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RESEARCH

Hispidulin can improve lipid parameters in the HepG2 cell line with metabolic dysfunction-associated steatotic liver disease

Hispidulin, metabolik disfonksiyonla ilişkili steatotik karaciğer hastalığı olan HepG2 hücre hattında lipid parametrelerini iyileştirebilir

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Abstract

Purpose: Metabolic dysfunction-associated steatotic liver disease (MASLD) is a significant health issue. Although its pathogenesis remains unclear, insulin resistance, steatosis, and inflammation play crucial roles. Research on alternative treatment agents is ongoing. This is the first study to investigate the effect of hispidulin, a flavonoid, in a MASLD model.
Materials and Methods: Non-toxic concentrations of hispidulin and oleic acid were determined using the MTT cytotoxicity assay. Cells were first treated with hispidulin, followed by the addition of oleic acid two hours later. The cells were incubated for 24 hours to induce lipolysis. The intracellular lipids were demonstrated both qualitatively and quantitatively using Oil Red O staining. Triglyceride and total cholesterol levels, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, and adenosine monophosphate-activated protein kinase (AMPK) and sirtuin 1 (SIRT1) levels were measured.
Results: Hispidulin at 40 µM significantly reduced triglyceride levels by 67%, total cholesterol levels by 53%, ALT levels by 66%, and AST levels by 36%. However, no increase in AMPK or SIRT1 levels was observed compared to the model group.
Conclusion: Hispidulin can reduce cellular lipid accumulation, improve lipid parameters, and lower aminotransferase enzyme levels in MASLD. However, this effect may not occur via the AMPK-SIRT1 pathway but rather through other mechanisms. Further studies are needed to elucidate the mechanisms of hispidulin's action in MASLD.
Keywords:. Hepatic steatosis; Cell Culture; Hispidulin; Oil Red O; AMPK; SIRT1

Öz

Amaç: Metabolik ilişkili steatotik karaciğer hastalığı (MASLD) önemli bir sağlık sorunudur. Patogenezi hala net olmasa da insülin direnci, steatoz, inflamasyonun önemli yeri vardır. Tedavi için alternatif ajan çalışmaları devam etmektedir. Bu çalışma, bir flavonoid olan hispidulinin MASLD modelindeki etkisini inceleyen ilk çalışmadır.
Gereç ve Yöntem: MTT sitotoksisite testi ile hispidulin ve oleik asidin toksik olmayan konsantrasyonları belirlendi. Hücrelere önce hispidulin uygulandı, 2 saat sonra ise oleik asit verildi. Lipogenez için hücreler 24 saat inkübe edildi. Oil Red O boyama yöntemi kullanılarak hücre içi lipitler, hem nitel hem de nicel olarak gösterildi. Trigliserid ve Total kolesterol düzeyleri, Alanin aminotransferaz (ALT) ve aspartat aminotransferaz (AST) düzeyleri ve Adenozin monofosfat-aktif protein kinaz (AMPK) ve Sirtuin 1 (SIRT1) seviyeleri ölçüldü.
Bulgular: 40 µM Hispidulin grubunda model grubuna kıyasla; trigliserid seviyesini %67, total kolesterol seviyesini %53, ALT seviyesini %66, AST seviyesini %36 oranında anlamlı ölçüde azalttı. Ancak AMPK ve SIRT1 seviyelerinde artış görülmedi.
Sonuç: Hispidulinin MASLD' da hücresel lipid birikimini azaltıp, lipid parametrelerini iyileştirebileceği ve aminotransferaz enzim seviyelerini azaltabileceği belirlenmiştir. Fakat bu etkinin AMPK-SIRT1 yolu üzerinden değil de başka yolaklar üzerinden olabileceği düşünülmektedir. Hispidulinin MASLD' daki etki mekanizmalarını belirlemek için daha ileri araştırmalara ihtiyaç vardır.

Anahtar kelimeler: Hepatik steatoz, hücre kültürü, hispidulin, Oil Red O, AMPK, SIRT1

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a prevalent condition that was renamed to Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) last year. This change was made because the term NAFLD was considered stigmatizing and did not accurately reflect the nature of the disease[1,2]. MASLD is associated with obesity, insulin resistance, type 2 diabetes mellitus, and metabolic syndrome, and its prevalence is steadily increasing. It is predicted to become the leading cause of liver transplantation by 2030[3,4]. Experimental studies have demonstrated potential benefits from drugs such as glucagon-like peptide-1 receptor agonists, metformin, THR-β agonists, PPAR agonists, and FXR agonists[5]. While Phase 3 trials of various compounds and drugs were ongoing, resmetirom became the first drug approved by the U.S. Food and Drug Administration (FDA) for this condition[6,7]. MASLD is characterized by the accumulation of hepatic triglycerides (TG) and cholesterol[8], with de novo lipogenesis identified as the primary source of this accumulated lipid. While de novo lipid synthesis does not exceed 5% in healthy individuals, this rate has been reported to reach 26% in MASLD patients[9]. The buildup of lipids triggers inflammation within hepatocytes[10], playing a crucial role in the development of MASLD and contributing to insulin resistance, elevated lipid levels, and macrophage infiltration. This inflammation is also linked to fibrosis, leading to parenchymal damage[1]. Alanine and aspartate aminotransferase (ALT, AST) enzyme levels increase as a result of parenchymal damage. While ALT is a liver-specific enzyme, AST is also released from extrahepatic tissues. Studies have shown that AST levels in MASLD patients are associated with disease progression[11].

Due to the role of de novo lipogenesis in the pathophysiology of MASLD, the AMP-activating Protein Kinase (AMPK) and Sirtuin 1 (SIRT1) pathway, known to be effective in de novo lipogenesis, has been investigated[12]. Studies have shown that AMPK and SIRT1 levels have significantly decreased in MASLD patients and increase again with the recovery of MASLD[13-15]. AMPK and SIRT1 are two important interrelated molecules involved in hepatic lipid metabolism[16]. SIRT1, also known as the NAD-dependent deacetylase Sirtuin 1, regulates protein activation through the deacetylation of several proteins that play significant roles in the pathophysiology of metabolic diseases. SIRT1 activates AMPK, an essential energy sensor in the cell, which is activated when the cell's energy demand increases[14,15]. AMPK activation suppresses fatty acid synthesis by inhibiting Acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), while also reducing cholesterol synthesis through the inhibition of HMG-CoA reductase. As a result, lipogenesis decreases, and beta-oxidation increases[17-20].

Hispidulin is a natural flavonoid used in traditional Chinese medicine and is found in Saussurea involucrata, Grindelia argentina, Arrabidaea chica, Crossostephium chinense and various Salvia species. It is one of the main components of Anatolian sage (Salvia fruticosa), which grows in Bodrum and Marmara Island in Turkey[21]. Hispidulin is a flavonoid that inhibits CYP2E1 activity[15,21], which has been implicated in the pathogenesis of MASLD[22,23]. Hispidulin has antioxidant and anti-inflammatory[18,24] effects. It has also been shown to have antiadipogenic and hepatoprotective effects and is a PPARα agonist like fibrates used in the treatment of dyslipidemia[25-27].

In this study, we tested the hypothesis that hispidulin - a natural flavonoid reported to be a PPARα agonist with anti-adipogenic, anti-inflammatory, and hepatoprotective effects - could ameliorate MASLD through the AMPK-SIRT1 pathway in an oleic acid-induced MASLD model in the HepG2 cell line. The study found that hispidulin was effective in MASLD, improving aminotransferase enzyme levels and lipid parameters. It was concluded that hispidulin is a potential therapeutic candidate for MASLD, though not through the AMPK-SIRT1 pathway.

MATERIALS AND METHODS

All experiments in this study were carried out in the faculty of medicine of Gaziantep University in the laboratories of the departments of Medical Biochemistry and Physiology. Since the HepG2 human hepatoma cell line used in this study was commercially available, ethics committee approval was not required. Hispidulin and oleic acid (OA) were purchased from Cayman (USA). Dulbecco's Modified Eagle's Medium (DMEM) and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) were obtained from Sigma (USA). Fetal Bovine Serum (FBS) and Bovine Serum Albumin (BSA) were purchased from Hyclone (USA). Penicillin-streptomycin-amphotericin B was sourced from Gibco (USA). The Oil Red O staining kit was obtained from Abcam (UK). Triglycerides (TG) and Total Cholesterol (TC) kits were purchased from Elabscience (USA). ALT (Alanine Aminotransferase), AST (Aspartate Aminotransferase), AMPK (Adenosine Monophosphate-activated Protein Kinase) and Sirtuin 1 (SIRT1) kits were sourced from FineTest (China).

Cell culture, preparation of treatment and creation of the MASLD model

Cells were maintained in a medium containing 10% FBS and 1% antibiotic-antimycotic in an incubator at 37°C with 95% humidity. The medium was changed 2-3 times a week, and cells were passaged when 80-90% confluency was reached [28]. Hispidulin was dissolved in Dimethyl sulfoxide (DMSO) to prepare a stock solution, and dilution was performed with PBS before the experiment. OA was prepared as a stock solution by dissolving it in ethanol and diluted with medium before the experiment until the final ethanol concentration was below 0.2% [29]. The treatment groups were pretreated with 20 and 40 μM hispidulin. After 2 hours, the control group was treated with BSA, while the model and treatment groups were treated with BSA and oleic acid. All cells were then incubated for 24 hours. The study consisted of three independent experiments, each with a sample size of n=3.

Increased plasma levels of free fatty acids, such as oleic acid and palmitic acid, have been reported in MASLD[30]. Therefore, oleic acid and palmitic acid are frequently used separately or in combination in free fatty acid-induced in vitro MASLD models. Since oleic acid is more steatogenic and less apoptogenic than palmitic acid[31], it was preferred in this study[32]. According to the literature, oleic acid concentrations ranging from 0.25 mM to 1.25 mM are not cytotoxic to the HepG2 cell line over a 24-hour period[33]. In our study, a 0.5 mM oleic acid concentration was used, which was found to be non-toxic and effective in preliminary experiments. Additionally, a high glucose (25 mM) medium was used because it has been shown that lipid accumulation has significantly increased when high glucose medium is used with free fatty acids in adiposity models, compared to normal or low glucose medium.

Cell viability assay

For the MTT cytotoxicity assay, cells were seeded in a 24-well plate at a density of $1.25x10^5$ cells per well and allowed to adhere. Hispidulin was then added at concentrations of 10 μM, 20 μM, and 40 μM. After 2 hours, 0.5 mM OA and BSA were added to the wells, and the cells were incubated for 24 hours. Following incubation, 9 mg of MTT was dissolved in 2.3 ml PBS, and 100 μl of the MTT solution was added to each well. After a 4-hour incubation, DMSO was added, and absorbance was measured at 570 nm.

Preliminary experiments using Oil Red O (ORO) staining and triglyceride (TG) level measurements were conducted to determine the concentrations at which the MASLD pattern was induced and the treatment response was observed. A concentration of 10 μM hispidulin was excluded due to its ineffectiveness. OA was found to be effective and non-toxic at a concentration of 0.5 mM. Based on these findings, four experimental groups were established: Control, OA, hisp20 + OA, and hisp40 + OA. The group treated with OA alone was referred to as the model group.

Oil red O staining

Cells were seeded in 24-well plates at a density of $1.25x10^5$ cells/well. When the cells reached 80% confluence, they were treated with hispidulin, followed by OA 2 hours later, and then incubated for 24 hours. The medium was removed, and staining was performed using the ORO staining kit (Abcam, ab150678, Cambridge, UK) as described in reference[34]. Briefly, the cells were washed with PBS, fixed with 4% paraformaldehyde, incubated with propylene glycol, stained with ORO, washed with propylene glycol and then water, and photographed with a 40x objective after drying. For absorbance measurement, 0.3 ml of 100% isopropanol was added to each well, and 100 μl was taken from each well, then transferred to a 96-well plate, and the absorbance was read at 490 nm using a microplate reader.

Quantification of intracellular triglyceride and cholesterol levels

Each flask was seeded with 2.5×10^6 cells and incubated for 2 days to reach 80% confluence. Hispidulin was then added, and 2 hours later, OA was added and incubated for 24 hours. At the end of the 24-hour incubation, the cells were removed with PBS and transferred to Eppendorf tubes. The tubes were centrifuged at 1000×g for 10 minutes. The supernatant was discarded, and 450 µl of isopropanol was added and vortexed. After centrifugation at 10,000×g for 10 minutes, the supernatant was removed and stored at -80°C until measurement. TG and TC measurements were performed using a commercial kit. For TG measurement (Elabscience-ELK8169, Texas, USA), absorbance was read at 510 nm on a microplate reader. For TC measurement (Elabscience-ELK8420, Texas, USA), absorbance was measured at 510 nm using a spectrophotometer. Results were normalized to protein concentration and expressed as milligrams of TG/TC per milligram of protein.

Enzyme - linked immunosorbent assay

Samples stored at -80°C were thawed at room temperature. The sandwich Enzyme-Linked Immunosorbent Assay (ELISA) method was performed to measure ALT (FineTest-EH0770, Wuhan, China), AST (FineTest-EH2671, Wuhan, China), AMPK (FineTest-EH2622, Wuhan, China), and SIRT1 (FineTest-EH3785, Wuhan, China). Absorbance was read at 450 nm. The results were normalized to protein concentration and expressed as nanograms of ALT/AST/AMPK/SIRT1 per milligram of protein.

Statistical analysis

SPSS software was used for statistical analysis. The homogeneity of variances was assessed; homogeneous variables (MTT, TG, ORO, ALT) were evaluated using one-way ANOVA and post hoc Tukey tests, while non-homogeneous variables (TC, AST, AMPK, SIRT1) were evaluated using one-way ANOVA and post hoc Tamhane tests. The results are expressed as mean ± standard deviation (SD). A significance level of $p < 0.05$ was considered statistically significant.

RESULTS

When cells were exposed to hispidulin at concentrations of 20 µM and 40 µM, as well as OA at 0.5 mM for 24 hours, no significant decrease in cell viability was observed (Figure 1).

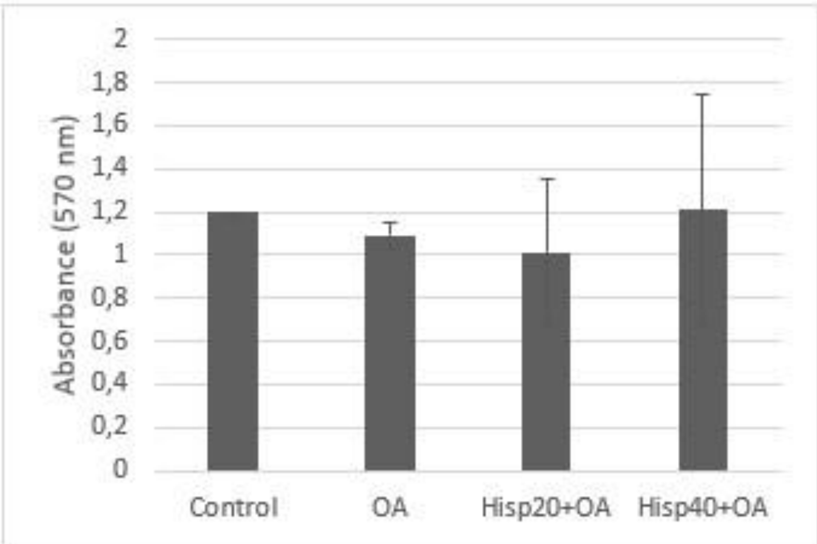


Figure 1. Effect of hispidulin on HepG2 cell viability. After treating HepG2 cells with hispidulin and oleic acid for 24 hours, MTT cytotoxicity testing was performed.

Values are expressed as mean ± SD from three different experiments. Hisp: Hispidulin, OA: Oleic acid.

The 40 µM hispidulin group showed a significant decrease of 67% in TG levels and 53% in TC levels compared to the model group ($p < 0.05$) (Figure 2). Furthermore, treatment with 20 µM and 40 µM hispidulin reduced intracellular lipid accumulation, as shown by ORO staining, by 47% and 42%, respectively, compared to the model group ($p < 0.05$) (Figure 3).

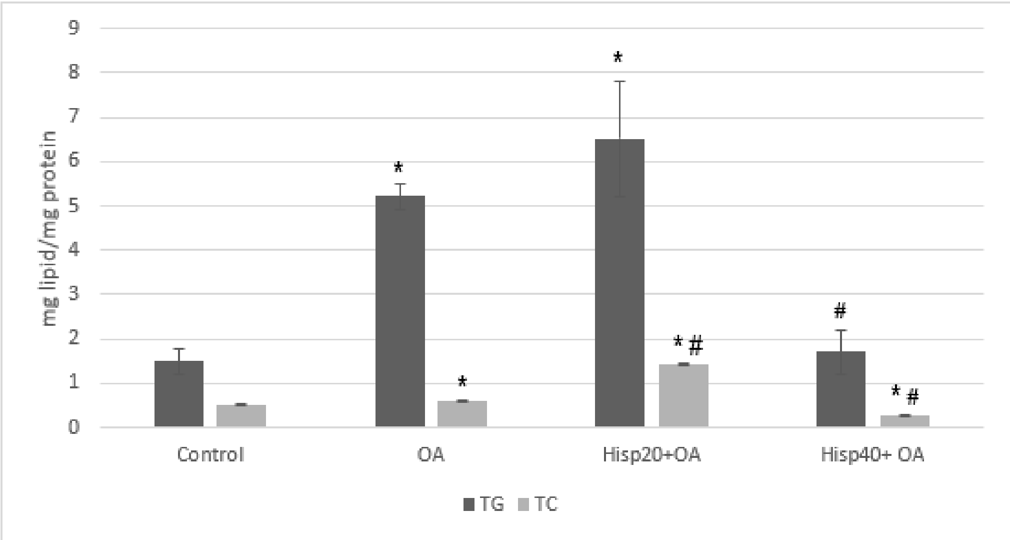
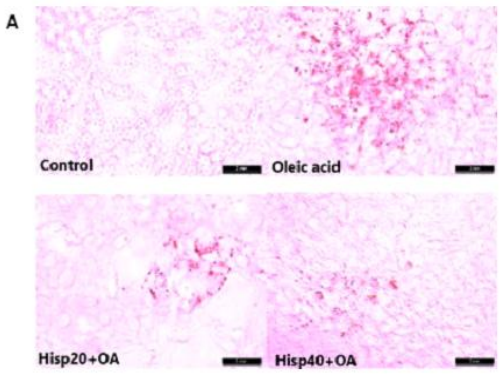


Figure 2. TG and TC Results.

Values are expressed as mean ± SD from three different experiments. *p< 0.05 denotes significance compared to the control group, # p < 0.05 denotes significance compared to the oleic acid group. Hisp: Hispidulin, OA: Oleic acid.



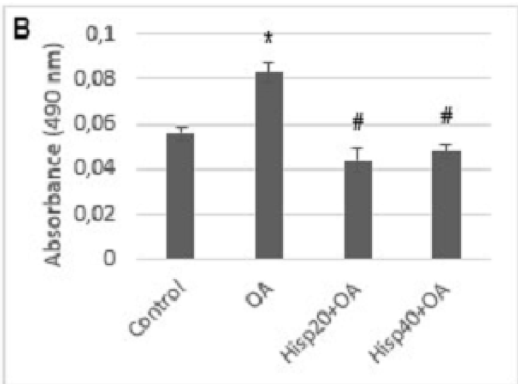


Figure 3. Oil Red O Staining Results. A. Oil red O staining images were taken under an inverted microscope with a 40x objective. Arrows indicate oil drops. Scale bar in the lower right corners of the figures shows the value of 2 mm. B. Oil Red O staining absorbance results.

Values are expressed as mean ± SD from three different experiments. *p< 0.05 denotes significance relative to the control group, # p< 0.05 denotes significance relative to the oleic acid group. Hisp: Hispidulin, OA: Oleic acid.

The ALT enzyme level was significantly decreased by 55% in the 20 μM hispidulin group and by 66% in the 40 μM hispidulin group compared to the model group ($p < 0.05$). The AST enzyme level was not significantly decreased in the 20 μM hispidulin group but was reduced by 36% in the 40 μM hispidulin group compared to the model group ($p < 0.05$) (Figure 4).

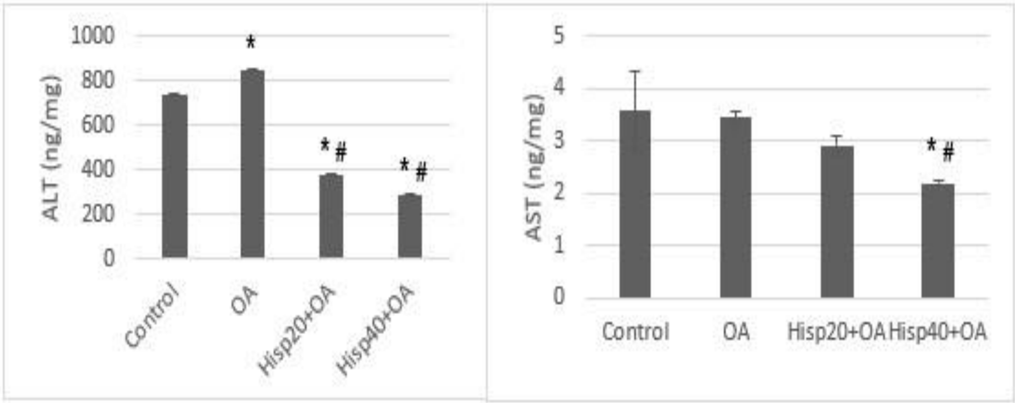


Figure 4. ALT and AST Results.

Values are expressed as mean ± SD from three different experiments. *$p < 0.05$ denotes significance relative to the control group, # $p < 0.05$ denotes significance relative to the oleic acid group. Hisp: Hispidulin, OA: Oleic acid.

In the 20 µM hispidulin group, AMPK levels increased compared to the model group ($p < 0.05$). No significant difference was observed in the 40 µM hispidulin group. Regarding SIRT1 levels, no significant difference was found in the 20 µM hispidulin group compared to the model group, whereas a decrease was detected in the 40 µM hispidulin group ($p < 0.05$) (Figure 5).

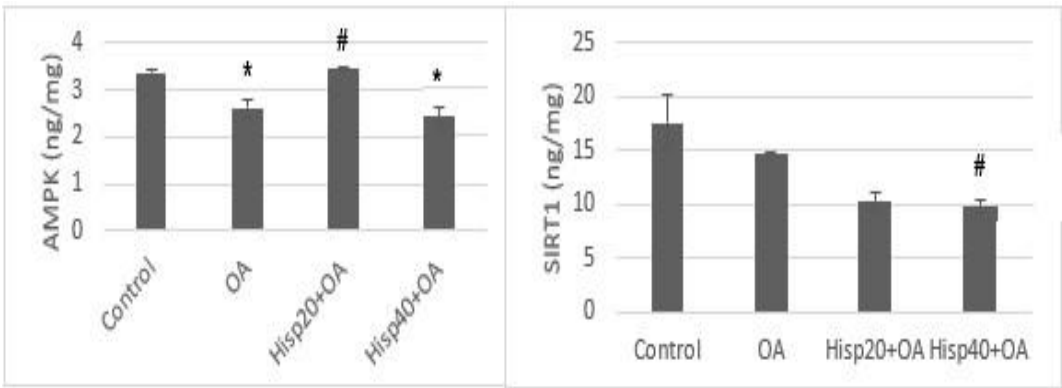


Figure 5. AMPK and SIRT1 results.

Values are expressed as mean ± SD from three different experiments. *$p < 0.05$ denotes significance relative to the control group, # $p < 0.05$ denotes significance relative to the oleic acid group. Hisp: Hispidulin, OA: Oleic acid.

DISCUSSION

In our study, compared to the model group, the hispidulin 40 µM group showed a decrease in intracellular lipid content, TG and TC levels, ALT and AST levels in the HepG2 cell line, where the MASLD model was induced with oleic acid, indicating the therapeutic effect of hispidulin.

The HepG2 cell line is a well-characterized cancer cell line that retains many functions of healthy hepatocytes, including cholesterol, triglyceride, lipoprotein, and glycogen metabolism[35], and it has been used for many years in MASLD models[36]. There are similar studies in the literature where the HepG2 cell line was used alone without a healthy cell line[37].

In the literature, two studies were found in which hispidulin was applied to the HepG2 cell line. In these studies, hispidulin was applied at concentrations between 50-200 µM for 24, 48, and 72 hours, and it was found to be significantly cytotoxic at concentrations of 50 µM and above within 24 hours. A study using a different cell line, 3T3-L1 preadipocyte cells, showed that hispidulin did not affect cell viability when applied at concentrations of 10, 20, and 40 µM for 24 hours[26]. In our study, consistent with the literature, no significant toxicity was observed at concentrations of 20 µM and 40 µM within 24 hours.

In a study where hispidulin was administered alone or in combination with p-synephrine to investigate its antiadipogenic effect in 3T3-L1 preadipocytes, it was found that hispidulin inhibited adipocyte differentiation and significantly reduced lipid accumulation in ORO staining at concentrations of 20 µM and 40 µM[38]. Similarly, in our study, hispidulin significantly reduced intracellular lipid accumulation in ORO staining at concentrations of 20 µM and 40 µM. The 40 µM hispidulin group also significantly reduced TG and TC levels. Hispidulin demonstrated antiadipogenic effects consistent with the literature and reduced lipid levels.

Hispidulin combined with synephrine, octopamine, and HCl was reported to reduce body weight and decrease TC and ALT levels in mice fed a high-fat diet[39]. In another study, using a bromobenzene-induced hepatotoxicity model in mice, hispidulin inhibited lipid peroxidation significantly lowered ALT levels, and reduced hepatotoxicity was observed[27]. ALT and AST do not always increase together in model groups. In a similar study examining the effect of oleic acid and chicoric acid in modeling MASLD in HepG2 cells, AST levels increased significantly in the OA group compared to the control group, while ALT levels did not increase[33]. In another study where HepG2 cells were exposed to 0.1 mM OA for 24 hours to induce lipid accumulation, the TG, TC, ALT, and AST levels were significantly increased in the OA group compared to the control group. When Alpha-naphthoflavone was administered as treatment and incubated for another 24 hours, the TG, TC, ALT, and AST levels significantly decreased in the treatment group compared to the OA group[36]. ALT and AST levels are variable in MASLD, and their elevation does not rule out the disease. ALT is specific to the liver and is elevated in about one in three patients with MASLD, whereas AST is an enzyme released from other tissues, and its elevation has been associated with the histopathologic progression of the disease[11,40,41]. In this study, ALT levels were significantly increased in the OA group compared to the control group, while AST levels did not show a significant increase. However, a significant decrease in both ALT and AST levels was observed in the 40 µM hispidulin group compared to the OA group, and these results are consistent with similar studies. Hispidulin significantly decreased ALT and AST enzyme levels compared to the model group, demonstrating hepatoprotective effects consistent with the literature.

In the literature, in vitro models of MASLD, AMPK and SIRT1 levels were usually significantly decreased in the model group compared to the control group and significantly increased again with treatment. In mice fed a high-fat diet supplemented with Salvia-Nelumbinis extract, hepatic SIRT1 and AMPK levels gradually decreased in the model group. SIRT1 was significantly increased in the model group compared to the control group at week 4, but it decreased significantly from week 12 onward. A significant decrease in AMPK activity was observed in the model group at week 16. AMPK and SIRT1 levels increased again with Salvia-Nelumbinis treatment[42]. However, there are also studies where AMPK and SIRT1 were not significantly reduced together. In a study where an oleic acid-induced MASLD model was used in HepG2 cells, no significant decrease in AMPK and SIRT1 levels was observed in the model group compared to the control group. However, AMPK was activated, and SIRT1 levels increased with Ginkolide C treatment [37]. Studies investigating the relationship between hispidulin and AMPK reported that hispidulin may activate AMPK[43-48]. In a study where GBM8401 and GBM8901 cell lines were treated with 40 µM and 60 µM hispidulin for 48 hours, respectively, it was shown that hispidulin activated AMPK, suppressed FAS and ACC, and decreased lipid synthesis. In the study, AMPK activation was evident for 48 hours[49]. AMPK needs to phosphorylate the ACC enzyme to exert its lipid-lowering effects, and this phosphorylation may take time[50]. In this study, a significant decrease in AMPK levels was observed in the model group compared to the control group, which is consistent with the literature. Hispidulin treatment at 20 µM and 40 µM decreased TG and TC levels but did not increase AMPK levels at 40 µM compared to the model group. The fact that hispidulin significantly increased

AMPK levels at 20 μM but did not decrease TG levels may be related to the insufficient time required to observe the effect of AMPK increase on lipid levels.

In this study, no significant increase in SIRT1 levels was observed at 20 μM hispidulin, while SIRT1 levels significantly decreased in the 40 μM hispidulin group. Similar to studies in the literature, it is noteworthy that SIRT1 levels were expected to increase but instead decreased in the treatment group. The catalytic activity of SIRT1 is regulated by nutritional, hormonal, and environmental factors that can alter cellular levels of NAD+, and the level and activity of SIRT1 protein may not always be parallel. Therefore, it has been reported that not only the protein level of SIRT1 but also its activity should be considered [51]. The fact that hispidulin at 40 μM, which was effective in this study, did not increase SIRT1 levels may be attributed to the measurement of protein expression. The lack of examination of SIRT1 activity is a limitation of this study. On the other hand, the significant reduction of lipid accumulation by 40 μM hispidulin, despite the decrease in SIRT1 levels, may indicate a specific effect of hispidulin on HepG2 cells or that hispidulin may have inhibited lipogenesis via another pathway.

Although the absence of a healthy cell group is a limitation, similar studies can be found in the literature. Since there was no FDA-approved treatment for MASLD at the time of this study, no positive control was used. Hispidulin also has anti-inflammatory effects, and the inability to analyze inflammatory parameters is a limitation of this study. Additionally, SIRT1 activity could not be measured.

In conclusion, this study established a MASLD model by exposing the HepG2 cell line to oleic acid for 24 hours. Oil Red O staining, along with TG and TC measurements, indicated that hispidulin had a therapeutic effect on MASLD by improving lipid parameters and decreasing ALT and AST levels. However, this effect was not mediated through AMPK and SIRT1. SIRT1 may not have been able to activate AMPK. Contrary to expectations, however, the SIRT1 level decreased significantly at a 40 μM hispidulin concentration, despite significant improvement in lipid parameters. These results suggest that hispidulin may have acted through a pathway other than the SIRT1-AMPK pathway. In one study, it was reported in silico that hispidulin may also act via the NF-κB and CYP450 enzymes in MASLD and provide a hepatoprotective effect [52]. Given the anti-inflammatory effects of hispidulin, it is necessary to conduct a study using a MASLD model in experimental animals to evaluate the systemic effects of hispidulin and to determine whether it exerts its effects through the nuclear factor-kappa B (NF-κB) pathway.

Author Contributions: Concept/Design : BA, HU; Data acquisition: BA, HU, MT; Data analysis and interpretation: BA, DSK; Drafting manuscript: BA; Critical revision of manuscript: DSK; Final approval and accountability: BA, DSK, HU, MT; Technical or material support: -; Supervision: DSK; Securing funding (if available): n/a.
Ethical Approval: As the study is conducted on cell lines, there is no need for ethical clearance.
Peer-review: Externally peer-reviewed.
Conflict of Interest: Authors declared no conflict of interest.
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