

Full Length Research Paper

Effects of Kelulut honey from *Trigona* sp. on zebrafish (*Danio rerio*) embryo that mimics human embryonic development

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Kelulut honey (KH) is a type of honey with various pharmacological properties that can be found in Malaysia. Nevertheless, the safety aspects of this honey have not been adequately addressed. This study evaluated the developmental toxicity of KH from *Trigona* sp on zebrafish (*Danio rerio*) embryos. Viable zebrafish embryos at 3 hours post fertilization (hpf) (early stage) and 24 hpf (organ development stage) were treated with KH (1 to 20 mg/mL). The embryos were examined for morphological abnormalities and viability until 96 h of KH treatment. Coagulated embryos were identified after treatment with KH (≥ 10 mg/mL) for 3 hpf group and KH (≥ 12 mg/mL) for 24 hpf group. The LC₅₀ values of KH at 96 h of exposure for the 3 hpf and 24 hpf group were 12.52 and 16.36 mg/mL, respectively. The maximum allowable concentration (MAC) for KH on 3 hpf and 24 hpf group were 0.63 and 0.82 mg/mL, respectively. The irregular cardiac rate of the embryos was noted at ≥ 10 mg/mL for 3 hpf group and ≥ 13 mg/mL of KH for 24 hpf group. In summary, the early stage embryo (3 hpf group) was more sensitive to KH than the one of later stage (24 hpf group). It indicates that serious precautions should be taken into account in the use of any material including natural product, be it food or supplement, especially in the early stage of life.

Key words: Kelulut honey, *Trigona* sp., toxicity, zebrafish (*Danio rerio*) embryos.

INTRODUCTION

Honey is one of the natural products that has been widely used for various therapeutic purposes. There are several common varieties of honey that can be found in Malaysia

such as Tualang, Gelam, Kelulut Putih and Kelulut Hitam (Ghazali, 2009). Honey is produced by bees from a process of regurgitation and evaporation that chemically

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converts nectar into honey (Hamid et al., 2015). In general, honey contains 80% of carbohydrates (35% glucose, 40% fructose and 5% sucrose) and 20% water (Akhtar et al., 2014). The composition of honey depends on the flowers foraged by the honey bees and climatic factors (Rahman et al., 2010; Getu and Birhan, 2014; Umarani et al., 2015).

Kelulut honey (KH) is produced by small size stingless bees, which usually form a complex social colony. Kelulut bees often build and develop their nests on roots or wooden trees that have been chopped down. In Malaysia, there are about 33 species of stingless bee, several of which could be domesticated for honey, propolis and bee bread production. The most common species that have been widely commercialized in the country are *Trigona itama*, *T. terminate* and *T. thoracica* (Resnick and Mann, 2014). There are differences between ordinary honey and KH in terms of the colour, smell, taste, physical characteristics and properties. The colour of KH is clearer compared with other forest honeys. The taste is sweet and a bit sour (Roowi et al., 2012). Besides, Roowi et al. (2012) found seven types of phenolic acids in KH through gas chromatography-mass spectrometry (GCMS) analysis. There are benzoic acid, phenylpropanoic acid, 4-hydrobenzoic acid, 4-hydroxyphenyl acetic acid, vanillic acid, protocatechuic acid and coumaric acid. KH has been demonstrated to exhibit various pharmacological effects such as antibacterial (Zainol et al., 2013), antioxidant (Yusof and Pui, 2014), anti-inflammatory (Nurhayati et al., 2015) and anti-ageing (Afrouzan et al., 2007). Recently, KH from *Trigona* species has been reported to exhibit chemopreventive properties in rats induced with azoxymethane (Latifah et al., 2016).

There is a misconception that everything that comes from nature is always safe, harmless and without risk. The stringent challenge faced by natural products is the lack of scientific evidence for their mode of action and safety profile *in vivo*. The safety of natural products has become a major concern to national health authorities, urging sufficient studies to be conducted before a product can be consumed. Therefore, toxicological assessment is required to identify the adverse effects and to determine the limits of exposure level at which such effects occur (Ifeoma and Oluwakanyinsola, 2013). Prior to clinical tests for toxic effects in human, a range of toxicity tests performed in non-human experimental models are needed. These involve *in vitro* (the use of cell lines) and *in vivo* evaluation (the use of animal models) (Ifeoma and Oluwakanyinsola, 2013).

It is difficult to assess the harmful effects of chemicals in the present mammalian models because their embryonic development is longer and the mother has to be sacrificed in order to get the embryos (Padje, 2007). Of advantage, the accessibility and transparency of zebrafish embryos simply allow scoring of teratological

and embryological toxicity (He et al., 2014). In addition, 75% of zebrafish genes encoding the proteins and organs are similar to that of a human being and makes it an appropriate model (Hsu et al., 2007). Latifah et al. (2016) demonstrated earlier that KH was not toxic to Sprague Dawley rats. In the present study, KH was evaluated for its developmental toxicity on zebrafish (*Danio rerio*) embryo as a model.

MATERIALS AND METHODS

Fish care and egg collection

Adult zebrafish (*D. rerio*) were purchased and raised according to the Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia (Ethical approval reference number: UPM/IACUC/AUP-R065/2016). The temperature was maintained at $26 \pm 1^\circ\text{C}$ (Organisation for Economic Cooperation and Development - OECD, 2013). The photoperiod was regulated and maintained at 14:10 h (light:darkness) (Westerfield, 1993). The fish were fed three times daily with a mixture of Hikari Micropelets and Hikari Microwafers (Japan). The adult zebrafish with a ratio of 2 (male):1 (female) were placed in an aquarium tank (OECD, 2013). Sufficient air was supplied through an external pump without any disturbance in the flow of water. For production of eggs, the mature zebrafish were kept in the tank up to the age of six months old. A specialized egg/embryo collection box was placed in the aquarium tank a day before use. Marbles and artificial plants were provided to stimulate spawning of the fish (OECD, 2013). The eggs were collected from the tank 3 h after the onset of light. The viable normal dividing spherical eggs were washed and placed in a petri dish containing the 1X E3 medium at $26 \pm 1^\circ\text{C}$.

Treatment of Kelulut honey

The fertilized eggs (zebrafish embryos) were selected by using a stereomicroscope (Labomed, US) and divided into two groups, which were 3 h post fertilization (hpf) (early stage) and 24 hpf (organ development stage). The fertile embryos will always look clear and symmetrical. The embryos at 24 hpf were placed in the 1X E3 medium with 1 mg/mL pronase for five minutes at room temperature, then gently agitated with a plastic pipette until the embryos were freed from the disrupted chorions. The dechorionated embryos were washed, placed in fresh 1X E3 medium and incubated at $26 \pm 1^\circ\text{C}$. Kelulut honey (KH) was provided by Marbawi Food Trading and Processing, Kuala Kangsar, Perak, Malaysia. Both of the groups (3 hpf and 24 hpf) were treated with various concentrations of KH (1 to 20 mg/mL) with the final test volume of 2 mL in 24-well plates (OECD, 2013). Twenty embryos ($n=20$) were used for each concentration in triplicate. Total number of 600 embryos were used in the study. The 1X E3 medium was used as negative control and 3, 4-dichloroaniline of 4 mg/L was used as positive control (OECD, 2013). The plates were incubated at $26 \pm 1^\circ\text{C}$. Assessments of toxicological endpoints were performed using a Labomed stereomicroscope (Labomed, USA) at 24, 48, 72 and 96 h of KH exposure.

Assessment of toxicological endpoints

Lethality of zebrafish embryo

Lethality was recorded based on the number of zebrafish embryos

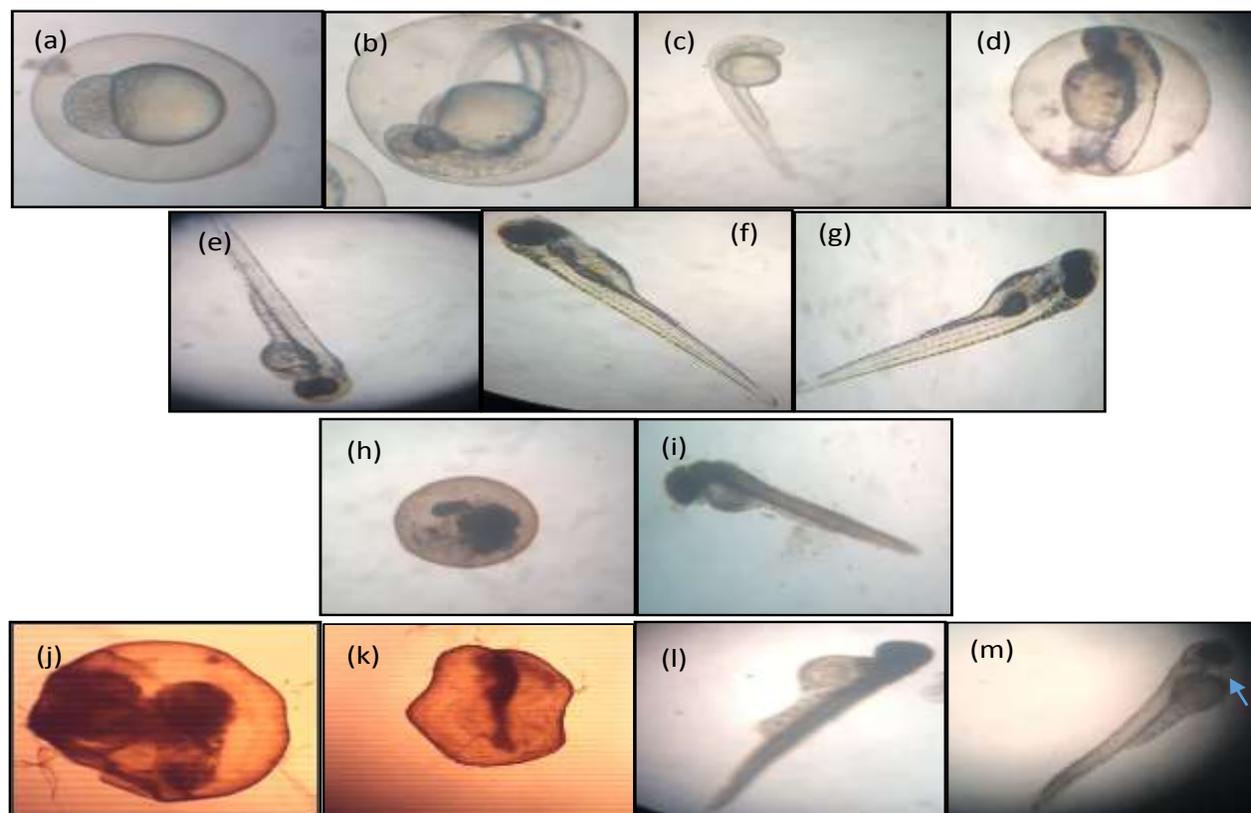


Figure 1. Normal and abnormal development of zebrafish embryos. (a) At 3 hpf from negative control group (normal development, with chorion); (b) and (c) = at 24 hpf from negative control group (normal development, with and without chorion, respectively); (d) and (e) = at 48 hpf from negative control group (normal development, with and without chorion, respectively); (f) = at 72 hpf from negative control group (normal development, without chorion); (g) = at 96 hpf from negative control group (normal development, without chorion); (h) and (i) = at 48 hpf from positive control group (coagulated, with and without chorion, respectively); (j) and (k) = at 3 hpf, treated with 10 mg/mL of KH for 24 and 48 h, respectively (coagulated, with chorion); (l) = at 24 hpf, treated with 14 mg/mL of KH for 24 h (coagulated, without chorion); (m) = at 48 hpf, treated with 17 mg/mL of KH for 24 h (without cardiac pulse (heart beats were undetected) and absence of chorion (blue arrow)).

that is coagulated and lacks somite formation with non-detachment of the tail and without cardiac pulse (OECD, 2013). A graph of percentage of mortality versus KH concentration was plotted. The LC_{50} value (the concentration of KH that caused 50% zebrafish embryos lethality in comparison with the negative control) was determined. From the LC_{50} value obtained, the safe concentration of KH or maximum allowable concentration (MAC) was calculated using the formula

$$MAC = \text{Application factor (0.5)} \times LC_{50} \text{ (at the exposure time)} \text{ (Boyd, 2005).}$$

Abnormalities of zebrafish embryo

Abnormalities that include body curvature, changes of eyes (microphthalmia), head (microcephaly), oedema and hemorrhagic occurrence of the KH-treated zebrafish embryos were recorded every 24 h. The cardiac rate of the viable zebrafish embryos at 48 hpf was measured by counting the beats in 15 s and converted to beats per minute (Hoage et al., 2012). Observations were performed using a Labomed, USA stereomicroscope.

Statistical analysis

Data were analyzed using one-way ANOVA followed by Dunnett's test using Social Science (SPSS) version 21 and presented as mean \pm SEM, where $p < 0.05$ was considered as significant.

RESULTS

Lethality of zebrafish embryo

The analyzed parameters for lethality were coagulation, lack of somite formation with non-detachment of the tail and no cardiac pulse; and as results we observed that the development was compromised as depicted in Figure 1.

The percentage of coagulated 3 hpf zebrafish embryos treated with KH for 24 and 48 h is presented in Figure 2(a). Based on the data, 12 to 87% coagulated zebrafish

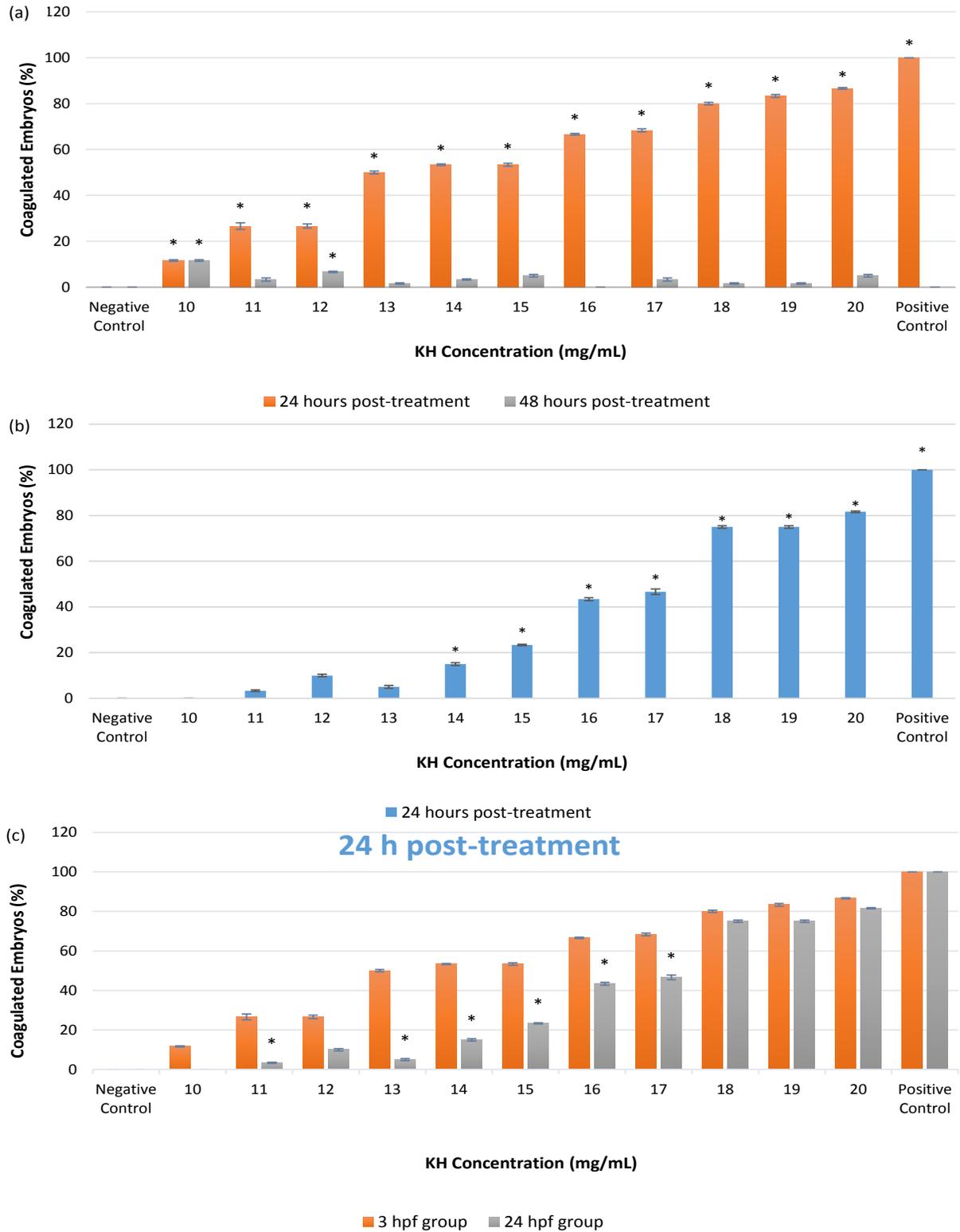
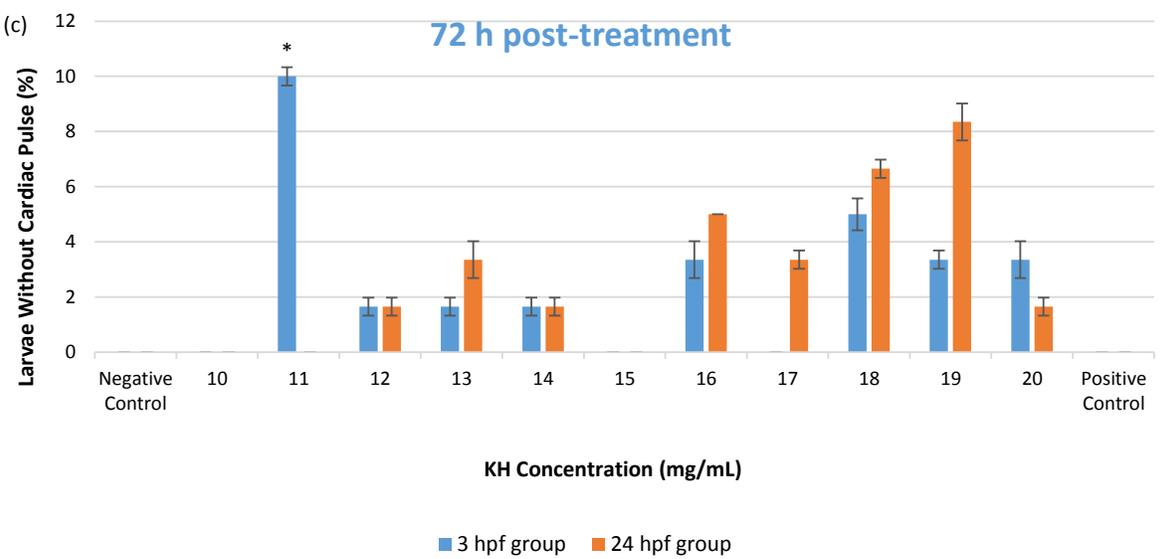
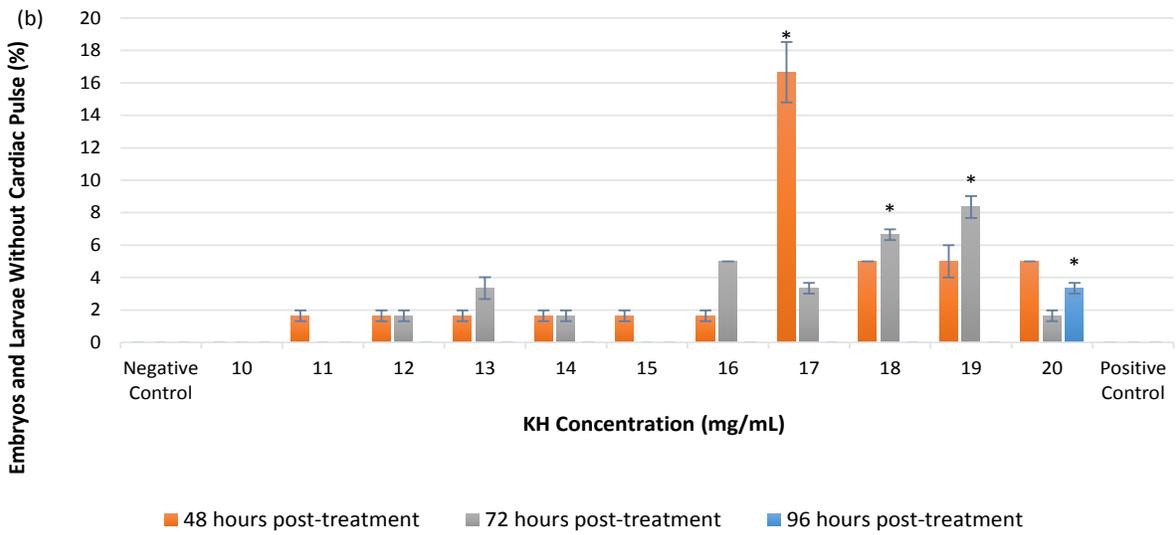
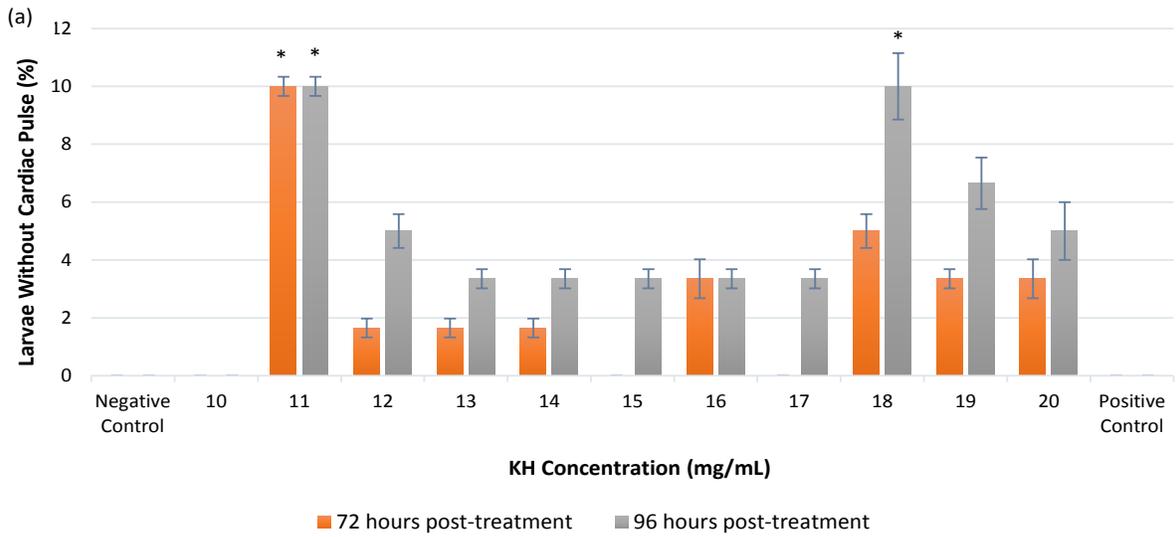


Figure 2. (a) Coagulated zebrafish embryos for 3 hpf group treated with KH for 24 and 48 h. *indicates the significant difference ($p < 0.05$) compared to the negative control. (b) Coagulated zebrafish embryos for 24 hpf group treated with KH for 24 h. *indicates the significant difference ($p < 0.05$) compared to the negative control. (c) Coagulated zebrafish embryos for 3 hpf and 24 hpf groups treated with KH for 24 h. *indicates the significant difference ($p < 0.05$) compared to the 3 hpf group.



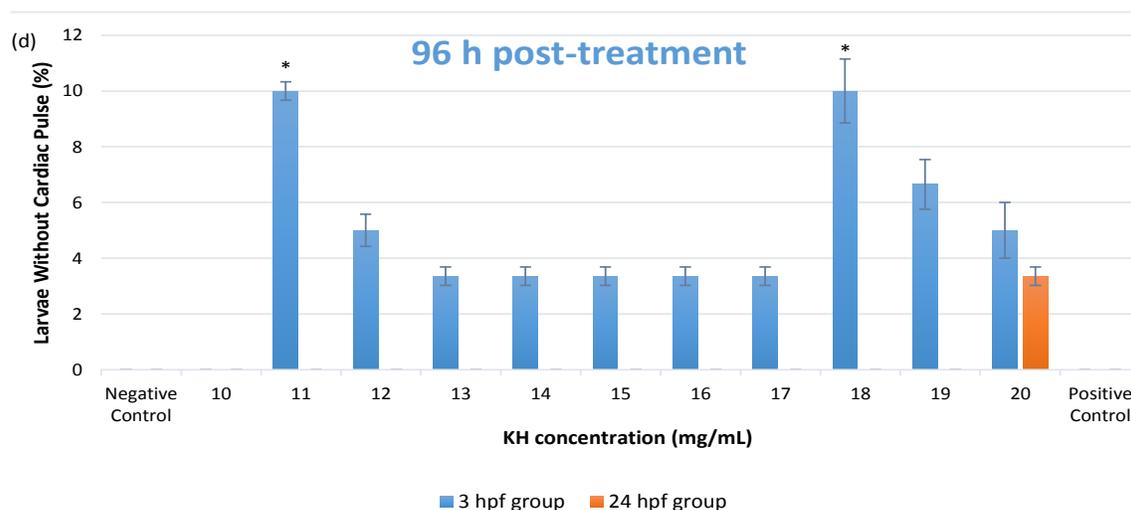


Figure 3. (a) Zebrafish larvae without cardiac pulse for 3 hpf group treated with KH for 72 and 96 h. *indicates the significant difference ($p < 0.05$) compared to the negative control. (b) Zebrafish embryos and larvae without cardiac pulse for 24 hpf group treated with KH for 48, 72 and 96 h. *indicates the significant difference ($p < 0.05$) compared to the negative control. (c) Zebrafish larvae for 3 hpf and 24 hpf groups treated with KH for 72 h. *indicates the significant difference ($p < 0.05$) compared to the 24 hpf group. (d) Zebrafish larvae for 3 hpf and 24 hpf groups treated with KH for 96 h. *indicates the significant difference ($p < 0.05$) compared to the 24 hpf group.

embryos were noted at the treatment of 10 to 20 mg/mL of KH at 24 h. The percentages reduced to 12 and 7%, respectively, after treatment with KH for 48 h ($p < 0.05$). The percentage of coagulated 24 hpf zebrafish embryos treated with KH for 24 h is shown in Figure 2(b). The coagulation was observed only at 24 h KH post-treatment, which was because the rest of the embryos survived until the end of experiment. Based on the data, 15 to 82% coagulated zebrafish embryos were noted at the treatment of 14 to 20 mg/mL of KH. The percentage of coagulated 3 hpf and 24 hpf zebrafish embryos treated with KH for 24 h is depicted in Figure 2(c). Based on the data, the percentage of coagulated embryos was less in 24 hpf compared to 3 hpf group at the treatment of 11–17 mg/mL of KH at 24 h ($p < 0.05$).

Figure 3(a) shows the percentage of zebrafish larvae without cardiac pulse after treatment with KH for 72 and 96 h. In the 3 hpf group, the embryos without cardiac pulse were not detected at both 24 and 48 h KH post-treatment. The ones without cardiac pulse were noted at 72 h (10% at 11 mg/mL of KH) and 96 h (10% at both 11 and 18 mg/mL of KH). Figure 3(b) depicts the incidence of 24 hpf embryos and larvae without cardiac pulse after treatment with KH for 48 h (17% at 17 mg/mL of KH), 72 h (7 and 8% at 18 and 19 mg/mL of KH, respectively) and 96 h (3% at 20 mg/mL of KH). In the 3 hpf group, 10% of zebrafish larvae without cardiac pulse were noted at 11 mg/mL of KH after treatment for 72 h (Figure 3(c)). In the 3 hpf group, 10% of zebrafish larvae without cardiac pulse were noted at 11 and 18 mg/mL of KH after

treatment for 96 h (Figure 3(d)).

The LC_{50} and MAC value of KH for 3 hpf zebrafish embryos were 12.52 and 0.63 mg/mL, respectively. The LC_{50} and MAC value of KH for 24 hpf zebrafish embryos were 16.36 and 0.82 mg/mL, respectively.

Abnormalities of zebrafish embryo

Figure 4 depicts the 48 hpf zebrafish embryos with normal morphology/development and abnormality (body curvature). Figure 5(a) shows the percentage of 3 hpf zebrafish larvae with body curvature after treatment with KH for 72 and 96 h. The percentages at 11–19 mg/mL of KH for both treatment hours were 17, 22, 30, 38, 35, 22, 27 and 5%. Figure 5(b) shows the percentage of 24 hpf zebrafish embryos and larvae with body curvature after treatment with KH for 24, 48, 72 and 96 h. The percentages of body curvature were 7% at 24 h (at 18 and 19 mg/mL of KH) and 23, 35, 38, 30, 12 and 10% at 48, 72 and 96 h (at 14–19 mg/mL of KH). Figure 5(c) shows that at 72 and 96 h, 17, 22, 30 and 38%; and 22% of 3 hpf larvae experienced body curvature at 11–14 and 16 mg/mL of KH post-treatment, respectively.

Cardiac rate of zebrafish embryo

Cardiac rate of zebrafish embryos after treatment with KH for 48 h is depicted in Figure 6. The average cardiac rate

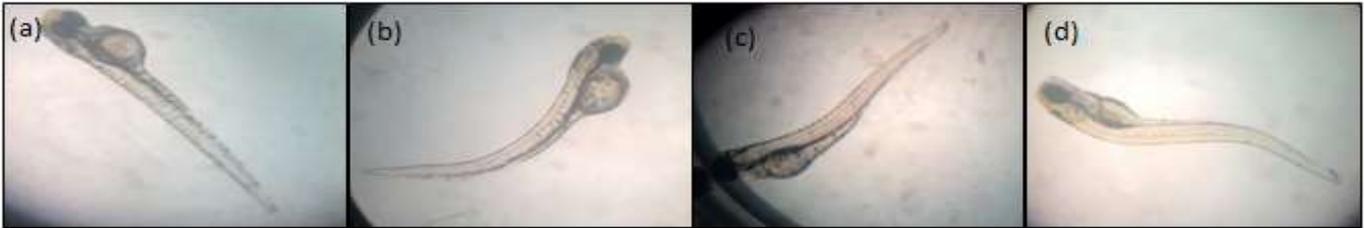


Figure 4. Zebrafish embryos with normal morphology (a) and abnormality (body curvature) ((b) – (d)) at 48, 72 and 96 hpf, respectively.

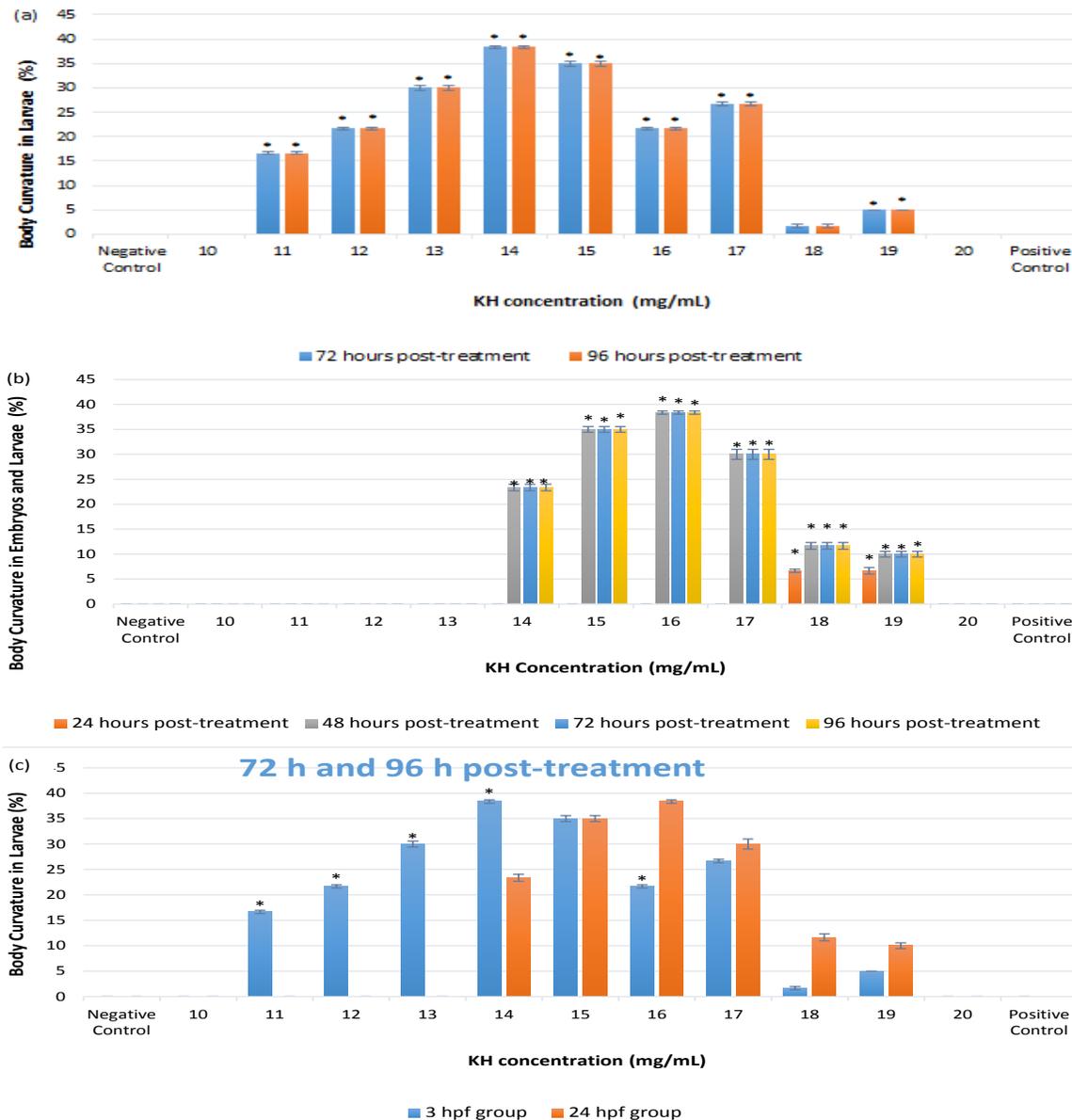


Figure 5. (a) Zebrafish larvae with body curvature for 3 hpf group treated with KH for 72 and 96 h. *indicates the significant difference ($p < 0.05$) compared to the negative control. (b) Zebrafish embryos and larvae with body curvature for 24 hpf group treated with KH for 24, 48, 72 and 96 h. *indicates the significant difference ($p < 0.05$) compared to the negative control. (c) Zebrafish larvae with body curvature for 3 hpf and 24 hpf groups treated with KH for 72 and 96 h. *indicates the significant difference ($p < 0.05$) compared to the 24 hpf group.

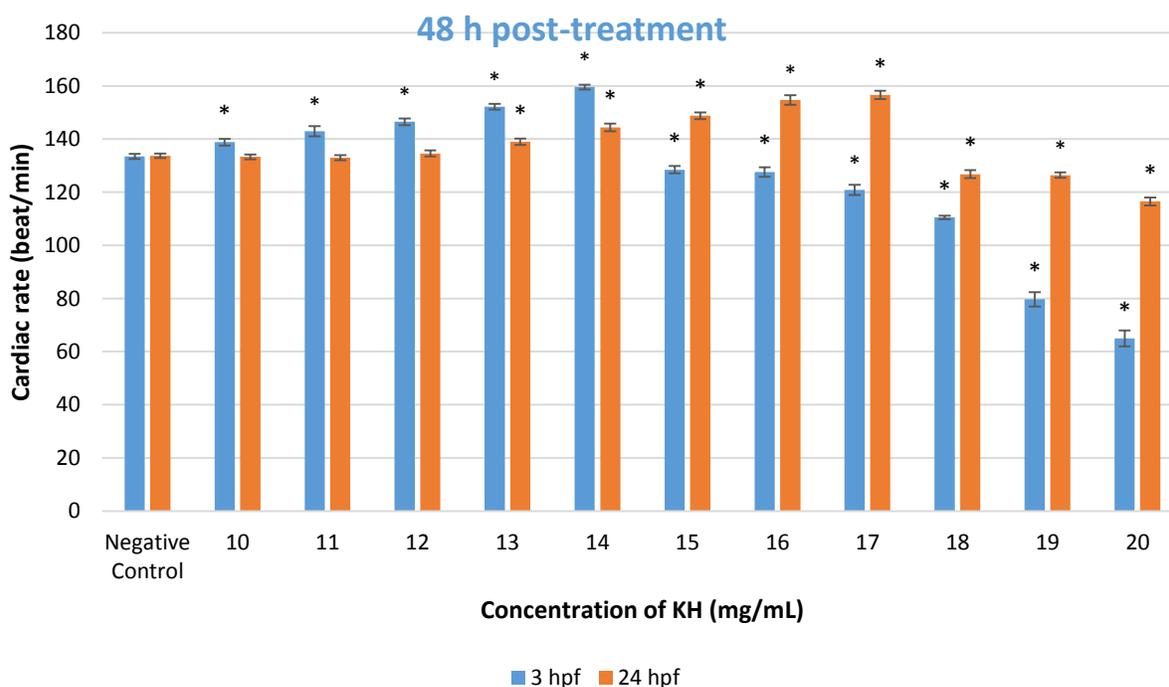


Figure 6. Cardiac rate of zebrafish embryos for 3 hpf and 24 hpf groups treated with KH for 48 h. *indicates the significant difference ($p < 0.05$) compared to the negative control.

of negative control group (untreated) for both 3 hpf and 24 hpf groups was 134 beats per minute. The cardiac rate increased with the increase in the dose of KH. There was a significant difference ($p < 0.05$) in the cardiac rate between the treatment groups when compared to the negative control starting from 10 mg/mL of KH for 3 hpf embryos group. The cardiac rates were 139, 143, 147, 152 and 160 beats per minute at 10–14 mg/mL of KH, respectively. The cardiac rate decreased to 129, 128, 120, 111, 80 and 65 beats per minute, respectively, at higher concentrations of KH (15–20 mg/mL).

There was a significant difference ($p < 0.05$) in the cardiac rate between the treatment groups when compared to the negative control starting from 13 mg/mL of KH for 24 hpf embryos group. The cardiac rates were 139, 145, 149, 155 and 157 beats per minute at 13–17 mg/mL of KH, respectively. The cardiac rate decreased to 127 and 117 beats per minute, respectively, at higher concentrations of KH (18–20 mg/mL).

DISCUSSION

This study took advantage of the uniqueness and convenience of zebrafish as a means to explore the developmental toxicity of KH. It has been reported that zebrafish is an excellent model for that purpose since it

mimics the complexity of interactions in the human body due to its high degree of homology to human genome and biological systems. Its embryos can be used to monitor phenotypic and genotypic abnormalities upon exposure to toxic agents (Lee et al., 2017). Toxic-response similarities between zebrafish and mammals have been renowned for small molecules that cause endocrine disruption, reproductive toxicity, behavioral defects, teratogenesis, carcinogenesis, cardiotoxicity, ototoxicity, liver toxicity and others (Zon and Peterson, 2005). Organs and structures of the fish react specifically to certain types of toxicants. In cardiotoxicity study, for instance, Milan et al. (2003) have developed an automated, high-throughput assay for bradycardia in zebrafish embryos to correlate with QT prolongation in humans. Drug-induced prolongation of the cardiac QT interval can lead to fatal arrhythmia (irregular heartbeat), and QT prolongation has become a leading cause of failure during drug development (Zon and Peterson, 2005). They have tested 100 compounds in the assay and showed that 22 from 23 drugs known to cause prolongation in humans caused bradycardia in zebrafish (Milan et al., 2003).

The zebrafish development is divided into four major stages, which are embryo, larvae, juvenile and adult (Parichy et al., 2009). The zebrafish eggs are considered as embryos until they hatch to become larvae and attain

protruding mouth at 72 hpf. At the larval stage (72 hpf-15 dpf (days post fertilization)), morphogenesis takes place. The larvae will develop into juvenile from 4 weeks until 12 weeks post fertilization depending on strain and rearing conditions. At this stage, most adult characteristics (loss of larval fin fold and acquired scales) have been acquired in the absence of sexual maturity. Adult zebrafish has developed the secondary sexual characteristics (urogenital papillae, body colour, body shape and anal fin) and is capable of producing viable eggs. It is estimated that 99% of the embryonic-essential zebrafish genes are identical in human embryonic development (Amsterdam et al., 2004; Howe et al., 2013). In this study, we selected zebrafish embryos at 3 hpf to represent early embryonic development (consists of relatively few cells) and at 24 hpf to represent organ development (organogenesis) that mimic the human embryonic growth (Ali, 2007).

There were two types of lethality identified in the zebrafish embryos in the study, which are coagulation and absence of cardiac pulse. Coagulation occurs when a toxicant significantly disrupts the embryo and finally leads to death (Ali, 2007). The frequency of coagulation increased with the increase in KH concentration. Less number of coagulated embryos was observed in 24 hpf group compared to 3 hpf group. The survival rate of the embryos increased over the treatment time. The coagulation was less in 48 h post-treatment compared to 24 h post-treatment. In general, any agent (KH in this case) has its own tolerable uptake limit. During organogenesis (24 hpf), the increasing number of cells makes the embryo particularly less susceptible to toxicants as well as permits survival of the embryo even after significant damage (Ali, 2007). Embryos without cardiac pulse were noted in both 3 hpf and 24 hpf groups but only at certain KH concentrations. Therefore, it is believed that cardiotoxicity is not a major effect induced by KH. Cardiotoxicity may arrest development of embryos at early stage that leads to the absence of cardiac pulse (Romagosa et al., 2016).

The 24 hpf embryos had better survival upon exposure to KH compared to the ones of 3 hpf based on the LC_{50} value. The difference in sensitivity and susceptibility between early developmental stage and later stage embryos may be due to several factors such as surface area/volume ratio, greater uptake of toxicant from the environment and immature immune systems (Mohamed, 2013). The maximum allowable concentration (MAC) of KH, which is the concentration that has no negative effect on fish in the experimental period (Daryoush and Ismail, 2012), below 0.63 and 0.82 mg/mL for 3 hpf and 24 hpf, respectively, indicating that the honey is not inducing harmful effect on the zebrafish embryos.

In this study, zebrafish embryos with body curvature have been identified in both 3 hpf and 24 hpf groups after treatment with KH. According to Nellore and Nandita

(2015), curved spine (body curvature) is a type of neurodegenerative phenotype that is associated with central nervous system (CNS) development. Floor plate is a specialized stripe of large cuboidal structure in the ventral neural tube of CNS (Brand et al., 1996). Reduction or absence of this structure might cause defects in the midline of the underlying CNS that lead to body curvature (Brand et al., 1996). KH at 11-19 mg/mL for 3 hpf group and KH at 14-19 mg/mL for 24 hpf group appeared to interrupt the normal development of the floor plate of CNS. The incidence of body curvature in 24 hpf group was identified at higher concentrations of KH compared to the 3 hpf group. It indicates that early developmental stage embryo has higher susceptibility to the complex composition of water mixture (KH in this case) (Gellert and Heinrichsdorff, 2001). As only a single morphological change (body curvature) was identified in the study, KH cannot be classified as a toxicant. It is because body curvature is also a common phenomenon occurring during development of zebrafish. In a study on developmental neurotoxicity of pyrethroid insecticides in zebrafish embryos, DeMicco et al. (2010) found that the curvature did not appear to be the result of morphological effect on either the spinal cord or musculature, as the experiment conducted did not reveal any clear changes.

The normal embryonic cardiac rate in zebrafish is much closer to that of human, at 120–180 beats per minute (De Luca et al., 2014). In this study, higher doses of KH (≥ 18 mg/mL) caused a decrease in the zebrafish embryos cardiac rate to abnormal count (≤ 120 beats per minute). A decrease in cardiac rate to an abnormal level is primarily due to a steady increase in the incidence of complete absence of ventricular contraction (Antkiewicz et al., 2005). On the other hand, the increase in cardiac rate in the embryos is speculated to be due to the presence of cardiac glycoside compounds in KH (Bhuvaneswari et al., 2014). Cardiac glycoside plays an important role in inhibition of $Na^+/K^+-ATPase$, thus raises the level of sodium ions in cardiac myocytes, which leads to an increase in the level of calcium ions and an increase in cardiac contractile force (Prassas and Diamandis, 2008).

Conclusion

KH exhibited some toxic effects towards the zebrafish embryos, which were depending on its concentration and the embryonic developmental stage. The higher the concentration of KH, the higher the incidence of lethality and abnormality. The zebrafish embryos at early developmental stage (3 hpf) were more sensitive and susceptible to KH compared to the later one (24 hpf) as evidenced by lower survival rate. This study provides beneficial information about developmental toxicity for evaluation of other natural products.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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