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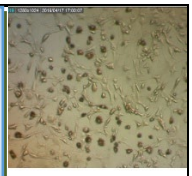

Developmental Toxicity of Methanol in Zebrafish Embryos for Establishing Safe Solvent Concentrations

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Abstract

Methanol (MeOH) is widely used as a solvent for extracting bioactive compounds from plant materials, but its inherent toxicity may confound biological assays if not properly controlled. This study aimed to evaluate the developmental toxicity of MeOH in zebrafish embryos to determine the maximum concentration that can be safely used as a solvent without causing adverse developmental effects. Zebrafish embryos were exposed to a range of MeOH concentrations (0.39% to 24.98% v/v in embryo media). Survival rate, mortality, hatching rate, heartbeat rate, and teratogenic effects were assessed over 120 hours post-fertilization (hpf). Embryo survival decreased with increasing MeOH concentrations. The highest survival (91.7%) was observed at 0.39% MeOH, followed by 83.3% at 0.78%, and 50% at 1.56%. Concentrations ≥3.13% caused 100% mortality. The calculated LC50 was 1.58% MeOH. Hatching and heartbeat rates followed similar concentration-dependent trends. Teratogenic effects, including oedema, failure of tail detachment, and curved body, were observed at sub-lethal concentrations. Methanol exhibits significant developmental toxicity in zebrafish embryos at concentrations above 1.56%. This study establishes 1.56% MeOH as the maximum concentration tolerable without severe toxicity, providing a safe threshold for its use as a solvent in future bioassays involving plant extracts or other biological samples.

INTRODUCTION

MeOH is widely used as a solvent in scientific and industrial applications, particularly for extracting bioactive compounds from plant materials. Its ability to solubilise a broad spectrum of compounds makes it a preferred choice for preparing methanolic extracts from leaves, callus tissues, and other plant sources. However, MeOH itself poses toxicity risks, as it can metabolise into harmful byproducts such as formaldehyde and formic acid, which disrupt normal cellular functions [1; 2]. This raises concerns, especially in toxicity studies where plant extracts are dissolved in MeOH, as the observed toxicity may originate from the solvent rather than the bioactive compounds. Therefore, it is crucial to establish the maximum concentration of MeOH that does not cause developmental toxicity. This ensures that any toxicity observed in subsequent assays using methanolic plant extracts can be confidently attributed to the extract itself rather than residual MeOH. This study addresses this need by evaluating the developmental toxicity of MeOH in zebrafish embryos to define safe solvent thresholds for bioactivity testing. Zebrafish embryos are increasingly employed as a model organism in developmental toxicity studies due to their advantageous characteristics. Their transparent bodies allow for non-invasive visualisation of internal developmental processes, including organ formation and circulation [3].

The rapid development of zebrafish embryos, with key milestones occurring within a few days, makes them particularly suited for acute toxicity studies. Furthermore, the genetic conservation between zebrafish and higher vertebrates, including humans, enhances the relevance of findings for understanding potential impacts on human health [4]. Methanol exposure during early zebrafish development induces dose-dependent ocular and developmental defects. Moderate (3%) and high (4%) concentrations result in a small eye phenotype,

with high concentrations specifically causing morphological abnormalities in the retinal pigment epithelium and photoreceptors. Additionally, methanol inhibits retinal cell differentiation and proliferation. While low concentrations affect photoreceptor function without altering retinal structure, higher concentrations disrupt retinal patterning [5]. Methanol exposure also increases embryonic mortality and causes growth retardation. Embryos treated with 3% and 4% methanol exhibit reduced spontaneous tail movement at 24 hours post-fertilisation (hpf) and decreased locomotor activity at 120 hpf. These effects, particularly at higher concentrations, indicate methanol-induced neurodevelopmental impairment and motor dysfunction [6].

In light of these considerations, this study aims to investigate the developmental toxicity of methanol on zebrafish embryos by exposing them to various concentrations of MeOH. The research will assess survival and mortality rates, hatching rates, heartbeat frequencies, and teratogenic effects to provide a comprehensive evaluation of methanol's impact. By analysing how different concentrations of methanol affect these developmental parameters, the study seeks to establish a detailed toxicity profile and identify safe concentration thresholds for methanol use in research and industrial applications.

METHODOLOGY

Toxicity test

The Danio Assay Kit for toxicity studies was obtained from Danio Assay Laboratories Sdn. Bhd, Malaysia. The kit included zebrafish embryos, 96-well plates, Danio-embryo media (EM) containing 0.1% DMSO (500 mL), and an instruction manual. In this study, zebrafish embryos were used in accordance with experimental protocols approved by the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (UPM/IACUC/AUPU002/2021).

Preparation of zebrafish embryo

The embryos were placed in a petri dish filled with Danio-Sprint^{M} Embryo Media (EM). Prior to the toxicity test, the quality of the embryo was assessed by examining its appearance. Milky, white, and opaque embryos, indicating unfertilised eggs and coagulated embryos were removed. Only viable and healthy embryos were selected for further testing. The old EM was replaced with fresh EM in the Petri dish. At 4 hours post fertilisation (hpf), healthy embryos were transferred into a 96-well plate using a sterile pipette with a 1 mL micropipette tip. Each well was populated with one embryo, and 100 µL of EM was quickly added to each well to prevent drying [6]. The plate was then labelled as the embryo plate for further use.

Preparation of methanol Preparation of Methanol Control and Embryo Toxicity Testing

The embryos were exposed to embryo medium (EM) containing varying concentrations of MeOH. To prepare the initial concentration of MeOH (100% v/v), a 1:2 ratio of MeOH (2 mL, 99.99% v/v) to EM (4 mL) was used, resulting in a final working concentration of 47.5% MeOH in EM. This solution was then pipetted into a 96-well plate and subjected to serial dilution. The final concentration of MeOH in each well is detailed in **Table 1**. The embryos were observed under a stereomicroscope every 24 hours for a total exposure period of 120 hours.

The endpoints criteria, including coagulation, heartbeat, oedema, body curvature, tail detachment, somite formation, and hatching, were assessed [6].

Table 1. Final concentrations of MeOH in each well.

Well	Embryo Media (EM) + MeOH
A	EM with 0.1 % DMSO + 24.98 % MeOH
B	EM with 0.1 % DMSO + 12.48 % MeOH
C	EM with 0.1 % DMSO + 6.24 % MeOH
D	EM with 0.1 % DMSO + 3.13 % MeOH
E	EM with 0.1 % DMSO + 1.56 % MeOH
F	EM with 0.1 % DMSO + 0.78 % MeOH
G	EM with 0.1 % DMSO + 0.39 % MeOH
H	EM with 0.1 % DMSO

RESULTS

Toxicity effects of methanol on zebrafish embryos

In this study, zebrafish embryos served as a model vertebrate to assess the developmental toxicity of various concentrations of MeOH, a solvent used for extracting bioactive compounds from plants.

Survival rate of zebrafish at different concentrations of methanol

Based on the results presented in **Fig. 1**, the survival rate of the zebrafish embryos increased as the concentration of methanol decreased. The lowest concentration of methanol in EM (0.39 % MeOH + EM) exhibited the highest survival rate (91.7%), followed by 0.78 % MeOH + EM (83.3 %) and 1.56 % MeOH + EM (50%). Meanwhile, no embryos survived in the presence of 3.13 % MeOH + EM, 6.24 % MeOH + EM, 12.48 % MeOH + EM, and 24.98 % MeOH + EM. In contrast to the positive control, all embryos survived in EM without the addition of methanol after 120 hpf.

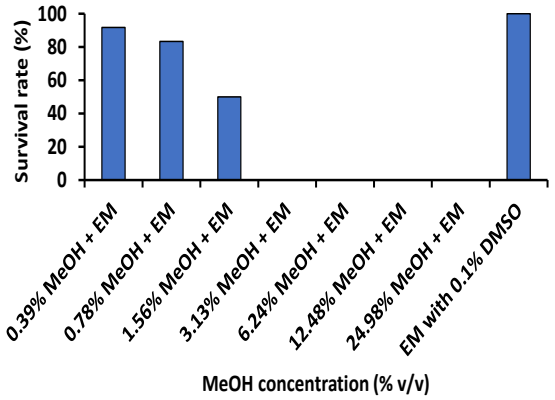

Fig. 1. Survival rate of zebrafish embryos treated with different concentrations of methanol (MeOH) at 120 hpf. The EM used contained 0.1% DMSO.

The effect of the methanol exposure on the mortality rate of zebrafish embryos is shown in **Fig. 2**. The mortality rate of the embryos increased when exposed to high concentrations of methanol, while zero mortality was recorded in the control. The lowest mortality rate was obtained in embryos treated with 0.39 % MeOH + EM, at 8.3 %, followed by 0.78 % MeOH + EM (16.7 %), while the rest of the treatments recorded a 100 % mortality rate. Consequently, the percentage mortality data were used to determine the LC_{50} value, which was obtained to be 1.58 µg/mL.

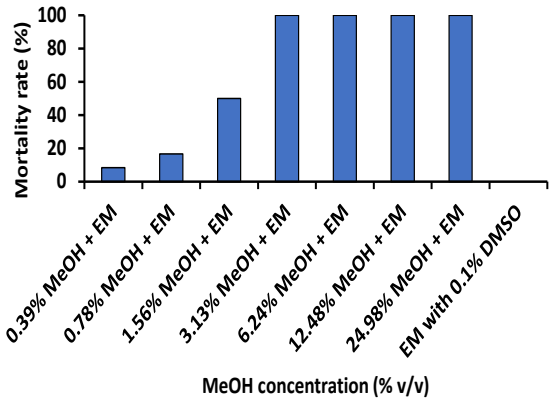

Fig. 2. Mortality rate of zebrafish embryos treated with different concentrations of methanol (MeOH) at 120 hpf. The EM used contained 0.1% DMSO.

Hatching rate of zebrafish at different concentrations of methanol

Based on the result in **Fig. 3**, the hatching rate of the zebrafish embryo at 120 hpf increased as the concentration of methanol decreased. The lowest concentration of methanol in EM (0.39 % MeOH + EM) exhibited the highest hatching rate (91.7%), followed by 0.78 % MeOH + EM (91.7 %) and 1.56 % MeOH + EM (75.0 %). No embryos hatched in higher concentrations of MeOH, including 3.13 % MeOH + EM, 6.24 % MeOH + EM, 12.48 % MeOH + EM, and 24.98 % MeOH + EM. In contrast to the positive control, all the embryos survived in EM without the addition of MeOH after 120 hpf.

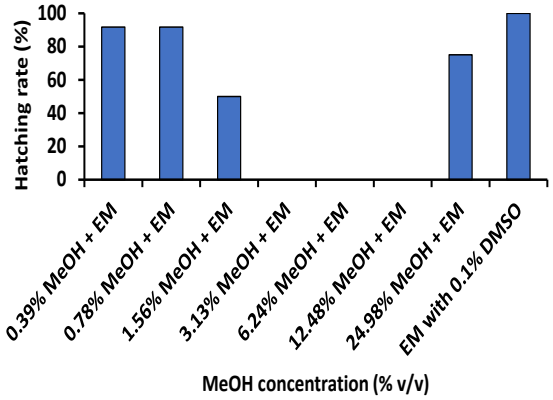

Fig. 3. Hatching rate of zebrafish embryos treated with different concentrations of methanol (MeOH) at 120 hpf. The EM used contained 0.1% DMSO.

Heartbeat rate of zebrafish at different concentrations of methanol

Based on the results in **Fig. 4**, the heartbeat rate of the zebrafish embryos at 120 hpf was observed in the embryos treated with a lower concentration of MeOH (0.39 % MeOH + EM to 1.56 % MeOH + EM).

No heartbeat was observed at higher concentrations (3.13 % MeOH + EM to 24.98 % MeOH + EM) due to the coagulation of the embryos. The lowest concentration of MeOH in EM (0.39 % MeOH + EM) resulted in the highest heartbeat rate (147 heartbeat/min), followed by MeOH at 0.78 % in EM (143 heartbeat/min) and 1.56 % in EM (127 heartbeat/min). The values obtained were slightly lower than the heartbeat rate of the positive control (152 beats per minute).

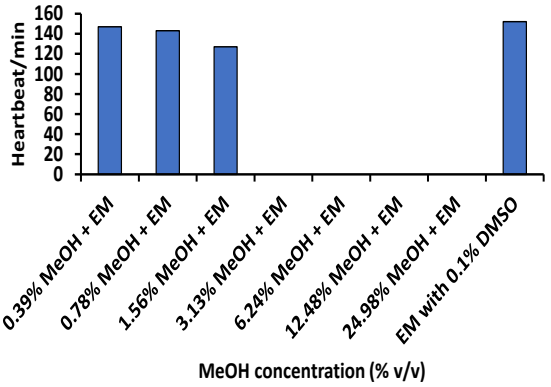

Fig. 4. Heartbeat rate of zebrafish embryos treated with different concentrations of methanol (MeOH) at 120 hpf. The EM used contained 0.1% DMSO.

Teratogenic effects of methanol on zebrafish embryos

Table 2 describes the teratogenic effects in embryos treated with methanol at different concentrations. Meanwhile, **Fig. 5** illustrates the morphological characteristics evaluated as a parameter to determine the embryotoxicity and teratogenicity at different time frames (24 hpf – 120 hpf). At higher concentrations, no teratogenic effects were observed, as the embryos were dead or coagulated and these data were recorded as a percentage of mortality. Meanwhile, the embryos that showed developmental deficiencies such as oedema, non-detachment of tail, lack of somite formation, and curved body with the presence of heartbeat were taken as a percentage of survival. These toxicity assessments revealed that methanol did exhibit toxic effects on the zebrafish embryos at high concentrations.

Table 2. Teratogenic effects of zebrafish embryos treated with different concentrations of methanol (MeOH) at 120 hpf.

Concentration of Methanol (MeOH)	Survive (%)	Teratogenic (%)	Died (%)
EM + 24.98% MeOH	0.0	0.0	100.0
EM + 12.48% MeOH	0.0	0.0	100.0
EM + 6.24% MeOH	0.0	0.0	100.0
EM + 3.13% MeOH	0.0	66.7 (OE); 50 (NT); 91.7 (LS)	0.0
EM + 1.56% MeOH	50.0	91.7 (OE); 8.3 (LS)	50.0
EM + 0.78% MeOH	16.7	91.7 (OE); 8.3 (LS)	83.3
EM + 0.39% MeOH	8.3	91.7 (OE); 8.3 (NT); 8.3 (LS); 8.3 (BC)	91.7

Key:
EM:embryo media; OE:oedema; NT:non-detachment of tail; LS:lack of somite; BC:curved body

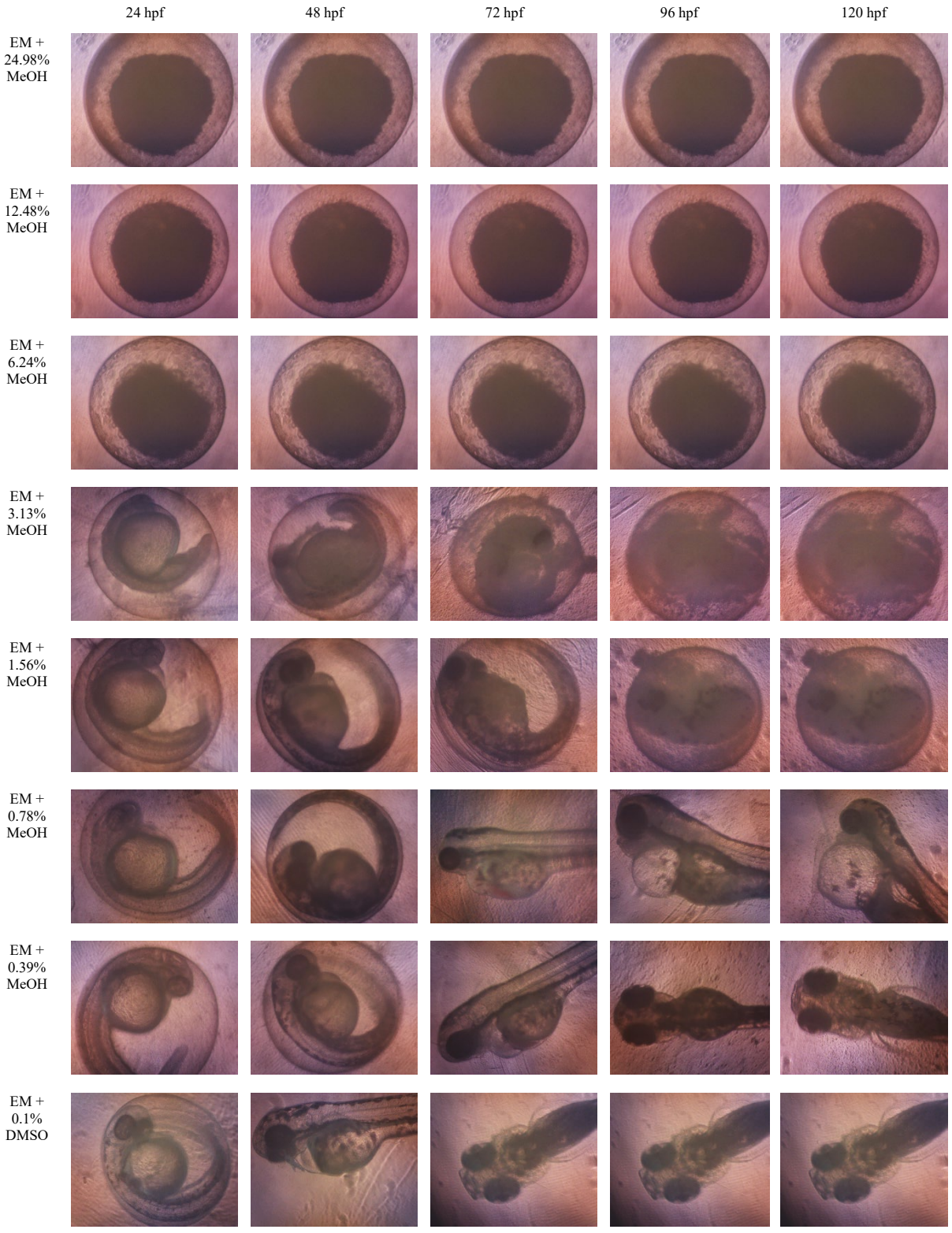

Fig. 5. Morphological response of zebrafish embryos to different concentrations of methanol in different time frames (24 hpf – 120 hpf).

DISCUSSION

Toxicity effects of different concentrations of methanol on zebrafish, *Danio rerio* embryos

Methanol is a common organic solvent that is colorless, poisonous, and volatile. It has an odor similar to alcohol and can be poisonous when it enters the bloodstream through the skin, gastrointestinal tract, or respiratory system. This polar solvent is commonly used in analytical studies involving HPLC and is also a preferred choice for plant secondary metabolite extraction [7; 8]. Methanol toxicity can lead to potentially lethal poisoning, requiring specialized care [6]. In this study, methanol was used to assess its toxicity effects on zebrafish embryos.

The LC_{50} was determined using the zebrafish mortality rate to assess the safe concentration threshold for consuming various sources of plant extracts. A higher LC_{50} value indicates lower toxicity, as it requires a higher concentration to cause mortality in 50% of the organisms [9]. According to OECD (2013) guidelines, toxicants are classified as harmful if the LC_{50} value falls between 10 and 100 mg/mL, toxic if it ranges from 1 to 10 mg/mL, and highly toxic if it is less than 1 mg/mL. Therefore, since the LC_{50} value obtained was less than 1 mg/mL, methanol can be considered highly toxic to the development of zebrafish embryos. Higher mortality rates were observed in zebrafish embryos exposed to higher concentrations of methanol ($\geqslant$ 3.13%). This can be explained the ability of alcohol to induce oxidative stress, disrupting normal biological functions during development or promoting defects in developing organs [10]. In this study, zebrafish embryos were able to tolerate methanol concentrations up to 1.56% in embryo media (EM) without exhibiting severe developmental toxicity. This observation is consistent with findings by [8], who reported that zebrafish embryos could tolerate methanol concentrations between 1.5% and 2.5%. This threshold is critically important for downstream toxicity assays involving plant methanolic extracts. Maintaining methanol concentrations at or below 1.56% ensures that any observed toxicological effects can be confidently attributed to the bioactive compounds within the extract, rather than to residual methanol present as a solvent.

The findings indicate that exposure to higher concentrations of methanol led to increased mortality rates, reduced survival rates, delayed hatching rates, and lower heartbeat rates. These findings align with a study reported by [6], which reported that higher concentrations of methanol (4% to 6%) caused mortality, morphological abnormalities, delayed hatching, and reduced of embryonic autonomous movement in zebrafish embryos. A similar conclusion was drawn by [11], where a high concentration of methanol (60%) was more lethal to embryos in the early stages of development than a lower concentration (40%). The higher mortality rate in zebrafish embryos could be associated with the inherent characteristic of the chorion, which exhibits high permeability to substance intake [11,12].

Methanol is considered one of the teratogens that could cause malformations in developing embryos, such as oedema, non-tail detachment, and a lack of somite formation. Zebrafish embryos rely on the yolk sac for a continuous supply of nutrients. When edema occurs, it disrupts the nutrient supply to the embryos, preventing them from absorbing vital nutrients and leading to malnutrition and coagulation [13]. The presence of somites in zebrafish embryos is indicative of their ability to develop muscle fibres, which contribute to the formation of the axial skeleton and trunk skeletal muscle [14]. The absence of somite formation limits the ability of embryos to release themselves from the chorion and inhibits the hatching process [15]. As emphasized by [6], high concentrations of methanol significantly reduce the autonomous movement of zebrafish embryos, impacting the hatching process. The hatchability of zebrafish embryos relies on their locomotor capabilities and the secretion of chorionase enzymes. The reduced hatching rate of embryos suggests that methanol inhibits the hatching process. A similar conclusion was drawn by Pinheiro da [16], where ethanol disrupted the hatching process by interfering with choriolytic enzyme functions and embryonic movement activity. Heartbeat rate is a critical parameter in toxicological assessments. The normal heartbeat rate in zebrafish embryos is comparable to the human heartbeat, which falls within the range of 120-180 beats per minute (bpm) [17]. Generally, methanol is considered cardiotoxic when embryos' heartbeats fall below the normal range. According to [18], a lower heartbeat rate likely indicates heart dysfunction, potentially due to blockages in blood vessels that disrupt blood flow throughout the zebrafish body.

In conclusion, our study demonstrates that methanol induces significant developmental toxicity in zebrafish embryos, with adverse effects becoming increasingly pronounced at higher concentrations. Specifically, higher levels of methanol resulted in reduced survival rates, decreased hatching rates, compromised heartbeat functionality, and increased incidence of teratogenic effects. The findings underscore the critical need for careful consideration of methanol concentrations in research and industrial applications, particularly in processes involving bioactive compound extraction from plant materials.

CONCLUSION

This study demonstrates that methanol induces significant developmental toxicity in zebrafish embryos, with adverse effects becoming increasingly severe at higher concentrations. Specifically, increased methanol exposure led to reduced survival and hatching rates, impaired heartbeat, and a higher incidence of teratogenic effects. Importantly, the findings establish that zebrafish embryos can tolerate methanol concentrations up to 1.56% in embryo media without severe developmental toxicity. This threshold is critical for ensuring that subsequent toxicity assessments of plant methanolic extracts reflect the effects of the bioactive compounds rather than solvent-induced toxicity. These results provide a practical guideline for researchers using methanol as a solvent in bioassays, particularly in natural product research. Future studies should apply this solvent threshold to toxicity evaluations of plant extracts and explore whether similar thresholds are applicable across different biological models or alternative solvents.

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