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ORIGINAL PAPER

FRESH GARLIC JUICE VERSUS AGED GARLIC EXTRACT. DETERMINATION OF LETHAL CONCENTRATION (LC_{20} AND LC_{50}) VALUES ON ZEBRAFISH (*DANIO RERIO*) EMBRYOS AND LARVAE*

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Abstract

Developmental abnormalities due to fresh garlic (*Allium sativum* L.) juice exposure have been previously reported on zebrafish (*Danio rerio*) embryos. Freshly made garlic juice (FGJ) containing allicin as a main ingredient and aged garlic extract (AGE) containing mainly S-allyl-L--cysteine were evaluated to investigate the lethality during a 5-day assay using zebrafish embryos, starting from 2 hours post fertilization (hpf), in a static system at an garlic concentration range of 0.00001-0.1%. In addition, lethality comparison between embryos and 3 days of post fertilization (dpf) larvae was performed. The results indicate that the mortality rate is positively correlated to the concentration of FGJ. Lethal concentration for the 24-h LC_{20} and 24-h LC_{50} in zebrafish embryos exposure was determined as 0.034% and 0.021%. The corresponding values for larval forms were significantly higher, 0.081% and 0.073% ($P < 0.01$), suggesting a greater sensitivity of developing embryos to FGJ treatment, as well as no protection from an embryo's chorion. Below the FGJ concentrations of 0.02% (for embryos) and 0.06% (for larvae), no increase in mortality was observed over the entire exposure time. The mortality of AGE-treated embryos did not differ significantly ($P = 0.571$) from the control regardless of exposure duration and AGE concentration. The overall results indicate that zebrafish embryos constitute a reliable model for testing the developmental toxicity of garlic, and that different types of garlic preparations have different pharmacologic properties.

Keywords: Aged garlic extract, allicin, *Danio rerio*, fresh garlic juice, hydrogen sulfide, S-allyl--L-cysteine.

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INTRODUCTION

The medicinal use of garlic (*Allium sativum* L. family *Amaryllidaceae*) is well documented for more than 5,000 years. There are several reports providing pharmacological evidence for its curative properties, such as antiatherogenic (Gonen et al. 2005), antiatherosclerotic (preventive) (Lau et al. 2013), hypoglycaemic (Liu et al. 2006, Ashraf et al. 2011), hypolipidemic (Saravanan, Ponmurugan 2013), antioxidant (Colín-González et al. 2012, Chen et al. 2014), anti-inflammatory (Ginter, Simko 2010), antimicrobial (Lemar et al. 2002, Goncagul, Ayaz 2010), antitumor (Ishikawa et al. 2006, Tanaka et al. 2006, Galeone et al. 2009) and cardio-protective effects (Sharma et al. 2012, Majewski 2014). This is due to the chemical complexity of garlic and synergistic activity of its active ingredients.

Aqueous extracts of garlic inhibit blood coagulation and platelet aggregation (Bordia et al. 1998) by reducing the formation of thromboxane. Thromboxane inhibits the phospholipase activity and lipoxygenase formation in thrombocytes (Banerjee et al. 2002). It has also been found that garlic has a free radical scavenging action and can inhibit oxidative modification of low-density lipoproteins (Xu et al. 2015). Earlier studies have shown that administration of aged garlic extract (AGE) lowers blood pressure (Ashraf et al. 2004, Ried, Fakler 2014), causing vasodilation of vascular smooth muscle cells in both *in vitro* and *in vivo* studies. The mechanism appears to be mediated through functional endothelium and through the release of endogenous gasotransmitters (Ashraf et al. 2004, Benavides et al. 2007). Earley et al. (2009) reported that endothelium -dependent cerebral artery dilatation is connected with transient receptor potential cation channel A1(TRPA1) and Ca^{2+} – activated K^{+} channels. Garlic also exerts its therapeutic effect by increasing nitric oxide (NO) production and the production of the vascular gasotransmitter hydrogen sulphide (H_2S) (Bełtowski 2015, Bhuiyan et al. 2015). Both NO and H_2S cause relaxation of vascular tissue. Moreover, H_2S functions include neuromodulation, regulation of gastrointestinal and cardiovascular system and it is a mediator of inflammation (Olas 2015).

Allicin, one of the active compounds found in fresh garlic juice (FGJ), is produced immediately when a garlic bulb is crushed, minced or chewed (Lawson, Gardner 2005, Nwachukwu, Asawalam 2014). Allicin oxidizes thiol groups in proteins and glutathione due to its sulphur composition (Gruhlke, Slusarenko 2012), and is able to shift the cellular redox potential and induce apoptosis in animal cells (Oommen et al. 2004). Moreover, allicin may also increase the permeability of cell membranes (Gruhlke et al. 2015).

Over the last years, zebrafish (*Danio rerio*) model has become a useful tool for studying toxicity (Spitsbergen, Kent 2003). Similar efforts were made to screen environmental chemicals as well as drugs for possible embryotoxic and teratogenic effects.

To our knowledge no studies have compared the lethality of FGJ and AGE in developing zebrafish.

MATERIAL AND METHODS

Preparation of fresh garlic juice and aged garlic extract

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 performed according to Majewski et al. (2017). Briefly, 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.

AGE was obtained as follows: garlic cloves were sliced and soaked in a water/ethanol mixture (30% ethanol) and naturally extracted/aged for 15 month at room temperature under light protection.

The dilution was performed by addition of garlic extract/juice to E3 medium to obtain 0.00001-0.1% concentrations as vol/vol. In another set of experiments, the increasing concentrations of garlic extract/juice 0.01-0.2% (vol/vol) in E3 medium were prepared.

HPLC determination of allicin and other water soluble compounds

Garlic cloves were blended with HPLC grade water in a ratio of 5 mL of water per 1 g of garlic, and analyzed according to earlier methods (Rybak et al. 2004, Chowdhury et al. 2008). The blended mixture was allowed to stand for 10 min in order to ensure a complete enzymatic reaction of allicin with the allicinase enzyme. The mixture was filtered thoroughly first through a Buchner funnel with filter paper, then for 30 s centrifuged at 3,500 rpm and filtered again with a syringe filter. The HPLC mobile phase was set as a 60:40 ratio of water to methanol. The UV wavelength was set at 254 nm. The conditions for the HPLC analysis were: initial volume of 20 μL, flow of 1 μL min^{-1} and maximum pressure of 35 MPa at ambient temperature. The HPLC conditions were set to best accommodate the available materials and the stability of allicin (Rahman et al. 2012).

Zebrafish husbandry

Zebrafish were maintained 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. Zebrafish were maintained in a recirculating, light and temperature controlled facility on a standard 14/10-hour light/dark cycle in standard system fish water. Adult fish were fed twice daily with dry food and with *Artemia nauplii* once a day. 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 descri-

bed 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) could clearly be identified as fertilized. Confirmed fertilized eggs were 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 $CaCl_2 \cdot 2H_2O$, 0.33 mM $MgSO_4$). Within 2 hpf, fertilized eggs were separated from non-fertilized ones with a pipette using 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 28.5°C with a 14/10-hour light/dark cycle in a precision incubator. One embryo was set in one well to prevent any toxins being released from dead organisms. Garlic solutions were exchanged every 24 hours.

Range-finding preliminary experiments

Experiments were conducted to determine the waterborne median lethal concentrations (LC_{20} and LC_{50}) that produced mortality in 20% and 50% of the embryos and larvae exposed. The present study was divided into two parts. The first part was run on embryos from the 2 hpf and was carried up to the fifth day of development. The second part was done on 3 dpf larvae and was carried out for the next 48 hours. The aim of this study was to determine a group of organisms most prone to FGJ using a large range of concentrations. The preliminary experiments were conducted with a constant spacing factor of 10. The minimum and maximum concentration tested for FGJ and AGE were 0.00001% and 1.0% in E3 medium.

Lethality of embryos and larvae exposed

Concentration-response curves for the most sensitive endpoints were based on the number of affected embryos and larvae (increased lethality). Lethality was defined as an embryo or larva that lacked cardiac function and were unresponsive to touch, or were in a state of decomposition.

Further along in our study, the concentrations were narrowed to represent a range between the concentrations with the lowest observable lethal effect with 100% mortality and the highest concentration that caused less than 10% lethality. Solutions were replenished every day and nine concentrations of garlic (0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.08, 0.10, and 0.20%) were used.

Twenty-four embryos and larvae were used at each concentration and each experiment was repeated at least 3 times. Control experiments were also performed by the addition of carrier solvent alone (E3 medium).

Percent mortality of the embryos and larvae exposed to different concentrations of AGE and FGJ was recorded at 12-hour intervals.

Statistical analysis

Mean LC values were estimated after 24, 48, 72, 96 and 120 h of fish exposure using GraphPad Prism v.6 based on nonlinear regression. Values are expressed as mean ± standard error of the mean (SEM) for all experiments. One-way analysis of variance (ANOVA) and the *post-hoc* Bonferroni test were carried out to identify statistically significant differences between the treatments and control group. Homogeneity of variance was tested for all data using the Levene's test. For parametric variables, Student's *t*-test was used to compare two experimental groups. The threshold level of significance was set at P value < 0.05 using SPSS (version 24.00) software.

RESULTS

HPLC determination of allicin in fresh garlic juice

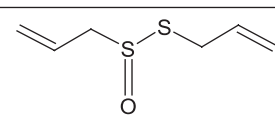
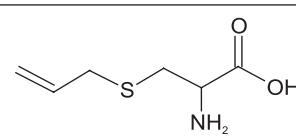
The yield of allicin and S-allyl cysteine (SAC) from several random bulbs is shown in Table 1.

Embryos and larvae lethality

Lethal effects recorded during the experiments carried out on zebrafish embryos and larvae are presented on Figure 1. All controls fulfilled the crite-

Table 1

Organosulphur compounds determined in the fresh garlic juice and aged garlic extract

Sulphur components	Chemical structure#	FGJ (μg mL^{-1})	AGE (μg mL^{-1})
Allicin (diallyl thiosulfinate)		3103 ± 109*	n.d.
S-allyl-L-cysteine (SAC)		70 ± 2.8*	1520 ± 68

Values are mean ± SEM (n = 6); *P < 0.05, compared with respective AGE group (analyzed with Student's *t*-test); n.d. – not determined, # the chemical structures were developed with the program ACD/ChemSketch Freeware. (http://www.acdlabs.com/resources/freeware/chemsketch/); Abbreviations: AGE – aged garlic extract, FGJ – fresh garlic juice.

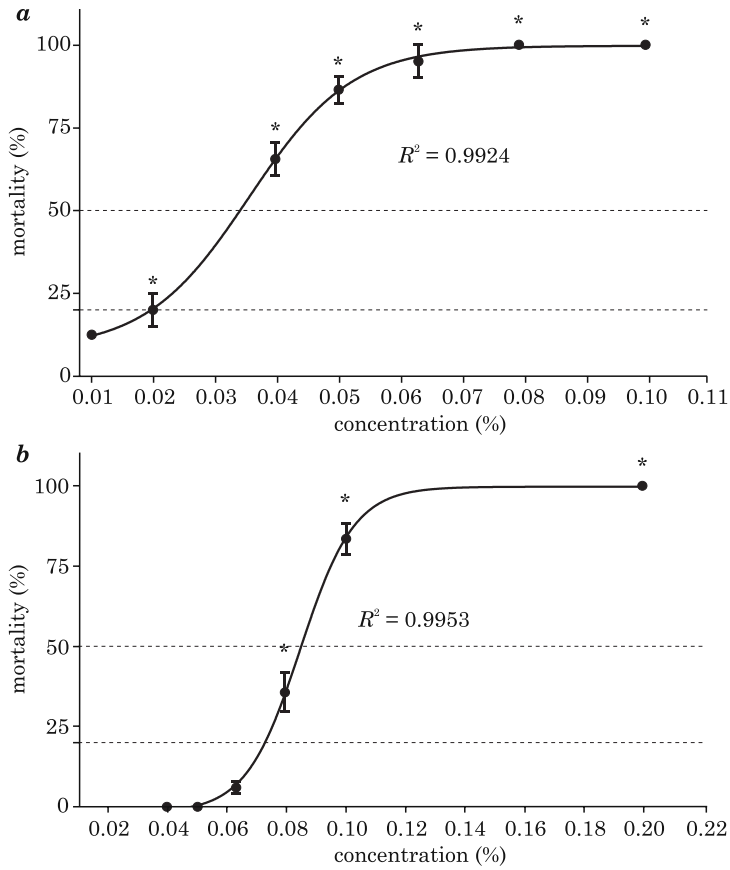


Fig. 1. Mortality of zebrafish embryos after 24 h of exposure to increasing doses of FGJ (0.01-0.10%). Embryos were introduced to the dilutions from 2 h post fertilization (*a*); Mortality of zebrafish larvae after 24 hours of exposure to increasing doses of FGJ (0.04-0.20%). Larvae were introduced to the dilutions from 3 days post fertilization (*b*). The lethal concentration for 20% (LC_{20}) and 50% (LC_{50}) exposure to acute FGJ were indicated with horizontal lines (GraphPad Prism v.6). Results are reported as mean ± SEM for 3-4 replicates and 24 eggs per replicate (analyzed with ANOVA with Bonferroni test). The mortality of AGE-treated zebrafish did not differ significantly from the control group (mortality <10%). Significant differences between FGJ and AGE are indicated by asterisks ($P < 0.05$)

ria of ≤ 10% affected embryos and larvae during the experimental period. Mortality was identified by lack of embryonic development and coagulation of the egg or by cessation of the heart rate in larval stage.

The mortality of AGE-treated embryos or larvae did not differ significantly ($P = 0.571$) from the control regardless of exposure duration and garlic extract concentration (data not shown). In contrast, the mortality of FGJ varied significantly from controls ($P < 0.05$) in a dose and time-dependent manner (Table 2). More than 90% of zebrafish embryos and larvae died within 60 min when treated with 1.0% FGJ. At the concentration of 0.1%, all

Table 2

The lethal concentrations calculated for 20% (LC_{20}) and 50% (LC_{50}) of FGJ tested on zebrafish embryos (120-h) and larvae (48-h) during the exposure. Embryos and larvae were introduced to the varying dilutions starting from 2 h post fertilization (2 hpf) and 3 days post fertilization (3 dpf), respectively

FGJ		Concentration (%) in each exposure tenure				
		24-h	48-h	72-h	96-h	120-h
Embryos (2 hpf)	LC_{20}	0.021 ± 0.001	0.021 ± 0.001	0.021 ± 0.001	0.024 ± 0.001	0.024 ± 0.001
	LC_{50}	0.034 ± 0.001	0.034 ± 0.001	0.034 ± 0.001	0.028 ± 0.001	0.028 ± 0.001
Larvae (3 dpf)	LC_{20}	0.074 ± 0.002*	0.072 ± 0.003*	-	-	-
	LC_{50}	0.083 ± 0.005*	0.079 ± 0.004*	-	-	-

Results are reported as mean ± SEM for 3-4 replicates and 24 eggs per replicate (analyzed with ANOVA with the Bonferroni test). Significant differences between corresponding LC values in embryos and larvae group are indicated by asterisks ($P < 0.05$).
Abbreviations: FGJ – fresh garlic juice, LC – lethal concentration.

embryos died within 12 h, as opposed to 3 dpf larvae, being less prone to FGJ, which resulted in only a 10% death rate within 12 h after the beginning of the experiment. During the first 24 h of exposure, garlic juice at the concentration of 0.08% resulted in 100% mortality in embryos and only 40% in developing larvae. Under the concentration of 0.06%, the results obtained were similar to the 0.08% FGJ only in developing embryos. However, in the larval forms, no mortality was observed during the 48 h of exposure to 0.06% of FGJ. Exposure to the concentration range of 0.01% and lower had no impact on embryos or larvae mortality.

Also, the LC_{20} and LC_{50} values were determined in both embryos and larvae at 24-hour intervals (Table 2). Probit analysis revealed that LC_{50} parameters at every 24-hour interval were not significantly different from each other inside each group. However, between the larvae and embryo groups, zebrafish embryos were more sensitive to the applied concentration, resulting in a higher mortality (Figure 1).

DISCUSSION

Toxicological activities of two garlic preparations (FGJ and AGE) were investigated using zebrafish embryos and larvae as a model in these studies. With increasing concentrations of FGJ, zebrafish mortality also increased, in particular at 24 and 96-hour exposure and no significant mortality was observed thereafter (up to 120-hour embryos' exposure). At the concentrations of 0.02% and lower, no significant increase in embryo mortality was observed over the entire exposure time, whereas a significant increase in mortality was observed at FGJ concentrations of 0.04, 0.06 and 0.08%.

Embryos exposed to the concentration of 0.06% showed a sharp decrease in survivorship at 12-hour and suffered 100% mortality at 24-hour exposure which is in accordance with our previous studies (Majewski et al. 2017).

Exposure to FGJ had less significant effects on the survival of the hatched larvae. In our study, no significant increase in mortality was observed with exposure to 0.04 or 0.06%, whereas exposure to 0.1% of FGJ caused almost all larvae to die by 48 hours. The results indicate that the embryo stage is more sensitive to FGJ stress than the larval stage. Although no obvious increase in mortality was found in larvae exposed to 0.1% FGJ for 12 h, almost all of the exposed embryos were dead. These results suggest that the chorion did not protect the exposed embryos against the environment and that allicin easily penetrates the cellular compartments in the biological systems.

Predictably, AGE did not cause significant mortality. In our study AGE was much less toxic than FGJ to early stages of aquatic organism.

As allicin is a volatile and unstable product which rapidly decomposes to other breakdown products such as (E/Z)-ajoene, diallyl disulphide or vinyldithiin, it is highly probable that allicin may not have been solely responsible for the results obtained. Amagase (2006) reported that allicin and diallyl disulphide present in fresh garlic have higher toxicity when compared to SAC (which is present at a high concentration in AGE). However, the chemical complexity of garlic leads to the production of formulations with varying degrees of efficacy and safety. Also, the processing of garlic such as ageing, drying, freezing and other methods of extraction, makes it feasible to obtain complex products (Majewski 2014).

Garlic pungency is attributed mainly to the presence of allicin, the major source of H_2S in fresh garlic (Macpherson et al. 2005). Allicin toxicity is attributed to the disruption of respiratory events. Recently, it has also been found that *Allium sativum* L. induces pain and inflammation by activation of TRPA1 and TRPV1 ion channels (Bautista et al. 2005, Macpherson et al. 2005, Koizumi et al. 2009, Yassaka et al. 2010). Induction of inflammation can have a significant impact on zebrafish mortality by causing a significant stress to the organisms. It is probable that hydrogen sulphide at a higher concentration may be the key to the observed effects. Allicin and allicin derivatives may also up-regulate the detoxifying enzymes, protecting from reactive oxygen species damage with an increase of the cellular glutathione level (Rabinkov et al. 2000, Horev-Azaria et al. 2009). However, the precise mechanism and the compound or compounds responsible for FGJ mortality have yet to be established.

CONCLUSIONS

There is a need for further investigation into other garlic juice constituents (apart from allicin) which have toxic effects, and to elucidate the precise mechanisms by which they exert their effects.

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.

The following abbreviations are used in this manuscript:

AGE – aged garlic extract; FGJ – fresh garlic juice; H_2S – hydrogen sulphide; LC_{20} – lethal concentration for 20%; LC_{50} – lethal concentration for 50%; NO – nitric oxide; SAC – S-allyl cysteine; TRP – transient receptor potential channel.

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