading to oxidative damage [14, 15, 28]. Indeed, our present study demonstrated that treatment of HepG2 cells with 500  $\mu\text{M}$  OA significantly increased intracellular malondialdehyde (MDA) levels, a characteristic marker of oxidative stress and lipid peroxidation. Interestingly, pretreatment with GME at both 3 and 10  $\mu\text{g}/\text{mL}$  markedly reduced MDA levels, suggesting that GME may exhibit strong antioxidant activity in this model. These results were consistent with previous reports highlighting the antioxidant properties of *G. montanum*, possibly attributed to the presence of stilbenoids and flavonoids in this plant [21, 29, 30].

The final consequence of excessive lipid accumulation and oxidative stress can result in liver cell damage and apoptosis. In this study, OA-induced lipotoxicity caused damage to the hepatocyte membrane, leading to increased leakage of AST and ALT enzymes into the culture media. GME significantly reduced AST release at both tested concentrations and lowered ALT activity at the higher dose (10  $\mu\text{g}/\text{mL}$ ), indicating its ability to protect membrane integrity under OA-induced lipotoxic conditions.

In addition, apoptosis was evaluated based on typical biomarkers, including the nuclear translocation of caspase-3 detected

by immunocytochemistry staining and DNA fragmentation assessed by TUNEL staining. Using both caspase-3 immunostaining and TUNEL assay to evaluate apoptosis provides a more comprehensive assessment due to the different characteristics of these methods. Caspase-3 is a key intracellular protease involved in the execution phase of apoptosis, and its translocation from the cytoplasm into the nucleus is considered an early marker of this process. In contrast, the TUNEL method detects cells at a later phase of apoptosis by identifying fragmented DNA. By combining the two methods, it is possible to monitor apoptosis progression at different stages, increasing the reliability and accuracy of the results. This dual method also helps avoid false negatives that might occur if only one marker is used. Treatment with OA resulted in a clear nuclear translocation of caspase-3 and a higher number of TUNEL-positive cells, reflecting a significant increase in apoptosis. Notably, pretreatment with GME reduced these effects in a dose-dependent manner, suggesting that GME may suppress OA-induced apoptotic pathways. The combined protective effects of GME, against oxidative stress, membrane damage, and apoptosis, highlight its potential for managing hepatic lipotoxicity.

Interestingly, GME at 10  $\mu\text{g}/\text{mL}$  appeared to exert a stronger effect than silibinin (30  $\mu\text{M}$ ) in reducing intracellular lipid droplet accumulation and markers of oxidative stress and hepatocellular injury (MDA, AST, and ALT levels). Notably, GME at both concentrations showed a greater reduction in intracellular triglyceride content than silibinin (Figs. 1-3). These observations highlight the promising hepatoprotective potential of GME. However, further studies are warranted

to confirm these findings and elucidate the underlying mechanisms.

The present study demonstrated that GME exerts significant protective effects against OA-induced lipid accumulation, oxidative stress, and apoptosis in HepG2 cells. These findings suggest that GME helps maintain hepatic lipid and redox balance under lipotoxic conditions. *Gnetum montanum* is known to contain several stilbene derivatives, particularly resveratrol, which possess well-documented antioxidant and metabolic regulatory properties. Therefore, it can be assumed that the combined activity of these constituents contributes to the biological effects observed in the present study. Although the precise molecular mechanisms underlying these actions remain to be clarified, previous studies have indicated that stilbene compounds can modulate hepatic lipid metabolism and oxidative signaling through pathways related to energy sensing and redox regulation [31-33]. Further studies are warranted to confirm these proposed mechanisms at the molecular level. In particular, future investigations will focus on examining the expression and activation of key proteins and genes involved in lipid metabolism and oxidative stress using assays such as Western blotting and RT-qPCR.

Although HepG2 cells are widely used for *in vitro* hepatic lipid accumulation studies due to their stable growth and maintenance of certain hepatocyte-like metabolic functions, several important limitations should be considered when interpreting the results. As a hepatocellular carcinoma-derived line, HepG2 exhibits cancer-related alterations such as dysregulated cell-cycle control, abnormal gene expression profiles, and

atypical signaling pathways, which may not accurately reflect the physiology of primary hepatocytes. Moreover, the level and pattern of lipid accumulation in HepG2 cells generally do not fully correspond to those observed in primary liver cells, and the line lacks key morphological and functional characteristics as well as the multicellular interactions present in native hepatic tissue. These factors restrict the capacity of HepG2 to model the complex physiological and pathological processes underlying hepatic steatosis. To overcome these limitations, future studies may incorporate alternative cell models with higher physiological relevance, such as primary mouse hepatocytes, primary human hepatocytes, or hepatocyte-like cells differentiated from induced pluripotent stem cells (iPSCs). Among these options, primary mouse hepatocytes offer a practical and accessible model, providing closer structural and functional similarity to native hepatocytes, high expression of metabolic enzymes, genetic homogeneity, and relatively low isolation cost. Future studies using animal models of hepatic steatosis are needed to confirm these results and to better understand the effects of GME *in vivo*.

## CONCLUSION

In summary, our study demonstrated that *Gnetum montanum* extract (GME) protected against oleic acid-induced lipid accumulation and lipotoxicity in HepG2 cells. GME significantly reduced intracellular lipid droplet formation and the accumulation of triglycerides, alleviated oxidative stress by lowering malondialdehyde (MDA) levels, and protected hepatocytes from membrane damage and apoptosis. These results show the potential of GME as a natural agent for inhibiting hepatic lipid accumulation and

protecting liver cells, providing a basis for further studies on its application in hepatic lipid metabolic disorders.

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#### CONFLICTS OF INTEREST

None.

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