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ORIGINAL PAPER

INFLUENCE OF FRESH GARLIC (*ALLIUM SATIVUM* L.) JUICE ON ZEBRAFISH (*DANIO RERIO*) EMBRYOS DEVELOPMENTAL EFFECTS

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Abstract

A higher sensitivity and the cytotoxic effects of garlic have been previously reported on culture cells. No studies, however, have investigated the developmental toxicity of fresh garlic in zebrafish model. In this study freshly made garlic juice, which chemically is a combination of sulphur containing compounds, was evaluated to investigate the developmental toxicity during a 5-day assay using zebrafish embryos, starting from 2 h post fertilization. The results showed that toxicity endpoints such as the hatching rate, survival rate, malformation rate and growth rate had a significant dose-response relationship with increasing concentrations of garlic juice. The average hatching rate and time to hatch of zebrafish exposed to lower concentrations (0.001% and less) were significantly increased around 48-60 h of treatment ($P < 0.05$), strongly emphasizing the acceleration of hatchability caused by garlic juice. Mortality was also increased with exposure to garlic, exhibiting the maximal toxicity after 12 h of exposure to the concentration of 0.08%. A significant reduction in the heart rate by 35.5% was recorded due to the higher concentrations of juice (above 0.01%) starting form 36-hour exposure ($P < 0.05$). Growth retardation, pericardial oedema with heart malformations, deformity of yolk, lack of pigmentation and body malformation have been noticed at the concentration of 0.03% starting from 48 h of exposure. No severe malformations in any of the experimental zebrafish were produced at concentrations less than 0.01% of garlic juice. The results indicate that developing zebrafish are sensitive to garlic juice and further studies are required to investigate the molecular basis of the observed effects that garlic constituents exert upon aquatic vertebrates.

Keywords: *Danio rerio*, developmental toxicity, fresh garlic juice.

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INTRODUCTION

A number of toxicological and clinical studies of different garlic extracts have been performed, both with garlic juice and raw garlic, with no detected severe effects upon humans. Recently performed tests with garlic juice have been made to evaluate acute and subacute toxicity, chronic toxicity, mutagenicity, general toxicity, teratogenicity, and cytotoxic properties. Also, clinical studies have been performed on more than 1,000 subjects, with no serious malformations to be reported (Amagase et al. 2001). Although garlic has been used safely for many years, some of the adverse effects associated with raw garlic consumption have been reported both in animals and in humans (Majewski 2014). Stomach disorders and diarrhoea (Nakagawa et al. 1980), growth retardation (Nakagawa et al. 1980), damage of the intestinal lining (Amagase et al. 2001, Hoshino et al. 2001, Chiang et al. 2006), decrease of serum protein and calcium ions (Miyamoto 1938, Shashikanth et al. 1986), haemolytic anaemia (Oboh 2004), asthma (Shin et al. 2013), contact dermatitis (Xu et al. 2014), inhibition of spermatogenesis (Hammami et al. 2013), swelling of various organs: the liver, spleen and adrenal glands (Nakagawa et al. 1980) and anaphylaxis (Ma, Yin 2012) have been reported in the literature so far. However, little is known regarding potential garlic developmental toxicity to aquatic vertebrates.

Over the last years, a zebrafish (*Danio rerio*) model has become a useful tool for studying toxicity (Spitsbergen, Kent 2003), owing to moderate transparency during early development and cost reduction. Also the organogenesis is largely completed by 48 h post fertilization – hpf (Kimmel et al. 1995). Gene expression in a developing zebrafish is a quick process, whereas enzyme activity is lengthy and involves complex processes, which are subjected to environmental changes (Wu et al. 2015).

Embryos and developing larvae are the most sensitive stages in the life cycle of zebrafish. Zebrafish embryos are also able to metabolize and activate pro-teratogenic substances without the addition of an exogenous metabolic activation system (Lele, Krone 1996). When comparing human and zebrafish CYP metabolism, the zebrafish might even be a better model for toxicity studies than some commonly used mammalian models (Rubinstein 2006).

As of yet, several studies investigating the developmental toxicity of garlic have reported no neurotoxic or hepatotoxic effects in rodents (Ghareeba et al. 2010). Sadeghi et al. 2013 reported protective effects of garlic juice from lead-induced neural damage in developing young rats. However, Nwachukwu, Asawalam (2014) highlighted the toxic effect of freshly prepared juice from garlic against some insects. Yoshida et al. (1984) reported severe damages of cultured cells due to garlic juice exposure. To our knowledge no studies have investigated the toxicity of FGJ in developing zebrafish.

MATERIAL AND METHODS

Preparation of fresh garlic juice

The garlic bulbs used for this study were purchased from a local main market between July and August 2015 (Poland). The process of yielding garlic juice was done according to AQEL et al. (1991) and ASHRAF et al. (2004). Briefly, the garlic cloves in good physical shape were peeled, weighed (276 ± 15 g) and finely ground in a mortar with a pestle. The process yielded 58.5 ± 8 mL of juice with an average solid content of 0.980 ± 0.14 g mL^{-1}. Each day of the experiment fresh garlic juice (FGJ) was produced in the same manner. Dilutions were prepared in an E3 medium on the day of the experiment. The dilution was performed by addition of garlic juice to E3 medium to obtain 0.00001-0.1% concentrations as vol/vol. In another set of experiments, the increasing concentration of garlic juice 0.02%, 0.03%, 0.04%, and 0.05% (vol/vol) in E3 medium were prepared.

Animal care and egg production

Zebrafish were handled according to standard animal care protocols and in accordance with the Animal Care guidelines. Healthy mature wild type Tubingen strain older than 6 months were used for egg production. The breeding stock were maintained in a recirculating housing system at temperature 27.5°C. A 14/10-hour light/dark cycle lighting was controlled. Adult fish were fed twice daily with dry food and with Artemia nauplii once a day. The concentrations of nitrate and nitrite were checked once per week, but were consistently < 100 mg L^{-1} and 1.0 mg L^{-1}, respectively. Mating and spawning took place within 30 min after turning on the lights in the morning. About 30 min after the onset of light, egg trays were removed and eggs were collected. Under the culture conditions described above, fertilized eggs underwent the first cleavage after approximately 15 min. Based on their transparency, the 2 hpf embryos (from 8- to 32-cell stage eggs) were clearly identified as fertilized and used for the study.

Embryo exposure

The experiment was executed for five consecutive days of post fertilization (dpf), as zebrafish larvae used in the study (up to 6 dpf) are exempt from ethical legislations. Eggs were first rinsed twice in glass Petri dishes with E3 medium (5 mM NaCl; 0.17 mM KCl; 0.33 mM $CaCl_2$ x $2H_2O$; 0.33 mM $MgSO_4$). Further, fertilized eggs were separated from the non-fertilized ones under a digital video stereomicroscope (Zeiss Discovery.V8, Germany) into a plastic Petri dish containing an E3 medium. At no more than 2.5 hpf, the embryos were exposed individually to the increasing concentrations of FGJ, in 24-well plates containing a final volume of 2 mL of dilutions per one well at temperature of 28.5°C with a 14/10-hour light/dark cycle in a precision incubator. One embryo was placed in one well to prevent any toxins

being released from dead organisms and influencing other embryos. Garlic dilutions were changed every 24-hour interval.

Evaluation of teratogenic effects

Embryos and larvae were scored for developmental abnormalities at 24-hour intervals using a Zeiss microscope. This was performed to obtain the information about the specific time point when the defined endpoints were observed. The 12 hpf time point served as a control step. This was to separate unfertilized eggs, which entered the test by accident. The final scoring was performed at 5 dpf on both embryos and larvae. Teratogenic effects were considered as fingerprint endpoints only when the following criteria were fulfilled: concentration–response relationship was present and the endpoint was observed in ≥ 50% of all zebrafish embryos showing teratogenic effects in subjected test groups.

Hatching (starting from 48 hpf)

In addition to the assessment of morphological changes, larval hatch rate and median hatching time (HT_{50}) were measured and compared to the control embryos. The hatching rate was calculated as the percentage of hatched egg reported to living eggs for a given sampling time. HT_{50} represents the time necessary for half of the eggs to hatch, and was calculated from log-logistic regression using MORSE models (REGTOX). The hatching rate on every tested concentration was monitored during the exposure period of 36-120 hours. Secondly, the HT_{50} for each tested concentration was calculated individually and compared with the control.

Heart rate measurement and body development

After 36 h of garlic exposure, the surviving embryos and larvae were transferred to glass slides, ensuring the presence of at least 100 µL of the liquid from the chambers. The counting was done using a stop clock while visually observing the embryos under 100x (10x objective lens and 10x eyepiece) magnification of a compound upright microscope. Each heart rate (f_H) measurement was done by counting the contractions of either of the two chambers for at least 30 seconds. Counting was performed at intervals of every 1 min, or longer depending on the treatment and time taken for complete heart cessation. For each measurement at least 7 embryos were used (Rana et al. 2010).

Statistical analysis

Values are expressed as means ± SEM for all experiments; n refers to the number of embryos. For comparison of the mean values, the t test for paired and unpaired data was used, as appropriate. When two or more groups were compared with the same control, one-way analysis of variance (ANOVA) followed by the Dunnett's test was used. The threshold level of significance was set at P values < 0.05 using SPSS software.

RESULTS

Developmental toxicity

The results indicate that FGJ did not induce any of the severe morphological abnormalities at a concentration below 0.001%. It was observed that the concentration of 0.02% resulted in moderate body malformations, causing the lack of swim bladder inflation and notochord curvature distortion with kyphosis (Figure 1). In turn, concentration value 0.03% of FGJ resulted in

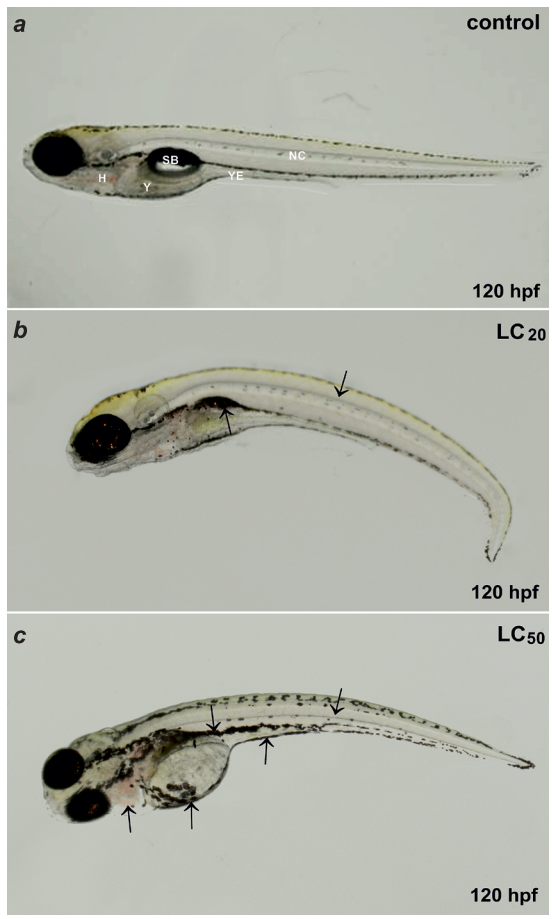

Fig. 1. Representative larva in the control (in E3 medium) – *a*; and larvae exposed to garlic at the concentrations of 0.02% (LC_{20}) – *b*, and 0.03% (LC_{50}) – *c*, under 120 h post fertilization (120 hpf). Zebrafish embryos were introduced to the dilutions from 2 h post fertilization and the experiment was carried for 5 days. The concentration of 0.02% resulted in moderate body malformations (arrows), affecting the swim bladder inflation and notochord curvature in comparison to the control (*b*). The concentration of 0.03% resulted in more severe body malformations (arrows), among which the most repeatable were the lack of swim bladder inflation, curved notochord, lack of yolk resorption, yolk extension deformation and pericardial oedema (*c*). SB – swim bladder, NC – notochord, Y – yolk, YE – yolk extension, H – heart

more severe malformations, among which the most frequent were pericardial oedema, lack of swim bladder inflation, curved notochord, lack of yolk resorption, yolk extension deformation and growth retardation (Table 1).

Table 1

The observed effects in developing zebrafish depending on the exposure time (12-120 h of treatment). Embryos were introduced to the concentration of 0.03% starting from 2 h post fertilization

Category	Physiological effects	Exposure tenure (h)					
		12	24	48	72	96	120
Lethal effect	coagulated egg	+	+	+	+	+	–
	heartbeat	–	–	+	+	+	+
Teratogenic effect	pericardial oedema	–	–	+	+	+	+
	depigmentation of body	–	–	–	+	+	+
	malformation of body	–	–	+	+	+	+
	malformation of tail	–	–	–	+	+	+
	malformation of tail tip	–	–	–	+	+	+
	kyphosis	–	–	–	–	+	+
	deformity of yolk	–	+	+	+	+	+
	growth retardation	–	–	+	+	+	+

"–" not observed, and "+" observed

Mortality

An increase in the number of the mortalities with an increase in the concentration of FGJ was observed. There was less than 10% mortality among the hatched larvae at low concentrations (0.02%) and in the control group. As the concentration of FGJ increased, fish mortality also increased, which indicates a direct proportional relationship between mortality and concentration of garlic juice. The lethal concentration (LC_{50}) was 0.03%, where the lower limit was 0.028% and the upper limit was 0.034%. Garlic juice exhibited the maximal toxicity after 12 h of exposure to the concentration of 0.08%.

Hatching (2 hpf zebrafish embryos)

Similar to the developmental toxicity profiles, concentration-response curves were generated in order to compare the individual concentrations on the hatching profile (see Figure 2*a*, *b*). Under the standard conditions, 85-95% of all larvae hatched, starting from 48 to 72 h of treatment. In the experiment, the per cent of hatched embryos treated with FGJ at the concentration range between 0.02% and 0.05% was significantly lower than that of the control ($P < 0.001$, $n = 72$) – Figure 2*a*. Also, the median hatching time (HT_{50}) for each tested concentration significantly differed from the control. At

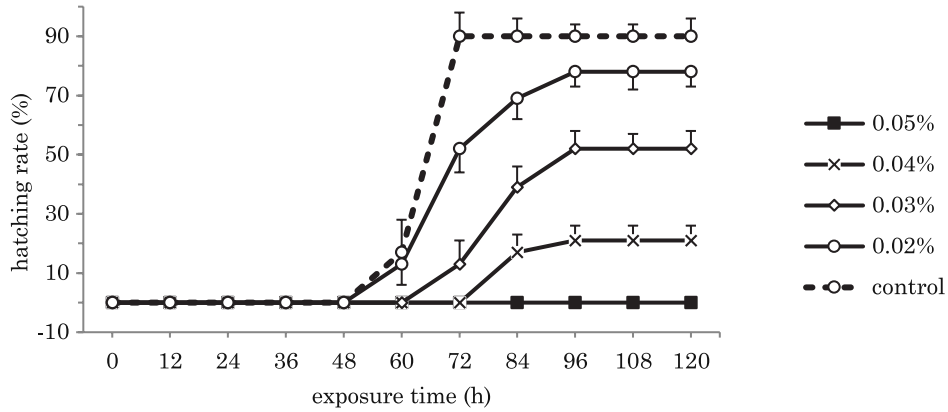

Fig. 2*a*. Inhibitory effects of garlic on the hatching profile of zebrafish embryos. Embryos were introduced to the increasing dilutions (0.02-0.05%) from 2 h post fertilization during 120-hour exposure. Means ± SEM of n = 72 embryos in each group. In a few cases the error bars were covered by the data marker

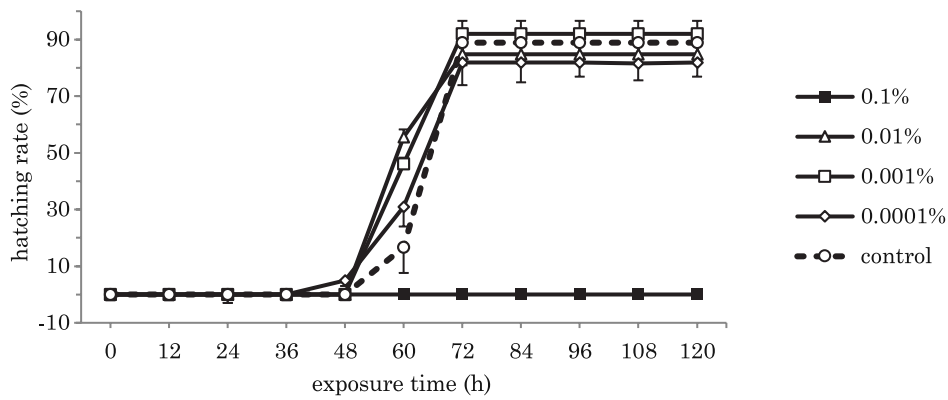

Fig. 2*b*. The effects of garlic on the hatching profile of zebrafish embryos. Embryos were introduced to the increasing dilutions (0.0001-0.1%) from 2 h post fertilization during 120-hour exposure. Means ± SEM of n = 72 embryos in each group. In a few cases the error bars were covered by the data marker

the concentration 0.03%, the median hatching time was delayed (HT_{50} = 78 ± 3 hours) in comparison to the control (HT_{50} = 66 ± 2 h, $P < 0.001$, n = 72).

In contrast, a concentration range between 0.01% and 0.001% resulted in the accelerated hatching during 48 h of treatment with a maximum acceleration at 0.01% (HT_{50} = 60 ± 2 h, $P < 0.01$) – Figure 3. Also, a significant difference in the hatching rate was observed starting from 60 hours of exposure to garlic. Under the observed concentrations of FGJ (0.01% and 0.001%) more than 50% of developing larvae hatched ($P < 0.01$). At the concentration of 0.0001% and below, hatchability was similar to the control.

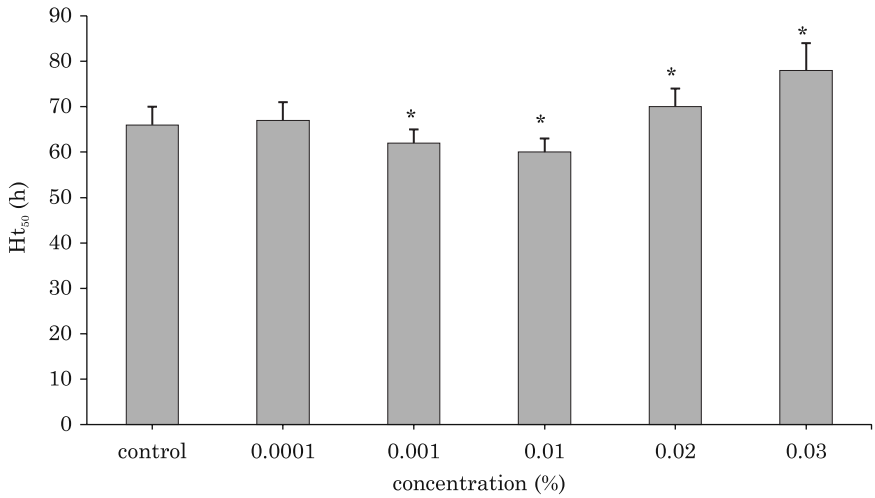

Fig. 3. Median hatching time (HT_{50}) in control zebrafish larvae and in response to garlic under increasing concentrations of FGJ. Means ± SEM of n = 72 embryos in each group. *$P < 0.05$

Basal heart rate (f_H) and heart development

Development of the heart and its function was assessed by counting the heart rate (f_H) in response to exposure to varying concentration of FGJ (0.00001-0.04%) – Figure 4.

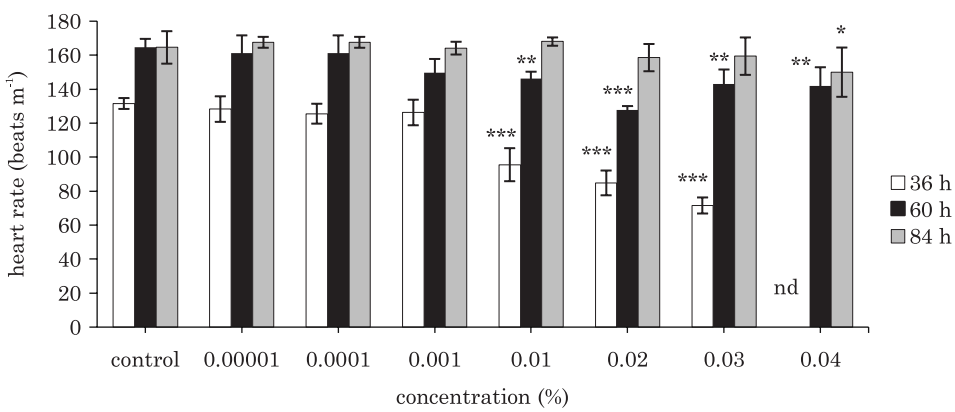

Fig. 4. Heart rate measured in developing zebrafish embryos following exposure to increasing concentrations of FGJ during 36, 60 and 84 h of exposure. Embryos were introduced to the dilutions from 2 h post fertilization. Means ± SEM of n = 7-10 embryos in each group. Different symbols indicate significant differences compared to the control at each time point at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, nd – underdeveloped heart

36-hour exposure

Zebrafish embryos treated with FGJ at the concentration of 0.01% reve-

aled a significant decrease in the f_H from 131.6 ± 3.2 to 95.6 ± 9.7 beats min^{-1}. At the higher concentrations of FGJ, the heart rate was decreased to 84.9 ± 7.3 beats min^{-1} (for concentration of 0.02%) and 71.6 ± 4.7 beats min^{-1} (0.03% FGJ). In addition, mild defects in the heart structure were observed in few cases. Exposure to the concentration of 0.04% resulted in a severe delay in heart development.

60-hour exposure

In our study, concentrations of FGJ above 0.01% caused a decrease in f_H during 60 h exposure. FGJ exposure to 0.02% caused a decrease in f_H in treated fish (from 164.6 ± 5.0 to 127.7 ± 2.4 beats min^{-1}) and 0.04% (to 141.8 ± 11.0 beats min^{-1}).

Pericardial oedema and heart deformation were also noticed in developing zebrafish treated with FGJ (0.03%) – Table 1.

84-hour exposure

A significant decrease in the heart rate from 164.0 ± 9.5 to 150 ± 4.4 beats min^{-1} was seen in the group subjected to FGJ at the concentration of 0.04%.

DISCUSSION

The present findings suggest that freshly prepared garlic juice did not produce severe malformations in any of the experimental zebrafish (*Danio rerio*) embryos or larvae at low concentrations (0.0001-0.01%). However, as the concentrations were increased, the growth rates decreased. More concentrated extracts (0.03% and more) demonstrated a greater inhibitory effect and pericardial oedema with heart malformations, deformity of yolk, lack of pigmentation and body curvature with kyphosis have been noticed starting from 48 h of exposure. Similar findings on cultured cells, with growth inhibition and morphological changes due to garlic juice, were observed by Yoshida et al. (1984).

In our study, delayed hatchability was connected with retardation in the development of embryos at higher concentrations (0.02-0.05%) of garlic juice. The hatching rate in zebrafish embryos was concentration dependent and decreased significantly, as the mortality was increased. The effects observed might be due to changes in tetraspanin CD63 gene coding (Trikić et al. 2011), which is responsible for the secretion of proteolytic enzyme that softens the chorion membrane, or interactions with many different proteins. Also, the lack of pigmentation might be related with depletion of AP-3 dependent transport of CD63 (Pols, Klumperman 2009).

On the other hand, FGJ at a concentration below 0.01% stimulated both

the hatching rate and time to hatch between 48 and 60 h of exposure. The increased hatching at a lower concentrations of FGJ (0.01% and 0.001%) could have several possible explanations. This increase can be attributed to the weakening of the chorionic membrane, increased embryo motility or inducing the activity of chorionase enzyme. Garlic constituents *in vivo* may also up-regulate the detoxifying enzymes, protecting from reactive oxygen species damage with an increase of the phase II detoxifying enzymes and increasing the cellular glutathione level (Rabinkov et al. 2000, Horev-Azaria et al. 2009). Hatching requires secretion of proteolytic enzymes from the hatching gland to soften the chorion and later larval movement to break free. If either of these processes is inhibited, hatching cannot occur. In the absence of overt developmental changes, an altered hatching would suggest a disruption in one of these processes.

Similarly to hatchability, heart development and functionality have also been shown to be concentration-dependant. As cardiac functioning may be impaired by the underdeveloped heart or pericardium, this could induce an abnormal heartbeat and circulation failure, with subsequent body growth retardation caused by deficiency of nutrients. The low concentrations (less than 0.001%) had the least effect on cardiac functioning and heart development. However, a significant reduction in the heart rate was observed over the 36 and 60-hour exposure. The results revealed that garlic juice may influence the embryonic heart development in a concentration-dependent manner (at concentrations of 0.01% and more), starting from 36 hours of exposure onwards. It should be noted that the developing zebrafish's heart might be an important potential target for FGJ toxicity.

Limitations

The authors focused on FGJ rather than on individual juice constituents. It is necessary to perform further research including behavioural effects of acute exposure in adult zebrafish of the sulphur containing compounds, such as allicin, which is possibly responsible for described mechanism of action. In addition, the enzyme activities of SOD, CAT, and GPx, as well as the mRNA expression levels of genes encoding for the antioxidants should be measured. Moreover, apoptosis in embryos and larvae should be monitored.

CONCLUSIONS

1. The abnormalities including growth retardation, pericardial oedema with heart malformations, deformity of yolk and body malformation could be induced in zebrafish embryos and larvae by garlic juice at higher concentrations.

2. The results showed a significant dose-response relationship between toxicity endpoints (hatching rate, survival rate, malformation rate and gro-

wth rate) and FGJ concentration.

The mechanisms of action of garlic constituents *in vivo* still require much research. An individual compound responsible for the described malformations is yet to be established.

Conflict of interests

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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