

Clinacanthus nutans leaf extract prevents metabolic dysfunction-associated steatotic liver (MASL) to metabolic dysfunction-associated steatohepatitis (MASH) progression in western diet-fed C57BL/6 mice

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Abstract

The phytochemical profile and hepatoprotective, antioxidant, and lipid-modulating effects of the ethanolic extract of *Clinacanthus nutans* leaves were examined in male C57BL/6 mice fed a cholesterol-supplemented western diet. After 15 weeks of feeding, the untreated mice developed steatosis, lobular inflammation, and ballooning hepatocytes, with NAFLD activity scores ≥ 5 , indicative of metabolic dysfunction-associated steatohepatitis (MASH). Treatment with *Clinacanthus nutans* extract at doses of 100, 200, and 400 mg/kg from week 8 to 15 significantly reduced the liver-to-body weight ratio and NAFLD activity score, with dose-dependent histological improvement, particularly reduced lobular inflammation. AST, ALT, and hepatic malondialdehyde levels were significantly lowered in the treated groups, with effects exceeding those of atorvastatin. However, *Clinacanthus nutans* extract did not attenuate the elevated LDL-to-HDL ratio. In conclusion, *Clinacanthus nutans* extract effectively prevented the progression from simple steatosis to steatohepatitis, likely through antioxidative mechanisms attributable to its high tannin content.

Keywords

antioxidant, hepatitis, NAFLD activity score, tannin, western diet

Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD) has emerged as a global epidemic, affecting approximately 1.27 billion people worldwide and causing nearly 170,000 deaths annually (Chen et al. 2022; Feng et al. 2025). Its prevalence is projected to rise from 38% currently to over 55% of adults by 2040 (Younossi et al. 2025). MASLD is the updated nomenclature for the condition formerly known as non-alcoholic fatty liver disease (NAFLD). This change reflects a shift in clinical focus toward identifying and managing cardiometabolic risk factors in affected patients (Hong et al. 2024). It is now the most prevalent chronic liver disease and the leading indication for liver transplantation (Younossi et al. 2025).

The pathological process of MASLD begins with substantial fatty accumulation in hepatocytes, a condition termed simple steatosis or MASL. This presumably benign, reversible condition may advance into an inflammatory state called steatohepatitis or MASH, which puts the liver at a greater risk of permanent damage with the progression into fibrosis, cirrhosis, and hepatocellular carcinoma (Lekakis and Papatheodoridis 2024). In addition to liver-related complications, MASH is associated with a greater risk of cardiovascular events compared with MASL (Sanyal et al. 2024).

The progression from MASL to MASH is driven by multiple, simultaneously interacting insults, as conceptualized by the multiple hits theory. Among these, lipotoxicity-induced oxidative stress plays a central role in sustaining hepatocellular injury (Syed-Abdul 2023). Damaged hepatocytes release damage-associated molecular patterns (DAMPs), which amplify inflammation through the activation of innate immune responses. This persistent inflammatory cascade, compounded by ongoing metabolic dysregulation, promotes a pro-fibrogenic state (Parthasarathy et al. 2020).

The global burden of MASLD has risen exponentially; however, effective pharmacotherapeutic options remain limited (Branković et al. 2024; Hasanatuludhhiyah et al. 2025a). While weight reduction and cardiometabolic risk management are central to halting MASLD progression, their overall efficacy is hindered by poor adherence to lifestyle modifications in today's westernized society (Clemente-Suárez et al. 2023; Fan et al. 2024). Plant-based nutraceuticals, rich in secondary metabolites with antioxidant, anti-inflammatory, and metabolic regulatory activities, are potentially beneficial for MASH prevention (Vrentzos et al. 2025).

Clinacanthus nutans (Burm. f.) Lindau leaf is a source of a traditional remedy to treat inflammation and diabetes in the Southeast Asia region (Alam et al. 2016). It is rich in antioxidant properties that are plausibly advantageous for the amelioration of liver pathology in MASLD (Ng et al. 2022). Complementing this notion, an *in silico* investigation demonstrated that bioactive compounds from the aerial parts of *Clinacanthus nutans* exhibit significant interactions with key molecular targets implicated in MASLD pathomechanisms, suggesting their potential role in modulating disease-related signaling pathways (Hasa-

natuludhhiyah et al. 2025b). Experimental evidence from diabetic models indicates that *Clinacanthus nutans* administration improves metabolic, oxidative stress, and inflammatory parameters (Mustika et al. 2023; Susanti et al. 2024a). Water and methanolic leaf extract of *Clinacanthus nutans* were shown to enhance the antioxidant capacity of the liver and improve the markers of insulin resistance in rats induced by the high-fat, high-cholesterol diet (Sarega et al. 2016a, 2016b). Furthermore, the water extract of *Clinacanthus nutans* demonstrated anti-inflammatory and hepatoprotective effects in the hepatitis B mice model by improving metabolomic profiles of the liver and gut microbiota (Zhang et al. 2023). The protective effect of *Clinacanthus nutans* in preventing the progression of MASL to MASH warrants investigation. Therefore, this study aimed to examine the effect of the ethanolic extract of *Clinacanthus nutans* L. on liver histology, liver function, lipid profiles, and hepatic malondialdehyde levels in male C57BL/6 mice fed with the cholesterol-supplemented western diet.

Materials and methods

Extract preparation

Dried leaves of *Clinacanthus nutans* (Burm. f.) Lindau (batch number: 220525. DGS.FMMB.002) was obtained from Balai Materia Medica, Batu, East Java, Indonesia (−7.867547063098887, 112.51965390340497), where the plant was cultivated, and the voucher specimen was deposited in its herbarium. The maceration method was employed for extraction. The leaves were ground into fine powder, subsequently soaked in 70% ethanol for 24 hours at room temperature, then stirred periodically, and filtered to obtain the residue. This procedure was repeated twice, and the residue was evaporated in a water bath using a rotary evaporator to yield the extract.

Phytochemical analysis

The total phenolic content was determined using the Folin–Ciocalteu colorimetric method (Pérez et al. 2023). An aliquot of *Clinacanthus nutans* extract (1 mL) was mixed with Folin–Ciocalteu reagent (diluted in water by 7.5-fold). Following 8 minutes of resting, 4 mL of 1% NaOH was added to the mixture. This was incubated in the dark at room temperature for 1 hour. The absorbance was measured at $\lambda = 770$ nm using a UV-Vis spectrophotometer. Gallic acid was used to generate the calibration curve, and the results were expressed as mg gallic acid equivalents (GAE)/g extract. The total flavonoid content was assessed using the aluminum chloride (AlCl₃) colorimetric assay (Nicolescu et al. 2025). A total of 0.5 mL of the extract was mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. The mixture was incubated at room temperature for 30 minutes. Absorbance was recorded at 425 nm. Quercetin was used as the reference standard. Results were ex-

pressed as mg quercetin equivalents (QE)/g extract. The tannin content was estimated using a modified Folin–Denis method (Elgailani and Ishak 2014). An aliquot (0.5 mL) of the extract was mixed with 3.75 mL of distilled water and 0.25 mL of Folin–Denis reagent. After 3 minutes, 0.5 mL of saturated sodium carbonate was added. The mixture was incubated for 30 minutes at room temperature. The absorbance was measured at 760 nm. Tannic acid was used to construct the standard curve. Results were expressed as mg tannic acid equivalents (TAE)/g extract.

The total saponin content (TSC) and alkaloid content were quantified using gravimetric assays (Nimyel and Lori 2023). A 1 g portion of the sample was refluxed with 50 mL of *n*-hexane at 60–80 °C for 30 minutes. After cooling, the *n*-hexane was discarded, and the residue was dissolved in 50 mL of ethyl acetate. The solution was transferred to a separatory funnel, and the ethyl acetate layer was separated. The remaining residue was extracted three times with 50 mL of *n*-butanol. The combined *n*-butanol extracts were evaporated to dryness using a rotary evaporator. The residue was dissolved in 10 mL of methanol and added dropwise to 50 mL of *n*-hexane with stirring to precipitate saponins. The precipitate was collected on a pre-weighed filter paper, dried to a constant weight, and the saponin content was determined by the weight difference. For the quantification of the alkaloid content, 10 g of *Clinacanthus nutans* powder was mixed with 100 mL of methanol and 10 mL of ammonia. The mixture was heated using a water bath for 30 minutes. After heating, the mixture was filtered to separate the liquid extract. This process was repeated three times. Subsequently, 50 mL of 1 N hydrochloric acid was added to the pooled filtrate. The acidic mixture was then concentrated by evaporation until the volume was reduced to approximately 25 mL. The concentrate was filtered and transferred into a separatory funnel. The filtrate was basified using ammonia solution and then extracted three times with 25 mL of chloroform. The chloroform layers containing the free alkaloids were combined and evaporated at 50 °C. The resulting residue was further dried in an oven at 100 °C until a constant weight was achieved. The final dried residue was weighed and recorded as the total alkaloid content, expressed as a percentage of the sample weight.

Experimental animals

Healthy, eight-week-old, male C57BL/6 mice, weighing approximately 25 g, were purchased from Kemuning Breeding Center, Karanganyar, Indonesia, and housed in the Animal Laboratory of the Faculty of Medicine, Universitas Airlangga. They were kept in polypropylene cages under a 12-hour light-dark cycle, room temperature of 22 ± 2 °C, and humidity of $50 \pm 15\%$. The animals were acclimatized for 2 weeks before commencing feeding for MASH induction.

The sample size was calculated using G*Power, with an estimated effect size of 0.60, alpha of 0.05, and power of 0.80 (Zhang and Hartmann 2023). Therefore, 42 mice were randomly allocated into six groups. The healthy control was fed a chow diet and given tap water. Five other

groups were fed a western diet containing 21.1% fat (lard-sourced), 41% sucrose, and 1.25% cholesterol (Dyet) and were given a drink of mixed glucose (18.9 g/L) and fructose (23.1 g/L) solution. The diets were provided by the Animal Feed Laboratory, Faculty of Veterinary Medicine, UNAIR. The treatments were started after 8 weeks of feeding. The healthy control and WD groups were administered CMC Na suspension containing a placebo. The WD+atorvastatin group was treated with atorvastatin at 4 mg/kg BW. The WD+CN1, WD+CN2, and WD+CN3 groups were treated with *Clinacanthus nutans* extract at 100, 200, and 400 mg/kg BW per day, respectively. The extract was mixed with aquadest and CMC Na, making up a suspension. All treatments were administered orally, 10 mL/kg BW daily by orogastric sonde for 7 weeks, along with continued WD feeding until 15 weeks. Three additional mice allocated to the WD+placebo group were sacrificed before 8 weeks for histological evaluation of the liver biopsy. The animals' weight was evaluated weekly. All experimental procedures were carried out following the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the Committee of Health Research Ethics, Faculty of Medicine, Universitas Airlangga, Indonesia (94/EC/KEPK/FKUA/2024).

Biochemical analysis

Blood specimens were collected from the tail vein after 8 weeks of feeding and at the end of treatment for lipid assay. Liver function assays, including AST, ALT, and albumin levels, were carried out on intracardiac blood at the end of treatment. The blood samples were collected in 0.5 mL tubes containing EDTA K3 and then analyzed by dry chemical analyzers (Jiangsu Konsung). The animals were fasted from food and sugar solution for 6 hours before blood collection.

Histopathological examination

The mice were euthanized by a ketamine:xylazine anesthetic cocktail and intracardiac blood puncture on the day following the last treatment. The liver of each mouse was carefully removed and washed in cold PBS, then dried with tissue paper and weighed. The liver tissue was halved and cut for a histological slide and a malondialdehyde colorimetric assay. The liver cut was soaked in 10% formaldehyde in phosphate buffer solution (PBS) for 24 hours. On the subsequent day, it was immersed in 70% ethanol before being embedded in a paraffin block. The block was cut using a Leica RM 2235 microtome (Leica Biosystems, Wetzlar, Germany) at 3 µm thickness. The liver sections were then mounted onto slides and stained with hematoxylin and eosin (HE). The pathological features were observed under a light microscope (Olympus, USA) connected to a digital camera (Optilab, Miconos, Indonesia) and a computer (software cellSens). The liver slides were evaluated by an independent pathologist who was blinded to the experiment. Scoring of liver pathologies was

performed using the NASH Clinical Research Network (CRN) criteria in 20 fields of view at 200× magnification. The NAFLD activity score (NAS) was calculated as the sum of steatosis (0–3), lobular inflammation (0–3), and hepatocellular ballooning (0–2) scores. An NAS ≥ 5 was considered definitive NASH (Takahashi 2014).

Measurement of malondialdehyde

The liver was minced and homogenized in PBS with a glass homogenizer on ice. The tissue homogenate was centrifuged, and the supernatant was collected and stored. It was subsequently heated in a water bath at 80 °C for 15 minutes. After cooling, it was centrifuged at 3000 rpm for 15 minutes. The supernatant was reacted with the thio-barbituric acid (TBA) reagent. The intensity of supernatant absorbance was measured using a spectrophotometer (Boeco S22 UV/Vis) at $\lambda = 532$ nm.

Statistical analysis

The mean differences among groups were analyzed by one-way ANOVA followed by the least significant difference (LSD) post hoc test, provided that the parametric data were normally distributed and homogeneous. Otherwise, the data were analyzed using the Kruskal-Wallis test, followed by the Mann-Whitney U test. A p-value <0.05 indicated a statistically significant difference. IBM SPSS 27 was employed for the statistical analyses.

Results

Phytochemical analysis

The phytochemical content of *Clinacanthus nutans* leaf was quantitatively analyzed for five major compound groups (Table 1). Among the compounds analyzed using the colorimetric method, tannin was found at the highest content (29.1 mg/g), while flavonoid was found at the lowest (8.6 mg/g). Quantification of saponins and alkaloids carried out using the gravimetric method yielded values of 39 mg/g and 23.8 mg/g, respectively. These findings indicate that *Clinacanthus nutans* leaves contain a significant amount of secondary metabolites, particularly tannins and saponins, which may contribute to their pharmacological properties.

The average body weight of animals in each group at baseline, at the start, and at the end of treatment, as well as the change in body weight, is presented in Fig. 1. There was no significant difference in body weight among the groups at baseline, at the start, and at the end of treatment. An increase in body weight was found in the chow diet and WD-fed groups within the first 8 weeks. The healthy control group exhibited a reduction in body weight, while all WD-fed groups maintained relatively stable weights from week 8 to 15. Body weight change from the start to the end of treatment was also not significantly different between the groups.

The average total cholesterol and LDL levels of the healthy control group were considerably lower than those of the WD-fed groups at week 8. However, the western diet

Table 1. Phytochemical quantification of *Clinacanthus nutans* leaf.

Compound	Content (mg/g)	RPD(%)	Method	Standards
Polyphenol	28.8	0.9	Folin–Ciocalteu	Galic acid
Flavonoid	8.3	5.7	Aluminum chloride	Quercetin
Tannin	29.0	0.7	Folin–Denis	Tannic acid
Saponin	39.0	0.2	Gravimetry	–
Alkaloid	23.8	0.4	Gravimetry	–

RPD: relative percentage difference

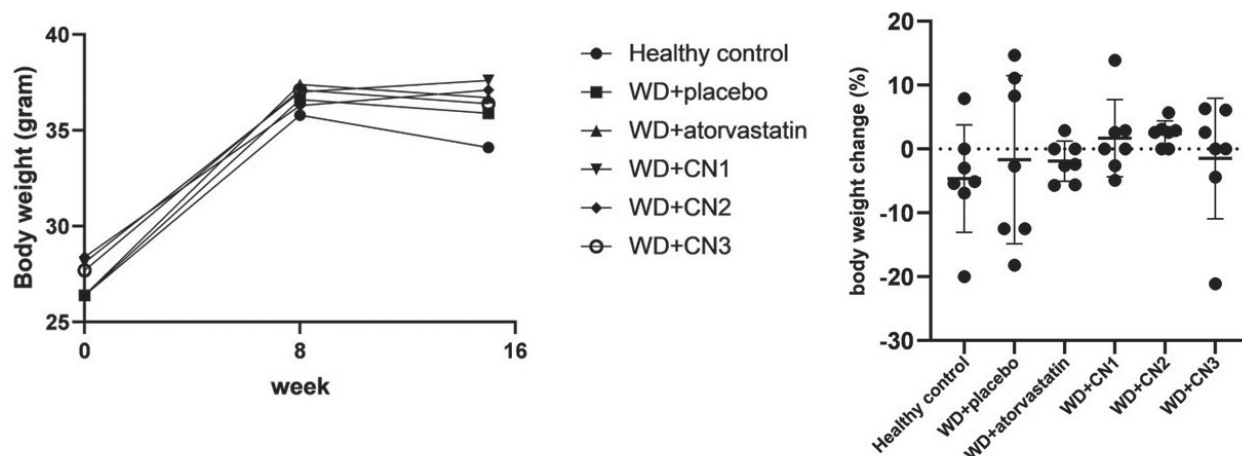


Figure 1. Body weight (left); change of body weight from week 8 to week 15 (right) start of feeding (week 0), start of treatment (week 8), and at the end of treatment (week 15). CN, *Clinacanthus nutans* extract; WD, western diet.

did not result in statistically significant alterations in blood levels of total cholesterol, LDL, and HDL at either 8 or 15 weeks of feeding. In comparison with other WD-fed groups, the atorvastatin-treated group tended to show a greater reduction in total cholesterol and LDL levels from 8 to 15 weeks. The western diet-fed groups exhibited a significantly higher LDL-to-HDL ratio compared with the healthy control group at week 8 ($p < 0.05$), an effect that was no longer evident at week 15. Atorvastatin- and CN2-treated groups tended to show a reduction in the LDL-to-HDL ratio from week 8 to 15. In comparison with the healthy control group, blood triglyceride levels were significantly reduced in all WD-fed groups at week 15 ($p < 0.05$), but not at week 8. The average triglyceride level in the healthy control group increased at week 15 compared with week 8, while all WD-fed groups displayed a decline, with the WD+placebo group showing the most drastic reduction (Fig. 2).

The untreated WD group showed a significant increase in AST and ALT levels, suggesting a deterioration of liver function compared with the healthy control group ($p < 0.05$). Meanwhile, the WD groups treated with atorvas-

tin or *Clinacanthus nutans* extracts exhibited significantly reduced AST and ALT levels to an extent comparable with the healthy control group ($p < 0.05$), suggesting the effect of *Clinacanthus nutans* extract in improving liver function. The WD+placebo group tended to show lower blood albumin levels compared with the other groups; several subjects in this group had albumin levels below the normal range (albumin reference: 2.6–5.4 g/dL), suggesting impaired liver synthetic function. However, there was no statistically significant difference among the groups in albumin levels (Fig. 3).

The macroscopic features and liver-to-body weight ratio of each group are presented in Fig. 4. A significantly increased liver-to-body weight ratio was demonstrated in the untreated WD group compared with the healthy control group ($p < 0.05$), indicative of liver enlargement associated with inflammation. In contrast, WD-fed groups treated with atorvastatin and *Clinacanthus nutans* extract at all tested doses displayed significantly reduced liver-to-body weight ratios ($p < 0.05$), with no significant differences relative to the healthy control group, suggesting a preventive action against liver enlargement.

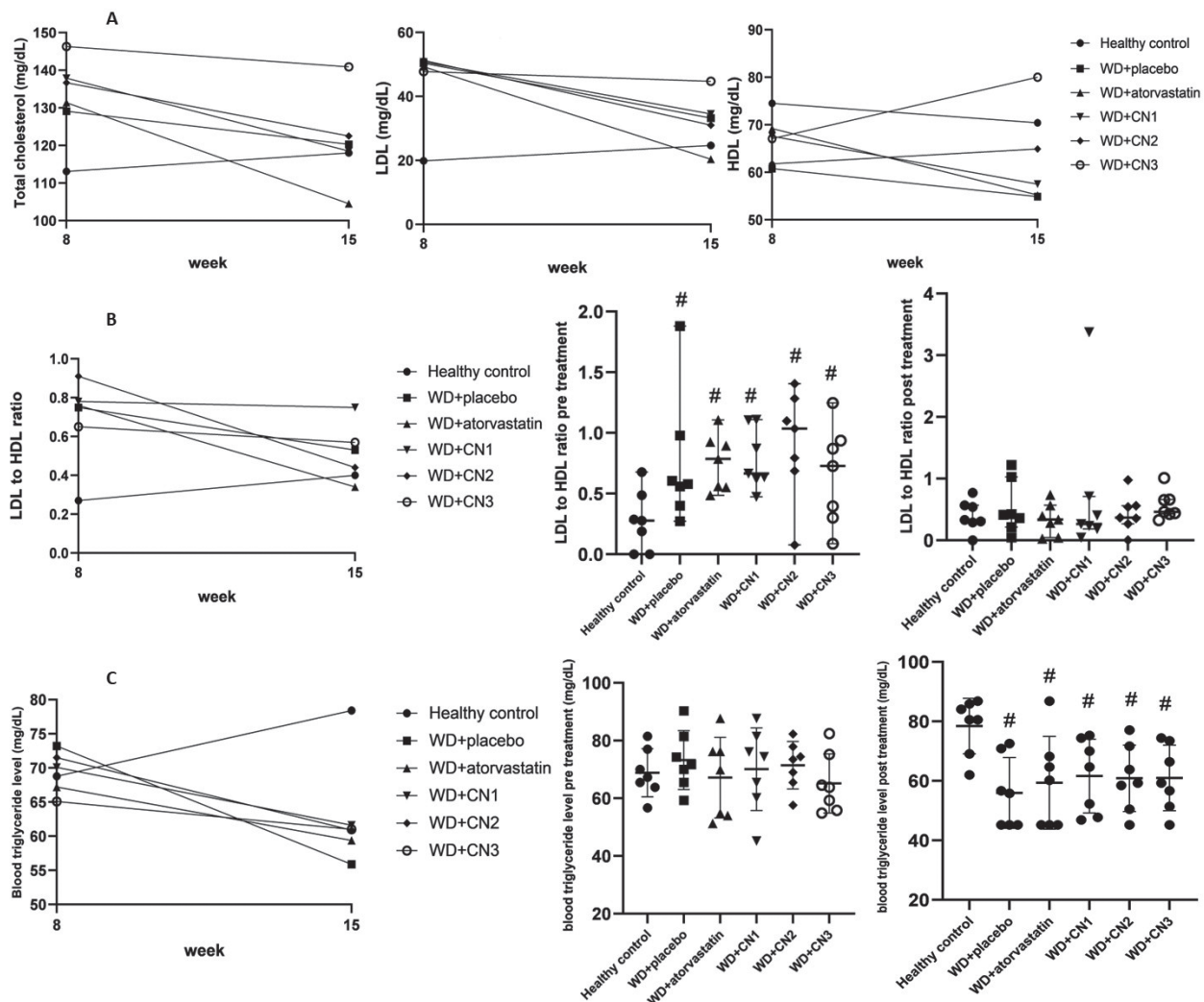


Figure 2. A. Total cholesterol, LDL, and HDL levels; B. LDL-to-HDL ratio. # $p < 0.05$ compared with the healthy control group, Mann-Whitney; C. Blood triglyceride levels. # $p < 0.05$ compared with the healthy control group, LSD.

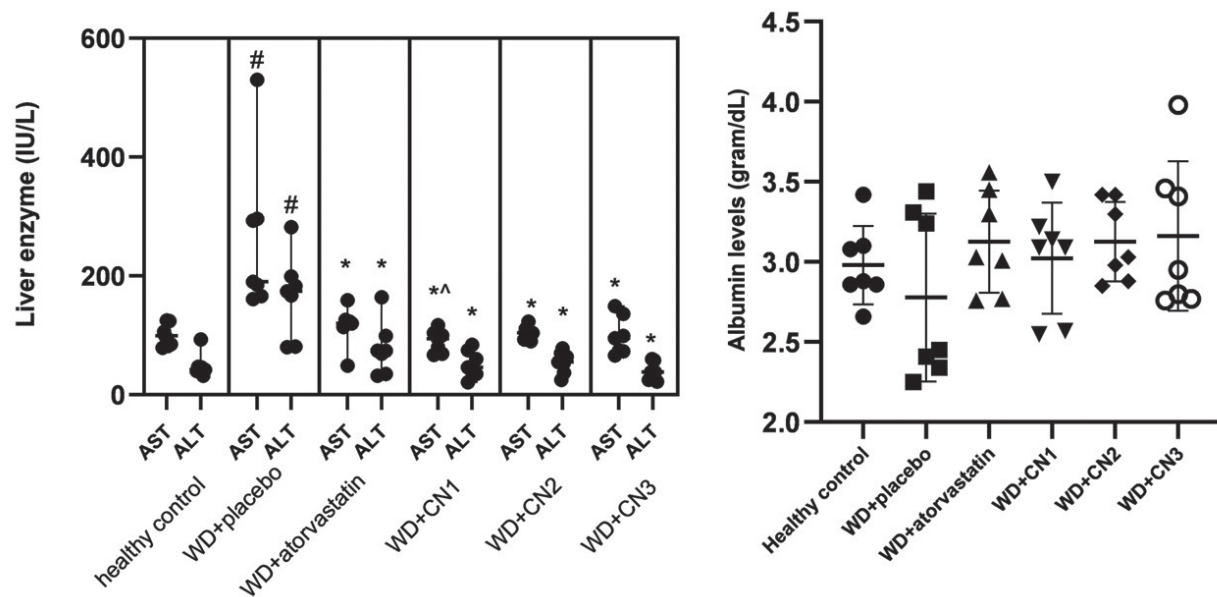


Figure 3. Blood levels of liver enzymes (AST and ALT) and albumin (n = 7). # p < 0.05 compared with the healthy control group; * p < 0.05 compared with the WD+placebo group; ^ p < 0.05 compared with the WD+atorvastatin group, Mann-Whitney. ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Western diet feeding for 8 weeks induced microvesicular and macrovesicular steatosis, with minimal features of lobular inflammation and ballooning. By week 15, prominent azonal macrovesicular steatosis was observed, along with inflammatory foci of leukocyte infiltration in necrotic areas, ballooned hepatocytes, and the presence of ropey, eosinophilic cytoplasmic inclusions of Mallory–Denk bodies (Suppl. material 1). All WD-fed groups demonstrated a significant increase in NAS compared with the healthy control group (p < 0.05). Treatment with either atorvastatin or *Clinacanthus nutans* extract at 100, 200, and 400 mg/kg improved liver histology, as indicated by significantly lower NAS relative to the placebo group (p < 0.05). Administration of *Clinacanthus nutans* extract at 400 mg/kg resulted in significantly lower NAS compared with atorvastatin, suggesting a comparable or potentially superior pharmacological effect in preventing the progression of MASL to MASH. Although the differences in NAS among CN-treated groups were not statistically significant, the WD+CN3 group tended to show the most pronounced histological improvement, as presented in Fig. 5. *Clinacanthus nutans* extract administration led to significant reductions in the pathological features of MASH. In particular, *Clinacanthus nutans* extract at 400 mg/kg/day administered for 7 weeks ameliorated liver histological features, as shown by significant decreases in steatosis, lobular inflammation, and ballooning scores compared with the placebo group (p < 0.05), with no significant differences relative to the healthy control group. Furthermore, the WD+CN3 group exhibited a significantly lower lobular inflammatory score than the WD+CN1 group, suggesting a dose-dependent effect of the *Clinacanthus nutans* extract.

In addition to inducing the liver pathologies characteristic of MASH, WD feeding significantly elevated hepat-

ic malondialdehyde (MDA) levels, indicating increased lipid peroxidation resulting from oxidative stress. Treatment with *Clinacanthus nutans* extract markedly reduced this effect (p < 0.05), as illustrated in Fig. 6. Notably, the WD+CN2 and WD+CN3 groups exhibited significantly lower hepatic MDA levels compared with the WD+atorvastatin group (p < 0.05), highlighting the superior antioxidative potential of the *Clinacanthus nutans* extract.

Discussion

The *Clinacanthus nutans* leaf extract investigated in this study was found to contain a substantial amount of secondary metabolites, which may contribute to its protective effects against liver pathology in WD-fed mice. The total phenolic and flavonoid contents of the *Clinacanthus nutans* extract in this study were higher than those reported in a previous study using a similar extraction method (Susanti et al. 2023). However, these were lower than in a Vietnamese study (Ngoc et al. 2023). The alkaloid content in the *Clinacanthus nutans* leaf extract was higher than that of a Malaysian study, but the tannin and saponin contents were lower. These variations may be attributed to differences in the solvent used for extraction and in the assay methods employed (Kong and Abdullah Sani 2019).

In our study, daily feeding with a western diet supplemented with cholesterol and a high-sugar solution successfully induced MASL within approximately 8 weeks, with progression to steatohepatitis (MASH) by week 15; however, no overt features of metabolic syndrome were observed. Although the WD-fed groups exhibited trends toward elevated total cholesterol and LDL levels and reduced HDL levels at week 8, these differences did not

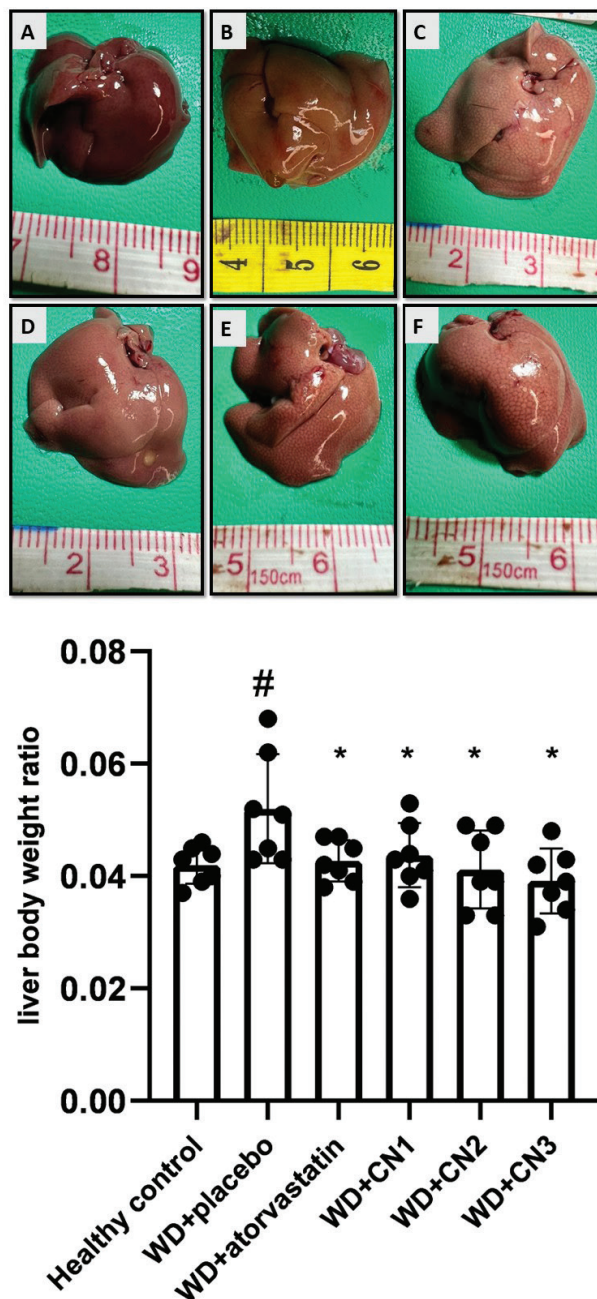


Figure 4. Macroscopic features of the liver. Above: A. Healthy control; B. WD+placebo; C. WD+atorvastatin; D. WD+CN1; E. WD+CN2; F. WD+CN3. Below: Liver-to-body weight ratio of all groups (n = 7). # p < 0.05 compared with the healthy control group; * p < 0.05 compared with the WD+placebo group, LSD.

reach statistical significance. Several factors may underlie this observation, including the use of wild-type mice, which are known to possess metabolic resilience; the moderate duration of dietary exposure; and thermoneutral housing conditions, all of which may have mitigated the development of overt metabolic disturbances despite the hepatic pathology (Vacca et al. 2024).

Interestingly, a significantly higher LDL-to-HDL ratio was observed in the WD-fed groups compared with the healthy control group at week 8. This index is commonly

employed in clinical settings as a reliable estimator of coronary heart disease risk (Lemieux et al. 2001) and has recently been shown to outperform individual lipid parameters in predicting atherosclerotic disease (Sun et al. 2022). The association between MASLD and atherosclerotic cardiovascular disease is well established in both clinical and epidemiological studies (Arslan and Yenerçag 2020). In preclinical settings, this dual pathology is frequently observed in genetically modified murine models subjected to dietary interventions, whereas it is rarely seen in wild-type animals (Van Herck et al. 2017). Whether the elevated LDL-to-HDL ratio observed in this study can accurately predict cardiovascular risk in MASLD mice remains to be determined and warrants further investigation. However, by 15 weeks, a significantly higher LDL-to-HDL ratio in the WD-fed groups was no longer observed. Presumably, at a more advanced stage, the synthetic function of the liver started to decline, including very low-density lipoprotein (VLDL) synthesis. This agrees with a finding from a US population-based study suggesting an association between both low LDL and high LDL with higher odds of elevated AST and ALT (Jiang et al. 2014).

Although hypertriglyceridemia is recognized as a metabolic risk factor for the development of MASLD, most dietary models of the disease do not induce elevated circulating triglyceride levels (Vacca et al. 2024). In the present study, a significant reduction in plasma triglyceride concentrations was observed in the WD-fed groups compared with the healthy control group after 15 weeks, despite marked intrahepatic lipid accumulation. This paradoxical finding may be attributed to impaired VLDL secretion, potentially driven by endoplasmic reticulum (ER) stress and mitochondrial dysfunction in hepatocytes (Kawano and Cohen 2013; Chiang Morales et al. 2022). Interestingly, while elevated triglycerides are implicated in the onset of MASLD, lower fasting triglyceride levels have been linked to advanced disease states of MASH and indices of liver fibrosis (Jiang et al. 2015).

The significant elevation of AST and ALT levels observed in this study reflects marked hepatocellular injury, most likely driven by lipotoxicity, a key pathogenic mechanism underlying the progression from MASL to MASH (Geng et al. 2021). One of the critical consequences of lipotoxicity is mitochondrial dysfunction, which promotes the generation of reactive oxygen species (ROS). These ROS target cellular macromolecules and induce lipid peroxidation (Allameh et al. 2023), as evidenced by the increased MDA levels detected in the liver tissues of the untreated WD-fed group in the present study. Notably, the administration of *Clinacanthus nutans* extract effectively attenuated this oxidative damage, underscoring its hepatoprotective potential. The antioxidant activity of *Clinacanthus nutans* extract is likely attributable to its polyphenolic constituents, particularly tannins, which may act through direct free radical scavenging or by activating nuclear factor erythroid 2-related factor 2 (Jing et al. 2022).

The ethanol extract of *Clinacanthus nutans* leaf prevented the progression of MASL to MASH, as indicated

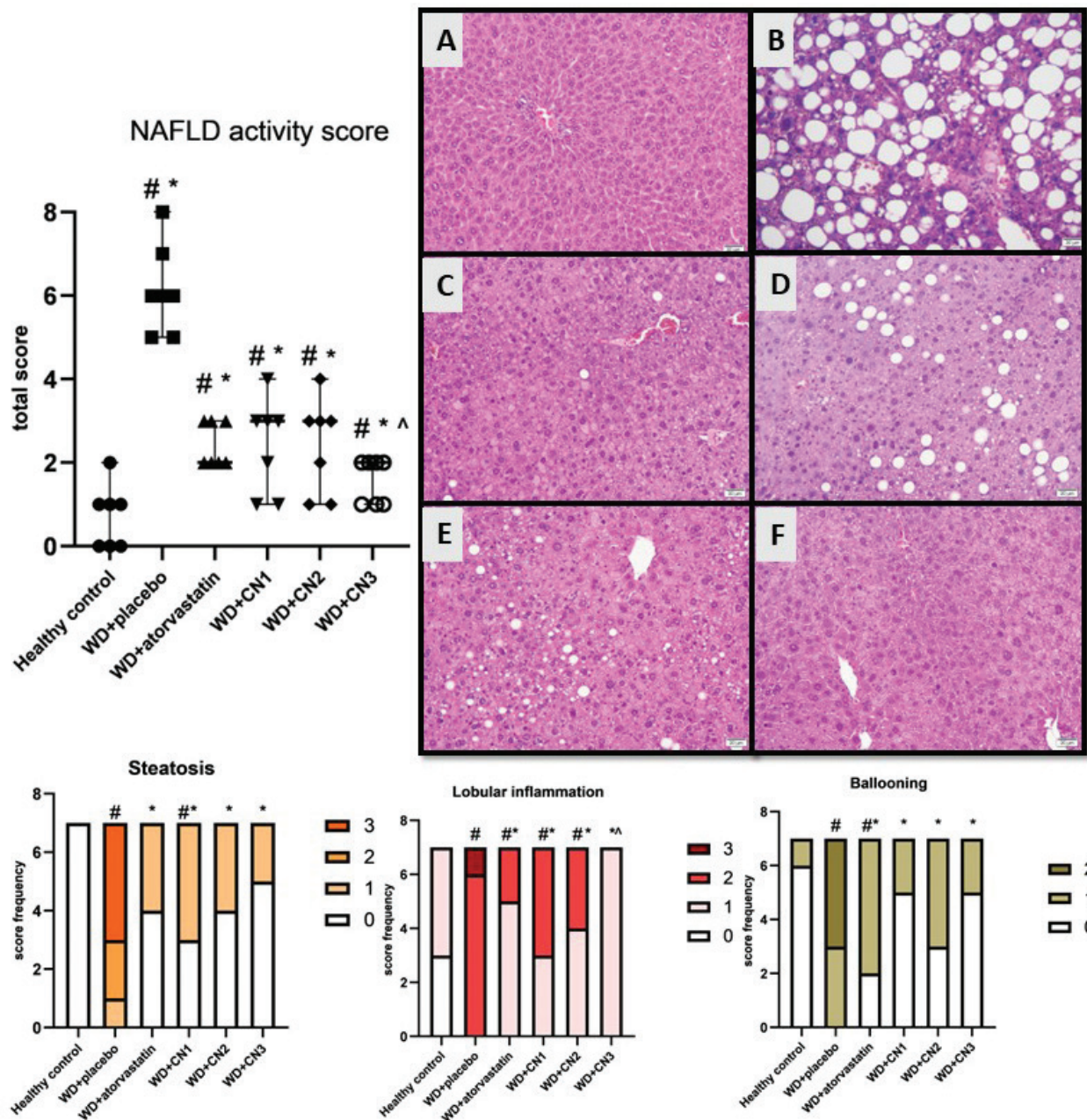


Figure 5. Histological features of the liver. Above: left, NAFLD activity score as the total of steatosis, lobular inflammation, and ballooning scores. # $p < 0.05$ compared with the healthy control group; * $p < 0.05$ compared with the WD+placebo group; ^ $p < 0.05$ compared with the WD+atorvastatin group, Mann–Whitney U test. Right: HE slides of liver sections across all groups (light microscopy, 200 $\times$). A. Healthy control; B. WD+placebo; C. WD+atorvastatin; D. WD+CN1; E. WD+CN2; F. WD+CN3. Bottom (left–right): Frequency of mice according to the scores of steatosis, lobular inflammation, and ballooning features. # $p < 0.05$ compared with the healthy control group; * $p < 0.05$ compared with the WD+placebo group; ^ $p < 0.05$ compared with the WD+CN1 group, Mann–Whitney U test.

by the significantly reduced AST and ALT enzymes in the CN-treated groups versus the untreated WD-fed group. These enzyme levels were comparable with those of the healthy control group, with AST reduction even surpassing that achieved with atorvastatin treatment. Statins, such as atorvastatin, are currently recommended in the management of MASLD, particularly for patients with coexisting dyslipidemia, to mitigate cardiometabolic risks (Fan et al. 2024). Moreover, extensive preclinical and clin-

ical studies have highlighted the efficacy of statins in improving liver-related outcomes (Zhang et al. 2024; Yun et al. 2025). Although concerns have been raised regarding their hepatic safety due to the potential for mild and transient elevations in transaminase enzymes (Liu et al. 2010), a recent meta-analysis has reaffirmed the favorable safety profile of statins in this context (Pastori et al. 2022).

Improvement in both macroscopic and microscopic liver features was observed in the WD-fed groups treated

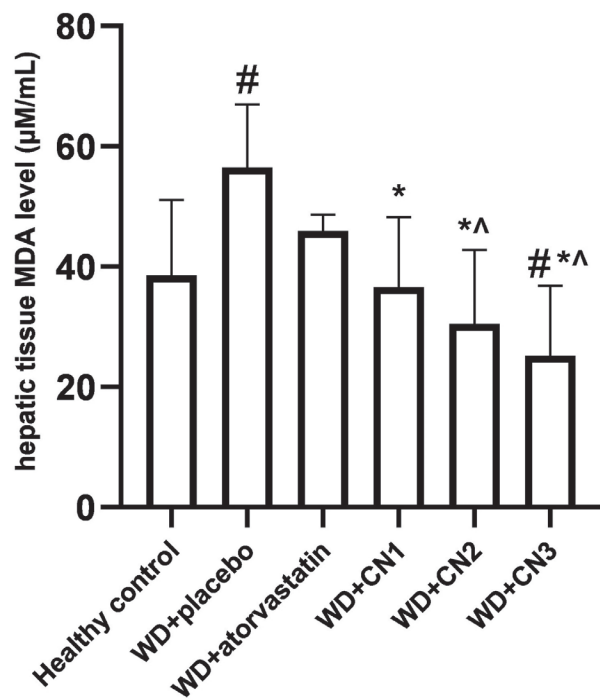


Figure 6. Hepatic malondialdehyde (MDA) levels across all groups. #, $p < 0.05$ compared with the healthy control group. *, $p < 0.05$ compared with the WD+placebo group. ^, $p < 0.05$ compared with the WD+atorvastatin group, $n = 7$, LSD.

with *Clinacanthus nutans* extract at all tested doses. This was evidenced by significantly lower liver-to-body weight ratios, NAFLD activity scores, and reductions in steatosis, lobular inflammation, and hepatocyte ballooning compared with the placebo group. Although the improvements generally exhibited a dose-dependent trend, a statistically significant difference between doses was only observed in lobular inflammation scores, specifically between the 400 mg/kg and 100 mg/kg groups. These hepatoprotective effects are consistent with findings from previous studies. Daily administration of the ethanol extract of *Clinacanthus nutans* leaves at doses of 100, 200, and 400 mg/kg for 28 days was shown to improve liver morphology and function in streptozotocin-induced diabetic rats. This was indicated by reductions in AST and ALT serum levels along with attenuation of hepatocyte degeneration and necrosis. Furthermore, increased hepatic MDA levels and decreased superoxide dismutase (SOD) levels were observed, suggesting that the antioxidant activity of *Clinacanthus nutans* may be a key mechanism underlying its hepatoprotective effects. However, it should be noted that streptozotocin induces type 1 diabetes mellitus, which rarely gives rise to MASLD (Susanti et al. 2024b).

In contrast to a previous study on Sprague–Dawley rats fed a high-fat, high-cholesterol diet, where aqueous and aqueous methanolic extracts of *Clinacanthus nutans* leaves reduced body weight (Sarega et al. 2016a), our study found no significant weight-lowering effect with *Clinacanthus nutans* extract. In this MASLD model, *Clinacanthus nutans* extract did not improve lipid metabolic

parameters. Beyond its antioxidative effects, *Clinacanthus nutans* extract may also exert benefits through modulation of gut microbiota. A previous study reported that the aqueous extract of *Clinacanthus nutans* leaf prevented liver deterioration in hepatitis B virus-infected BALB/c mice, an effect likely mediated by improvements in gut microbial balance (Zhang et al. 2023).

This study offers new insights into the potential utilization of *Clinacanthus nutans* extract for preventing the progression of MASLD to its severe form of steatohepatitis. *Clinacanthus nutans* has long been used as a traditional remedy with efficacy and safety claims for various conditions that have been scientifically proven. It is also appreciated as one of the richest sources of naturally occurring antioxidants (Chia et al. 2021). Therefore, its potential benefit in MASLD might be comparable with other hepatoprotective plant-based compounds (Vrentzos et al. 2025). This study has several limitations to take into account. First, liver fibrosis was not evaluated, since observation on HE slides hardly detects early fibrosis. Other methods, such as Sirius red staining and α -SMA immunohistochemistry, are better for identifying early-stage fibrosis (Carpino et al. 2005; Arjmand et al. 2020). Furthermore, at least 32 weeks of high-cholesterol WD feeding is required to induce fibrosis in C57BL/6 mice (Vacca et al. 2024). A duration of 15 weeks was not sufficient to induce fibrosis. Thus, the MASLD model in this study corresponds to early MASH in non-obese individuals. Second, we only measured hepatic MDA levels, while oxidative stress-targeting proteins or DNA were not examined. Third, as a preliminary study, mechanistic pathways were not explored and should be addressed in future investigations.

Conclusion

The ethanolic extract of *Clinacanthus nutans* leaf demonstrated hepatoprotective activity in male C57BL/6 mice subjected to a cholesterol-supplemented western diet feeding, effectively preventing the progression of liver damage from metabolic dysfunction-associated steatotic liver (MASL) to metabolic dysfunction-associated steatohepatitis (MASH). This protective effect is likely mediated by its antioxidant properties, as evidenced by a reduction in malondialdehyde (MDA), a marker of lipid peroxidation. The high tannin content of this indigenous Southeast Asian plant may contribute to its ability to attenuate oxidative stress and ameliorate liver pathology, highlighting its potential relevance in the management of MASLD.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statements

The authors declared that no clinical trials were used in the present study.

The authors declared that no experiments on humans or human tissues were performed for the present study.

The authors declared that no informed consent was obtained from the humans, donors or donors' representatives participating in the study.

Experiments on animals: Committee of Health Research Ethics, Faculty of Medicine, Universitas Airlangga, Indonesia (94/EC/KEPK/FKUA/2024).

The authors declared that no commercially available immortalised human and animal cell lines were used in the present study.

Use of AI

No use of AI was reported.

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Author contributions

Concept and design: Nurina Hasanatuludhhiyah, Arifa Mustika. Data acquisition: Nurina Hasanatuludhhiyah. Data analysis/interpretation and drafting manuscript: Nurina Hasanatuludhhiyah, Arifa Mustika, Viskasari Pintoko Kalanjati. Critical revision of manuscript: Hirokazu Wakuda. Funding: Nurina Hasanatuludhhiyah. Supervision: Arifa Mustika, Viskasari Pintoko Kalanjati, Naoto Uemura, Muhammad Miftahussurur. Final approval: Nurina Hasanatuludhhiyah, Arifa Mustika, Viskasari Pintoko Kalanjati, Hirokazu Wakuda, Muhammad Miftahussurur, Naoto Uemura.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

The characteristic features of the MASH of the liver section in the WD-fed groups

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Data type: jpg

Explanation note: Left: prominent azonal macrovesicular steatosis (HE, 40X); Center: inflammatory foci of leukocyte infiltration in the necrotic area indicated by red arrow (HE, 200X); Right: ballooned hepatocytes indicated by blue arrow with the presence of ropey, eosinophilic cytoplasmic inclusions of Mallory-Denk bodies indicated by black arrow (HE, 400X).

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