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Galbanic acid of *Ferula assa-foetida* L, as a regulator of the AMPK pathway in reduction of lipid accumulation in HepG2 cells

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Abstract

Introduction: Hepatic fat accumulation is a complication of non-alcoholic fatty liver disease (NAFLD). An AMP-activated protein kinase (AMPK) reduces the synthesis of fatty acids by inhibiting sterol regulatory element-binding transcription factor 1-c (SREBP1-C) and acetyl COA carboxylase (ACC).
Objectives: We aimed to investigate the impress of galbanic acid (Gal) from the Ferula plant on AMPK and its inhibitory effect on lipogenic enzymes in HepG2 cells.
Materials and Methods: In an applied-fundamental study, HepG2 cells were treated for 24 hours with Gal in palmitate (Pal). Resveratrol (RSV) was conducted as a positive control. Fatty acid synthase (FAS) and SREBP1-C gene expression were evaluated by reverse transcription polymerase chain reaction (RT-PCR). FAS, phospho-acetyl-CoA carboxylase (P-ACC), P-AMPK, AMPK, SREBP-1c, and ACC protein levels were measured by western blotting. Lipid accumulation was investigated qualitatively and semi-quantitatively with oil red.
Results: The semi-quantitative results of oil revealed a substantial reduction ($P<0.004$) in lipid accumulation for treatment with Gal. The significant increase in the protein level of P-AMPK ($P<0.001$) and P-ACC ($P=0.054$) and significant decrease in FAS ($P<0.003$), SREBP-1c ($P<0.001$) and ACC ($P<0.011$) due to the effect galbanic acid was observed. FAS gene expression decreased significantly ($P<0.009$), while the decrease in SREBP-1c gene expression was not significant ($P=0.303$).
Conclusion: These findings direct that galbanic acid can be a new regulator of AMPK. Hence, the present study may introduce galbanic acid as a new plan to positively regulate the AMPK pathway, which leads to the regulation of various cellular processes.

Introduction

Non-alcoholic fatty liver disease (NAFLD) usually occurs without the excessive consumption of alcohol and has become one of the most common medical problems. Hepatic fat accumulation is associated with NAFLD. This disease is generally found in people with diabetes who are obese (1). NALFD develops in two stages; first, the accumulation of large amounts of lipids in the liver causes insulin resistance and then inflammatory damage occurs. Since, it can lead to liver cancer, investigating the underlying molecular mechanism for treatment is essential (2). Adipogenesis is usually regulated through complex changes in gene expression and hormone sensitivity by various transcription

Key point

In this study, the effect of galbanic acid on the expression of AMP-activated protein kinase (AMPK) and lipogenesis enzymes was shown. The protective effect of galbanic acid on intracellular lipid accumulation can be used in the design of therapeutic methods for the prevention and treatment of fatty liver.

factors (3). The sterol regulatory element-binding transcription factor (SREBP) family of transcription factors has a crucial role in the regulation of cellular lipid metabolism. Fatty acid synthase (FAS) is usually regulated by SREBP1-C (4). AMP-activated protein kinase is one of the main proteins to regulate carbohydrate and lipid metabolism and

energy control. According to the previous investigations, hyperglycemia results in hepatic AMPK disorder and directs a fundamental mechanism for the accumulation of hepatic fat and hyperlipidemia in diabetic patients (5). AMP-activated protein kinase reduces the expression of enzymes required to synthesize fatty acids by inhibiting the SREBP-1 protein. AMPK, on the other hand, phosphorylates and inactivates acetyl COA carboxylase (ACC), which is an essential enzyme in the synthesis of fatty acids and cholesterol (6). Due to the lower activity of AMPK in conditions of inflammation, obesity and diabetes, increasing the activity of AMPK is considered a practical treatment strategy to reduce NAFLD. For developing new anti-lipidemic medications having low side effects and high efficacy, attention has recently been paid to herbal medicines (6). *Ferula assa-foetida* L. is one of the medicinal plants that has been used for a long time in the treatment of diseases due to the presence of various chemical compounds. The resin part of this plant consists of ferulic acid with its esters, coumarins (including galbanic acid and farnesol), sesquiterpene, and other terpenoids (7). Several studies demonstrated a significant fat reduction in diabetic rats ministered with the extract of this plant. Sesquiterpene terpene coumarins are highly important among these compounds due to their extensive biological properties. In previous studies, the effects of several sesquiterpene coumarins, including galbanic acid, on serum triglyceride and cholesterol levels in the blood, liver and aorta were investigated, which showed that these compounds prevent the elevation of serum cholesterol and triglyceride levels (8).

Objectives
On this evidence and the widespread use of this medicinal extract in Asia, this study was therefore conducted to evaluate the effects of Gal in *Ferula* plant on AMPK regulator and its inhibitory effect on lipogenesis enzymes and inhibition of HepG2 cells' lipid accumulation.

Materials and Methods
Cell culture and treatment
HepG2 cells (Iranian Biological Resources Center, Tehran, Iran) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS), and 1% pen/strep (All three were purchased from Dena Zist Asia, Mashhad, Iran), at 37°C, and cells at 5% CO2 were incubated. When the cell density reached 70-80%, the treatment was performed on the cells. To prepare a stock solution, Gal (GolEXIR, Mashhad, Iran) and resveratrol (RSV) (GolEXIR, Mashhad, Iran) were dissolved in Ddimethyl sulfoxide (DMSO) (Sigma–Aldrich, USA). The DMSO maximum final concentration was 0.1%. Subsequently, the cells were treated for 24 hours. The subgroups tested in cell culture were: 1; treatment with palmitate (Pal) (Sigma–Aldrich, USA), 2; treatment with Pal and Gal. 3; positive control (treatment with palmitate

and RSV). The experiments were done in triplicate.

Cell viability assessment
Around 2×10^4 HepG2 cells were inoculated in 96-well plates. The concentration of total albumin (Sigma–Aldrich, USA) in all groups in the culture medium was 0.7 mM, which was similar to the concentration of total human albumin (16). The cells were then incubated in the different palmitate concentrations (0 as control, 0.2, 0.4, 0.5, 0.8, and 1 mM) for 24 hours. Pal and Gal in the combined treatments were treated in a separate group. The following day, each well was inoculated with fresh-medium containing different Gal concentrations (12.5, 25, 50, 100, 200, 300, 400, and 800 μM). Moreover, MTT (Sigma–Aldrich, USA) solution (50 μg/well) was poured to each well and set for about four hours, after which the resulting purple crystals were dissolved in DMSO solution. Furthermore, using a plate reader (BioTek Instruments, USA), the absorption of the wells was read at 540 nm wavelength. Cell viability was calculated as the percentage of treated cell absorbance as compared to the control group.

Staining
At first, the HepG2 cells were inoculated to 6-well plates. At 80-90% density, the culture medium inside the wells was replaced with a renewed medium containing palmitate (at concentrations of 0, 0.2, 0.4, 0.5, 0.8, 1 mM). The resulting cells were washed with phosphate-buffered saline (PBS) after an hour with a 1% paraformaldehyde solution and 60% isopropanol and then incubated with a complex solution [Oil Red-O (ORO) with isopropanol 60%] for 30 minutes. To prepare the above solution, 350 mg of ORO (Sigma–Aldrich, USA) in 100 mL of 100% isopropanol was dissolved and filtered. After incubating with ORO, the cells were washed with distilled water and imaged using an inverted microscope (19). In the combined cases, the final concentration of palmitate with the final concentration of Gal/RSV was performed for ORO staining to evaluate the total lipid content of the cell.

FAS, and SREBP1C gene expression by RT-PCR
Using TRIzol from kit GeneAll, total cell RNA was extracted. Total RNA concentration was measured by optical density and A260/A280 ratio by Nanodrop 1000 spectrophotometer (Wilmington, DE, USA). Geneall kits were used for cDNA synthesis. The RT-PCR protocols were performed by SYBR green-based polymerase chain reaction (PCR) Master Mix by Yekta Tehiz kit (Iran), and MIC system (Australia) was used to evaluate gene expression. The following PCR primer sequences were conducted for amplification (Table 1): ΔΔCt method with efficiency correction according to Pfaffl technique was used for quantitative data interpretation and CT values (cycle threshold) were standardized compared to β-actin expression.

Table 1. Primer sequences for polymerase chain reaction amplification

Primers	Forward	Reverse
β-Actin	5´- AAACAGAGCCTCGCCTTTGC -3´	5´- ACACGATGGAGGGGAAGACG -3´
SREBP-1c	5´-CACCGAGAGCAGAGATGGC-3´	5´-AAGGAGACGAGCACCAACAG-3´
FAS	5´- GTGTCCACCAGCAACATCAG -3´	5´- AGCCATCTCTCAAGACCACG -3´

Western blot analysis

Protease/phosphatase inhibitor cocktail with RIPA buffer (containing 2 mM EDTA, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 100 mM PMSF, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) was conducted to lyse HepG2 cells. The sample total protein was measured by the kit of bicinchoninic acid protein concentration (Thermo Fisher Scientific Inc. USA). Forty micrograms of the proteins of each sample were separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) technique and then transferred to PVDF (polyvinylidene dichloride membranes) (Buckinghamshire, UK). BSA (bovine serum albumin; 5% w/v)) in saline Tris-buffered was use for blocking, then incubated with primary antibodies (Santa Cruz, CA, USA) overnight at 4°C. In the next step, it was incubated for one hour at room temperature with HRP-conjugated secondary antibodies (Santa Cruz, USA). Imaging was done by enhanced chemiluminescence (ECL) detection reagent using a quantitative luminescence imaging system (Vilber Lourmat). At the end, densitometric analysis was performed using ImageJ software.

Statistical analysis

Data analysis was conducted by analysis of variance (ANOVA) and Tukey's post hoc tests using GraphPad Prism 8.0 software (San Diego, CA, USA). The experiments were in three repetitions and reported as mean ±SD. P values < 0.05 was considered significant.

Results

Cell viability and induction of lipid accumulation in Pal-treated HepG2 cells

Figure 1A, reveals the effect of treating with different palmitate concentrations on the viability of cells after 24 hours. As shown in Figure 1A, a decrease in cell survival was observed at higher concentrations of palmitate, therefore at concentrations above 0.5 mM, a reduction of 50% was observed (P < 0.004). To confirm the development of steatosis, intracellular lipid content was observed with ORO staining. Microscopic examination of stained HepG2 cells showed a difference in the accumulation of fat droplets in the morphology of cells treated with palmitate compared to the group without treatment (Figure 1B). In addition, the amount of red dye absorbed by lipid droplets was semi-quantitatively measured by spectrophotometry. The results were expressed as the amount of light absorption of each sample compared to the control group. As Figure 1C shows, treatment with 0.4

to 1 mM of palmitate can cause a considerable elevation in lipid. Therefore, the lowest significant concentration of palmitate (0.4 mM) with no effect on cell survival was conducted to evaluate the impacts of Gal and RSV.

Gal-treated HepG2 cell viability and the effect of Gal/RSV on intracellular total lipid content

After determining the appropriate palmitate concentration, total intracellular lipid content was measured to evaluate the effect of Gal/RSV on lipid accumulation in fatty liver. Initially, the assessment of Gal cell survival was conducted. For this purpose, cells were treated with different concentrations of Gal with 0.4 mM Pal for 24 hours. The results of the MTT test show that at concentrations above 400 µM, a 50% reduction in cell survival was observed (P < 0.001, Figure 2A); therefore, the highest Gal concentration below IC50 was 300 µM for further studies. About 50 µM RSV was selected as a positive control in all studies, followed by a qualitative and semi-quantitative
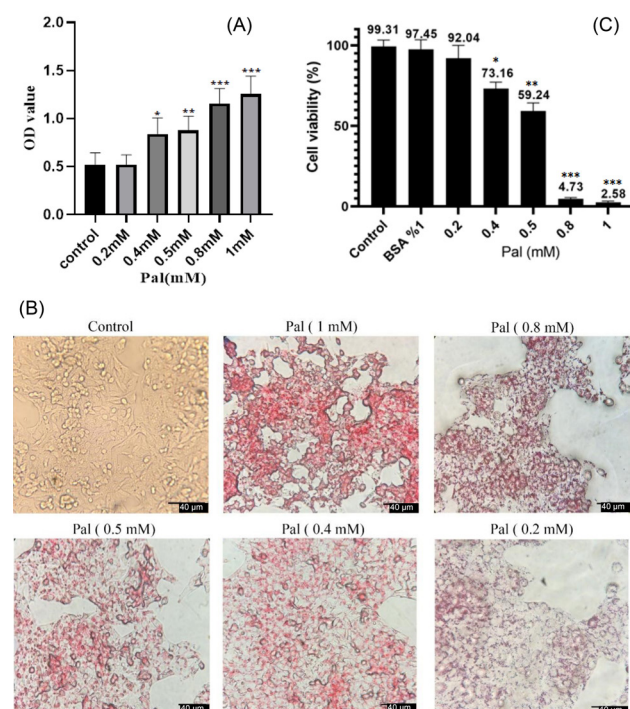

Figure 1. Effect of palmitate on HepG2 cells. Different concentrations of palmitate were treated to HepG2 cells for 24 hours. A) Effect of palmitate on HepG2 cell survival. B) Effect of palmitate on lipid accumulation in HepG2 cells. C) Quantitative results of ORO staining from the effect of Pal on the induction of lipid accumulation in HepG2 cells. Results are Mean ± SD from 3 independent experiments. *P <0.05, **P <0.01, and ***P <0.001 compared with the untreated control.
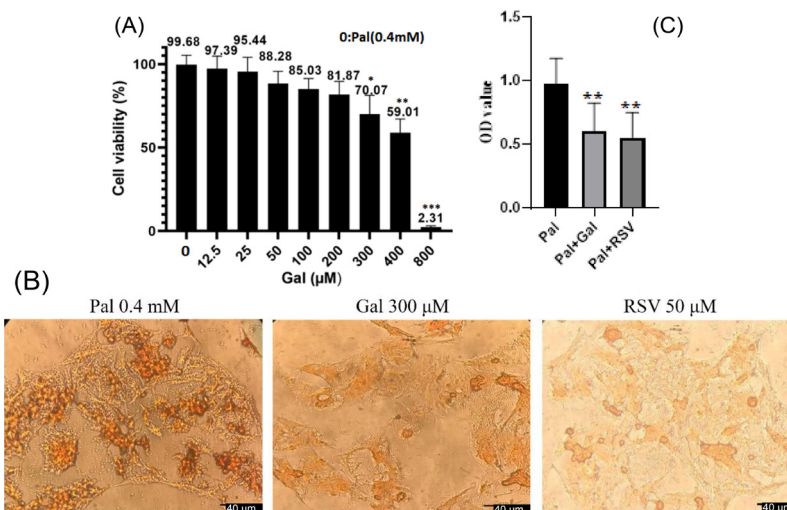
Figure 2. Effect of Gal/RSV on HepG2 cells. **(A)** Dose-dependent effect of Gal on HepG2 cell survival. Cells with the desired concentrations of Gal were treated for 24 hours and then MTT was performed. **(B)** effect of Gal/RSV on intracellular total lipid content of HepG2 cells with 0.4 mM Pal alone, with 300 µM Gal, and with 50 µM RSV were treated with 0.4 mM palmitate for 24 hours. **(C)** shows the semi-quantitative results of ORO staining of the effect of Gal/RSV on the induction of lipid accumulation in HepG2 cells. Results are mean ± SD from three independent experiments. *P <0.05, and **P <0.01 compared with the untreated control.

ORO test for Gal/RSV effects. Microscopic examination showed a decrease in cell staining for both Gal/RSV effects (Figure 2B). The semi-quantitative results as the amount of light absorption of each sample compared to the palmitate group (0.4 mM) showed a significant reduction in lipid accumulation for both treatments with Gal (P < 0.004) and RSV(P < 0.001) in combination with palmitate (0.4 mM).

Effect of Gal /RSV on the expression of SREBP-1c and FAS genes

To evaluate the expression of the studied genes, the effect of Gal/RSV on the expression of FAS and SREBP-1c genes after induction of steatosis was determined in comparison with treatment with palmitate alone. Gal had no significant effect on SREBP-1c expression (P = 0.303) but had a significant reduction in FAS gene expression (P < 0.009; Figure 3). RSV was considered a positive control for cells and changes in gene expression in RSV treatment were compared to Gal and palmitate alone. Treatment of HepG2 with RSV was observed in SREBP-1C expression (P = 0.127) and FAS (P < 0.004) in steatosis conditions similar to Gal (Figure 3).

Effect of Gal/RSV on AMPK, P-AMPK, SREBP-1c, ACC, p-ACC, and FAS proteins in steatosis

Figure 4 shows the results of P-AMPK, AMPK, ACC, SREBP-1c, P-ACC and FAS proteins by Western blotting. In each group, band intensity was compared with the beta-actin band. According to the figure, the amounts of AMPK, P-AMPK, and SREBP-1c proteins, and ACC, P-ACC and FAS cells treated with concentrations of Gal/RSV with palmitate were compared with those cells only receiving palmitate. Significant enhancement in the amounts of and P-ACC (P < 0.054) and P-AMPK (P < 0.001) proteins were revealed by treatment with Gal with palmitate in

comparison to the cells treated with palmitate alone. In contrast, protein levels of SREBP-1c (P < 0.001), FAS (P < 0.003), and ACC (P < 0.011) showed a significant decrease. Similarly by treatment with RSV with palmitate a significant raise in the amount of P-AMPK (P < 0.001) and P-ACC (P < 0.006) proteins was observed. In contrast, protein levels of SREBP-1c (P < 0.001), FAS (P < 0.001), and ACC (P < 0.011) showed a significant decrease.

Discussion

The pathology of NASH is mainly related to lipid and carbohydrate disorders (9). Researchers are trying to prevent lipogenesis in the human body by discovering and introducing new drugs. Gal from *Ferula* species is biologically active with a variety of biological properties such as anticancer, cancer chemo-preventive and anticoagulant function (10).
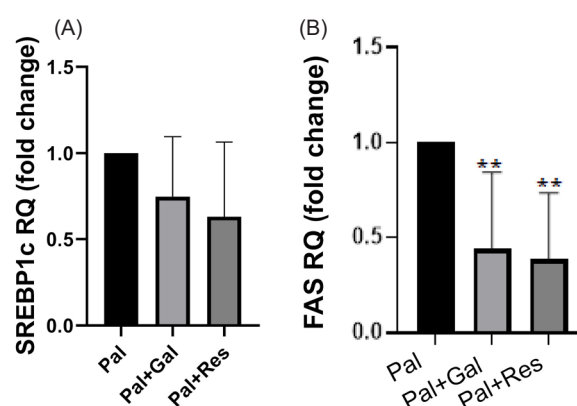

Figure 3. Effect of Gal (300 µM)/RSV (50 µM) on FAS expression and SREBP-1c in HepG2 cells, were treated with 0.4 palmitate mM, for 24 hours. (A) Effect of Gal/RSV on SREBP-1c expression in steatosis. (B) Effect of Gal/RSV on FAS expression in steatosis. Results are expressed as mean ± SD from three independent experiments. *P < 0.05 and ** P <0.01.
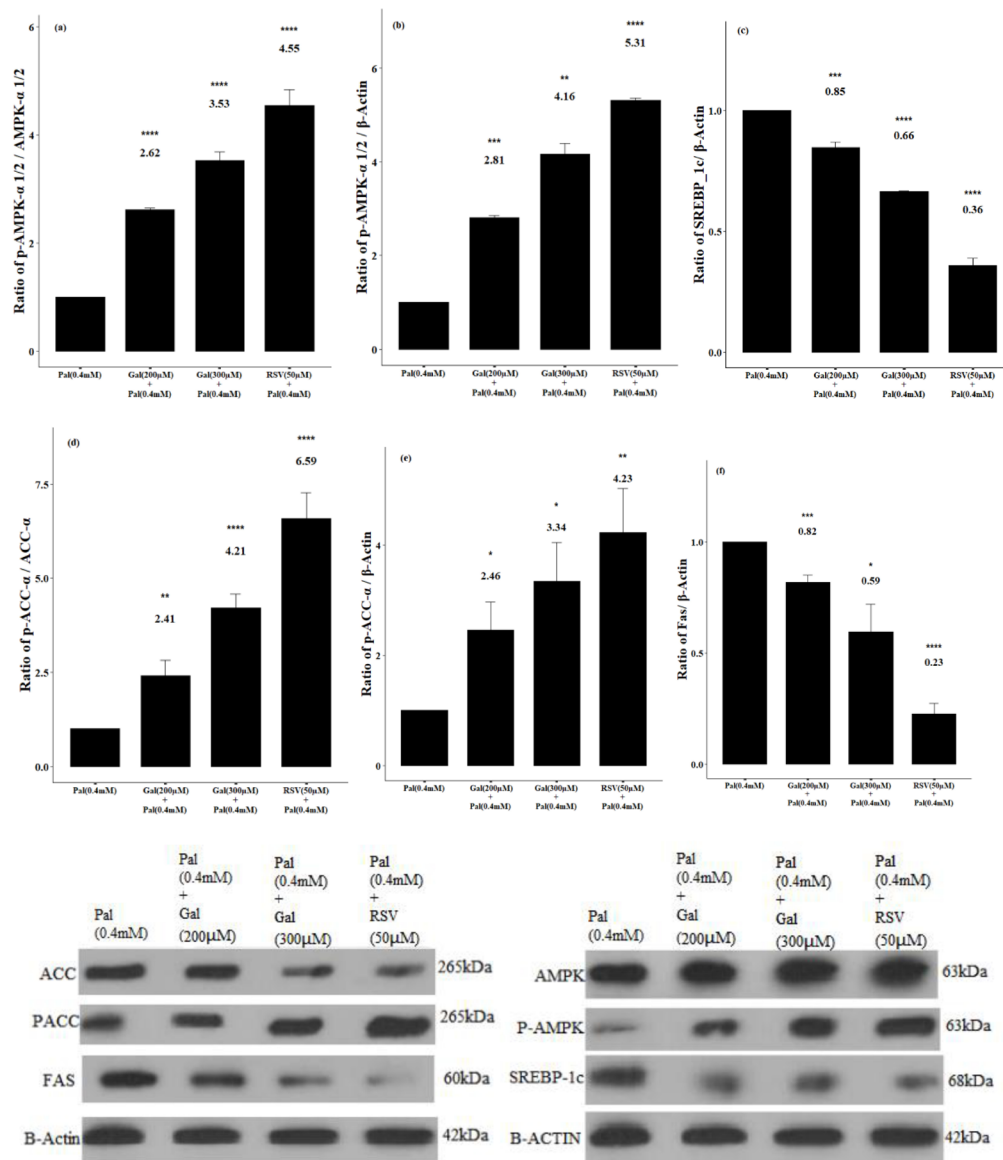
Figure 4. Effect of Gal/RSV on AMPK, P-AMPK, SREBP-1c, ACC, P-ACC, and FAS proteins. HepG2 cells were treated with Gal/RSV for 24 h. Graphs: a) Effect of Gal/RSV on P-AMPK/AMPK; b) Effect of Gal/RSV on P-AMPK/B-Actin; c) Effect of Gal/RSV on SREBP-1c/B-Actin protein content; d) Effect of Gal/RSV on P-ACC/ACC content; e) Effect of Gal/RSV on P-ACC/B-Actin protein content; f) Effect of Gal/RSV on FAS/B-Actin content. Results are expressed as Mean ± SD from three independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$, and ****$P<0.0001$ relative to control.

In this study, first cell viability and induction of lipid accumulation in Pal-treated HepG2 cells were investigated. Our results showed that treatment with palmitate, HEPG2 cells viability decreases, and lipid accumulation in these cells shows a significant increase in comparison to the control group. Moreover, treatment with 300 µL of Gal and 50 µL of RSV led to a significant reduction of FAS gene and protein expression. The SREBP-2 gene expression decreased in comparison with the control group but this change was not significant while SREBP-2 protein expression significantly decreased. FAS association with apoptosis was determined previously (11). It seems that Gal via FAS expression reduction in gene and protein levels counteracts the effects of Pal on hepatocytes and prevents apoptosis which causes via palmitate treatments. This result was confirmed by our previous investigation.

SREBP-1c is a factor with an important function in metabolic diseases such as dyslipidemia, type 2 diabetes, hepatic steatosis and hyperhomocysteinemia (12). SREBP-1c, involved in glucose metabolism and fatty acid synthesis, is considered an important target for treatment in pathological conditions (13). It seems that Gal does not have a significant effect on the gene expression of SREBP-1c, and exerts its positive effects by reducing the protein expression, which is responsible for functional activity. The up-regulation of SREBP-1c following hepatic steatosis has been decreased by coumarin. These results show that coumarin reduces hepatic fat accumulation by regulating SREBP-1c and thus reduces the expression of genes related to fatty acid synthesis (14). On the other hand, other factors can be involved in the positive effects of Gal on hepatocytes, which will be discussed below.

To determine the molecular mechanism of Gal on hepatocytes treated with palmitate, we investigated AMPK, P-AMP, P-ACC, and ACC. AMPK is a key energy sensor activated via phosphorylation and plays an important function in regulating adipogenesis, so can be a potential therapeutic target. AMPK act as an inhibitor of ATP-consuming and as an inducer of ATP-production procedure including fatty acid, cholesterol biosynthesis, glucose uptake and fatty acid oxidation respectively (15). Moreover, AMPK activation led to the regulation of key proteins involved in fatty acid and cholesterol biosynthesis such as the phosphorylation of ACC, inhibiting SREBP-2, and HMGCR (16). There was a significant elevation in the amount of p-AMPK and p-ACC proteins due to treatment with Gal/RSV with Pal compared to hepatocytes treated with palmitate alone.

Studies show that flavonoids such as Gal increase the phosphorylation levels of AMPK, ACC and GSK3β in our study. In this study, the expression level of SREBP-2 and HMGCR decreased in HepG2 cells treated with palmitate. Overall, the results show that the anti-lipid effect of flavonoids on HepG2 cells treated with palmitate is caused by the activation of AMPK by flavonoids (17). Along with our study, Syrov et al reported the positive effect of Gal in hepatitis by anti-oxidant properties and improved restoration of the intensity of bilirubin synthesis, bile acids, bile secretion, and cholesterol excretion compared to the control group (18). Also, like Gal, flavonoids hold an anti-adipogenic effect through the activation of AMPK and glycogen synthase, inhibition of SREBP-2, and increase P-ACC in palmitate-treated HepG2 cells as reported by Rajan et al (17). Recently, a phenolic glycoside with an herbal source named niazirin has also improved lipid metabolism through a reduction in fatty acid synthesis and induction of fatty acid oxidation via AMPK pathway activation in an animal model (19). Moreover, another herbal agent named s-petasioleic acid-induced NASH through reduction in lipid and triacylglycerol levels, induced phosphorylation of AMPKα and ACCα, inhibition of lipogenesis, increase triacylglycerol turnover by down-regulation of FAS and stearoyl-CoA desaturase and upregulation of adipose triglyceride lipase which led to AMPK signaling activation and regulation of transcriptional factors, Forkhead (FKHR) and SREBP-1 was reported by Guo et al (20). The use of medicinal plants in the prevention and treatment of diseases has long been considered. Our results and other studies show that herbal medicines have agents that can prevent liver disease caused by disorders of lipid and carbohydrate metabolism. In this regard, berberine improve glucose and lipid metabolism in Hepg2 cells via AMPK signaling pathway. So, AMPK is an important pathway in herbal agent effect in NASH and related diseases (21). These results recommend that Gal has protective effects on Pal-treated HepG2 cells via regulation of the AMPK signaling pathway and can act as a potential therapeutic agent in hepatic steatosis.

Conclusion
It is concluded that galbanic acid exerts its effect on HepG2 treated with palmitate by reducing the expression of FAS enzyme as the most important enzyme of the lipogenesis pathway, reducing the activity of ACC enzyme and the SREBP-1c factor. Galbanic acid exerted its effect on P-AMPK and p-ACC proteins by increasing their expression. These findings show that galbanic acid can be a new regulator of AMPK pathway. Hence, the present study might introduce galbanic acid as a new strategy with the aim of positive regulation of the AMPK pathway, which leads to the regulation of various cellular processes.

Limitations of the study
A limitation of this study is not investigating the inhibition of AMPK activity by AMPK inhibitor. If this investigation was conducted, we could express the galbanic acid effect on the AMPK signaling pathway with more certainty.

Authors' contribution
Conceptualization: HM, SM.
Methodology: HM, AB.
Validation: HM, SM, AKH.
Formal analysis: HM, AKH, HSH.
Investigation: HM, SM, AKH, HSH.
Resources: HM, ZM.
Data curation: HM, AB.
Writing–original draft preparation: HM.
Writing–review and editing: HM, SM, AKH.
Visualization: HM, SM, AKH.
Supervision: HM, SM, AKH.
Project administration: HM, SM, AKH.
Funding acquisition: HM, SM, AKH.

Conflicts of interest
The authors declare that they have no competing interests.

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