

Sex differences in the effects of *Auricularia auricula-judae* ethanolic extracts on the life span of *Drosophila melanogaster* during stress and non-stress conditions

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ABSTRACT

Antioxidant enzymes can help to increase the life span of flies by allowing the organisms to adapt during the stressful conditions. Edible mushrooms are good sources of antioxidant compounds. *Auricularia auricula-judae*, locally known as “tenga ng daga” is a popular edible mushroom in the Philippines. We investigated the effects of chronic supplementation of wild and cultivated *A. auricula-judae* crude ethanolic extracts in the life span of adult *Drosophila melanogaster* in the presence and absence of environmental stress such as thermal stress and starvation stress. 2,2-diphenyl-1-picrylhydrazyl assay revealed a higher antioxidant activity of cultivated *A. auricula-judae* crude ethanolic extract (CAEE) compared with wild *A. auricula-judae* crude ethanolic extract (WAE). WAE had no effect on the life span of female flies but it increased the life span of male flies. CAEE decreased the life span of both male and female flies. Heat and starvation stress significantly reduced the life span of flies for both sexes. Life-span reduction was more evident in the female flies. Supplementation with CAEE and WAE showed a profound sex-specific effect on the life span of *D. melanogaster* under stress. Our results showed that CAEE and WAE did not increase the life span of male flies but significantly increased the life span of female flies under starvation and heat stress.

INTRODUCTION

Aging is a biological phenomenon manifested by the progressive decline of physiological integrity and function within living organisms over time. This decline associates aging with the progressive increase in mortality and age-related diseases such as cardiovascular disorder and diabetes (Iliadi and Boulianne, 2010). Aging is natural and unavoidable, but experimental research studies have determined that there are genetic and non-genetic factors altering the normal aging process (Magwere *et al.*, 2004). The fruit fly, *Drosophila melanogaster*, is one of the most popular

model organisms in aging research because of its short life span and low-maintenance requirements. Its genome was fully sequenced with more than 50% homologs in humans. Almost 75% of known human genetic disorders have fly homologs (Sun *et al.*, 2013).

Dietary interventions have been shown to increase life span of various animal species including flies. Sex differences in the life span of male and female flies have been reported which may be attributed to the fundamental differences in the amount of energy needed by both sexes (Hunt *et al.*, 2011; Magwere *et al.*, 2004; Velasco and Medina, 2014). Natural products with high antioxidant levels have been reported to increase the life span based on studies on *D. melanogaster* (Kumar *et al.*, 2015; Lin *et al.*, 2014; Rawal *et al.*, 2014). Mushrooms, harboring numerous bio-active components, appear to be a promising anti-aging agent (Azizur Rahman *et al.*, 2017). Edible mushrooms are good sources of antioxidants (Acharya *et al.*, 2005; Boonsong *et al.*, 2016; Luo *et al.*, 2009). *Auricularia auricula-judae*, locally known as “tenga

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ng daga,” is a popular edible mushroom in the Philippines. It is widely used in Chinese folk medicine for dysentery, numbness, and as a topical treatment of a sore throat, conjunctivitis, and tonsillitis (Kadnikova *et al.*, 2015). Several studies have reported high antioxidant activity of *A. auricula-judae* (Asatiani *et al.*, 2007; Kho *et al.*, 2009; Zou *et al.*, 2015). Other study shows that an extract from *A. auricula* extended the life span of *D. melanogaster* in a dose- and sex-dependent manner (Huang *et al.*, 2011). It was also reported to exhibit many medicinal properties such as anti-tumor activity, blood pressure regulation, and a decrease in the concentration of lipid and cholesterol in the blood (Wasser and Weis, 1999).

Auricularia auricula-judae has been a part of the diet of Filipinos, especially among some indigenous tribes. Thus, we explored the potential effects of *A. auricula-judae* crude ethanolic extracts in life span using *D. melanogaster*. We investigated the antioxidant activities of wild and cultivated *A. auricula-judae* and determined their effects in the life span of adult *D. melanogaster* in the presence and absence of environmental stress such as heat stress and starvation stress.

MATERIALS AND METHODS

Preparation of mushroom extracts

Wild *A. auricula-judae* was collected from Hermosa, Bataan, Philippines, while cultivated *A. auricula-judae* was bought from a commercial establishment in Chinatown, Manila. Voucher specimens were submitted to the Botany Department of the National Museum of the Philippines for taxonomic identification.

Wild *A. auricula-judae* was washed with water three times: twice with tap water then once with distilled water. Then, they were chopped to smaller pieces, dried under shade at room temperature, and powdered using an electric blender. The cultivated *A. auricula-judae* was already dry when purchased and was directly pulverized using an electric blender.

The resulting powders were then subjected to ethanolic extraction using 95% ethanol (Belman Laboratories; Philippines) as a solvent. One hundred grams of the wild and cultivated mushroom samples were then soaked separately in 200 ml of 95% ethanol for 72 hours. Each mixture was filtered using Whatman No. 1 filter paper, then the filtrate was subjected to a rotary evaporator (Rotavap R-200, Buchii, Switzerland) at 38°C. The resulting extracts were collected in an evaporating dish, then labeled as wild *Auricularia* ethanolic extract (WAEE) and cultivated *Auricularia* ethanolic extract (CAEE), respectively. The extracts were then subjected to a 40°C water bath inside a fume hood for a few days or until the complete evaporation of the solvent. Both WAEE and CAEE were stored inside the refrigerator at 4°C.

Preparation of fly media

The sweet potato yeast agar media consisted of 10 g of yeast (Saf-Instant Gold, Lessafre, France), 15.4 g of agar (Agar Powder, Titan Biotech Limited, India), and 1,000 ml distilled water for every 500 g of sweet potato, *Ipomoea batatas* (L.) Lam.

The sweet potato was bought from a local market in Pasay, Philippines and then washed, peeled, and boiled in distilled water until soft and tender. Water was drained and the sweet potato was then mixed with yeast, agar, and distilled water. The

resulting mixture was allowed to boil and cool down to 60°C before adding 0.08% propionic acid (Propionic Acid Laboratory Grade, Qualikems, India). The final mixture was placed in their final containers before adding the yeast paste superficially. Yeast paste consisted of 1 part yeast (Saf-Instant Gold, Lessafre, France) and 1 part 0.08% propionic acid.

Test animals

Wild-type *D. melanogaster* was obtained from the Department of Biochemistry and Molecular Biology, College of Medicine, University of the Philippines Manila. The flies were bred according to the protocol established by Velasco and Medina (2014). Flies were age-synchronized by the daily removal of newly emerged flies from a general breeding population and then placed in a fresh new media labeled as Day 0 flies. On Day 3, flies were immobilized and anesthetized in a fly pad using carbon dioxide gas (Alvimco Company Inc., Philippines) and then sorted by sex using a stereomicroscope.

Chronic lethality test

Chronic lethality test served as an optimization assay in order to determine the sublethal and non-toxic concentrations of the ethanolic extracts of *A. auricula-judae* to be used as a daily food supplementation to the fly media for the experimental setups. Three increments of WAEE and CAEE concentrations were supplemented to the fly media for this assay specifically: 0.1, 0.5, and 1.0 mg/ml. For each concentration of the extract, 20 male flies and 20 female flies were used. Percent survival of the flies was noted every day for 5 days. Observed mortality must be due to the chronic effects (>48 hours) and not as acute effects (<48 hours) of the mushroom extracts. A fly was considered dead if it no longer exhibits any movement after some time.

Concentrations with percent survival higher than 90% were considered sublethal. The highest sublethal concentration was considered as the high dosage. Middle and low experimental dosages were calculated as a 10-fold lower and 100-fold lower of the high dosage, respectively. High, middle and low dosages were used as the experimental concentrations treated as food supplements for the life span assay (LSA), heat stress assay (HSA), and starvation assay (SA).

Life span assay

Three-day-old flies were anesthetized with CO₂, sorted according to sex, and placed in *Drosophila* vials in groups of 10. There were a total of seven setups: one negative control and three experimental setups for each of the WAEE and CAEE. There were 60 flies per setup: 30 males and 30 females. Each group of 10 flies was placed in a *Drosophila* vial with the corresponding breathable plug (*Drosophila* vials, Biologix, USA). The setup was maintained at room temperature and the mortality per vial was recorded daily until all flies were dead. The flies were carefully transferred to newly prepared vials every 3 days until the end of their lifetime.

Heat stress assay

The HSA was a modified LSA. All *Drosophila* vials were placed inside a 37°C incubator 1 hour every day. Mortality per vial was recorded every day until all the flies died. Flies were also transferred to new media every 3 days.

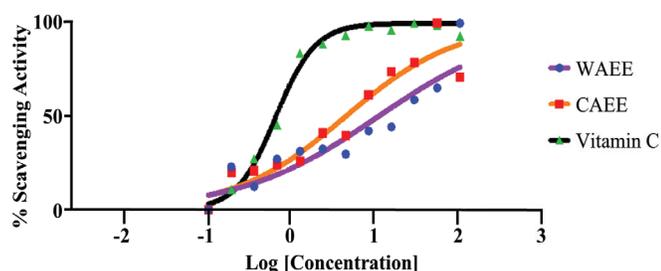


Figure 1. Dose-response curves of normalized values of % activity vs. concentration of *A. auricula-judae* ethanolic extracts and a commercial antioxidant (Vitamin C).

Starvation assay

The SA was also a modified LSA. Instead of transferring the flies to fresh media after the first 3 days, the flies were placed in a *Drosophila* vial containing only moist cotton. All of the *Drosophila* vials were kept in room temperature. Hourly mortality per vial was counted until all flies were dead.

DPPH free radical scavenging assay

The total antioxidant activities of WAE and CAEE were measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay. The DPPH radical (DPPH, Sigma-Aldrich, Singapore) used was at 0.2 mM in 95% ethanol. Aliquots of the WAE and CAEE dissolved in dimethyl sulfoxide were plated out in triplicates in a 96-well microtiter plate. Ethanol was used as a negative control, while vitamin C was used as a positive control. The ethanolic DPPH solution was added to alternating columns of the test samples. The plate was shaken for 2 minutes and incubated for 20 minutes in darkness at 37°C in a water bath. The difference in absorbance values was obtained using a spectrophotometer (Bio Rad Microplate Reader 550, Bio Rad, China) at 517 nm. The percentage of scavenging activity on DPPH radicals was calculated using the following formula

$$\%SA = \left[\frac{(Abs_{sample} - Abs_{control})}{Abs_{control}} \right] \times 100$$

The effective concentration (EC₅₀), the concentration of the sample scavenging 50% of the initial DPPH radical, can be calculated by interpolating the $[(Abs_{sample} - Abs_{control})]$ into a calibration line generated by the absorbance of DPPH at different concentrations.

Statistical analysis

Standard survival curves were constructed using the online application for survival analysis 2 (OASIS 2) (Han *et al.*, 2016). The average life span of flies in control and treatment groups was calculated and analyzed using the Wilcoxon-Breslow-Gehan Test. *p* value of 0.05 and below was considered significant. *p* value of 0.001 was considered as highly significant. One-way analysis of variance with Tukey's post-hoc analysis was done using IBM SPSS Statistics Version 21.0 to determine the difference in the antioxidant activity of the crude extracts.

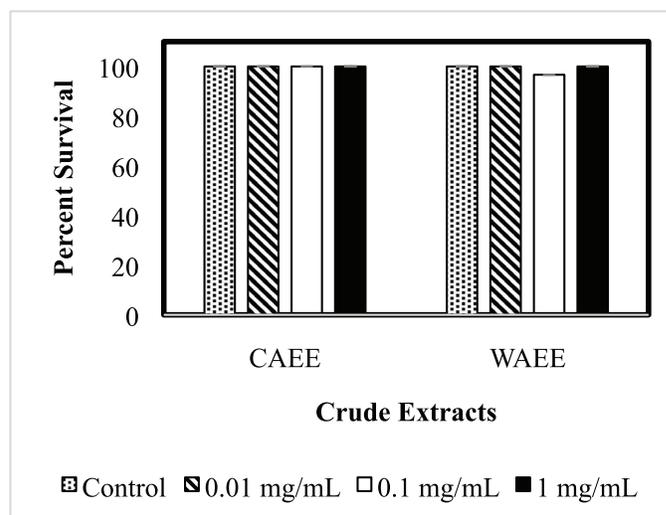


Figure 2. Survival rates of *D. melanogaster* supplemented with CAEE and WAE during the chronic lethality assay.

RESULTS AND DISCUSSION

DPPH free radical scavenging assay

Results show that CAEE neutralizes DPPH radicals more than WAE but less effectively than does Vitamin C (Fig. 1). Using the DPPH free radical assay, the EC₅₀ values were 16.64 µg/ml for WAE, 6.20 µg/ml for CAEE, and 0.76 µg/ml for Vitamin C. Both WAE and CAEE exhibited high antioxidant activity but not significantly comparable to that of Vitamin C (*p* < 0.001). There was an eight-fold decrease in half-maximal effective concentration for CAEE and a 22-fold decrease for WAE when compared with Vitamin C. This means, the cultivated *A. auricula-judae* has a higher antioxidant activity compared with wild *A. auricula-judae*.

Chronic lethality test

All concentrations of WAE and CAEE used in this study exhibited close to 100% survival rates for male and female flies after 5 days of supplementation (Fig. 2). WAE and CAEE were non-lethal to adult male and female flies. Nearly, 1 mg/ml was assigned as the high dose, 0.1 mg/ml as the middle dose, and 0.01 mg/ml as low dose. These sublethal doses were used in all subsequent assays in this study.

Life-span assay

The LSA revealed that WAE and CAEE have different effects on the life span of male and female adult flies in the long-term supplementation of extracts. WAE has neutral effects while CAEE reduces the life span. The control flies already showed a difference in the life span between male and female flies. Female flies were shown to have higher average life span compared with male flies. Female flies supplemented with WAE had an average life span comparable with that of the control group. Male flies supplemented with the low dose (0.01 mg/ml) of WAE significantly increased the life span of flies (*p* < 0.05; Fig. 3).

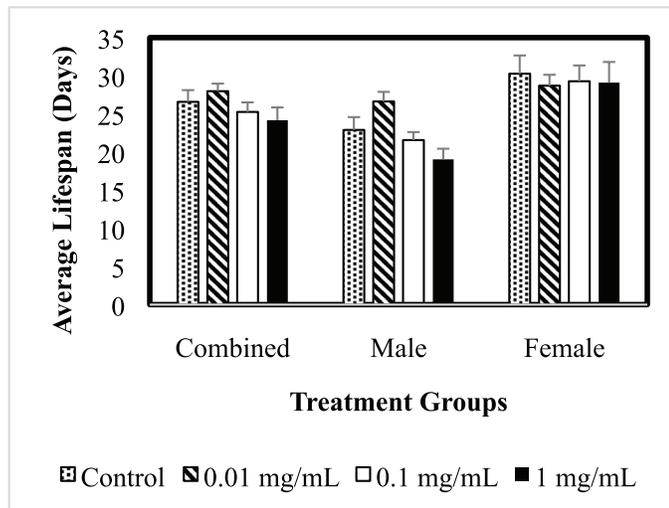


Figure 3. Life span of WAE-supplemented *D. melanogaster* under normal conditions.

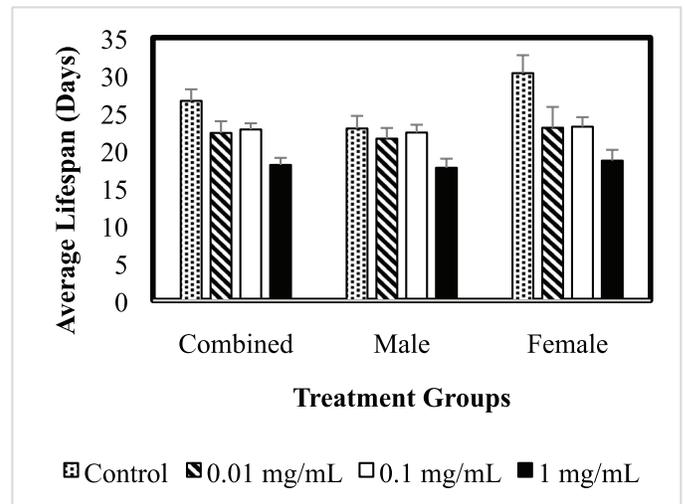


Figure 4. Life span of CAEE-supplemented *D. melanogaster* under normal condition.

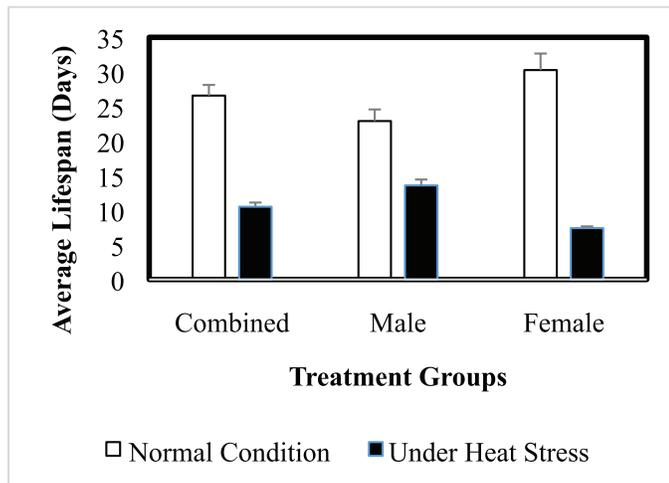


Figure 5. Life span of control flies under normal condition and heat stress condition.

Flies supplemented with CAEE had a significant life span reduction compared with the control group. The decrease in life span was more evident in female flies since all concentrations of CAEE resulted in a significant decrease in the life span. However, in male flies, only the high dose (1 mg/ml) of CAEE resulted in a significant decrease in the life span ($p < 0.05$; Fig. 4).

Stress assays

When supplemented flies were exposed to stressful conditions, the life span of male flies decreased but the life span of female flies increased. The stressful conditions were induced by subjecting flies to heat stress and starvation stress. Heat stress significantly reduced the life span of flies for both sexes; however, it was more evident in female flies under heat stress. Male flies had a 40.62% reduction in life span, while female flies had a 75.45% reduction in life span (Fig. 5).

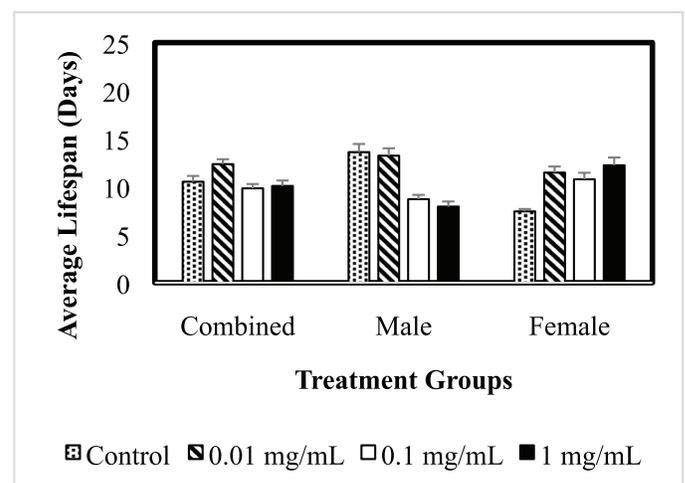


Figure 6. Life span of WAE-supplemented *D. melanogaster* after the presence of daily heat stress.

Supplementation with WAE showed a profound sex-specific effect on the life span of *D. melanogaster* under heat stress. There was an observed significant life span reduction in male flies. However, WAE significantly preserved the life span of female flies. It even significantly increased the life span of female flies as compared with the control flies under heat stress (Fig. 6).

Supplementation with CAEE preserved the life span of male flies under heat stress and it significantly increased ($p < 0.05$) the life span of female flies across all concentrations; however, no dose-response relationship was observed (Fig. 7).

The energy deprivation significantly decreased the life span of control flies in both sexes. Reduction in life span was more evident in female flies with 52.96% reduction in life span, while there was only 29.21% reduction in the life span of male flies (Fig. 8).

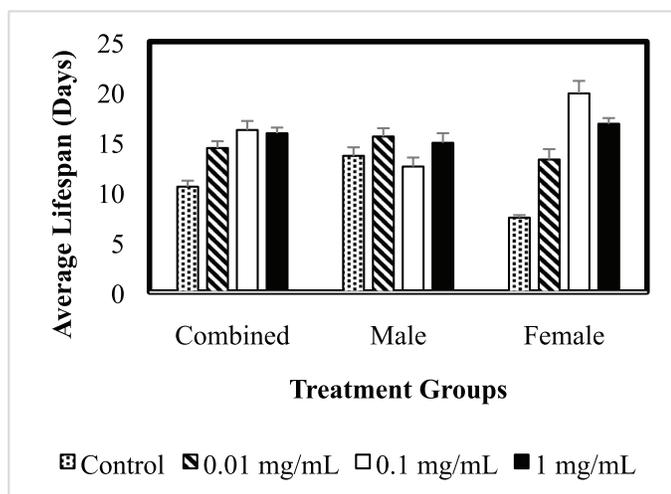


Figure 7. Life span of CAEE-supplemented *D. melanogaster* after the presence of daily heat stress.

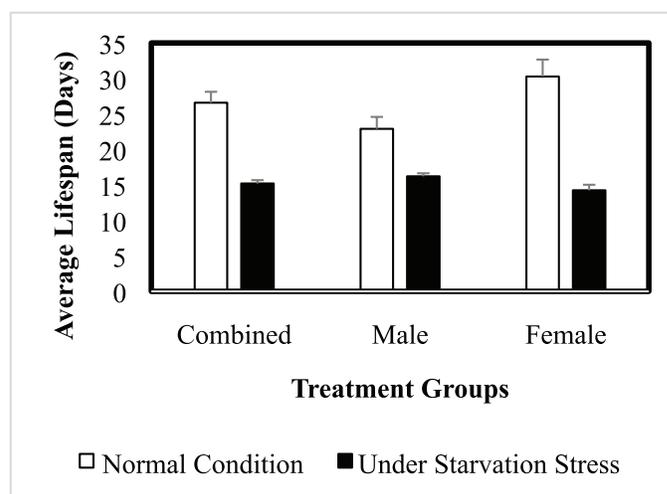


Figure 8. Life span of control flies under normal condition and energy-deprived condition.

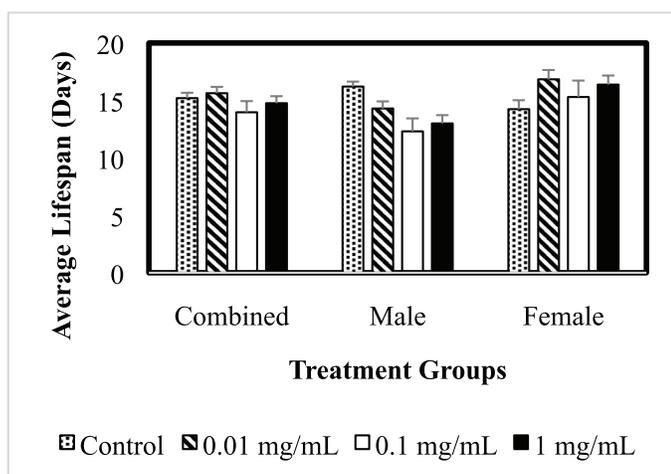


Figure 9. Life span of WAE-supplemented *D. melanogaster* after starvation stress.

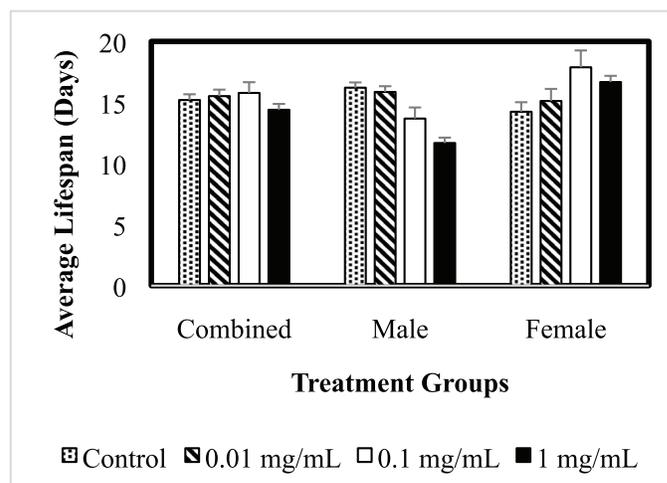


Figure 10. Life span of CAEE-supplemented *D. melanogaster* after starvation stress.

The supplementation with WAE increased the life span of female flies compared with control flies (Fig. 9). However, it significantly decreased the average life span of male flies. No dose-response relationship was observed in the effects of WAE in the life span of both male and female flies.

The supplementation with CAEE significantly decreased the life span of male flies under starvation (Fig. 10). A dose-response relationship was observed since higher concentrations of CAEE resulted in a greater decrease in the life span. However, CAEE significantly increased the life span of female flies under starvation.

Differential antioxidant activity of cultivated and wild *A. auricula-judae* ethanolic extracts

The differences in the nutritional values and concentration of bioactive compounds may explain the differences in the antioxidant activity of wild and cultivated *A. auricula-judae* (Boonsong *et al.*, 2016). These differences may cause the variances

in the antioxidant activities of wild and cultivated mushrooms. The controlled environment of cultivated *A. auricula-judae* was seen to be beneficial in terms of antioxidant activity. The previous study also showed a higher antioxidant activity of the cultivated mushroom compared with mushroom from the wild (Wasser and Weis, 1999). These results suggested that cultivating edible mushrooms in a controlled environment did not decrease the antioxidant activity of the bioactive compounds present in the wild source. In fact, it may even enhance the antioxidant activity of the edible mushroom.

During non-stressful conditions, *A. auricula-judae* ethanolic extracts decreased the life span of flies

The results of the LSA have revealed the potential toxic effect of cultivated *A. auricula-judae* to *D. melanogaster*. High concentrations of CAEE and WAE resulted in a significant decrease in the life span of male flies. WAE did not decrease the life span of female flies while CAEE resulted in the reduction in life span. Female flies feed more frequently

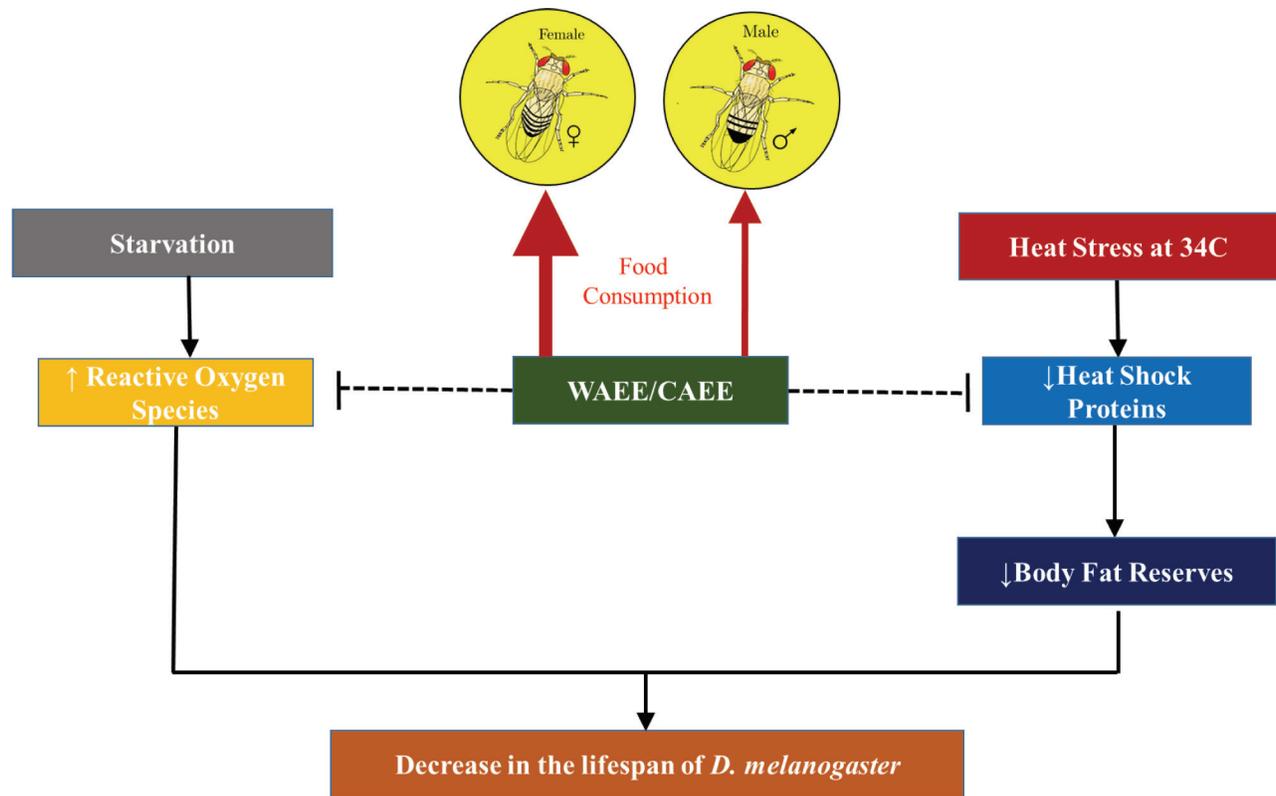


Figure 11. Proposed mechanism of action of CAEE and WAEE in the life span of *D. melanogaster* under normal condition and under starvation and heat stress.

than males (Wong *et al.*, 2009). Since the antioxidant activity of CAEE is three-fold higher compared with WAEE, female flies have a higher intake of the food containing antioxidant compounds. Antioxidants at high concentration can cause pro-oxidative effects to the flies.

There are several antioxidants that do not increase the life span of flies (Le Bourg, 2001). It was hypothesized that several antioxidants may have pro-oxidative effects and be potentially toxic to flies if there is a failure to utilize and clear these antioxidants from the body (Velasco and Medina, 2014). Similar effects were seen in a previous study where they reported a dosage-dependent decrease in the life span of *D. melanogaster* (Dahomey strain) fed with exogenous antioxidants mixed with the regular diet of the flies (Magwere *et al.*, 2004). Another possible explanation is the high levels of protein content in the mushroom extracts. The previous study observed that an increase in protein content can decrease longevity (Sanchez *et al.*, 2015).

Female flies are more susceptible to life span reduction from heat stress and starvation

Mild heat stress (exposure to 34°C) tends to increase life span more in males than in females, at least in *Drosophila*. One possible explanation is that female flies are more sensitive to heat stress compared with male flies. Heat resistance increased in male but only slightly in female flies (Sørensen *et al.*, 2007). This may explain the difference in the response of male and female flies to heat stress. Female flies have a more profound decrease in their life span upon exposure to heat stress because of higher sensitivity and lower resistance to heat stress.

The same trend was observed in male and female flies under starvation. A previous study showed that female flies showed a steeper decrease in starvation resistance compared with male flies. Male flies showed a decrease in the rate of metabolite utilization and lower energy requirement to survive under starvation stress as compared to female flies. The inability to decrease metabolic rate and energy requirement in female flies resulted in the more profound decrease in the life span in female flies (Aggarwal, 2014).

Flies used in this study were all virgin flies. It was noted in a previous study that mated female flies have higher starvation resistance compared with virgin female flies; however, the absence of mating did not decrease the starvation resistance of male flies. Thus, virgin female flies are expected to be more sensitive to starvation stress compared with virgin male flies. This explained the more pronounced decrease in the life span of female flies under starvation stress compared with male flies (Goenaga *et al.*, 2012).

Effect of CAEE and WAEE in the life span of *D. melanogaster* under normal condition and under starvation and heat stress is shown in Figure 11.

Starvation and heat stress decreased the life span of flies but the effect was more profound in female flies. Heat stress decreased heat shock proteins and body fat reserves, while starvation impaired the cellular antioxidant system which increased the levels of reactive oxygen species (ROS). These processes decreased the life span of *D. melanogaster*. The observed increase in the life span of flies treated with CAEE and WAEE may be explained by the antioxidant activity of the extracts, which can decrease the levels of ROS and enhance the levels of heat shock proteins. However, without starvation and heat stress, CAEE and

WAEF decreased the life span of flies and this may be attributed to pro-oxidative effects of antioxidant compounds when they are not utilized and have accumulated in the body.

Under starvation and heat stress, CAEE and WAEF decreased the life span of male flies but prolonged the life span of female flies

The effect of the supplementation of WAEF and CAEE on the life span of *D. melanogaster* provides an additional knowledge on the understanding of the role of antioxidants in the physiology and metabolism of flies during heat stress. Exposure to high temperatures around 29°C and above can result to damage in heat shock proteins in flies. Heat shock response is essential to protect flies from body fat decline. Heat stress can also increase cellular stress and damage which can promote apoptosis of fat body cells. These cellular damage, decrease in body fat reserve, and damage in heat shock protein may explain the decrease in the life span of flies exposed to heat stress (Klepsatel *et al.*, 2016). A previous study showed that the increased levels of heat shock proteins are associated with increased life span in flies (Hercus *et al.*, 2003). Antioxidant compounds can increase the levels of heat shock proteins which can help prevent damage from oxidative stress (Geiger and Gupte, 2011). Based on these two studies, we hypothesized that the high antioxidant activity of CAEE and WAEF increased the heat shock proteins in female flies which resulted in higher average life span in female flies compared with male and control flies. This area will be the focus of future studies.

The antioxidant activity of the extract can be considered as stress enzymes. The previous studies have hypothesized that they can protect the flies during stressful conditions but they are of no use under normal conditions (Le Bourg, 2001). CAEE did not increase the life span of flies under normal conditions but it significantly increased the life span of male and female flies under starvation and heat stress. Although supplementation with WAEF and CAEE did not prevent the reduction of life span brought by the heat stress in male flies. However, flies supplemented with WAEF and CAEE still have higher average life span compared with the control flies. The presence of antioxidant compounds from WAEF and CAEE may have compensated for the decreased resistance to oxidative stress resulting to higher average life span in female flies. Since female flies feed more frequently than male flies, they have a higher intake of WAEF and CAEE containing antioxidant compounds, this may explain the greater increase in the life span of female flies.

There are reports that antioxidant enzymes help to increase the life span of flies by allowing the organisms to adapt during stressful conditions (Wong *et al.*, 2009). Prolonged starvation can also result in oxidative stress and failure in cellular antioxidant defenses. This failure can probably be caused by inhibition of G6PDH activity. Starvation increased the ROS generation, and the antioxidant system of *D. dentex* was not able to totally scavenge the free radical which leads to lipid peroxidation (Morales *et al.*, 2004). Since CAEE and WAEF have high antioxidant activity, it may have allowed the flies to cope with starvation stress and counter the oxidative stress from prolonged starvation. The higher life span of female flies may be due to higher intake of food supplemented with CAEE and WAEF.

CONCLUSIONS

Our data showed the sexually dimorphic effects of CAEE and WAEF in the life span of *D. melanogaster* under normal, starvation, and heat stress conditions. CAEE exhibited a higher antioxidant activity than WAEF in DPPH assay. Under normal condition, CAEE did not have an effect in the life span of male flies but it decreased the life span of female flies while WAEF showed no effects in the male and female flies. The life span reduction due to CAEE supplementation may be due to the pro-oxidative effects of antioxidants in female flies. Starvation and heat stress significantly decreased the life span of male and female flies. Excessive production of ROS and a decrease in heat shock proteins were hypothesized to be the cause of the observed life span reduction. Supplementation with CAEE and WAEF countered the life span reduction in female flies but not in male flies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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