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Wu-Mei-Wan retards NAFLD progression by ameliorating obesity and regulating serum metabolites in high-fat diet-fed mice

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Abstract: **Background**: Non-alcoholic fatty liver disease (NAFLD), closely associated with obesity and metabolic syndrome, lacks universally effective therapies. Wu-Mei-Wan (WMW), a traditional Chinese medicine formula, has been used to treat metabolic disorders, but its efficacy and mechanism against NAFLD remain unclear. **Objectives**: This study investigated the therapeutic potential of WMW in a high-fat diet (HFD)-induced NAFLD mouse model. **Methods**: After 24 weeks of HFD feeding, mice were allocated to control, model, low/middle/high-dose WMW (L-/M-/H-WMW), or metformin groups. Physiological parameters and liver pathology were assessed. Untargeted serum metabolomics was employed to identify altered metabolic pathways. **Results**: H-WMW treatment significantly alleviated NAFLD phenotypes, reducing body weight, hepatic steatosis, dyslipidemia, and insulin resistance. Metabolomic analysis identified vitamin B6 metabolism as a key target, with H-WMW restoring levels of pyridoxal 5'-phosphate and 4-pyridoxic acid to near-normal. Mechanistically, WMW coordinately modulated interconnected purine and vitamin B6 metabolic networks. **Conclusion**: WMW demonstrates significant efficacy against NAFLD, likely through multi-target reprogramming of hepatic metabolism. These findings position WMW as a promising phytopharmaceutical candidate worthy of further clinical investigation.

Keywords: Liver injury; NAFLD; Obesity; Serum metabolism; Wu-Mei-Wan

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INTRODUCTION

NAFLD is a chronic liver disorder characterized by excessive fat accumulation in the liver, commonly associated with obesity, diabetes, and dyslipidemia. The prevalence of NAFLD in the United States has risen sharply from 83.1 million cases in 2017 (~ 25% of the population) to an estimated 100.9 million by 2030. In China, the prevalence was about 15.0% (Kalligeros *et al.*, 2024; Teng *et al.*, 2023; Estes *et al.*, 2018; Hochreuter *et al.*, 2022). The incidence of NAFLD can be as high as 75% in individuals who are obese or have type 2 diabetes (T2DM) (Yun and Ko, 2021; Tilg *et al.*, 2017). The progression of NAFLD involves a detrimental cycle of hepatic lipid accumulation, oxidative stress, and inflammatory responses, which can lead to severe liver diseases. This condition often exacerbates oxidative stress and promotes systemic inflammation. Over the past decade, NAFLD has been recognized as a systemic disorder, presenting with various metabolic, cardiovascular, skin, and digestive symptoms, alongside advanced complications. The underlying mechanisms primarily involve insulin resistance, lipid metabolism disorders, and the release of inflammatory factors (Israelsen *et al.*, 2024; Younossi *et al.*, 2018).

Currently, the treatment of NAFLD primarily relies on pharmacological therapies and lifestyle modifications. Engaging in regular physical exercise and reducing the intake of high-sugar and high-fat foods have proven effective in decreasing hepatic fat accumulation and inflammation (Su *et al.*, 2023; Byrne and Targher, 2015). Pharmacological interventions focus on managing blood glucose and lipid levels, including the use of the farnesoid X receptor (FXR) agonist obeticholic acid, the glucagon-like peptide-1 (GLP-1) receptor agonist liraglutide, and statin-based lipid-lowering medications. A significant challenge in contemporary medicine is the absence of FDA-approved drugs specifically designed to treat NAFLD, highlighting the urgent need for innovative therapeutic options (Harrison *et al.*, 2023; Friedman *et al.*, 2018).

In recent years, traditional Chinese medicine (TCM), which primarily consists of herbal ingredients formulated into decoctions, has gained considerable attention from researchers for its natural, gentle, and holistic regulatory properties. Studies have shown that TCM can effectively alleviate NAFLD by improving liver function, decreasing lipid droplet accumulation, and modulating immune cell activity (Lan *et al.*, 2022; Chen *et al.*, 2021b). One notable TCM formula is WMW, composed of ten plants and derived from the ancient text *Treatise on Cold Damage Diseases* (Chinese: *Shanghanlun*), written by Zhongjing

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Zhang during the Eastern Han Dynasty (approximately 200-205 CE). With a history spanning over 2,000 years, WMW has been traditionally used to treat diarrhea and colitis. Recent evidence suggests that WMW may protect pancreatic beta cells and improve insulin sensitivity (Chen *et al.*, 2025; Yang *et al.*, 2019). However, the cellular and molecular pharmacological mechanisms by which WMW influences NAFLD remain insufficiently understood and warrant further exploration. In this study, we aimed to determine whether WMW alleviates NAFLD by modulating liver pathology. To achieve this, we explored WMW's potential to ameliorate NAFLD by modulating hepatic pathological conditions. Utilizing comprehensive serum metabolomic sequencing, we conducted a thorough analysis of the systemic metabolic effects of WMW in a NAFLD mouse model.

MATERIALS AND METHODS

Herbal materials and preparation of WMW

The composition of WMW is detailed in Table S1. The Ningxia Institute of Traditional Chinese Medicine was responsible for the authentication, storage, and formulation of WMW. *Prunus mume* was immersed in vinegar overnight, whereas *Typhonium giganteum* was preboiled for two hours. The remaining herbs were soaked in water for 60 minutes. After this, the mixed herbs were boiled for an additional two hours. A further boiling phase of 60 min followed before filtration, and the extracts were stored in a refrigerator at 4 °C until use.

Compositional analysis of WMW

The components of WMW were systematically identified using the Traditional Chinese Medicine Systems Pharmacology Database (TCMSP) by searching for their respective names. The processed samples underwent analysis via LC-MS according to established protocols. Initially, the samples were homogenized and then 100 μL of the homogenate was combined with 900 μL IS solution (4 μg/mL in water) in 1.5 mL tubes. This mixture was vortexed for 1 minute and subjected to ultrasonication for 60 minutes in an ice-water bath.

Following this, the mixture was centrifuged at 12,000 rpm for 10 min at 4°C to separate the supernatant. A total of 200 μL of the supernatant was then collected for LC-MS analysis. The analysis was performed using an ACQUITY UPLC I-Class HF/QE HF MS system equipped with an HSS T3 column (2.1×100 mm, 1.8 μm). The LC analysis conditions included a column temperature of 45°C, a mobile phase composed of water and acetonitrile (0.1% FA) at a flow rate of 0.35 mL/min, an injection volume of 5 μL and PDA detection set to a wavelength range of 210-400 nm.

Animal husbandry and experimental grouping

Male C57 BL/6 J mice, six weeks old, were housed in the specific pathogen-free (SPF) animal facility of the Experimental Animal Center at Ningxia Medical University. The breeding environment was maintained at a temperature of 22 ± 2°C, with a 12 hours light-dark cycle. The relative humidity was maintained between 40% and 60%. The *in vivo* study was approved by Ningxia Medical University's Animal Care and Use Committee (IACUC-NYLAC-2023-011) and was conducted in accordance with the 3R principles (Replacement, Reduction, Refinement). Analgesia was provided for all invasive procedures. After a week of adaptive feeding, the mice were divided into a normal group and a model group, with the latter receiving a high-fat diet for 24 weeks.

After 24 weeks on a high-fat diet, the model mice were divided into five groups: the Model group, H-WMW group (1.92 g/kg/d), M-WMW group (0.96 g/kg/d), L-WMW group (0.48 g/kg/d), and Metformin group (250 mg/kg/d). These groups underwent treatment for an additional 7 weeks. In the 8th week, the mice were fasted for 12 hours before undergoing a glucose tolerance test. Following the test, the mice were euthanized through cervical dislocation. The livers were fixed in a 4% paraformaldehyde (PFA) solution, while the residual tissues were quickly frozen in liquid nitrogen and stored at −80°C for future analysis.

Oral glucose tolerance test

On the third day of the final experimental week, an oral glucose tolerance test was performed. The mice were fasted overnight before being administered glucose (2 g/kg). Blood samples were collected from the lateral tail vein at 0, 15, 30, 60 and 120 minutes and glucose levels were measured using a Yuyue glucose meter. The area under the curve (AUC) for blood glucose levels was calculated using trapezoidal approximation.

Measurement of serum parameters

Blood levels of critical biomarkers, including triglycerides (TG), total cholesterol (TC), LDL cholesterol (LDL-C), glucose ,and liver enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), catalase (CAT), and malondialdehyde (MDA), were quantified using standardized commercial assay kits, following the manufacturer's protocols. Assessments for insulin (INS), tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β) levels were measured using ELISA reagents.

Histopathology analysis

Fresh liver samples were preserved in 10% neutral-buffered formalin, then embedded in paraffin, sliced, and stained with hematoxylin and eosin (H&E) before undergoing Oil Red O staining for microscopic examination. The slides were observed using whole-slide digital scanning, and the pathology was scored by experienced pathologists.

Untargeted serum metabolomics analysis

Serum samples were extracted using 80% methanol, following established protocols (Chen *et al.*, 2022; Want *et al.*, 2013). Quality control (QC) samples were prepared by

pooling equal amounts of the extracted supernatants and were analyzed at consistent intervals (every six samples). A 10 μL aliquot of each sample was injected into a Vanquish UHPLC System equipped with a Hypersil Gold column. The analytical procedure for HPLC adhered to the parameters specified by the manufacturer and was based on previous methodologies (Wu *et al.*, 2022; Jin *et al.*, 2019).

The data analysis was conducted using Compound Discoverer 3.3, which facilitated peak alignment, key feature extraction, and metabolite quantification. Normalization was primarily based on peak area measurements. Following this, bioinformatics analyses were performed using the R package in conjunction with mzCloud (http://www.mzcloud.org/), mzVault,and the Mass List database to ensure the accuracy of annotations. To identify comprehensive metabolic alterations, we employed PCA and OPLS-DA techniques. We employed a dual approach to identify key metabolites, utilizing variable importance (VIP > 1.0) and univariate t-test screening ($P < 0.05$). For pathway analysis, the KEGG database was leveraged, using both Goatools and KOBAS 2.1.1 to elucidate metabolic enrichment patterns. Furthermore, integrated transcriptomic and metabolomic profiling was performed through iPath 3.0 to identify significant changes in metabolic pathways.

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA (GraphPad Prism 9.5), with significance set at $P < 0.05$. Tukey's test was used for multiple comparisons across all groups.

RESULTS

Identification of chemical components in WMW

To assess the composition of WMW, we utilized LC-MS for identification. This analysis revealed seven key components, including jatrorrhizine, [6]-gingerol, citric acid, isoferulic acid ,and ginsenoside Rb1. The HPLC results are presented in Fig. 1A and 1B. The major constituents of WMW were categorized into phenylpropanoids, terpenes, flavonoids, alkaloids, carbohydrates, and glycosides, as detailed in Fig. S1 A. Notably, the main bioactive compounds identified in WMW included allocryptopine, epiberberine, jatrorrhizine, protopine, and citric acid, with the top 10 components listed in Fig. S1 B.

Effects of WMW on obesity in NAFLD mice

The experimental workflow for the animal study is illustrated in Fig. 2A. After 24 weeks of HFD modeling, diet-induced obesity was observed in the model group (Fig. 2B). Notably, the H-WMW intervention resulted in a significant reduction in the body weight of the mice. Conversely, no significant changes were observed in the L-WMW and M-WMW groups (Fig. 2C-D).After 7 weeks, the H-WMW group exhibited a reduction in abdominal circumference compared to the model group, indicating a significant decrease in abdominal fat and fat pads.

Conversely, the L-WMW and M-WMW groups showed no statistically significant differences (Fig. 2E-G). In contrast, H-WMW treatment effectively improved blood glucose levels in the NAFLD mice (Fig. 2H-J), while the L-WMW and M-WMW groups showed no noteworthy changes. In summary, both L-WMW and M-WMW failed to show significant differences in blood glucose, body weight, or abdominal fat within the NAFLD model when compared to the H-WMW group. Therefore, considering the effectiveness of the doses and the cost-benefit ratio of the treatments, L-WMW and M-WMW were deemed ineffective for further experiments. As a result, H-WMW was selected as the optimal dosage for subsequent studies.

WMW alleviates hepatic steatosis in NAFLD mice

Notably, significant changes were observed in the livers of obese mice, characterized by a pale coloration indicative of fatty liver compared to normal liver tissue. Following WMW treatment, levels of ALT, AST, oxidative stress markers MDA and CAT, and inflammatory markers TNF-α and IL-1β were significantly restored compared to the model mice. After 24 weeks on a high-fat diet, the experimental mice demonstrated substantial liver changes compared to the control group, as illustrated in Fig. 3A. Specifically, the liver weights in the WMW group were significantly lower than those in the model group (Fig. 3B). Furthermore, the WMW group showed significantly reduced levels of AST and ALT compared to the model group ($P < 0.05$), indicating that the WMW intervention effectively mitigates liver injury (Fig. 3C-D). H&E and ORO staining were employed to assess the morphological alterations in NAFLD liver tissues. In the control group, hepatocytes were uniformly distributed and organized in a cord-like structure, displaying distinct nuclei without lipid droplets or inflammatory aggregates in the cytoplasm. In contrast, the hepatocytes in model group were hypertrophied and contained substantial lipid droplets, with some cells exhibiting predominantly bullous steatosis. Conversely, hepatocytes in the WMW group demonstrated improved structural uniformity and a significant reduction in lipid accumulation when compared to the control cohort (Fig. 3E, G).

WMW reduces liver inflammation, lipid accumulation, and oxidative stress in NAFLD mice

The model group mice exhibited a significant accumulation of merged red lipid droplets within their liver cells, in stark contrast to the control group. In contrast, the WMW and metformin-treated mice exhibited a marked reduction in liver lipid droplets (Fig. 3F, H). Serum insulin level measurements revealed that WMW treatment significantly lowered insulin levels in the obese mice (Fig. 3I). Furthermore, WMW intervention appeared to alleviate

oxidative stress by reducing MDA levels while enhancing catalase (CAT) activity in the livers of the treated mice (Fig. 3J-K). Serum low-density lipoprotein (LDL) levels in the mice showed a notable decline, accompanied by reduced concentration of triglyceride and cholesterol (Fig. 3L). Following WMW treatment, key pro-inflammatory cytokines such as TNF-α and IL-1β exhibited significant reductions compared to the control group, indicating that WMW may effectively reduce liver inflammation and oxidative stress in mice with NAFLD (Fig. 3M-N).

WMW intervention and its effect on serum metabolism in NAFLD mice

To further investigate the effects of WMW on NAFLD mice, we assessed various metabolic serum parameters. We analysed the serum from high-dose WMW mice, comprising six samples from both the control and model groups. Our analysis identified five main types of metabolites: lipids and related compounds, organic acids and their derivatives, organic heterocyclics, oxygen-containing organic compounds, and benzenoid derivatives, as illustrated in Fig. S1 D. The PCA analysis clearly indicated a distinct separation between the control and model groups, with the WMW group positioned primarily between the two, suggesting that high-fat diets significantly altered the serum metabolomic profiles of the mice. Furthermore, the WMW intervention appeared to partially counteract these diet-induced changes (Fig. 4A). The QC group, positioned between the two main groups, confirmed the stability and reliability of the data collected in this experiment, a finding that was further supported by PLS-DA validation (Fig. S1 E).

In negative mode, the model group exhibited an upregulation of 74 metabolites and a downregulation of 103 metabolites compared to the control group. In positive mode, there were increases in 37 metabolites and decreases in 133 (Fig. 4B). A comparative analysis between the model group and the WMW group highlighted significant differences in metabolite expression levels. Under negative ionization conditions, 44 metabolites showed increased activity, while 72 displayed decreased levels. In the positive ionization mode, 72 metabolites were elevated and 75 were reduced. These results underscored substantial metabolic variations between the two groups across both detection methods (Fig. 4C). The comparison of WMW versus Model and Model versus Control groups revealed a total of 175 overlapping metabolites between the two analyses (Fig. 4D). Post-treatment with WMW, the metabolic profiles shifted towards those seen in the normal group, suggesting a tendency for most serum metabolites to normalize (Fig. 4E). Furthermore, KEGG pathway analysis indicated that WMW treatment primarily influenced metabolic pathways associated with fatty acid metabolism, tyrosine metabolism and other relevant metabolic pathways (Fig. 4F).

Correlation between serum metabolites and NAFLD-related indicators

To explore the variations in metabolite levels among different groups, we set VIP thresholds above 1 and P-values below 0.05 as our analytical criteria. This approach allowed us to compare the model and control groups effectively. Our results indicated that 236 metabolites showed decreased expression. Using the same screening parameters, we also compared the model and WMW groups, which led to the identification of 88 upregulated metabolites. Furthermore, applying more stringent criteria of VIP > 1.5 and $P < 0.05$, we identified 16 metabolites that were reduced by high-fat diets but were significantly restored following the WMW intervention. The functional annotation of these 16 metabolites is detailed in Table 1. The differences among these metabolites primarily pertain to vitamin B6 metabolism, biotin metabolism and associated metabolic pathways (Fig. 5A). To further investigate the relationships between the 17 metabolites responsive to the WMW intervention and NAFLD-related indicators in mice, we conducted a Spearman correlation analysis (Fig. 5B). Notably, TNF-, MDA, blood glucose, LDL and ALT exhibited negative correlations with LPC O-14:0. Additionally, body weight and abdominal fat weight were negatively correlated with methandrostenolone. Conversely, MDA, blood glucose, LDL, and ALT displayed a positive correlation with methandrostenolone, thereby establishing a connection between these metabolites and the progression of NAFLD.

DISCUSSION

Our study demonstrated that WMW medication, a well-established botanical formulation, significantly mitigated the progression of high-fat diet-induced NAFLD in mice. This improvement was achieved by reducing liver fat accumulation, enhancing glucose regulation, and alleviating liver damage. These findings support previous research highlighting the effectiveness of natural compounds and herbal formulations in modulating metabolic disorders by regulating serum metabolites. Our results suggest that the WMW intervention not only reduced body weight and abdominal fat but also restored serum lipid profiles and hepatic function markers (AST, ALT), indicating the presence of complex therapeutic mechanisms.

In a mechanistic context, WMW significantly reduced body weight in obese mice fed a high-fat diet, while also decreasing serum levels of total cholesterol, triglycerides, and LDL cholesterol. The increase in body weight and adiposity leads to hepatic overload, and clinical studies have linked obesity to NAFLD (Fan *et al.*, 2017; Machado and Cortez-Pinto, 2023). Preserving liver function is crucial for maintaining overall metabolic balance; disruptions in this function can lead to health complications such as obesity and insulin resistance (Benedé-Ubieto *et al.*, 2024).

Table 1: Key metabolites after WMW intervention.

Name	HMDB ID	Class
LPC O-14:0	--	--
Biocytin	HMDB0003134	Carboxylic acids and derivatives
Monobutyl phthalate	HMDB0013247	Benzene and substituted derivatives
4-tert-Amylphenol	HMDB0246582	Benzene and substituted derivatives
6-Keto-prostaglandin f1alpha	HMDB0002886	Fatty acyls
6-Ketoprostaglandin F1α	HMDB0002886	Fatty acyls
12-epi Leukotriene B4	--	--
2-[6-(1H-benzo[d]imidazol-2-yl)-2-pyridyl]-1H-benzo[d]imidazole	--	--
Hydrocinnamic acid	HMDB0000764	Phenylpropanoic acids
p-Mentha-1,3,8-triene	HMDB0037013	Prenol lipids
CAR 20:4	--	--
Pyridoxine	HMDB0000239	Pyridines and derivatives
Acetyl-L-carnitine	HMDB0240773	Fatty acyls
19-Nortestosterone	HMDB0002725	Steroids and steroid derivatives
1-[4-hydroxy-3-(3-methylbut-2-en-1-yl) phenyl] ethan-1	--	--
LPC O-14:0	--	--

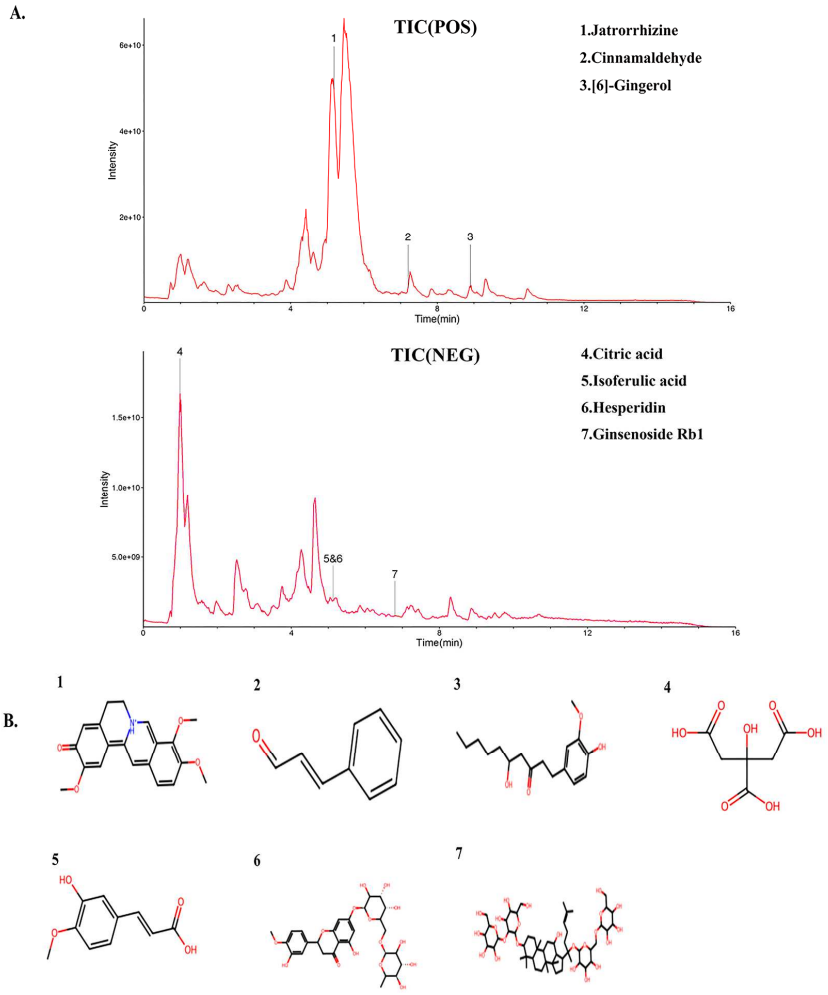

Fig. 1: HPLC analysis of WMW.
(A) Representative HPLC chromatograms of WMW extracts. (B) The structural formula of chemical compounds in WMW: 1 Jatrorrhizine; 2 Cinnamaldehyde; 3 [6]-Glngerol; 4 Citric acids; 5 Isoferulic acid; 6 Hesperetin; 7 Ginsenoside Rb1.

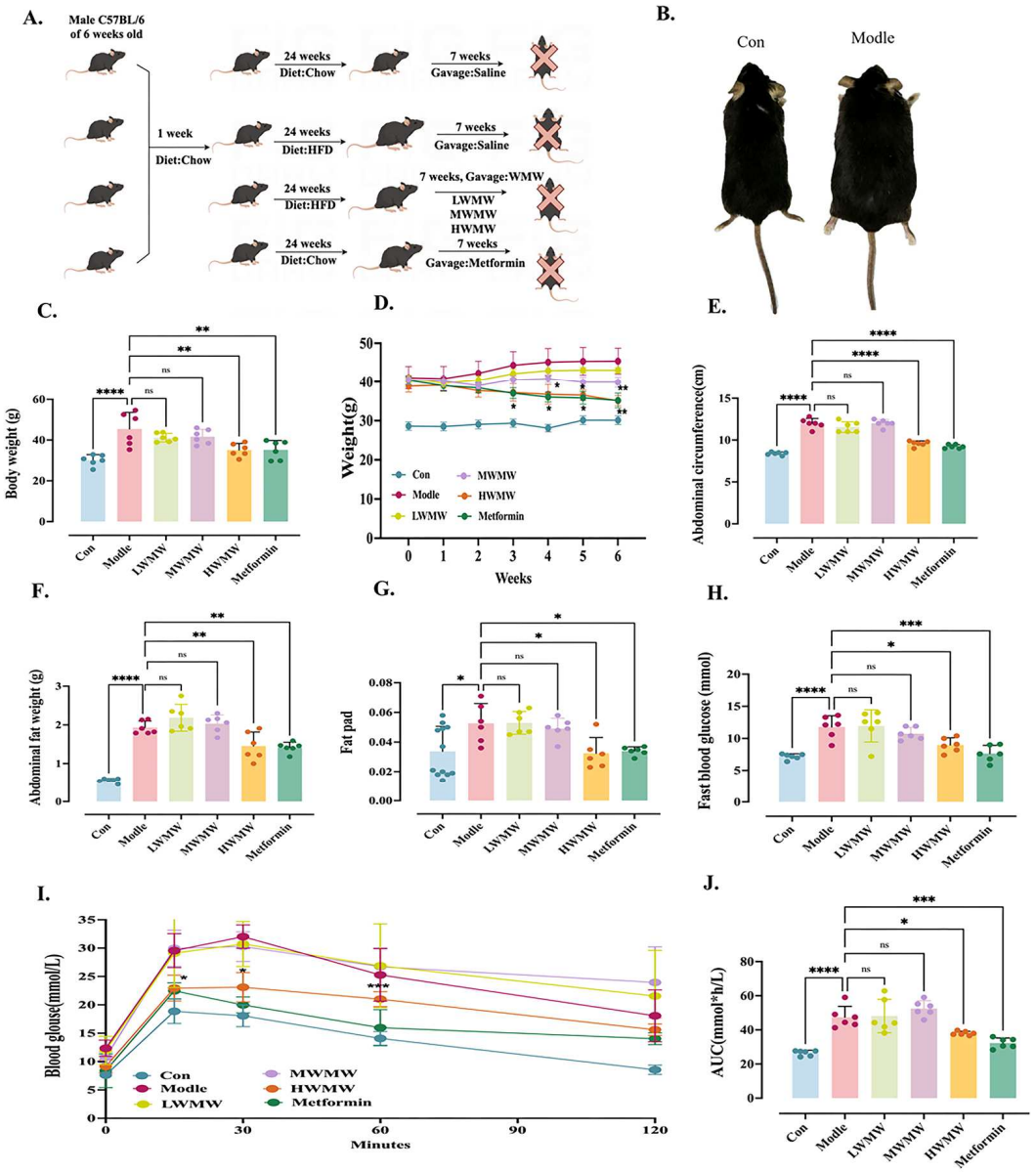

Fig. 2: Effects of WMW reduced body weight and improved dyslipidaemia.
(A) Schematic representation of animal experiment process. (B) Comparison chart of mouse body types. (C) End point change in body weight. (D) Curve chart showing weekly weight changes. (E) Changes in abdominal circumference. (F) Weight of abdominal fat. (G) Fat pad ratio. (H) Fasting blood glucose. (I) Oral glucose tolerance test (OGTT). (J) AUC of OGTT. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

The liver-protective effects of WMW were clearly evidenced by several indicators: a significant reduction in fat accumulation in liver tissue, as demonstrated by Oil Red O staining; improved liver function test results, shown by lower ALT and AST levels; and enhanced antioxidant activity (evidenced by increased catalase levels and decreased malondialdehyde. Collectively, these findings suggest that WMW effectively supports liver health by addressing lipid metabolism, cellular damage, and oxidative stress. Furthermore, our results suggest that WMW may exhibit synergistic effects, thereby enhancing its bioactivity compared to its individual components. The differing efficacy among dosage groups highlights the importance of optimizing dosages in herbal formulations, with 1.92 g/kg/d identified as the therapeutic threshold. WMW's ability to improve insulin sensitivity and glucose tolerance is consistent with findings from studies on dietary fibers and polyphenols. The significant reduction in TNF-α and IL-1β levels in WMW-treated mice underscores its anti-inflammatory properties, likely through the enhancement of antioxidant defenses, such as MDA and GSH-Px, as documented in models of metabolic endotoxemia (Abulikemu *et al.*, 2023; Yu *et al.*, 2023).

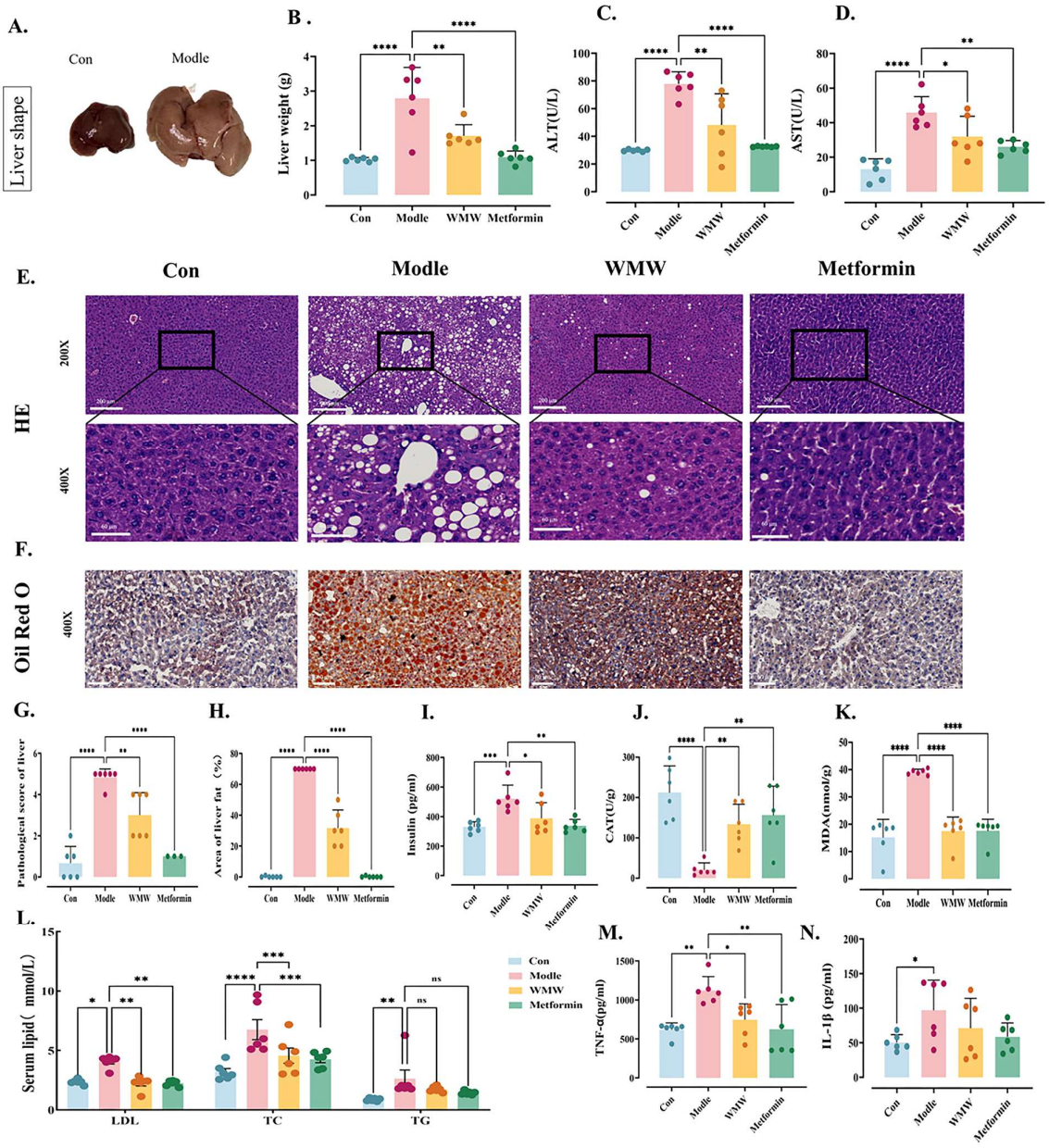

Fig. 3: WMW alleviated the inflammation, oxidative stress and lipid accumulation of NAFLD mice.
(A) Comparison of liver sizes among the groups. (B) Comparison of liver weight across the indicated groups. (C-D) ALT and AST levels in serum. (E) Histological examination of liver tissue from different groups. 200 X (bar=200 μm), 400 X (bar=60 μm). (F) Oil Red O Staining, 400 X (bar=60 μm). （G）HE staining pathological score for the indicated groups. (H) Area of liver fat, ORO staining score. (I) Level of insulin in serum. (J) CAT levels in liver. (K) MDA levels in liver. (L) LDL-C, TC, TG levels in serum. (M-N) Level of IL-1β, TNF-α in serum. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

Regulating metabolism has proven to be an effective strategy for managing metabolic syndrome. WMW was able to reverse the changes in serum metabolites observed in mice with this condition, primarily affecting metabolic pathways related to tyrosine, fatty acids, biotin, D-glutamine, D-glutamate, vitamin B6, and porphyrins. Research conducted by Momen highlighted a significant link between tyrosine and other branched-chain amino acids, such as leucine and isoleucine, in relation to obesity and insulin resistance (Momen *et al.*, 2024; Newgard *et al.*, 2009). This suggests that disturbances in tyrosine metabolism may play a role in the development of metabolic syndrome. Furthermore, recent studies have demonstrated that plasma amino acid levels, particularly tyrosine, are associated with visceral obesity, insulin resistance, and the potential development of diabetes and cardiovascular diseases (Sun *et al.*, 2021; Wang *et al.*, 2011).

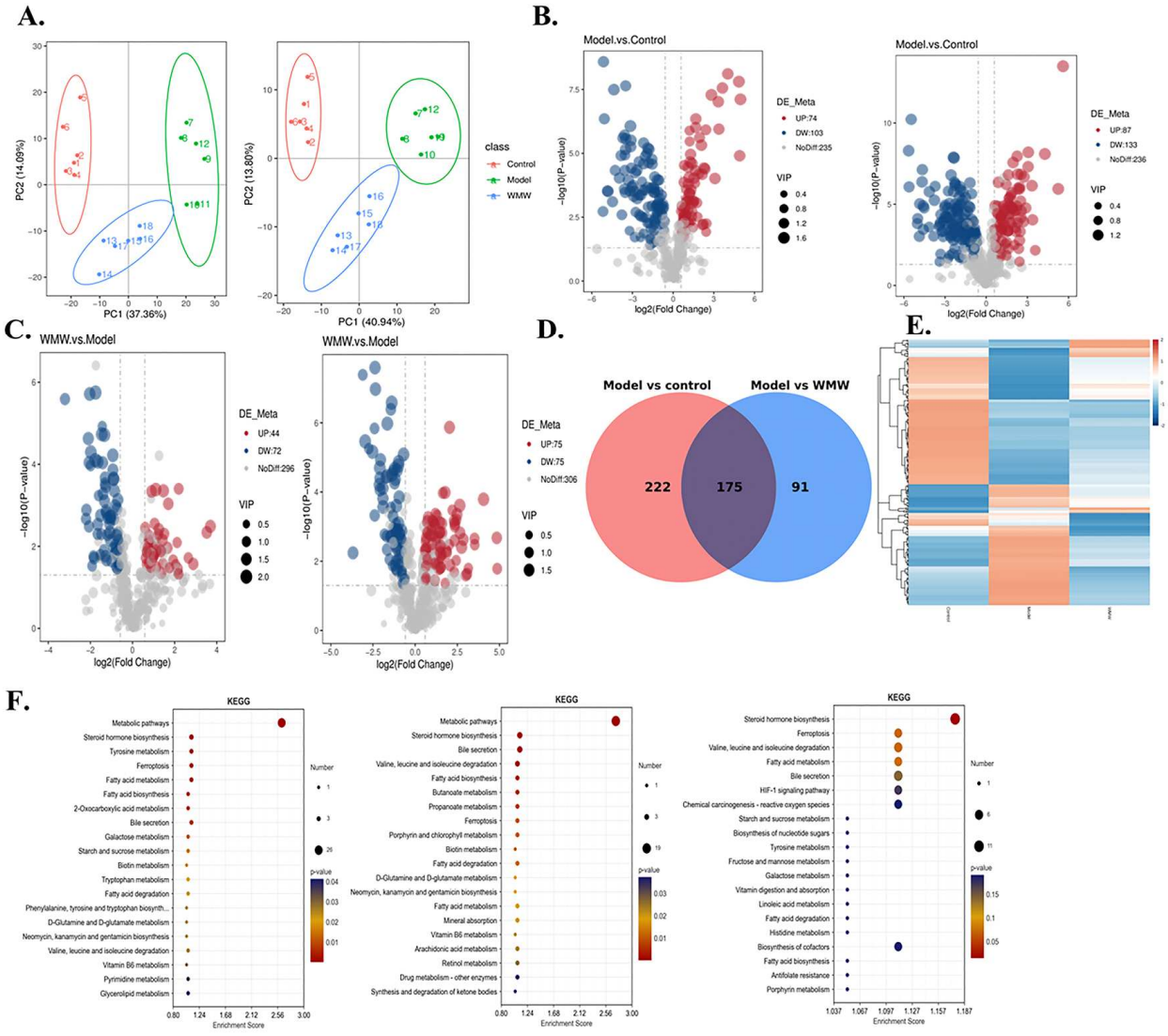

Fig. 4: WMW reversed the serum metabolism in NAFLD mice.
(A) PCA score plots of blood profile following WMW treatment recorded in the negative mode (left) and positive mode (right). (B) The distribution of differential metabolites for Model *vs* Control. (C) The distribution of differential metabolites for WMW *vs* Model. (D) Venn diagram revealing the intersection of differential metabolites between the Model *vs* Control groups and WMW *vs* Model groups. (E) Heat map showing the identified differential metabolites among control, model and WMW groups. (F) KEGG analysis between Control *vs* Model groups (left), Model *vs* WMW groups (middle) and two groups of intersecting target proteins (right).

A survey by Lecoutre investigated the effects of L-glutamine on insulin sensitivity and overall metabolic health, demonstrating that L-glutamine supplementation improved metabolic parameters in obese individuals (Lecoutre *et al.*, 2024; Gleeson *et al.*, 2011). Moreover, D-glutamic acid is believed to play a significant role in NAFLD. The WMW treatment notably increased metabolites such as LPC 19:2, LPC 22:5, and LPC O-14:0, which recent five-year studies suggest may serve as indicators of metabolic health (Zhao *et al.*, 2024).

Serum metabolites associated with WMW reversal, such as biocytin, 12-epi Leukotriene B4, CAR 20:4 and acetyl-L-carnitine, are linked to oxidative stress, inflammation and obesity (Chen *et al.*, 2021a; Mouskeftara *et al.*, 2024; Yu *et al.*, 2022; Jakubs *et al.*, 2008; Meyers and Berk, 1990). The progression of NAFLD is intrinsically related to these metabolic reactions. The WMW intervention not only effectively reversed metabolic disturbances caused by a high-fat diet but also restored essential serum metabolites such as LPC O-14:0 and methandrostenolone. These metabolites showed strong inverse correlations with indicators of NAFLD progression, including TNF-α, MDA and LDL-C. This aligns with earlier studies that indicated methandrostenolone's role in increasing hepatic toxicity, which contributes to hepatic steatosis and fibrosis, while steroid metabolites may influence insulin sensitivity (Gronert *et al.*, 2023; Zheng *et al.*, 2025).

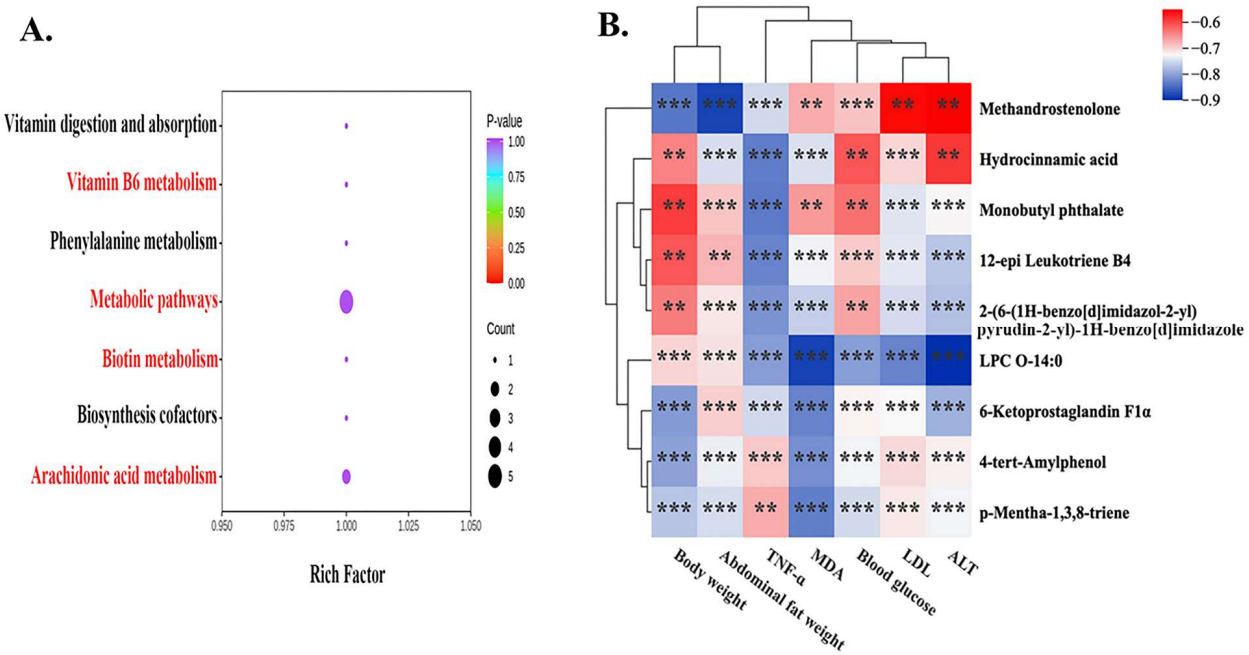

Fig. 5: The improved serum metabolites of WMW were linked to the vitamin B6 metabolism, metabolic pathways, biotin metabolism and arachidonic acid metabolism.
(A) KEGG analysis of key metabolites after WMW intervention. (B) Heatmap showing the association between serum metabolites and NAFLD-related parameters

WMW successfully alleviated methandrostenolone-induced hepatotoxicity, resulting in decreased ALT levels, with a robust correlation underscoring the varied efficacy of WMW treatments for NAFLD.

The normalization of vitamin B6-related metabolites, specifically pyridoxamine and pyridoxic acid, is noteworthy due to vitamin B6's recognized role in reducing oxidative stress and improving lipid profiles in metabolic disorders (Kobayashi *et al.*, 2021). Following the WMW intervention, the vitamin B6 metabolic pathway in mice was activated. Research by Fanghua G indicated that elevated liver vitamin B6 levels activate the PPAR signaling pathway, which in turn inhibits the TLR4/NF-κb inflammatory pathway, leading to reduced fat accumulation and oxidative stress in the liver (Guo *et al.*, 2023). These findings deepen our understanding of WMW's therapeutic effects, revealing its potential to extend beyond traditional applications and identifying new metabolic targets for managing NAFLD.

However, this study has certain limitations. Our study demonstrated the impact of WMW on serum metabolites and liver pathology. The precise molecular targets and signaling networks require further validation through transcriptomic and proteomic analyses. Future studies should integrate gut microbiome and hepatic kinome profiling to better understand the systemic mechanisms involved in this process. These findings reposition WMW as a modulator of metabolic networks, offering a phytochemical framework for multi-target therapy in NAFLD. Additional research could employ metagenomic sequencing to identify relevant bacterial taxa and confirm their functions through fecal microbiota transplantation. Our study also indicated that elevated concentrations of WMW are essential for significant metabolic improvements. These dose-dependent effects raise important questions regarding bioavailability and optimal clinical dosing. This study revealed that WMW influences serum metabolites and liver pathology; however, the precise molecular targets and signaling networks involved have not been identified, which need to be clarified through transcriptomic and proteomic analyses. In the future, researchers should integrate gut microbiome and hepatic kinome profiling tools to elucidate their systemic mechanisms. These findings reposition WMW as a metabolic network modulator, providing a phytochemical framework for developing multi-target therapies for NAFLD. Moreover, researchers should use metagenomic sequencing to identify specific bacterial taxa and confirm their functions via fecal microbiota transplantation.

CONCLUSION

This study positions WMW as a systems-level regulator of NAFLD, merging traditional herbal therapies with modern metabolomic and mechanistic approaches. It enhances hepatic lipid management and insulin sensitivity, reduces oxidative stress and inflammation,and reprograms metabolic pathways by modulating vitamin B6 and fatty

acid metabolism. Overall, WMW emerges as a promising candidate for further exploration as a natural adjunct therapy for obesity-related liver diseases.

Acknowledgments
Not applicable

Authors' contributions
Mengting Guo: Data curation, Formal analysis, Methodology. Xue Zhou: Data curation, Investigation. Haodong Zhang: Software. Investigation. Xinna Huyan: Validation. Yang Zhao: Supervision. Xiaocui Wang: Conceptualization, Funding acquisition, Resources

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Data availability statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical approval
All animal experiments were approved by the Experimental Animal Center of Ningxia Medical University (Approval number: IACUC-NYLAC-2023-011) and all procedures were conducted in accordance with the center's animal welfare regulations. This study was performed in adherence with the ARRIVE guidelines. See supplementary file for the ARRIVE checklist.

Conflict of interest
The authors declare no competing financial interests or personal relationships that could influence the work reported in this paper.

Supplementary data
https://www.pjps.pk/uploads/2026/04/SUP1775907814.pdf

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