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Toxicity and teratogenicity evaluation of ethanolic extract from *Momordica charantia* fruit using zebrafish (*Danio rerio*) embryo model

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Abstract

Zebrafish (*Danio rerio*), a freshwater fish, has become a favoured animal model to assess the teratogenicity effects of various compounds. *Momordica charantia* is a fruit traditionally used as a functional food to treat various ailments. In the present work, 80% ethanolic extract of *M. charantia* fruit was investigated for its teratogenicity effects on the zebrafish embryos. The embryos of 12 h post-fertilisation were immersed in the ethanolic extract at various concentrations of 250, 500, 750, 1,000, and 1,250 mg/L prepared in 2% DMSO. Microscopic observation was carried out every 24 h. Results showed an increased mortality rate, and a delayed hatching rate with increasing concentration. Some of the deformities observed included hyperactivity, crooked backbone, reduced pigmentation, awkward positioning, and coagulation at the highest concentration. Probit analysis resulted in 725.90 mg/L as the median lethal concentration (LC_{50}). Chromatographic analysis revealed the presence of propanedioic acid, malic acid, contrunculin-A, glutamine, D-fructose, sorbopyranose, xylitol, galactonic acid, D-mannitol, and mannose. These compounds may contribute to the deformities observed in a concentration-dependent manner. Therefore, *M. charantia* fruit must be consumed with caution and within the recommended amount.

Introduction

Medicinal plants and their phytoconstituents are continuously explored for their healing properties. One of the medicinal plants that have been reported to exhibit various pharmacological effects and traditionally consumed as a functional food is *Momordica charantia* Linn. This plant belongs to the Cucurbitaceae family, and is also known as bitter melon, bitter gourd, balsam pear, *condemnor* (South America), *karela* (India), and *carilla* (Jamaica), and *peria katak* (Malaysia) (Lee *et al*., 2009; Kumar *et al*., 2010). According to Raman and Lau (1996), *M. charantia* is widely distributed throughout the tropics, particularly in India, China, East Africa, and South America. The proximate analysis of the fruit has been reported to contain high crude protein as compared to cucumber and tomato (Jules, 2003). Other nutritional elements found in *M. charantia* fruit include carbohydrates, vitamins, iron, zinc, calcium, magnesium, phosphorus, and ascorbic acid. It has numerous medicinal properties such as antidiabetic, antimicrobial, antioxidant, antimalarial, anticarcinogenic, and anti-obesity (Virdi *et al*., 2003; Grubben, 2004). Besides possessing diverse metabolites that have the potentials to cure ailments, some of the fruit's constituents are considered toxic, carcinogenic, mutagenic, and teratogenic.

In the present work, the teratogenicity effects of *M. charantia* fruit extracts were assessed by using

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zebrafish (*Danio rerio*) embryos. Various animal models have been used in the investigation of plant extract and toxicity effects. Zebrafish embryo is an emerging reliable animal model to assess teratogenicity effects of multiple components. Zebrafish is a freshwater fish of tropical species that belongs to the Cyprinidae family. The use of zebrafish embryos in toxicity analysis is mainly due to its simple and easy maintenance in the laboratory as well as cost-effectiveness (Andrade *et al.,* 2017). Besides, the developmental process of the embryo is rapid, and its embryonic development is similar to the higher forms of vertebrates. The present work thus aimed to utilise this model to assess the toxicity and teratogenicity effects of *M. charantia* fruit extracts by exposing the embryos to various treatment concentrations.

Materials and methods

Plant material and extract preparation

Momordica charantia var. *muricata* fruits of 12-week maturity were randomly harvested from a farm located in Perak, Malaysia. The sample was deposited for species verification and authentication at the Herbarium of Kulliyyah of Pharmacy, International Islamic University Malaysia with the voucher number of PIIUM 0215. After harvest, the fruits were deseeded, washed, and lyophilised in liquid nitrogen. After freeze-drying, the fruits were ground into a fine powder, and extracted following a method described by Javadi *et al*. (2014) by soaking in 80% ethanolic solvent (1:3, w/v). The extract was then filtered, and the residue was extracted two times with fresh solvent. The filtrates were combined and evaporated at 40 ± 1°C using a rotary evaporator (Buchi®, Flawil, Switzerland). The extract was freeze-dried and preserved at -80°C until further treatments and analyses.

Zebrafish maintenance

The experiment using zebrafish embryos was carried out at the Zebrafish Laboratory under the management of Central Research Animal Facility (CREAM) following the Institutional Animal Care and Use Committee (IIUM) guidelines with the ethical approval number of IIUM/ IACUC Approval/2016/(12)(85). Zebrafish were purchased from a local pet store, and maintained under a 10:14 h (dark:light) cycle in a pH range of 6.5 - 7.5 in 9 L acrylic tank. The tank was kept in a closed multi-rack aquatic housing system (Aquaneering®, San Diego, California, USA) at 27 ± 1°C (optimum temperature = 26 - 28°C). The water in the recirculating water system was aerated continuously with an aquarium air pump. The fish was fed twice per day with Zeigler Adult Zebrafish Complete Diet composed of approximately 55% crude protein, 15% crude fat, 1.5% crude fibre, and 12% moisture, together with some ash and phosphorus (Ali *et al*., 2011; Avdesh *et al*., 2012).

Zebrafish spawning and embryo care

Healthy and active adult female and male zebrafish (1:2 ratio) were selected for spawning by placing them in a glass aquarium with a continuous recirculation system at 12:12 h dark-to-light cycle and a temperature of 27 ± 1°C. Embryos were collected from the spawn tank after the completion of fertilisation and transferred into clean Petri dishes containing embryo water. The embryo water was prepared by dissolving 0.21 g of Instant Ocean® salt in 1 L of Milli-Q water, and embryos were allowed to grow for 6 h post-fertilisation (hpf) (Pamanji *et al*., 2015). The embryos were then washed three times to remove debris, and unhealthy and dead embryos were removed by aspiration using a plastic dropper. The selected healthy embryos of 6 hpf were observed under a microscope to view the embryonic development before the treatment (He *et al*., 2014).

Extract preparation and treatment procedure

The treatment procedure was carried out based on the Organisation for Economic Cooperation and Development (OECD) guideline (OEDC, 2013) whereby 24 embryos (12 embryos in each group × 2 plates) were used for each variable. The fertilised healthy embryos were transferred into a 96-well plate with each well containing one embryo. The treatment was carried out using two sets of plates with 12 embryos in each group along with a set of a control group on each plate. Each well contained 150 µL of embryo water aspirated with an embryo at 12 hpf and 150 µL of 80% ethanolic *M. charantia* fruit extract prepared in 2% DMSO (Fisher Scientific, Leicester, UK) with a final volume of 300 µL. The final concentrations of the extract were 250, 500, 750, 1,000, and 1,250 mg/L. A control group was prepared by exposing the embryos to 2% DMSO without the extract. All plates were incubated in a temperature-controlled room (27 ± 1°C) (Pamanji *et al*., 2015; Andrade *et al.,* 2017).

Microscopic observation

The developmental process of the embryos will be significant at 24, 48, 72, and 96 hpf. Therefore, the teratogenic effects of the samples on the embryos were observed at these periods. The observation was carried out using an inverted microscope (Nikon Eclipse TS 100). The sublethal endpoint assessment that was observed and recorded included the hatchability (Eq. 1) and mortality (Eq. 2) counts, delayed development, reduced body length, oedema, and other morphological abnormalities. Another important parameter was the heartbeat rate using a DanioScope (version 1.1; Noldus Information Technology, Wageningen, The Netherlands), a non-invasive software that provides measurement via descriptive analysis method (mean ± standard deviation). Probit analysis was used to determine the median lethal concentration (LC_{50}) of *M. charantia* fruit extract (Finney, 1971; He *et al*., 2014).

$$Percentage\ of\ Hatchability\ (\%) = \frac{No.\ of\ hatched\ embryos}{Total\ embryos} \times 100\% \quad \text{(Eq. 1)}$$

$$Percentage\ of\ Mortality\ (\%) = \frac{No.\ of\ dead\ embryos}{Total\ embryos} \times 100\% \quad \text{(Eq. 2)}$$

Metabolite profiling using GC-MS

An Agilent 6890 gas chromatograph connected to an Agilent 5973 quadrupole and mass selective detector using an electron impact ionisation with a complement autosampler were used to analyse the 80% ethanolic *M. charantia* fruit extract. A DB-5MS (5% phenyl methyl siloxane) column of 250 × 0.25 µm with helium gas as the carrier gas (flow rate = 1.0 mL/min) was used in the experiment to separate 1 µL of the sample in a splitless mode with a scan mass range of 50 to 550 m/z after 6 min solvent delay. The initial oven temperature was set at 50°C (hold time of 6 min), then the temperature was increased to 180°C at a rate of 10°C/min (hold time of 25 min), and reached a final temperature of 315°C at a rate of 50°C/min (hold time 14 min). The setting yielded a total running time of 60.70 min. Data were processed and analysed using Agilent ChemStation G1701DA software and The National Institute of Standards Technology (NIST; Gaithersburg, MD, USA) 2014 library database (Javadi *et al*., 2014).

Results

Lethal concentration and teratogenicity effect

The lethal median concentration (LC_{50}) value was determined using the probit analysis which estimates the toxicant concentration (mg) per body weight (kg) that can cause 50% death of the tested animal population (Finney, 1971). The LC_{50} result is displayed in Figure 1. Based on OECD guideline (OECD, 2013), LC_{50} values that ranges from 10 to 100 mg/L are harmful, 1 to 10 mg/L are toxic, and any value less than 1 mg/L are highly toxic. The LC_{50} value of the 80% ethanolic *M. charantia* fruit extract was calculated to be 725.90 mg/L, which was more than the cut-off value suggested by the guideline, thus indicating that the extract could be considered safe.

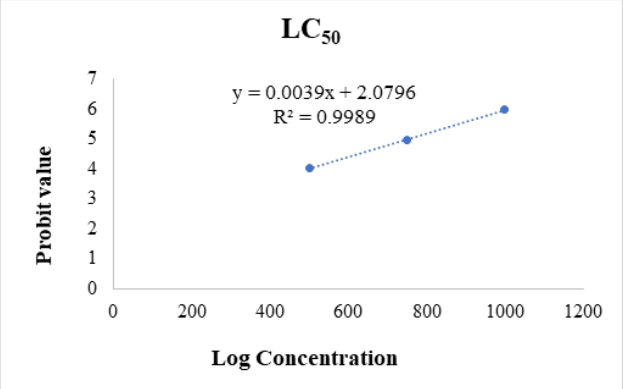

Figure 1. Median lethal concentration (LC_{50}) value of 80% ethanolic *Momordica charantia* fruit extract based on probit analysis.

Figure 2 shows the morphology of the normal embryo and embryos treated with different concentrations of 80% ethanolic *M. charantia* fruit extract at concentrations of 250, 500, 750, 1,000, and 1,250 mg/L after 48 h of treatment (48 h post-treatment, hpt). Observation showed that embryos treated with 250 mg/L concentration (Figure 2B) appeared to be normal. Embryos treated with 500 mg/L concentration showed less pigmentation (Figure 2C) and hyperactive movement with only three embryos developed into larvae. Awkward positioning of the embryos was observed in Figure 2D. Embryos in Figures 2E and 2F were dead with embryo in Figure 2E showing defects with crooked backbone in 1,000 mg/L concentration.

Table 1 shows the teratogenicity parameters observed for the normal embryo and embryos treated with different concentrations of 80% ethanolic *M. charantia* fruit extracts at 48 hpt. Growth retardation is one of the important parameters in teratogenicity assay. Embryos in normal and lowest concentration (250 mg/L) showed the absence of the parameters.

Embryos treated with 500 mg/L concentration showed less pigmentation. Hyperactivity in movement with only three embryos developed into larvae due to delayed hatching was observed in embryos treated with 750 mg/L concentration. Awkward positioning was observed in embryos treated with 750 and 1,000 mg/L concentrations. A few of the embryos in 1,000 and 1,250 mg/L concentrations were dead, with some of the embryos in 1,000 mg/L were observed with crooked backbone.

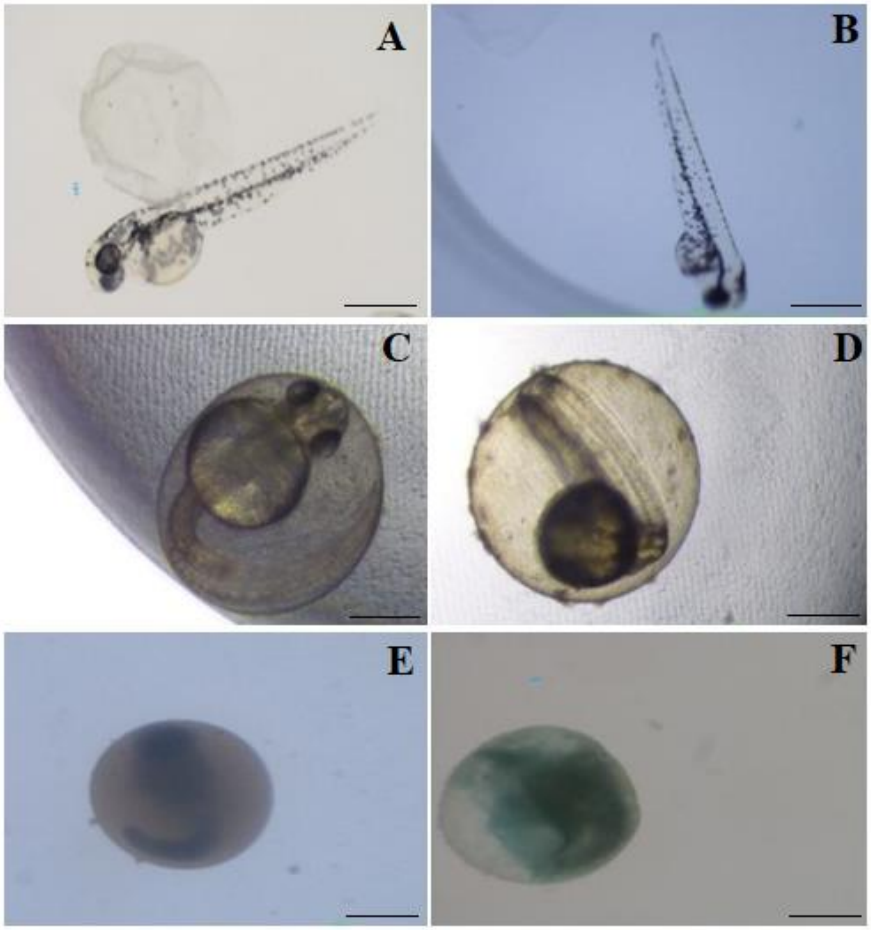

Figure 2. Morphology of zebrafish embryos; normal **(A)** and embryos treated with different concentration of 80% ethanolic *Momordica charantia* fruit extract [250 **(B)**, 500 **(C)**, 750 **(D)**, 1,000 **(E)**, and 1,250 mg/L **(F)**] at 48 hpt. Scale bar: **(A)** = 300 µm, **(B)** = 250 µm, and **(C - F)** = 100 µm.

Table 1. Teratogenic effects of different concentrations of 80% ethanolic *Momordica charantia* fruit extract at 48 hpt in zebrafish embryos.

Extract concentration (mg/L)	Teratogenicity parameter				
	Hyperactive	Delayed hatch	Crooked backbone	Less pigmentation	Awkward position
250	–	–	–	–	–
500	–	–	–	✓	–
750	✓	✓	–	✓	✓
Control	–	–	–	–	–

Hatchability and heartbeat rate

Table 2 shows the hatchability in percentage and heartbeat beat rate per min for the normal embryo and embryos treated with different concentrations of 80% ethanolic *M. charantia* fruit extracts after 48 hpt. The hatchability of the embryos was observed to be normal and complete in the control group and the group treated with 250 mg/L concentration. Further, all embryos treated with the lowest concentration survived and successfully turned into larvae. As

compared to the control group, there were no hatched embryos observed in 750, 1,000, and 1,250 mg/L concentrations after 48 hpt. Some of the embryos had heartbeats despite failing to hatch within the first 24 hpt as observed in 750 mg/L concentration. The success of embryonic development is indicated by hatching. There were no hatched embryos in 1,250 mg/L due to a 75% mortality rate with coagulation as early as 24 hpt.

Table 2. Hatchability at 48 hpt and heartbeat rate of zebrafish embryos treated with different concentrations of 80% ethanolic *Momordica charantia* fruit extract.

Extract concentration (mg/L)	Hatchability (%)	Mortality (%)		Heartbeat rate (BPM) (mean ± SD)
		24 hpt	48 hpt	
250	100	0	0	140.54 ± 38.37
500	12.5	0	12.5	122.11 ± 7.34
750	0	33.3	50.0	96.72 ± 4.26
1,000	0	50.0	100	NH
1,250	0	75.0	100	NH
Control	100	0	0	143.78 ± 14.16

BPM = beats per minute; and NH = no heartbeat.

There was zero mortality rate for the lowest concentration in both timelines. Embryos treated with 500 mg/L concentration also showed zero mortality at 24 hpt. However, a 12.5% mortality rate was observed after 48 hpt. In 750 mg/L concentration, the mortality rate increased from 33.3% at 24 hpt to 50.0% at 48 hpt. Concentrations of 1,000 and 1,250 mg/L recorded a 100% mortality rate after 48 hpt, thus indicating toxicity effect at this concentration. Coagulation and the absence of heartbeat of zebrafish embryos are indicative of mortality. Coagulation can be seen in Figures 2E and 2F that showed the absence of heartbeat and mortality in embryos treated with 1,000 and 1,250 mg/L concentration, respectively.

The normal heartbeat rate of zebrafish embryos ranges from 120 - 180 per min (Abdillah *et al.*, 2019). The mean heartbeat rates of zebrafish embryos exposed to different concentrations of 80% ethanolic *M. charantia* fruit extracts at 48 hpt are presented in Table 2. Control embryos had the highest heartbeat rate of 143.78 per min followed by 140.54 beats per minute in 250 mg/L, 122.11 beats per minute in 500 mg/L, and 96.72 beats per minute in 750 mg/L concentration. There was no heartbeat observed in embryos treated with 1,000 and 1,250 mg/L concentrations, which could be due to the early arrested growth and development.

GC-MS metabolites profiling

Table 3 shows the GC-MS spectrometry profile of 80% ethanolic *M. charantia* fruit extracts. Metabolites found abundantly were propanedioic acid, malic acid, contrunculin-A, glutamine, D-fructose, sorbopyranose, xylitol, galactonic acid, D-mannitol, and mannose. The assignment of peaks was performed after NIST library comparison with more than 80% similarity index.

Table 3. Metabolites in 80% ethanolic *Momordica charantia* fruit extract.

No	RT (min)	Peak area	Detected compound	Similarity index
1	9.0	0.0754	Propanedioic acid	94
2	10.4	0.1936	Malic acid	99
3	10.6	0.3442	Contrunculin-A	95
4	13.6	0.1848	Glutamine	95
5	14.6	0.3745	D-Fructose	83
6	14.7	0.2742	Sorbopyranose	94
7	16.2	2.751	Xylitol	90
8	16.6	29.7448	Galactonic acid	96
9	17.6	2.8101	D-Mannitol	86
10	20.4	1.8438	Mannose	91

Discussion

The use of zebrafish vertebrate model in *in vivo* analyses of drug toxicity and efficacy, and chemical toxicity and safety is increasing in research. The embryogenesis of zebrafish completes in the first 72 h as compared to the other animal models. Besides, the transparency of the embryos eases the observation and evaluation procedures, whereby the development

stages from fertilisation up to hatching takes only three days. Therefore, zebrafish is a suitable model to assess toxicants and their cellular effects (De Luca *et al*., 2014). In general, the level of toxicity can be expressed by the state of adverse effects exhibited by the interaction between the toxic components and cells, which may occur on the cell surface and intracellular or extracellular matrix.

Previous studies have reported the presence of polypeptides, triterpenoids, flavonoids, alkaloids, phenolics, saponins, steroids, and sterols in *M. charantia* fruit that may have contributed to its pharmacological activities (Virdi *et al*., 2003; Jumaat *et al.*, 2017). However, some studies have also reported that compounds exerting toxic effects are also used as anticancer agents. Some constituents found in *M. charantia* fruit extract have been reported to exert toxic effects including lectin, cucurbitacin, and α-momorcharin (Abdillah *et al*., 2019).

Patel *et al.* (2010) suggested that the ethanolic extract of *M. charantia* is considered safe up to 2,000 mg/kg. The extract did not exhibit any toxicity or behavioural changes in mice following oral administration, and no mortality was observed above 5,000 mg/kg. Another study administered ethanolic extract of *M. charantia* orally to rats at 300 and 2,000 mg/kg of body weight (Husna *et al*., 2013). After 30 min of force-feeding, dizziness and depression were observed in both treatment groups with no significant differences in the feeding patterns (water and food intakes) or changes in the body weight. Haematology analysis of the rats treated with 2,000 mg/kg showed reduced red blood cells count (RBC) and packed cell volume (PCV) percentage as compared to the control group.

Another study using methanolic extract of *M. charantia* administered orally to mice at 200, 400, 800, 1,600, and 3,200 mg/kg concentrations showed no significant effects on the general condition and behaviour. The mice showed tolerance up to 3,200 mg/kg with no mortality or visible clinical signs throughout the acute toxicity study (Ofuegbe *et al.*, 2017). Despite the presence of some toxic components in *M. charantia,* some of the acute toxicity studies suggest that the alcoholic fruit extract is safe. This could be due to the use of low concentration, treatment duration, or synergistic reaction between the compounds.

The toxicity effects of *M. charantia* fruit extract, however, have not been discussed at the cellular level. The present work showed teratogenic effects of 80% ethanolic *M. charantia* fruit extract by causing abnormalities in the developing embryos. Except in 250 mg/L concentration, the other concentrations displayed movement hyperactivity, less pigmentation, awkward position, and crooked backbone. The highest concentration led to coagulation which indicated death. This could be due to the presence of α-momorcharin, which has been reported to cause cellular toxicity and can depress embryonic growth. Another compound found to be prominent in *M. charantia* fruit extract is lectin, a carbohydrate-binding protein that may inhibit DNA and protein synthesis in human peripheral blood lymphocytes of normal and leukemic cells. It is also known to hamper the growth of the embryo, which explains the deformities observed in the developing embryos (Husna *et al*., 2013).

Cucurbitanes and tetracyclic triterpenoids consist of numerous highly unsaturated keto-, hydroxyl-, and acetoxy-groups, hence the bitterness of *M. charantia* fruit. The compound is known to possess antidiabetic properties; however, it also exhibits high toxicity effects, and can be fatal. In some cases, poisonings occurred due to exposure to substantial quantities of the compounds (Kaushik *et al*., 2015). Apart from that, ingestion of more than 250 g of *M. charantia* fruit is reported to cause abdominal pain and diarrhoea in diabetic patients. At the cellular level, in an *in vitro* analysis, more than 600 µg/mL of *M. charantia* fruit extract is toxic to the fibroblast and keratinocyte (Husna *et al*., 2013).

Cardiac glycosides are specific types of toxic glycosides in plants that affect the cardiac muscle, thus leading to fatal toxicosis. Cardiac glycosides increase the contraction force of the heart by inhibiting the myocardial Na-K ATP-ase, which can lead to cardiac arrest. Some of the toxic substances that have been reported to be present in *M. charantia* fruit extracts are cucurbitane glycoside derivatives (kuguaoside A), momordicosides I, F1, and K, as well as goyaglucosides b and d. These compounds have been reported to have moderate toxicity activity (Jose *et al*., 2016).

Based on the GC-MS profiling, some of the compounds identified in the ethanolic extract might have caused teratogenic effects on the embryos. These included the non-halogenated dicarboxylic acids named propanedioic acid, which could exert a carcinogenic effect in the female rat sub-model (Moudgal *et al.*, 2000). Tsubuku *et al*. (2004) have reported the adverse effects of administering

glutamine, a semi-essential amino acid found in dietary supplements, with minor elevation in the urine and serum parameters. Meanwhile, Garlick (2001) reported that high glutamine intake was reported to cause vomiting, nausea, and death to adults, while it induced neurological damage in preterm infants. The amino acid is known to be metabolised into glutamate and ammonia, thus causing neurological effects, while psychological and behavioural testing may be important. These compounds could have been the reason behind the toxic effects in the treated embryos.

Recently, an *in vivo* experiment was conducted using zebrafish embryos to examine the toxicity of the Indian and Chinese *M. charantia* fruits' aqueous extract (Thiagarajan *et al*., 2019). The extract caused decreased hatchability and survival rate with increasing concentrations, which is aligned with the findings obtained in the present work. The Chinese *M. charantia* yielded LC_{50} value of 251.19 µg/mL, while the Indian variety yielded LC_{50} value of 199.53 µg/mL. At 1,000 µg/mL concentration, it caused a major defect of scoliosis with inconsistent heart rate. They concluded that the aqueous extract exhibited a mild toxicity effect at higher concentrations potentially due to the presence of cardiac glycosides.

The *M. charantia* fruit extract can be safe at moderate dosages or concentrations. However, it may produce adverse effects at high concentration due to the presence of certain toxic compounds. The toxic effects of *M. charantia* fruit extracts on the developing embryos of zebrafish were found to be directly proportional to the exposure time and concentrations of the extract. Despite having LC_{50} more than 100 mg/L, which is considered safe, some of the embryos treated with higher concentrations have shown some morphological defects. This could be due to the toxic compounds in the extract at higher concentrations. The hatching of the embryos was affected by the different concentrations of the extracts, in which higher concentration decreased the percentage of hatchability. Low hatchability and delayed hatching indicate growth retardation. The reason for delayed hatching could be due to the developmental abnormalities of the zebrafish embryos that are unable to break the chorion. This is possibly explained by the morphological abnormalities observed in the embryos with limited hatchability.

Overall, the LC_{50} value obtained in the present work was considered safe as the value was much higher than the cut-off range stated in the OECD guideline (OECD, 2013). The results agree with Patel *et al.* (2010) and Husna *et al*. (2013) who reported that the ethanolic extract of *M. charantia* fruit is considered safe up to 5,000 mg/kg, with no mortality observed in their animal model. A clinical study has indicated the effectiveness of *M. charantia* fruit upon consumption in various forms among diabetic patients without any significant adverse effects. The diabetic patients have shown improved glucose tolerance and a significant reduction in glycosylated haemoglobin level upon consumption of 50 mL of the fruit juice or 0.23 kg fried *M. charantia* fruit. However, the preparation of *M. charantia* fruit for consumption can vary the results, and there are insufficient clinical findings on the lethal concentration of *M. charantia* fruit extract (Tiwari, 2007; Ghorbani, 2013).

Based on the obtained results, *M. charantia* fruit is safe to be consumed as a functional food for its pharmacological benefits. The toxicity effects observed in the present work occurred at higher concentrations for zebrafish embryos. This is due to their fragile morphology that can be defected even at the smallest concentration of the extract containing potential toxicity-exerting compounds. Therefore, *M. charantia* fruit should be consumed in appropriate amounts as recommended to avoid any adverse effects that may occur by overconsumption (Thiagarajan *et al.*, 2019).

Conclusion

The present work demonstrated the toxic effects of 80% ethanolic *M. charantia* fruit extract on the developing zebrafish embryos; and the median lethal concentration (LC_{50}) was 725.90 mg/L at 48 hpt. The observed effects were dependent on the exposure time and concentrations of the extract. At higher concentrations, the extract caused some morphological defects such as less pigmentation, dented tail, spinal curvature, oedema, malformed yolk sac, as well as reduced hatchability and growth retardation thus indicating the presence of toxicant(s). Therefore, further research on *M. charantia* fruit's metabolites should be carried out before any nutraceutical or pharmaceutical applications. As functional food, the fruit should be consumed with caution and in moderation to avoid overconsumption which could lead to adverse side effects.

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