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Effect of lead acetate on livability of zebrafish embryo/larvae and amelioration by garlic aqueous extract (GAE)

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Abstract
Lead is a well known non-biodegradable toxic heavy metal in the environment and now, it has become a global issue. Lead is a cumulative toxicant that affects multiple body systems. Garlic, *Allium sativum* L. is a member of the Alliaceae family, has been widely recognized as a valuable spice and a popular remedy for various ailments. Hence in this study the effect of lead acetate on zebrafish embryo/larvae and amelioration by garlic aqueous extract was assessed. Normally dividing 5 hpf (hours post fertilization) embryos were allotted into different groups, in six well plates. Two control groups one with plain embryo water and the other with garlic aqueous extract. Lead acetate was exposed at three dose levels 0.1, 0.5 and 1 ppm to next three groups. The other three groups were exposed with lead acetate at three levels along with 1 µg garlic aqueous extract. To evaluate the livability, embryos and larvae were counted at 5, 24, 48, 72, 96 hpf and 5, 6, 7 and 8 dpf (days post fertilization). From 24 hpf to 8dpf control and drug control groups differ significantly ($P \leq 0.01$) from all other groups. At all hours and days post fertilization, the livability of the embryo/larvae was affected dose dependently in the lead acetate treated groups and as well the garlic aqueous extract has protective effect, in dose dependant manner.

Keywords: Lead, zebrafish, garlic, livability

Introduction
Heavy metal toxicity is one of the major current environment health problems and is potentially dangerous because of bio-accumulation through food chain and this can cause hazardous effects on livestock and human health (Aycicek *et al.*, 2008) [1]. Among them, lead is ubiquitous, non-biodegradable and one of the earliest metals discovered by the human race (Flora *et al.*, 2012) [2]. Lead is a highly toxic heavy metal occurring naturally in the Earth's crust (as lead sulphide). Lead has been found in at least 1,272 of the 1,684 National Priority List (NPL) sites identified by the United States Environmental Protection Agency (EPA) (Sanders *et al.*, 2009) [3].

Unique properties of lead like softness, high malleability, ductility, low melting point and resistance to corrosion, have resulted in its widespread usage such as mining, smelting, refining and informal recycling of lead; use of leaded petrol; production of lead acid batteries and paints; jewellery making, soldering, ceramics and leaded glass manufacture in informal and cottage industries; electronic waste and use in water pipes and solder. Other sources of lead in the environment include natural activities, such as volcanic activity, geochemical weathering and sea spray emissions, and remobilization of historic sources, such as lead in soil, sediment and water from mining areas (Flora *et al.*, 2012, WHO, 2010) [2, 4].

As lead is an element, once it is released into the environment, it persists. Because of lead's persistence and potential for global atmospheric transport, atmospheric emissions affect even the most remote regions of the world (WHO, 2010) [4]. There is no such level of lead that appears to be necessary or beneficial to the body and no 'safe' level of exposure to lead has been found. Lead toxicity is a potential insidious hazard with the potential of causing irreversible health effects (Flora *et al.*, 2012) [2]. Lead exposure is estimated to account for 0.06% of the global burden of disease, with the highest burden in developing regions. Lead is a cumulative toxicant that affects multiple body systems, including the neurological, haematological, gastrointestinal, cardiovascular and renal systems (WHO, 2010) [4].

Garlic (*Allium sativum*) is a medicinal plant that has been inseparable part of Indian culinary for over 5000 years. Besides its use as a condiment, it is credited to have remarkable therapeutic and pharmacological properties like antimicrobial, antithrombotic, antihypertensive, antiatherosclerotic, antihyperglycemic and anticancer. Also it is known that

garlic acts as an antioxidant by enhancing cellular antioxidant enzymes and inhibiting lipid peroxidation. Many studies have reported the efficacy of garlic extract in reducing the lead burden from various tissues like kidney, liver and bone. A lot of useful health-associated features of garlic have been attributed to its main effective element organosulphur substance 'allicin' (thio-2-propene-1-sulfinic acid S- allyl ester) (Saleh *et al.*, 2017) [5].

The zebrafish, a robust tropical fish that has long been a common feature in home aquariums, has recently attainted a pre-eminent position in biomedical research. The Zebrafish possesses a number of strengths as a test species in developmental toxicity studies including an abundance of embryos developing *ex utero*, presenting ease in chemical dosing and microscopic assessment at all early developmental stages. Zebrafish is also listed as a recommended test species in the 'Fish early life stage Toxicity test' (OECD Test guideline TG210) and the 'Fish short term toxicity test on embryo and sac-fry stages' (OECD Test guideline TG212) for determination of lethal and sub lethal effects on chemicals (Selderslaghs *et al.*, 2009) [6].

Hence this study was undertaken to evaluate the livability of zebrafish embryos/larvae that were exposed to lead acetate and its amelioration by garlic aqueous extract.

Materials and Methods

Zebrafish and tank

Wild type zebrafish (*Danio rerio*) were procured from local fish breeders and maintained in aerated standard fish rearing glass tanks. The stocking density was 5 fish/litre of water. Water without chlorination and Reverse osmosis water in the ratio of 4: 1 at the temperature of 29 °C, at a pH of 7.6 – 8.4, hardness between 50 – 100 mg/L and electrical conductivity between 360-520 µs was used for rearing the adult zebrafish. The temperature, pH and electrical conductivity of the zebrafish rearing water samples were estimated automatically by Multiparameter tester (PCST) in the department of Livestock Production and Management, Veterinary College and Research Institute, Namakkal. The hardness of the water was tested by EDTA titration method (Westerfield, 2000) [7].

Rearing and Breeding of zebrafish

Male and female zebrafishes were maintained in separate tanks under the light: dark period of 14:10 hours and were fed with standard food pellets twice a day. The male and female fish were separated one week before breeding and fed with protein rich freeze dried worms twice a day. A specially self designed breeding tank was used. The mice cage was used as a breeding tank, where a window mosquito net was placed 1/3rd above the surface of water. Two sets of female and male zebrafishes in the ratio of 2: 1 were placed above the mosquito net, so that the adult zebrafish will not have the access to eat the eggs. The male and female fishes were placed in the breeding tank overnight. The eggs were laid in the morning following the first flash of light (Westerfield, 2000) [7].

Egg collection and embryo water

The live eggs will be small, transparent and round in shape. The dead eggs will be milky white in colour. The live eggs were aspirated using pasture pipette and transferred to petridishes containing embryo water. The embryo water was prepared by adding 0.06 gram of ocean salt in one litre of reverse osmosis water. The collected zebrafishes were washed twice in the embryo water. Normally dividing and spherical embryos at 5 hour post fertilization (hpf) were selected and utilized for the study (Truong *et al*., 2011) [8].

Preparation of Garlic Aqueous Extract (GAE)

The garlic aqueous extract was prepared as per Zakaria, 2003 [9]. 100 gram of peeled garlic was ground by adding 100 ml of cool deionised water. The ground material was filtered through the filter paper to get the clear fresh extract. From this extract required concentrations of garlic aqueous extract (GAE) were prepared.

Experimental Design

Normally dividing 5 hpf embryos were allotted into different groups as follows, in six well plates. Group I - Control (embryo water), Group II - Drug control (GAE 1 µg), Group III - 0.1 ppm lead acetate, Group IV - 0.5 ppm lead acetate, Group V - 1.0 ppm lead acetate, Group VI - 0.1 ppm lead acetate + GAE 1 µg, Group VII - 0.5 ppm lead acetate + GAE 1 µg and Group VIII - 1.0 ppm lead acetate + GAE 1 µg. Twenty embryos were allotted for a group with three replicates.

Assessment of livability of zebrafish embryo/larvae

The embryos and larvae of zebrafish were assessed for their livability through the inverted microscope at the department of Veterinary Microbiology, Veterinary College and Research Institute, Namakkal. To evaluate the livability embryos and larvae were counted at 5, 24, 48, 72, 96 hpf and 5, 6, 7 and 8 dpf (days post fertilization). Dead embryos were removed from the plates immediately and the embryo water was changed daily (Guo *et al*., 2015) [10].

The data were analyzed by Kruskal Wallis test followed by Mann whitney u test procedure using SPSS® 20.0 software package for windows (Snedecor and Cochran, 2007) [11].

Results and Discussion

The livability of the zebrafish embryo/larvae was given in the Table. 1 and the pictures of live and dead embryos/larvae are shown in Fig. 1. At 5 hpf, the control groups have significantly higher survivability when compared to lead acetate treated groups (III, IV and V) but not with lead acetate and GAE treated groups. At other time points of observation, control groups (I and II) differ significantly ($P \leq 0.01$) with higher survivability when compared to all other treatment groups including lead acetate and GAE treated groups.

At 24, 48, 72, 96 hpf and 5 and 6 dpf group V (1 ppm lead acetate) has lower survivability and it differ significantly ($P \leq 0.01$) from all other groups. At the remaining time point of observations group V did not differ significantly from group IV (0.5 ppm lead acetate), but differ significantly ($P \leq 0.01$) from all other groups. Group III (0.1 ppm lead acetate) did not differ significantly from group IV up to 72 hpf and at remaining time points of observation the group has significantly ($P \leq 0.01$) higher survivability when compared to group IV. At all time points of observation group III differ significantly ($P \leq 0.01$) from group V which explains the dose dependant toxic effect of lead acetate on survivability of zebrafish embryo/larvae.

When comparing the lead acetate alone and lead acetate and GAE treated groups, group VI and VIII significantly ($P \leq 0.01$) showed better survivability when compared to group III and V at all time points of observation. Group VII showed better survivability when compared to group IV numerically at all time points of observation and significantly ($P \leq 0.01$) at 5, 24, 96 hpf and 5, 6, 7 and 8 dpf.

Similar results of lead effect on livability of zebrafish embryo/ larvae were reported in other studies. Zhang *et al.*, (2012) [12] exposed zebrafish embryo to lead acetate at the doses of 0, 0.1, 0.5, 2.5 and 12.5 µmol/L, respectively, from 1 hour post fertilization (hpf) to embryonic hatching or death. The embryonic mortalities of exposure groups were significantly increased dose dependently compared to the control group ($P < 0.05$) with a higher mortality at the early stage of embryonic development. Hongwei Tu *et al.*, (2017) [13] exposed zebrafish to lead at the concentrations of 2.5, 5.0 and 10.0 µM concentrations for 5 dpf. There was dose dependant increase in mortality rate.

Huyen Nguyen Thi Thuong *et al.*, (2014) [14] examined the effects of lead at different concentrations (20, 40, 60, 80, 100, 120 and 140 ppb) on the life of larval zebrafish from 1-7 days old. They concluded that the minimum concentration of lead that affects the survival rate of larval zebrafish was 40 ppb. The survival rate of larval zebrafish was affected significantly at 6th and 7th days as the lead penetrated via gills, skin and mouth whereas only through skin up to 5 days. Similar to their results in this study also the survival rate was severely affected from 6 dpf especially at the dose of 1 ppm lead acetate.

Garlic can prevent oxidative stress in lead toxicity by chelating lead ions and scavenging free radicals (Flora *et al.*, 2012) [2].

Sulfur-containing amino acids such as cysteine have already been reported for their chemoprophylactic use in lead toxicosis. The efficiency of garlic was perhaps due to the presence of the compounds having sulfur-containing amino acids, free carboxyl and amino groups in their structures like allicin, S-allyl-cysteine, diallyl-di-sulfide, and diallyl-sulfide. The mechanism of garlic mediated chelation of lead acetate might include formation of ionic bonds between sulfur-containing compounds and lead. Garlic components with sulfur moieties have been documented to act as active Lewis acids with electron affinity and therefore have a tendency to form compounds with positively charged ions. In contrast, lead is a highly electropositive metalloid exhibiting ionic states of +2 and is an active Lewis base. It thus possesses an affinity for negative ions and forms stable compounds with them. Thus these biologically active compounds might have chelated lead and resulted in partial amelioration of lead toxicity in zebrafish embryos/larvae (Arti Sharma *et al.*, 2010) [15].

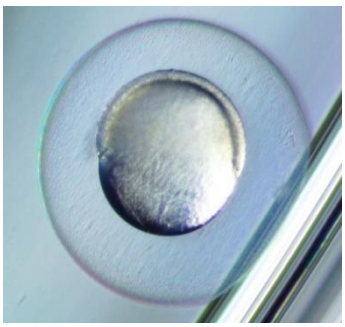
Garlic was already proved to have protective effects against lead in rat by Jarad (2012) [16], Hamid Abdulraouf Saleh *et al.*, (2017) [17] and Galal *et al.*, (2019) [18].

Table 1: Effect of lead acetate on livability of zebrafish embryos / larvae and ameliorative effect by GAE

S. No	Age	Group I (Control)	Group II (Garlic control)	Group III (0.1 ppm LA)	Group IV (0.5 ppm LA)	Group V (1.0 ppm LA)	Group VI (0.1 ppm LA + GAE 1 µg)	Group VII (0.5 ppm LA + GAE 1 µg)	Group VIII (1.0 ppm LA + GAE 1 µg)
1.	5 hpf	$20.00^{a} \pm 0.00$	$20.00^{a} \pm 0.00$	$18.50^{bc} \pm 0.58$	$17.00^{cd} \pm 1.16$	$16.50^{d} \pm 0.58$	$20.00^{a} \pm 0.00$	$19.50^{ab} \pm 0.58$	$19.00^{abc} \pm 1.16$
2.	24 hpf	$20.00^{a} \pm 0.00$	$20.00^{a} \pm 0.00$	$17.00^{d} \pm 0.00$	$16.50^{d} \pm 0.58$	$14.50^{e} \pm 0.58$	$19.00^{b} \pm 0.00$	$18.50^{bc} \pm 0.58$	$17.50^{cd} \pm 0.58$
3.	48 hpf	$20.00^{a} \pm 0.00$	$20.00^{a} \pm 0.00$	$16.75^{c} \pm 0.50$	$16.50^{c} \pm 0.58$	$14.50^{d} \pm 0.58$	$18.50^{b} \pm 0.58$	$18.00^{bc} \pm 1.16$	$16.50^{c} \pm 0.58$
4.	72 hpf	$20.00^{a} \pm 0.00$	$20.00^{a} \pm 0.00$	$16.75^{c} \pm 0.00$	$16.50^{c} \pm 0.58$	$14.50^{d} \pm 0.58$	$18.50^{b} \pm 0.58$	$18.00^{bc} \pm 1.16$	$16.50^{c} \pm 0.58$
5.	96 hpf	$20.00^{a} \pm 0.00$	$20.00^{a} \pm 0.00$	$16.75^{c} \pm 0.00$	$15.00^{d} \pm 1.16$	$12.00^{e} \pm 1.15$	$18.50^{b} \pm 0.58$	$18.00^{bc} \pm 1.16$	$16.50^{cd} \pm 0.58$
6.	5 dpf	$20.00^{a} \pm 0.00$	$20.00^{a} \pm 0.00$	$16.50^{cd} \pm 0.58$	$14.00^{e} \pm 1.16$	$11.50^{f} \pm 0.58$	$18.50^{b} \pm 0.58$	$17.50^{bc} \pm 0.58$	$16.00^{d} \pm 0.00$
7.	6 dpf	$20.00^{a} \pm 0.00$	$20.00^{a} \pm 0.00$	$15.50^{c} \pm 0.58$	$10.50^{e} \pm 1.73$	$8.00^{f} \pm 1.16$	$17.50^{b} \pm 0.58$	$16.50^{bc} \pm 0.58$	$13.50^{d} \pm 0.58$
8.	7 dpf	$20.00^{a} \pm 0.00$	$20.00^{a} \pm 0.00$	$12.00^{c} \pm 1.16$	$6.50^{de} \pm 1.73$	$4.50^{e} \pm 0.58$	$17.00^{b} \pm 1.16$	$13.00^{c} \pm 1.16$	$8.50^{d} \pm 0.58$
9.	8 dpf	$20.00^{a} \pm 0.00$	$20.00^{a} \pm 0.00$	$8.50^{d} \pm 0.58$	$3.50^{f} \pm 0.58$	$2.50^{f} \pm 0.58$	$15.50^{b} \pm 0.58$	$11.00^{c} \pm 1.16$	$6.50^{e} \pm 0.58$

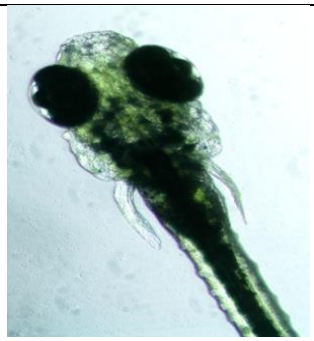
n = 4

Overall mean bearing different superscripts between columns differ significantly ($P \leq 0.01$)

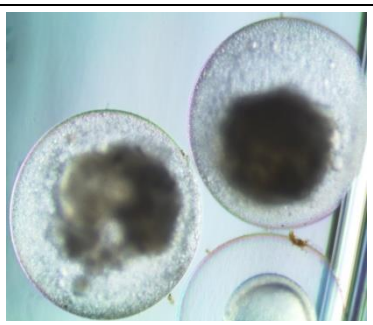


Group I – 5hpf (live)

Group I - 48 hpf (live)

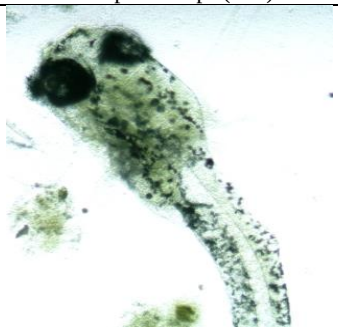


Group II – 5dpf (live)



Group V - 5 hpf (dead)

Group V – 48 hpf (dead)



Group V – 5 dpf (dead)

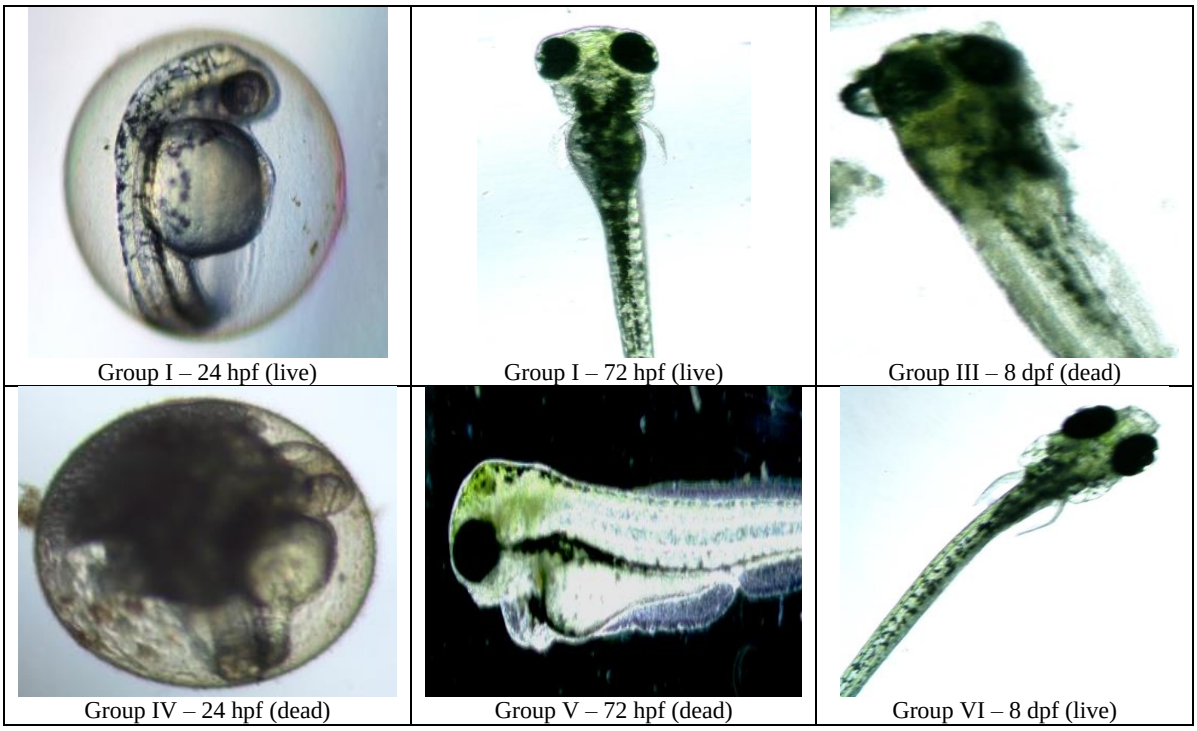


Fig 1: Live and dead embryos/larvae of different groups

Conclusion

Thus the lead induced dose dependant mortality of the zebrafish embryos/larvae and the partial protective effect of garlic aqueous extract in dose dependent manner on lead acetate treated embryos was evident. The amelioration by garlic was better in 0.1 ppm lead acetate treated group followed by 0.5 and 1.0 ppm lead acetate treated groups respectively.

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