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Triterpenoid-Enriched Bitter Melon Leaf Extract Mitigates Hepatic Lipotoxicity and Necroptosis in Diet-Induced Obese Zebrafish

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ABSTRACT

Background: Metabolic dysfunction–associated steatotic liver disease (MASLD) is closely linked to obesity, lipid dysregulation, and hepatic oxidative stress. Wild bitter melon (WBM, *Momordica charantia* L. var. *abbreviata* Ser.) leaves contain triterpenoids that have demonstrated anti-inflammatory and hepatoprotective activities.

Methods: A diet-induced obesity (DIO) zebrafish model was established using a hyperphagic feeding regimen. The effects of a triterpenoid-enriched extract (TEE) derived from WBM leaves were evaluated. Metabolic parameters, hepatic lipid accumulation, oxidative stress markers, and gene expression profiles were analyzed.

Results: TEE treatment significantly reduced fasting blood glucose and cholesterol levels without affecting body weight or body mass index. Hepatic lipid accumulation and triglyceride levels were markedly decreased, accompanied by downregulation of genes associated with lipogenesis and cholesterol metabolism. TEE also reduced hepatic lipid peroxidation, as indicated by decreased thiobarbituric acid–reactive substances (TBARS), while reduced glutathione levels remained unchanged. In addition, expression of necroptosis-related genes was suppressed.

Conclusions: TEE exerts hepatoprotective effects in a zebrafish DIO model by improving lipid and glucose metabolism and attenuating lipid-associated oxidative damage. These findings highlight the potential of TEEs as natural interventions for MASLD and support the use of zebrafish as a model for studying metabolic improvements independent of weight reduction.

1 | Introduction

Metabolic dysfunction–associated steatotic liver disease (MASLD) [1], formerly known as nonalcoholic fatty liver disease (NAFLD), is the most prevalent chronic liver condition globally, affecting approximately 25%–30% of adults [2, 3]. MASLD encompasses a spectrum of hepatic pathologies ranging from simple steatosis to metabolic dysfunction–associated steatohepatitis (MASH), fibrosis, and hepatocellular carcinoma [4]. Its pathogenesis is multifactorial, with obesity, insulin resistance, and excessive dietary fat intake serving as major contributors to hepatic lipid accumulation, oxidative stress, and chronic inflammation [5, 6].

Recent studies have identified necroptosis, a regulated form of necrotic cell death mediated by receptor-interacting protein kinases RIPK1 and RIPK3, as a critical driver of hepatic inflammation and fibrogenesis in MASLD [7]. Despite advances in

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understanding these molecular mechanisms, effective therapeutic options remain limited, prompting growing interest in natural compounds with hepatoprotective properties.

Momordica charantia L., commonly known as bitter melon, is a traditional medicinal plant widely used in Asia for its antidiabetic, antioxidant, and anti-inflammatory effects [8, 9]. While most research has focused on the fruit, emerging evidence suggests that wild bitter melon (WBM) leaves are particularly rich in cucurbitane-type triterpenoids. Our previous studies have demonstrated that key triterpenoids, such as kuguacin R and 3β,7β,25-trihydroxycucurbita-5,23-dien-19-al, exhibit potent anti-inflammatory and antifibrotic activities in models of periodontal and chemically induced liver injury [10, 11]. However, their efficacy in mitigating diet-induced hepatic lipotoxicity and necroptosis remains unclear.

Zebrafish (*Danio rerio*) have emerged as a powerful vertebrate model for studying metabolic diseases because of their genetic homology with humans and conserved lipid metabolism [12, 13]. Adult zebrafish subjected to diet-induced obesity (DIO) develop hyperglycemia, hypercholesterolemia, and hepatic steatosis, closely resembling aspects of human MASLD, making them an attractive system for evaluating hepatoprotective interventions [14–17].

In this study, we examined the effects of a triterpenoid-enriched extract (TEE) derived from WBM leaves on hyperphagia-induced hepatic lipotoxicity and necroptosis in obese zebrafish. By elucidating the protective mechanisms of WBM-derived triterpenoids, we aimed to explore their potential as natural therapeutic agents for preventing and managing MASLD.

2 | Materials and Methods

2.1 | Experimental Animals

Five- to six-month-old adult AB male zebrafish were maintained in a recirculation aquaculture system. The aquaculture system was maintained at 28.5°C ± 0.5°C under a light/dark cycle of 14/10 h [18, 19]. To induce obesity, all subjects were subjected to a seven-meal hyperphagia regimen (every 2 h during the light cycle, 70 mg/fish/day) with dry commercial food (crude protein: 50.18%; crude fat: 10.54%; energy density: 4658 kcal/kg) for 2 weeks. After the DIO phenotype was established, zebrafish were randomly assigned to two groups receiving either TEE dissolved in olive oil (10 mg/kg body weight) or olive oil only (vehicle control, 8.19 cal) via oral gavage once a day [20], while the seven-meal hyperphagia regimen continued for an additional 2 weeks. All experimental procedures in this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of National Taiwan University (NTU-110-EL-00129) and performed in accordance with the approved guidelines.

2.2 | TEE Preparation

In our previous studies, the major cucurbitane-type triterpenoids present in this fraction, including kuguacin R and 3β,7β,25-trihydroxycucurbita-5,23-dien-19-al (TCD), were identified and structurally characterized using chromatographic and spectroscopic analyses [11]. Briefly, fresh WBM leaves (Hualien No.1, Hualien, TW) were washed with water, air-dried, and ground using a blender. The resulting leaf powder was extracted with ethanol (1:20, w/v) at room temperature for 24 h with continuous stirring. The mixture was filtered through Whatman No. 1 filter paper under suction, and the solvent was removed using a rotary evaporator (EYELA, Tokyo, JP) to obtain the crude ethanol extract. The crude extract was redissolved in ethyl acetate and fractionated by open-column chromatography using n-hexane/ethyl acetate (80/20, v/v) as the eluent. Fractions were pooled based on thin-layer chromatography profiles (Fraction 1–5). The triterpenoid content of each fraction was quantified using vanillin-sulfuric acid colorimetric assay [21], with protopanaxadiol as the calibration standard. Fraction 5, which consistently exhibited the highest triterpenoid content, was collected and redissolved in acetone before final evaporation to yield TEE. The yield of TEE was 313-mg protopanaxadiol equivalents per g. This study utilized the same batch of Fraction 5 as characterized in our earlier reports [11].

2.3 | Body Measurements and Blood Biochemistry

To assess the effects of obesity and TEE on overall physiological condition, each test subject's body weight and length were measured and recorded at 0, 7, 10, 14, 18, 21, 25, and 28 days. Briefly, after being anesthetized with 0.016% ethyl 3-aminobenzoate methanesulfonate (MS-222, Sigma-Aldrich, St. Louis, MO, USA), excess water was removed using tissue paper, and the subjects were weighed on a precision balance (PJ3600, Mettler-Toledo, Columbus, OH, USA), photographed, and immediately returned to the water. The body length of the fish was measured using ImageJ to determine the body mass index (BMI) [22, 23]. At the time of sacrifice, blood was collected from the fish's heart, and fasting blood glucose and cholesterol levels were determined using a glucometer (Accu-Chek, Fora).

2.4 | Histology

After being sacrificed, whole zebrafish were fixed in Davidson's fixative solution (BioTnA Biotech, Kaohsiung, TW) for 24 h and then transferred to a 10% formalin solution (Sigma-Aldrich). To evaluate the progress of MASLD caused by obesity, anatomically comparable cryosections of the zebrafish liver were stained for 20–30 min with freshly prepared Oil-Red-O (BioTnA Biotech) following a standard protocol. The cryosections were fully immersed in Mayer's hematoxylin for 1–2 min for nuclei counterstaining. Staining intensity was analyzed using microscopy and ImageJ [22].

2.5 | Protein, Cholesterol, and Triglyceride (TG) Measurements

To assess the lipid content in the liver, half of the liver was collected in a tube containing 200 μL of lysis solution. The homogenized liver was centrifuged, and the supernatant was collected to quantify cholesterol, TG, and protein levels. Cholesterol (Cholesterol/Cholesterol Ester-Glo Assay Kit, Promega, Madison, WI, USA) and TG (Triglyceride-Glo Assay kit, Promega) levels were evaluated following the manufacturer's recommendations by measuring the luminescence using a plate-reading luminometer (GLOMAX, Promega). For total protein levels (T-Pro BCA Protein Assay kit, T-Pro Biotechnology, New Taipei County, TW), the absorbance at 562 nm was measured (CLARIOstar, BMG Labtech, Offenburg, DE) to determine the total protein concentrations of each sample for data normalization.

2.6 | Assessment of Hepatic Oxidative Stress

To evaluate hepatic oxidative stress, the hepatic lysate's glutathione (GSH) and thiobarbituric acid reactive substances (TBARS) were quantified. Briefly, half of the liver from each tested subject was collected in a tube, and the tissue was perfused with PBS containing heparin to remove blood and clots. The liver was homogenized on ice with PBS containing 2-mM EDTA. The supernatants were collected to analyze GSH and protein after centrifugation at 10,000 × g for 5 min. GSH levels were assessed using the GSH-Glo Glutathione Assay kit (Promega) following the manufacturer's protocols. Luminescence was then measured using a plate-reading luminometer (GLOMAX, Promega) to determine the GSH level.

For TBARS measurement, the liver of each tested fish was collected in a tube, and blood was removed from the tissue by perfusion with PBS containing heparin. The liver was resuspended in PBS at a concentration of 50–100 mg/mL. To prevent further oxidation, 100 × BHT solution was added to achieve a final concentration of 1×. The liver was homogenized on ice and spun at 10,000 × g for 5 min to collect the supernatant. The supernatant was directly assessed for its TBARS level according to the manufacturer's protocols (OxiSelect TBARS Assay Kit, Cell Biolabs, San Diego, CA, USA). The absorbance at 532 nm was measured using a CLARIOstar (BMG Labtech). The GSH and TBARS levels were normalized based on the amount of protein in each sample.

2.7 | Quantitative Reverse Transcription PCR (qRT-PCR)

To extract the total RNA, the liver of one fish was homogenized in 500-μL GENEzol Reagent (Geneaid, New Taipei County, TW). The homogenate was mixed with 50 μL of 1-bromo-3-chloropropane and then centrifuged at 16,000 × g for 15 min at 4°C. The aqueous phase was transferred into a new tube and mixed with an equal volume of isopropanol. The mixture was incubated for 10 min and centrifuged at 16,000 × g for 10 min at 4°C. The pellet was then washed with 1 mL of 75% ethanol, briefly air-dried, and dissolved in DEPC-treated water. Total RNA was treated with DNase I for 30 min at 37°C following precipitation with LiCl. The single-stranded cDNA was synthesized from 2 μg of total RNA with random primers and SuperScript III reverse transcriptase (Invitrogen, Waltham, MA, USA). To quantitatively analyze the expression levels of target genes, 4 μL of 10 × diluted cDNA was mixed with 5 μL of iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and 1 μL of primer set mix. To amplify the fragments of target genes, the PCR reaction was as follows: 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 15 s, followed by 72°C for 30 s. The sequences of primer pairs are listed in Table 1.

2.8 | Statistical Analysis

To analyze the effect of TEE on body weight and BMI, two-way analysis of variance (ANOVA) followed by the Bonferroni test was used. To analyze the effect of TEE on fasting blood glucose and cholesterol, one-way ANOVA followed by Tukey's post hoc test was performed. The incidence of metabolic abnormalities, including hyperglycemia and hypercholesterolemia, was compared between groups using the Chi-square test with Yates' correction. All other comparisons between TEE and vehicle control were analyzed using the Mann–Whitney U test. A p value of < 0.05 was considered statistically significant. All data are presented as the mean ± standard error of the mean (SEM).

3 | Results

3.1 | TEE Improves Glucose and Cholesterol Homeostasis Without Affecting Body Weight

To evaluate the metabolic effect of TEE, a DIO zebrafish model was established using a hyperphagia feeding regimen for 4 weeks as previously described [14] (Figure 1). Compared with the normal diet control group (Ctrl), zebrafish subjected to hyperphagia exhibited significantly increased body weight and BMI after 11 and 7 days of feeding, respectively (Figures 1(a) and 1(b)). In addition, fasting blood glucose levels were significantly elevated after 2 and 4 weeks of hyperphagia feeding (Figure 1(c)). Histological examination of the liver using Oil Red O staining further revealed marked hepatic lipid accumulation in DIO zebrafish compared with the Ctrl group (Figure 1(d)). These results confirm the successful establishment of the DIO model in our laboratory.

After 2 weeks of hyperphagia feeding to induce obesity, zebrafish were randomly assigned to receive either TEE dissolved in olive oil (TEE) or olive oil alone as the vehicle control (VEH), while the hyperphagia regimen continued for an additional 2 weeks. During this period, both groups exhibited significant increases in body weight (Figure 2(a), $p < 0.0001$, two-way ANOVA) and BMI (Figure 2(b), $p < 0.0001$, two-way ANOVA) over time. However, no significant differences in body weight ($p = 0.7791$) or BMI ($p = 0.9066$) were observed between the groups.

Interestingly, fasting blood glucose levels were significantly lower in the TEE group than in the VEH group and were comparable to those of the Ctrl group (Figure 2(c)). Based on previously reported fasting blood glucose levels in healthy adult zebrafish (59 ± 5.13 mg/dL) [24], hyperglycemia was defined as values exceeding two standard deviations above the mean (69.26 mg/dL). Under this criterion, none of the fish in the TEE group were classified as hyperglycemic ($n = 17$), whereas 8 of 19 fish in the VEH group exceeded this threshold ($p = 0.0085$, Chi-square with Yates' correction). A similar classification was obtained using the threshold calculated from our Ctrl group (75.58 mg/dL; dotted line in Figure 2(c)).

Consistent with the improvement in glucose homeostasis, fasting blood cholesterol levels were also significantly lower in the TEE group than in the VEH group (Figure 2(d)). The cholesterol levels in the TEE-treated zebrafish were comparable to those observed in the Ctrl group (214.60 ± 13.01 mg/dL, dotted line in Figure 2(d)) and to previously reported normal levels in zebrafish (6.20 ± 0.50 mmol/L; 239.33 ± 12.13 mg/dL) [25].

3.2 | TEE Supplementation Ameliorates Hepatic Lipid Accumulation

Since the liver is among the most essential organs governing the metabolic homeostasis, it is intriguing to investigate the effect of TEE supplementation on the liver in DIO. Histological examination of Oil Red O staining revealed drastic neutral lipid accumulation in the liver of the VEH-treated DIO zebrafish (Figure 3(a)). In contrast, the livers of DIO zebrafish

TABLE 1 | The oligo sequences used in this study.

Gene name	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
acaca	ACGCTTTGTTGTCATGGTGA	TTCTTGTGCAGCAATTCAGG
acacb	GTGGCTGGAAAACAACCTGT	CCATGTGAATGATGCAGTCC
fasn	GCACCGGTACTAAGGTTGGA	ACACAACCGACCATCTGTCA
scd1	GCAGGCGTTCACAGATTGTGG	CATAGATGTCATTCTGGAAGGCC
fads2	CCCGTGGAGTATGGCGTTA	TGTGGCTCATCTGTGTGACC
acadl	GCTGGATGAGTGATGTTGTGA	TTTTGGTAGCCCTTAGTGCC
acadm	AGGTTTTGAGGGCAGGTGTT	TCTGCTGCTCGGTTAGTTCA
acox1	CCTGGAGATTCTTACAAACG	TGAAATACACCCCTCACAAC
acsl1	AGCAGGAAAGAAGCGGAA	AAGTGAGGACGGTTGGAGAC
cpt1aa	CGATGAGGAGCACCAAAGAA	GTGGGAAAAGCGTAAAGAAAGT
Hmgcra	CTGAGGCTCTGGTGGACGTG	GATAGCAGCTACGATGTTGGCG
Hmgcrb	CCTGTTAGCCGTCAGTGGA	TCTTTGACCACTCGTGCCG
cyp7a1	GCAGCAGACAGAAGAAGCATG	ACGGATGACAGCATGGTAGG
abca1a	AAGGAACAATCATTAAGGAGCAA	CCACAACAGTAAACCCAACTGA
abca1b	GCCATGGTACTTCCCTTTCA	TCCTCTTCAATGCAAACAGC
abcg1	TCTTCAGAGGGATGCCACA	CCGCAAGTGAGTCAACACC
gck	TGAGGATGAAGAGCGAGGC	AGAGAAGGTGAATCCCAGTG
pck1	ATCACGCATCGCTAAAGAGG	CCGCTGCGAAATACTTCTTC
pfkla	AGGTATGAACGCAGCCATCC	TGCCAATCACTGTTCCTCCC
pfklb	TTTGAGCACAGGATGCCGAA	TCGATGCTAAGGGTTCGACG
ripk1	TCCTGGACCAAACCATCAGC	ACTGACTCCTCAATTCGGGC
ripk3	AGCAAAGCCTTCGACGTGTA	CATCCAGTCCTTCGGTCTCG
casp9	AAATACAGAGCAAGGCAACC	CACAGGGAATCAAGAAAGG
casp3a	CCGCTGCCCATCACTA	ATCCTTTCACGACCATCT
casp8	GGAATGATCTGGAAGCCTGGG	TCCGGCAAAAGGCAGTGTAA
actin	ATGGATGAGGAAATCGCTGCC	CTCCCTGATGTCTGGGTCGTC

supplemented with TEE showed minimal neutral lipid accumulation (Figure 3(b)). Accordingly, colorimetric quantification of Oil Red O staining indicates a significantly lower neutral lipid accumulation in the livers of the TEE group (Figure 3(c)).

One of the hallmarks of nonalcoholic fatty liver in humans is the accumulation of TGs in hepatocytes. Accordingly, a significantly higher level of TG was found in the liver of the VEH group than in the liver of the TEE group (Figure 4(a)). In line with the TG level, TBARS, a well-established marker of lipid peroxidation and oxidative stress, was also found to be significantly higher in the liver of the VEH group than in the TEE group (Figure 4(b)). However, reduced GSH was comparable between the two groups (Figure 4(c)). Unlike the blood cholesterol profile, the total cholesterol, free cholesterol, and cholesteryl esters were also similar between the two groups (Figures 4(d), 4(e), and 4(f)).

3.3 | TEE Supplementation Attenuates Hepatic Expressions of Lipogenic and Lipolytic Genes

To probe for the potential mechanisms underlying the lower TG accumulation in the TEE group zebrafish, RT-qPCR was performed to evaluate gene expression related to lipid metabolism. In accordance with the lower TG accumulation, the expressions of genes encoding acetyl-CoA carboxylase alpha (*acaca*), fatty acid synthase (*fasn*), and fatty acid desaturase 2 (*fads2*) were significantly lower in the TEE group compared with the VEH group (Figure 5(a)). In addition, the expressions of genes encoding acyl-CoA synthetase long-chain family member 1 (*acsl1*) and acyl-CoA dehydrogenase medium chain (*acadm*), which play important roles in acyl-CoA synthesis and lipid β-oxidation, were significantly lower in the TEE group (Figure 5(b)). In accordance with the lower fasting blood cholesterol level, the key enzyme for cholesterol *de novo* synthesis, 3-hydroxy-3-methylglutaryl-CoA reductase b (*hmgcrb*), was significantly lower in the TEE group, as well as the gene encoding for ATP-binding cassette subfamily A member 1 (*abca1*), which plays a role in hepatocyte cholesterol efflux (Figure 5(c)). In addition, Cytochrome P7a1 (*cyp7a1*), the rate-limiting enzyme that converts cholesterol to bile acid, was also significantly lower in the TEE group. In line with the lower fasting blood glucose in the TEE group, the expression of phosphoenolpyruvate carboxykinase 1 (*pck1*) was also significantly and drastically lower in the TEE group.

3.4 | Effect of TEE on Hepatic Inflammasome

Recent studies showed that inflammation and the necroptotic pathway play pivotal roles in liver damage during the progression

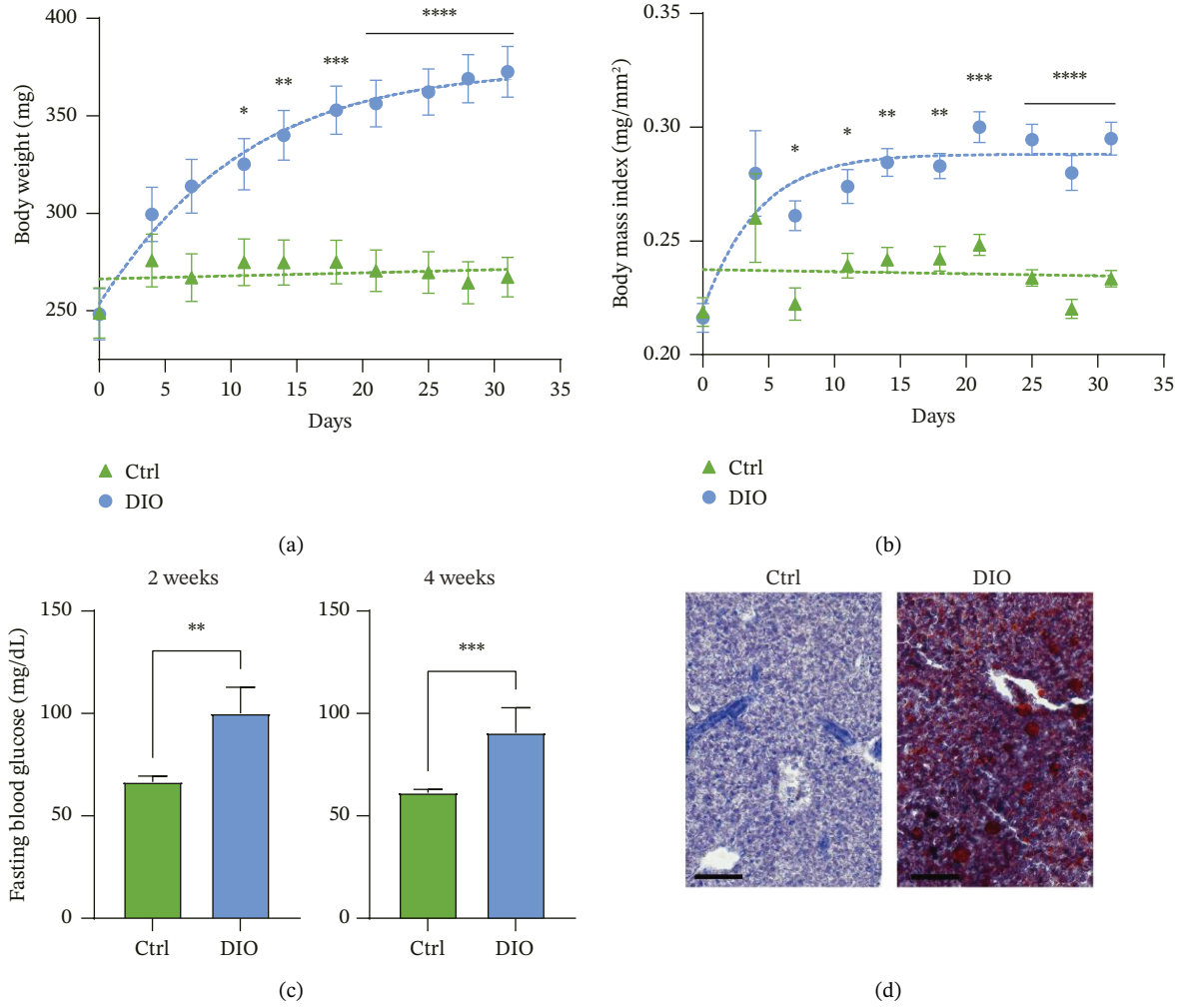


FIGURE 1 | Establishment of diet-induced obesity (DIO) in zebrafish. Zebrafish subjected to the hyperphagia feeding regimen exhibited significant increases in (a) body weight and (b) body mass index (BMI). The fasting blood glucose (c) was significantly elevated after 2 and 4 weeks hyperphagia feeding. The hepatic lipid accumulation (d), as determined by Oil Red O staining, was increased in DIO fish but not in normal diet control (Ctrl) fish.

of MASLD to MASH and potentially fibrosis and cirrhosis [7]. Accordingly, qPCR analysis of the liver tissue showed significantly lower expression levels of receptor-interacting serine/threonine-protein kinase 3 (*ripk3*) and caspase 8 (*casp8*), the key regulator and effector of necroptosis, in the TEE group than in the VEH group (Figure 6). Interestingly, the expression levels of *ripk1*, *casp3*, and *casp9* remained comparable between the two groups, suggesting that apoptosis is less involved in the liver damage correlated with lipid accumulation.

4 | Discussion

In this study, we demonstrated that treatment with a TEE derived from WBM (*Momordica charantia* L.) leaves significantly improved fasting blood glucose and cholesterol levels in a DIO zebrafish model without affecting body weight or BMI (Figure 1). Although both TEE-treated and vehicle-treated groups exhibited significant weight gain over time, no significant difference in body weight was observed between the groups. These findings indicate that TEE confers metabolic benefits independent of weight reduction.

This phenotype is conceptually related to the phenomenon of metabolically healthy obesity (MHO), which describes individuals who meet the anthropometric criteria for obesity but maintain relatively preserved metabolic homeostasis, including normal glucose regulation and lipid profiles [26]. However, zebrafish-specific criteria for MHO have not yet been established. Therefore, our findings should be interpreted as indicating that TEE improves metabolic parameters in obese zebrafish without altering body weight, a phenotype that resembles certain metabolic features of MHO. These results further support the utility of the zebrafish DIO model as a platform for investigating metabolic improvements independent of weight loss.

The normalization of fasting glucose levels in the TEE-treated group is particularly noteworthy, as none of the treated fish exceeded the hyperglycemia threshold, whereas a substantial proportion of vehicle-treated zebrafish did (Figure 2(a)). This suggests that TEE exerts protective effects against diet-induced hyperglycemia. Previous transcriptomic studies in overfed zebrafish have shown that high-fat diets induce insulin resistance and dysregulate genes involved in glucose metabolism [17, 27]. TEE may modulate these pathways, potentially through mechanisms such as AMP-activated protein kinase (AMPK) activation and suppression of gluconeogenic enzymes, as reported for *Momordica charantia*–derived compounds [9, 28].

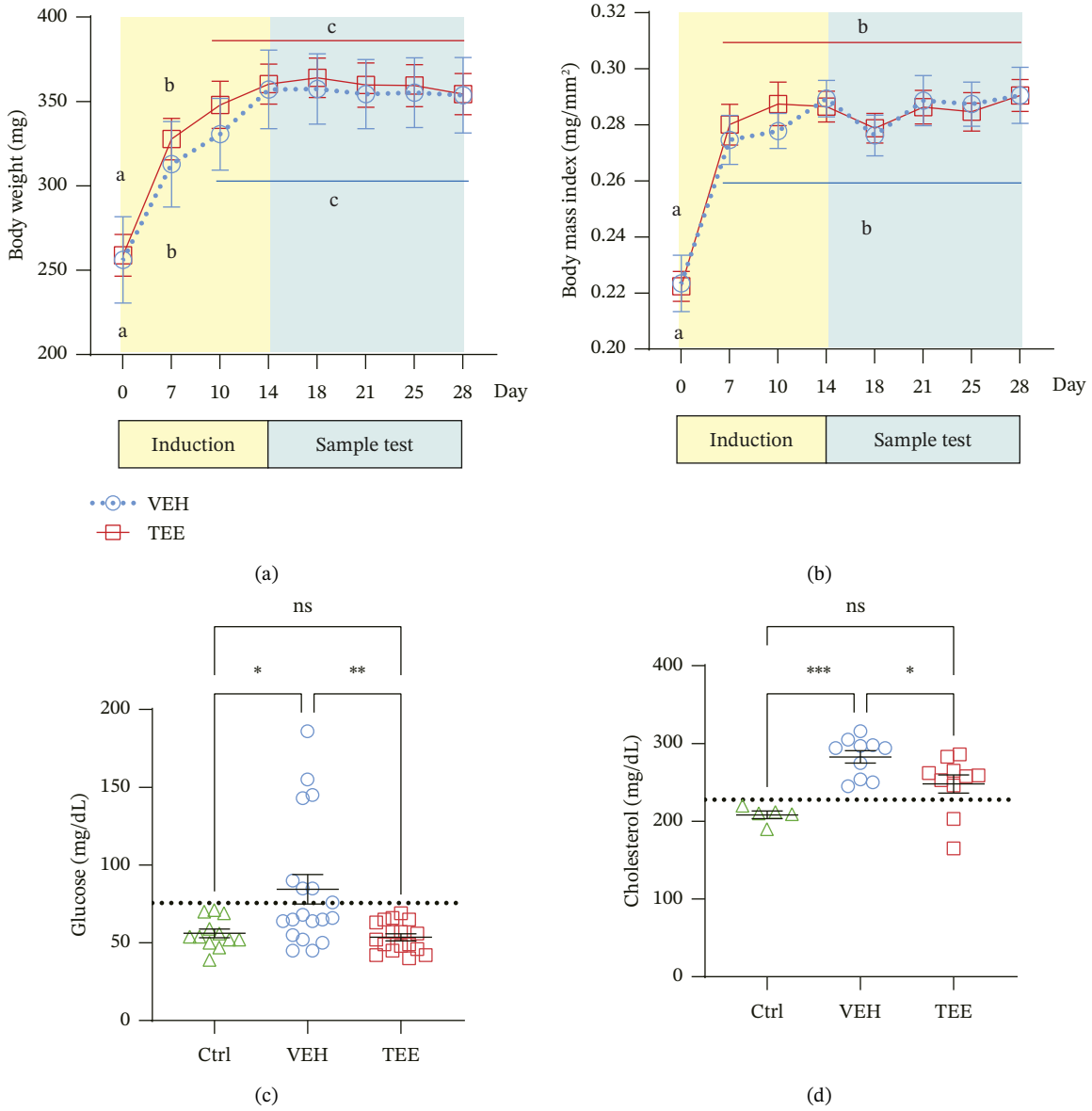


FIGURE 2 | TEE does not affect body measurements but improves fasting blood glucose and cholesterol in DIO zebrafish. Adult zebrafish were subjected to hyperphagic feeding to establish a diet-induced obesity (DIO) model and treated with olive oil (vehicle, VEH) or TEE dissolved in olive oil (TEE). (a) Body weight and (b) body mass index (BMI) increased significantly in both groups during the induction period ($p < 0.0001$, two-way ANOVA, F-test). However, no significant differences were detected between TEE and VEH group fish for either body weight ($p = 0.7791$) or BMI ($p = 0.9066$). Data are presented as mean ± SEM. Different letters indicate significant differences between time points within a group ($p < 0.05$, Bonferroni post hoc test). (c) Fasting blood glucose was significantly lower in the TEE group compared with VEH groups and was comparable to normal diet control (Ctrl). The dotted line indicates the hyperglycemia threshold calculated from uninduced control zebrafish (75.58 mg/dL). No TEE group fish were hyperglycemic (0/17), whereas 8 of 19 VEH group fish exceeded the threshold ($p = 0.0085$, chi-square with Yates' correction). (d) Fasting blood cholesterol was significantly reduced in the TEE group compared with the VEH group and was similar to normal diet control (Ctrl). The dotted line indicates the mean cholesterol level in uninduced controls (214.60 ± 13.01 mg/dL). Data are presented as mean ± SEM.

Similarly, fasting blood cholesterol levels were significantly reduced in the TEE-treated group and approached levels observed in uninduced controls (Figure 2(b)). Given that hypercholesterolemia is a hallmark of metabolic syndrome and commonly observed in zebrafish obesity models [29], this finding further supports the metabolic benefit of TEE. The cholesterol-lowering effect of TEE may result from reduced cholesterol biosynthesis or enhanced systemic clearance, although further mechanistic studies are required.

Importantly, these metabolic improvements occurred without concurrent reductions in body weight, highlighting the therapeutic potential of TEE for metabolic syndrome beyond conventional weight-loss strategies. The zebrafish model provides a robust and translational system for evaluating such interventions due to its conserved metabolic pathways and genetic tractability [13, 30, 31].

The liver plays a central role in systemic lipid and glucose homeostasis, and hepatic steatosis is a defining feature of MASLD.

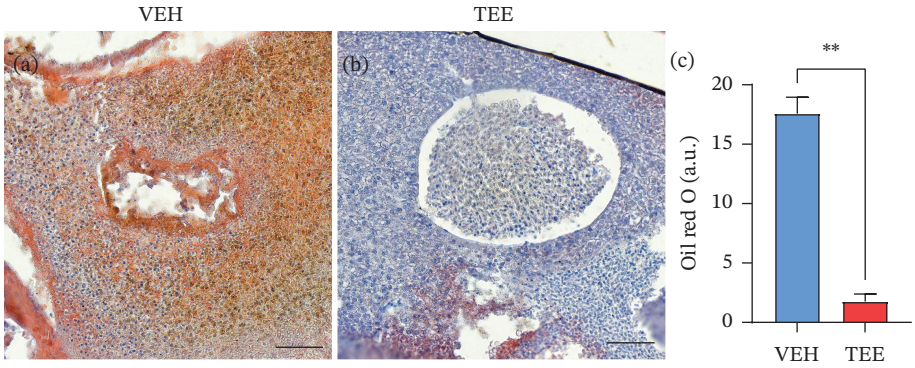


FIGURE 3 | TEE supplementation ameliorates the hepatic lipid accumulation in DIO zebrafish. Representative Oil Red O staining of liver sections from (a) vehicle-treated DIO zebrafish (VEH) and (b) TEE-supplemented DIO zebrafish (TEE). Extensive neutral lipid accumulation was observed in livers of the VEH group, whereas livers of the TEE group showed minimal lipid deposition. (c) Quantification of Oil Red O staining revealed a significant reduction in hepatic neutral lipid accumulation in the TEE group compared with the VEH group. Data are presented as mean ± SEM. $p < 0.01$. Scale bars: 60 μm.

Consistent with previous studies [32], DIO zebrafish in our study developed pronounced hepatic lipid accumulation, as evidenced by Oil Red O staining and elevated hepatic TG levels (Figures 3 and 4(a)). TG accumulation in hepatocytes is a hallmark of early-stage MASLD and a precursor to inflammation and fibrosis [6]. TEE supplementation significantly reduced both neutral lipid deposition and hepatic TG content, indicating a clear hepatoprotective effect.

Oxidative stress is a key driver of MASLD progression, contributing to hepatocellular injury and inflammation [5]. In the present study, hepatic TBARS levels, an indicator of lipid peroxidation, were significantly elevated in DIO zebrafish and markedly reduced following TEE treatment (Figure 4(b)). In contrast, reduced GSH levels remained unchanged between groups, suggesting that the antioxidant effects of TEE are not primarily mediated through GSH-dependent mechanisms. Instead, the reduction in hepatic lipid accumulation likely decreases upstream lipotoxicity, thereby indirectly attenuating oxidative stress. Future studies incorporating antioxidant enzyme activities, such as superoxide dismutase and catalase, will help clarify whether TEE also modulates endogenous antioxidant defense systems.

Despite improvements in hepatic TG and TBARS levels, hepatic cholesterol fractions, including total cholesterol, free cholesterol, and cholesteryl esters, remained unchanged. This discrepancy with circulating cholesterol reduction suggests that TEE may primarily affect systemic cholesterol metabolism or hepatic cholesterol efflux rather than intrahepatic storage. Similar selective effects have been reported for other natural compounds, such as epigallocatechin gallate [33]. Collectively, these findings indicate that TEE ameliorates hepatic steatosis and oxidative stress primarily through modulation of lipid metabolism.

Gene expression analysis further supported this mechanism. TEE significantly downregulated key lipogenic genes, including acetyl-CoA carboxylase alpha (*acaca*), fatty acid synthase (*fasn*), and fatty acid desaturase 2 (*fads2*) (Figure 5(a)), consistent with the suppression of de novo lipogenesis and reduced hepatic TG accumulation [34]. Interestingly, genes involved in fatty acid activation and β-oxidation, including acyl-CoA synthetase long-chain family member 1 (*acsl1*) and acyl-CoA dehydrogenase medium chain (*acadm*), were also downregulated (Figure 5(b)), suggesting a coordinated reduction in lipid turnover rather than a simple shift toward increased oxidation. This pattern may reflect a rebalancing of hepatic lipid homeostasis under TEE treatment.

TEE also downregulated *hmgcrb* (Figure 5(c)), the rate-limiting enzyme in cholesterol biosynthesis, aligning with the observed reduction in circulating cholesterol. This is consistent with previous findings that dietary interventions or natural compounds can modulate *hmgcr* expression to regulate cholesterol levels [35]. Decreased expression of *abca1* and *cyp7a1* (Figure 5(c)) may represent a compensatory response to reduced cholesterol availability [36]. In parallel, TEE suppressed pck1, a key gluconeogenic enzyme (Figure 5(d)), consistent with improved glycemic control and reduced hepatic glucose output [37].

The biological activities of triterpenoids suggest that these transcriptional changes may be mediated through key metabolic pathways, including AMPK, PPARs, and SREBPs. Previous studies have shown that triterpenoids can activate AMPK signaling, modulate PPAR activity, and regulate SREBP-dependent lipogenesis [38–41]. Although protein-level validation was not performed in this study, the observed transcriptional patterns are consistent with these established mechanisms. Future studies should confirm these pathways at the protein level and evaluate TEE's therapeutic potential in mammalian models and clinical settings.

Necroptosis, a regulated form of necrotic cell death mediated by receptor-interacting protein kinases (RIPK1, RIPK3) and caspase 8 (CASP8), has emerged as a key contributor to the progression of MASLD toward steatohepatitis, fibrosis, and cirrhosis [7, 42, 43]. In this study, TEE significantly downregulated hepatic expression of *ripk3* and *casp8* (Figure 6), suggesting attenuation of necroptotic signaling.

Caspase-8 plays a dual role as both an initiator of apoptosis and a suppressor of necroptosis; its dysregulation can shift the balance toward RIPK3-dependent necroptotic signaling, thereby promoting inflammation [44]. In our study, TEE downregulated both *ripk3* and *casp8*, suggesting an overall dampening of necroptotic potential. Importantly, necroptosis, oxidative stress, and

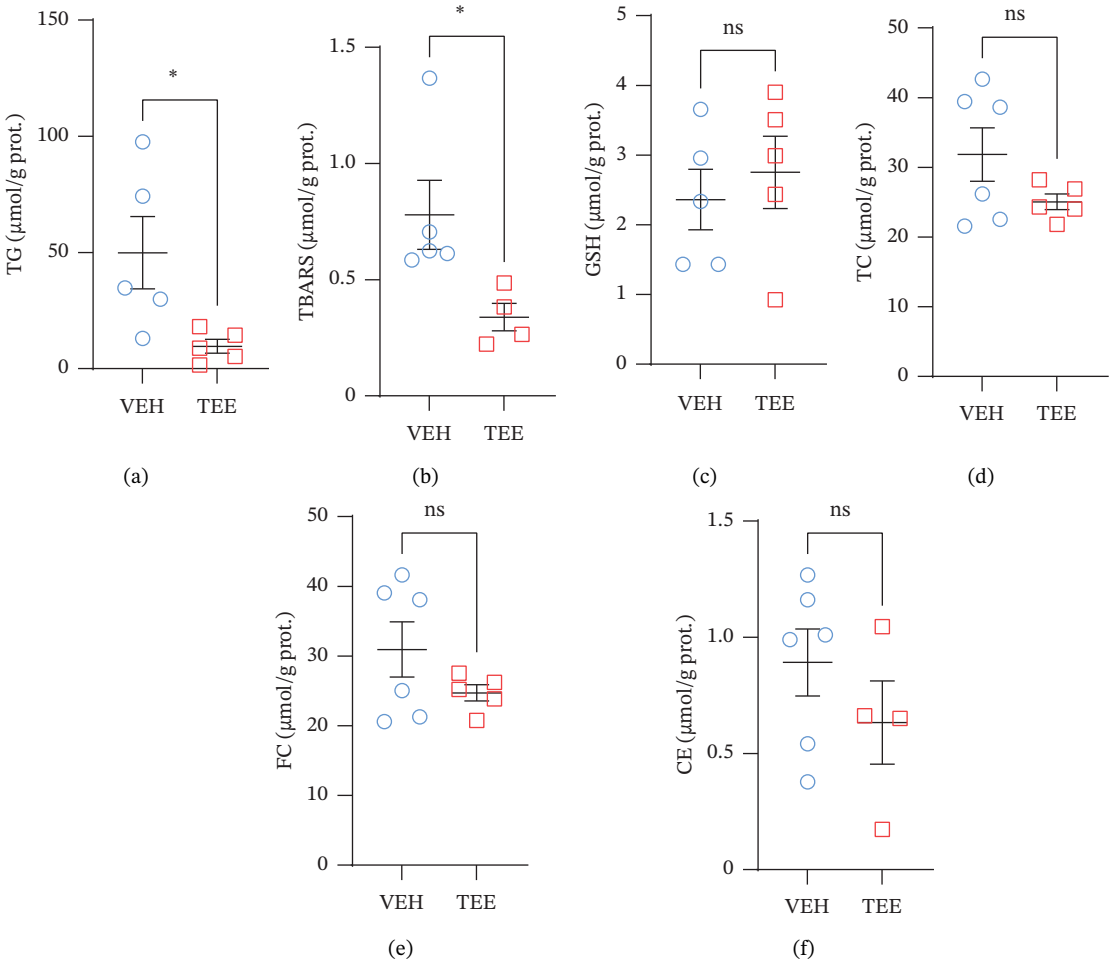


FIGURE 4 | TEE reduced hepatic TG accumulation and oxidative stress in DIO zebrafish. (a) Hepatic triglyceride (TG) levels were significantly lower in the TEE group zebrafish compared with the VEH group. (b) Thiobarbituric acid reactive substances (TBARS), a marker of lipid peroxidation, were also significantly reduced in the TEE group. (c) Hepatic reduced glutathione (GSH) levels did not differ between groups. (d–f) Hepatic total cholesterol (TC), free cholesterol (FC), and cholesteryl esters (CE) were comparable between the VEH and TEE groups. Data are presented as mean ± SEM. $^{*}p < 0.05$; ns, not significant.

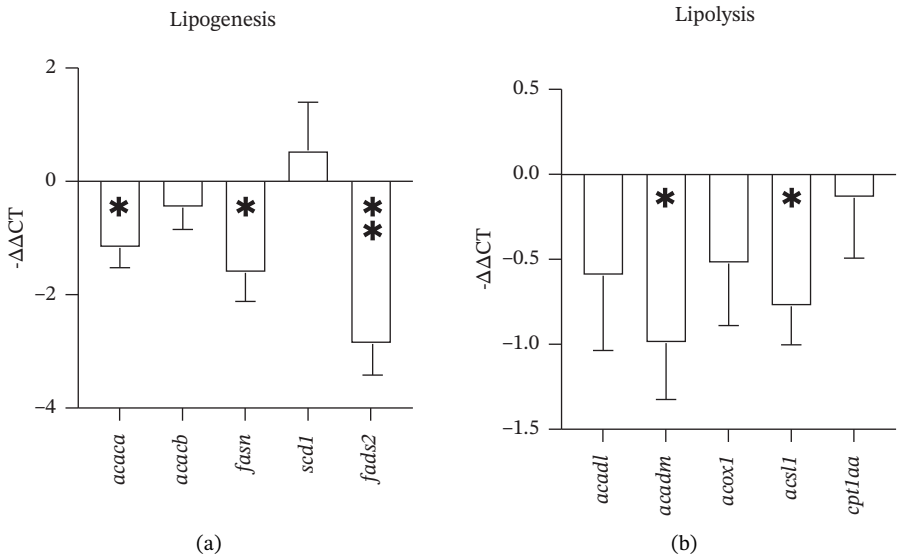


FIGURE 5 | (Continued)

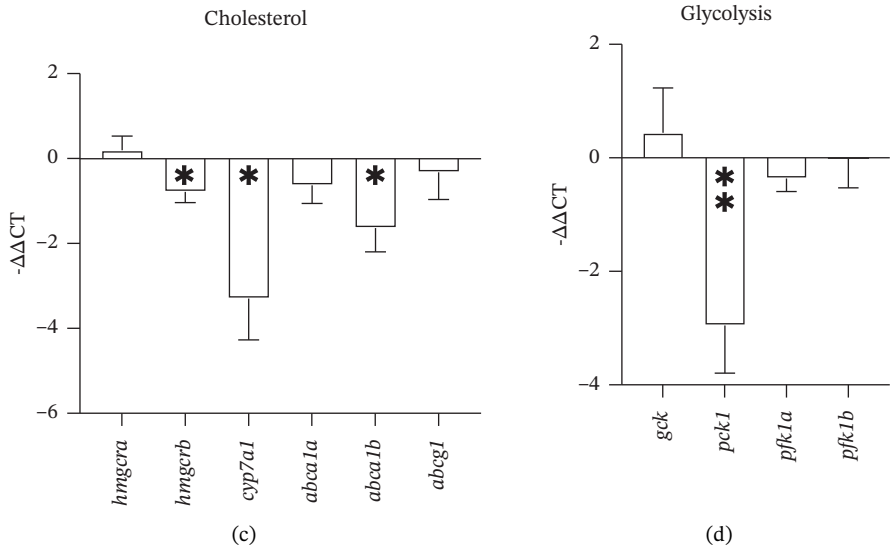


FIGURE 5 | TEE supplementation alters hepatic gene expression involved in lipid and glucose metabolism in DIO zebrafish. RT-qPCR analysis was performed on liver samples to evaluate the expression of genes related to lipid and glucose metabolism. (a) Lipogenesis-related genes, including *acaca*, *fasn*, and *fads2*, were significantly downregulated in the TEE group zebrafish. (b) Genes involved in lipid activation and *β*-oxidation, *acsl1* and *acadm*, were also significantly reduced in the TEE group. (c) In cholesterol metabolism, the key biosynthetic enzyme *hmgcrb*, the cholesterol efflux transporter *abca1*, and *cyp7a1*, the rate-limiting enzyme for bile acid synthesis, were significantly decreased in the TEE group fish. (d) In line with improved fasting glucose, the gluconeogenic enzyme *pck1* was markedly downregulated in the TEE group. Data are presented as mean ± SEM. $^{*}p < 0.05$.

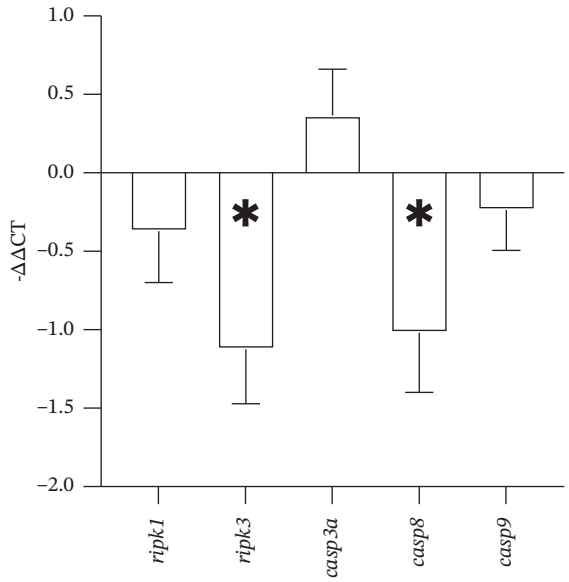


FIGURE 6 | TEE supplementation suppresses cell death–associated gene expression in DIO zebrafish. RT-qPCR analysis of liver tissue showed significantly lower expression of *ripk3* and *casp8*, key regulators of necroptosis, in the TEE group zebrafish compared with the VEH group. In contrast, the expression levels of *ripk1*, *casp3a*, and *casp9* were unchanged, suggesting that apoptosis is less involved in hepatic injury associated with lipid accumulation. Data are presented as mean ± SEM. $^{*}p < 0.05$.

lipid accumulation are tightly interconnected in MASLD, where excess TG deposition promotes ROS production and hepatocyte death, while necroptotic injury amplifies inflammation and further disrupts lipid homeostasis. The concurrent reductions in TG content, oxidative stress, and necroptotic markers observed in the TEE-treated zebrafish suggest that this cycle was attenuated, although the primary site of TEE's action remains to be determined.

Notably, apoptotic markers (*casp3*, *casp9*) were unchanged, implying that apoptosis was not activated as a compensatory pathway. This selective modulation is consistent with the concept of PANoptosis, in which pyroptosis, apoptosis, and necroptosis are interconnected cell death programs whose relative dominance depends on the inflammatory context [45]. By dampening necroptotic signaling without inducing apoptosis, TEE may shift hepatic cell death signaling toward a less inflammatory state.

Zebrafish provide a robust model for studying liver inflammation and cell death [31]. The suppression of necroptotic markers in the TEE-treated DIO zebrafish underscores the hepatoprotective and anti-inflammatory potential of triterpenoid-rich extracts. These findings align with previous reports that triterpenoids can inhibit inflammatory signaling cascades and protect against liver injury [46–48]. Future studies should investigate upstream regulators, including tumor necrosis factor-alpha, Toll-like receptors, or inflammasome components [49], to further elucidate the molecular mechanism underlying TEE-mediated protection and its potential to prevent progression from steatosis to steatohepatitis and fibrosis.

5 | Conclusion

This study demonstrates that TEE exerts hepatoprotective effects in a DIO zebrafish model by improving lipid and glucose metabolism and attenuating necroptosis-associated signaling. TEE reduced hepatic TG accumulation, circulating cholesterol, and fasting blood glucose, while selectively suppressing necroptotic markers without activating apoptosis. These findings highlight the potential of TEEs as multitargeted modulators of

metabolic dysfunction and liver injury, supporting their further development as therapeutic strategies for MASLD.

Author Contributions

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Disclosure

All the authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

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

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