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5,7,3′,4′,5′-pentamethoxyflavone (PMF) exhibits anti-obesity and neuroprotective effects in an obese zebrafish model

Muhammad Sufyan Vohra [a], Bilal Ahmad [b,1], Emerald R. Taylor [c], Khaled Benchoula [a], Isabel Lim Fong [d,e], Ishwar S. Parhar [f], Satoshi Ogawa [f], Christopher J. Serpell [g,*], Eng Hwa Wong [a,h,**]

[a] School of Medicine, Faculty of Health and Medical Sciences, Taylor's University Lakeside Campus, 47500, Subang Jaya, Selangor Darul Ehsan, Malaysia
[b] School of Biosciences, Faculty of Health and Medical Sciences, Taylor's University Lakeside Campus, 47500, Subang Jaya, Selangor Darul Ehsan, Malaysia
[c] School of Chemistry and Forensic Science, Ingram Building, University of Kent, Kent, Canterbury, CT2 7NH, United Kingdom
[d] Department of Paraclinical Sciences, Faculty of Medicine and Health Sciences, 94300, Kota Samarahan, Malaysia
[e] Universiti Malaysia Sarawak, Malaysia
[f] School of Medicine and Health Sciences, Monash University, Sunway Campus, PJ 46150, Selangor, Malaysia
[g] Department of Pharmaceutical and Biological Chemistry, School of Pharmacy, University College London, 29-39 Brunswick Square, London, WC1 1AX, United Kingdom
[h] Digital Health and Medical Advancement Impact Lab, Taylor's University Lakeside Campus, 1, Jalan Taylor's, Subang Jaya, Selangor, 47500, Malaysia

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ABSTRACT

Obesity is a multi-chronic illness characterized by superfluous fat accumulation, contributing to significant metabolic and neurological complications. Current therapeutic approaches have limited efficacy and notable side effects, underscoring an urgent demand for novel, safer alternatives. This study is the first to investigate the anti-obesity potential of 5,7,3′,4′,5′-pentamethoxyflavone (PMF) *in vivo* using a zebrafish model. Our findings demonstrate that PMF administration exerts pronounced anti-obesogenic effects, evidenced by reductions in blood glucose, plasma triglycerides, total cholesterol, hepatic low-density lipoproteins (LDL), and high-density lipoproteins (HDL). Mechanistically, PMF suppressed hepatic adipogenic and lipogenic gene expression while promoting lipid catabolism through activation of peroxisome proliferator-activated receptor-alpha (PPAR-α) and its downstream enzymes, including acyl-CoA oxidase 1 (ACOX1), medium-chain acyl-CoA dehydrogenase (ACADM), and carnitine palmitoyl transferase 1B (CPT-1β). Additionally, PMF markedly mitigated oxidative stress by lowering malondialdehyde (MDA) and nitric oxide (NO) levels, accompanied by increased antioxidant enzyme activities, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and glutathione S-transferase (GST). Notably, PMF effectively prevented obesity by suppressing food intake, down-regulating orexigenic genes, and enhancing anorexigenic signals. Furthermore, PMF exhibited neuroprotective properties by elevating brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin receptor kinase B2 (TrkB2), revealing a novel link between metabolic and neurological regulation. This study provides pioneering, comprehensive *in vivo* evidence supporting PMF as a promising therapeutic candidate with dual beneficial roles in metabolic health and neuroprotection.

1. Introduction

Obesity is an increasingly widespread disorder, affecting all ages and genders across the globe (Afolabi et al., 2020; Cizza, 2022). The Atlas report from the World Obesity Federation (WOF) stated that in 2020 approximately 770 million adults globally were adversely affected by obesity (Lobstein et al., 2022). Obesity is caused by a decrease in energy expenditure and/or increased energy intake for a prolonged period,

* Corresponding author.
** Corresponding author. School of Medicine, Faculty of Health and Medical Sciences Taylor's University Lakeside CampusDigital Health and Medical Advancement Impact Lab, Taylor's University Lakeside Campus, 1, Jalan Taylor's, Subang Jaya, Selangor, 47500, Malaysia.
E-mail addresses: chris.serpell@ucl.ac.uk (C.J. Serpell), EngHwa.Wong@taylors.edu.my (E.H. Wong).
[1] Current address: Division of Endocrinology, Metabolism and Lipid Research, Washington University in St. Louis, Missouri USA.

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resulting in an energy imbalance. Environmental factors such as consumption of highly caloric food and/or a sedentary lifestyle are widely recognized as the main causes of this ailment. Obesity is associated with several life-threatening non-communicable diseases including neurodegenerative diseases, cardiovascular diseases, diabetes and many types of cancer (colon, breast, endometrium, oesophagus, kidney and liver) (Xie et al., 2009; Dietz et al., 2015; Boleti et al., 2023; Avgerinos et al., 2019). The central nervous system (CNS) and cognitive functions are also adversely affected by obesity. Clinical and experimental studies on obesity revealed that a fat rich diet can result in systemic inflammation of the brain causing learning, memory and executive functioning deficits (Castro et al., 2017; Sabia et al., 2009). Recent meta-analyses have identified a strong correlation between obesity and neurological disorders including dementia, Parkinson's disease (PD), and Alzheimer's disease (AD) (Pedditizi et al., 2016; Costello et al., 2020; Martin-Jiménez et al., 2017). Understanding obesity, and hence moving forward to develop effective interventions is now a high priority for global public health. Due to the urgent and global nature of the problem, the discovery of a suitable solution to combat obesity in both developed and underdeveloped countries is critically needed.

Obesity is caused by the excessive accumulation of fats in the adipocytes. Adipocytes are specialized cells within adipose tissue that undergo differentiation and become fully functional through a well-orchestrated process via adipogenesis and lipogenesis, giving them the ability to store excess fat in lipid droplets (Audano et al., 2022). This complex process comprises of numerous stages extensively regulated by the various genes, proteins, and transcription factors involved in adipocyte development. Inhibition of these genes and transcriptional factors accelerates lipolysis (fat catabolism). This is a crucial strategy in the fight against obesity and its associated disorders. Obesity is also regulated through neuronal wirings situated in the CNS, specifically the arcuate nucleus (ARC) of the hypothalamus (Vohra et al., 2022). The ARC is a virtual central platform that identifies hormonal and nutrient-associated signals and inform the body about the energy state of the organisms. Orexigenic AgRP/NPY-expressing and anorexigenic Proopiomelanocortin (POMC)-expressing neurons send chemical signals of hunger and satiety, respectively (Vohra et al., 2022; Deem et al., 2022). These neuronal peptides are crucial in regulating the organism's energy homeostasis. The disruption or breakdown of these central neuronal populations may act as a potential strategy to control body weight gain.

Despite being the fifth leading cause of death across the globe, there is still no satisfactory medicine available for the treatment of obesity. Existing anti-obesity drugs, although somewhat effective in managing weight, frequently induce serious adverse effects, including damage to vital organs, limiting their clinical utility (Van Houten et al., 2018; Gura, 2003; Tai et al., 2018; Freitas et al., 2019). Therefore, there is an urgent and unmet need for novel therapeutics that provide safer and more effective alternatives for obesity management.

Natural resources have long been a rich repertoire of bioactive molecules. There is a great untapped potential arising from these resources against a range of indications which could be treated, with further benefits in availability through agriculture rather than chemical synthesis, and anticipated safety (Payab et al., 2018; Mir et al., 2019; Moro et al., 2000). In particular, flavones are a large group of biomolecules which have demonstrated promising activities coupled with limited adverse reactions on the human subjects (Vajdi et al., 2021; Sudhakaran et al., 2020).

Previously, we demonstrated potent anti-adipogenic effects of four flavones *in vitro*, specifically using 3T3-L1 adipocytes, and identified 5,7,3′,4′,5′-pentamethoxyflavone (PMF) as the most effective candidate (Ahmad et al., 2023a, 2023b). Building on these foundational findings, the present study is the first to explore the anti-obesity effects of PMF (Fig. 1) *in vivo*, utilizing a zebrafish model. The investigation provides a comprehensive assessment of the anti-obesogenic properties of PMF, including its underlying mechanisms involving antioxidative and neuroprotective effects. By exploring PMF's efficacy at both metabolic and neurological levels, this study establishes novel evidence supporting its therapeutic potential, positioning PMF as a promising therapeutic compound for future clinical translation and obesity treatment.

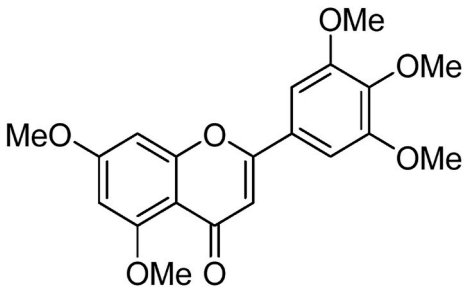

Fig. 1. Structure of 5,7,3′,4′,5′-pentamethoxyflavone, used in the study.

2. Material and methods

2.1. PMF

The compound 5,7,3′,4′,5′-pentamethoxyflavone was synthesized (purity ≥99 %) following previously reported protocols (Ahmad et al., 2023a, 2023b).

2.2. Husbandry of zebrafish

Wild-type zebrafish of 3-months old were purchased from a local supplier (in Malaysia) and housed in 9 L tanks for a settling period of 9 days before testing was initiated. The water was replaced daily, well-aerated, dechlorinated, and kept at 22–23 °C, pH 6.8–7.5, with a minimum oxygen air saturation of 80 %, in 12-h light and 14-h dark cycles. The experimental conditions and procedures complied with the guidelines of The Zebrafish Book (Harper et al., 2016). All animal experimental work was performed and approved by Commission of Ethics at Monash University, Malaysia (Project ID: 28100, Review Reference: 2021-28100-66770), in accordance with internationally approved European Community guidelines (EEC Directive of 1986; 86-609-EEC) for laboratory animal use and care.

2.3. Acute and chronic toxicity study of PMF (1st phase)

Our animal study was designed in two phases. In the first phase, we carried out an *in vivo* toxicity study to find the safest, non-toxic dose of PMF for zebrafish, as PMF has previously not been studied in zebrafish. This information was used as a base for the classification and hazard assessment of PMF. We carried out a 4-day acute and 14-day chronic toxicity assay of PMF using the semi-static method in accordance with OECD Test Guideline 203 and 204, respectively (OECD, 2019; Co-operation, 1984). In the acute toxicity assay, the median lethal dose (LD-50) of PMF was computed based on cumulative mortality (%) of fish using Probit analysis (Software: IBM SPP Version 27). In the chronic toxicity assay, we compared prolonged effects of the compound on body length, weight, and fasting blood glucose levels in male and female zebrafish. For these assessments, the fish were fasted overnight and anesthetized using cold water (Zhao et al., 2021). We used <1 % of methanol to dissolve PMF in water, and therefore a solvent control group was also tested to ensure that the methanol was not the source of the effects.

2.4. Establishment and validation of high fat diet (HFD)-induced zebrafish model (2nd phase)

Obesity was induced in adult zebrafish through overfeeding using a

high fat diet (HFD) following a published diet protocol modifications (Landgraf et al., 2017). In the HFD group, zebrafish (n = 60) were overfed for 6 weeks with the HFD containing 30 mg/fish of egg yolk powder (32 % proteins, 59 % fat, 2 % carbohydrates) 3 times/day, and 5 mg/fish of artemia (44 % proteins, 22 % fat, 16 % carbohydrates) 2 times/day. In the normal diet (ND) group, zebrafish (n = 60) were fed once a day using artemia (5 mg/fish). The fish were maintained in 9 L tanks in groups of 30.

After 6 weeks of feeding, measurement of phenotypic parameters and blood analysis were used to confirm the successful establishment of the obesity model. Fig. 2 shows the experimental design for the development of the HFD-induced obese zebrafish model. The blood glucose levels were measured via direct cardiac puncture. Triglycerides (TG) and total cholesterol (TC) levels were measured from zebrafish plasma, and for the analysis of lipid parameters (HDL-C and LDL-C), we used the zebrafish liver (Babaei et al., 2013; Gupta et al., 2010). Plasma of zebrafish were collected according to the protocol described by Fatemah et al. (2013) (Fatemeh et al., 2013). Fish tissues were excised and homogenized with tissue ruptor II (Qiagen, USA), and the supernatant was collected for assessments. All these kits were certified for appropriate analysis of zebrafish plasma and fish tissues and used according to the manufacturer instructions (Elabscience® Kit, Texas, USA).

2.5. Treatment of obesity in HFD induced zebrafish with PMF

Based on the two-phase study, we selected 10 mg/L (low PMF dose) as the safest, non-toxic dose; and 25 mg/L (high PMF dose) which showed <20 % mortality to examine the potential effects of a higher dose. To test the effectiveness of PMF in the HFD-induced obesity model, we divided the wild-type zebrafish into six groups given their diet and treatment protocol, as shown in Table 1. Again, solvent control groups were used to check if methanol posed any effects on obesity (Fig. 3).

2.6. Food intake measurements in PMF treated zebrafish

The food intake of each group was monitored for 3 consecutive days every week during the treatment period. An aliquot of 50 mg/fish of food was given in each tank. Once the fish had eaten, the food intake was measured by calculating the weight of the artemia left in the tank (Oka et al., 2010).

Table 1
Obesity treatment design showing zebrafish experimental groups with high and low PMF dose.

Groups	Description	Procedure/Treatment
ND (Group 1)	Normal diet (ND)	5 mg/fish of artemia, once daily
HFD (Group 2)	High fat diet (HFD)	30 mg/fish of egg yolk powder for 3 times per day + 5 mg/fish of artemia for twice a day
ND-methanol (Group 3)	Normal diet treated with 50 μL/L methanol	30 mg/fish of egg yolk powder for 3 times per day + 5 mg/fish of artemia for twice a day →Treatment overnight with 50 μL/L methanol
HFD-methanol (Group 4)	HFD treated with 50 μL/L methanol	30 mg/fish of egg yolk powder for 3 times per day + 5 mg/fish of artemia for twice a day →Treatment overnight with 50 μL/L methanol
High PMF Dose (Group 5)	HFD treated with 25 mg/L PMF	30 mg/fish of egg yolk powder for 3 times per day + 5 mg/fish of artemia for twice a day →Treatment overnight with 25 mg/L PMF
Low PMF Dose (Group 6)	HFD treated with 10 mg/L PMF	30 mg/fish of egg yolk powder for 3 times per day + 5 mg/fish of artemia for twice a day →Treatment overnight with 10 mg/L PMF

2.7. Measurement of body length and weight, BMI and blood glucose levels in PMF treated zebrafish

After 6 weeks, BMI was calculated for all treated groups and fasting blood glucose levels were determined through direct cardiac puncture, measuring the blood with glucometer (Benchoula et al., 2019; Vargas et al., 2017).

2.8. Estimation of triglycerides, total cholesterol, and lipid parameters levels in PMF treated zebrafish

We used zebrafish plasma to study the TG and TC levels, and zebrafish liver to assess lipid parameters including LDL-C and HDL-C (Babaei et al., 2013; Gupta et al., 2010). All the kits used in this study were suitable for zebrafish plasma and tissue examination and conducted according to the manufacturer instructions (Elabscience® Kit,

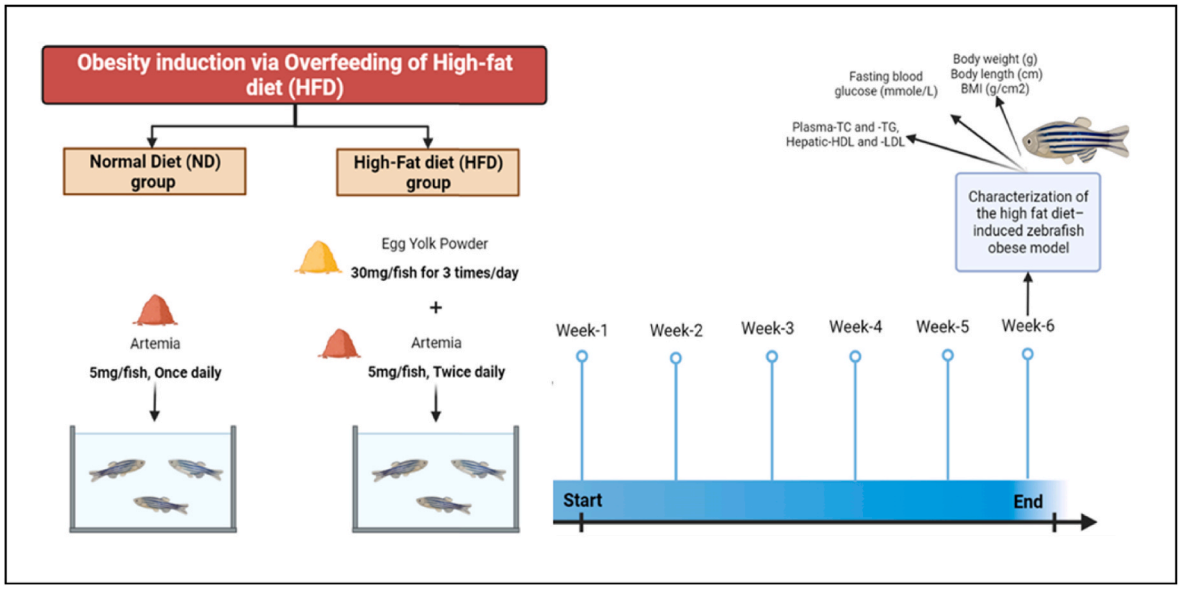

Fig. 2. Development of the obesity model in adult zebrafish via overfeeding with a high fat diet.

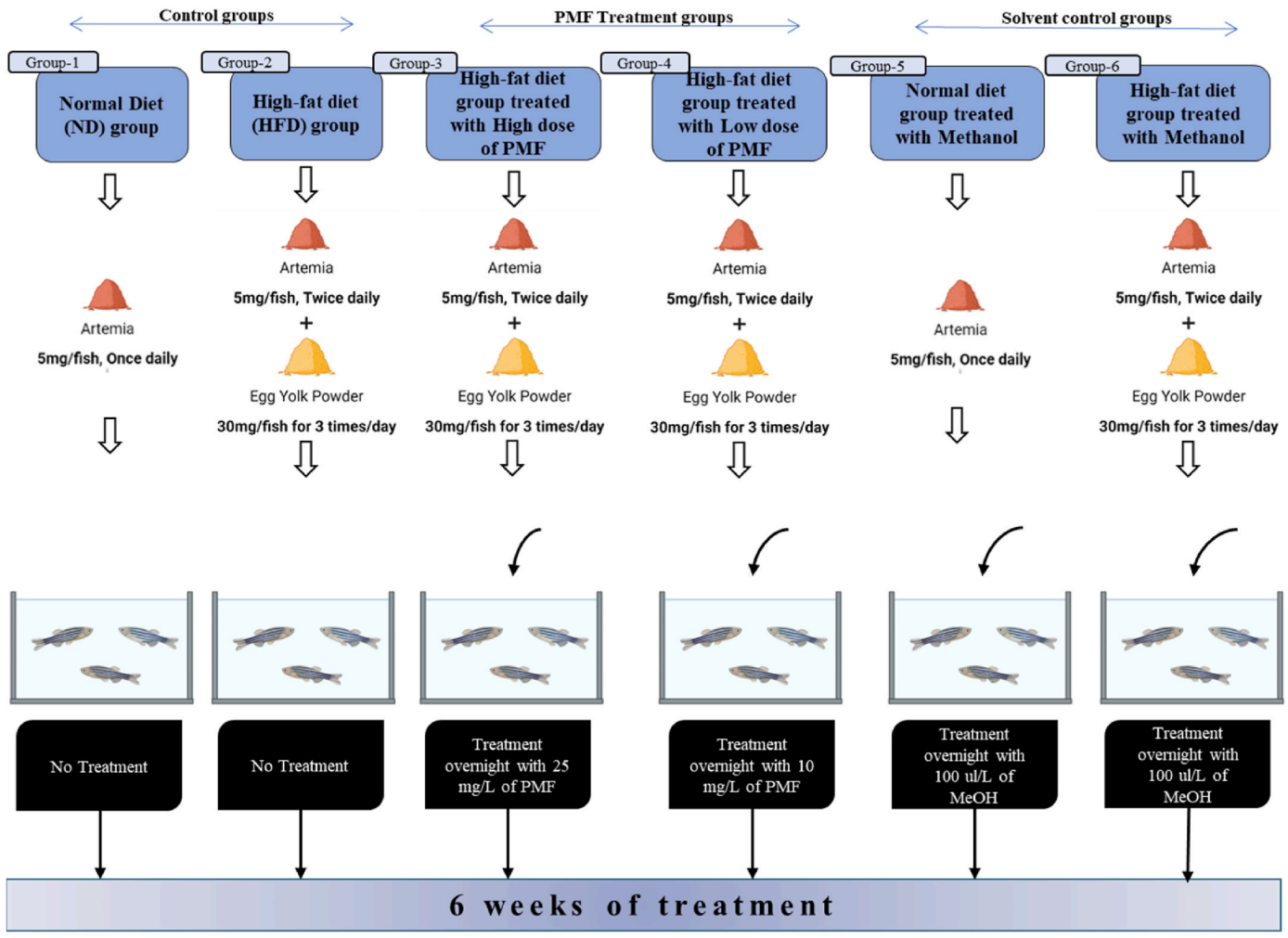

Fig. 3. Schematic diagram of PMF treatment design of obesity in HFD induced zebrafish model.

Texas, USA).

2.9. Assessment of oxidative and anti-oxidative enzymes capacities in PMF treated zebrafish

Oxidative stress damages cellular structures. In combination with under-production of anti-oxidant enzymes, this leads to development of obesity-related complications (Furukawa et al., 2017; Ighodaro et al., 2018). Oxidative stress was examined by measuring MDA and NO levels in liver and brain of zebrafish. Furthermore, anti-oxidative enzyme capacities were assessed to identify effects of PMF under obese conditions using the following parameters: total levels of SOD, CAT, GSH-Px and GST. Fish brain and liver tissues were homogenized. After centrifugation, the supernatant was collected for the final examinations. The total protein content was quantified according to Bradford assay using commercially available kits (Elabscience® Kit, Texas, USA).

2.10. Gene expression analysis

The effects of PMF on gene expression were analysed through RT-qPCR. The liver and brain tissues from each dietary group were excised, mechanically disrupted and homogenized in QIAzol lysis reagent (Qiagen, USA) at full speed for 1.5 min with tissue ruptor-II. The total RNA was extracted using RNeasy® Plus Universal kit (Qiagen, Germany). For each sample, 1 μg of RNA was reverse transcribed to first strand cDNA using QuantiTect® Reverse Transcription kit (Qiagen, Germany) with gDNA eliminator solution according to the manufacturer's protocol in a thermocycler (BIO-RAD T100™).

The cDNA was amplified using gene-specific forward and reverse primers (Table 2) to investigate the expression levels of adipogenic, lipogenic, lipolytic, appetite- and neuroprotective-related genes in RT-qPCR (Eppendorf Realplex^2 Master cycler, Germany) using QuantiNova™ SYBR® Green PCR kit (Qiagen, Germany). The samples were activated at 95 °C for 2 min (1 cycle), then denaturation was performed for 5 s at 95 °C. Lastly, combined annealing and extension was conducted at 60 °C for 1 min with a total of 40 cycles. The relative expression of target genes was calculated using the ΔΔT method and was normalized to an internal control β-actin (housekeeping gene).

2.11. Statistical analysis

GraphPad Prism 9.0 software (GraphPad, San Diego, USA) was used to analyze all the data. Statistical analyses were performed using IBM SPSS 27.0 software (IBM SPSS Inc. Chicago, USA) to calculate LD-50 value. All the values and data were expressed as the mean (±SD and ±SEM). All the phenotypic and genotypic parameters were statistically evaluated using One-Way and Two-Way ANOVA. A paired *t*-test, Tukey post-test and Bonferroni post-test were applied for the comparisons between the groups. A value of $P<0.05$ was considered statistically significant and indicated as $*P<0.05$, $**P<0.01$, $***P<0.001$ and $****P<0.0001$.

Table 2
List of the targeted marker genes and their primers sequences.

Gene Name	Forward primer sequence (5' - 3')	Reverse primer sequence (5' - 3')	Product size (bp)	Locus ID
PPAR-γ	CCGCTGATATGGTGGACACG	AGCGTGGAGAAGGGCTTGAT	121	NM_131467.1
C/EBP-α	GGGAGCGCAACAACATAGCC	CTGCCTGAAGATGCCCCGTA	167	NM_131885.2
C/EBP-β	GCAATCCGGCGGGTGTTAAG	CTGGCTGCTTGCATACACCG	121	NM_131884.2
SREBF1	GGCTGTGACCCGCACTCTAA	CGTGGTAAATGCTCCACCGC	116	NM_001105129.1
ACACB	ATCATCCCACCCAAACAGAC	CCCATCACAGAAGGTGGAAC	250	XM_678989
FASN	ACTCAACGGCTGCTTCGGAT	ACCGCCTGTCGTCTTCTGTG	170	XM_009306807.3
SCD	CGCGTATTTCATCCCGACGC	TCCATCCGTACTCGCTGGTG	215	NM_198815.2
PPAR-α	TGTGTGTTCGGATCGGGCAT	ACGGATGGCATTGTGGGACA	208	NM_001102567.1
ACOX1	ACAAACGTGCTGGATGGTGG	GGACACCTAGGGGCTCTTGA	250	NM_001005933.2
ACADM	GAGATTGTTCCAGCTGCCCC	CCCCAGAGAGTTCGCCTCAA	213	NM_213010.2
CPT1A-A	TGTATCACCGGGGCCGTTAC	GTGCCATGTCGCAAGTAGGC	202	NM_001044854.1
Lep-A	CAGCTCTCCGCTCAACCTGT	TGCCCGTCAATGTGTTCCCT	135	NM_001128576.1
POMC	GCAAATCGACCTCCTCGCAC	ACACCTTGATGGGTCTGCGT	161	NM_181438.3
MC4R	ACCTGACCAACCGTGAGAGC	GTTGTGGTAGCGCAAAGCGT	149	NM_173278.1
AGRP	CCTGGGACGTGAGCACTACA	TGCGTCTCAGGTGTGGATGA	111	NM_001328012.1
NPY	AGCACTAAGACACTACATCAACC	GATGAGATCACCATGCCAAATG	149	NM_131074.2
GHRL	GTGTTTCTCTTTCCTTGTGTCTC	TTCTTTGATCACTGGTATCTCTGG	140	XM_021476948.1
B-Actin 1	TTGCCCCGAGGCTCTCTT	AGTTGAAGGTGGTCTCGTGGAT	74	NM_131031
ELFA	ACCGTCGCTCTGGGAAGAAG	CCCCCTTGATGACACCCACT	183	NM_001017795.2
GAPDH	TGGTATGGCCTTCCGTGTCC	TGGATGAACGGCAATCCCCA	194	NM_001115114.1
BDNF	CGCCGTTACTCTTTCTCTTGG	CCATTAGTCACGGGGACCTTC	214	NM_001308649.1

3. Results

3.1. Acute toxicity and LD-50 determination of PMF

The acute toxicity of PMF was evaluated in adult zebrafish at concentrations ranging from 10 to 40 mg/L over a 96-h exposure period. Toxicity for PMF was observed starting between 30 mg/L and 40 mg/L, with the LD-50 value at 32.59 mg/L (31.24 mg/L (lower limit)-33.88 mg/L (upper limit)) based on the 96-h toxicity results (Fig. 4A). Zebrafish exposed to higher PMF concentrations exhibited clear signs of toxicity, including significantly reduced locomotive activity and prolonged periods spent at the top or bottom of tanks, indicative of distress. In contrast, no abnormal behaviour, altered swimming patterns, or mortality were observed in either the untreated control or solvent control groups. Representative images demonstrating dose-dependent toxic manifestations, such as spinal curvature, body discoloration, lethargy, internal hemorrhaging (indicated by red arrows), and mortality within 24–72 h post-exposure, are shown in Fig. 4B.

3.2. Chronic toxicity results of PMF

Following a 14-day exposure of PMF, no lethal effect was found at 5 mg/L, 10 mg/L, 15 mg/L, and 20 mg/L PMF concentration in either male or female zebrafish respectively. However, mortality was recorded

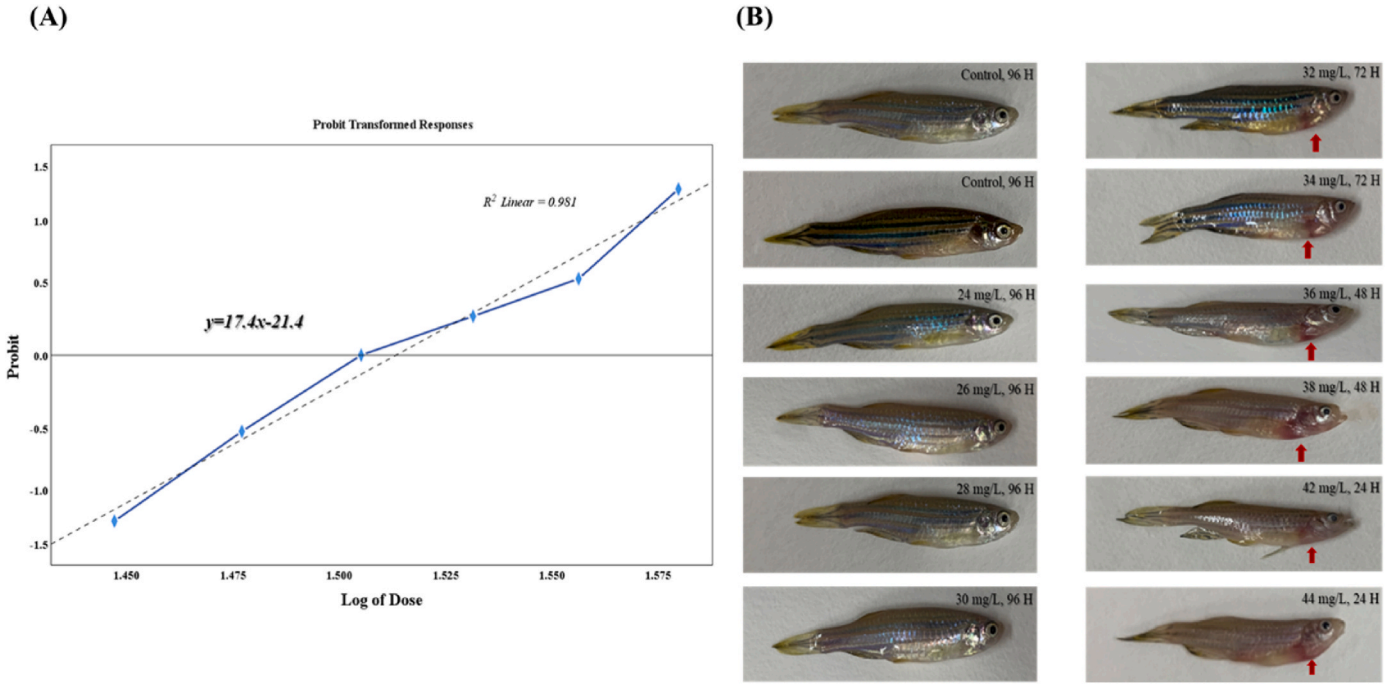

Fig. 4. Quantification of acute toxicity of PMF in adult zebrafish. **(A)** 96-h probit analysis showing dose-dependent mortality following exposure to increasing concentrations of PMF. **(B)** Representative images illustrating acute toxicity effects of PMF. Zebrafish exposed to control (0 mg/mL), 24 mg/mL, 26 mg/mL, 28 mg/mL, and 30 mg/mL PMF displayed normal morphology and behaviour after 96 h. In contrast, exposure to higher concentrations (32–44 mg/mL) induced clear toxic effects characterized by reduced locomotive activity, spinal curvature, discoloration, lethargy, internal hemorrhaging (red arrows), and mortality observed between 24 and 72 h post-treatment.

as 20 % at 25 mg/L, 40 % at 30 mg/L and 60 % at 35 mg/L. No evident differences were recorded between the blank and solvent control groups (Fig. 5 A).

After PMF exposure, we examined the effects based on the gender of the zebrafish. A significant reduction *(P < 0.05)* in length at 25–35 mg/L of male zebrafish and 20–35 mg/L in length of female zebrafish was observed when compared to control group (Fig. 5 B). The 20–35 mg/L PMF groups showed significant decrease *(P < 0.05)* in weight of both male and female zebrafish respectively when compared to the control group (Fig. 5 C). Likewise, 10–35 mg/L PMF resulted in changes *(P < 0.05)* to fasting blood glucose levels in both male and female zebrafish respectively (Fig. 5 D). Thus, the current toxicity studies suggest that gender differences do not significantly influence the toxicological effects of PMF. Additionally, our findings demonstrate that PMF effectively reduces body length, body weight, and blood glucose levels in zebrafish, supporting its potential as a therapeutic candidate for obesity treatment. However, given that this study represents the first *in vivo* toxicity evaluation of PMF, comprehensive safety assessments are imperative to rule out potential adverse effects and establish a robust safety profile prior to clinical application.

3.3. Successful development of HFD induced obese model

After 6 weeks of overfeeding, a drastic rise *(P < 0.05)* in body weight (BW) (0.74 g ± 0.09, n = 30) and length (4.19 ± 0.19 cm, n = 30) was found when compared to control group (0.27 g ± 0.05 and 3.37 cm ± 0.09) respectively (Fig. 6 A). Overfeeding group also gave much higher BMI value (0.045 g/cm^2) and fasting blood glucose level (7.55 mmol/L) when compared to ND zebrafish (0.024 g/cm^2 and 3.30 mmol/L) respectively (Fig. 6B and C). Fig. 6 D provides exemplary images of zebrafish in each of the analysed dietary groups at the 6-week endpoint. All these outcomes suggest a successful establishment of HFD induced obesity zebrafish model.

3.4. Effects of HFD on triglycerides (TG), total cholesterol (TC), and lipid parameter levels in zebrafish model

The lipid profiles were further analysed to confirm the successful establishment of HFD induced obesity zebrafish model. Plasma TG (142.2 ± 5.5 mg/dL), plasma TC (392 ± 3.8 mg/dL) and hepatic LDL-C (191.6 ± 1.3 mg/dL) levels in HFD group after 6-weeks overfeeding showed a significant increase trend (73.50 ± 2.9 mg/dL, 198 ± 5.8 mg/dL) respectively (Fig. 7 A and C). However, no significant effects *(P>0.05)* were found in hepatic HDL-C (Fig. 7D). Hence, these results suggest that the obese zebrafish model were diagnosed with dyslipidemia disorders including hyperglycaemia, hypertriglyceridemia, and hypercholesterolemia.

3.5. PMF treatment in HFD induced zebrafish model

To assess the anti-obesity activity of PMF, groups of HFD induced zebrafish were separately exposed to 25 mg/L and 10 mg/L of PMF overnight for a period of 6 weeks. PMF-treated zebrafish (high and low doses) showed significant decrease *(P < 0.05)* in body length (cm) and weight (g) compared to the HFD group, while no significant effects *(P>0.05)* were found in solvent-treated groups (ND and HFD group treated with methanol) (Fig. 8 A).

A lower BMI value (0.028 g/cm^2) was determined in zebrafish treated with 25 mg/L of PMF. However, no statistical difference *(P>0.05)* was detected in BMI value of 10 mg/L of PMF. These results were compared with BMI (0.045 g/cm^2) of HFD group. We also found that 25 mg/L of PMF significantly reduced *(P < 0.05)* the BMI of HFD

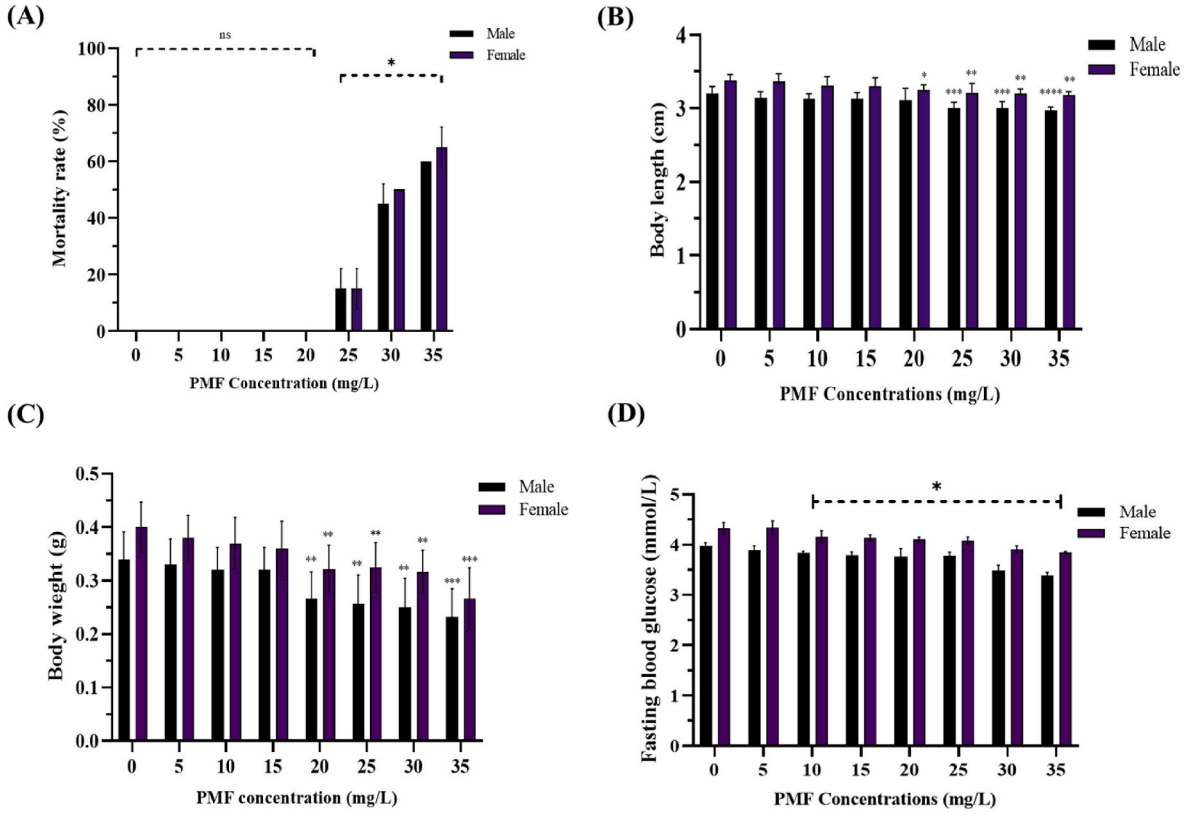

Fig. 5. Quantification of chronic toxicity of PMF in male and female zebrafish treated with different concentrations (mg/L) of PMF respectively. **(A) A** 14-day fish prolonged mortality results (Duplicates, n = 10/gender). **(B)** Body length (Duplicates, n = 10/gender). **(C)** Wet body weight (Duplicates, n = 10/gender). **(D)** Fasting blood glucose levels (Triplicates, n = 5). All data were expressed as means (±SD). Statistical analyses were performed using Two-Way ANOVA followed by Šidák multiple comparison and significant differences were indicated as *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$.

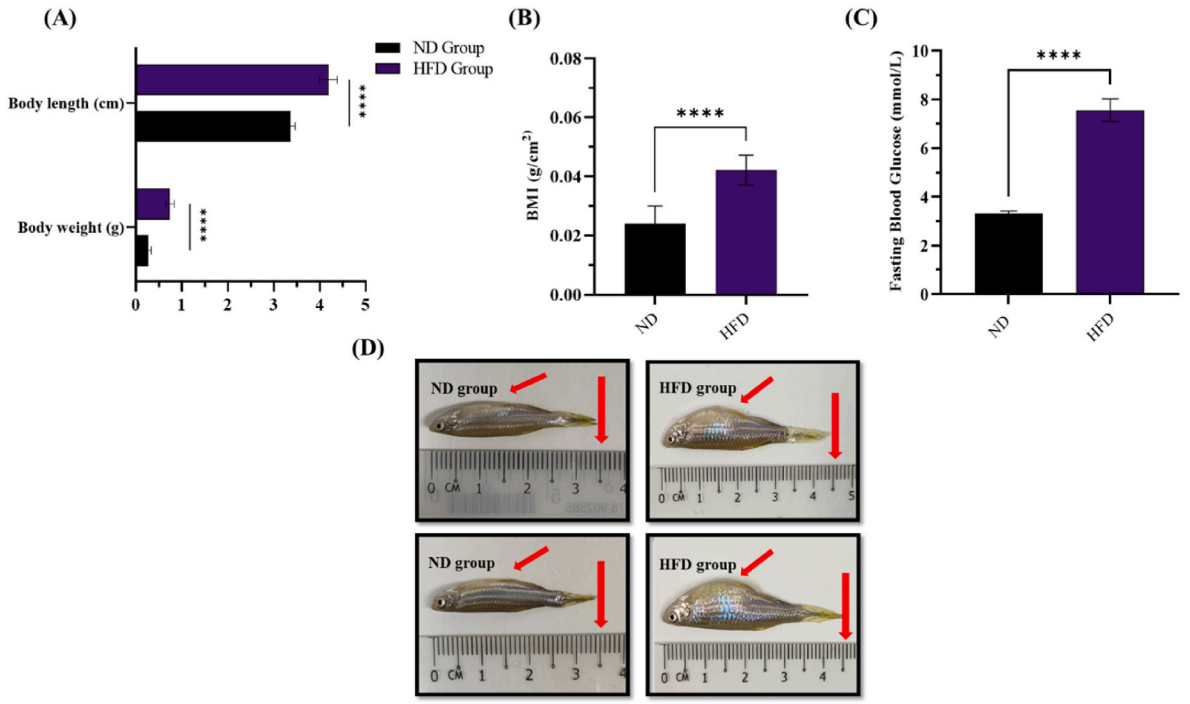

Fig. 6. Development of obese zebrafish groups following overfeeding with HFD and ND. **(A)** Body weight and length (n = 30). Statistical analysis was performed using Two-Way ANOVA followed by Bonferroni post-test and significant differences are indicated as *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$. **(B)** BMI (n = 30). **(C)** Fasting blood glucose levels (n = 10). All data expressed as means (±SD). Statistical analyses were performed using One-Way ANOVA followed by Paired *t*-test and significant p-values were indicated as *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****P < 0.0001. **(D)** Exemplary images of zebrafish included in each of the analysed dietary groups at 6 weeks endpoint. Red arrows point to the body length and abdominal region. *BMI: Body mass index, ND: Normal diet, HFD: High-fat diet.*

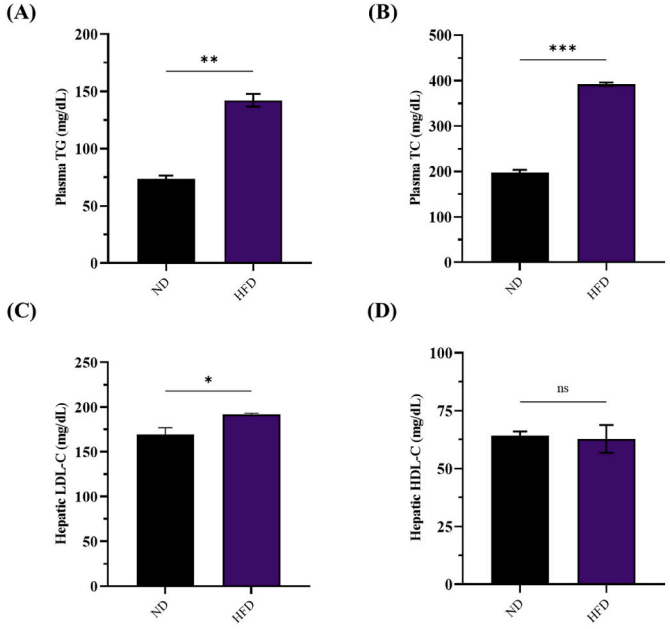

Fig. 7. Biochemical and lipid assessment in ND versus HFD zebrafish groups. **(A)** TG levels (n = 4). **(B)** TC levels (n = 6). **(C)** LDL-C levels (n = 6). **(D)** HDL-C levels (n = 5). All the values are expressed as means (±SD) in triplicates. Statistical analyses were performed using One-Way ANOVA followed by Paired *t*-test and significant p-values were indicated as *$P<0.05$, **$P<0.01$, ***$P<0.001$ and *'ns' indicates no significant difference, ND: normal diet, HFD: high-fat diet.*

zebrafish treated with methanol (0.043 g/cm^2) (Fig. 8B) Both high (7.07 ± 0.05 mmol/L) and low (7.17 ± 0.07 mmol/L) doses of PMF treated zebrafish showed a significant decreased trend in blood glucose levels when compared to HFD group (7.55 ± 0.4633 mmol/L) respectively (Fig. 8 C). Hence, these results indicate that PMF is a potentially effective compound for high blood glucose implications.

3.6. *Effects of PMF on triglycerides (TG), total cholesterol (TC), and lipid parameters in HFD induced zebrafish model*

To further examine the effects of PMF, we assessed the impact on biochemical parameters. When compared with values from HFD group (TG: 142.2 ± 5.52 mg/dL and TC: 392.0 ± 3.86 mg/dL), an efficacious reduction in plasma-TG (high PMF dose: 120.8 ± 4.93 mg/dL and low PMF dose: 128.8 ± 0.62 mg/dL) and plasma-TC (high PMF dose: 366.8 ± 1.83 mg/dL and low PMF dose: 373.1 ± 5.14 mg/dL) were recorded respectively (Fig. 9A and B).

As for lipid parameters, the high dose of PMF significantly decreased the hepatic-LDL level (178.6 ± 7.19 mg/dL vs. HFD: 191.6 ± 1.3 mg/dL, *P-value=0.048*). However, no significance *($P>0.05$)* was found with lower dose of PMF (refer in Fig. 9 (C)). No significant effects were found with the hepatic-HDL levels (Fig. 9 (D)). These findings indicate that PMF exposure may improve dyslipidaemia, including hyperglycemia, hypertriglyceridemia, and hypercholesterolemia conditions.

3.7. *Effects of PMF on food intake in HFD induced zebrafish model*

As seen in Figs. 10 and 6 weeks of PMF exposure affected the feeding patterns in adult zebrafish. High PMF dose considerably *($P<0.05$)* reduced the food intake in HFD fish. No significant difference in feeding pattern was found in low PMF dose treatment of zebrafish.

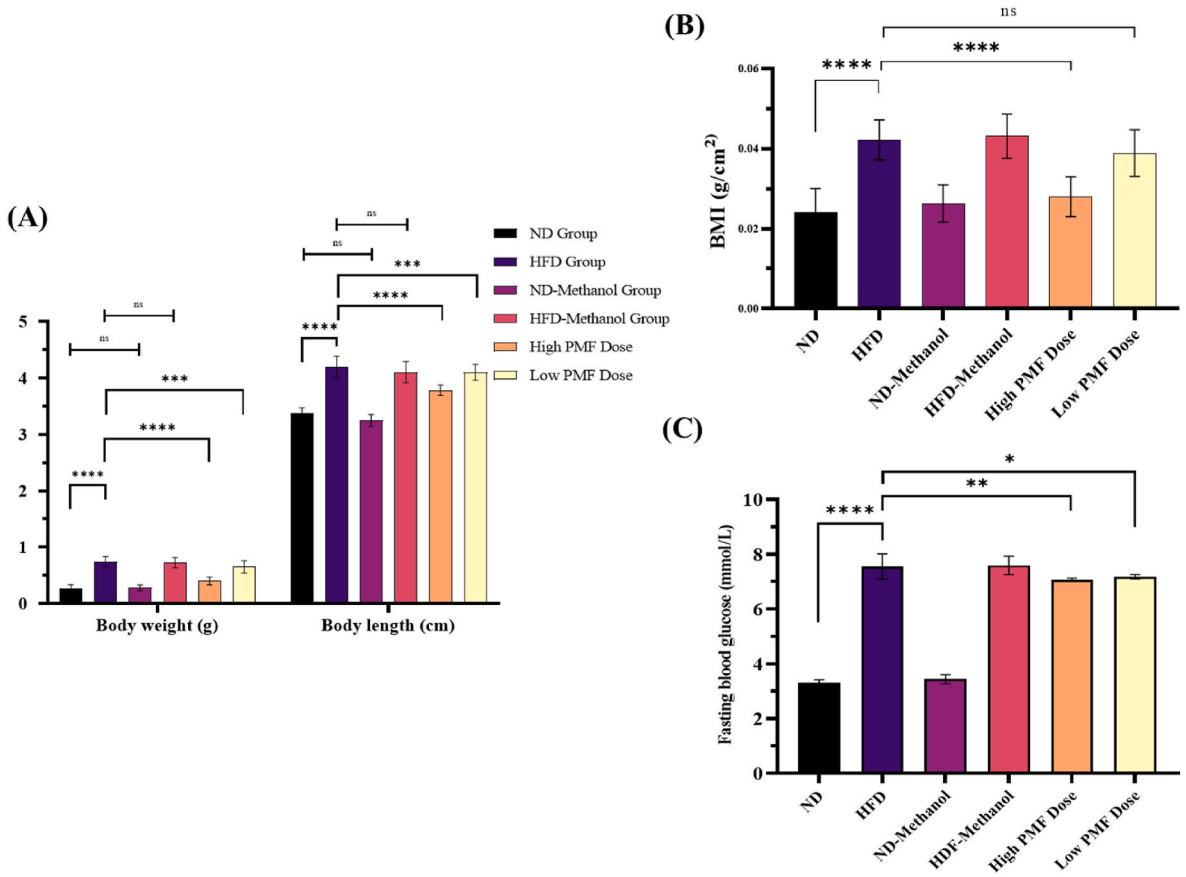

Fig. 8. Effects of PMF on BMI and fasting blood glucose levels of all treatment groups at 6-week endpoint. **(A)** Body weight and length (n = 30). **(B)** BMI (n = 30). **(C)** Fasting blood glucose levels (n = 10). All the values are expressed as means (±SD). Statistical analyses were performed using One-Way and Two-Way ANOVA followed by Tukey-test, and significant p-values were indicated as *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$. *ns: no significant difference, BMI: body mass indes, ND: normal diet, HFD: high-fat diet.*

3.8. Effects of PMF on oxidative stress in HFD induced zebrafish model

To assess the protective effect of PMF against HFD-induced oxidative stress in the livers and brains of zebrafish, MDA and NO levels were evaluated. Overfeeding resulted in significant increase *($P<0.05$)* of MDA levels in liver (7.9 nmol/mg protein) and brain (10.8 nmol/mg protein) of HFD group respectively. However, PMF treatment was able to significantly lower the hepatic (high PMF dose: 6.2 nmol/mg protein and low PMF dose: 6.8 nmol/mg protein) and brain (high PMF dose: 6.9 nmol/mg protein and low PMF dose: 7.6 nmol/mg protein) MDA activities in a dose-dependent manner, in comparison to positive control HFD group (Fig. 11A and B).

On the other hand, the NO activities in liver of HFD and ND zebrafish, and PMF treated zebrafish were highly variable, and patterns like those for MDA did not reach statistical significance *($P>0.05$)* (Fig. 11 C). However, overfeeding did result in greater NO levels (5.4 μmol/g protein) in the brain for the HFD group. The high PMF dose significantly reduced NO (3.5 μmol/g protein) content in brain to those approaching normal levels, although the lower dose of PMF did not *($P>0.05$)* (Fig. 11 D). These outcomes suggest that PMF exposure may have the ability to reduce oxidative stress (OS) in the liver and brain of HFD zebrafish.

3.9. Effects of PMF on antioxidant enzymes capacities in HFD induced zebrafish model

The effects of PMF on the anti-oxidative enzyme activities of Total-SOD, CAT, GSH-Px and GST were measured. The results demonstrated that overfeeding significantly alters enzyme profiles in the liver and brain of HFD-induced obese zebrafish. The PMF-treated groups displayed significant improvement in Total-SOD (high PMF dose: 38.5 U/mg protein and low PMF dose: 35.3 U/mg protein) levels in liver (vs. HFD: 28.7 U/mg protein) (Fig. 12 (A)), and the higher PMF dose resulted in a significant increase in T-SOD levels in brain as compared to HFD-Methanol group (Fig. 12 B). Likewise, CAT (Fig. 12C and D) and GSH-Px (Fig. 12E and F) activities showed significant improvement *($P<0.05$)* in both liver and brain. Only the HFD group treated with 25 mg/L of PMF showed increased *($P<0.05$)* recovery in GST activity in liver (32.5 U/mg protein), while PMF treatment with both doses significantly *($P<0.05$)* restored GST contents in brain (Fig. 12 G). Significant effects *($P<0.05$)* were found in GST levels in brain of ND vs ND-Methanol group (Fig. 12 H). PMF may therefore have the ability to enhance the activity of antioxidant defense enzymes to cope with obesity-induced consequent oxidative stress.

3.10. Effect of PMF on expression of adipogenic marker genes

To confirm the mechanism by which PMF exerts anti-obesity effects, we examined the expression of adipogenic genes (PPAR-γ, C/EBP-α and β, and SREBF1). The mRNA expression levels of PPAR-γ and C/EBP-α were observed to be significantly upregulated (>60 %) in HFD zebrafish as compared to ND (control) zebrafish. However, no significant effect was found in mRNA expression of C/EBP-β between the HFD and ND groups. PMF treatment in HFD zebrafish markedly down-regulated PPAR-γ mRNA expression by 51.6 % under high dose and by 45.2 % under low dose regimes, and C/EBP-α by 47.1 % via high dose and 45.0 % via low dose as compared to the positive control zebrafish respectively. No significant effect *($P>0.05$)* was found in C/EBP-β expression in the liver of PMF-treated and HFD groups (Fig. 13). PMF treatment in

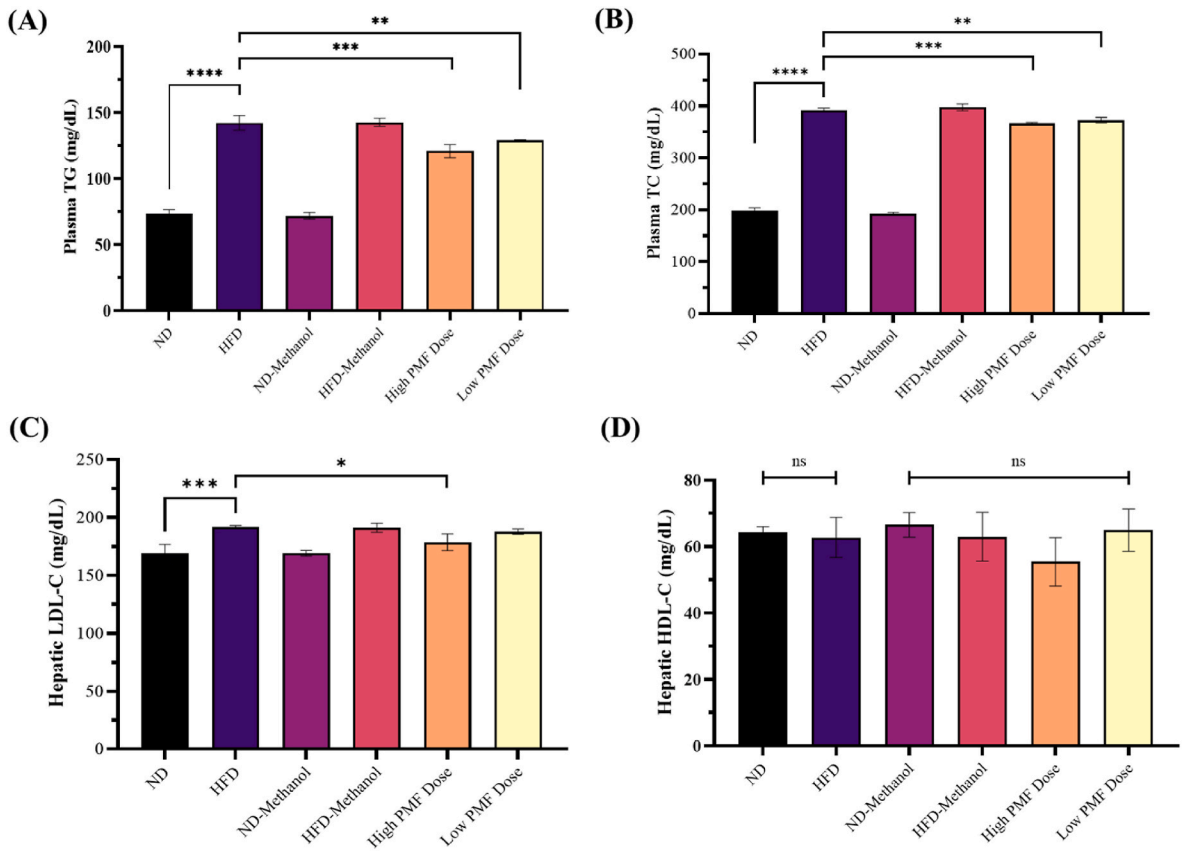

Fig. 9. Effects of PMF on blood and lipid parameters. **(A)** TG levels (n = 4). **(B)** TC levels (n = 6). **(C)** Hepatic LDL-C levels (n = 6). **(D)** Hepatic HDL-C levels (n = 6). All the values are expressed as means (±SD) in triplicates. Statistical analysis was performed using One-Way ANOVA followed by Tukey-test, and significant p-values were indicated as *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P < 0.0001$. *ns: no significant difference, ND: normal diet, HFD: high-fat diet.*

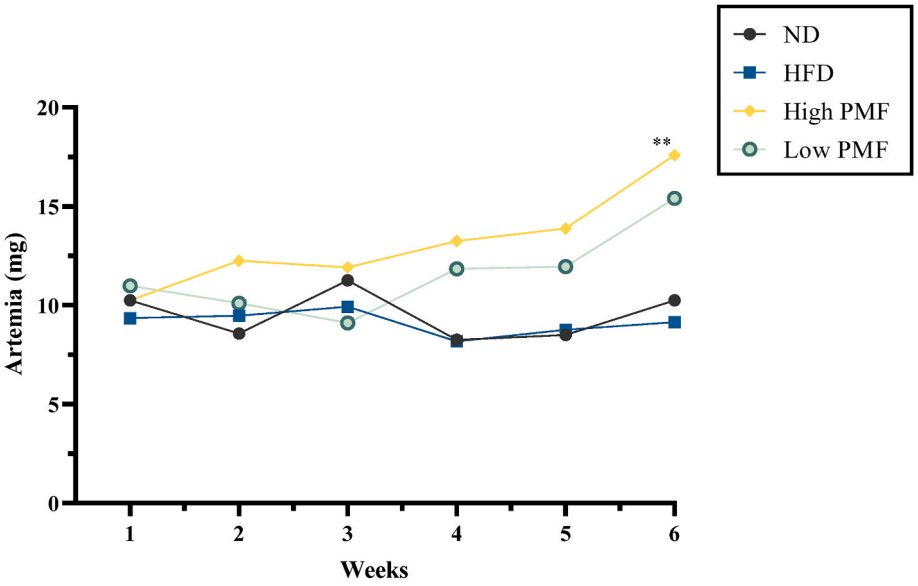

Fig. 10. Measurement of feeding intake (mg). The values are showed as means (±SD), n = 10, Feeding = 5mg/fish. Statistical analysis was performed using One-Way ANOVA followed by post-test Tukey multiple comparison and significant p-values were indicated as *$P<0.05$ and **$P<0.001$. *ND: normal diet, HFD: high-fat diet.*

HFD zebrafish also significantly reduced SREB-F1 mRNA expression by 44.3 % with high dose PMF and 36.9 % with low dose PMF in hepatocytes. These results indicate that PMF shows anti-adipogenic effects that are consistent with the earlier results obtained by phenotypic and biochemical quantification.

3.11. PMF promotes expression of lipolytic genes and inhibits that of lipogenic genes

After confirming the anti-adipogenic effects of PMF, we next investigated its effects on the expression of lipogenic and lipolytic genes.

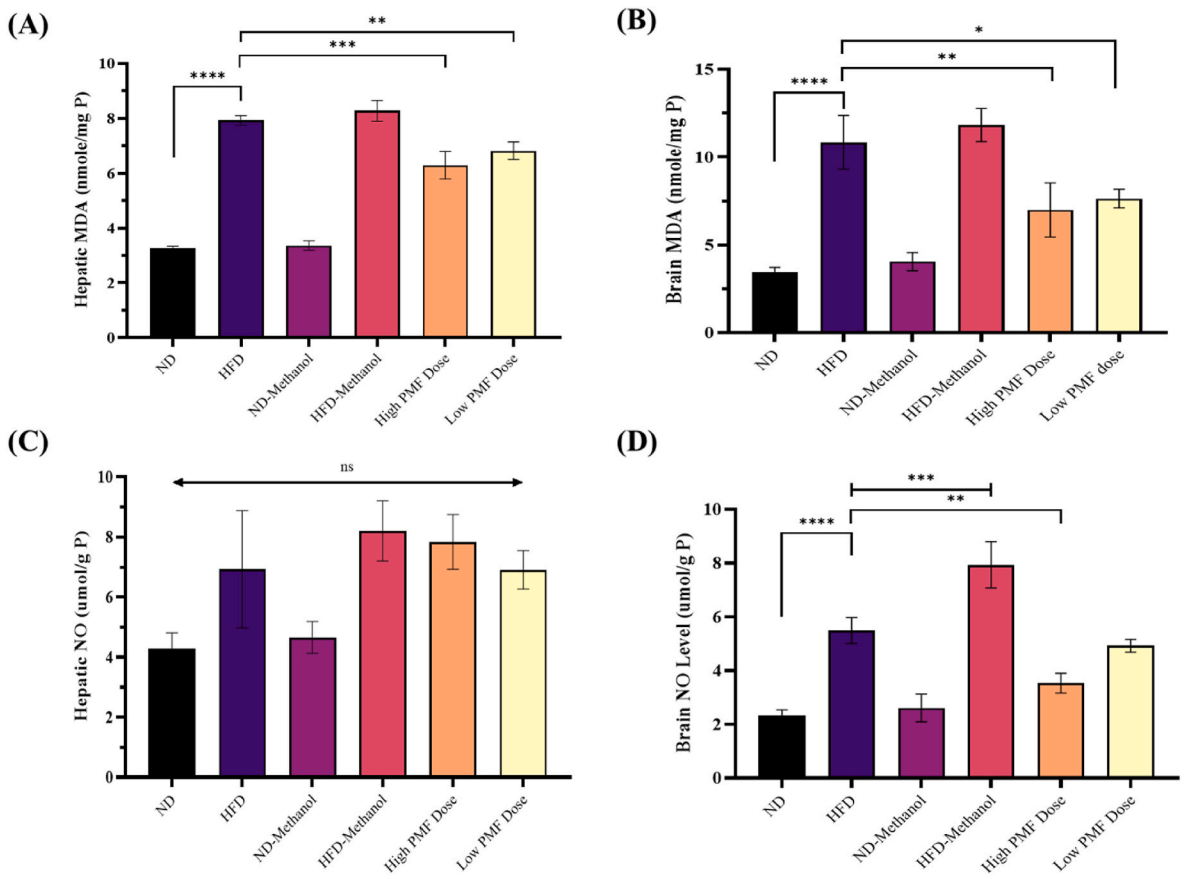

Fig. 11. Effects of PMF on Oxidative stress (OS) markers. **(A & B)** Liver and brain MDA levels (Liver: n = 4, Brain: n = 3). **(C–D)** Liver and brain NO levels (Liver: n = 6, Brain: n = 4). Values are means (±SD) in triplicates. Statistical analyses were performed using One-Way ANOVA followed by Tukey-test and significant p-values were indicated as *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P < 0.0001$. *ND: normal diet, HFD: high-fat diet, ns: no significant difference.*

Treatment of zebrafish with 25 mg/L of PMF significantly decreased the expression of ACACB by 44 % and SCD by 39 % respectively. Additionally, PMF at 25 mg/L and 10 mg/L significantly downregulated FASN expression (41 % via high dose and 35 % via low dose) than those of HFD zebrafish (Fig. 15).

For lipolysis, the transcription factor PPAR-α (responsible for lipid clearance) was significantly upregulated *($P<0.001$)* by PMF treatment, and its target gene, ACOX1, a peroxisomal β-oxidation enzyme expression was also significantly upregulated (>35 % vs. HFD group) when given PMF treatments in HFD zebrafish. Another PPAR-α-target gene, ACADM was enhanced by 42 % via high PMF dose and by 38 % via low PMF dose (*$P<0.001$* vs. HFD) respectively (Fig. 14). CPT-1β, a rate-limiting enzyme for the catalysis of long-chain fatty acid also showed a remarkable increase in expression by 41 % in high dose PMF and 37 % in low dose PMF treatments respectively. In summary, this data suggests that PMF promotes lipolysis and inhibits lipogenesis and thus may be effective in reducing obesity.

3.12. PMF modulates obesity via stimulation of anorexigenic signals and inhibition of orexigenic signals

In addition to measurement of adipogenic, lipogenic and lipolytic gene expression, the mRNA expression of orexigenic (AgRP, NPY, GHRL (Ghrelin)) genes that trigger hunger sensation and hence, an increase in food-intake and anorexigenic (POMC, MC4R, Lep-A) genes that decrease food-intake were also assessed in four experimental groups.

HFD zebrafish showed significant enhanced expression of AgRP, NPY and GHRL in the brain when compared with ND zebrafish. Six weeks of treatment with PMF failed to show significant effects on the expression of AgRP in the brain when compared to the HFD control group. However, PMF treatment in HFD zebrafish significantly downregulated expression of NPY by 48 % in high dose and 40 % in low dose PMF, and GHRL by 45 % in high dose and 39 % in low dose PMF in brain when compared to HFD group indicating that PMF can inhibit food intake by downregulation of orexigenic genes in the brain, bringing these levels closer to those of the ND zebrafish. With respect to anorexigenic genes, PMF treatment of HFD zebrafish significantly enhanced the expression of POMC by 0.52 factor with high dose and 0.34 factor with low dose and MC4R by 0.48 factor via high dose and 0.17 factor via low dose in the brain respectively when compared to HFD zebrafish. However, the HFD group displayed no significant effect on expression of POMC and MC4R in the brain when compared to ND zebrafish. PMF treatment in HFD zebrafish showed no significant effect on expression of Lep-A in the brain when compared to HFD group (Fig. 15).

3.13. Neuroprotective effects of PMF

The expression levels of brain-derived neurotrophin growth factor (BDNF) and its tropomyosin kinase receptor B2 (TrkB2), astrocyte biomarker glial fibrillary acidic protein (GFAP) and microglia biomarker Intergrin (CD11b) genes were assessed in the brains of each zebrafish group. CD11b expression in HFD zebrafish showed significant effects as compared to the ND group. PMF treatment group at 25 mg/L suppressed expression of CD11b when compared to HFD group. However, PMF treatment did not affect GFAP expression. In the brain, BDNF mRNA levels significantly increased with PMF treatment (High dose: *$P=0.0137$* and Low dose: *$P=0.0219$*) with respect to HFD control group (Fig. 16). Similarly, TrkB2 mRNA expression was also significantly upregulated *($P= 0.0042$)* in the brain of high dose PMF group. These findings suggest that PMF might suppress obesity induced inflammation and

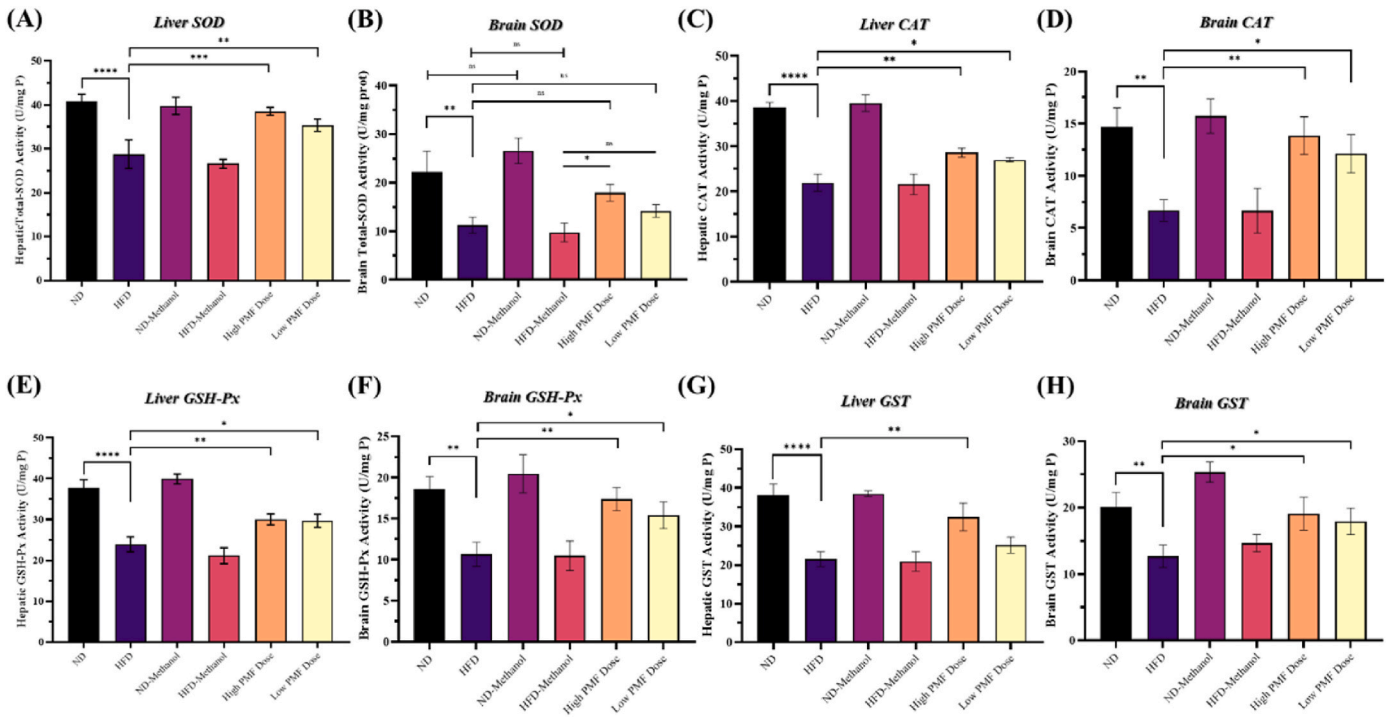

Fig. 12. Effects of PMF on Antioxidative enzyme activities. **(A–B)** Liver and brain Total-SOD levels (Liver: n = 4, Brain: n = 5). **(C–D)** Liver and brain CAT levels (Liver: n = 5, Brain: n = 4). **(E–F)** Liver and brain GSH-Px levels (Liver: n = 7, Brain: n = 4). **(G–H)** Liver and brain GST activity (Liver: n = 6, Brain: n = 4). All values are means (±SD) in triplicates. Statistical analyses were performed using One-Way ANOVA followed by post-test Tukey multiple comparison and significant p-values were indicated as $*P<0.05$, $**P<0.01$, $***P<0.001$ and $****P < 0.0001$. *ND: normal diet, HFD: high-fat diet.*

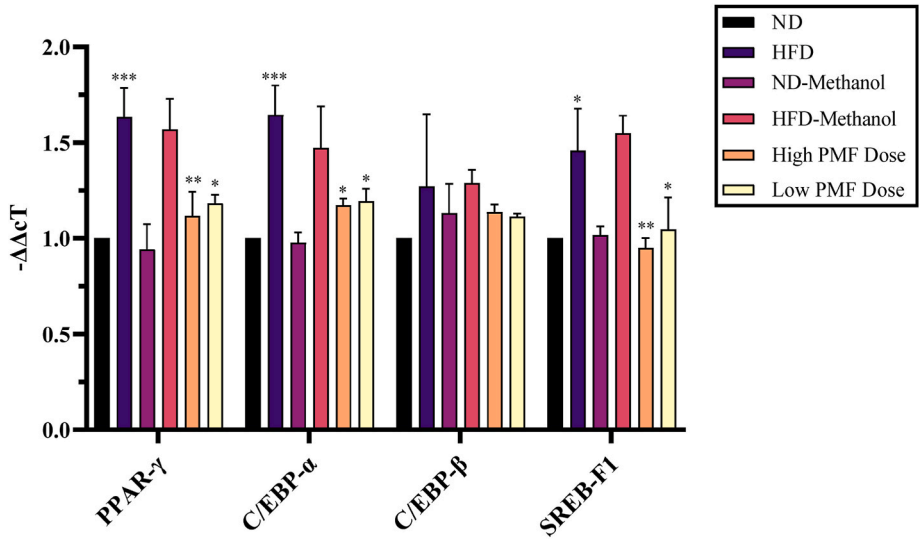

Fig. 13. Effects of PMF exposure on the expression of positive adipogenic factor genes in hepatocytes of zebrafish overfed for 6 weeks. Data represented as means (±SEM), n = 6. Statistical analysis was performed using Two-Way ANOVA followed by Bonferroni post-test and significant p-values indicated as $*P<0.05$, $**P<0.01$ and $***P<0.001$. mRNA expression values were normalized with β-Actin expressed as a ratio of the ND group. *ND, normal diet; HFD, high fat diet.*

neuroprotective action via BDNF/TrkB2 signalling in the brains of zebrafish.

To further confirm the neuroprotective effect of PMF, BDNF protein levels in the brain were assessed via ELISA. The overfed group showed decreased BDNF (7.3 ± 0.67 pg/mL) when compared to ND group (9.07 ± 0.71 pg/mL). In contrast, treatment with high and low PMF significantly increased (11.23 ± 0.62 pg/mL) and (9.09 ± 0.46 pg/mL) the BDNF protein level in brain of zebrafish respectively when compared to the HFD group (7.3 ± 0.67 pg/mL) (Fig. 17).

4. Discussion

Obesity is a global pandemic impacting the health of adults and children in both developed and underdeveloped nations. It is associated with multiple peripheral diseases such as diabetes, liver, kidney, cardiovascular and central nervous system diseases, and is also linked with the diagnosis of over 13 different types of cancers (Pierobon et al., 2013; Forny-Germano et al., 2019; Murtha et al., 2022; Karra et al., 2022). Research shows that these health complications drastically reduce quality of life and expectancy, while increasing health-care costs. An

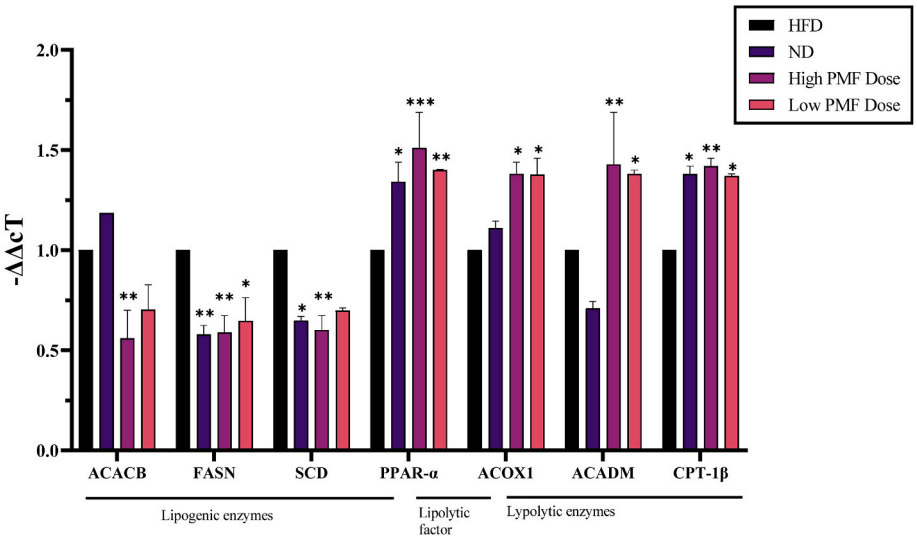

Fig. 14. Effects of PMF exposure on the expression of lipogenic and lipolytic (factors and direct targeted) genes in hepatocytes of zebrafish overfed for 6 weeks. Data represented as means (±SEM), n = 6. Statistical analysis was performed by Two-Way ANOVA followed by Bonferroni post-test and significant p-values indicated as *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. The mRNA expression values were normalized with β-Actin expressed as a ratio of the HFD group. *ND, normal diet; HFD, high fat diet.*

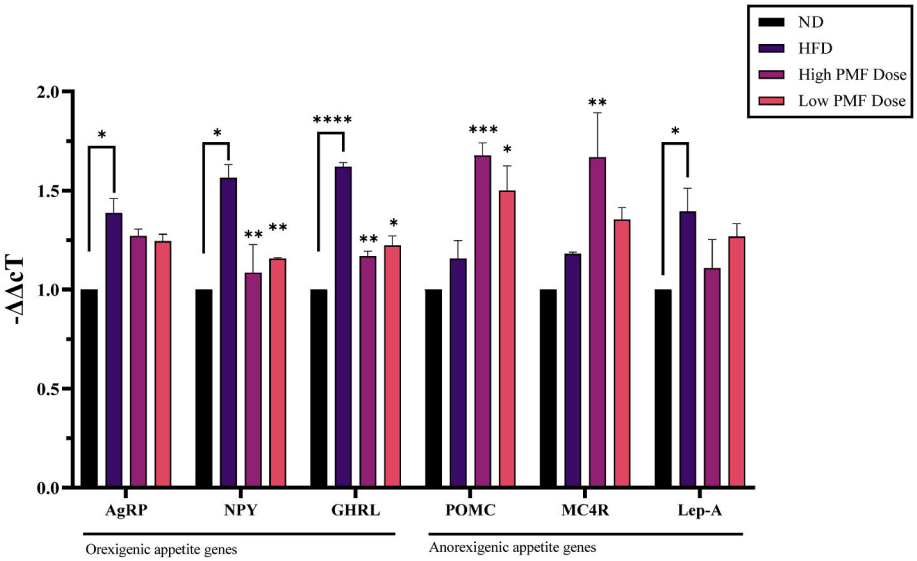

Fig. 15. PMF can modulate the expression of selected appetite responsible-orexigenic and -anorexigenic genes in zebrafish brain overfed for 6 weeks. Data represents as means (±SEM), n = 8. Statistical analyses were performed by Two-Way ANOVA followed by Bonferroni post-test and significant p-values indicated as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$. mRNA expression values were normalized with β-Actin expressed as a ratio of the ND group. *ND, normal diet; HFD, high fat diet.*

effective anti-obesity drug with minimal or no side effects represents an urgent medical need. This study examined the efficacy of PMF using an HFD-induced adult zebrafish model to determine its anti-obesogenic properties.

Zebrafish have emerged as a highly effective model for studying metabolic diseases due to their genetic, physiological, and functional similarities to humans. Notably, their lipid metabolism closely mirrors that of humans, making them a valuable system for investigating glucose homeostasis, leptin signalling, and insulin regulation (Oka et al., 2010; Zang et al., 2018). The structural and functional conservation of key metabolic organs, including the liver, adipose tissue, and brain, further enhances their relevance in metabolic research (Schlegel et al., 2007; Howe et al., 2013). In addition, zebrafish share fundamental neuroendocrine mechanisms that regulate appetite and food intake with mammals, including the hypothalamic circuits involved in energy homeostasis (Bessesen, 2008; Nishio et al., 2008; Shams et al., 2018). These conserved pathways make zebrafish a powerful tool for investigating obesity and neurological disorders linked to metabolic dysfunction. Studies using zebrafish have provided critical insights into metabolic and neurobehavioral regulation, including the effects of genetic mutations, pharmacological interventions, and environmental factors on metabolic health (Zang et al., 2018; Howe et al., 2013; Popiolek-Barczyk et al., 2018).

Despite these advantages, the translational applicability of zebrafish findings requires careful validation. While zebrafish share key metabolic and neurological features with humans, their physiology differs in aspects such as adipose tissue distribution, thermoregulation, and the complexity of their central nervous system. Therefore, findings from

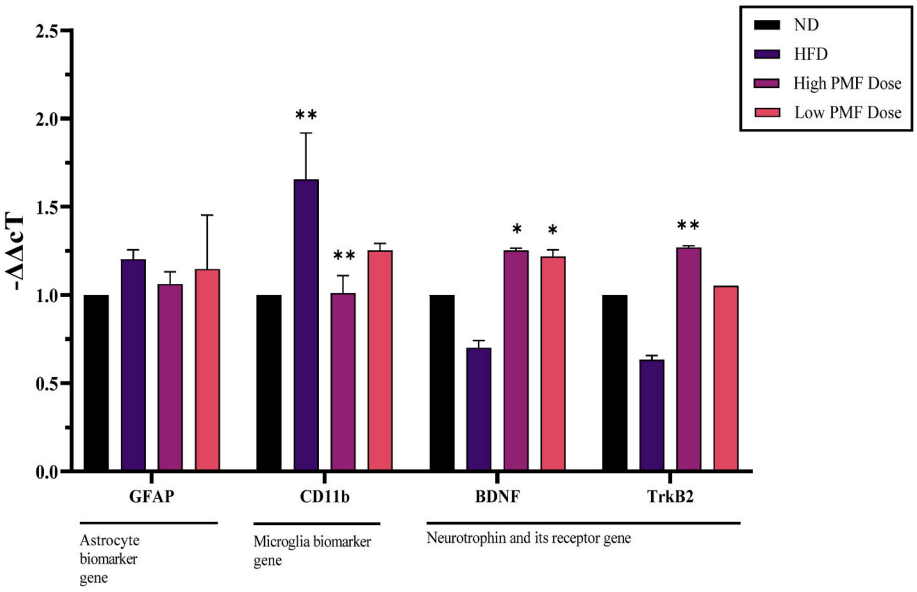

Fig. 16. PMF can reduce the expression of inflammation-related gene and provides neuroprotective effect in zebrafish. Data represented as means (±SEM). (BNDF and TrkB2, n = 8). (GFAP and CD11b, n = 10). Statistical analysis was performed by Two-Way ANOVA followed by Bonferroni post-test and significant p-values were indicated as $*P<0.05$ and $**P<0.01$. mRNA expression values were normalized with β-Actin expressed as a ratio of the ND group. *ND, normal diet; HFD, high fat diet.*

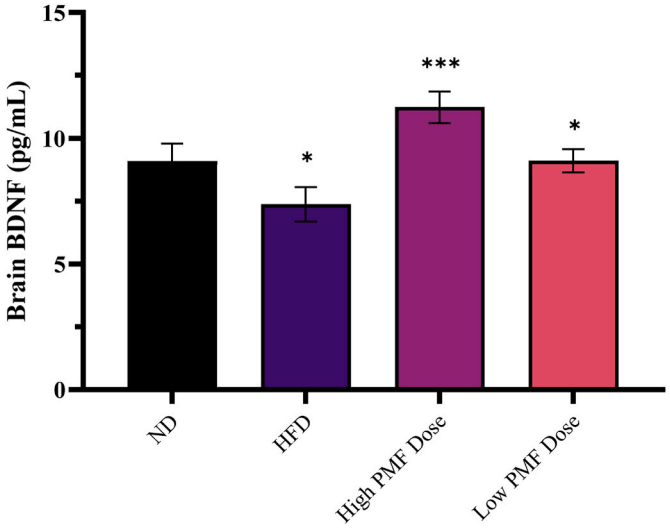

Fig. 17. Brain BDNF contents of non-treated and treated zebrafish with high (25 mg/L) and low doses (10 mg/L) of PMF following HFD and ND for 6 weeks. Values are means (±SD) in triplicates, n = 8. Statistical analysis was performed using One-Way ANOVA followed by post-test Tukey multiple comparison and significant p-values were indicated as $*P<0.05$, $**P<0.01$ and $***P<0.001$. *ND; normal diet, HFD; high-fat diet.*

zebrafish studies should be complemented by research in mammalian models, particularly rodents, and ultimately validated through clinical studies to ensure their relevance to human health. This multi-tiered approach strengthens the reliability of preclinical research and enhances the potential for developing effective therapeutic strategies.

Landgraf and colleagues have reported the development of a HFD-overfeeding zebrafish obese model over a period of 8 weeks, presenting high BMI, hyperglycaemic characteristics, and liver fibrosis via ectopic lipid accumulation, in addition to altered lipid metabolism marker gene expression (Landgraf et al., 2017). For our study, we were able to develop the same metabolically unhealthy obese zebrafish model over just 6 weeks by overfeeding with a HFD (30 mg of egg yolk powder 3 times/day along with 5 mg of artemia twice/day). This feeding regime provides a more rapid (and therefore cost effective) method for induction of metabolically distinct obesity phenotypes in zebrafish for future studies of metabolic regulatory mechanisms. Compared to other animal models, the zebrafish system has practical advantages. For instance, research suggests that mice require approximately 4–6 months to develop obesity (Fan et al., 2021; Wu et al., 2021). Zebrafish are therefore a time efficient and practical model to study obesity and related lipid metabolism.

We first explored the toxicity of PMF through *in vivo* acute and chronic toxicity tests in zebrafish over 4 and 14 days respectively, to determine the safest dose of PMF in adult zebrafish. The results indicated that PMF is toxic at higher concentrations, with an LD-50 value of 32.6 mg/L (Fig. 16). Comparing this value with acute toxicity rating scale provided by Fish and Wildlife Service (FWS) (Reed et al., 2019; El-Harbawi, 2014), it was found to be "slightly toxic" (for context, super toxic is defined as having a LD-50 of 0.01–0.1 mg/L, highly toxic at 0.1–1 mg/L, and moderately toxic at 1–10 mg/L respectively). Following a 14-day prolonged toxicity study, the body length, weight, and blood glucose levels of adult zebrafish were significantly reduced in higher PMF groups. This implies that PMF delays growth parameters and reduces blood glucose levels. A lower dose (10 mg/L) and higher dose (25 mg/L) of PMF were identified for testing the efficacy of the compound in established HFD induced zebrafish. Our results demonstrated that PMF treatment of overfed groups had inhibitory effects on body weight and length as compared to positive control (HFD) group; as well as significantly reduced BMI when compared to positive and solvent control groups. This indicates that PMF could effectively attenuate BW gain in HFD-induced obese zebrafish, highlighting its positive effects as a treatment of obesity.

Food intake was measured weekly during the PMF treatment period in terms of the average number of artemia consumed. The results were significantly different between HFD and PMF treated group (Fig. 10), indicating that PMF can suppress appetite. This result suggests that the effects of PMF on improving BW loss may occur, at least in part, via appetite regulation. Notably, similar flavonoids, such as quercetin and orange juice (OJe) extracts, have been reported to influence central appetite regulation, leading to reduced food intake and improved metabolic homeostasis (Russo et al., 2023; Rufino et al., 2021).

After overfeeding for 6 weeks, we measured the phenotypic, biochemical (fasting blood glucose, plasma TG and TC levels), hepatic

lipids (LDL and HDL) parameters as well as genotypic expression profiles of the zebrafish model to examine the effects on both ND and HFD zebrafish. The results demonstrated that body length, weight, and BMI of HFD zebrafish were significantly higher than ND control group. This also corresponded to a significant increase in fasting blood glucose levels, plasma TG, and TC levels in the HFD group. This implies that uncontrolled overfeeding with HFD results in hyperglycaemia, hypertriglyceridemia, and hypercholesterolemia in the obese model (Rufino et al., 2021). We also explored glycolipid metabolism in our HFD-induced zebrafish model, analysing lipid parameters including LDL and HDL. A considerable increase in the value of hepatic LDL content in HFD group as compared to ND group was recorded. However, no significant effect was found in hepatic HDL levels. Sevearal studies stated that reduced levels of HDL in obese states are associated with increased risk for the development of coronary artery disease (CAD) (Rashid et al., 2007; Stadler et al., 2021). Our HFD model was designed to mimic the progression of hyperglycaemia, hypertriglyceridemia, and hypercholesterolemia in zebrafish with excess calories. Our developed obese model showed parallel results to previous studies, where HFD-induced obese zebrafish also showed similar pathophysiological conditions of obesity (Oka et al., 2010; Leibold et al., 2015).

Obesity driven dysregulation of metabolic processes causes metabolic syndrome (MetS) conditions such as cardiovascular, type 2 diabetes, fatty liver, kidney diseases, insulin resistance and dyslipidaemia (Wu et al., 2020; Jam et al., 2022). To confirm the potency of PMF in preventing metabolic syndrome, we examined biochemical parameters such as plasma TG and TC levels, lipids parameters including hepatic LDL and HDL levels, and fasting blood glucose levels in HFD induced zebrafish. As shown in Fig. 9 the overfed HFD-induced zebrafish showed an increase of expected biochemical parameters (TG and TC) and blood glucose levels after 6 weeks. The HFD zebrafish groups exposed to 10 mg/L and 25 mg/L of PMF demonstrated a significant reduction in plasma TG, plasma TC, and fasting blood glucose levels as compared to the positive control. Similarly, PMF treatment also significantly reduced the LDL levels, however, hepatic HDL levels revealed no significant effects in all groups. LDL is responsible for cholesterol transportation to cells whilst HDL helps excess cholesterol return to the liver and then removes it from the body (Zhang et al., 2024; Luo et al., 2022). The observed reduction in hepatic LDL levels with PMF treatment, despite the lack of significant effects on HDL, suggests that PMF may selectively modulate specific lipoprotein metabolism pathways. This selectivity could be attributed to its regulatory effects on hepatic lipid homeostasis, favoring LDL clearance while maintaining HDL stability. Mechanistically, PMF may enhance LDL receptor (LDLR)-mediated uptake and degradation of LDL in the liver, leading to its reduced accumulation. Therefore, it is understood that consumption of PMF increases blood glucose disposal and normalizes the impact of triglycerides, and total cholesterol in the blood, and efficiently delivers cholesterol to liver through low-density lipoprotein. Consequently, PMF exposure ameliorates hypertriglyceridemia and hypercholesterolemia, contributing to improved lipid homeostasis. Additionally, PMF exerts a blood glucose-lowering effect, potentially mitigating overfeeding-induced diabetic complications, thereby demonstrating its anti-hyperglycemic potential.

We further determined the expression levels of the genes associated with lipogenesis, and lipolysis. We observed downregulation in the expression of PPAR-γ and C/EBP-α following PMF exposure compared with the HFD zebrafish group as shown in Fig. 14. PPAR-γ and C/EBP-α are the central transcriptional regulators of adipogenesis. These genes regulate lipid metabolism in adipose tissue and the liver, the ultimate effector organs of adipogenesis. Downregulation of these transcriptional factors triggers loss of fat and enhanced lipolysis (Wilson-Fritch et al., 2003; Yogosawa et al., 2013). In our study, PMF significantly reduced the expression of PPAR-γ and C/EBP-α indicating that PMF is capable of lowering the activity of fat cells in liver. In adddition, PMF also reduced mRNA expressions of lipogenic genes (SREB-F1, ACACB, FASN and SCD) in the liver of HFD-induced zebrafish model, and enhanced the expression of lipolytic genes (ACOX1, ACADM, and CPT-1β. ACOX1). SREB-F1 is the most important transcription regulator of lipogenesis, crucial for energy storage, and enhances *de novo* lipid synthesis in the liver. ACACB, FASN, and SCD are key enzymes in lipid synthesis. ACOX1 is a peroxisomal β-oxidation, ACADM is a mitochondrial β-oxidation enzyme, and CPT1β is a long FA transporting enzyme (Tahri-Joutey et al., 2021). A study with polymethoxyflavones have been tested in mice model that shown to inhibit obesity via lipid metabolism (Jin et al., 2022). Another studies demonstarted that 5-methoxyflavone (5 MF) on HFD fed mice, resultant in alleviates fat accumulation via hepatic adipogensis while improving the glucose metabolism (Zhang et al., 2023). Our study indicates that PMF possesses both anti-adipogenic and anti-lipogenic properties and enhances lipolysis in the liver of the HFD zebrafish model.

PMF-induced activation of PPAR-α and its downstream lipid catabolic enzymes, such as ACOX1, ACADM, and CPT1β, likely promotes enhanced fatty acid oxidation and LDL utilization. In contrast, HDL levels may remain unchanged due to the differential regulation of reverse cholesterol transport (RCT) pathways, where PMF may does not significantly affect ApoA-I expression or cholesterol efflux mechanisms involving ATP-binding cassette transporters. These findings indicate that PMF exerts a targeted lipid-modulating effect, primarily influencing LDL metabolism. Further studies are warranted to elucidate the precise molecular mechanisms underlying this selective lipoprotein regulation.

Obesity is also characterized by chronic low-grade inflammation with permanently increased oxidative stress (Rosenthal et al., 2021). In our study, it was found that HFD overfeeding in zebrafish significantly changes anti-oxidant enzyme activities as well as oxidative stress markers (MDA and NO content) in the liver and brain of zebrafish. In this study, PMF treatment considerably ameliorated both hepatic and brain MDA and NO activities, and enhanced total-SOD, CAT, GSH-Px, and GST activities in the liver and brain. A 3′,4′,5,7-tetrahydroxyflavone (luteolin) has been shown to regulate multiple aspects of adipose biology including interference with the expression of adipogenic transcription factors (PPAR-γ and C/EBP-β), possesses anti-inflammatory properties (TNF-α and IL-6) and provide anti-oxidant activities (Sharma et al., 2025). Similarly, we have found that PMF is a potent agent that can reduce oxidative stress and increase anti-oxidant activities in zebrafish models. Nonetheless, it warrants further in-depth study.

There is growing evidence linking brain pathology to obesity through mechanisms such as neurotransmitter imbalance, neuroendocrine dysregulation, inflammation, increased OS, decreased neurotrophic growth factors, and neurodegeneration. Importantly, obesity and related metabolic conditions significantly impact neural pathways involved in meal size regulation and food intake mechanisms(Arkes, 2012; DC et al., 2012; Nguyen et al., 2013). Specifically, orexigenic AgRP/NPY-expressing neurons stimulate hunger, while anorexigenic POMC/MC4R-expressing neurons signal satiety (Vohra et al., 2022; Deem et al., 2022). Our current findings indicate that PMF exhibits the potential to modulate food intake neuropeptides by downregulating orexigenic genes such as NPY and GHRL while upregulating anorexigenic genes including POMC and MC4R within zebrafish brains. AgRP and NPY have been implicated in appetite stimulation, and sustained expression of these peptides promotes increased food foraging, hoarding, and overall intake, contributing significantly to obesity pathogenesis (Teubner et al., 2012; Zagmutt et al., 2018). Conversely, suppressing these peptides has shown promise in obesity treatment (Vohra et al., 2021).

Recent studies have highlighted intricate interactions between lipid metabolism and neuroprotection, particularly involving orexigenic and anorexigenic genes. For instance, NPY is not only a powerful hunger-inducing peptide but also influences lipid metabolism by enhancing adipogenesis and lipogenesis in peripheral tissues. Moreover, NPY reduces mitochondrial activity through decreased oxidative phosphorylation proteins, thereby inhibiting lipid oxidation and energy

expenditure. This dual role of NPY suggests a direct impact on lipid storage and utilization, with potential consequences for neuronal health (Sousa et al., 2023). Similarly, AgRP functions as an endogenous antagonist of MC4R, contributing to increased appetite and reduced metabolism, leading to lipid accumulation. Dysregulation of melanocortin signaling, particularly involving MC4R, has been strongly linked to obesity through increased lipid uptake and triglyceride synthesis in adipose tissues, affecting neuronal health (Nogueiras et al., 2007; Han et al., 2022).

On the other hand, POMC neurons produce α-melanocyte-stimulating hormone (α-MSH), an MC4R agonist that promotes satiety and increases energy expenditure. Activation of POMC neurons reduces food intake and promotes lipid mobilization, whereas their inhibition leads to decreased energy expenditure and elevated adiposity. Therefore, maintaining a balanced interaction between POMC and AGRP/NPY neurons is essential for both lipid metabolism and neuronal integrity (Han et al., 2022; Lieu et al., 2020). GHRL, another critical orexigenic hormone, promotes appetite via activation of NPY/AgRP neurons and modulates lipid metabolism through increased adipogenesis and reduced lipid oxidation. Excessive lipid accumulation linked to ghrelin's activity can lead to neuroinflammation and oxidative stress, underscoring the role of lipid metabolism in neuroprotection (Lieu et al., 2020).

The crosstalk between these orexigenic and anorexigenic genes and lipid metabolism presents significant implications for neuroprotection. Dysregulation of pathways involving NPY and AgRP can enhance lipid accumulation, mitochondrial dysfunction, and oxidative stress, negatively impacting neuronal health. Conversely, maintaining homeostatic equilibrium within the POMC and MC4R signaling pathways promotes lipid mobilization, enhances energy expenditure, and supports neuronal integrity and function. Nevertheless, the precise molecular mechanisms underlying the interactions between lipid metabolic processes, neuroprotective pathways, and PMF protective effects remain incompletely defined. Future research focusing on elucidating the intricate molecular crosstalk between lipid metabolism, neuroprotection, and PMF activity—particularly via modulation of orexigenic and anorexigenic gene expressions—could provide crucial mechanistic insights and identify novel therapeutic strategies for obesity-related neurodegenerative conditions.

Lastly, we investigated the effects of PMF on the expression of neuroprotective-related genes, specifically BDNF and its receptor, TrkB2, in the zebrafish brain. BDNF is a critical neurotrophic factor essential for neuronal growth, maintenance, and differentiation. It plays a pivotal role in promoting neuronal survival by enhancing synaptic transmission and motor performance(You et al., 2020; Tejeda et al., 2019; Palasz et al., 2020). Our findings indicate that PMF treatment led to a significant upregulation of BDNF and TrkB2 mRNA levels, along with an increase in BDNF protein expression in the brains of HFD-fed zebrafish. This suggests that PMF enhances BDNF gene and protein expression, potentially exerting a protective effect against obesity-associated neuronal damage. In support of these observations, prior research has shown that 7,8-dihydroxyflavone (7,8-DHF), a potent small-molecule agonist of the TrkB receptor, effectively mitigates obesity in female mice. This beneficial effect has been mechanistically associated with increased energy expenditure within skeletal muscle, emphasizing the pivotal regulatory role of BDNF-TrkB signaling in metabolic control and obesity prevention (Youn et al., 2015).

Additionally, we evaluated the expression of neuroimmune markers associated with resident immune cells in the brain, including Glial Fibrillary Acidic Protein (GFAP) for astrocytes and CD11b for microglia. Both GFAP and CD11b are established neuroinflammation biomarkers linked to brain injury and neuronal disorders (Wang et al., 2022; Gonçalves et al., 2008). Previous studies have shown that CD11b expression is elevated under obesogenic conditions (Wang et al., 2020, 2022; Ghaddar et al., 2020). In our study, PMF treatment significantly suppressed CD11b expression in HFD zebrafish, while GFAP levels remained unaffected. These findings indicate that PMF mitigates obesity-induced neuroinflammation, likely through the modulation of BDNF/TrkB2 signaling, thereby conferring neuroprotective effects in the zebrafish brain.

5. Conclusion

To determine the safest dose of PMF in our animal model, a toxicity study established its LD_{50} value at 32.59 mg/L. Prolonged toxicity assessments revealed no significant sex-dependent differences in body length, weight, or fasting blood glucose levels, indicating a stable toxicity profile.

In the HFD-induced obese zebrafish model, PMF demonstrated anti-obesity effects by modulating key metabolic pathways. Specifically, it reduced adipogenesis via PPAR-γ and C/EBP-α downregulation while suppressing lipogenesis by inhibiting SREBP-1, ACACB, FASN, and SCD expression. Additionally, PMF promoted lipolysis by upregulating PPAR-α, ACOX1, ACADM, and CPT-1β, suggesting an enhanced fatty acid oxidation process. However, the precise molecular mechanisms underlying these effects remain to be fully elucidated. Future studies should investigate whether PMF mediates its metabolic benefits through the WNT/β-catenin pathway or AMPK signaling.

Furthermore, PMF mitigated obesity-induced OS and inflammation in the liver and brain by enhancing the activity of key anti-oxidant enzymes, including Total-SOD, CAT, GSH-Px, and GST. It also regulated appetite by promoting anorexigenic pathways (upregulating POMC and MC4R) while suppressing orexigenic pathways (downregulating NPY and GHRL), thereby reducing hunger signals. Additionally, PMF exhibited neuroprotective effects, as evidenced by CD11b downregulation, indicating reduced microglial activation and neuroinflammation. It also enhanced neuronal survival via BDNF/TrkB2 signaling, which is crucial for neuroprotection and synaptic plasticity. However, further research is required to elucidate the precise molecular mechanisms underlying these neuroprotective and anti-inflammatory effects.

A limitation of this study is the lack of evaluation of PMF's antibacterial activity. Given that flavones have demonstrated antibacterial properties beneficial for health maintenance and the management of obesity-associated infections (Tang et al., 2025), future research should investigate whether PMF exerts similar antimicrobial effects.

Despite the advantages of zebrafish models in studying metabolic and neurological disorders, their translational applicability requires validation. Differences in adipose tissue distribution, thermoregulation, and brain complexity necessitate supplementary studies in mammalian models (e.g., rodents) and clinical trials to confirm human relevance. This multi-tiered validation strengthens the robustness of preclinical findings and enhances the potential for therapeutic development.

PMF emerges as a promising anti-obesity compound with protective effects against obesity-associated metabolic and neurological disorders. These findings suggest that PMF could serve as a potent alternative medicine for the prevention and management of metabolic syndromes related to obesity. Further molecular studies and clinical validation are warranted to fully establish its therapeutic potential.

CRediT authorship contribution statement

Muhammad Sufyan Vohra: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Bilal Ahmad:** Writing – review & editing, Writing – original draft, Investigation. **Emerald R. Taylor:** Writing – review & editing, Investigation. **Khaled Benchoula:** Writing – review & editing, Investigation. **Isabel Lim Fong:** Writing – review & editing, Conceptualization. **Ishwar S. Parhar:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Satoshi Ogawa:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Christopher J. Serpell:** Writing – review & editing, Supervision, Project administration, Funding

acquisition, Conceptualization. **Eng Hwa Wong:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors have no interests to declare.

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Data availability

Data will be made available on request.

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