RESEARCH ARTICLE

Effects of 2,3,5,6-Tetramethylpyrazine on alcohol-induced injury in liver cells and on the early life stages of zebrafish (Danio rerio Hamilton, 1822)

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ABSTRACT

Background: Alcoholic liver disease (ALD) is a major health problem referring to the collection of liver damage caused by excessive alcohol intake. The search for effective and safe alternatives for compounds from plants to protect the liver from extensive damages and delay the progress to a disease is still a big effort done in the scientific community. 2,3,5,6-Tetramethylpyrazine (TMP) is a compound found in a Chinese herbal medicinal plant, *Ligusticum chuanxiong* Hort and in some other plants.

Objective: This study was done to assess the hepatoprotective effects of TMP against ALD using histopathological analysis of zebrafish livers subjected to different exposure groups. TMP has been mainly used for the treatment of cardio- and cerebrovascular diseases due to its antioxidant, anti-inflammatory, and anti-apoptotic properties.

Methodology: Adult male zebrafish were exposed to three TMP concentrations (40, 60, and 80 mg/L TMP) and to 1% v/v of ethanol. The dissected livers of the zebrafish were processed for fixing on glass slides using the H&E stains and were observed under the light compound microscopes for scoring. The safety of the TMP to the early life stages of the zebrafish was tested using the Zebrafish Embryotoxicity Test (ZFET).

Results: Results showed that TMP was able to dose-dependently decrease mean scores for the four parameters diagnostic of ALD, i.e., steatosis, inflammation, cell death, and ballooning degeneration. These scores were comparable to those of the untreated group (no ethanol + no treatment) and positive control (ethanol + Hepasil DTXTM), with all groups' scores being statistically different from those of the negative control group (ethanol + no treatment) ($p<0.05$). Results for the ZFET showed that incidence of embryo
mortality as well as teratogenic malformations of embryos exposed to TMP were significantly lower compared to the positive control group.

Conclusion: The hepatoprotective role of TMP was implied because anomalies such as cholestasis, vessel congestion, and hemorrhage were only observed in the ethanol-treated group and not in the other groups. In the analysis of the early development of the embryos using the Zebrafish Embryotoxicity Test (ZFET), TMP was found to be non-toxic and non-teratogenic at concentrations used for liver treatment. These initial findings on TMP provided justification for its plausibility as a hepatoprotective compound against alcoholic liver diseases (ALD).

Keywords: *Alcoholic liver disease (ALD), Zebrafish Embryotoxicity Test (ZFET), Tetramethylpyrazine (TMP)*

Introduction

Alcohol consumption beyond safe limits has long been identified as a risk factor for developing liver diseases not only in Western countries but also in Asian countries such as Japan, China, and the Philippines [1-5]. The World Health Organization in 2011 has reported an estimate of 2.5 million alcohol abuse-related deaths per year, inclusive of alcoholic liver disease. Among the diseases linked to alcohol abuse, alcoholic liver disease is the leading cause of morbidity and mortality. Alcoholic liver disease (ALD) refers to the collection of liver damage caused by excessive alcohol intake. There are three widely recognized stages of this condition: alcoholic fatty liver (steatosis), acute alcoholic hepatitis (steatohepatitis), and cirrhosis [6]. Alcoholic fatty liver or steatosis is the earliest abnormality associated with alcohol-induced liver injury. It is defined as the accumulation of lipids within the cytoplasm of the hepatocytes which may be in

the microvesicular, macrovesicular, or mixed form, and the continuous accumulation of lipids inside the cells might cause cell rupture and elicit local inflammatory response [7-8]. The second stage of ALD is steatohepatitis which involves ballooning degeneration and necro-inflammatory changes brought about by the infiltration of immune cells as a response to the presence of cellular debris [9-10]. It is also associated with progressive steatosis and variable fibrosis [11]. The third phase, liver cirrhosis, is a consequence of long-term alcohol abuse. It is characterized by septa formation resulting in the generation of nodules, parenchymal extinction, and dilation of blood vessels [12,13]. ALD is primarily caused by oxidative stress because of changes in the hepatocytes' redox state, acetaldehyde adduct formation, hypoxia, reduction of antioxidant levels, formation of reactive oxygen species (ROS), and activation of Kupffer cells that release proinflammatory cytokines such as tumor necrosis-alpha (TNF-α), interleukin (IL)-1, and IL6 [1,14-20].

The primary treatment for alcoholic liver disease is complete abstinence from alcohol. Other medical treatments include intake of nutritional supplements and anti-inflammatory compounds. However, the latter has yet to be studied to be considered a primary treatment for alcoholic liver disease. The search for alternative treatment for ALD has banked on using components extracted from plants. An important component of the Chinese herbal medicinal plant, *Ligusticum chuanxiong* Hort, is a compound known as 2,3,5,6-Tetramethylpyrazine (TMP) [21]. It can also be found in the herb *Ligusticum wallichii* Franch [22] and *Jatropha podagrica* [23].

For several years, TMP has been mainly used for the treatment of both cardio- and cerebrovascular diseases due to its anti-inflammatory and vasodilatory effects [24,25]. Several studies have determined that this anti-inflammatory property of TMP is a result of its ability to block calcium channels [24-27]. In a study done in 2002 by Co *et al.*, TMP was shown to have a hepatoprotective effect by preventing lipid peroxidation in mice that were induced to have liver injury by thioacetamide [28]. Another similar study that investigated the hepatoprotective as well as the therapeutic roles of TMP was done using rats as models whose livers were injured by the administration of various doses of econazole [29]. The use of zebrafish as an animal model or as a tool for liver disease research has been reviewed extensively and commended for use in future studies [30,31]. The use of zebrafish in the study of human liver diseases is commendable since there are similarities in the livers of both humans and zebrafish such as hepatic cellular composition, function, signaling, and response to injury as well as the cellular processes that mediate liver diseases. The genes are also highly conserved between humans and zebrafish, making them a useful system to study the basic mechanisms of liver disease [31].

However, to date, there is no study yet regarding the possible hepatoprotective effects of TMP against ALD using the zebrafish model. Therefore, this study aimed to explore the possible effects of TMP on zebrafish induced to have alcoholic liver disease (ALD) by the administration of ethanol. To further evaluate the plausibility of TMP for possible liver injury treatment, its toxicity was also evaluated. The toxicity of TMP, however, is rarely studied because herbal products (with which TMP is an active component) are being sold as nutraceuticals and are, thus, exempted from pre-clinical efficacy and toxicity screening [32].

Despite the growing market demand for herbal medicines, concerns related to their safety remains questionable since strict quality control measures are not usually adhered to [33]. Available toxicological studies for TMP suggest that it is relatively safe for the following reasons: (1) it has a high oral LC50 value of 1910 mg/kg in rats; (2) it has no component present that at levels greater than or equal to 0.1% is identified as a human carcinogen (International Agency for Research for Cancer [IARC] as cited in M&U International); and, (3) it does not induce any pathological damage in organs as proven by hematological examinations and urine analyses [34,35]. However, these toxicological studies are rather limited such that other kinds of toxicity are not yet assessed. In fact, to date, there is no sufficient data available for TMP's developmental toxicity [36,37]. Developmental toxicity refers to the "adverse effects induced during pregnancy, or as a result of parental exposure, manifested at any point in the life span of the organism". Screening of this parameter should be made to complement previous toxicological studies on TMP. As such, the Zebrafish Embryo Toxicity Test (ZFET) was employed in this study to determine whether TMP possesses any hazardous effect in the development of the zebrafish.

Methodology

Animals and Treatments

Zebrafish Procurement and Maintenance

Wild type, sexually mature zebrafish (Danio rerio) were obtained from the Bureau of Fisheries and Aquatic Resources (BFAR) in Taal Lake, Batangas. They were housed in 20 x 10 x 12 inches aquaria with a 10-gallon capacity. Aerating pumps and sponge filters were provided for the maintenance of proper conditions. Feeding was done twice a day with

TetraMinTM flakes, supplemented with brine shrimp (Artemia sp.). The following control conditions in the aquaria were maintained: 26°± 10°C, dissolved oxygen of >6 mg/L, conductivity of ~300µS, chlorine, nitrates, nitrite content of ~0 mg/L, and pH of 7.5±0.5. A 12-h light/12-h dark photoperiod was also strictly followed throughout the experiment.

Chemicals

Ninety-eight percent (98%) TMP was purchased from Sigma-Aldrich (Science Park, Singapore) and delivered through Chemline Scientific Corporation in Tandang Sora, Quezon City. Absolute ethanol of technical grade was purchased from Duksan Reagents. USANA Hepasil DTXTM was bought from a trusted distributor.

Lethality Test

A preliminary test was done to determine which concentrations are sublethal and/or potentially effective to produce significant hepatoprotective effects against alcoholic liver disease. Fifty (50) male adult zebrafish were divided into ten groups of five, each group was exposed to TMP concentrations ranging from 10 to 100 mg/L. Survival rates were observed for 7 days. Results revealed mortality at concentrations 90 and 100 mg/L. Since no deaths were observed at 80 mg/L, it was recorded as the maximum tolerable concentration (MTC).

Liver Toxicity and Treatment

Zebrafish Exposure Set-ups

Adult male zebrafish were randomly selected and divided into six groups (A-F) of 10-15 individuals each. Each group was housed in different tanks. Only male zebrafish were used in this study to avoid possible gender-related differences in the alcohol metabolism between males and females since it is known that females have enhanced sensitivity to alcohol following chronic alcohol exposure [38]. The zebrafish in Group A were not exposed to 1% v/v ethanol nor any treatments (untreated group). The rest of the groups, however, were exposed to 1% v/v ethanol for 8 weeks, to induce liver injury. Exposure time is in accordance with the study of Lin *et al.* [39]. The fishes in Group B served as the negative control since the fishes in this group were not co-treated with any hepatoprotective chemical. Group C served as the positive control since they were co-treated with 60 mg/L USANA Hepasil DTXTM, a well-known water-insoluble liver supplement. Hepasil comes in the form of tablets and was thus crushed and dissolved initially in 2% dimethyl sulfoxide (DMSO) and were redissolved with TMP in a v/v ratio according to the treatment doses. Groups D, E, and F were the experimental groups such that they were exposed to ethanol together with 40, 60, and 80 mg/L TMP which were dissolved in .0008%, .0012%, and .0016% of DMSO, respectively. Note that these concentrations were based on the determined MTC serving as the high dosage group. All solutions were replaced every day to replenish the respective concentration of the treatments per tank, especially since ethanol is a volatile compound. The zebrafish were treated and used humanely during the experimental procedures as well as at the end of the experiment wherein the fishes were euthanized by submerging them in ice-cold water, as approved by the Institutional Animal Care and Use Committee of the University of the Philippines Manila.

Liver Histopathology

Ten (10) zebrafish per group were euthanized via submersion in ice-cold water for approximately five minutes. Their livers were immediately dissected as per protocol [40]. The fixation of the liver tissues was done in 10% neutral buffered formaldehyde. The fixed liver samples were brought to the University of the Philippines, College of Medicine, Department of Pathology for slide preparations using the H&E stain.

A scoring system for hepatic steatosis, inflammation, cell death, and ballooning degeneration [12,41] (with slight modifications) was used to semi-quantitatively assess the degree of damage brought about by alcohol (Table 1).

Table 1. *Scoring system for steatosis, inflammation, cell death, and ballooning degeneration for the liver**

Score	Steatosis (% hepatocyte involvement)	Inflammation (lobular)	Cell death	Ballooning degeneration
0	< 5%	No foci	Absent	None
1	5 – 33%	> 2 foci	Focal apoptosis (few acidophil bodies)	Few ballooned cells
2	33 – 66% >	2 – 4 foci	1 necrotic focus	Many ballooned cells
3	66%	> 4 foci	> 2 necrotic foci	

*Based on Nanji *et al*. (2002) and Yip *et al.* (2006)

Steatosis was evaluated under oil-immersion field while inflammation, cell death, and ballooning degeneration were evaluated under high power magnification. Three randomly selected fields were observed for each histopathological section and the scores were averaged for each. The scoring of the histological sections of the liver samples was done by the researchers of this study, but partiality was minimized because the slides obtained from the Philippine General Hospital were not labelled accordingly with the experimental groups used in this study and had to be observed one by one.

Zebrafish Embryotoxicity Test (ZFET)

Zebrafish Spawning and Egg Collection

Three days prior to spawning, brine shrimp (Artemia sp.) were fed daily ad libitum to sexually mature zebrafish while maintaining a 12-h light/12-h dark photoperiod for optimal mating. A breeding chamber was equipped below with a spawning tray covered with a mesh net with a grid size of 2.55 mm. Approximately 24 hours prior to mating, twelve (12) adult zebrafish of 2:1 male to female ratio were placed inside the chamber. Mating was triggered upon illumination and eggs were collected after about 30 minutes.

The collected eggs were washed with reconstituted water and were transferred to respective Petri dishes of varying treatments using a micropipette with a large tip. Spawned eggs were screened under a stereomicroscope (25x magnification) to identify normal, fertilized eggs. The selected eggs were subsequently transferred respectively to 96-well plates for each treatment.

Zebrafish Embryo Exposure to Test Chemicals

Five hundred four (504) viable embryos were collected for exposure to five TMP concentrations (20, 40, 60, 80, and 100 mg/L) with 24 embryos for each concentration used. Note that these concentrations were based on the concentrations used during the liver toxicity test with additional two concentrations. Prior to the transfer of embryos, the wells were pre-saturated with their respective concentration for 24 hours. The placement of the embryos followed a 1:1 embryo to well ratio to exclude mutual influences. The positive control that was used to induce embryotoxicity effects was 3.5% ethanol, while the negative control was reconstituted water. The average of three readings for each of the experimental groups was computed and was used to represent the results of the experiment.

Data Evaluation

Egg development was recorded at time points 24, 48, 72, and 96 hours post-fertilization (hpf). Observations were scored using developmental parameters such as lethal and sublethal endpoints. The lethal endpoints are distinct aberrations at 24 and 48 hpf that immediately indicate the death of a supposedly developing embryo. These include coagulation of the embryo, non-detachment of the tail, non-formation of the somites, and non-detection of heartbeat. The sublethal endpoints, on the other hand, are morphological aberrations during larval and hatching stages of 72 hpf which lower the chances of survival of the developing organism. Sublethal endpoints assessed in this study include spinal curvature, yolk sac edema, pericardial edema, heart rate, and body length. The heart rate was obtained by calculating the average of three readings of the number of heartbeats per minute. Video recordings were also made for backup purposes.

Data Processing and Analysis

All data were expressed as mean ± standard error of the mean (SEM). The statistical software IBM SPSS version 20 was used to analyze the data. Comparison of means among groups was done using One-way ANOVA or Kruskal-Wallis test (in cases wherein assumptions for normality were not met). Comparison of means between levels of independent variable was done using Tukey's post hoc test or Mann-Whitney U test (in cases wherein assumptions for normality were not met). Statistical significance was accepted at 95% confidence interval ($p<0.05$) for all tests.

Results

Lethality Test

All male zebrafish were noted to have survived until the 5th day as shown in Figure 1. Mortality was, however, noted at concentrations 90 mg/L and 100 mg/L TMP at days 6 and 7; with the lowest survival rate (40%) observed at the highest concentration. This study, then, used the maximum tolerable concentration (MTC) of 80 mg/L as the concentration for the high dosage group. The mid and low dosage were determined in increments of 20 from 80 mg/L, that is 60 mg/L for the mid dosage group and 40 mg/L for the low dosage group.

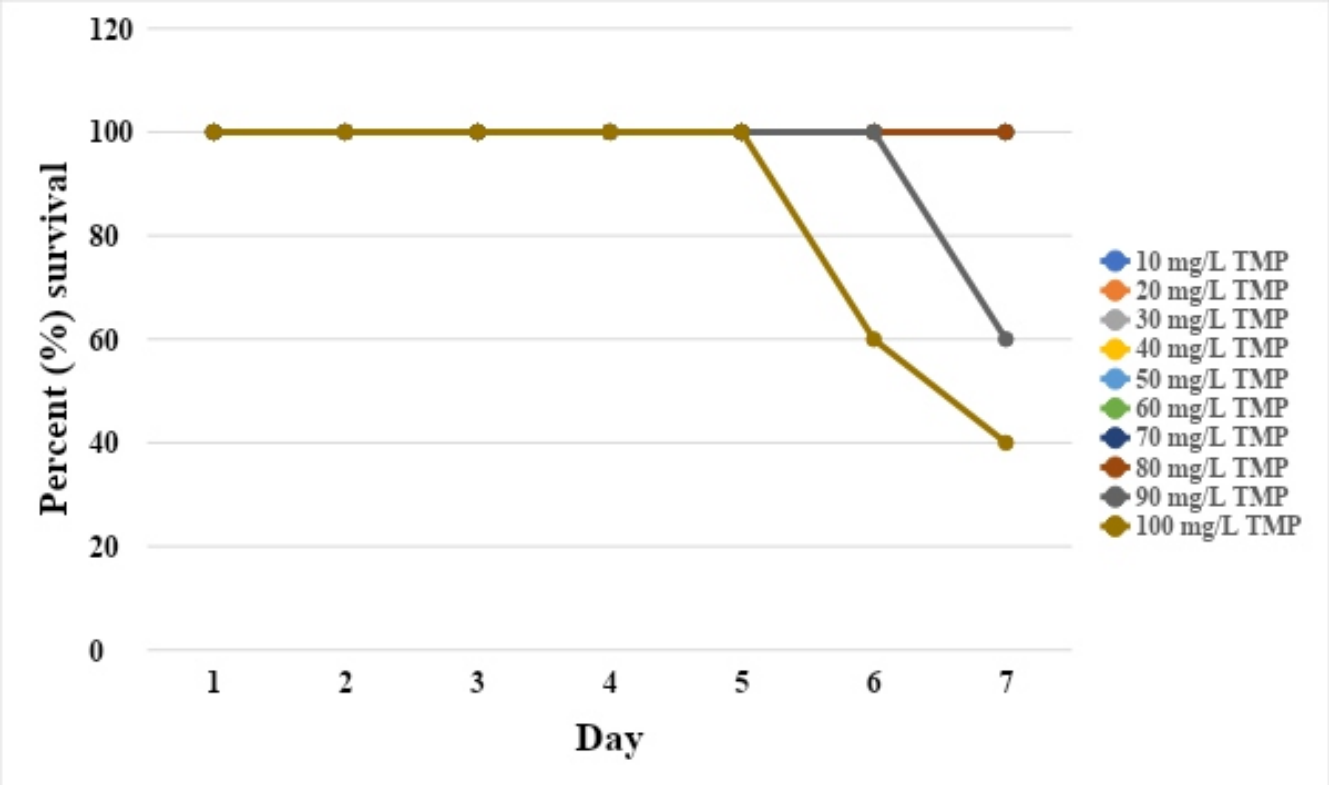

Figure 1. *Survival rates of adult male zebrafish exposed to varying concentrations of TMP for seven days.*

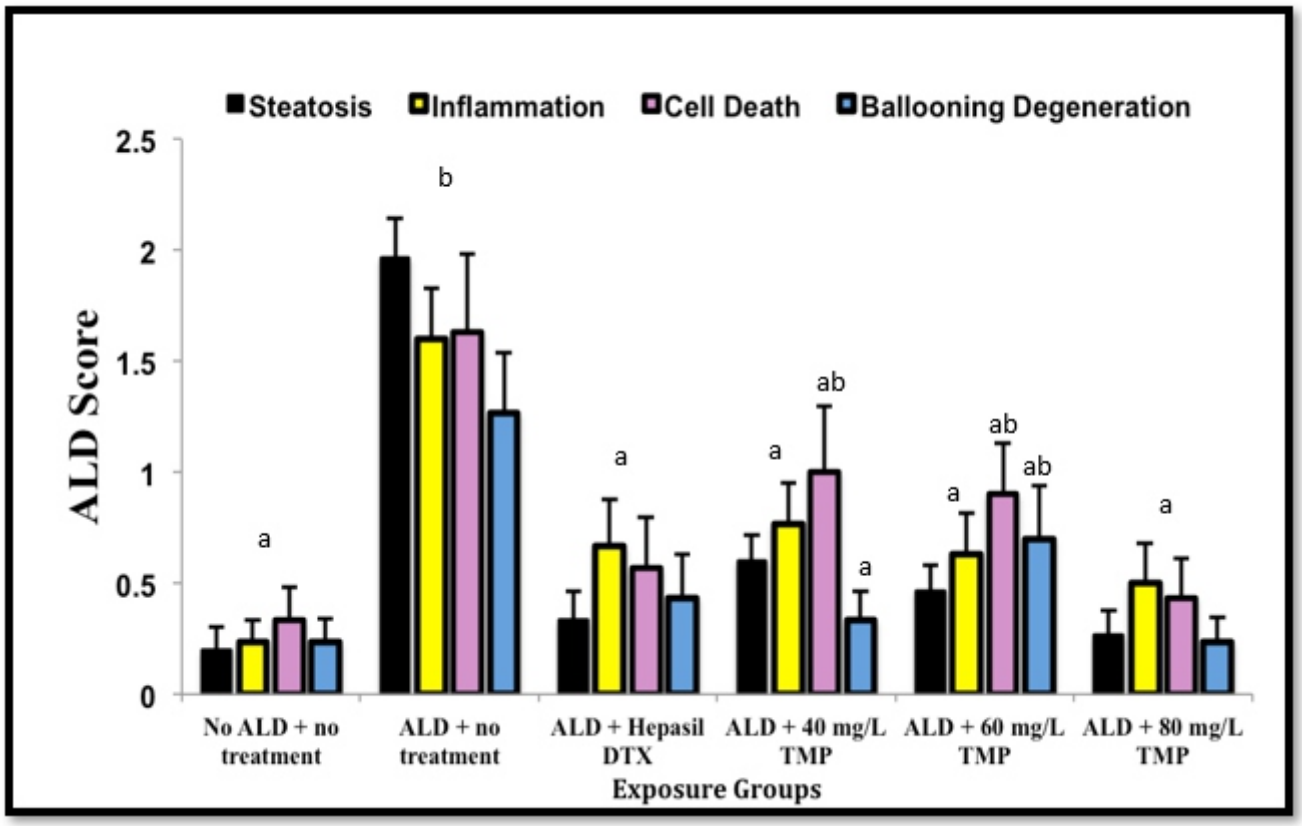

Figure 2. *Effects of TMP on the livers of male adult zebrafish with ALD. The ALD scores were based on steatosis, inflammation, cell death, and ballooning degeneration. Values represent mean score ± SEM. Treatments labeled with the same letter (per parameter) do not significantly differ at α = 0.05.*

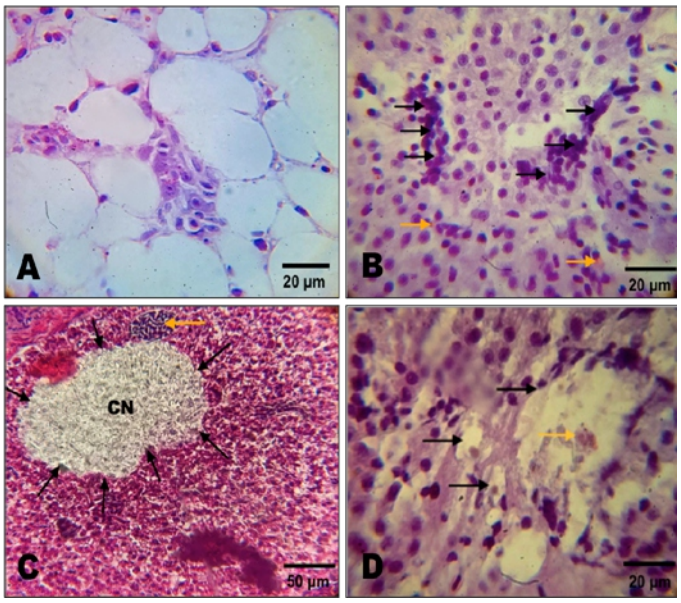

Figure 3. *Representative histological sections of zebrafish liver exposed to 1% ethanol with no Hepasil nor TMP treatments given to the fishes. The hallmarks of Alcohol Liver Disease are evident in the A – D sections as viewed in a 1000x magnification of a compound microscope:*
(A) Macrovesicular steatosis is shown wherein the hepatocytes have a single fat vacuole filling up the cell and the nucleus of each cell is pushed to the periphery.
(B) Mononuclear aggregates as pointed by the black arrows may indicate inflammation and the megamitochondria pointed by the yellow arrows may indicate liver injury/disease.
(C) Focal, coagulative necrosis (CN) as bounded by black arrows shows a dissolution of the hepatocyte and the associated inflammatory infiltrate pointed by the yellow arrow. The infiltrates might be the coagulated neutrophils.
(D) Ballooning degeneration as pointed by the black arrows is a loss of cell shape of the liver cells presumed to be caused by damage in the membrane. A Mallory-Denk body as pointed by the yellow arrow is considered to be an inclusion in the hepatocyte and may indicate liver injury.

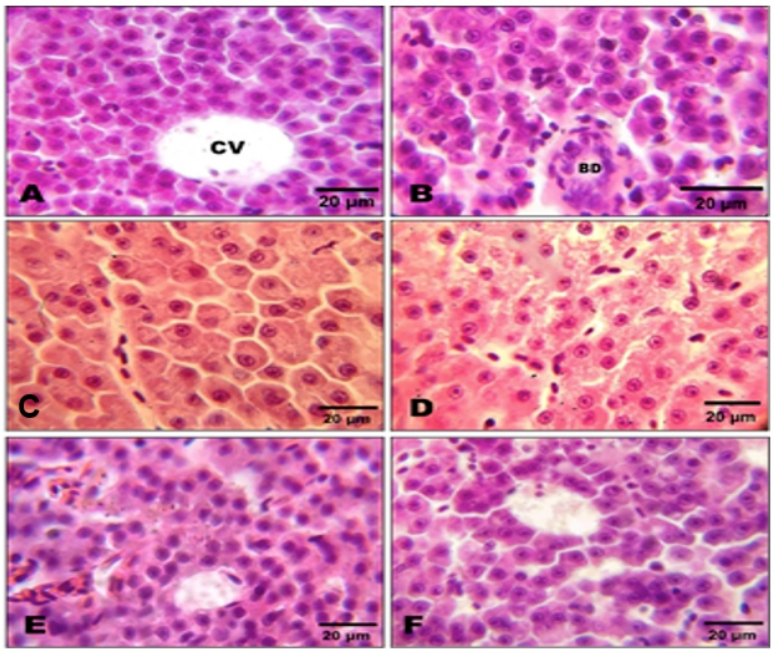

Figure 4. *Photomicrographs of representative zebrafish liver sections from group (A) with no ALD and no treatments given. Intact hepatocytes and a firm central vein (CV) are seen in this section. In (B) which is the group with induced ALD and no treatments given, shows some of the hepatocytes losing their usual polygonal shape and the bile ductule (BD) cells losing their distinct cuboidal shape. In (C) which is the liver section from Hepasil-DTX-treated group shows hepatocytes with intact shapes like in group A. The TMP-treated groups are shown in D-F. The low dosage group of 40 mg/L TMP is shown in D, the mid dosage group of 60 mg/L is shown in E and the high dosage group of 80 mg/L TMP all shows almost normal and intact liver architecture. All photos are viewed in 1000x magnification.*

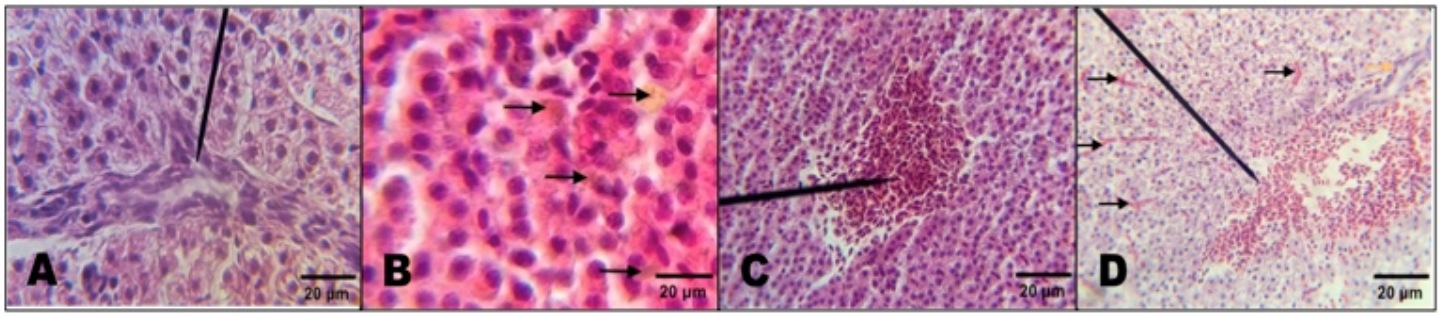

Figure 5. Photomicrographs of zebrafish livers showing (A) fibrosis (solid pointer), (B) cholestasis (black arrows), (C) congestion (solid pointer), and (D) hemorrhage (black arrows) [pointer = dilated sinusoid; yellow arrow = cluster of Kupffer cells]. A and B are viewed under HPO, C and D are viewed under OIO.

Effects of 2,3,5,6-Tetramethylpyrazine (TMP) against alcohol liver disease (ALD)

The effects of TMP on the zebrafish with alcohol-induced liver injury are shown in Figure 2. Results followed the trend in which the highest average ALD score for each of the four parameters was noted in group B (ALD + no treatment), whereas the lowest mean score was observed in group A (no ALD + no treatment). The TMP treatment groups generally showed a dose-dependent decrease in each of the parameters. Both TMP treatment groups (D-F) and group C (positive control treated with 60 mg/L Hepasil) showed no significant ($p<0.05$) difference in mean scores with those of the untreated group, suggesting high efficacy of TMP in terms of hepatoprotection. The representative histology of livers obtained from the ethanol-induced liver injury group with no Hepasil nor TMP treatments are shown in Figure 3. The liver sections obtained from group A (no ALD/no treatments), group B (with ALD/no treatments), group C (with ALD/Hepasil treatment), and groups D-F (TMP treatment groups) are shown in Figure 4. Other liver alterations such as fibrosis, cholestasis, vessel congestion, and hemorrhage have been observed in the ethanol group, but not for the other groups as seen in Figure 5. The negative control group was found to have the highest mean score of 1.967 ± 0.175, with all other groups significantly different from this ($p<0.05$). TMP dose-dependently decreased lipid droplet formation, although statistical analysis revealed that there is no significant difference between TMP groups and the positive control.

Zebrafish Embryotoxicity Test (ZFET)

The observations of lethal and sublethal endpoints in zebrafish embryos after exposure to treatments are shown in Table 2. The incidence of lethal and sublethal endpoints were mostly observed in the fishes exposed to 3.5% ethanol, the accepted concentration of ethanol that can induce embryotoxic effects to zebrafish embryos. The slowest heart rate and shortest body length were observed also in the 3.5% ethanol group. Though some were also observed in the TMP treatment groups (D-F) and group A control group, there was no statistically significant ($p<0.05$) difference between their means, whereas they all significantly differ from group C (Hepasil treated), suggesting the non-toxicity of TMP at these concentrations. Photomicrographs of zebrafish embryos showing different lethal and sublethal endpoints are shown in Figures 6 and 7.

Table 2. *Mean ± SEM of lethal and sublethal endpoints across different treatments in three independent trials. Results labeled with the same letter (per parameter) have no statistically significant difference at α = 0.05.*

	Lethal Endpoints (% Mortality)	Sublethal Endpoints				
		Yolk Sac Edema (% occurence)	Pericardial Edema (% occurence)	Spinal Curvature (% occurence)	Heart Rate (beats/min)	Body length (mm)
Reconstituted Water	5.56±3.67$_a$	0.00^a	1.39±1.39^a	0.00^a	198.65±1.77^{ac}	3.60±0.02^a
3.5% Ethanol	51.39±10.02^b	25.00±6.36^b	50.00±10.49b	19.44±1.39^b	174.13±5.84^b	2.50±0.05^b
20 mg/L TMP	4.17±0.00^a	0.00^a	0.00^a	0.00^a	180.64±1.74^b	3.54±0.02^{ac}
40 mg/L TMP	9.72±2.78^a	0.00^a	0.00^a	0.00^a	192.00±2.24^{ac}	3.44±0.02^{cd}
60 mg/L TMP	15.28±6.94^a	0.00^a	1.39±1.39^a	1.39±1.39^a	196.25±2.58^{ad}	3.34±0.03^{de}
80 mg/L TMP	4.17±2.41^a	2.78±2.78^a	6.49±3.67^a	6.49±3.67^{ac}	207.14±2.34^c	3.20±0.05^e
100 mg/L TMP	9.72±5.01^a	6.94±5.01^a	16.67±6.36^a	11.11±3.67^{bc}	203.94±2.28^{cd}	2.97±0.06^f

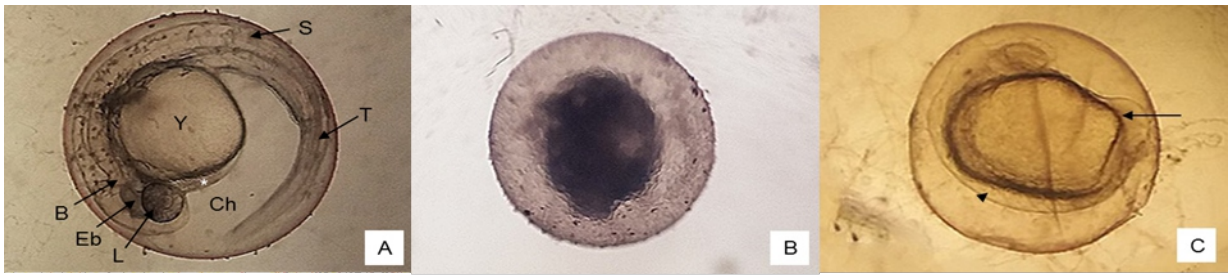

Figure 6. *Photomicrographs of zebrafish embryos approximately 24 hpf. Normal development of control embryo (A) immersed in reconstituted water in comparison to the observed lethal endpoints developed by embryos exposed to 3.5% ethanol even if there is TMP treatment (B, C). The embryo in A shows a visible brain (B) earbud (Eb), lens (L), yolk (Y), chorion (Ch), blood cells (asterisk), somites (S), and tail (T). Coagulation of the embryos are observed in (B) which were exposed to 3.5% ethanol. Non-detachment of the tail (C, black arrow) and non-formation of somites (C, arrowhead) were recorded from embryo exposed to 100 mg/L TMP.*

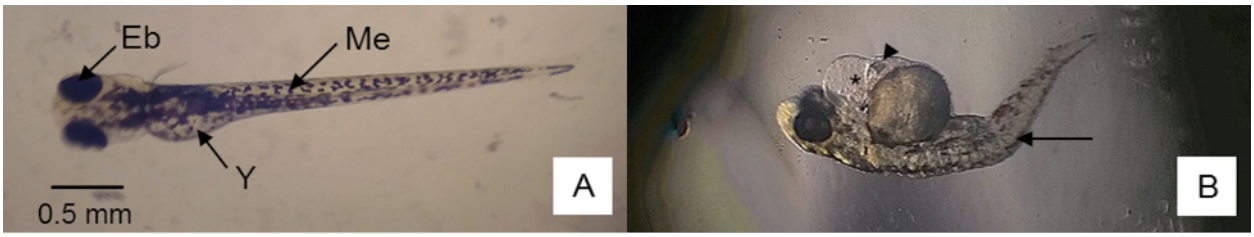

Figure 7. *Photomicrographs of zebrafish larva approximately 96 hpf. Normal control embryo (A) in comparison to the observed morphological abnormalities developed by embryos exposed to 3.5% ethanol (B). The 96 hpf embryo in A is with visible eye bud (Eb), yolk (Y), and melanophores (Me). Bar is approximately 0.5 mm. The larva in (B) 96 hpf shows pericardial edema (asterisk), yolk sac edema (arrowhead), and spinal curvature (arrow) as observed in an embryo exposed to 3.5% ethanol.*

Discussion

Effects of 2,3,5,6-Tetramethylpyrazine (TMP) against Alcoholic Liver Disease (ALD)

This study evaluated the hepatoprotective effects of 2,3,5,6-Tetramethylpyrazine (TMP) against alcoholic liver disease (ALD) using the zebrafish as the animal model. The zebrafish has many similar systems, organs, and tissues as those of mammals [42,43] that is why this was chosen as a model for studying the effects of TMP as a hepatoprotective agent. This disease of the liver is a multi-stepped process that includes steatosis, steatohepatitis, fibrosis, and cirrhosis [44,45]. Steatosis is the accumulation of lipids within hepatocytes and is considered the earliest histological abnormality manifested in ALD [12].

Results showed that TMP can significantly lessen steatosis in the zebrafish liver. The negative control group was found to have the highest mean score of 1.967 ± 0.175, with all other groups significantly different from this ($p<0.05$). TMP dose-dependently decreased lipid droplet formation, although statistical analysis revealed that there is no significant difference between TMP groups and the positive control, suggesting that these treatment groups had comparable effects.

As seen in Figure 3, group B (ALD but no TMP treatments) exhibited severe macrovesicular steatosis, with some microvesicular steatosis observed in this study. Some of the lipid droplets were seen to be diffusely distributed, while many of which were observed to be near the central veins (described as pericentral or zone 3; although acinar zones may be vague since portal triads are not distinct in the fish liver [46]. Acinar zone 3 is the site of maximal alcohol dehydrogenase activity. It is also the site of the central veins making this zone the most hypoxic, and therefore highly susceptible to injury [2]. Treatment with Hepasil DTXTM (group C) and TMP-treated groups (D-F) significantly decreased the amount of fat droplets.

Although the mechanism by which TMP has ameliorated steatosis was not determined in this study, it could be explained by its ability to: (1) downregulate PAQR3 and (2) inhibit SCAP/SREBP1 signaling pathway [47]. Sterol regulatory enzyme binding proteins (SREBPs) are important enzymes needed for the biosynthesis of fatty acids and cholesterol, while SREBP cleavage-activating protein (SCAP) is SCREBP's escort protein [48,49]. It was shown that TMP can down-regulate the mRNA and protein expressions of both SCAP and SREBP in the liver and heart of mice fed with high-fat diet [47]. Also, PAQ3, a member of the progestin

and adipoQ receptors superfamily has been shown to interact with SCAP and SREBP to promote SCAP/SREBP complex formation, and thus, enhance lipid synthesis [50]. Interestingly, PAQR3's protein expression could likewise be reduced by TMP treatment [47]. It is further suggested that the induction of PI3K/Akt/mTORC1 signaling pathway may be involved in the TMP mediated amelioration of excessive lipid synthesis by inhibiting the SCAP/SREBP signaling pathway [47]. It must be noted that studies on the ability of TMP to reduce abnormal lipid metabolism are rather limited. Therefore, it is of interest to explore and inquire on other mechanisms, for instance, on the effect of TMP on PPAR-α and AMPK, and NADH expression and production, which are also affected in alcohol liver injury besides the SCREBP pathway [48,51,52].

Chronic alcoholism induces oxidative stress one manifestation of which is lipid peroxidation and eventually reactive aldehyde formation contributing to liver cell damage. Lipid peroxidation refers to the oxidative degradation of lipids, in which free radicals "steal" electrons from cell membranes, and whose process proceeds in a free radical chain reaction mechanism [53,54]. The attenuation of hepatic damage as observed in this study may also be attributed to TMP's potent ability to inhibit lipid peroxidation. A study on the inhibitory activity of TMP in mice liver tissues on lipid peroxidation has shown that TMP exhibited a notable and dose-dependent inhibition on FeCl2-induced lipid peroxidation as indicated by a significant decrease in malondialdehyde (MDA) formation, a reactive end-product of peroxidation [55]. In a similar study, the same results were obtained, however, streptozotocin-induced diabetes in the mice liver tissues was used [56]. Furthermore, TMP has been shown to have a more potent free radical scavenging activity than Vitamin E, as indicated by the cytochrome-c test using liver homogenates [55]. TMP could also restrain mitochondrial ROS generation and upregulate the expression of PGC1, NRF1, and Tfam, reflecting mitochondrial biogenesis and reduced oxidative damage [57].

The presence of megamitochondria (Figure 3B) was also observed in the groups exposed to ethanol. Megamitochondria are eosinophilic, intracytoplasmic inclusions representing enlarged mitochondria with decreased membrane potential, and thus, compromised ability to synthesize ATP [58]. The formation of megamitochondria may be an adaptive process wherein mitochondria try to decrease reactive oxygen species (ROS) levels by decreasing oxygen consumption [59]. Megamitochondria were not observed in any of the TMP groups, which indicates that the TMP had protected the cells against oxidative damage.

The next stage of ALD is steatohepatitis or alcohol hepatitis which is characterized by necro-inflammatory changes with or without steatosis and may be associated with variable degrees of fibrosis [12]. Steatosis is linked to steatohepatitis because fatty hepatocytes may rupture, and the consequent release of lipids may elicit inflammatory responses. Another important component of alcoholic hepatitis is varying degrees of degenerative change such as hydropic ballooning of hepatocytes, which is described as cells being larger than their adjacent counterparts because of increased cytosolic fluid [60].

The exposure to TMP decreased the inflammation of the hepatocytes caused by alcohol in the zebrafish liver. As seen in Figure 3B, there were prominent mononuclear infiltrates within the lobules of the livers in the negative control group. Consequently, this group recorded the highest score based on inflammation (1.600 ± 0.232). Treatment groups and positive control groups had shown significantly less inflammation. The TMP groups dose-dependently decreased inflammation; however again, differences within TMP groups were not statistically significant.

The dramatic decrease in the mean inflammation scores in TMP groups is likely due to the TMP's anti-inflammatory properties, which are so far, established for the cardiovascular system, although not so much for the liver [61]. It has been shown that by using immunofluorescent assay, the TMP was able to dose-dependently decrease the expression of pro-inflammatory signal molecules, TNF-α, NLRP3, NF-kB, and IL-1β [62]. The potential role of NOD-like receptor protein 3 (NLRP3) inflammasome in alcoholic and non-alcoholic steatohepatitis, hepatitis, nanoparticle-induced liver injury, and other liver diseases has been attracting widespread attention [63,64]. It is suggested that the decrease in the mean inflammation scores of the hepatocytes treated with TMP may implicate the PDGF/BR/NLRP3/caspase1 pathway that is involved in TMP's anti-inflammatory effect in the liver [62]. The application of TMP may have reversed the trend dose-dependently, suggesting disruption of the pathway.

The TMP also reduced the incidence of cell injury and death (Figure 3C). Through the combined effects of (1) regulating lipid metabolism; (2) suppressing the expression of inflammatory cytokines (especially TNF-α, which is directly associated with cell death); (3) protecting against lipid peroxidation; and (4) countering ROS via free radical scavenging activity, cell injury (in the form of ballooning degeneration) and death (necrosis and apoptosis) are

lessened with TMP treatment. Indeed, the results of this study indicate that mean cell death scores were less than those of the negative control ($p<0.05$).

Necrosis or cell death is a process in which viable cells become nonviable, resulting in the dissolution of the cell contents, and in livers, there are several patterns of necrosis [65]. The results of this study also showed that the TMP treatment groups (D-F) and group C (Hepasil-treated group) had only a few spotty necrosis. In spotty necrosis, only minute clusters of hepatocytes, usually in association with lymphocytes, exhibit necrosis [64]. In contrast to group B (no TMP), it had higher counts of focal necrosis and even showed massive, coagulative necrosis. In focal necrosis, necrosis occurs in large groups of hepatocytes within a lobule [66]. The liver samples of the zebrafish in group B where there was no TMP given also showed coagulative necrosis, wherein hepatocytes still retained their cellular architecture despite the cytoplasm being lysed and the nuclei no longer distinct. In the groups of the zebrafish with TMP treatments (groups D-F), majority of the liver samples did not exhibit necrosis, and apoptosis was, thus, limited to few hepatocytes. Normal liver structure and function depend on the balance between hepatocyte death and regeneration. The presence of few apoptotic bodies in the groups of zebrafish with ALD but were given high doses of TMP (40, 60, and 80 mg/L TMP) may indicate regeneration of the hepatocytes without significant adverse effects on the liver as compared to the several focal necrosis seen in the ethanol group [67].

Ballooning degeneration, a form of liver injury used in the context of an inflamed liver, was also revealed to be lessened as evidenced by a significant decrease of mean scores compared to group B, where no treatments were given to fishes with induced ALD ($p<0.05$). The TMP treatment groups (D-F) also registered comparable scores with those of group C (Hepasil-treated) and group A (untreated group) at $p>0.05$. Liver sections in the 3.5% ethanol-treated group had many ballooning hepatocytes some of which were near the point of bursting, after which, lytic necrosis (lysis of adjacent liver cell membranes in ballooning hepatocytes) is expected to occur. Figure 3D, distinctly shows a Mallory-Denk body in one of the ballooned hepatocytes, representing damaged intermediate filament proteins and related glycoproteins due to altered proteosomic capacity [12]. Mallory bodies (MB), also known as Mallory-Denk bodies (MDB), are cytoplasmic hyaline inclusions of hepatocytes, once thought to be specific for alcoholic hepatitis now occur in other liver diseases which include non-alcoholic steatohepatitis (NASH), cholestatic liver diseases, primary biliary cirrhosis (PBC), and hepatocellular carcinoma (HCC) [65]. It must be noted that for the ballooning degeneration parameter, the low dose group (40 mg/L TMP) recorded a lower score than the mid-dose group (60 mg/L TMP), although the difference is not significant. It is possible that some of the hydropic hepatocytes observed in the mid dosage group may not be due to alcohol injury and are instead attributed to the glycogen or neutral fat content often found in swollen, but nonetheless normal cells, of cultured fish [46].

The final stage of alcohol-induced liver injury is fibrosis, or the thickening of connective tissue as a result of injury. Hepatic inflammation drives the activation and proliferation of hepatic stellate cells (HSCs), which synthesize and lay down collagen and other matrix proteins [46]. HSCs exposed to inflammatory microenvironment can release inflammatory factors, which then enhance the migration capacity of HSCs, and inflammatory cells to lesions, thus exaggerating hepatic fibrosis [12, 68].

Fibrosis was observed in group B (fishes with ALD but no treatments given) as shown in Figure 5A, but not for any other groups, including the Hepasil-treated group. Scoring was not done for this parameter because visualization of fibrosis was rather limited to using only the H&E stain. The Masson-Trichome stain [69] should have been utilized to observe fibrosis readily and clearly. Nonetheless, considering that the inflammation (the trigger for fibrosis) is lessened with the treatment with the TMP, it is reasonable to state that the TMP had prevented the development of fibrosis in TMP-treated zebrafish livers. This finding is consistent with previous studies which indicate that the TMP could lessen liver fibrosis as evidenced by it's ability to significantly decrease four biomarkers in mice with liver fibrosis. These biomarkers include blood hydroxyproline, α-SMA, procollagen, and fibronectin, all of which are elevated in mice with liver fibrosis [62,68].

Other histological abnormalities had been observed in the TMP-untreated group (group B), but not in any other treatment groups including the Hepasil-treated group (group C). These include cholestasis, congestion, and hemorrhage. Cholestasis refers to the decrease in bile flow due to the impaired secretion of hepatocytes or obstruction of bile flow through intra- or extrahepatic bile ducts [67]. It can be seen in Figure 5B that some of the canalicular bile plugs between individual hepatocytes had a brownish-green stippled appearance. Sufficient pressure, via bile accumulation, may cause bile ducts to rupture and ultimately cause hepatic

necrosis [58]. Congestion and hemorrhage are manifestations of disturbances in the liver circulation. Passive congestion is associated with the decrease in venous outflow due to tissue torsion, tumors, or other compressive events. In this condition, there is impediment in blood flow and stasis of several red cells within dilatated sinusoids as shown in Figure 5C. Hemorrhage, on the other hand, refers to the escape of blood from the vascular system as shown in Figure 5D [42]. Both anomalies impair oxygen supply to the hepatocytes through the blockage of the sinusoids, ultimately leading to liver cell ischemia with atrophy and eventual parenchymal extinction [58]. These manifestations are not pathognomonic to alcohol liver disease. However, the fact that these conditions were not found in any of the TMP treatment groups (D-F) strengthens the claim that TMP can protect the liver from injury—even from those which are not exclusive to ALD.

Taken together, this part of the study provided histopathological justification for the hepatoprotective effects of the TMP as evidenced by significantly lower ALD scores based on steatosis, inflammation, cell death, and ballooning degeneration, compared to the negative control. Similarly, the ALD scores of all TMP groups were comparable to both the untreated or no ALD (group A) and Hepasil-treated (group C), indicating a high efficacy of TMP in terms of hepatoprotection. Furthermore, other liver alterations which are not diagnostic of ALD were found in the 3.5% ethanol group but not in the TMP-treated groups (D-F), further implying the high hepatoprotective ability of the compound. This ability may be attributed to TMP's antioxidant, anti-inflammatory, and anti-apoptotic properties.

Zebrafish Embryotoxicity Test (ZFET)

Having established the potential of TMP as treatment for alcoholic liver disease, TMP doses previously used for liver injury treatment, as well as two other concentrations (20 and 100 mg/L) were evaluated based on their toxicity in zebrafish embryos. The negative control group (without embryotoxic effects) was reconstituted water, whereas the positive control was 3.5% ethanol (with known embryotoxic effects). A maximum tolerable concentration range of 0.5 to 1.5% ethanol was suggested for zebrafish embryos [70], however, other studies have used higher concentrations to imitate human consumption [71-73]. In this study, a concentration of 3.5% ethanol was set to standard as the positive control, since higher concentrations led to 100% population mortality as revealed by a series of range-finding experiments. This concentration led to lower population mortality while significantly increasing the incidence of morphological defects.

Analysis of ZFET results revealed that TMP is relatively not embryotoxic nor teratogenic at the concentrations tested. There was no trend found in the incidence of embryo mortality in varying concentrations of TMP, although the incidence of sublethal endpoints was observed to be gradually increasing with the increase in TMP concentration. Nonetheless, the overall lethal and sublethal effects in all concentrations of the recorded TMPs showed no statistically significant difference from the control group.

The heart rate was observed to slightly increase with increasing concentrations of TMP, although several studies have shown that the TMP was a vasodilator and thus causing a decrease in blood pressure and consequent heart rate [74,75]. TMP was also observed to increase heart rates [76], however, results were not significantly different from the negative control group. Body length, on the other hand, was observed to gradually decrease with an increase in TMP concentration; this may be attributed to the increase in antioxidant concentration, too much of which may elicit the production of pro-oxidants [77] which may then pose problems in the embryos' growth. Lower concentrations were not statistically significant with the control group as opposed to higher concentrations.

The highest mean mortality was observed in the ethanol-exposed group. Morphological abnormalities such as yolk sac edema, pericardial edema, and spinal curvature were also mostly seen in the ethanol-exposed group. It also recorded the slowest average heart rate, which is significantly different from all other treatments except for the lowest concentration of TMP with 20 mg/L. Moreover, embryos exposed to ethanol were also observed to have the shortest body length, which is also significantly different from all other treatments. Most embryos in this group were observed to have spinal curvatures which may explain their stunted length. Although the incidence of spinal curvature in the ethanol group and in the group with the highest concentration of TMP (100 mg/L) was revealed to have no statistically significant difference, the overall incidence of lethal and sublethal effects of ethanol showed a statistically significant difference from all other treatment groups suggesting the high toxicity of ethanol. Ethanol is a common solvent present in drugs and other materials intended for human use. However, it is also a known psychoactive ingredient and teratogen [70].

Several *in vivo* and *in vitro* studies in humans, mice, and zebrafish have been done in order to assess the toxicity of ethanol. Apoptotic neurodegeneration in different areas in the murine brain after prenatal ethanol exposure was

observed [78]. Other studies have consequently shown that neurodegeneration may lead to morphological abnormalities, unusual behavior, and learning deficits in ethanol-exposed mice [79-81]. Similar results have been reported regarding the effects of ethanol but, this time, on humans [82]. It was hypothesized that the mechanism behind ethanol teratogenicity is by the inductive capacity of ethanol on oxidative stress [83]. They explained that the increase in fetal consumption of nitric oxide causes vasoconstriction and abnormal blood flow. Motor and reflex development delays, pre- and post-natal growth deficiencies, and cranial, facial, joint, and cardiac abnormalities in infants were reported to have fetal alcohol syndrome [84].

Zebrafish embryos exposed to ethanol have been reported to exhibit similar defects as those in infants with the said fetal alcohol syndrome such as eye development defects, heart rate abnormalities, neurodegeneration, skeletal morphogenesis delays, and locomotion deficits [85,86]. Other morphological abnormalities observed in ethanol-treated zebrafish embryos include cyclopia, somite defects, and edema [70].

In general, there was no statistically significant difference between varying concentrations of TMP and the negative control group but the TMP significantly differed from the ethanol group. These suggest that the TMP is relatively non-toxic at the doses used for liver treatment in the alcohol-exposed adult zebrafish. The general absence of deleterious effects or abnormalities on embryos exposed to varying concentrations of TMP is a good indicator of its safety while being used as treatment for ALD. This can be attributed to its protective (e.g., anti-inflammatory, antioxidant, and anti-apoptotic) properties. Although some abnormalities were found in the highest concentration, the following must be considered (1) the frequencies of which are not statistically significant and (2) the TMP is traditionally used at effective concentrations up to 8-20 times lower [62] than the ones used in this study. Thus, it is unlikely that this drug could pose significant embryotoxic or teratogenic effects given that proper dosages are given to women who are pregnant with history of ALD.

Conclusion

The findings of this study provided evidence that the zebrafish is an effective model for alcoholic liver disease (ALD) because hallmarks of this condition have been successfully induced, consistent with previous studies. More importantly, this study demonstrated that TMP has a significant hepatoprotective effect against ALD. The dose-dependent reduction in hepatic lesions may be explained by TMP's inherent antioxidant, anti-inflammatory, and anti-apoptotic properties.

A series of trials of the zebrafish embryotoxicity test also revealed that the doses used for the treatment of alcoholic liver disease in this study are relatively non-toxic to the early life stages of the zebrafish. Moreover, since the TMP is normally used at doses lower than those used for this study, it is rather improbable that this compound may pose embryotoxic or teratogenic harm provided that proper dosages are recognized. Considering other tests such as immunohistochemistry, polymerase chain reaction, and biochemical analysis of blood serum are invaluable to support the findings of this study. These shall assess the biochemical and molecular basis of ALD and the precise mechanism by which TMP could ameliorate this condition. More concentrations should be used for the ZFET to accurately determine the LC50 of TMP.

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