EXCELLENT PUBLISHERS

International Journal of Current Research in Biosciences and Plant Biology

ISSN: 2349-8080 Volume 1 Number 4 (November-2014) pp. 35-42

www.ijcrbp.com

Original Research Article

Crude Anthocyanin Extract (CAE) from Ballatinao Black Rice Does Not Alter Longevity and Increases Stress Susceptibility of *Drosophila melanogaster*

Adrian Jonathan D. Velasco[1] and Paul Mark B. Medina[2]*

[1]College of Medicine, University of the Philippines – Manila, Philippines, 1000
[2]Department of Biochemistry and Molecular Biology, College of Medicine, University of the Philippines – Manila, Philippines, 1000

**Co-first author and Corresponding author. E-mail:* pmbmedina@post.upm.edu.ph

Abstract

Black rice varieties are rich in antioxidants such as anthocyanin compounds. In this study, **C**rude **A**nthocyanin **E**xtract (CAE) from Ballatinao black rice was tested for its effects on longevity, heat-stress and energy-deprivation stress in *Drosophila melanogaster*. We show that the CAE had no significant effect on the longevity of fruit flies. Under heat-stress, CAE reduced the mean life span by 18%, 18% and 23% for 10, 20 and 30 mg/ml concentrations respectively. Furthermore, under energy-deprived conditions, CAE significantly reduced the survival of male flies ($p<0.05$) but had no significant effect on female flies. To our knowledge, this is the first demonstration that a crude anthocyanin extract decreases the longevity of *D. melanogaster* when placed under heat and energy deprivation stress. We proposed a model to explain anthocyanin toxicity during non-oxidative stress.

Keywords

Anthocyanin
Ballatinao black rice
Crude anthocyanin extract
Heat stress
Longevity

Introduction

Oryza sativa (rice) is an important crop in the developing world, particularly in Asia. A wide gamut of antioxidants can be found in both pigmented and non-pigmented rice varieties (Goufo and Trindade, 2014). These antioxidants fall under phenolic acids, flavonoids, anthocyanins, proanthocyanidins, tocopherols, tocotrienols, γ-oryzanols and phytic acids (Goufo and Trindade, 2014). Notably, pigmented rice varieties possess higher antioxidant activities when compared to non-pigmented rice varieties (Goufo and Trindade, 2014; Walter and Marchesan, 2011; Seo et al., 2013; Saikia et al., 2013; Gunaratne et al., 2013; Pitjia et al., 2013).

Black rice varieties have been the subject of exploratory research for its potential biomedical applications. There is evidence that correlate black rice consumption with improvement of cardiovascular health status (Sangkitikomol et al., 2010) and inhibition of cancer invasion (Chen et al., 2006). The anthocyanin compounds in black rice include cyanidin-3-O-glycoside and peonidin-3-O-glycoside (Zhang et al., 2006). In particular, cyanidin-3-O-glycoside has been shown to have antioxidant activity 3.5 times more potent than Trolox, a vitamin E analog (Zuo et al., 2012).

Previous work have demonstrated that black rice extract (1) induces superoxide dismutase (SOD), catalase (Chiang, 2006) and gluthathione peroxidase activities (Auger et al., 2002); (2) suppresses reactive oxygen species (ROS) and nitric oxide (NO) radicals (Hu, 2003) and (3) inhibits xanthine oxidase, one of the generators of superoxide anions (Zuo et al., 2012).

Drosophila melanogaster has been widely used as a model organism for longevity, aging, neurodegenerative diseases and drug discovery (Pandey and Nichols, 2011). The advantages of using this organism include: low cost, convenient diet formulation, well-characterized physiology, as well as a known genome (Pandey and Nichols, 2011). Zuo et al. (2012) has demonstrated that black rice extract prolonged the mean life span of fruit flies by 14%. Furthermore, this effect is accompanied by an upregulation of CuZnSOD (SOD1), MnSOD (SOD2) and catalase (Zuo et al., 2012).

Most studies about black rice are done on varieties cultivated in China (Zuo et al., 2012), Thailand (Sangkitikomol et al., 2010), and to some extent, Korea (Seo et al., 2013). In northern Philippines, a black rice variety, known locally as Ballatinao rice, is consumed widely in the Mountain Province, Benguet and other neighboring provinces. Chemical analysis of Ballatinao rice showed that it has the highest levels of anthocyanin, vitamin B, crude protein, total phenolics and fatty acids when compared to red (Chochoros) and non-pigmented (NSIC Rc 160) rice varieties (Romero et al., 2012).

This study explored the effect of Ballatinao rice **C**rude **A**nthocyanin **E**xtract (CAE) on the longevity of *D. melanogaster*. Furthermore, this study also determined the effect of the extract on *D. melanogaster* longevity under heat stress and energy-deprivation stress. We found that the CAE from Ballatinao rice do not significantly alter the mean life span of fruit flies. However, when exposed to daily heat stress, the mean life span of *Drosophila* at CAE concentrations of 10, 20 and 30 mg/ml were reduced by 18, 18 and 23% respectively (p<0.05). Under energy deprivation stress, CAE reduces survival of male flies (p<0.05). These data suggest that the CAE from Ballatinao black rice increase susceptibility of fruit flies to heat stress and energy-deprivation stress.

Materials and methods

Crude extraction of back rice anthocyanin

The Ballatinao black rice, cultivated in Bontoc, Mountain Province, was acquired from Baguio city. A total of 1.0 kg of the Ballatinao black rice was submerged in 4000 ml of 60% ethanol (with 0.1% HCl) for 6 to 8 hrs with moderate shaking at room temperature. The rice grains were strained out and the ethanol from the extraction solvent was removed using a rotary evaporator, BÜCHI Rotavapor R-200. Finally, the crude anthocyanin extract was lyophilized using a centrifugal evaporator, DNA Speed Vac® DNA110. The lyophilized CAE was stored at -4°C in a dark bottle.

Fruit fly culture media and yeast paste formulation

The fruit fly culture medium was prepared by boiling 500 g of orange sweet potato cubes in 1000 ml dH_2O. The softened sweet potato cubes and accompanying infusion were homogenized using a blender. After blending, 15.4 g agar and 10g yeast were added. The mixture was allowed to boil for 5 min. with constant stirring to incorporate the agar and the yeast powder. The entire mixture was allowed to cool down to 60ºC before adding 400 μl of glacial acetic acid (to prevent growth of bacteria and molds) and was poured into containers before solidifying. The fruit fly culture medium was used in rearing several generations of *D. melanogaster* and was also used in the control set-up. The experimental set-ups were prepared by adding appropriate lyophilized CAE such that the final concentrations will be 10 mg/ml, 20 mg/ml and 30 mg/ml CAE in the experimental culture media.

The fruit fly media was supplemented with yeast paste. Approximately 5 g Baker's yeast powder was mixed with 1 ml 0.04% acetic acid to achieve a pasty consistency. Yeast paste made with 10 mg/ml, 20 mg/ml and 30 mg/ml CAE solutions were also prepared.

Life span assay

For the life span assay, 2-day old flies (males and females) were collected and aged for 2 more days in a common culture bottle. The flies were anesthetized with CO_2 and were sorted according to sex. There were a total of four set-ups: 10 mg/ml, 20 mg/ml and 30 mg/ml CAE concentrations and a control set-up with no CAE. The flies were distributed in 50 ml conical tubes (cotton-plugged) such that each tube had 10 flies of the same sex. Overall, each set-up had 4 tubes, 2 of which had 10 male flies and 2 had 10 female flies. These set-ups were maintained at ambient laboratory temperature and the live flies were counted everyday and the flies were transferred to newly prepared culture tubes every 4 days.

Heat stress assay

The heat stress assay was done as a modified life span assay. Every day, the flies were placed in an incubator set at 37^{o}C for 1 h. The live flies were counted before and after the heat stress incubation.

Energy-deprivation assay

The energy-deprivation assay was also done as a modified life span assay. For three days, the flies were fed with the following diets: 10 mg/ml, 20 mg/ml and 30 mg/ml CAE and control (no CAE). On the third day, the flies were transferred to a new conical tube containing only cotton ball moistened with dH_2O. The live flies were counted every six hours until all flies were dead.

Statistics

For the life span assay and the stress experiments (heat stress and energy deprivation stress), the Kaplan-Meier test was used to assess the differences in the life span curves of each treatment group, as detailed by Rich et al. (2010). Independent Z-test was employed to determine the differences in the mean life span of each treatment group, a difference at $p<0.05$ was considered significant. The StatsDirect statistical software was used for these statistical tests.

Results

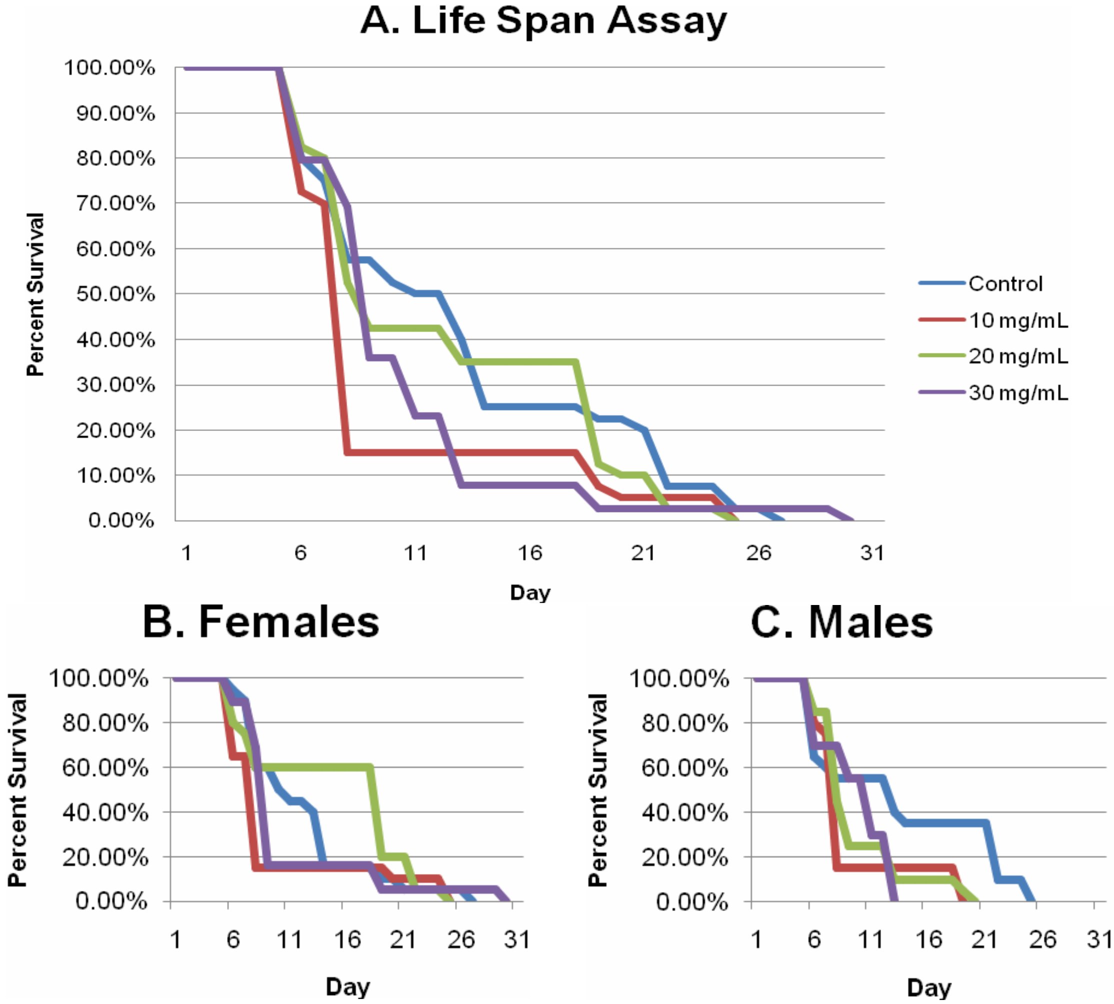
CAE does not increase mean life span of *Drosophila*

Previous studies have shown that antioxidants are able extend the longevity of *Drosophila*, as demonstrated in the antioxidants from cucurmin (Suckow and Suckow, 2006) and from apple (Peng et al., 2011). Furthermore, anthocyanins were also able to prolong the life spans of organisms such as in *Caenorhabditis elegans* (Chen et al., 2013) and in *Drosophila* (Zuo et al., 2012).

Contrary to the expectations set by these studies, our research demonstrated that CAE from Ballatinao black rice did not increase life span of *D. melanogaster*, as shown in Fig. 1. The lack of significant difference between the CAE-fed flies and the controls may be attributed to the use of low CAE concentrations in the experimental set-ups. We originally based our CAE concentrations on the study of Zuo et al. (2012), however, it should be noted that they used a pure extract, whereas we used a crude extract. Thus, the effective concentration of the active components in our crude extract may not have been enough to elicit any significant increase in mean life span, if any.

Another possible reason for the lack of significant increase in longevity may be due to the presence certain chemicals in our CAE that may have neutralized the anthocyanin's effect. We purposely chose to use a crude extract over a pure extract because it more closely resembles the manner by which Ballatinao black rice is consumed. Lastly, there is a possibility that the anthocyanins in our CAE may not have any effect on *D. melanogaster* longevity under normal physiological conditions. There are antioxidants that have been shown to have no effect on *D. melanogaster* longevity as exemplified by melatonin and epithalamin (Anisimov et al., 1997). While some anthocyanin extracts show life span-prolonging activity, this study suggests that this may not be true for all anthocyanins.

Fig. 1: Kaplan-Meier survival curves from life span assay.



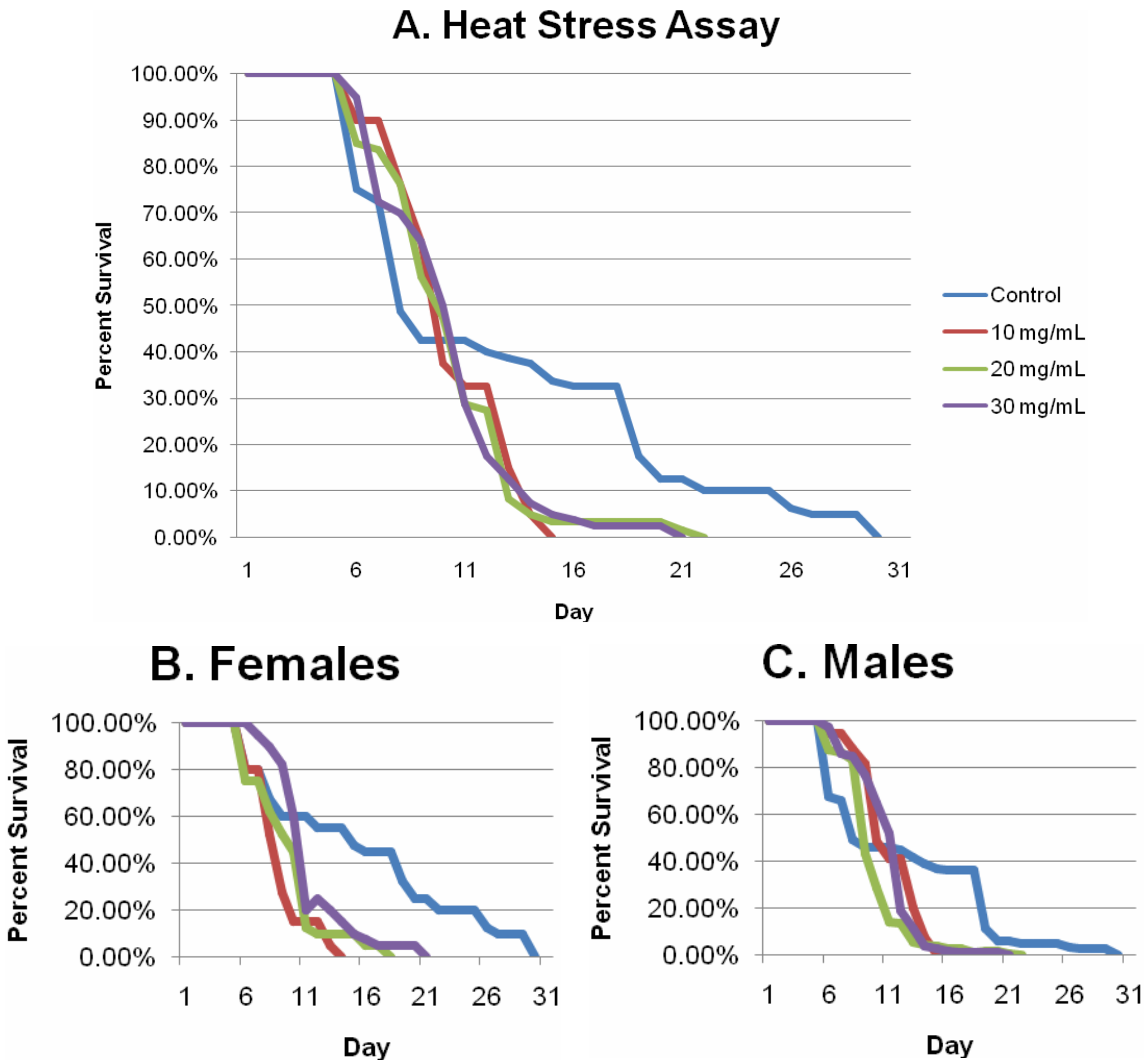
(A) Consolidated data for both male and female flies from control (no CAE) and experimental set-ups with 10 mg/ml, 20 mg/ml and 30 mg/ml concentrations of CAE from Ballatinao black rice; (B) Data from female set-ups only; (C) Data from male set-ups only (n=20 for males per treatment group, n=20 for females for no CAE group and 10 mg/ml and 20 mg/ml treatment groups, n=19 females for 30 mg/ml treatment group).

CAE reduces the mean life span of Drosophila under heat stress

Although Ballatinao CAE did not alter *D.melanogaster* longevity under ambient conditions as previously shown in Fig. 1, interestingly, it significantly reduced the mean life span across the three CAE experimental concentrations when exposed to heat stress. As depicted in the Kaplan-Meier survival curves in Fig. 2, the mean life spans of *D. melanogaster* at CAE concentrations of 10, 20 and 30 mg/ml were reduced by 18, 18 and 23% respectively (p<0.05). This suggests that the CAE may interfere with the physiology of the *D. melanogaster* heat stress response which leads to a reduced life span.

Metabolism is down regulated as a normal response to stress in *D. melanogaster* (Sørensen et al., 2005). Glucose, along with other compounds, is the major carbon source for nerves. During the early stages of *D. melanogaster* heat stress response, the importance of glucose as an energy source for nervous tissues becomes more pronounced as pathways for other energy sources are down regulated (Sørensen et al., 2005). This phenomenon could have prevented the flies from modifying and clearing CAE which could lead to an increased accumulation to potentially toxic levels. Previous studies show that other antioxidants such as pyrogallol and thiodipropionic acid can reduce life span of *D. melanogaster* (Le Bourg, 2001; Massie, 1988).

Fig. 2: Kaplan-Meier survival curves from heat stress assay.

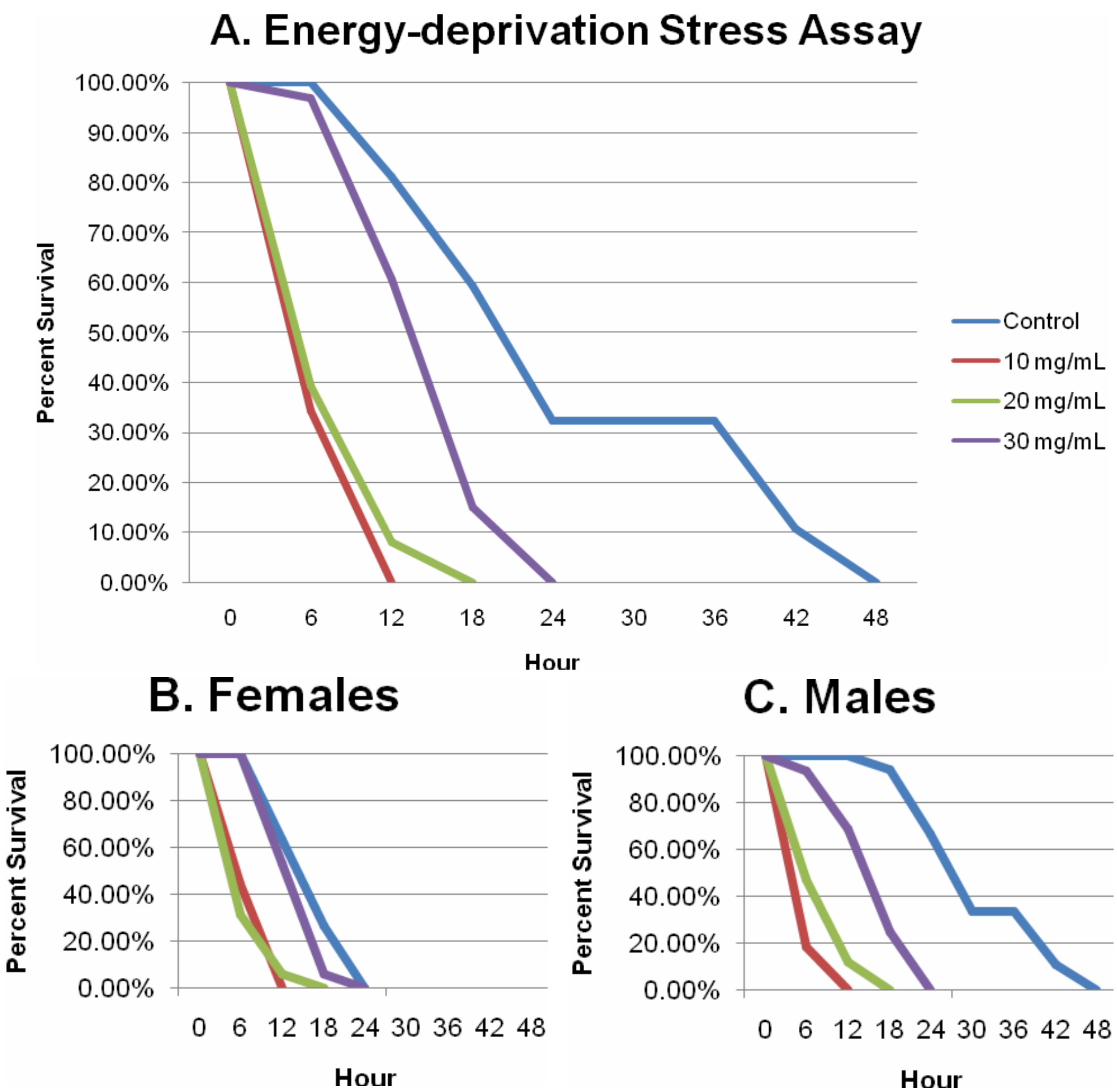


(A) Consolidated data for both male and female flies from control (no CAE) and experimental set-ups with 10 mg/ml, 20 mg/ml and 30 mg/ml concentrations of CAE from Ballatinao black rice. The mean life spans of *Drosophila* were reduced by 18, 18 and 23% for 10, 20 and 30 mg/ml CAE concentrations respectively; (B) Data from female set-ups only; (C) Data from male set-ups only (n=20 for males per treatment group, n=20 for females per treatment group).

CAE reduces survival of *Drosophila* under energy-deprivation stress

Previous studies that involve dietary restriction have shown to increase life span in *C. elegans* (Lee et al., 2006) and in *D. melanogaster* (Partridge et al., 2005). Furthermore, *C. elegans* undergoing dietary deprivation (the removal of food source) demonstrate an increased life span, enhanced thermotolerance and a resistance to oxidative stress (Lee et al., 2006). Currently, there is no record of *Drosophila* behavior during energy deprivation stress. This study showed that *D. melanogaster*, pre-exposed for two days to CAE, have a significantly reduced survival time across the three experimental concentrations ($p < 0.05$), when placed under energy deprivation stress (Kaplan-Meier survival curves in Fig. 3A). The reduced survival time may be due to the longer persistence of anthocyanin in *D. melanogaster* as a result of lowered oxidative stress during energy deprivation, which would have otherwise decreased the amount of anthocyanin. A steady elevated level anthocyanin may be toxic to *D. melanogaster*. In the sex-specific survival curves (Fig. 3B and 3C), it was revealed that the significant reduction of survival is only observed in males and not in females.

Fig. 3: Kaplan-Meier survival curves from energy-deprivation stress assay.



(A) Consolidated data for both male and female flies from control (no CAE) and experimental set-ups with 10 mg/ml, 20 mg/ml and 30 mg/ml concentrations of CAE from Ballatinao black rice. Across all concentrations of CAE, the survival curves of fruit flies decreased significantly (p<0.01); (B) Data from female set-ups only. There appears to be no significant difference in the survival curves; (C) Data from male set-ups only. The survival curves of the flies across all concentrations of CAE were significantly reduced (p<0.01) (n=20 for males per treatment group, n=20 for females per treatment group).

The difference between the sex-specific survival curves could be due to fundamental differences in the amount of energy requirement in male and female flies. Physiologically, females have a higher energy demand than males. Thus under energy deprived conditions, females are more susceptible to the detrimental effects of starvation than males. This explains why female flies fed with CAE survived just as long as their negative control counterparts while males fed with CAE died earlier than their corresponding negative controls.

In summary, we propose that in normal physiological conditions, the CAE from Ballatinao black rice neither prolong nor shorten the life span of fruit flies. However, when the flies are exposed to heat stress or energy deprivation stress, Ballatinao CAE significantly reduces life span possibly by increasing susceptibility to these stressors.

Conclusions

To our knowledge, this is the first demonstration that anthocyanin, an established antioxidant, can potentially be detrimental during stressful conditions, at least in *D. melanogaster*. This suggests that not all anthocyanins are beneficial and extend longevity.

We propose a model where heat and energy deprivation stressors may lead to reduced metabolic activity, which in turn reduces oxidative stress. With a lowered oxidative stress level, the utilization of anthocyanin in the system will likewise diminish, allowing anthocyanins to persist longer, or even accumulate. Although anthocyanins are generally beneficial due to their antioxidant activity, there may exist a level above which it becomes toxic to the organism.

We mentioned earlier that we chose a crude anthocyanin extract over a pure extract for our assays because it more closely reflects the manner by which Ballatinao black rice is consumed. However, we strongly recommend testing a purified anthocyanin of Ballatinao to conduct studies that will unravel mechanisms of its action with regards to longevity and stress responses. We also recommend the use of other animal model organisms such as *C. elegans* and mice in exploring the effect of Ballatinao black rice anthocyanins on longevity and stress response. This will determine if our observations on *D. melanogastor* are consistent with other species. Furthermore, other black rice varieties and other anthocyanin sources can also be tested in future experiments. This will enable us to evaluate the potential effects on longevity and stress responses of anthocyanins from different black rice varieties and other anthocyanin-rich sources.

Acknowledgements

We thank the Department of Biochemistry and Molecular Biology, College of Medicine, University of the Philippines Manila laboratory personnel for providing the materials and equipment used in conducting the experiments, and its administration for granting us permission to use its laboratory facilities.

References

Anisimov, V. N., Mylnikov, S. V., Oparina, T. I., Khavinson, V. K., 1997. Effect of melatonin and pineal peptide preparation epithalamin on life span and free radical oxidation in *Drosophila melanogaster*. Mech. Ageing Develop. 97, 81–91.

Auger, C., Caporiccio, B., Landrault, N., Teissedre, P. L., Laurent, C., Cros, G., Besançon, P., Rouanet, J., 2002. Red wine phenolic compounds reduce plasma lipids and apolipoprotein B and prevent early aortic atherosclerosis in hypercholesterolemic golden Syrian hamsters (*Mesocricetus auratus*). J. Nutr. 132, 1207-1213.

Chen, P., Kuo, W., Chiang, C., Chiou, H., Hsieh, Y., Chu, S., 2006. Black rice anthocyanins inhibit cancer cells invasion via repressions of MMPs and u-PA expression. Chem. Biol. Interact. 163, 218-229.

Chen, W., Müller, D., Richling, E., Wink, M., 2013. Anthocyanin-rich purple wheat prolongs the life span of *Caenorhabditis elegans* probably by activating the DAF-16/Foxo transcription factor. J. Agricult. Food Chem. 61, 3047-3053.

Chiang, A. N., Wu, H. L., Yeh, H. I., Chu, C. S., Lin, H. C., Lee, W. C., 2006. Antioxidant effects of black rice extract through the induction of superoxide dismutase and catalase activities. Lipid. 41, 797-803.

Goufo, P., and Trindade, H., 2014. Rice antioxidants: phenolic acids, flavonoids, anthocyanins, proanthocyanidins, tocopherols, tocotrienols, γ-oryzanol, and phytic acid. Food Sci. Nutr. 2(2), 75–104.

Gunaratne, A., Wu, K., Li, D. Q., Bentota, A., Corke, H., Cai, H. Z., 2013. Antioxidant activity and nutritional quality of traditional red-grained rice varieties containing proanthocyanidins. Food Chem. 138, 11153–11161.

Hu, C., Zawistowski, J., Ling, W., Kitts, D.D., 2003. Black rice (*Oryza sativa* L. *indica*) pigmented fraction suppresses both reactive oxygen species and nitric oxide in chemical and biological model systems. J. Agricult. Food Chem. 51, 5271-5277.

Le Bourg, E., 2001. Oxidative stress, aging and longevity in *Drosophila melanogaster*. FEBS Lett. 498, 183-186.

Lee, G. D., Wilson, M. A., Zhu, M., Wolkow, C. A., de Cabo, R., Ingram, D. K. and Zou, S. (2006). Dietary deprivation extends lifespan in *Caenorhabditis elegans*. Aging Cell, 5(6): 515-524.

Massie, H.R., 1988. *Drosophila* as a Model Organism for Ageing Studies. Blackie, Glasgow. pp.69-70.

Pandey, U.B., Nichols, C. D., 2011. Human disease models in *Drosophila melanogaster* and the role of the fly in therapeutic drug discovery. Pharmacol. Rev. 63(2), 411–436.

Partridge, L., Piper, M. D. W., Mair, W., 2005. Dietary restriction in *Drosophila*. Mech. Ageing Develop. 126(9), 938-950.

Peng, C., Chan, H. Y. E., Huang, Y., Yu, H., Chen, Z. Y., 2011. Apple polyphenols extend the mean lifespan of *Drosophila melanogaster*. J. Agricul. Food Chem. 59(5), 2097–106.

Pitjia, K., Nakornriab, M., Sriseadka, T., Vanavichit, A., Wongpornchai, S., 2013. Anthocyanin content and antioxidant capacity in bran extracts of some Thai black rice varieties. Int. J. Food Sci. Technol. 48(2), 300-308.

Rich, J. T., Neely, J. G., Paniello, R. C., Voelker, C. C. J., Nussenbaum, B., Wang, E. W., 2010. A practical guide to understanding Kaplan-Meier curves. Otolaryngol. Head & Neck Surg. 143(3), 331–336.

Romero, M. V., Ramos, N. C., Soco, O. C., Mamucod, H. F., 2012. Characterizing the nutraceutical content and enhancing the utilization of pigmented rice in the Philippines. Philippine Rice R & D Highlights. pp.19-22.

Saikia, S., Dutta, H., Saikia, D., Mahanta, C.L., 2012. Quality characterisation and estimation of phytochemicals content and antioxidant capacity of aromatic pigmented and non-pigmented rice varieties. Food Res. Internat. 46, 334–340.

Sangkitikomol, W., Tencomnao, T., Rocejanasaroj, A., 2010. Antioxidant effects of anthocyanins-rich extract from black sticky rice on human erythrocytes and mononuclear leukocytes. African J. Biotechnol. 9(48), 8222–8229.

Seo, W. D., Kim, J. Y., Song, Y. C., Cho, J. H., Jang, K. C., Han, S. I., Ra, J. E., Oh, S. H., Kang, H. J., Kim, B. J., Baek, N. I., Jeong, R. H., Nam, M. H., 2013. Comparative analysis of physicochemicals and antioxidative properties in new red rice (*Oryza sativa* L. cv. Gunganghongmi). J. Crop Sci. Biotechnol. 16, 63–68.

Sørensen, J. G., Nielsen, M. M., Kruhøffer, M., Justesen, J., Loeschcke, V., 2005. Full genome gene expression analysis of the heat stress response in *Drosophila melanogaster*. Cell Stress Chaperon. 10(4), 312–328.

Suckow, B. K., Suckow, M. A., 2006. Lifespan extension by antioxidant cucurmin in *Drosophila melanogaster*. Int. J. Biomed. Sci. 2(4), 402-405.

Walter, M., Marchesan, E., 2011. Phenolic compounds and antioxidant activity of rice. Brazilian Arch. Biol. Technol. 54, 371–377.

Zhang, M., Guo, B., Zhang, R., Chi, J., Wei, Z., Xu, Z., Zhang, Y., Tang, X., 2006. Separation purification and identification of antioxidant compositions in black rice. Agricult. Sci. China 5, 431–440.

Zuo, Y., Peng, C., Liang, Y., Ma, K. Y., Yu, H., Chan, H. Y., Chen, Z. Y., 2012. Black rice extract extends the lifespan of fruit flies. Food Funct. 3(12), 1271–1279.