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The Improvement Effect of Magnolia officinalis Oil Extract on Non-alcoholic Fatty Liver Disease and Its Mechanism of Regulating Inflammatory Pathways and Oxidative Stress in HepG2 cells

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Abstract

Background: Non-alcoholic fatty liver disease (NAFLD) is a prevalent metabolic disorder characterized by lipid accumulation and hepatic inflammation. Magnolia officinalis, a traditional herbal medicine, has shown potential therapeutic effects in various metabolic conditions. However, its mechanisms of action in NAFLD remain unclear. **Methods:** HepG2 cells were treated with oleic and palmitic acid to establish an NAFLD model and then exposed to water, ethanol, or ethyl acetate extracts of Cortex Magnoliae Officinalis at varying concentrations for 6 or 24 hours. The SIRT1/AMPK pathway was investigated using shRNA knockdown and plasmid overexpression, with transfection efficiency confirmed via RT-qPCR and fluorescence microscopy. Gene expression, cell viability, and cytokine levels were analyzed using RT-qPCR, CCK-8 assay, and ELISA, respectively, with statistical analyses to evaluate significance. **Results:** Our findings suggest that Magnolia officinalis ethanol extracts activate the SIRT1/AMPK pathway, leading to improved lipid metabolism and reduced oxidative stress and hepatic inflammation. These effects appear to modulate pro-inflammatory cytokines and alleviate metabolic dysregulation associated with NAFLD. **Conclusions:** Magnolia officinalis extracts exhibit promising therapeutic potential in NAFLD management by targeting the SIRT1/AMPK pathway and reducing inflammation. Further in vivo studies are required to confirm these findings, investigate long-term safety and bioavailability, and explore synergistic effects with other therapeutic compounds. This research bridges traditional natural medicine with advanced scientific methods, providing novel insights into NAFLD treatment.

Keywords

Non-alcoholic Fatty Liver Disease (NAFLD); Magnolia Officinalis; SIRT1/AMPK Pathway; Lipid Metabolism

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a metabolic disorder characterized by the accumulation of excess fat in liver cells in individuals who consume little or no alcohol. It is the most common liver disorder worldwide, affecting

approximately 25% of the global population [1]. The mortality of NAFLD was 10% in the past decades [2]. The direct cost for patients with NAFLD is about 103 billion dollars. In average, each patient affords 1,613 dollars.

Magnolia officinalis, a traditional Chinese medicinal herb, has been studied for its potential therapeutic effects on hepatic ailments, including hepatoprotective and anti-inflammatory properties. Recent research has demonstrated that extracts from Magnolia officinalis can significantly reduce lipid accumulation in hepatocytes, suggesting a promising role in the treatment of hepatic steatosis in non-alcoholic fatty liver disease (NAFLD) [3]. Moreover, studies indicate that these extracts may modulate key metabolic pathways involved in NAFLD, potentially mitigating progression to more severe forms like non-alcoholic steatohepatitis (NASH) [4]. Magnolia Officinalis is a traditional Chinese medicine that is the root bark and branch bark of a plant. It mainly works by conditioning the liver to improve NAFLD.

The metabolic disturbances in NAFLD, including insulin resistance, increased free fatty acid (FFA) release, and enhanced triglyceride (TG) storage, are illustrated in Figure 1. Metabolic syndrome leads to elevated glucose and insulin levels, promoting FFA synthesis and storage. Insulin resistance exacerbates this process, contributing to oxidative stress, ketone body production, and the generation of toxic fatty acid metabolites. Key pathways, such as ChREBP and SREBP-1c, increase fatty acid synthesis, TG accumulation, and the formation of very low-density lipoproteins (VLDL). An imbalance between FA synthesis and lipophagy further aggravates NAFLD's metabolic dysfunction. NAFLD is characterized by excessive fat accumulation in the liver, known as hepatic steatosis, which can progress to non-alcoholic steatohepatitis (NASH), leading to inflammation and liver cell damage. Factors like oxidative stress, lipid toxicity, and inflammatory processes play a crucial role [4, 5]. The condition is closely associated with obesity and insulin resistance; when liver fat content surpasses 5%, it is classified as steatosis, a metabolic disorder [6, 7]. As NAFLD progresses, macrovesicular steatosis accumulates in liver cells, potentially resulting in cirrhosis, fibrosis, and eventually NASH, posing a significant economic and clinical burden [8, 9].

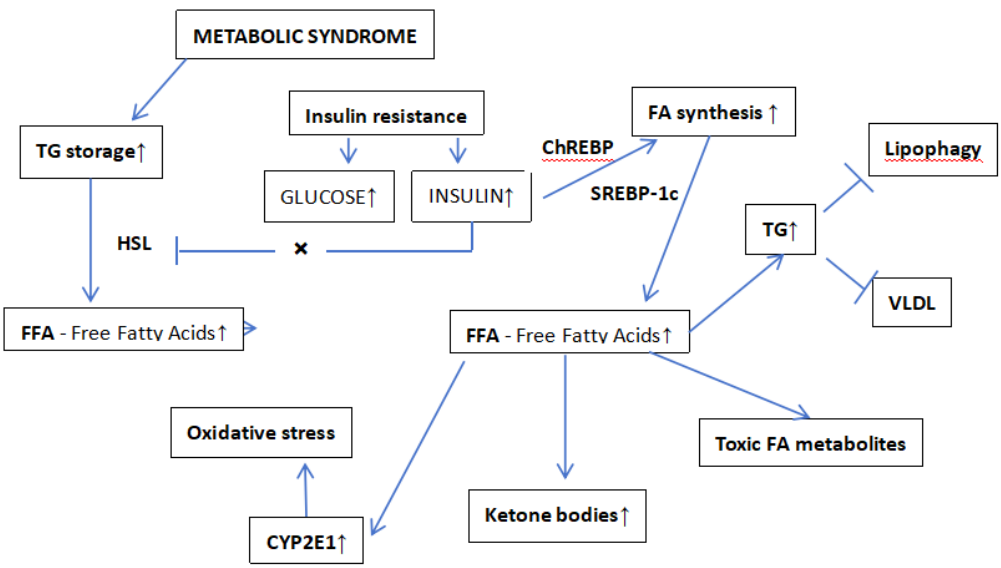

Figure 1. Pathophysiological Mechanisms Linking Metabolic Syndrome to NAFLD Progression Through Lipid Metabolism Dysregulation.

Non-alcoholic fatty liver disease (NAFLD) is a global health concern characterized by excessive fat accumulation in the liver, potentially progressing to non-alcoholic steatohepatitis (NASH) [10]. Oxidative stress and free radicals play a critical role in the pathogenesis of NAFLD, underscoring the need for effective treatments [11]. Due to the current lack of specific pharmacological options, research has shifted toward exploring natural compounds with multi-target therapeutic effects. Cortex Magnoliae Officinalis, known for its anti-inflammatory, antioxidant, and hepatoprotective effects, has shown potential in this regard [12, 13]. Extracts from Magnoliae Officinalis using water, ethanol, ethyl acetate, n-butanol, and petroleum ether were applied to a high-fat-induced HepG2 cell model to assess their efficacy in reducing lipid accumulation and metabolic dysfunctions related to NAFLD [14, 15]. The study focused on key biomarkers of lipid metabolism,

inflammation, and oxidative stress, exploring how the SIRT1/AMPK pathway is regulated, providing insights into the molecular mechanisms of NAFLD [16-18].

Our preliminary experiments suggest that Magnolia officinalis extracts may activate the SIRT1/AMPK pathway, potentially improving metabolic dysregulation and alleviating hepatic inflammation associated with NAFLD. By targeting this pathway, the extracts might offer a multi-target approach to managing NAFLD symptoms, including the modulation of lipid metabolism and oxidative stress. Based on these observations, we hypothesize that Magnolia officinalis extracts exert therapeutic effects on NAFLD primarily by modulating the SIRT1/AMPK pathway. The research bridges traditional natural medicine with advanced scientific methods, providing novel insights into NAFLD management [19, 20].

2. Methods and Materials

2.1 Cell Model and Treatment

HepG2 cells (purchased from ATCC, USA) were used to establish the NAFLD model through induction with a mixture of oleic acid (Sigma-Aldrich, USA) and palmitic acid (Sigma-Aldrich, USA) at a 2:1 ratio. The concentration range was set to 0.25–1 mM for a treatment duration of 24–72 hours. Different extracts of Cortex Magnoliae Officinalis were prepared using water (Thermo Fisher Scientific, USA), ethanol (Sigma-Aldrich, USA), and ethyl acetate (Merck, Germany). Cells were divided into different groups for treatment: a control group (untreated), an NAFLD model group, and groups treated with varying concentrations (10 μM, 50 μM) of each extract. The treatments lasted for either 6 or 24 hours, depending on the experimental requirements.

2.2 Cell Transfection

For the third experiment investigating the SIRT1/AMPK pathway, HepG2 cells were transfected with small hairpin RNA (shRNA) to silence specific genes (sh-SIRT1, sh-AMPK) and with plasmids to overexpress specific genes (p-SIRT1). The shRNA constructs for gene silencing were obtained from a commercial provider (e.g., Dharmacon, USA or GenePharma, China), while the plasmid for gene overexpression (p-SIRT1) was sourced from Addgene (USA). Transfection was carried out using Lipofectamine 3000 (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Negative control shRNAs (sh-NC) and control plasmids (p-NC) were included to ensure the specificity of gene silencing and overexpression effects. After transfection, cells were incubated for 24–48 hours before treatment with the extracts. To assess transfection efficiency, a parallel set of cells was transfected with a fluorescent control shRNA (e.g., shRNA-GFP) and visualized using fluorescence microscopy (Nikon, Japan). The efficiency was quantified by calculating the percentage of GFP-positive cells in random fields of view. Additionally, quantitative RT-PCR was used to measure the expression levels of target genes to confirm successful gene knockdown or overexpression before proceeding with further treatments.

2.3 RT-qPCR

Table 1. Primer sequences in this study

Gene	Sequence
CPT1	GCGCCCCTTGTTGGATGAT
	CCACCATGACTTGAGCACCAG
PPARα	ATGGTGGACACGGAAAGCC
	CGATGGATTGCGAAATCTCTTGG
Caspase-3	CATGGAAGCGAATCAATGGACT
	CTGTACCAGACCGAGATGTCA

Total RNA was extracted from treated HepG2 cells using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. RNA concentration and purity were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). cDNA synthesis was performed using the PrimeScript RT reagent kit (Takara Bio, Japan). Quantitative PCR was carried out using SYBR Green Master Mix (Applied Biosystems, USA) on a QuantStudio 5 Real-Time PCR system

(Thermo Fisher Scientific, USA). Target genes included CPT1, PPARα, caspase-3 with GAPDH serving as the internal control. Primer sequences were designed based on published data or obtained from primer banks (PrimerBank, USA). The PCR cycle conditions consisted of an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative gene expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method, normalized to GAPDH.

2.4 CCK-8 Assay

The CCK-8 assay (Dojindo Laboratories, Japan) was employed to assess cell viability and cytotoxicity. HepG2 cells were seeded in 96-well plates (Corning, USA) at a density of 5×10^3 cells/well and treated with different concentrations of Cortex Magnoliae Officinalis extracts for 6 or 24 hours. After treatment, 10 μL of CCK-8 solution was added to each well, followed by incubation for 2 hours at 37°C. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad, USA). The percentage of cell viability was calculated relative to the control group.

2.5 ELISA

The levels of inflammatory cytokines (TNF-α, IL-6) in cell culture supernatants were quantified using ELISA kits (Abcam, UK) according to the manufacturer's instructions. Briefly, supernatants were collected after the treatment period, centrifuged to remove debris, and added to ELISA plates (Nunc, Thermo Fisher Scientific, USA) coated with specific antibodies. The plates were incubated, washed, and treated with HRP-conjugated secondary antibodies, followed by the addition of TMB substrate (Thermo Fisher Scientific, USA) for color development. Absorbance was measured at 450 nm using a microplate reader (Bio-Rad, USA). Cytokine concentrations were calculated using standard curves generated from known concentrations of recombinant proteins (Abcam, UK).

2.6 Statistical Analysis

Data distribution was analyzed using the Shapiro-Wilk test. Measurement data were expressed as mean ± SD. Comparisons among three or more groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A p-value of <0.05 was considered statistically significant. All calculations were performed using SPSS 22.0 (IBM, USA) and GraphPad Prism 10.0 (GraphPad Software, USA).

3. Results

3.1 The Effects of Different Concentrations of Magnolia officinalis Extract on Induced NAFLD Cells

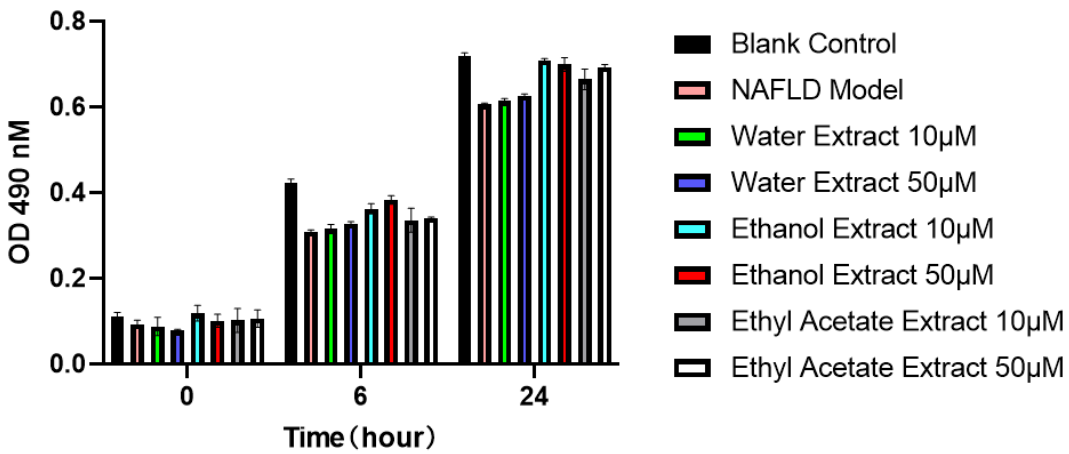

Figure 2. Effect of Different Extracts on Cell Viability in NAFLD Model Over 24 Hours as Measured by CCK-8 Assay.

Based on the results presented in the images, a two-way ANOVA analysis ($\alpha = 0.05$) was conducted to evaluate the effects of different extracts and time on the cell viability (measured as OD at 490 nm). The analysis showed that both the interaction between the factors, the row factor (time), and the column factor (treatment) were statistically significant ($P < 0.0001$).

The variation was primarily driven by the row factor (97.79% of total variation), indicating that time significantly impacted the observed effects. The bar graph illustrates that the Blank Control group consistently had the highest OD values at all time points (0, 6, and 24 hours). The NAFLD model exhibited reduced OD values, confirming decreased cell viability. Treatment with various extracts, including water, ethanol, and ethyl acetate, demonstrated differing effects on OD values. Notably, the ethanol extracts (both 10 μM and 50 μM) showed a more significant improvement in cell viability compared to the water and ethyl acetate extracts, particularly at the 24-hour mark.

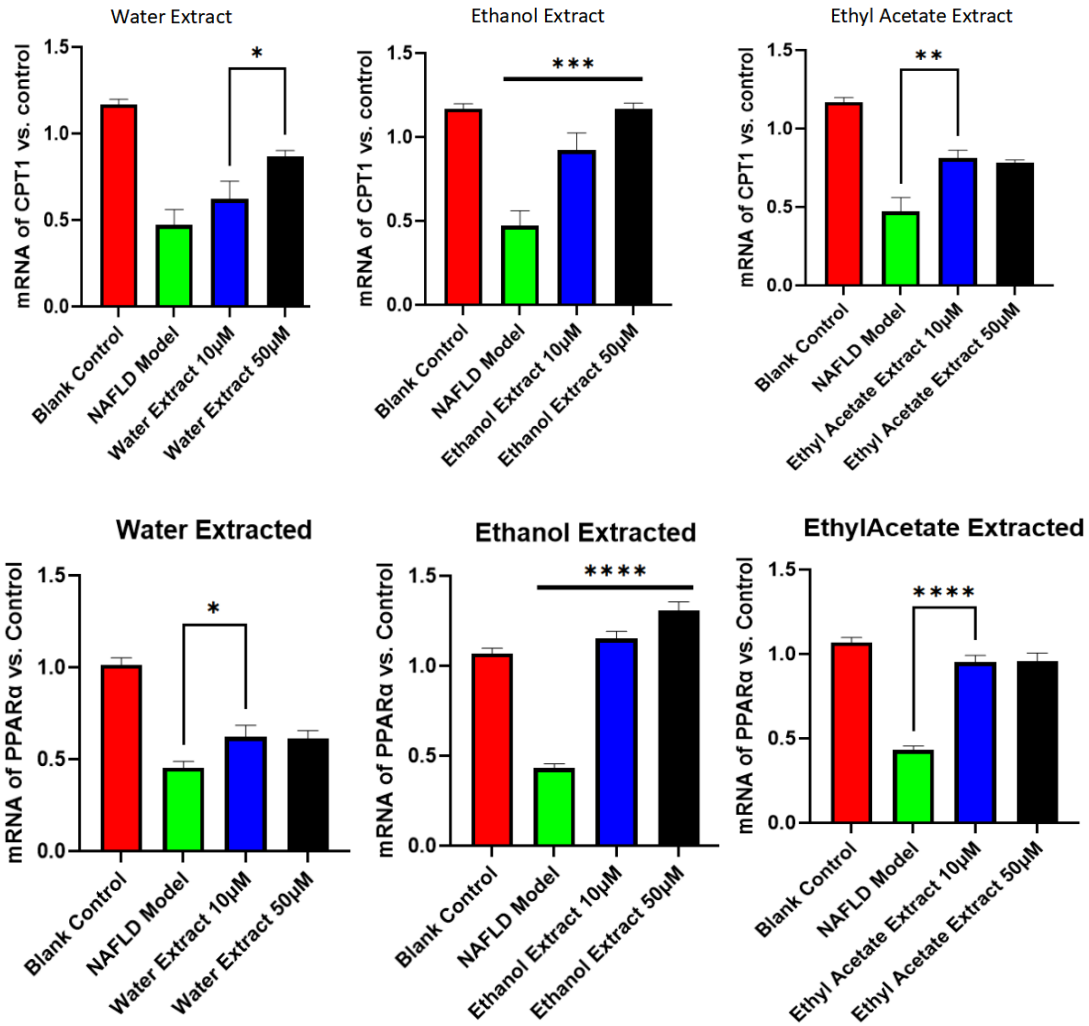

Figure 3. RT-qPCR results show the expression of CPT1, PPARα. Experiments are repeated in triplicate (n = 3). ***P<0.001, **P<0.01, *P<0.05.

The results in Figure 3 demonstrate the effects of different extracts (water, ethanol, and ethyl acetate) on the mRNA expression of CPT1 and PPARα in an NAFLD model. RT-qPCR analysis reveals that the Blank Control group exhibited the highest expression levels for both genes, while the NAFLD model significantly reduced these levels. Treatment with water extract led to a slight increase in gene expression, with a significant improvement at the 50 μM concentration for both CPT1 and PPARα (*P < 0.05). However, this effect was weaker than that of the other extracts. Ethanol extract notably increased gene expression at both 10 μM and 50 μM concentrations, with the 50 μM showing the most substantial effect (***P < 0.001). Ethyl acetate extract displayed a moderate effect, improving CPT1 and PPARα expression significantly more than the water extract (**P < 0.01) but less effectively than the ethanol extract. The raw data corroborate these findings, indicating that extract type and concentration critically influence gene expression.

3.2 The Dose-Dependent Inhibitory Effects of Different Magnolia officinalis Extracts on Apoptosis in NAFLD Model Cells

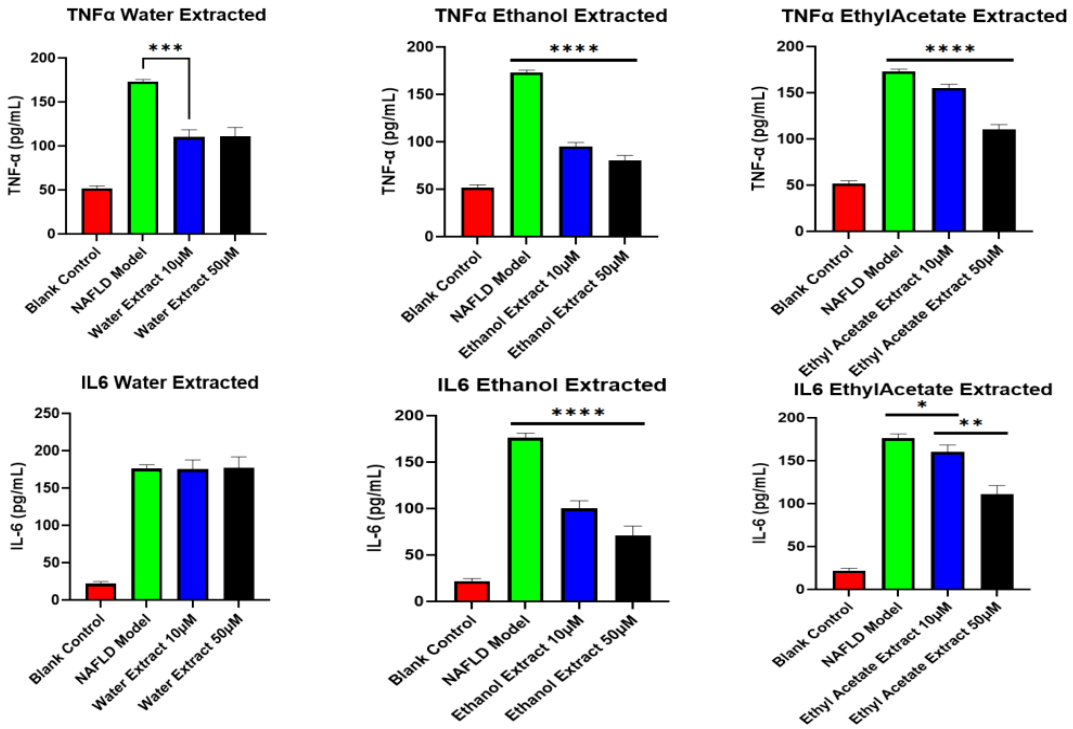

Figure 4. ELISA for levels of pro-inflammatory cytokines TNF-α and IL-6 after reoxygenation. Experiments are repeated in triplicate (n = 3). ***P<0.001, **P<0.01, *P<0.05.

Figure 4 shows the effects of water, ethanol, and ethyl acetate extracts on TNF-α and IL-6 levels after reoxygenation, measured by ELISA. The NAFLD model significantly increased both cytokines compared to the Blank Control (***P < 0.001). Water extract treatment led to a moderate reduction, but not statistically significant at higher concentrations. Ethanol extract, especially at 50 μM, significantly reduced both TNF-α and IL-6 levels (****P < 0.0001), indicating strong anti-inflammatory effects. Ethyl acetate extract also reduced cytokine levels (**P < 0.01) but was less effective than ethanol. Overall, ethanol extract showed the highest anti-inflammatory activity, followed by ethyl acetate, while water extract was the least effective.

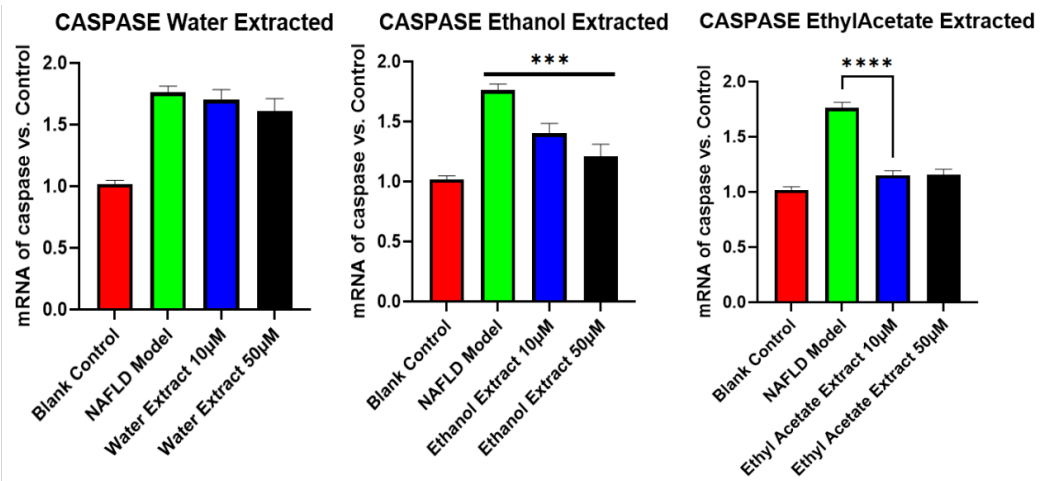

Figure 5. RT-qPCR results show the expression of CASPASE. Experiments are repeated in triplicate (n = 3). ***P<0.001, **P<0.01, *P<0.05.

Figure 5 shows the impact of different extracts on CASPASE mRNA expression in the NAFLD model. The NAFLD model significantly upregulated CASPASE expression compared to the Blank Control (***P < 0.001), indicating increased apoptosis. Ethanol extract, especially at 50 μM, significantly reduced CASPASE levels (***P < 0.001), demonstrating a strong anti-apoptotic effect. Ethyl acetate extract also reduced CASPASE expression, notably at 50 μM (**P < 0.01), but was less effective than ethanol. The water extract had minimal impact, showing no significant change. In conclusion, ethanol extract has the most potent anti-apoptotic effect, followed by ethyl acetate, while the water extract is the least effective.

3.3 Different Magnolia officinalis Extracts Improve NAFLD by Regulating the SIRT1/AMPK Pathway

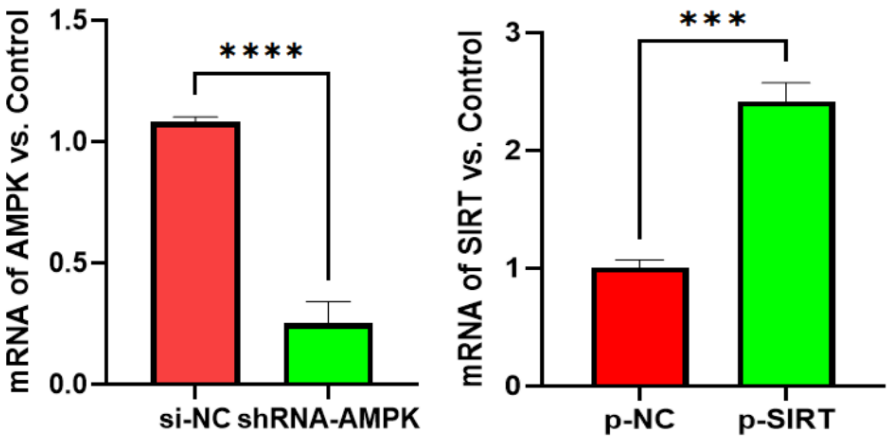

Figure 6. Transfection efficiency of siRNA and overexpression plasmid by RT-qPCR. ***P<0.001, **P<0.01.

Figure 6 illustrates the transfection efficiency of shRNA and overexpression plasmids in altering AMPK and SIRT expression levels, as measured by RT-qPCR. The results indicate that shRNA-AMPK significantly reduced AMPK mRNA expression compared to the si-NC control group (P < 0.001). Similarly, the overexpression plasmid (p-SIRT) resulted in a significant increase in SIRT mRNA levels compared to the p-NC control (P < 0.01). These findings confirm the successful transfection and modulation of target gene expression, validating their use in subsequent experiments.

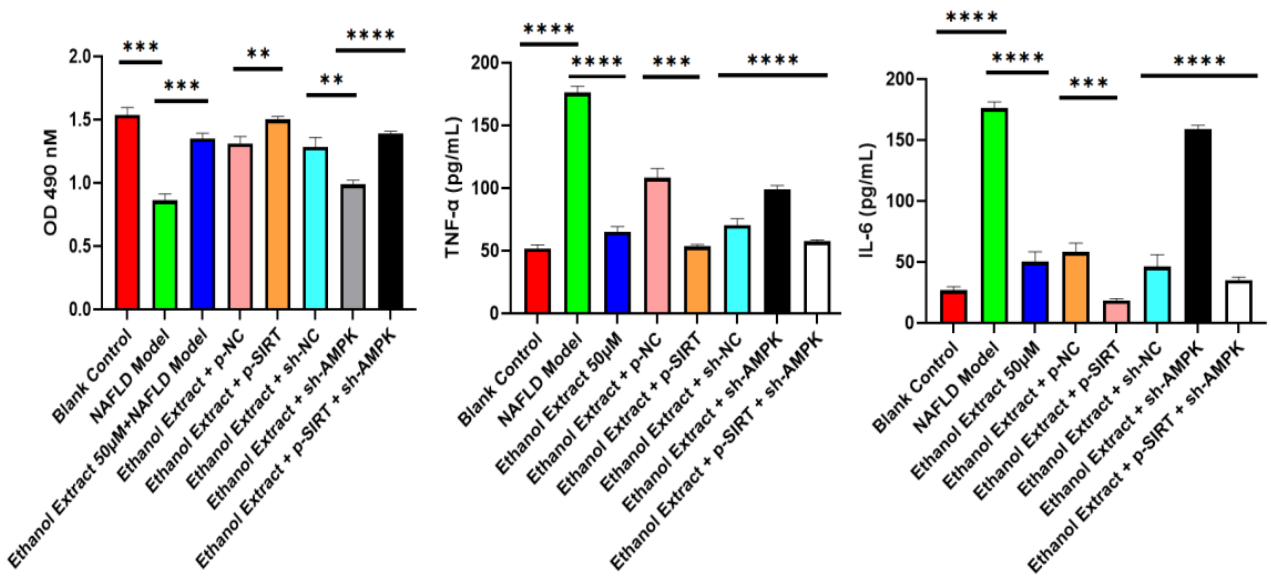

Figure 7. CCK-8 (A) for cell viability measured at 72 hours post-treatment following the establishment of the NAFLD model, and ELISA (B) for levels of pro-inflammatory cytokines TNF-α and IL-6 after reoxygenation. Experiments are repeated in triplicate (n = 3). ***P<0.001, **P<0.01, *P<0.05.

Figure 7 presents the results of cell viability (CCK-8) and cytokine levels (ELISA) in the NAFLD model treated with various ethanol extracts. The NAFLD model group showed significantly reduced cell viability compared to the Blank Control (**P < 0.01). Ethanol extract treatment at 50 μM notably improved cell viability (**P < 0.01). Further, p-SIRT overexpression significantly enhanced viability (****P < 0.0001), while sh-AMPK knockdown reduced it (****P < 0.0001). In terms of cytokine levels, the NAFLD model had a significant increase in both TNF-α and IL-6 (****P < 0.0001). Ethanol extract treatment reduced TNF-α and IL-6 levels, especially in the p-SIRT group. Combined treatment (p-SIRT + sh-AMPK) also decreased cytokine levels, although less effectively than p-SIRT alone. In conclusion, ethanol extract, particularly when combined with SIRT1 overexpression, effectively improves cell viability and reduces inflammation in the NAFLD model, suggesting its therapeutic potential.

4. Discussion

Non-alcoholic fatty liver disease (NAFLD) is increasingly recognized as a global health concern, affecting over a quarter of the adult population worldwide. It is characterized by excessive fat accumulation in the liver, unrelated to alcohol consumption, and is frequently associated with metabolic syndrome, obesity, and insulin resistance. NAFLD can progress to non-alcoholic steatohepatitis (NASH), cirrhosis, and even hepatocellular carcinoma, posing significant risks to patient health. The pathogenesis of NAFLD is multifactorial, with oxidative stress and chronic inflammation playing key roles in disease progression. Studies have shown that oxidative stress markers are elevated in NAFLD patients, correlating with the severity of the disease and its related cardiometabolic risks [21]. Despite the growing prevalence of NAFLD, effective therapeutic interventions remain limited, emphasizing the need for novel strategies to manage this condition.

In traditional Chinese medicine, Magnolia officinalis has been extensively utilized for its therapeutic properties. The plant's bark contains bioactive neolignans with anti-inflammatory, antioxidant, anti-anxiety, and antidepressant effects [22]. The therapeutic potential of Magnolia officinalis is harnessed through different solvent extractions, which yield distinct phytochemical compositions and biological activities. The solvent extracts, including ethanol, ethyl acetate, water, and n-butanol, have been shown to possess various pharmacological properties, making them promising candidates for NAFLD treatment. Specifically, ethanol extracts of Magnolia officinalis have demonstrated potent anti-inflammatory and antioxidant effects, which could counteract the oxidative stress and lipid metabolism dysregulation observed in NAFLD [23, 24]. Moreover, these extracts have been reported to modulate lipid metabolism by reducing triglyceride accumulation in hepatocytes, which is crucial in preventing the progression of NAFLD to NASH [25, 26]. However, while the potential benefits of Magnolia officinalis extracts are evident, the precise mechanisms through which they exert their effects, particularly in the context of NAFLD, are not fully understood.

This research addresses a critical gap in understanding how Magnolia officinalis solvent extracts, particularly ethanol extracts, regulate the molecular pathways involved in NAFLD. Previous studies have shown that NAFLD pathogenesis involves a complex interplay between metabolic, oxidative, and inflammatory processes. Yet, the exact role of key signaling pathways, such as the SIRT1/AMPK pathway, in response to herbal interventions remains underexplored. The SIRT1/AMPK pathway is a central regulator of cellular metabolism, influencing lipid oxidation, mitochondrial function, and insulin sensitivity. Dysregulation of this pathway is associated with the development and progression of NAFLD [27, 28]. Hence, investigating how Magnolia officinalis extracts interact with this pathway could provide insights into their therapeutic potential for NAFLD. Our study hypothesized that Magnolia officinalis extracts might improve NAFLD by modulating the SIRT1/AMPK pathway, thereby reducing oxidative stress, inflammation, and lipid accumulation in hepatic cells.

To explore this hypothesis, we examined the effects of Magnolia officinalis ethanol extracts on the SIRT1/AMPK pathway in an NAFLD model. Our findings reveal that the ethanol extract significantly activates SIRT1, leading to subsequent phosphorylation of AMPK. Activation of the SIRT1/AMPK pathway is known to enhance fatty acid oxidation, reduce lipid accumulation, and improve insulin sensitivity. This aligns with previous research indicating that Magnolia officinalis compounds can positively influence metabolic pathways in hepatic cells [29-31]. Additionally, the ethanol extract demonstrated a notable reduction in pro-inflammatory cytokines such as TNF-α and IL-6, which are often elevated in NAFLD, suggesting an anti-inflammatory effect. These observations support the premise that Magnolia officinalis extracts have multifaceted roles in managing NAFLD, potentially through mechanisms involving SIRT1/AMPK pathway modulation and suppression of oxidative stress markers [32].

In conclusion, our study advances the current understanding of NAFLD treatment by highlighting the therapeutic potential of Magnolia officinalis ethanol extracts. Our findings suggest that these extracts can effectively modulate the

SIRT1/AMPK pathway, thereby reducing hepatic lipid accumulation and inflammation. This research fills a critical knowledge gap regarding the molecular mechanisms underlying the efficacy of traditional Chinese medicine in NAFLD management. It also opens avenues for further investigation into the use of Magnolia officinalis as a complementary therapy for NAFLD. Future research should explore the long-term effects of these extracts in clinical settings and investigate their potential synergies with other dietary polyphenols, such as those found in cranberry [33-35]. By uncovering these molecular pathways, we move closer to developing targeted, effective treatments for NAFLD that are both natural and accessible.

5. Limitations and Future Plans

Despite promising findings on the therapeutic effects of Magnolia officinalis extracts in NAFLD, several limitations exist. First, while ethanol extracts were shown to modulate the SIRT1/AMPK pathway and reduce pro-inflammatory cytokines, other pathways such as mTOR and Nrf2 were not explored. Second, the study was conducted in vitro using HepG2 cells, which may not fully replicate the in vivo liver environment, necessitating further validation in animal models and clinical trials. Lastly, although different solvent extracts (e.g., ethyl acetate, water) were examined, the mechanisms underlying their effects remain unclear, requiring more detailed research to optimize their clinical application.

Future research should validate the in vitro findings of Magnolia officinalis extracts in NAFLD using animal models, focusing on liver histology, lipid metabolism, and oxidative stress. Exploring effects on pathways like mTOR and Nrf2 will provide a more holistic view. Investigating the long-term safety and bioavailability of different solvent extracts is essential. Additionally, combining these extracts with other compounds, such as polyphenols, could uncover synergistic effects. Lastly, analyzing their influence on gene expression could identify the most effective extracts and optimal dosages for therapeutic use.

6. Conclusion

In conclusion, Magnolia officinalis extracts, particularly ethanol extracts, show promising therapeutic potential in managing NAFLD by modulating the SIRT1/AMPK pathway and reducing pro-inflammatory cytokines. However, further in vivo studies are necessary to confirm these effects and explore other metabolic pathways involved in NAFLD. Additionally, investigating the long-term safety, bioavailability, and potential synergistic effects with other compounds will be crucial for optimizing their clinical application in NAFLD treatment.

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