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Toxicity Assessment of Ethanolic *Moringa oleifera* Leaf Extract (MOLE) Using Zebrafish (*Danio rerio*) Model

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ABSTRACT

Moringa oleifera, locally known as 'Kelor' in Malay, is a medicinal plant valued in Malaysia and other countries for its therapeutic bioactive compounds and health benefits, including anticancer and anti-inflammatory properties. However, the toxicity profile of ethanolic *M. oleifera* leaf extract (MOLE) has not been well explored. This study aims to assess the toxicity profile of MOLE using a zebrafish (*Danio rerio*) model. Zebrafish embryos were subjected to MOLE ($n \geq 15$; 24 h post-fertilisation [hpf]) at a concentration of 5-1000 µg/mL, and the survival rate, hatching rate, heart rate, and morphological development of zebrafish embryos were monitored daily for up to 72 h. Embryo media was used as a control. MOLE treatment was shown to be safe at concentrations ≤ 400 µg/mL with LC_{50} values of 1186 ±7 µg/mL, 560.1 ±7 µg/mL, and 445.1 ± 7 µg/mL at 24, 48, and 72 h post-treatment, respectively. A significant mortality rate and low heartbeat were recorded in embryos exposed to > 800 µg/mL across the three-time points. MOLE did not affect the hatching rate. A significant difference in embryos treated with > 800 µg/mL at 72 h post-treatment was not attributable to MOLE effects, as the embryos had died before hatching. Scoliosis was predominantly observed in embryos subjected to MOLE concentrations ranging from 25 to 200 µg/mL. The present data demonstrated that MOLE exhibited concentration- and time-dependent toxicities. Further studies are needed to identify the effective concentrations of MOLE for therapeutic application in *in vitro* and *in vivo* models.

Keywords: *Moringa oleifera*, toxicity, zebrafish embryo

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INTRODUCTION

Recently, in pharmaceutical research, there has been a surge in public interest in exploring medicinal plants for their bioactive compounds, which may offer various health benefits that improve human health. *Moringa oleifera*, locally known as 'Kelor,' has garnered significant attention in the industry due to extensive research on its nutritional, phytochemical, and pharmacological properties. *M. oleifera* has been recognised as a 'Miracle Tree' and 'Superfood'. Its leaves contain high concentrations of essential vitamins, minerals, and antioxidants. Studies have demonstrated that *M. oleifera* contains higher concentrations of vitamin C than oranges and greater potassium levels than bananas (Rockwood et al., 2013). Furthermore, it exhibits a higher proportion of polyunsaturated fatty acids compared to saturated fatty acids (Cervera-Chiner et al., 2024). These properties provide substantial health benefits, including overcoming malnutrition and exhibiting anti-inflammatory, antioxidant and anticancer effects (Leone et al., 2015). One of the 13 members of the genus Moringa is *M. oleifera,* and it belongs to the family Moringaceae. It is indigenous to South Asia and was initially discovered in northeast Pakistan, extending to northern West Bengal and India (Azlan et al., 2022). It is usually cultivated in subtropical and tropical areas (Milla et al., 2021).

Previous studies have indicated that plants used in traditional medicine may present risks if consumed inappropriately, contradicting their perceived safety (Başaran et al., 2022; Ekor, 2014; Shamsi et al., 2021). It is frequently attributed to a lack of scientific evaluation and regulation, rendering them potentially less safe than prescription drugs, which have been associated with severe adverse effects such as congestive, allergic reactions, heart failure, renal failure, and liver toxicity (Lorenzo et al., 2015; Posadzki et al., 2013). *Moringa oleifera*, though widely used in traditional medicine and dietary supplements, lacks comprehensive toxicological data. This study aims to fill this gap by evaluating the potential toxicity of ethanolic *M. oleifera* leaf extract (MOLE), ensuring it can be used safely at various doses. Testing on zebrafish embryos allows for an initial assessment of developmental toxicity, helping to identify safe concentration levels and prevent potential health risks associated with overconsumption or high-dose exposure.

Several studies have investigated the cytotoxic effects of ethanolic MOLE in various cell lines. In one of the studies, the fibroblast cells derived from chicken embryos were exposed to ethanolic MOLE at concentrations ranging from 0.02 to 400 µg/mL. The results showed significant cytotoxicity at concentrations > 200 µg/mL but were safe at < 50 µg/mL. The same study also reported that the 50% cytotoxic concentration (CC_{50}) was 100 µg/mL (Ashraf et al., 2017). In another study, the cytotoxic effects of ethanolic MOLE on the human cervical carcinoma cell line (HeLa cells) were compared with callus extract using the MTT assay (Jafarain et al., 2014). It was noted that both MOLE and callus extracts showed a significant decrease in cell viability at concentrations of 100 to

500 µg/mL and 1000 to 3000 µg/mL, respectively, in a concentration-dependent manner. Notably, ethanolic MOLE exhibited higher cytotoxicity compared to the callus extract, as indicated by a lower 50% inhibitory concentration (IC_{50}) (Jafarain et al., 2014). Despite the therapeutic benefits of medicinal plants, the prolonged use of *M. oleifera* may be toxic. In rats, extended consumption of ethanolic MOLE at doses of 200 and 400 mg/kg per body weight resulted in dose-dependent weight gain and elevated serum alanine transaminase (ALT), aspartate transaminase (AST), blood urea nitrogen (BUN), and creatinine levels, indicating liver and kidney damage. These findings suggest chronic MOLE supplementation could induce hepatic and renal injuries (Oyagbemi et al., 2013). It highlights the importance of understanding the potential toxic effects of both short- and long-term use of medicinal plants. Thus, comprehensive toxicity studies on medicinal plants and their preparations are essential to ensure their safety for human consumption.

Moreover, *in vivo* models have been used to study the toxicity profile of MOLE. Administration of 70% ethanol-extracted *M. oleifera* leaves at a concentration of 150 mg/mL intraperitoneally to albino rats and rabbits every 5-min interval until mortality was reached resulted in lethal doses of acute toxicity (Osman et al., 2015). Histopathological examination revealed significant damage to the kidney, heart, and liver, including renal tubule degeneration, cardiac muscle haemorrhage, and hepatic cell necrosis. It suggests that fluid accumulation leads to cell swelling and rupture, indicating minimal toxicity of MOLE when administered in concentrated doses over a short period in rodents (Osman et al., 2015). Nevertheless, a comprehensive toxicity profile of ethanolic MOLE using zebrafish embryos as an *in vivo* model has not yet been reported.

Zebrafish (*Danio rerio*) serves as a valuable toxicity model, offering real-time, *in vivo* studies to evaluate the potential risks to human health associated with naturally occurring compounds (Abdullah et al., 2022; Bambino & Chu, 2017). This model has been extensively utilised in toxicity studies due to its numerous advantages, particularly in terms of technical and economic aspects, compared to other vertebrate models such as rodents. One key advantage is their small size, which makes them both cost-effective and technically easier to manage. Additionally, zebrafish exhibit high fecundity, producing many embryos per mating session. The transparency of embryos facilitates detailed imaging and observation of developmental processes (Rothenbücher et al., 2019). Moreover, the zebrafish genome exhibits approximately 70% similarity to the human genome compared to the human reference genome. This similarity shows the potential of using zebrafish to enhance our understanding of the precise roles of genes in human diseases, spanning both rare and common conditions (Howe et al., 2013). The Fish Embryo Toxicity (FET) test, which employs zebrafish, is widely recognised for its application in pre-screening drugs and diseases, including the evaluation of lethal concentrations for 50% of the population (LC_{50}). The zebrafish embryo model bridges the gap between the *in vitro* and *in vivo*

methods, providing essential insights before conducting biocompatibility and toxicity assessments in living organisms, thus diminishing the necessity for animal experimentation (Rothenbücher et al., 2019).

In this study, MOLE was assessed for its toxicity during zebrafish embryogenesis. While MOLE is recognised for its beneficial properties, including anticancer (Jafarain et al., 2014), anti-inflammatory (Arulselvan et al., 2016), and antibacterial (Farooq & Koul, 2020) activities, its toxic effects, particularly during the critical embryogenesis period, remain largely unexplored. Therefore, this study aims to evaluate the toxicity and teratogenicity of MOLE during zebrafish embryogenesis.

MATERIALS AND METHODS

Plant Sample Collection and Identification

Fresh leaves of the *M. oleifera* plant were acquired from Bukit Katil, Malacca. The plant was sent to the Institute of Bioscience (IBS) UPM to authenticate its species. The voucher number obtained was KM 0088/23.

Preparation and Extraction of *Moringa oleifera* Leaves

The extraction of *M. oleifera* leaves was conducted according to the method described by Ismail et al. (2020). Approximately 500 g of *M. oleifera* leaves were washed, air-dried to a constant weight, and then ground into powder at room temperature. For the maceration process, 80% (v/v) ethanol (Chemiz, Malaysia) was used to extract the powdered plant material over 72 h. The resulting crude ethanol extract was filtered through Whatman No. 1 filter paper (GE Healthcare, Singapore). The filtrate was then evaporated using a rotary evaporator (BUCHI Rotavapor R-200, Buchi Corporation, Switzerland), dried, and stored in an airtight container at 4°C until needed. This extraction process was repeated three times. The plant extraction yield obtained was 20.78%. The percentage yield of the extract was calculated using the equation below:

$$\text{Percentage of yield (\%)} = \frac{\text{Weight of concentrated extract (g)}}{\text{Weight of dried plan sample (g)}} \times 100\% \qquad [1]$$

Phytochemical Identification Using Liquid Chromatography Analysis

The sample was dissolved in methanol (1 mg/mL) (Fisher Scientific, UK) and filtered through a 0.22 µm PTFE membrane filter (Phenomenex, USA) prior to analysis to identify the phytochemicals contained in the *M. oleifera* leaf extract (MOLE). The sample was analysed using a Liquid Chromatography–Mass Spectrometry (LC-MS) system consisting of a Q Exactive Focus mass spectrometer with Ultimate 3000 photodiode array detector, Rapid Separation (RS) column compartment, RS pump and RS autosampler (Thermo

Scientific, USA). A Hypersil Gold Dim (1.9 µm) column was used throughout (100 mm × 2.1 mm), and for mobile phase elution, a gradient of two solvents denoted as 'A' and 'B' was employed. 'A' was 0.1% of aqueous formic acid, whereas 'B' was 0.1% formic acid in acetonitrile with a 400 µL/min flow rate. The initial condition consists of 95% of 'A' and 5% of 'B', with a linear gradient increase from 5 to 100% of 'B' at 30–34 minutes of elution time, and the analysis was conducted at the initial solvent ratio until 38 min of elution time. A mass spectrometer with an electrospray ion source was set to positive and negative ionisation modes using data-dependent automatic switching between the MS and MS/MS acquisition modes. The system was controlled using Xcalibur software (Vijayalakshmi et al., 2015).

Zebrafish Embryo Toxicity Testing

Zebrafish embryos were purchased from the AAALAC-accredited Zebrafish Satellite Animal Facility, Animal Experimental Unit, Faculty of Medicine, Universiti Malaya.

Briefly, 4 h post-fertilisation (hpf) healthy embryos were transferred into 96-well culture plates (TPP, Switzerland), one embryo in 200 µL of embryo medium (E3 media [Zebrafish Satellite Animal Facility, Malaysia]) (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, and 0.1% (*w*/*v*) methylene blue) per well and acclimatised at 28°C for 24 h in a 12-h light-12-h dark (LD) cycle. The following day, any dead or unfertilised embryos were removed, and fresh embryo media was added in place of the old one. The viable embryos were exposed to eight different MOLE concentrations (5-1000 µg/mL) for 72 h at 28°C in a semi-static environment (24 hpf to 96 hpf). Using an inverted microscope equipped with a camera (Leica, Germany), several toxicological endpoints, such as survival rate, heart rate, hatching rate, and morphological development, were monitored and recorded at 24-hour intervals. The treatment solutions were changed daily, and E3 media was used as a control (Ghafor et al., 2020; Mohamad Shariff et al., 2020; Shamsi et al., 2022).

The mortality of the embryos was indicated by the presence of coagulation and the absence of a heartbeat to assess the survival rate. The survival rate was determined by counting the number of live embryos or larvae at each observation point. Dead embryos were denoted as "1," while surviving embryos were labelled as "0". A similar approach was employed for the hatching rate observation. Heart rate was determined by observing heartbeats over a 15-second interval and then extrapolating the count to 60 seconds to establish the average beats per minute (bpm) (Shamsi et al., 2020). Additionally, to assess the impact of MOLE toxicity on embryos, their morphological development was examined at 24 h, 48 h, and 72 h post-treatment, including the presence of body axis curvature, yolk sac oedema, pericardial oedema, and scoliosis. The dead embryos were extracted daily during the observation period to prevent contamination. Three independent experiments were performed for each treatment group ($n \geq 10$ embryos per exposure group).

Statistical Analysis

One-way Analysis of Variance (ANOVA), a statistical hypothesis test, was performed with Dunnett post-hoc test comparison where indicated. The statistical significance of the results was defined by a $p \leq 0.05$. The data were analysed using GraphPad Prism version 10.2.3 statistical analysis software (GraphPad Software, USA). All experiments were independently repeated three times. Data was presented as mean ± standard error of the mean (SEM). A *p*-value of less than 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Phytochemicals Composition of MOLE

An LC-MS analysis was conducted using both positive and negative ionisation MS/MS modes to investigate the phytochemical composition of the ethanolic MOLE. Ethanolic extracts efficiently solubilise a wide range of bioactive compounds, facilitating thorough toxicity evaluation while maintaining the pharmacological attributes of the plant (Lee et al., 2024). The data were compared and validated using online databases, PubChem, and previously published studies. Table 1 lists some of the principal phytochemicals detected in MOLE, which may have influenced the extract's pharmacological activities and toxicity profile. These phytochemical compounds are well-documented for their diverse properties, such as antioxidant activity, which interferes with the cell cycle, induces apoptosis, and decreases oxidative stress (Shukla & Gupta, 2010). For example, alkaloids, notably cepharanthine, may possess significant antioxidant properties that facilitate the scavenging and prevention of radical formation, thereby safeguarding deoxyribonucleic acid (DNA) from damage induced by endogenously generated radicals during oxidative metabolism.

A study reported that cepharanthine exhibits potent anti-inflammatory activity by diminishing the expression of the NF-κB transcription factor. This effect is achieved by inhibiting the IκB kinase (IKK) pathway and reducing pro-inflammatory cytokines such as TNF-α and IL-1β (Bailly, 2019). In the IKK pathway, IκB normally inhibits cytoplasmic NF-κB. However, upon LPS stimulation, IκB is phosphorylated, allowing NF-κB to translocate into the nucleus and activate pro-inflammatory genes. Cepharanthine interrupted this process by inhibiting LPS-induced IκB phosphorylation, thereby blocking NF-κB activation. As a result, the secretion of cytokines and nitric oxide (NOx) is reduced, demonstrating its anti-inflammatory mechanism (Kudo et al., 2011). In addition, flavonoids exhibit anti-inflammatory properties by inhibiting NF-κB activation, exerting anti-inflammatory effects, and preventing lipid oxidation (Milla et al., 2021). The terpenoid derivative, emmotin A, was also detected in MOLE. Emmotin A is an anti-neuroinflammatory agent that showed strong binding interactions with AChE, BChE, α-glucosidase, α-amylase, and tyrosinase in an *in silico* molecular docking study, indicating its ability to maintain ACh levels and suppress neuroinflammation (Saleem et al., 2020).

Table 1
List of major phytochemicals in MOLE and its potential activity

Name of compound	Molecular formula	Class of bioactive compound	Potential activity	Ion (+/-)	Reference
Cepharanthine	$C_{37}H_{38}N_2O_6$	Alkaloids	Antioxidant, anti-inflammatory	[M+H]+	Bailly (2019)
Apigenin 5-glucoside	$C_{21}H_{20}O_{10}$	Flavonoids	Antioxidant, anti-mutagenic and anti-inflammatory properties	[M+H]+	Shukla & Gupta (2010)
Quercetin 3-(6"-malonylgalactoside)	$C_{24}H_{22}O_{15}$	Flavonoids	Anti-inflammatory, anticancer, cardioprotective, anti-tumour, anti-viral, anti-diabetic, antihypertensive, gastroprotective effects	[M+H]+	Milla et al. (2021)
Emmotin A	$C_{16}H_{22}O_4$	Terpenoid	Anti-neuroinflammatory	[M+H]+	Ngu et al. (2022)
3,4-dihydroxyphenylethanol-elenolic acid (3,4-DHPEA-EA)	$C_{19}H_{22}O_8$	Phenolic compounds	Antioxidant, antihypertensive, antibacterial	[M+H]-	Segade et al. (2016), Bisignano et al. (2014)
Trans-Stilbene	$C_{14}H_{12}$	Phenolic compounds	Antioxidant, cardioprotective, cancer chemopreventive, anti-inflammatory, anti-diabetic, antibacterial	[M+H]-	Treml & Šmejkal (2016)
Monocrotaline	$C_{16}H_{23}NO_6$	Pyrrolizidine alkaloid (PA) family of plant toxins	Induce developmental toxicity, hepatotoxicity, pulmonary lesions, and cancer	[M+H]-	Luo et al. (2019), Chen et al. (2010), Louisse et al. (2019), Sakamoto et al. (2017)

The negative ionisation results of aMS/MS revealed several bioactive compounds, including 3,4-dihydroxyphenylethanol-elenolic acid (3,4-DHPEA-EA). This isomer of oleuropein aglycone acts as an antioxidant, protecting cells from oxidative damage through radical scavenging, metal ion chelation, and induction of antioxidant enzymes. Additionally, 3,4-DHPEA-EA has antihypertensive activity and is a major component of virgin olive oil consumed to regulate blood pressure (Segade et al., 2016). It also shows antibacterial properties and is effective against gram-positive bacteria such as staphylococci,

suggesting its potential as a natural antimicrobial for treating bacterial skin infections (Bisignano et al., 2014). Additionally, trans-stilbene has been identified and is renowned for its antioxidant properties. In a lipoperoxidation inhibition assay, which measures the ability of a compound to prevent the oxidative degradation of lipids, trans-stilbene emerged as the most active compound (Treml & Šmejkal, 2016). Its other pharmacological properties include cardio-protection, cancer chemoprevention, anti-inflammatory effects, anti-diabetes, and antibacterial activity. Monocrotaline (MCT) was detected in the extract. It is an 11-membered macrocyclic diester classified as a pyrrolizidine alkaloid (PA), a plant toxin. Ingestion of MCT can severely affect humans, causing developmental toxicity, hepatotoxicity, pulmonary lesions, and cancer (Chen et al., 2010; Louisse et al., 2019; Luo et al., 2019). Consequently, MCT has been utilised in toxicity studies to induce pulmonary hypertension, aiding research on chronic pulmonary vascular diseases in humans. For example, a study showed that rats can experience significant and gradual lung damage from even small amounts of MCT (Sakamoto et al., 2017).

Survival Rate of Zebrafish Embryos Treated with MOLE

In the present study, the toxicity effects of MOLE were determined using a zebrafish embryo model as it is known to provide real-time *in vivo* studies to address possible health risks to humans resulting from naturally occurring compounds (Bambino & Chu, 2017). The survival rate of zebrafish embryos exposed to different concentrations of MOLE (5-1000 µg/mL), as observed at 24 h, 48 h, and 72 h post-treatment, is shown in Figure 1A-C. The lethal concentration for the embryos (LC_{50}) values of MOLE in zebrafish embryos exposure for 24 h to 72 h post-treatment are also presented in Table 2. The concentration range of MOLE

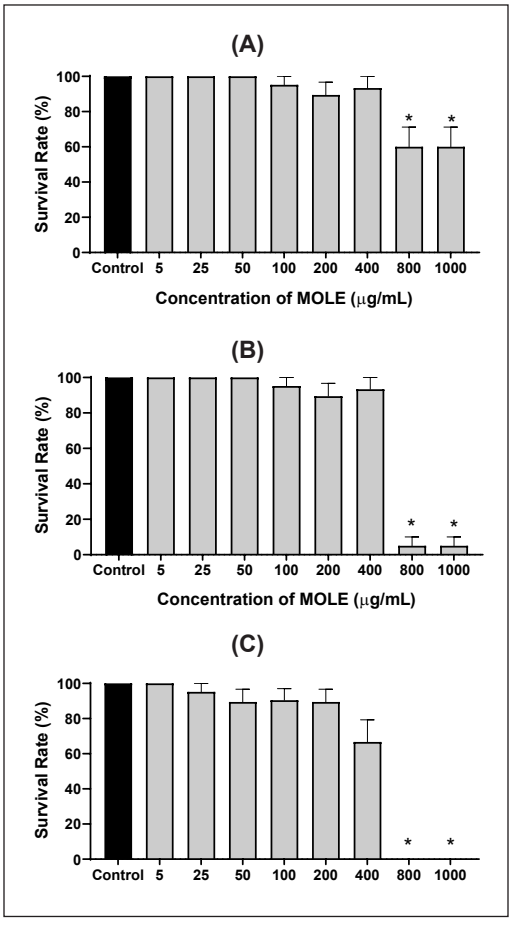

Figure 1. The effects of different concentrations of MOLE (5-1000 µg/mL) on survival of zebrafish (*Danio rerio*) at (A) 24 h, (B) 48 h, and (C) 72 h post-exposure ($n \geq 15$). Embryo media was used as control. Data were shown as mean ± SEM
Note. Significant difference to control is denoted by "*" (One-way ANOVA, followed by a post hoc test: Dunnet $p \leq 0.05$)

used in this study was determined based on previous findings indicating that embryos treated with aqueous ethanolic extract of MOLE exhibited better embryogenesis development compared to those treated with aqueous methanolic extract at equivalent concentrations (Mohamad Shariff et al., 2020).

Table 2
LC_{50} values of MOLE following its exposure to zebrafish embryos from 24 h to 72 h post-treatment

Time of exposure (post-treatment, hr)	LC_{50} of MOLE (µg/mL)
24	1186.0 ± 7
48	560.1 ± 7
72	445.1 ± 7

Survival analysis revealed that embryo survivability decreased with increasing MOLE concentrations in a time-dependent manner. A lower survival rate was recorded as the concentration of MOLE and the exposure times increased. Embryos treated with concentrations < 400 µg/mL of MOLE showed good survival rates over time, with > 80% of embryos surviving up to 48 h post-treatment before slightly declining at 72 h post-treatment (Figure 1). Although treatment with 400 µg/mL of MOLE caused a reduction in the survival rate to approximately 70% at 72 h post-treatment, it was not statistically significant. It was still comparable to the control group (Figure 1C). In contrast, the survival rate of zebrafish embryos treated with a higher concentration of MOLE (> 800 µg/mL) progressively decreased over the exposure period, from approximately 60% at 24 h post-treatment (Figure 1A) to less than 10% at 48h post-treatment (Figure 1B). By 72 h post-treatment, none of the zebrafish embryos in this group survived the study (Figure 7C). As expected, all embryos in the control group (E3 media) maintained 100% survival throughout the study.

This finding indicates that higher concentrations and prolonged exposure to MOLE significantly compromised the survivability of embryos. It is possible that MOLE is taken up by zebrafish embryos through the skin and gills in the early stages of embryo development (Sakeh et al., 2020). Furthermore, these results suggested that the ability of the surrounding compounds to penetrate the embryo became greater as the time of exposure lengthened, leading to its toxicity. It could be explained by the fact that the protective layer surrounding the embryo, called the chorion, which is present in the early stages of development, changes with age. Previous research has indicated that changes in the protein composition of the chorion could result in the expansion or enlargement of chorion pore channels, enabling a higher entry of external substances (Ali et al., 2017).

Hatching Rate of Zebrafish Embryos Treated with MOLE

The hatching rate is a critical stage during embryogenesis and an important parameter in toxicity studies. A zebrafish embryo is considered to have hatched once its body has completely emerged from the chorion (David et al., 2016), and it would usually begin at 48 h post-fertilisation (hpf) under normal conditions (Chen et al., 2018). In the present study, the

hatching rate of zebrafish embryos did not show significant differences when exposed to various concentrations of MOLE at any of the monitored time points (Figure 2), with all embryos showing comparable hatching rates to the control group. However, a significant difference in hatching rate was observed in embryos treated with > 800 µg/mL of MOLE at 96 hpf (Figure 2C). This reduction was not due to delayed hatching from the MOLE treatment but was attributed to high mortality rates at higher concentrations. Most embryos treated with 800 and 1000 µg/mL of MOLE died at 72 hpf (48 h post-treatment) (Figure 1B), explaining the similar hatching rates observed at both 72 hpf and hpf (Figure 1B-C). These findings show that MOLE did not affect the hatching rate at any time point or tested concentration.

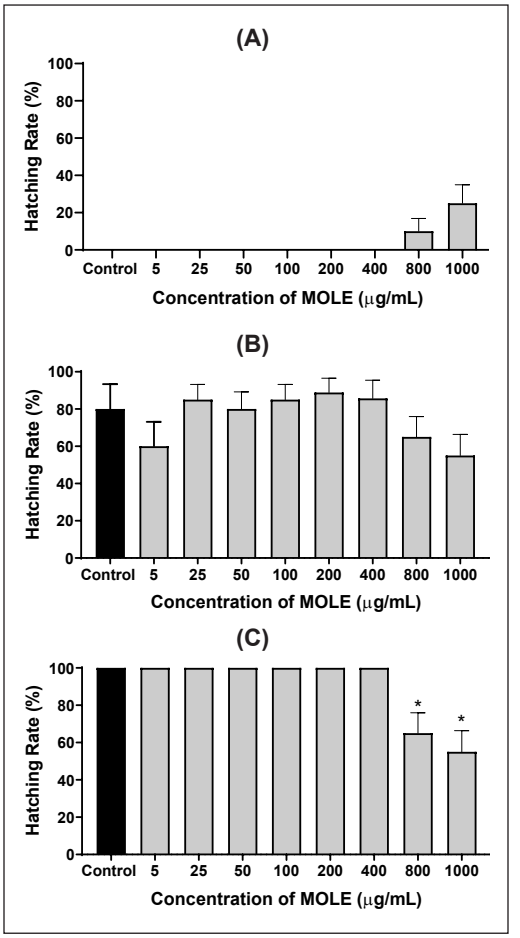

Figure 2. The effects of different concentrations of MOLE (5-1000 µg/mL) on the hatching rate of zebrafish (*Danio rerio*) at (A) 48 h, (B) 72 h and (C) 96 h post-fertilisation (hpf) ($n \geq 15$). Embryo media was used as a control

Note. Significant difference to control is denoted by "*" (One-way ANOVA, followed by a post hoc test: Dunnet $p \leq 0.05$)

Heart Rate of Zebrafish Embryos Treated with MOLE

The heart is the first organ to form and operate in zebrafish development. Zebrafish hearts have a transparency property that allows for single-cell resolution visualisation, making them a perfect subject for monitoring toxicity. Zebrafish embryos have a heart rate of 120–180 beats per minute (bpm) (Chahardehi et al., 2020; Ghafor et al., 2020).

The heart rates of the zebrafish embryos treated with different concentrations of MOLE are shown in Figure 3. There were no significant differences in the heart rate of zebrafish embryos treated with MOLE concentrations ≤ 400 µg/mL compared to the control group at all time points, as all rates fell within the normal range of between 120 and 180 bpm. At 72 h post-treatment, the heart rate of the 100 µg/mL concentration group appeared slightly above the normal range (>180 bpm). However, the difference was not statistically significant and comparable to the control group. The increase in heart rate could be due

to various factors, such as a response to sudden changes in temperature. A previous study has reported that a sudden decrease in temperature from 28°C to 18°C caused a 48% reduction in the heart rate of zebrafish (Lee et al., 2016).

In addition, abnormalities in the heart rate of zebrafish embryos might also result from exposure to compounds that influence sodium and potassium channels, particularly ATP-sensitive potassium (K_{ATP}) channels. For example, ouabain, which is a cardiac glycoside usually derived from *Stropanthus gratus*, primarily inhibits the Na^+/K^+-ATPase pump, leading to a disruption in the balance of sodium and potassium ions across the cell membrane (Rajanathan et al., 2023). These channels play crucial roles in heart rate control and adaptation to metabolic changes. Disruption of K_{ATP} channels through genetic manipulation or exposure to external compounds can lead to heart rate dysregulation (Aziz et al., 2018). Elevated heart rates can strain heart muscles, which could eventually cause damage to zebrafish hearts. Similar to other observations, higher concentrations of MOLE (> 800 µg/mL) significantly affected the heart rate of the treated embryos (Figure 3). A decrease in heart rate was noted at 24 h post-treatment, which gradually declined as exposure time increased. By the end of the observation period (72 h post-treatment), all embryos at these higher concentrations exhibited no heart rate, leading to mortality (Figure 3C). These findings suggest that MOLE may be harmful at higher doses that induce cardiac arrhythmia and embryonic bradycardia, which in turn causes severe hypoxia and foetal distress, contributing to the fatality observed in Figure 1 (Manjunatha et al., 2018).

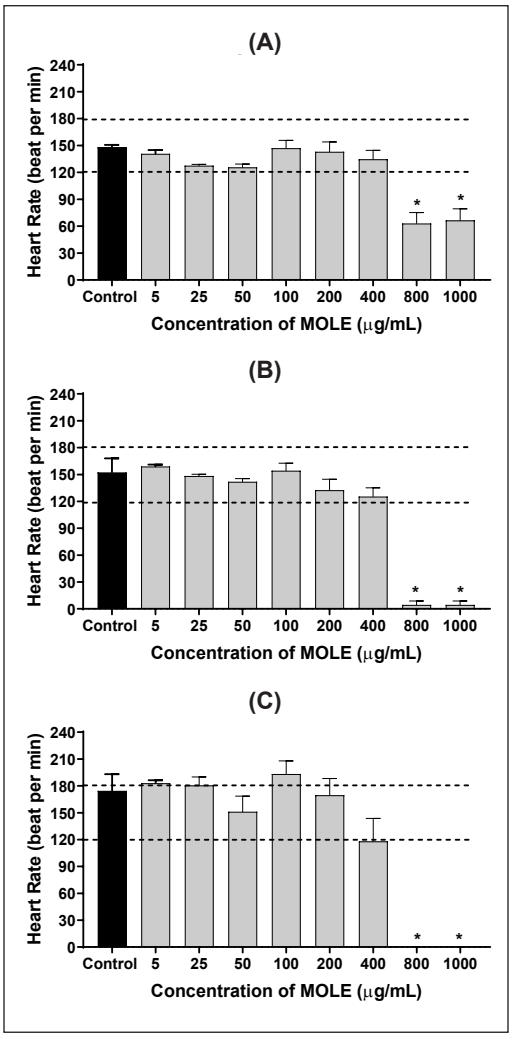

Figure 3. Heartbeats of zebrafish embryos in the presence of different concentrations of MOLE (5-1000 µg/mL) at (A) 24 h, (B) 48 h and (C) 72 h post-treatment ($n \geq 15$). Embryo media was used as control. Significant difference to control is denoted by "*" (One-way ANOVA, followed by a post hoc test: Dunnet $p \leq 0.05$). Dashed lines (---) represent the range of a normal heart rate for zebrafish embryos (120–180 beats per minute)

Morphological Assessments of Zebrafish Embryos Treated with MOLE

In the present study, the toxicity effects of MOLE were also assessed morphologically daily for up to 96 h post-treatment. The deformities or malformations detected in this study included scoliosis (S), pericardial oedema (PE), yolk sac oedema (YSE) and body axis curvature (BAC) (Figure 4). Scoliosis, which is the lateral curvature of the spine, was the only abnormality observed in embryos treated with 25 to 200 µg/mL of MOLE (Table 3), which might be caused by oxidative damage (Heredia-García et al., 2021). This oxidative damage may play a role in the development and progression of scoliosis, as elevated oxidative stress has been reported to lead to apoptosis and disrupt muscle tissue formation, potentially contributing to the pathological changes seen in patients with Idiopathic Scoliosis (IS). In zebrafish models, oxidative stress has been linked to muscle degeneration and spinal deformities, suggesting a possible mechanism by which oxidative damage influences scoliosis (Li et al., 2019). Interestingly, no other abnormalities were observed at this concentration range.

In contrast, pericardial oedema, yolk sac oedema, and body axis curvature were collectively observed in embryos treated with higher concentrations of MOLE (> 800 µg/mL). The formation of pericardial oedema during embryogenesis is most likely due to the weakening of the embryo chorion layer following exposure to the treatments, which allows a greater intake of MOLE and induces toxic effects on zebrafish over time (Ali et al.,

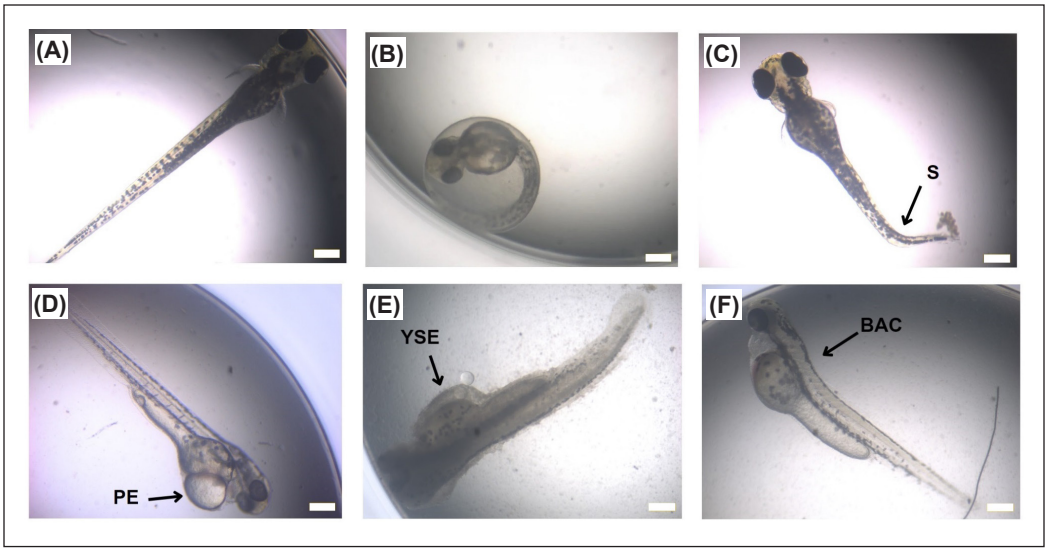

Figure 4. Microscope images showing the incidence of malformations in zebrafish embryos, as indicated by a black arrow. (A) Normal hatched zebrafish, (B) Normal zebrafish embryo, (C) Zebrafish with scoliosis, (D) Zebrafish with pericardial oedema, (E) Zebrafish with yolk sac oedema, (F) Zebrafish with body axis curvature *Note*. S = Scoliosis, PE = Pericardial oedema, YSE = Yolk sac oedema, BAC = Body axis curvature. Scale = 250 µm

2017). The yolk sac plays an important role in embryogenesis and normally stores nutrients. Impairment of the yolk sac, such as yolk sac oedema observed at higher concentrations, could impair nutrient absorption by the embryo, resulting in malnourished embryos that may eventually perish. Meanwhile, body axis curvature formations are potentially caused by chorion formation failure, which disrupts somite development and subsequently induces malformation in the body axis (Syahbirin et al., 2017).

Table 3
Malformation incidences in zebrafish embryos treated with different concentrations of MOLE at 24 to 72 h post-treatment

MOLE Concentration (µg/mL)	Malformation incidence (%)			
	Scoliosis (S)	Pericardial oedema (PE)	Yolk sac oedema (YSE)	Body axis curvature (BAC)
Control	0	0	0	0
5	0	0	0	0
25	14	0	0	0
50	15	0	0	0
100	35	0	0	0
200	52	0	0	0
400	0	0	0	0
800	25	8	33	50
1000	8	0	41	50

CONCLUSION

Moringa oleifera is rich in bioactive compounds with potential health benefits, particularly from its leaves. The toxicity assessment of ethanolic *M. oleifera* leaf extract (MOLE) on zebrafish embryos in this study revealed concentration- and time-dependent toxic effects on survivability, changes in heart rate and malformations during embryogenesis. Consequently, MOLE is considered safe at up to 400 µg/mL concentrations, as higher concentrations result in significant toxic effects. These findings indirectly highlight the effectiveness of the zebrafish embryo model in providing comprehensive toxicity profiles of medicinal plants, compared to other settings such as cell lines and rodents. Further toxicity assessments of MOLE are essential for comprehensive understanding and safe implementation as a human herbal therapeutic agent.

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