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Crude Anthocyanin Extract (CAE) from Ballatinao Black Rice Reduces Acute Lead Toxicity in *Daphnia magna*

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Abstract Lead commonly contaminates water systems and causes acute and chronic toxicity, and indirectly produces ROS causing oxidative stress, to organisms. In response, organisms produce antioxidant enzymes for protection from oxidative stress, and uptake antioxidants in the environment. Ballatinao black rice is rich in antioxidants, particularly anthocyanins, and this study examined the effect of the Crude Anthocyanin Extract (CAE) of Ballatinao black rice on the acute lead toxicity to *Daphnia magna* through acute toxicity assays. *D. magna* were exposed to 10, 15 and 20 ug/mL concentrations of CAE for 24-hours with and without 4.0 mg/L lead nitrate. Number of deaths were recorded each hour for 24 hours. Three additional treatment strategies of the same parameters were also designed. Heartbeat counts in relation to lead toxicity were also measured. A significant increase in *D. magna* survival (α=.01) was observed when exposed to increasing CAE concentrations both in untreated and lead-treated set-ups. Variations in pre- and post-exposure treatments to CAE and lead were also performed and found to be insignificant. Heartbeat counts during the toxicity assays dropped in the first hour and normalized at 12- and 24-hour time points. The study demonstrated that Ballatinao black rice CAE can reduce the toxic effects of lead exposure to *Daphnia magna.*

Keywords: *Ballatinao black rice, anthocyanin, acute lead toxicity, daphnia magna, heartbeat count*

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1. Introduction

Toxic metal wastes find their way to aquatic systems from industrial wastewaters [1]. Lead is not essential to living organisms, and uptake leads to bioaccumulation [2], [3]. This causes acute and chronic toxicity and may eventually lead to death of organisms [2].

Uptake of lead results to indirect production of reactive oxygen species (ROS) and induction of oxidative stress [4]. Organisms produce antioxidant enzymes [5] to counter oxidative stress. The pigmented variety of rice has been reported to have antioxidant activities [6,7]. In the Philippines, a black-to-purple variety known as Ballatinao rice (*Oryza sativa* cultivar) is cultivated in Bontoc, Mountain Province (277 km north of Manila). Ballatinao ranked first in anthocyanin levels and ranked second in antioxidant activity among the pigmented rice varieties in the Philippines [8]. Previous studies on Ballatinao CAE have shown that it decreases longevity of *Drosophila melanogaster* under non-oxidative stress [9], and reduces regeneration time of amputated *Dugesia hymanae* [10].

A number of mining areas which threaten land and water systems with lead contamination [3] can be found in Mountain Province, Philippines where Ballatinao rice is a staple food. To our knowledge, there is no published research on anthocyanin effects on lead toxicity. Thus, the effect of Ballatinao rice CAE on lead toxicity was investigated. *Daphnia magna*, among freshwater invertebrates, is the standard bioindicator used in evaluating aquatic pollution including toxic metals [11]. In this study, effect of CAE on the acute toxicity of lead to *Daphnia magna* was examined.

2. Materials and Methods

2.1. Specimen

Ballatinao black rice (*Oryza sativa* cultivar) cultivated in Bontoc, Mountain Province, Philippines was procured from the local market in Baguio City, Philippines.

Daphnia magna was obtained from Bulacan, Philippines and was cultured and maintained in filtered pond water from the University of the Philippines – Manila campus. Oxygenation was provided naturally using *Hydrilla* (aquatic plant), and photoperiod cycles followed a 12-hour light and 12-hour dark cycle. The *D.*

magna cultures were maintained and propagated at ambient laboratory temperature.

2.2. Extraction

Ballatinao rice from Bontoc was weighed at 200 g and soaked in 1 L extraction buffer (60% ethanol (with 0.1% HCl)) for 6 hours with gentle shaking. The solution was filtered using a Whatman© Grade 2 filter paper (Whatman (UK) Ltd, Maidstone, UK) and divided into five 200 mL portions. Each portion was concentrated in a rotary evaporator (BUCHI© Rotavapor R-200) at 40°C for two hours to remove excess ethanol. The concentrated extracts were pooled and lyophilized through a centrifugal evaporator (DNA Speed Vac© DNA110) and stored at 4°C in air-tight dark containers.

2.3. Acute Toxicity Test

Three-day neonates were acclimatized for 5 hours in reconstituted water consisting of 290 mg/L $CaCl_2.H_2O$, 120 mg/L $MgSO_4.7H_2O$, 65 mg/L NaHCO3, and KCl 6 mg/L (Sigma-Aldrich Corp., St. Louis, MO, USA) before each treatment. For each set-up, 10 neonates were transferred to glass vials containing 10 mL reconstituted water. Set-ups were exposed to varying concentrations of the CAE (10, 15 and 20 ug/mL) for 24 hours in the presence (co-exposure, **COE**) and absence (exposure to extract only, **EEO**) of 4.0 mg/L Pb(NO3)2 (Sigma-Aldrich Corp., St. Louis, MO, USA). The deaths were recorded each hour for 24 hours. Absence of *D. magna* voluntary movement upon gentle shaking was scored as dead. Three additional treatment strategies were also designed which included: 1) one hour pre-exposure to extract followed by lead only exposure (**EEL**); 2) one hour pre-exposure to lead only followed by co-exposure with extract (**ELC**) and; 3) one hour pre-exposure to extract only followed by co-exposure with lead (**EEC**).

2.4. Heartbeat Count

Ten-day adult *D. magna* were acclimatized in reconstituted water before each treatment. For each set up, an adult was transferred in a tube containing 5 mL reconstituted water. Set ups were exposed to varying concentrations of the CAE (10, 15 and 20 ug/mL) in the presence (co-exposure) and absence of 4 mg/L Pb(NO3)2. Each experimental set-up was done in three replicates. Heartbeat counts were determined at three different time points (1-, 12-, and 24-hour) by mounting each *D. magna* on a microscope (B-192 Optika© Light Microscope) under low power objective and immediately video recording for 30 seconds using a digital camera (Canon© IXUS 125 HS). A different *D. magna* individual was used for each video recording of heartbeats in the three time points. The video was viewed in slow motion to be able to count the number of heartbeats in 30 seconds.

2.5. Statistical Analysis

Data for survival plots were reported as counts. All analysis on comparison of populations were carried out in OpenEpi Version 3.01 online software (http://openepi.com/v37/Menu/OE_Menu.htm) using two-tailed z-test of independent samples at α=0.01 (99% confidence interval). Heartbeat counts were reported as means with standard error.

3. Results and Discussion

3.1. CAE Increases Survival of untreated and lead-treated *D. Magna*

Establishment of lead toxicity in *D. magna* at 4.0 mg/L is depicted in Figure 1A. Compared to the no extract/no Pb set-up, the number of deaths of *D. magna* in the lead-only (**LO**) treatment is significantly more (α=.01, z=4.14) at 90%. Previous studies showed the toxicity of lead to *D. magna* had been varied and inconsistent ranging from an LC50 of 0.15 mg/L to 4.92 mg/L [12,13,14]. These different documented lead toxicity levels can be attributed to the genetic background of the *D. magna* used in their assays and differences in their culture conditions. A similar reasoning was also expressed by Baird, et al. [15] when he tested nine toxic chemicals against *D. magna*. He discovered that different clones of *D. magna* have differences in their toxicity levels to these chemicals, and neonate performance during the toxicity assays varied depending on the culture environment. In our study, the 4.0 mg/L concentration of lead nitrate that we used falls within the LC50 range stated above. This was enough to cause 90% lethality in *D. magna* within a 24-hour period. Both the no extract/no Pb and lead-only treatment data served as reference plots for comparison to the experimental treatments.

Exposure of *D. magna* to 10, 15, and 20 ug/mL CAE were done in two ways: exposure to CAE only (EEO) and co-exposure of CAE with lead (COE). The survival plots of *D. magna* in the three EEO treatment concentrations were all higher, although not significantly, than the no lead/CAE set-up but significantly higher (α=.01, z=7.379, 8.433, 8.433 respectively) than lead-only treated set-up (Figure 1B-D). Contrary to previous studies that demonstrated a significant increase in longevity of organisms when exposed to anthocyanin [16,17], the CAE of Ballatinao black rice had no significant effect in the longevity of *D. magna*. This is consistent with the study of Velasco and Medina [9] that showed CAE of Ballatinao balck rice had no significant effect on the lifespan of *Drosophila melanogaster* .

The COE treatments for all three CAE concentrations displayed a significant improvement (α=.01, z=5.27, 7.379, 7.379 respectively) in the survival of *D. magna* when compared to the lead-only treatment set-up, with a survival increase of 50% to 70%. The significant survival outcome of *D. magna* may be attributed to the mitigation of lead toxicity via ROS, which, in turn, is scavenged by the antioxidant activities of anthocyanin in CAE [5].

Various antioxidant mechanisms are being utilized by an organism to counter oxidative stress caused by heavy metals. It is important that the balance between reduced GSH/ oxidized GSH be restored to maintain the redox environment of the cell [5]. If not, cellular metabolism maybe affected and even impaired. In this experiment, the CAE provided additional antioxidants aside from those produced by the organism. The black rice variety is known to be rich in anthocyanins such as cyanidin-3-glucoside, and peonidin-3-glucoside [18]. Acetylated procyanidins have also been found to be present in pigmented rice which are known to scavenge reactive oxygen species which would otherwise damage cellular integrity and activity [19], [20]. Additionally, Thai black rice extract was shown to be rich in anthocyanin and was able to reduce the oxidative stress in HepG2 cells [21].

The antioxidants in CAE may have reduced the burden of the oxidative stress (enhanced by the presence of lead) to the *Daphnia* by transforming the free radicals to inert products, thus increasing their survival in the lead treatments.

3.2. Duration of CAE Exposure Affects Survival of *D.* magna to Acute Lead Toxicity

Figure 1 revealed that CAE at 10, 15 and 20 ug/mL provides protection to lead toxicity in co-exposure experiments. To determine if this protection was modified in different pre-exposure and post-exposure scenarios, three additional treatment strategies were designed, which included: 1) one hour exposure to CAE followed by lead-only treatment (EEL); 2) one hour exposure to lead followed by co-exposure treatment with CAE (ELC) and; 3) one hour exposure to CAE followed by co-exposure treatment with lead (EEC).

The EEL treatment showed a trend similar to that of lead-only **(LO)** treatment across all concentrations (Figure 2A-C). Although the death count after 24 hours was a small difference of one or two, there was a big difference in the hour when first death occurred in the set-ups. The first death in the lead-only treatment took place after three hours, whereas the first deaths in 10, 15, and 20 ug/mL CAE in EEL transpired after seven, six, and seven hours, respectively (Figure 2D). This suggests that even a one-hour exposure to CAE prior to lead treatment offers short term protection that extends the time before *D. magna* succumbs to lead toxicity.

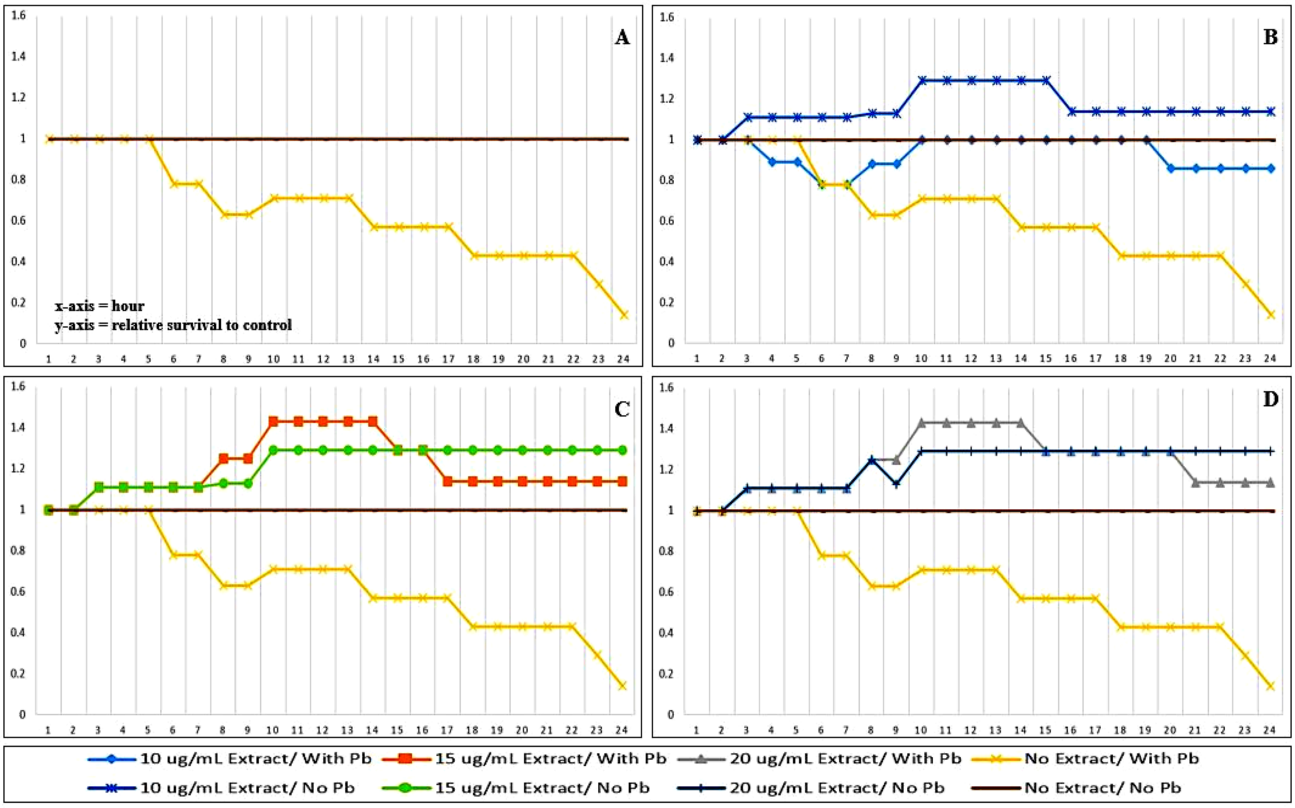


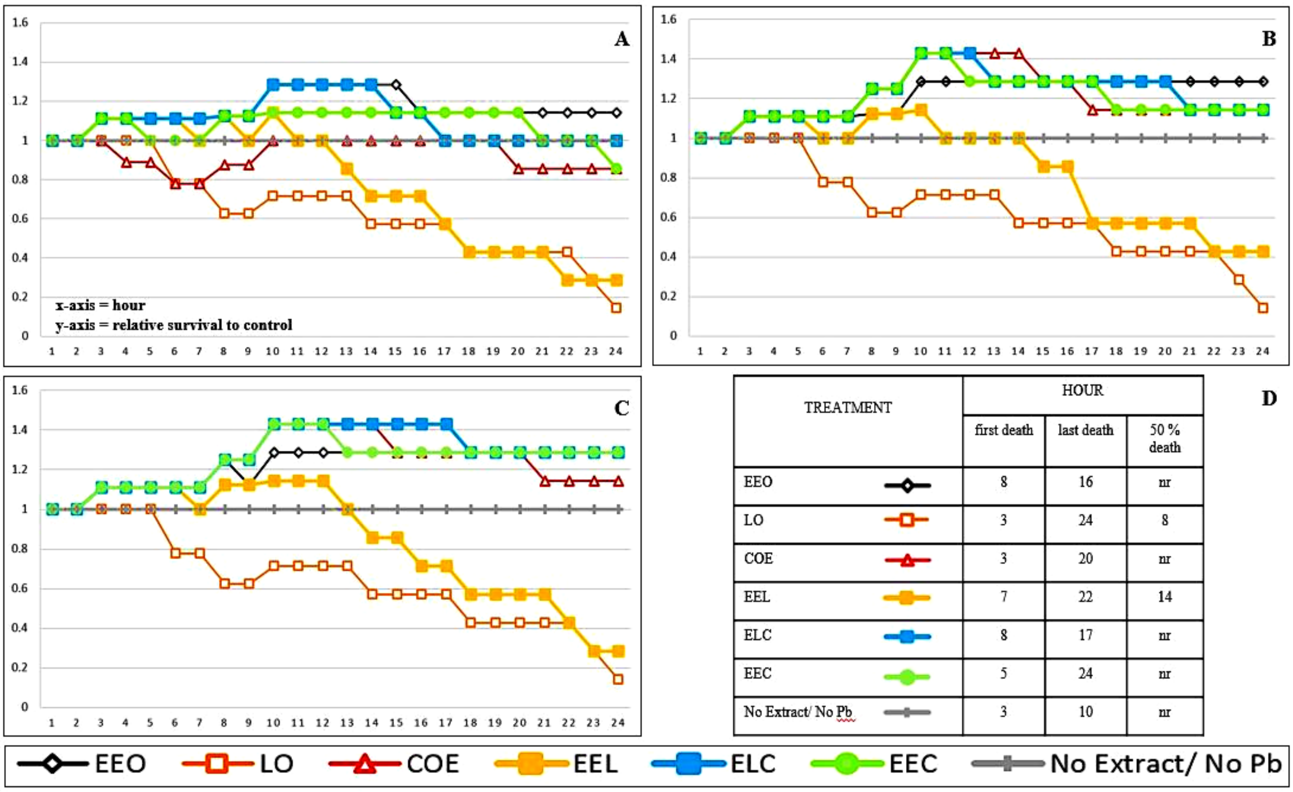
Figure 1. Ballatinao CAE reduced acute toxicity of lead. Ten 3-day old *Daphnia* neonates were used in the acute toxicity assays for each set-up, and the number of surviving neonates were recorded every hour for a 24-hour period. A) Survival plots of *D. magna* in the presence of lead nitrate and reconstituted water (negative control) were generated to serve as reference points for the succeeding co-exposure assays with CAE. These plots were superimposed in panels B to D. Co-exposure of lead with CAE and CAE exposure only were performed using B) 10, C) 15, D) 20 ug/mL CAE. All lead nitrate treatments were at 4.0 mg/L. Survival plots are normalized to no extract/ no Pb (control)

The ELC treatment displayed a drastic increase in the survival of *D. magna* comparable to CAE-only (EEO) treatment, and slightly higher than the no extract/ no Pb set-up (Figure 2A-C). Relative to the first death occurrence at three hours in lead-only treatment, the hours of first death in ELC are also extended to eight, thirteen, and eighteen hours in 10, 15, and 20 ug/mL CAE, respectively (Figure 2D). The rise in survival and the delay of first death imply that the detrimental effect due to a one-hour advanced exposure to lead is radically reduced when CAE is introduced in spite of continuing presence of lead.

The EEC treatment followed a similar pattern to ELC treatment as described above (Figure 2A-C), which indicates that a one-hour prior exposure to CAE-only, in a predominantly co-exposure treatment with lead, offered minimal additional protection to *D. magna* from lead toxicity.

3.3. Heartbeat Count of *D. magna* Poorly Correlates with Acute Lead Toxicity

Acute toxicity bioassays utilizing *D. magna* use mortality as the measured outcome. The study attempted to detect changes in heartbeat count since *Daphnia*'s heartbeat is particularly sensitive to changes in the environment. The heartbeat rate of *Daphnia* is one of the parameters considered when studying effects of various chemicals to the metabolism and physiology of an organism [22].



TREATMENT		HOUR		
		first death	last death	50 % death
EEO		8	16	nr
LO		3	24	8
COE		3	20	nr
EEL		7	22	14
ELC		8	17	nr
EEC		5	24	nr
No Extract/ No Pb		3	10	nr

Figure 2. Pre- and post-exposure of *D. magna* to CAE increases survival to lead toxicity. Ten 3-day old *Daphnia* neonates were used in the acute toxicity assays for each set-up, and the number of surviving neonates were recorded every hour for a 24-hour period. All the survival plots from Figure 1 are superimposed (with non-solid colored markers) in panels A to C for comparison with the assorted pre- and post-exposure assay designs in A) 10 ug/mL, B) 15 ug/mL, and C) 20 ug/mL CAE with survival plots normalized to no extract/ no Pb (control). D) Recorded hour of first and last deaths, and hour death reached 50% within the 24-hour observation period (nr = did not reach 50% death)

Four experimental set-ups were assembled, namely: no lead/CAE, lead-only, CAE-only (**CAE**) and co-exposure of lead (**COE**) and CAE. The heartbeat counts after the first hour in lead-only, CAE-only and co-exposure treatments showed a marked decline compared to the no lead/CAE treatment (Figure 3). These drops in the heartbeat counts during the first hour in the three experimental treatments and their corresponding recovery in the 12- and 24-hour time points suggest an early response of the *D. magna* to a sudden environmental change, and may not necessarily be directly due to the toxicity of lead.

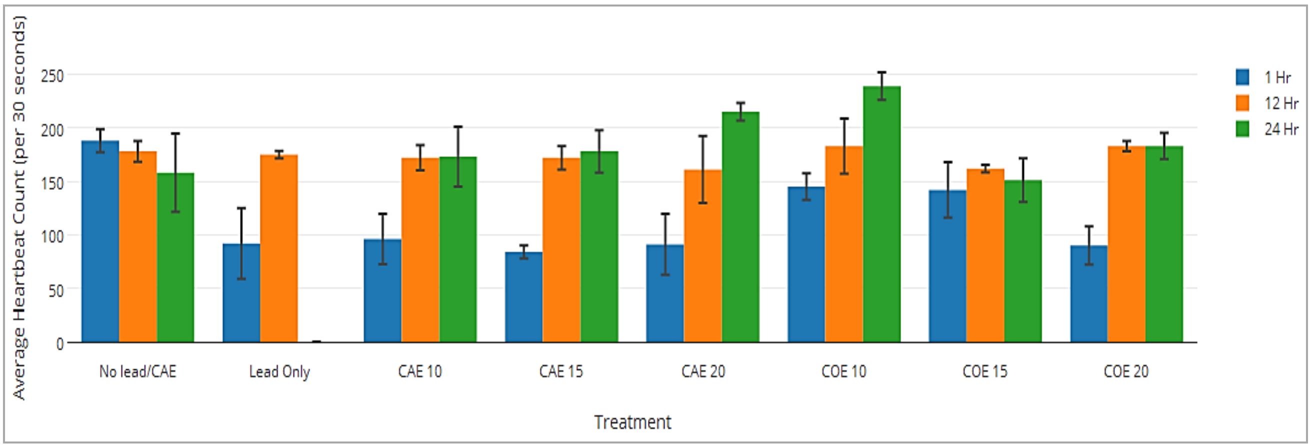


Figure 3. Lead treatment of *D. magna* does not affect heartbeat count. Ten-day old *D. magna* adults were placed in different treatments of lead and CAE, namely: No lead/CAE, lead-only, CAE and COE (co-exposure of lead and CAE) with 10, 15 and 20 representing concentrations of CAE in ug/mL. Heartbeat counts of three D. magna individuals per treatment were measured for 30 seconds during the 1st, 12th, and 24th hour periods. Different sets of *D. magna* were used for the three time points. (-) death of all test organisms

Comparison of the control (no lead/CAE) and the lead-only set-ups as seen in Figure 3 revealed lack of appreciable heartbeat count difference at the 12-hour time point. No heartbeat count values were obtained at the 24-hour time point because all the *Daphnia* organisms perished between the 12- and 24-hour time points. It should be noted that in the acute toxicity assay, at the 12-hour time point and using the same 4.0 mg/L of lead nitrate, 50% mortality was observed in *Daphnia*, thus, any surviving *Daphnia* was undergoing stress. This stress was not reflected in the recorded heart beat count.

The 12- and 24-hour heart beat counts in the CAE-only (Figure 3) were comparable to the control, except for the CAE-only with the highest concentration, which had a higher heart beat count than the control at the 24-hour time point. Little can be said to the implication of this increased heartbeat count because of insufficient supporting information, e.g., precise time the increase in heart beat count occurred, and how long it persisted.

On the other hand, for the COE treatments (Figure 3), there was no apparent trend (increase or decrease) in heart beat counts observed in COE relative to the control and CAE-only, and, thus, no correlations can be made.

Daphnia experience stress when exposed to lead, whether in the presence of CAE or not. However, the lead-induced stress did not translate to a perceptible change in heart beat count. Thus, this study showed that using heartbeat counts as a measure of acute lead toxicity in *Daphnia magna* is not recommended.

4. Conclusion

Anthocyanins are regarded as antioxidants owing to their abilities to scavenge ROS and free radicals. Lead and other heavy metals contribute to oxidative stress in aquatic organisms. This study was able to demonstrate a proof of principle that an antioxidant, such as anthocyanin, can reduce the toxicity of lead to *Daphnia*, perhaps through the reduction of oxidative stress. Furthermore, this study reveals that heartbeat count of *D. magna* is not a reliable indicator of acute lead toxicity.

It will be valuable to know if the protective nature of Ballatinao CAE to lead toxicity can be observed in other aquatic organisms, and if it can reduce toxicities of other heavy metals. Performing these two studies is essential to uncover the actual mechanism of Ballatinao CAE's protective effect against acute toxicity of lead. It will also be valuable to determine if Ballatinao CAE will have any protective effect on chronic lead exposure of *Daphnia*, in particular, assessing survival, fecundity and longevity parameters.

As far as we know, this is the first report documenting the protective ability of an antioxidant to a heavy metal, which has the potential to introduce a new line of research with antioxidants, and management of heavy metal toxicity.

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Statement of Competing Interests

There are no competing interests that exist between the authors.

List of Abbreviations

CAE	**C**rude **A**nthocyanin **E**xtract
ROS	**R**eactive **O**xygen **S**pecies
COE	**CO**-**E**xposure
EEO	**E**xposure to **E**xtract **O**nly
EEL	one hour pre-**E**xposure to **E**xtract followed by **L**ead only exposure
ELC	one hour pre-**E**xposure to **L**ead only followed by **C**o-exposure with extract
EEC	one hour pre-**E**xposure to **E**xtract only followed by **C**o-exposure with lead
LO	**L**ead **O**nly

References

[1] Cooper, N.L., Bidwell, J.R., Kumar, A., "Toxicity of Copper, Lead, and Zinc Mixtures to *Ceriodaphnia dubia* and *Daphnia carinata*," *Ecotoxicology and Environmental Safety*, 72. 1523-28. 2009.

[2] Berglind, R., Dave, G., Sjobeck, M., "The Effects of Lead on δ-Aminolevulinic Acid Dehydratase Activity, Growth, Hemoglobin Content, and Reproduction in *Daphnia magna*," *Ecotox Environ Safe*, 9. 216-29. 1985.

[3] Forstner, U., Wittman, G.T.W., *Metal Pollution in the Environment*, Springer Verlag, Berlin/Heidelberg/New York, 1979.

[4] Luschak, V.I., "Environmentally Induced Oxidative Stress in Aquatic Animals," *Aquat Toxicol*, 101. 13-30. 2011.

[5] Kim, S.H., Kim, S.J., Lee, J.S., Lee, Y.M., "Acute Effects of Heavy Metals on the Expression of Glutathione-related Antioxidant Genes in the Marine Ciliate *Euplotes crassus*," *Marine Pollution Bulletin*, 2014.

[6] Tananuwong, K., Tewaruth, W., "Extraction and Application of Antioxidants from Black Glutinuous Rice," *Food Science and Technology*, 43. 476-81. 2010

[7] Yawadio, R., Tanimori, S., Morita, N., "Identification of Phenolic Compounds Isolated from Pigmented Rices and their Aldose Reductase Inhibitory Activities," *Food Chemistry*, 101. 1616-25. 2007.

[8] Romero, M.V., Ramos, N.C., Soco, O.C., Mamucod HF., "Characterizing the Neutraceutical Content and Enhancing the Utilization of Pigmented Rice in the Philippines," *PhilRice R&D Highlights Natural Products and Value-Adding Systems*, 19-22. 2012.

[9] Velasco, A.J.D., Medina, P.M.B., "Crude Anthocyanin Extract (CAE) from Ballatinao Black Rice Does Not Alter Longevity and Increases Stress Susceptibility of *Drosophila melanogaster," Int. J. Curr. Res. Biosci. Plant. Biol.*, 1 (4). 35-42. 2014.

[10] Quebral, E.P.B., Medina, P.M.B., "Crude Anthocyanin Extract (CAE) from Ballatinao Black Rice Hastens Regeneration in *Dugesia hymanae*," *Pharm Biomed Sci*, 5 (02). 147-153. 2015.

[11] Altindag, A., Ergonul, M.B., Yigit, S., Baykan, O., "The acute toxicity of lead nitrate on *Daphnia magna* Straus," *African Journal of Biotechnology*, 2 (23). 4298-300. 2008.

[12] Le Blanc, G.A., "Laboratory Investigation into the Development of Resistance of *Daphnia magna* to Environmental Pollutants," *Environ Poll*, 27. 309-22. 1982.

[13] Beisinger, K.E., Christensen, G.M., Fiandt, J.T., "Effects of Metal Salt Mixtures on *Daphnia magna* Reproduction," *Environ Saf*, 11. 9-14. 1972.

[14] Gordillo, S., Fernandez-Pereira, A.C., Vale-Parapar, J.F., "Acute ecotoxicity Evaluation of Heavy Metals with *Daphnia magna*," *Ecotoxicol Environ Rest*, 1 (3). 3-12. 1998.

[15] Baird, D.J., Barber, I., Bradley, M., Soares, A.M.V.M., Calow, P., "A Comparative Study of Genotype Sensitivity to Acute Toxic

Stress Using Clones of *Daphnia magna* Strauss," *Ecotoxicology and Environmental Safety*, 9. 216-229. 1985.

[16] Chen, W., Muller, D., Richling, E., Wink, M., "Anthocyanin-rich Purple Wheat Prolongs Life Span of *Caenorhabditis elegans* Probably by Activating the DAF-16/FOXO Transcription Factor," *J Agric Food Chem*, 61 (12). 3047-53. 2013.

[17] Zuo, Y., Peng, C., Liang, Y., Ma, K.Y., Yu, H., Chan, H.Y.E., Chen, Z., "Black Rice Extract Extends the Lifespan of Fruit Flies," *Food Funct*, 3. 1271-79. 2012.

[18] Hou, Z., Qin, P., Zhang, Y., Cui, S., Ren, G., "Identification of Anthocyanins Isolated from Black Rice (*Oryza sativa* L.) and their Degradation Kinetics," *Food Res Int*, 50. 691-697. 2013.

[19] Oki, T., Masuda, M., Kobayash, M., Nishiba, Y., Furuta, S., Suda, I., Sato, T., "Polymeric Procyanidins as Radical-scavenging Components in Red-hulled Rice," *Journal of Agricultural and Food Chemistry*, 50. 7524-7529. 2002.

[20] Sutharut, J., Sudarat, J., "Total Anthocyanin Content and Antioxidant Activity of Germinated Colored Rice," *International Food Research Journal*, 19 (1). 215-221. 2012.

[21] Sangkitikomol, W., Tencomnao, T., Rocejanasarol. A., "Effects of Thai Black Sticky Rice Extract on Oxidative Stress and Lipid Metabolism Gene Expression in HepG2 Cells," *Genetics and Molecular Research*, 9 (4). 2086-2095. 2010.

[22] Villegas-Navarro, A., Rosas-L., E., & Reyes, J.L., "The Heart of *Daphnia magna*: Effects of Four Cardioactive Drugs," *Comparative Biochemistry and Physiology Part C*, 136. 127-134. 2003.