Phytochemical analysis of ionic liquid-Graviola (*Annona muricata*) fruit extract and its acute toxicity on zebrafish early-life stages

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Abstract. *Annona muricata*, commonly known as soursop and Graviola, is a member of the Annonaceae family. Some of its phytochemicals were reported to have a neurotoxicity effect causing neurodegenerative diseases. However, different parts of this tree have been used for ages in traditional medicine due to their biological activities, such as anti-inflammatory, antimicrobial, antioxidant and anticancer effects. This study aimed to qualitatively screen the crude ionic liquid-Graviola fruit extract (IL-GFE) phytochemical composition, assess its acute toxicity and determine the lethal concentrations using zebrafish (*Danio rerio*) embryos. IL-GFE contains acetogenins, alkaloids, phenols, terpenoids, tannins and flavonoids. Acute toxicity effects of IL-GFE on zebrafish embryos were observed from 24 to 120 hours of post fertilisation (hpf). The survival rate, LC₅₀, sublethal endpoints and effect of IL-GFE on the heart rate of zebrafish larvae were assessed. Results showed that the lethal concentration (LC₅₀) of the crude IL-GFE was 173.45 µg/mL. Interestingly, no significant changes on the morphology of the treated zebrafish were observed at a concentration of 125 µg/mL. However, the heart rate of zebrafish larvae at 96 hpf was significantly decreased by 33.76% after treated with crude IL-GFE at 125 µg/mL (119.00 ± 4.72 beats/min) as compared to the untreated group (179.67 ± 4.66 beats/min). This preliminary finding showed that crude ionic liquid-Graviola fruit extract and its phytoconstituents might have the potential to be developed as a food supplement or herbal product. However, further tests need to be conducted to evaluate its medicinal properties and adverse effects on organisms of higher orders.

Keywords: acute toxicity, *Annona muricata* (Graviola), phytochemicals, teratogenicity, zebrafish assay

INTRODUCTION

Medicinal plants and their constituents have been used for a considerable period by many communities and civilisations to help humankind to maintain good health and treat different diseases (Yunus *et al.*, 2010). Extensive studies are applied to evaluate the application and

formulation of medicinal plants and their bioactive compounds in folk medicine with significant concerns about their efficiency and safety. Despite all their essential pharmacological functions, some of the phytoconstituents could be causing potential toxicity, teratogenicity

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(teratogen agent can cause or induce morphological abnormalities), carcinogenicity, and mutagenicity as an individual compound or in combinations (Ng'uni *et al.*, 2018; Romagosa *et al.*, 2016). Thus, studying the toxic effect and teratogenicity of medicinal plants through their potential extracts is pivotal. The conventional animal models used for acute toxicity studies are mammals, including rats, mice and rabbits as well as flies and worms which are used for specific genetic and molecular studies (Lieschke and Currie, 2007).

Zebrafish (*Danio rerio*) has been used as an alternative model in biomedical and toxicology research due to the tremendous advantageous features among other vertebrates' model. Zebrafish is a small tropical freshwater fish, belonging to the minnow (Cyprinidae) family under the order of Cypriniformes (Parichy *et al.*, 2009). This fish is characterised by its transparency at the early stage of development, high embryos production number, quick maturation and high similarity with humans genome (over 80% genomic homology) as reported earlier by Oliver *et al.* (2011). Hence, higher genomic homology enables a significant correlation between the obtained data from the two species (human and zebrafish). Moreover, Ong *et al.* (2009) reported that zebrafish is widely used in disease studies and drug discovery due to the lower cost of handling compared to other models such as rodents; and they grow at the minimal environmental conditions and small space (e.g. aquarium). For these reasons, reducing animal trails is being necessary.

Reducing animal testing is one of the main goals of the Food and Drug Administration (FDA), which is stated in ISO 10993, hence zebrafish is therefore considered to be an optimum choice to replace rodent model and other animals testing. Since zebrafish can produce a thousand of embryos at one time compared to a rodent which produces at best only a dozen, consequently it is less time consuming and low amount of drug testing needed (Hill *et al.*, 2005). Recently, zebrafish have been used to identify the toxicity of nanoparticles (Abdul Ghafor *et al.*, 2020), antibiotics (Oliveira *et al.*, 2020) and metal (Bai and Tang, 2020). The acute toxicity of a substance on zebrafish can be conducted by a morphological observation to assess the lethal

endpoints such as malformations (death/coagulation, missing heartbeat, no tail detachment, missing somites, no spontaneous movement) and non-lethal malformations (spine deformation, yolk sac oedema, no pigmentation, low or missing of blood circulation and incomplete eye development) (Kapp *et al.*, 2006).

Annona muricata (Graviola) fruit is a member of the Annonaceae family that obtained much interest in the last decades due to its pharmaceutical potential. Previously, many studies reported that *Annona muricata* had been used in traditional medicine to treat many diseases such as diabetes, diarrhoea, fever, inflammation and stomach pain. Since then, the bioactivity and toxicity of this plant attracted the attention of many researchers as summarised by Coria-Téllez *et al.* (2018) and Daddiouaissa & Amid. (2018). Numerous techniques are used to extract bioactive compounds from Graviola fruit such as Soxhlet and maceration extraction (Ragasa *et al.*, 2012; Sun *et al.*, 2017). These methods have shown low efficiency, environmental pollution and a long time that is required for extraction (Dai *et al.*, 2011). On the other hand, a modern and more efficient technique of extraction such as microwave-assisted extraction (MAE) coupled with ionic liquids (ILs), has accelerated research in analytical chemistry as green and designer solvents. The structure of ionic liquids has a great influence on their physicochemical properties, which may have an impact on the extraction efficiency of targeted analytes (Tang *et al.*, 2012).

Several studies assessed the acute toxicity, mutagenicity and neurotoxicity of the Graviola leave, and its phytoconstituents on Wistar rats and Swiss albino mice (Escobar-Khondiker *et al.*, 2007; Guadaño *et al.*, 2000; Lannuzel *et al.*, 2003; Sousa *et al.*, 2010). Nevertheless, no research was conducted regarding the toxicity level and teratogenicity effects of the crude ionic liquid Graviola fruit extract (IL-GFE) on developing zebrafish embryos. Therefore, this study aimed to identify the phytoconstituents of the crude IL-GFE and to assess the toxicity level, determine the lethal concentration and measure the teratogenicity effects of this extract by using the apparent clinical signs and symptoms of developing zebrafish embryos.

MATERIALS AND METHODS

Chemicals and reagents

In this study, chemicals and reagents that were used are laboratory grade. They were utilised to investigate the phytochemical composition and acute toxicity effect of IL-GFE on developing zebrafish embryos. The ionic liquid, one-butyl-3-methylimidazolium chloride [C4MIM] Cl⁻ (96%) reagent was purchased from ALFA, (USA). DMSO and Keddie's reagent (3,5-Dinitrobenzoic acid) were obtained from Sigma-Aldrich (USA). Dragendorff reagent was purchased from Merk (Germany), sulphuric acid and ferric chloride were obtained from HmbG Chemical (Germany).

Plant collection and preparation

Samples of ripe Graviola fruit (DB3) were randomly collected during the season of 2017/2018 from the Adikafirdaus Resources Farm (4.5921° N, 101.0901° E), Perak state, Malaysia. The fruit sample was identified and deposited in the herbarium with voucher number KAED/HBL/S1A047/2018/706 at the Kulliyah of Architecture and Environmental Design (KAED), IIUM. The website (www.theplantlist.org) was used to check the name of the plant. Then, Graviola fruit samples were cleaned and the pericarp and seeds were removed. After that, the fruit pulp was lyophilised using a freeze drier (Christ Alpha, Germany) for 72 hrs to ensure initial sample texture is preserved. The dried sample was then ground to a powder form using a commercial blender (Kinematics, Swiss) and stored until further analysis (Gyamfi *et al.*, 2011).

Graviola fruit extraction

The Graviola fruit samples were extracted as described in our previous article (Daddionuissa *et al.*, 2019). Briefly, one gram of the dried powder of the fruit pulp was mixed with 20 mL of 1-butyl-3-methylimidazolium chloride at 0.5 mol/L. Then, the suspensions were heated in the microwave oven under irradiation power of 700W for 3 min. Next, the obtained extract (IL-GFE) was cooled down to 25°C and then filtered using Whatman No. 3 mm filter paper and transferred into a test tube. Then, lyophilised the sample

using a freeze drier for 72 hrs (Bhan *et al.*, 2017; Zhang *et al.*, 2014).

Phytochemical screening

According to the procedure of Harborne (1998) with slight modifications, the crude ionic liquid Graviola fruit pulp extract was subjected to qualitative determination of phytochemical constituents.

Detection of acetogenins

Keddie's reagent test was performed to detect the presence of acetogenins by using the method reported by Vyas. (2010). One drop of 90% ethanol in distilled water and two drops of 2% Keddie's reagent (3,5-dinitrobenzoic acid) were added to 2 mg of crude IL-GFE. Then, a 20% sodium hydroxide solution was added to alkalize the mixture. Formation of purple colour indicated the presence of β -unsaturated- γ -lactones that are commonly present in the annonaceous acetogenins.

Detection of alkaloids

Dragendorff reagent test was performed to detect the presence of alkaloids by dissolving 2 mg of the sample (IL-GFE) in diluted hydrochloric acid, then 1 mL of Dragendorff reagent was added to the mixture. Formation of reddish/brown precipitate indicates the presence of alkaloids (Harborne, 1998).

Detection of phenols

Ferric chloride test was applied to detect the presence of phenols by treating 2 mg of the sample (IL-GFE) with 1 mL of ferric chloride solution. Formation of bluish-black colour indicates the presence of phenols (Bhat *et al.*, 2009; Harborne, 1984).

Detection of flavonoids

Alkaline reagent test was employed to detect the presence of flavonoids as described by Evans. (1996). A few drops of 1 M sodium hydroxide (NaOH) solution were added to 2 mg of the extract (IL-GFE) in a centrifuge tube. A formation of an intense yellow colour which becomes colourless after adding a few drops of HCl indicated the presence of flavonoids.

Detection of saponins

An emulsion formation test was performed to detect the presence of saponins as described by Ejikeme *et al.* (2014) and Zakaria *et al.* (2019). About 200 μ L of distilled water was added to 2 mg of sample (IL-GFE). Then, approximately 1 mL of olive oil was added to the mixture and shaken vigorously. Formation of emulsion indicated the presence of saponins.

Detection of terpenoids

Salkowski test was applied to detect the presence of terpenoids as described by Ayoola *et al.* (2008). About 0.5 g of the sample (IL-GFE) was mixed with 2 mL of chloroform. Concentrated sulphuric acid (3 mL) was added to form a layer. Formation of a reddish-brown colour of the interface indicates the presence of terpenoids.

Detection of tannins

The diluted ferric chloride test was applied to detect the presence of tannins as described by Zohra *et al.* (2012) with slight modifications. About 2 mL of deionised water was added to the same volume of IL-GFE sample extract then, 1 to 2 drops of ferric chloride solution were added. The formation of a dark green or blue-green colour indicates the presence of tannins.

Acute toxicity of IL-GFE on zebrafish embryos/larvae

The acute toxicity of the crude IL-GFE was analysed using zebrafish embryos/larvae (24 to 120 hpf) screening assay in a 96-well plate to observe their morphological changes at *in vivo* level, determine the 50% lethality concentration (LC_{50}) and compare it to the standard LC_{50} of the plant extract on zebrafish embryos/larvae.

Zebrafish maintenance and embryo collection

After spawning, zebrafish embryos were collected from the adults' zebrafish of the wild type (AB strain). The zebrafish adults were raised and maintained by Danio Assay Laboratories Sdn. Bhd., Malaysia, under standard laboratory conditions of animal welfare in a flow-through tank with a light/dark photoperiod of 14/10 hrs at 28°C. The zebrafish embryos were handled in conformity with the related laws and institutional guidelines of the Institutional Animal Care and

Use Committee (IACUC), with an ethical approval number (UPM/IACUC/AUP-R059/2018), Universiti Putra Malaysia. The embryos were collected from a spawning tank after 1-hour post fertilisation (hpf), rinsed with egg water, staged and maintained in a Petri dishes at $28 \pm 2^\circ\text{C}$ until 24 hpf in which the time of exposure was started.

Zebrafish embryos/larvae toxicity

The embryos/larvae acute exposure assay was performed by following the guideline of OECD (2013) and Yan *et al.* (2016) with some modifications. After embryo quality control test was carried out, the live zebrafish embryos were randomly transferred into 96-well plates with one healthy embryo (24 hpf) per well and twelve embryos in each treatment group (3 replicates). Then, 200 μ L of Danio-Sprint M Embryo Media was added with either a crude IL-GFE sample or 0.1% DMSO as a control. The stock solution of the crude IL-GFE at a concentration of 50 mg/mL was dissolved in 10% v/v DMSO. Then, the IL-GFE stock solution was serially diluted to 2-fold at seven concentrations ranging from 7.81 μ g/mL to 500 μ g/mL. DMSO was maintained at 0.1% v/v in all sample concentrations. The vehicle of 0.1% v/v DMSO was considered as untreated (control) and was observed to be safe to the embryos. The exposure was continued in a temperature-controlled incubator at $28 \pm 1^\circ\text{C}$ at different stages of development from pharyngula period (24–48 hpf), hatching period (48–72 hpf) to early larval period (72–120 hpf) (Kimmel *et al.*, 1995). Embryos/larvae were observed every 24 hrs to assess the toxicological endpoints such as dead/coagulation, hatching rate, tail detachment, somite formation and body curvature using an inverted microscope (Olympus MVX10, Germany). The angle of curvature in the effected larvae was determined using digital images and compared to the linear spinal axis of the untreated group. The curvature of the spinal cord was measured and graded as low ($0\text{--}45^\circ$), medium ($46\text{--}90^\circ$), maximum ($91\text{--}135^\circ$) or severe ($136\text{--}180^\circ$) as described by Pamanji *et al.* (2015). The heart rate of the larvae was assessed at 96 hpf and recorded using an inverted microscope (Olympus MVX10, Germany) with a digital camera attached to the ocular lens and heartbeat was counted for 1 min using a stopwatch. The mortality and

hatching rates were determined using equations 1 and 2.

$$\text{Mortality rate (\%)} = \frac{\text{No. of dead embryos}}{\text{Total embryos number}} \times 100 \text{ (Eq 1)}$$

$$\text{Hatching rate (\%)} = \frac{\text{No. of hatched embryos}}{\text{Total embryos number}} \times 100 \text{ (Eq 2)}$$

Statistical analyses

The acute toxicity experiments were conducted in triplicates, and the data were represented as the overall mean of three independent experiments $\pm$ standard deviation (SD). The findings were analysed by GraphPad Prism (Version 7.00, GraphPad Inc., CA, USA) to carry out the statistical analysis. One-way ANOVA analysis of variance technique followed by Tukey's test was performed using Minitab software (Version 17, Minitab Inc., State College, PA, USA) to evaluate

the significant differences between samples with a certain interval of 95% and $P < 0.05$ was considered significant.

RESULTS

Phytochemical screening

The qualitative phytochemical analysis conducted on the crude IL-GFE revealed the presence of some biologically active compounds, including acetogenins, alkaloids, flavonoids, tannins, terpenoids, and phenols that exhibit many therapeutic activities (Table 1). They were detected based on the specific colour formation, froth formation, precipitation and the ring formation between the two layers (Ojezele *et al.*, 2016).

Table 1. Qualitative phytochemical components of the crude Graviola fruit extract.

Phytochemical constituents	Test name	Crude IL-GFE
Acetogenins	Keddie's reagent test	++
Alkaloids	Dragendorff reagent test	++
Phenols	Ferric chloride test	+
Flavonoids	Alkaline reagent test	++
Saponins	Emulsion formation test	-
Terpenoids	Sulphuric acid test	+
Tannins	Diluted ferric chloride test	+

Note: Negative (-) and positive (+) signs showed the absence and presence of metabolites in the crude IL-GFE, with (++) showed strong color and (+) mild color.

In vivo zebrafish embryo/larvae toxicity assay

The acute toxicity of the crude IL-GFE on developing zebrafish embryos was conducted by assessing the standard toxicological endpoints such as dead/coagulation, heartbeat, tail detachment, hatching rate, somite formation and body curvature from 24 to 120 hpf.

Effects of crude IL-GFE on the survival rate of zebrafish embryos

Embryos exposed to serial concentrations of IL-GFE were assessed daily and compared with the control group at 24, 48, 72, 96 and 120 hpf. The survival rates (%) of the embryos in egg water and the treated embryos with different concentrations of IL-GFE were shown in Figure 1. Concentrations above 250 $\mu\text{g/mL}$ of IL-GFE resulted in 100 % mortality at 96 hpf. The

concentrations of less than 62.5 $\mu\text{g/mL}$ did not show any significant mortality on developing zebrafish embryos until the end of exposure time at 120 hpf.

The LC_{50} value for IL-GFE was indicated using the statistical estimation of the extract ($\mu\text{g/mL}$) required to induce 50% of the dead population of developing zebrafish embryos using linear regression equation. Figure 2 represents the estimation of the LC_{50} value using the percentage of survival rate (%) of the embryos *vs* concentrations of crude IL-GFE ($\mu\text{g/mL}$) as described by Scarlett *et al.* (2013) and Yan *et al.* (2016). Based on the regression equation, the LC_{50} of the crude IL-GFE was calculated to be 173.45 $\mu\text{g/mL}$.

Figure 1. Effect of crude IL-GFE (7.81-500 µg/mL) on the survival rate of zebrafish embryos from 0 to 120 hpf. The sample was compared to the untreated group. The number of embryos tested for each concentration was 12 individually exposed embryos. The IL-GFE at a concentration of 31.25 µg/mL or less, showed no effect on the survival rate of zebrafish embryos. Superscripts with different letters (a-d) are significantly different ($p < 0.05$).

Figure 2. Effect of crude IL-GFE (7.81-500 µg/mL) on zebrafish embryos mortality rate at 120 hours post-fertilization (hpf). The LC₅₀ value of crude IL-GFE towards zebrafish embryos was 173.45 µg/mL. The number of embryos tested for each concentration was 12 individually exposed embryos.

Effects of crude IL-GFE on morphology and development of zebrafish embryos

Sublethal morphological and developmental effects were also assessed during the five days scoring period. The teratogenicity effects (Table 2) such as oedema (reversible side effect) and tail extension, somite formation, hatching rate and body curvature (non-reversible side effect) were observed for both control group and those treated with different concentrations of crude IL-GFE at 72 hpf. It can be seen that the crude IL-

GFE at a concentration of less than 250 µg/mL did not show any effect on the tested teratogenicity parameters at 72 hpf. However, all larvae treated at a concentration of 500 µg/mL died with a sign of body curvature.

The microscopy observation showed that the lowest concentrations of the crude IL-GFE (less than 62 µg/mL) did not affect the morphology (oedema, tail extension, somite formation, hatching rate and body curvature) of the treated larvae compared to the control group during the whole treatment period. In contrast, the crude IL-GFE at 500 µg/mL was found to be toxic to zebrafish embryos/larvae in which some embryos coagulated at 48 hpf, and all the larvae died at 96 hpf. Some larvae of these groups displayed an angle incidence of 0–45° of the spinal cord curvature, which is considered as low body curvature.

Hatching process of zebrafish embryos was also used to assess the effect of the crude IL-GFE on developing zebrafish embryos. Embryos hatching indicates the appropriate development of the embryos into larvae which usually occurs at 48 to 72 hpf (Murugesu et al., 2019). The treated embryos did not show any significant differences in hatching rate when compared to the control group (Figure 4). The hatching rate of embryos treated with 500 µg/mL could not be measured due to the 75% mortality rate with coagulation at 72 hpf.

Effects of crude IL-GFE on zebrafish heart rate

Embryos and larvae were considered dead if no heartbeat was observed. Generally, the heart rate of zebrafish embryos/larvae ranges between 120 and 180 beat/min (Luca et al., 2014). The mean heart rates of zebrafish larvae were recorded at 96 hpf and presented in Figure 5. The healthy larvae had a heart rate of 179.67 ± 4.66 beat/min which is the highest rate, followed by the other treated larvae with the crude IL-GFE at concentrations of 7.81, 15.63, 31.25, 62.5 and 125 µg/mL, having heart rates of 174.67, 165.67, 150.67, 134.00 and 119.00 beats/min, respectively while no heartbeat was recorded for the treated larvae with IL-GFE at the concentrations of 250 and 500 µg/mL due to early mortality.

Table 2. Teratogenicity effects of crude IL-GFE (7.81-500 µg/mL) on zebrafish development at 72 hpf.

Extract Concentrations (µg/mL)	Teratogenicity Parameters				
	Oedema	Delayed hatch	Not fully tail extension	Lack of somite formation	Body curvature
7.81	-	-	-	-	-
15.63	-	-	-	-	-
31.25	-	-	-	-	-
62.50	-	-	-	-	-
125.00	-	-	-	-	-
250.00	-	-	-	-	-
500.00	nd	nd	nd	nd	+
Control	-	-	-	-	-

(-): Not detected, (+): detected, (nd): Not determined.

Figure 3. Representative photographs of A: untreated zebrafish (control) and B: the treated zebrafish with crude IL-GFE (500 µg/mL) at a designated time point. Photographs were captured using an inverted microscope at 100X magnification. Scale bar = 250 µm.

Figure 4. Effect of crude IL-GFE (7.81-500 µg/mL) on zebrafish embryos hatching rate at 0 to 120 hpf. The number of embryos tested for each concentration was 12 individually exposed embryos. Superscripts with different letters (a-d) are significantly different ($p < 0.05$).

Figure 5. Heartbeat rates of zebrafish larvae treated with crude IL-GFE (7.81-500 µg/mL) at 96 hpf. Bars with different letters (a-d) are significantly different ($p < 0.05$).

DISCUSSION

Secondary metabolites from plant sources have shown to be a promising potential therapeutic in various human diseases (Viraraghavan and Shanmugam, 2018). Phytochemical analysis of the crude IL-GFE revealed the presence of a complex mixture of secondary metabolites that are reported to have various medicinal activities (Gajalakshmi *et al.*, 2012; Ojezele *et al.*, 2016). These phytochemicals are acetogenins, alkaloids, flavonoids, tannins, terpenoids, and polyphenols. This finding is in line with the previous studies conducted on the ethanolic seed extract (Ojezele

et al., 2016), ethanolic and water leaves extracts (Gavamukulya *et al.*, 2014) and hydro-alcoholic fruit extract (Viraraghavan and Shanmugam, 2018) of *Annona muricata* that reported the existence of many secondary metabolites such as alkaloids, saponins, triterpenoids, anthraquinones, flavonoids, tannins, and cardiac glycosides. These metabolites are produced in the plant tissue and considered as defence compounds (Gavamukulya *et al.*, 2014). Different identified metabolites from Graviola fruit such as acetogenins are believed to be mainly responsible for the anticancer activity of Graviola towards mammalian cancer cells (Mangal *et al.*, 2016). However, some of the annonaceous acetogenins (AGE) was reported to have a significant induction of atypical parkinsonism in Guadeloupe Island (Höllerhage *et al.*, 2015). These acetogenins are secondary metabolites deriving from the long-chained lipophilic in the polyketide pathway (Antony and Vijayan, 2016). They are inhibitors of the mitochondrial complex I by the inhibition of ubiquinone-linked NADH oxidase in the membrane (Escobar-Khondiker *et al.*, 2007) causing apoptosis in cancer cells.

The toxicological effect of crude ionic liquid-Graviola fruit extract was evaluated *in vivo* using developing zebrafish embryos as a model organism. The present study illustrates the usefulness of zebrafish as a useful model to rapidly assess the toxicity of crude IL-GFE. Many attributes make zebrafish as an attractive model for such studies. Zebrafish embryos grow up rapidly, and the major organs ultimately form within 48 hpf; hence, comprehensive toxicological assessment can be carried out within only a few days (Falcão *et al.*, 2018). With the transparency nature of the embryos, many toxicological endpoints can be evaluated non-invasively during the experiment. Lethality rate, which is defined as the most sensitive endpoint, was assessed entirely within 48 h (Frayssé *et al.*, 2006). Thus, influential dose-response studies are easily achieved through large sample sizes. This considerable supply of embryos also facilitates the assessment of toxicity of a significant number of materials in a short time.

Our present study demonstrated that crude IL-GFE had reduced the survival of zebrafish larvae at a relatively high dose of 250 µg/mL after 96 hpf while no significant effect was observed at

62.5 µg/mL and lower concentrations. Previously, it was reported that any toxicants displaying an LC₅₀ value range between 10 to 100 mg/L, 1 to 10 mg/L or less than 1 mg/L are considered highly toxic, toxic or harmful, respectively (Murugesu *et al.*, 2019). Based on this scale, the crude IL-GFE is found to be non-harmful with an LC₅₀ calculated to be 173.45 µg/mL on zebrafish embryo/larvae with bodyweight ranged between 58 and 79 µg at 0 to 96 hpf respectively (Hachicho *et al.*, 2015).

Heart rates were recorded at 96 hpf and showed a significant reduction of 33.76 % at 125 µg/mL as compared with the control group. A similar record was also registered in zebrafish larvae when treated with saxitoxin for 96 hrs by Lefebvre *et al.* (2004). They suggested that oedema might be a consequence of a lack of movement and the observed decrease in heart rates may be a secondary effect of oedema and the alteration of fluid balance related to paralysis. Hatching is an essential process in the life cycle of fish, and many biochemical and physical mechanisms are involved in this process (Jin *et al.*, 2009). In our study, the hatching rate of 48 hpf average was in line with the standard hatchability of the zebrafish model, which is between 48 and 72 hpf (Kimmel *et al.*, 1995). Body curvature is another sign of malformation that was observed in zebrafish larvae after treatment with crude IL-GFE at a high concentration of 500 µg/mL. Typically, there are three types of spinal curvature known as lordosis, kyphosis and scoliosis, that are classified based on the spinal bending degree. These spinal curvatures are caused by many factors, including toxins accumulation, inhibition of AChE enzyme activity and the deficit of neuromuscular coordination (Pamanji *et al.*, 2015). Ekrem *et al.* (2012) and Hayes *et al.* (2014) suggested that spinal deformities may be caused by reduced amounts of collagen in the spinal column, downregulation of the protein tyrosine kinase seven (ptk7) that plays a vital role as a regulator of Wnt signalling and alteration of the amino acid composition.

Several plant metabolites were evaluated for their toxicity in zebrafish embryos/larvae such as curcumin (Wu *et al.*, 2007), resveratrol (Pardal *et al.*, 2014), caffeine (Chen *et al.*, 2008), nicotine (Mora-Zamorano *et al.*, 2016), cannabinoid (Akhtar *et al.*, 2013), matrine and sophocarpine (Lu *et al.*, 2014). Previously, Wu *et al.* (2007) have

analysed the toxicity of curcumin (polyphenol derived from the rhizomes of *Curcuma longa*) on zebrafish embryos and larvae which displayed a significant decrease in survival and hatching rate at a dose of 7.5 µM or higher. Another study was conducted to assess the toxicity of *Millettia pachycarpa* (Leguminosae) on zebrafish embryos in which the aqueous extract of this plant exhibited an LC₅₀ of 3 µg/mL. In addition to that, many developmental abnormalities, including pericardial oedema, muscle defects and body curvature, were observed in a dose-dependent manner (Yumnamcha *et al.*, 2015).

Another study showed the toxicity of celastrol (terpenoid isolated from the Chinese herb *Tripterygium wilfordii*), which has been used traditionally as chemopreventive, neuroprotective and antioxidant agents (Kuchta *et al.*, 2017). The toxicity of celastrol, which was evaluated on zebrafish embryos, showed that the concentration of 0.5 µM or higher of celastrol induced many developmental abnormalities such as pericardial sac oedema, no blood flow and tail malformation. Also, the dose of 2.0 µM of celastrol had killed all the treated embryos within 24 hrs (Wang *et al.*, 2011). Furthermore, the exposure of zebrafish embryos to matrine and sophocarpine (alkaloids obtained from *Sophora alopecuroides*) at a dose of 250 and 180 mg/L, respectively exhibited frequent malformations at 96 hpf that includes the appearance of tail bending, lack of hatching and loss of movement (Tropepe and Sive, 2003). However, matrine and sophocarpine are commonly used as anti-tumour, anti-inflammatory, antioxidant and antiviral in Chinese traditional herbal medicine.

After analysing the data obtained from the acute toxicity studies conducted on mammals, Falcão *et al.* (2018) reported that the results of the toxicity test of cannabinoid exposure during pregnancy that was performed on zebrafish embryos (Akhtar *et al.*, 2013) were comparable with the one observed in rodents (Taylor and Fennessy, 1977) and humans (El Marroun *et al.*, 2010). They also found that cannabinoids can cause low birth weight, hyperactivity at small doses and low fetal growth in the treated animals. The use of these models in preclinical assays can be used to improve medicine through the identification of new pharmacological interventions at early stages of life.

CONCLUSION

Phytochemical analysis for the crude ionic liquid-Graviola fruit extract revealed the presence of many bioactive compounds such as acetogenins, alkaloids, phenols, flavonoids, tannins and terpenoids. These metabolites are known to have many therapeutic and physiological activities. The exposure to crude IL-GFE had reduced the survival of zebrafish larvae at a relatively high dose of 250 µg/mL after 96 hours post-fertilization (hpf), which make it non-harmful to the zebrafish model. Interestingly, no significant changes on morphology of the treated zebrafish embryos/larvae with the crude IL-GFE at (125 µg/mL) have been recorded. However, heart rates were significantly reduced in the treated larvae with crude IL-GFE at 125 µg/mL as compared with the control group and a sign of low body curvature at a high dose treatment of 500 µg/mL. Thus, further investigations are needed to understand and explain the specific effects of the Graviola fruit metabolites and build conclusions on human risk assessment.

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