




Ellagic acid prolongs the lifespan of *Drosophila melanogaster*

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Abstract Wild-type Canton-S flies of *Drosophila melanogaster* were treated with ellagic acid at 100 μM and 200 μM concentrations. Longevity assay showed male flies fed with 200 μM ellagic acid displayed longer mean lifespan and maximum lifespan than control flies. Female flies fed with 200 μM ellagic acid laid less number of eggs than control. The eclosion time was less in female flies fed with 200 μM ellagic acid. Ellagic acid fed female flies performed better than male flies and control flies for heat shock tolerance and starvation stress. Male flies treated with 100 μM ellagic acid recovered faster from cold shock compared with control flies. Male and female flies treated with ellagic acid displayed increased survival following exposure to 5% hydrogen peroxide. Gene expression studies displayed upregulated expressions of CAT, dFOXO, ATG1, and SOD2 in ellagic acid-treated male flies, and upregulated expressions of dFOXO, CAT, and SOD2 in ellagic acid-treated female flies. Results from these studies show the pro-longevity effect of ellagic acid on *Drosophila melanogaster*.

Keywords Ellagic acid · *Drosophila melanogaster* · Longevity · Stress · Gene expression

Introduction

Aging is a complex process determined by free radical species (ROS) that cause irreparable oxidative damage at cellular and molecular levels (Kregel and Zhang 2007). Gradual buildup of various stresses like heat shock, cold shock, calorie restriction, oxidative stress, and hypoxia for extended period demands flexibility of organism to adapt. This adaptation could happen through signaling molecules that generate complex profile of inducible and enzymatic protection to suit the particular condition (Pomatto and Davies 2018). The free radical theory of aging might suggest the influence of adaptive homeostasis to accelerate the aging process in young persons or slow down the aging process in old persons. The young persons might have the potential to expand their protective capacity at cellular and molecular levels when faced with multitude of adverse stresses, whereas the old ones show reduced ability and accumulate stress-induced damages to a larger extent (Pomatto and Davies 2017). Therefore, there is a distinct variation on how the young and aged organisms adjust and arrive at the homeostasis.

There are several plant-derived compounds and extracts available to promote longevity of lower model organisms via insulin-like growth factor (IGF) signaling pathway and anti-oxidant defense mechanism (Chattopadhyay and Thirumurugan 2018). Following a

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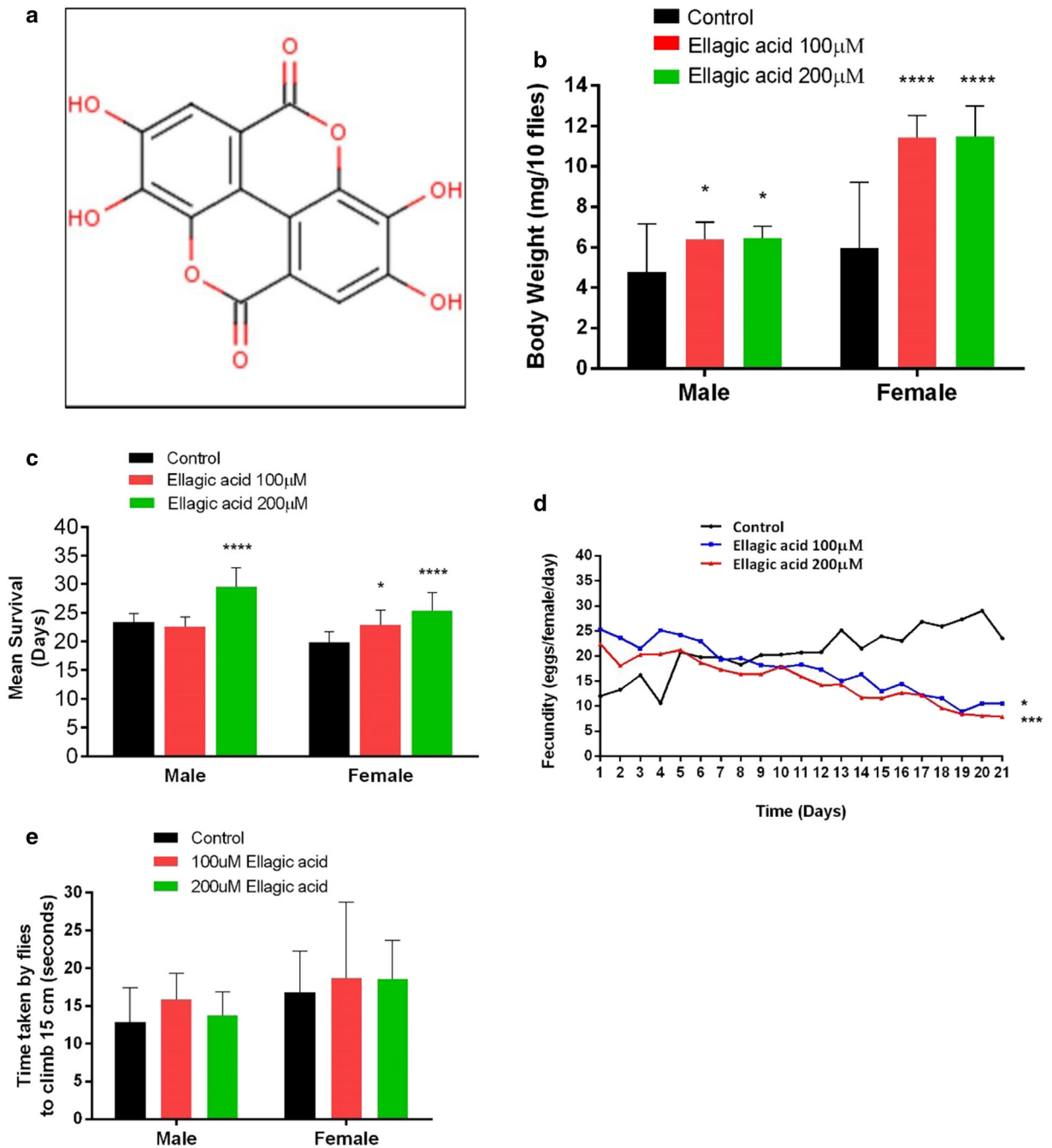
cue from early studies on the potential of natural compounds as anti-aging interventions, we had chosen ellagic acid for this study. Ellagic acid (EA) is a natural anti-oxidant (Fig. 1a) available in strawberry, blackcurrant, pomegranate, walnut, and grapes (Sepand et al. 2016; Sanadgol et al. 2017; Baeri et al. 2018). It is a tannic acid derivative having anti-oxidant, anti-inflammatory, and anti-depressant effects (Han et al. 2006; Rogerio et al. 2008; Feng et al. 2009; Uzar et al. 2012; Baeri et al. 2017). On oral administration of ellagic acid to mice, the microbiota present in the gut converts ellagic acid to urolithins for better gastrointestinal tract absorption (Saha et al. 2016). Ellagic acid is available more in the kidney and liver (Yan et al. 2014). Ellagic acid along with urolithin prevented cell proliferation of T24 cells in bladder cancer (Qiu et al. 2013). Addition of ellagic acid to phosalone-induced senescent rat embryonic fibroblast cells reduced the toxicity of phosalone through modulation of key inflammatory markers, and reducing gene and protein expressions of p38 and p53 (Baeri et al. 2017). Photoprotective effect of ellagic acid was studied on human skin cells exposed to ultraviolet-B light (Bae et al. 2010). Combination of ellagic acid with anesthetic adjuvant ketorolac reduced paw edema in Sprague-Dawley rats (Corbett et al. 2010). Ellagic acid inhibited renal activation of NF- κ B in high-fat diet/streptozotocin-induced Wistar albino rats. Ellagic acid acts like an anti-glycating agent through inhibition of carboxyethyl lysine (Muthenna et al. 2011). Though ellagic acid provides several benefits, its role in prolonging lifespan in fruit flies has not been reported and our study attempts to explore this.

The option of living longer is narrowing down with rapid consumption of unhealthy food combined with irregular lifestyle. Regular consumption of fruits and vegetables at affordable prices is a challenge for poorest sections of the human population. To find an affordable solution demands attention to work on lower model organisms like fruit flies. *Drosophila melanogaster* is a good model to study for lifespan extension due to its small body size, ease in handling, gastrointestinal tract hosting many microbiota, adaptation to change in media composition, and response to stress (Staats et al. 2018a). Male and female flies of *Drosophila* show extreme variations towards longevity (Austad and Fischer 2016). This may be due to following reasons: unmated females live longer than their counterparts, loss of gut integrity leading to fly mortality, reduced insulin signaling promoting female longevity (Giannakou et al.

Fig. 1 **a** Structure of ellagic acid. Molecular formula $C_{14}H_6O_8$. Molecular weight 302.19 g/mol. Effect of ellagic acid on various physiological parameters in flies. **b** Effect of ellagic acid on body weight of wild-type *Canton-S* flies. Data represented as body weight (mg/10 flies). Data were subjected to two-way ANOVA followed by Bonferroni's multiple comparisons test in the GraphPad Prism software v.6. Error bars denote standard deviation. Symbols represented as * $p < 0.05$ and **** $p < 0.0001$. Twenty vials containing 10 flies/vial were counted for each control, and treatment with EA 100 μ M and EA 200 μ M. The total number of flies used was 1200. **c** Effects of ellagic acid on fly longevity. Ten vials containing 30 flies/vial were monitored for lifespan. The total number of flies used was 1800. Mean lifespan was calculated by the Kaplan–Meier lifespan analysis in the OA-SIS2 software. Mean values of individual replicate of the control, and two treatments of male and female flies were fed into the GraphPad Prism v.6 software. Data were subjected to two-way ANOVA followed by Bonferroni's multiple comparisons test. Data represented as mean lifespan (in days) with error bars denoting SD. Symbols represented as * $p < 0.05$ and **** $p < 0.0001$. **d** Effect of ellagic acid on female fecundity. For each time point and each treatment, eggs from ten vials containing 1 male and 1 female flies/vial were counted to get the value for row means with SD. The mean number of eggs laid per female per day was used to get the fecundity curve. Data were subjected to one-way ANOVA followed by Holm–Sidak's multiple comparisons test in the GraphPad Prism software v.6. Significance $p < 0.005$. **e** Effect of ellagic acid on climbing efficiency in flies. Data represented as mean time (in seconds) taken by 10 flies per replicate per treatment to climb 15 cm, with error bars denoting SD. For each treatment and sex along with their respective control, twenty vials containing 10 flies/vial were counted. The total number of flies used was 1200. Data were subjected to two-way ANOVA followed by Bonferroni's multiple comparisons test in GraphPad Prism software v.6

2004). Also, there are factors like fecundity, mating status, and genotype that determine which sex to live longer (Austad and Fischer 2016). In addition, differences observed between male and female lifespans might be due to asymmetric inheritance of mitochondrial genomes, hormonal and metabolic variations, and maternal effects (Tower and Arbeitman 2009).

There was a recent paper on anti-aging potential of ellagic acid at low concentrations (0.1 to 1 μ M) on D-galactose-induced aging in human neuroblastoma cell line (Rahimi et al. 2018). This had prompted us to evaluate the role of ellagic acid to extend the lifespan of *Drosophila melanogaster* through longevity assay; fecundity; climbing ability; larval development; response to heat shock, cold shock, starvation stress, and oxidative stress; and gene expression. Molecular studies had shown upregulation and downregulation of several candidate genes in extending the lifespan of fruit flies



(Ma et al. 2018). Identification of these signature genes that correlate with lifespan extension might provide clues on signaling pathways and mechanisms involved in modulating longevity. Our results show increased longevity, reduced fecundity, reduced eclosion time, improved recovery time from cold shock, and increased

resistance to heat shock, cold shock, starvation stress, and hydrogen peroxide-induced oxidative stress in ellagic acid-treated flies. Gene expression studies showed upregulation of catalase (CAT), forkhead box transcription factor (dFOXO), autophagy (ATG1), and superoxide dismutase (SOD2) in ellagic acid-treated

male flies, and upregulation of dFOXO, CAT, and SOD2 in ellagic acid-treated female flies.

Materials and methods

Fly husbandry and diet preparation

Wild-type Canton-S flies of *Drosophila melanogaster* were reared at temperature $25\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ on 12:12-h light:dark cycle. Prior to assays, flies (25 males, 25 females) were bulk propagated in 300-ml polypropylene bottles having 30 ml of maintenance diet (10% semolina (w/v), 10% jaggery (w/v), 1.5% agar (w/v), 3% methylparaben (v/v), and 0.3% propionic acid (v/v)). Flies from propagation bottles were allowed to mate and lay eggs. Approximately 50 eggs were carefully collected and transferred to new 300-ml bottles having fresh maintenance diet. Egg collection was carried out within 24 h. Eggs were allowed to hatch in maintenance diet followed by larval pupation. For all assays, newly eclosed flies were collected over 24 h as 1-day-old flies. Flies were then segregated according to their sex and transferred to standard sugar–yeast (SY) diet (10% sucrose (w/v), 10% yeast extract (w/v), 2% agar (w/v), 3% methylparaben (v/v), and 0.3% propionic acid (v/v)) (Chattopadhyay et al. 2015). For ellagic acid supplementation, ellagic acid (EA) (HiMedia, India, RM2817) was prepared by dissolving EA in 5 ml of phosphate-buffered saline having 400 μl of 1 M NaOH. This was added into SY diet at final concentrations of 50 μM , 100 μM , 200 μM , 400 μM , 600 μM , and 800 μM by thorough mixing. Standard control diets contained only phosphate-buffered saline with 10 μl of 1 M NaOH as vehicle. For treatment of flies with ellagic acid, newly eclosed, adult young flies were collected from maintenance diet post mating within 24 h of eclosion and transferred to diet supplemented with ellagic acid. The flies were not virgin flies. For all assays except developmental and gene expression assays, flies were cultured in 50-ml polypropylene vials having 10 ml of diet with or without ellagic acid. For developmental and gene expression assays, larvae and/or flies were cultured in 300-ml glass bottles with 30 ml of diet with or without ellagic acid. Results from the pilot studies on ellagic acid for longevity prompted us to select only 100- μM and 200- μM doses.

Body weight

Newly eclosed flies were fed on SY diet with and without ellagic acid for 15 days. For each concentration of ellagic acid (100 μM , 200 μM) and control (without ellagic acid) and sex (male, female), twenty vials containing 10 flies/vial were counted. The total number of flies used was 1200. Body weight of 10 flies in a batch was measured using an analytical balance that measures to the nearest 0.01 mg weight. Data were subjected to two-way ANOVA followed by Bonferroni's multiple comparisons test in the GraphPad Prism software v.6. Error bars denote standard deviation.

Longevity assay

Newly eclosed flies (1 day old) were reared in SY diet with and without ellagic acid. Flies were transferred to fresh SY diet for every 2 days and the number of dead flies was recorded. The total number of days taken until the death of last fly was noted. For the pilot study, six treatments of ellagic acid were given at concentrations of 50 μM , 100 μM , 200 μM , 400 μM , 600 μM , and 800 μM . For each treatment and sex, three vials containing 20 flies/vial were monitored for lifespan. Following the results of the pilot study (Supplementary Figure 1), longevity assay was performed for only two concentrations of ellagic acid at 100 μM and 200 μM . For each treatment and sex along with their respective control, ten vials containing 30 flies/vial were monitored for lifespan. The total number of flies used was 1800. Mean lifespan was calculated by the Kaplan–Meier lifespan analysis in the OASIS2 software (<https://sbi.postech.ac.kr/oasis2/>) (Han et al. 2016). Chi-square and p value were obtained from log-rank test and Gehan–Breslow–Wilcoxon test, respectively. Mean values of individual replicate of control, and two treatments of male and female flies were fed into the GraphPad Prism v.6 software. Data were subjected to two-way ANOVA followed by Bonferroni's multiple comparisons test.

Measurement of fly fecundity

Newly eclosed flies (1 day old) were fed with SY diet with and without ellagic acid for 21 days. This long-term treatment is given to make the flies adapted to ellagic acid. Flies were transferred to fresh diets every day and number of eggs laid was counted every day. For two treatments (100 μM and 200 μM), along with

control, eggs from ten vials containing 1 male and 1 female were counted. For each time point and each treatment, eggs from ten vials were counted to get the value for row means with SD. The mean number of eggs laid per female per day was used to get the fecundity curve. Data were subjected to one-way ANOVA followed by Holm–Sidak’s multiple comparisons test in the GraphPad Prism software v.6.

Measurement of climbing efficiency in flies

Newly eclosed flies (1 day old) were fed with SY diet containing ellagic acid for 21 days. This long-term treatment is given to make the flies adapted to ellagic acid. Flies were transferred to empty tubes marked at 15 cm height. The tubes were tapped thrice to gather the flies at the bottom. Time taken by flies to climb the 15-cm mark was noted down using stopwatch. The assay was repeated three times with an interval of 30 min between each repetition. For each treatment and sex along with their respective control, twenty vials containing 10 flies/vial were counted. The total number of flies used was 1200. Data were subjected to two-way ANOVA followed by Bonferroni’s multiple comparisons test in the GraphPad Prism software v.6

Measurement of larval development

Freshly hatched, age-matched 1st instar larvae were collected from bulk fly stock cultures and carefully transferred to bottles with standard diet with or without ellagic acid. Larvae were allowed to rear and pupate. For control and each treatment, five replicates containing 40 larvae/replicate were used. Percentage pupation is $(\text{total number of pupae} / \text{total number of larvae}) \times 100$. Each pupa was numerically marked on the side of bottles and observed further. Percentage eclosion is $(\text{number of flies emerged} / \text{total number of pupae}) \times 100$. Time required by individual larvae to eclose as an adult fly was noted to know the eclosion time. Data were subjected to one-way ANOVA followed by Dunnett’s multiple comparisons test in the GraphPad Prism software v.6.

Starvation resistance

Newly eclosed flies (1 day old) were given with SY diet with or without ellagic acid for 21 days. This long-term treatment is given to make the flies adapted to ellagic acid. Then, the flies were kept in only 1% agar and

transferred to fresh agar every 5 h. During each transfer, the number of dead flies was recorded and filter paper strips dipped in sterile distilled water were kept to avoid death due to desiccation. For each treatment and sex along with their respective control, ten vials containing 20 flies/vial were counted. The total number of flies used was 1200. Mean lifespan was calculated by the Kaplan–Meier lifespan analysis in the OASIS2 software (<https://sbi.postech.ac.kr/oasis2/>) (Han et al. 2016). Mean values of individual replicate of control, and two treatments of male and female flies were fed into the GraphPad Prism v.6 software. Data were subjected to two-way ANOVA followed by Bonferroni’s multiple comparisons test.

Heat shock resistance

Newly eclosed (1 day old) male flies and female flies were fed on standard diet with or without ellagic acid for 21 days following which they were exposed to 37 °C. This long-term treatment is given to make the flies adapted to ellagic acid. The number of fly death was recorded every 4 h until all flies were dead. For each treatment and sex along with their respective control, ten vials containing 20 flies/vial were counted. The total number of flies used was 1200. Mean lifespan was calculated by the Kaplan–Meier lifespan analysis in the OASIS2 software. Mean values of individual replicate of control, and two treatments of male and female flies were fed into the GraphPad Prism v.6 software. Data were subjected to two-way ANOVA followed by Bonferroni’s multiple comparisons test.

Cold shock recovery

Newly eclosed flies (1 day old) were fed on SY diet with or without ellagic acid for 21 days, and then, they were exposed to 4 °C until all activity was stopped. This long-term treatment is given to make the flies adapted to ellagic acid. Time taken by flies to recover from cold shock was noted. For each treatment and sex along with their respective control, ten vials containing 20 flies/vial were counted. Assay was repeated three times for every vial with an interval of 1 h between each repetition. Mean values of individual replicate of control, and two treatments of male and female flies were fed into the GraphPad Prism v.6 software. Data were subjected to two-way ANOVA followed by Bonferroni’s multiple comparisons test.

Oxidative stress resistance

Newly eclosed flies (1 day old) were fed on SY diet with or without ellagic acid for 21 days. This long-term treatment is given to make the flies adapted to ellagic acid. Then, the flies were subjected to oxidative challenge by captivating them in a chamber containing filter paper strips dipped in 5% H₂O₂ in a 5% glucose solution. The number of dead flies was recorded every 4 h until all flies were dead. For each treatment and sex along with their respective control, ten vials containing 20 flies/vial were counted. The total number of flies used was 1200. Mean lifespan was calculated by the Kaplan–Meier lifespan analysis in the OASIS2 software. Mean values of individual replicate of control, and two treatments of male and female flies were fed into the GraphPad Prism v.6 software. Data were subjected to two-way ANOVA followed by Dunnett's multiple comparisons test.

Gene expression analysis

Newly eclosed adult flies (1 day old) were fed with standard diet with or without ellagic acid (100 µM, 200 µM) for 21 days. For control (male, female) and each treatment (male and female flies treated with ellagic acid (100 µM, 200 µM)), three vials were set up, with 30 flies/vial. Experiments were performed two times ($n = 3 + 3$). RNA was isolated and 1 µg of RNA was converted to cDNA. Real-time PCR was used to quantify mRNA of ATG1, CAT, TOR, FOXO, SOD2, and TSC1. Total RNA was extracted using RNAiso Plus (Takara Bio Inc., USA). Flies from each group were homogenized in 1 ml of RNAiso Plus solution and then centrifuged at 12,000g at 4 °C for 20 min. The supernatant was mixed with 500 µl of chloroform for 5 min and then centrifuged at 12,000g at 4 °C for 30 min. The upper layer was transferred to a new tube, and 600 µl of isopropanol was added to the supernatant and incubated for 2 min at 4 °C and centrifuged for 12,000g at 4 °C for 15 min. The pellet was saved and washed in 500 µl of 70% ethanol solution followed by re-centrifugation. The purity and concentration of RNA isolated were determined by using NanoDrop 2000 (Thermo Fisher Scientific).

The cDNA was constructed by using cDNA Reverse Transcription Kit (RT PrimeScript Takara Bio Inc., USA). For each reaction, 1 µg RNA was mixed together with RT PrimeScript buffer, RT PrimeScript reverse

transcriptase enzyme, random hexamers, and Oligo dT Primer. The final volume was adjusted to 10 µl. The cDNA was synthesized in the thermocycler Eppendorf AG 22331 (Hamburg) PCR system at 37 °C for 15 min followed by 95 °C for inhibition of enzyme for 5 s and stored at – 20 °C.

Gene expression was measured using a Real-time PCR system (Bio-Rad, USA) with 1:5 dilution of cDNA for one and half hours (first step, 95 °C for 3 min; second step, 95 °C for 10 s; third step, 60 °C for 30 s; fourth step, 72 °C for 20 s; steps 2–4 were repeated for 39 cycles; then, the melt curve was detected from 65 to 95 °C in every 5 s with precedence of 0.5 °C). Six target genes were used. Primers were designed using the Primer3 online tool. Primer sequences used for these genes are listed in Table 1. Rpl32 was used as a housekeeping gene and internal control. Gene expression was calculated on the basis of the relative threshold cycle (C_q) value. Levels of gene expression in all groups were normalized with the control group for each gene.

Statistical analysis

Mean lifespan was calculated by the Kaplan–Meier lifespan analysis in the OASIS2 software (<https://sbi.postech.ac.kr/oasis2/>). Log-rank test and Gehan–Breslow–Wilcoxon tests were used to estimate the significance of differences in survival data between samples. Remaining assays were analyzed by ANOVA with suitable post hoc tests. All data were analyzed using the GraphPad prism software. For all assays, p values for levels of significance are represented as * < 0.05 , ** < 0.01 , *** < 0.001 , and **** < 0.0001 .

Results

Effect of ellagic acid on body weight of flies

Ellagic acid at concentrations 100 µM and 200 µM showed a significant increase in body weight of both male and female flies compared with that of control flies (Fig. 1b) (Supplementary Table 1A). Male control flies had shown 4.8 mg, and female control flies had shown 6.0 mg, whereas flies fed with 100 µM ellagic acid weighed 6.4 mg ($p < 0.05$) in male and 11.4 mg ($p < 0.0001$) in female. Flies fed with 200 µM ellagic acid weighed 6.5 mg ($p < 0.05$) in male and 11.5 mg ($p < 0.0001$) in female.

Table 1 List of primers used in gene expression study

Target	Forward primer	Reverse primer
dATG1	CCGAGATAATCGCACACGGA	CGTAACACCACCACCGTCTT
CAT	ACTGCCCGTACAAGGTGAAG	CATTCTGGGGACCGTTGAA
dTOR	CACCGACTTCCAGACGGAAA	CACTGGCAGAGGTTCTCGTT
dFOXO	TTCGCTTGCGGTTAAAAGCC	GGGCCTCAAAGATCACTGC
SOD2	ATAAGCATCTGCGGATCGGA	ACGCCTTGTGAAGCGTTATC
dTSC1	TGAACGCAACTACCGCTAT	CATGCTGTTTCAGCACATCCG
RPL32	GCCCACCGGATTCAAGAAGT	CTTGCCTTCTTGGAGGAGA

Effect of ellagic acid on fly longevity

Longevity assay was performed to know the influence of ellagic acid on lifespan extension of flies. Male and female flies were reared on SY diet containing 50- μ M, 100- μ M, 200- μ M, 400- μ M, 600- μ M, 800- μ M concentrations of ellagic acid. In this pilot study, compared with control flies (for male group) that displayed 31 days of mean survival, male flies treated with 100 μ M ellagic acid showed 39 days, and male flies treated with 200 μ M ellagic acid showed 37 days. Other concentrations showed a range of 31 to 35 days (Supplementary Figure 1). Female flies followed the same trend. Control flies displayed 27 days of mean survival; female flies fed with 100 μ M ellagic acid showed 35 days, and those fed with other concentrations showed a range of 30 to 32 days (Supplementary Figure 1). Survival curve of male flies treated with 800 μ M ellagic acid showed 84% survival at 52nd day (Supplementary Figure 2A). Female flies treated with 50 μ M ellagic acid had shown 88% survival at 54th day (Supplementary Figure 2B).

The pilot study had displayed a decline in the mean survival of both male and female flies treated with higher concentrations of ellagic acid (above 200 μ M). Therefore, we have used only 100- μ M and 200- μ M concentrations of ellagic acid for further analysis. The mean lifespan of male flies treated with 100 μ M of ellagic acid did not differ significantly as compared with that of control flies (Fig. 1c). Increased survival in male flies was observed at the ellagic acid concentration of 200 μ M with mean lifespan of 30 days (30% increase, $p < 0.0001$) when compared with mean lifespan of 23 days in control (Fig. 1c) (Table 2). Mean lifespan of female flies

treated with 100 μ M of ellagic acid showed significance (15% increase, $p < 0.05$) (23 days) compared with that of control flies (20 days) (Fig. 1c). Female flies treated with 200 μ M ellagic acid displayed lifespan increase by 25% (mean survival 25 days, $p < 0.0001$) compared with control flies (20 days) (Table 2). Survival curve given in Supplementary Figure 3A showed 55% survival of male flies treated with 200 μ M ellagic acid. These flies lived for 52 days compared with control flies (42 days). Survival curve given in Supplementary Figure 3B showed 53% survival of female flies treated with 200 μ M ellagic acid. These flies lived for 46 days compared with control flies (36 days).

Effect of ellagic acid on fecundity and locomotion in flies

To know the relation between longevity and fecundity, the mean number of eggs laid per female per day was recorded up to 21 days. Control flies had laid 21 eggs; female flies fed with ellagic acid had shown a reduction in the number of eggs laid compared with control (Fig. 1d). Flies fed with 100 μ M ellagic acid had shown 17 eggs ($p < 0.05$); and flies fed with 200 μ M ellagic acid produce 15 eggs ($p < 0.005$).

Male control flies had taken 13 s to climb 15 cm. Male flies fed with 100 μ M ellagic acid had taken 16 s. When treated with 200 μ M ellagic acid, they climbed at 14 s (Fig. 1e) (Supplementary Table 1B). Female control flies had taken 17 s to climb 15 cm. Male flies fed with 100 μ M and 200 μ M ellagic acid had taken 19 s (Fig. 1e) (Supplementary Table 1B). Both male and female flies did not show any significant change in time taken to climb the 15-cm mark.

Table 2 Effect of ellagic acid on the lifespan of *Drosophila melanogaster*

Group	Mean survival (days)	Standard error	Age in days at 100% mortality (days)	Number of flies	Log rank χ^2	<i>p</i> value	Wilcoxon χ^2	<i>p</i> value
Male								
Control	23.41	0.66	42	300				
Ellagic acid 100 μ M	22.55	0.64	42	300	2.08	0.1489	0.78	0.3775
Ellagic acid 200 μ M	29.55	0.69	52	300	59.32	0	37.33	0
Female								
Control	19.83	0.59	36	300				
Ellagic acid 100 μ M	22.89	0.65	42	300	23.65	0.0000012	11.61	0.0007
Ellagic acid 200 μ M	24.78	0.7	46	300	53.43	0	26.41	2.8e ⁻⁷

Combined survival data of 10 individual cohorts of 30 flies. The OASIS2 software (<https://sbi.postech.ac.kr/oasis2/>)

Effect of ellagic acid on larval development

In the life cycle of *Drosophila*, larval stage is very important as it might have influence on other stages of the fly. We have analyzed the effects of different concentrations of ellagic acid on percentage pupation, percentage eclosion, and eclosion time. There is no significant change in percentage pupation of all flies (control as well treated) (Fig. 2a). Also, there is no significant change in percentage eclosion of control and treated flies (Fig. 2b). Time taken for fly emergence from pupae was reduced in flies fed with 200 μ M ellagic acid (187 h) compared with that in control flies (235 h) (Fig. 2c).

Effect of ellagic acid on stress resistance in flies

Flies fed with ellagic acid might show resistant to heat shock, cold shock, starvation stress, and oxidative stress. Both male and female flies fed with ellagic acid showed increased mean survival following heat stress (Fig. 3a). Female flies have shown a mean lifespan of 24.7 h ($p < 0.001$) at 100 μ M ellagic acid, and 23.9 h ($p < 0.01$) at 200 μ M ellagic acid. Control flies had shown 17.4 h (Fig. 3a) (Supplementary Table 1C). Male flies have shown a mean lifespan of 22 h ($p < 0.05$) at 100 μ M ellagic acid, and 18.5 h at 200 μ M ellagic acid. Control flies had shown 17.6 h (Fig. 3a) (Supplementary Table 1C).

Recovery time following cold shock was significantly faster in male flies treated with ellagic acid compared with that in control (Fig. 3b). Male flies treated with 100 μ M ellagic acid had taken 1.28 min to recover ($p < 0.01$) compared with control flies (2.22 min). Male flies fed with 200 μ M ellagic acid had taken 1.46 min ($p < 0.05$) compared with control flies (Fig. 3b) (Supplementary Table 1D). Female flies treated with 100 μ M ellagic acid had taken 1.38 min to recover compared with control flies (1.46 min). Female flies fed with 200 μ M ellagic acid had taken 1.53 min compared with control flies (Fig. 3b) (Supplementary Table 1D).

Flies were evaluated for their response to starvation stress. Control flies had shown a mean survival of 23 h following starvation, whereas male and female flies fed with ellagic acid and then undergone starvation had shown a longer survival (Fig. 3c). Compared with male flies, we observe increased resistance to starvation stress in female flies fed with 100 μ M (36 h, $p < 0.001$) and 200 μ M ellagic acid (34 h, $p < 0.01$). Male flies fed with

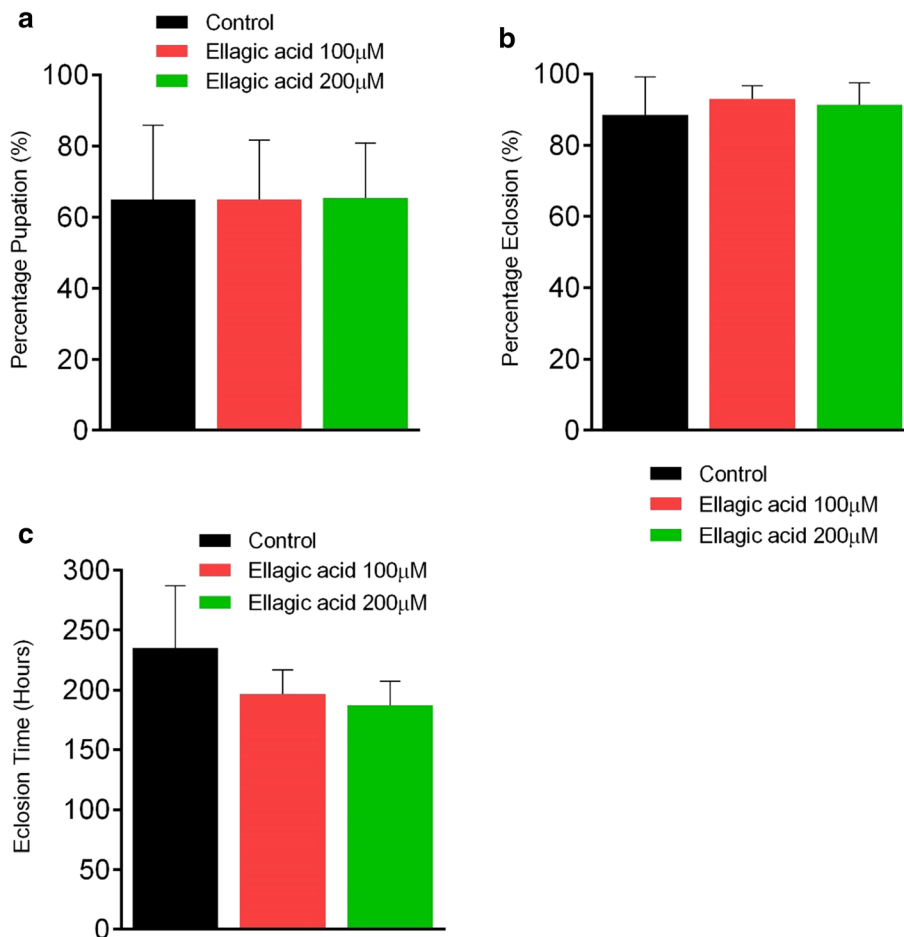


Fig. 2 Effect of ellagic acid on larval development. For control and each treatment, five replicates containing 40 larvae/replicate were used. **a** Percentage pupation of larva reared on diet supplemented with different ellagic acid concentrations. Percentage pupation = (total number of pupae/total number of larvae) \times 100. Data represented as percentage pupation with error bars denoting confidence intervals at $p = 0.05$. **b** Percentage eclosion of larva reared on diet supplemented with different ellagic acid concentrations. Percentage eclosion = (number of flies emerged/total

number of pupae) \times 100. Data represented as percentage eclosion with error bars denoting confidence intervals at $p = 0.05$. **c** Eclosion time of larvae reared on diet supplemented with different ellagic acid concentrations. Time required by individual larvae to eclose as an adult fly was noted to know the eclosion time. Data represented as mean eclosion time in hours with error bars denoting SD. Data were subjected to one-way ANOVA followed by Dunnett's multiple comparisons test in the GraphPad Prism software v.6

100 μ M ellagic acid had shown a longer survival (31 h, $p < 0.05$) than control (Supplementary Table 1E).

We have evaluated the flies for their potential to survive when treated with 5% H_2O_2 . Compared with control, both male and female flies showed a longer survival ability when treated with ellagic acid at 100 μ M and 200 μ M (Fig. 3d). Female flies fed with ellagic acid when subjected to oxidative stress with 5% H_2O_2 had shown a better mean survival than male flies fed with ellagic acid both at 100 μ M and at 200 μ M. The mean survival of female flies was 27.6 h ($p < 0.0001$) at 100 μ M ellagic acid and 27.4 h ($p < 0.0001$) with

200 μ M of ellagic acid as compared with 14.6 h in H_2O_2 -only control (Fig. 3d) (Supplementary Table 1F). The mean survival of male flies was 26.8 h ($p < 0.0001$) at 100 μ M ellagic acid and 22.8 h ($p < 0.01$) with 200 μ M of ellagic acid as compared with 18.4 h in H_2O_2 -only control (Fig. 3d) (Supplementary Table 1F).

Real-time PCR

In our study, male flies treated with 100 μ M ellagic acid showed upregulated expressions of CAT (23.2-fold

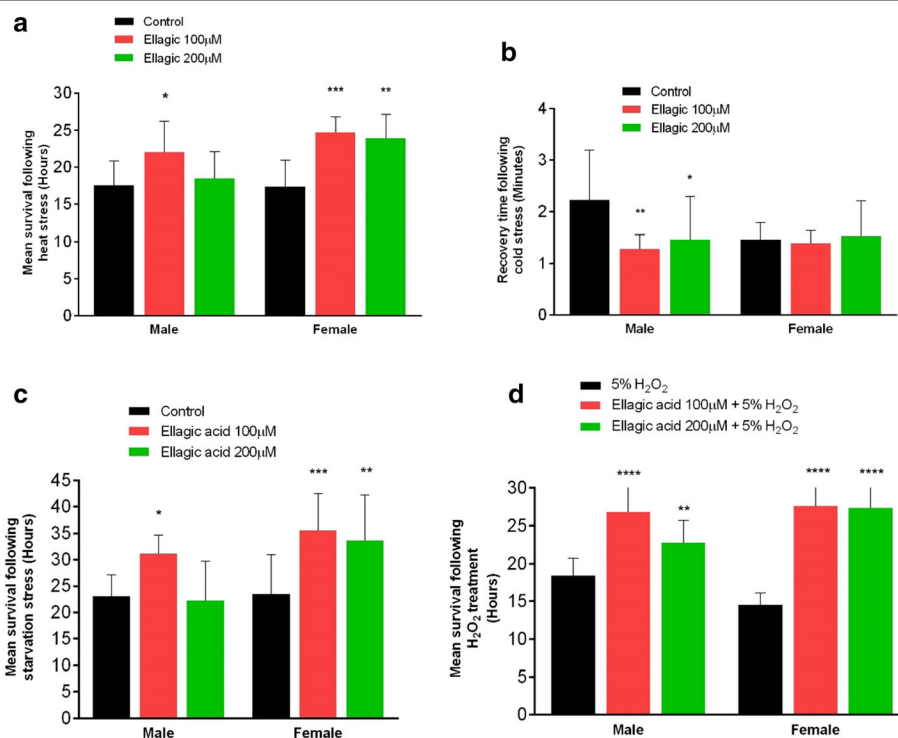


Fig. 3 Effect of ellagic acid on stress resistance in flies. **a** Effect of ellagic acid on resistance to heat stress in flies. Mean lifespan was calculated by the Kaplan–Meier lifespan analysis in the OASIS2 software. Mean values of individual replicate of control, and two treatments of male and female flies were fed into the GraphPad Prism v.6 software. Data represented as mean lifespan (in hours) following exposure to heat stress in flies, with error bars denoting SD. Data were subjected to two-way ANOVA followed by Bonferroni’s multiple comparisons test. Symbols represented as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. For each treatment and sex along with their respective control, ten vials containing 20 flies/vial were counted. The total number of flies used was 1200. **b** Effect of ellagic acid on resistance to cold shock in flies. Data represented as mean recovery time of flies (in minutes) following cold shock, with error bars denoting SD. Mean values of individual replicate of control, and two treatments of male and female flies were fed into the GraphPad Prism v.6 software. Data were subjected to two-way ANOVA followed by Bonferroni’s multiple comparisons test. Symbols represented as * $p < 0.05$ and ** $p < 0.01$. For each treatment and sex along with their respective control, ten vials containing 20 flies/vial were counted. **c** Effect of ellagic acid on resistance to starvation in flies. Mean lifespan

was calculated by the Kaplan–Meier lifespan analysis in the OASIS2 software. Mean values of individual replicate of control, and two treatments of male and female flies were fed into the GraphPad Prism v.6 software. Data represented as mean lifespan (in hours) following exposure to starvation stress in flies, with error bars denoting SD. Data were subjected to two-way ANOVA followed by Bonferroni’s multiple comparisons test. Symbols represented as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. For each treatment and sex along with their respective control, ten vials containing 20 flies/vial were counted. The total number of flies used was 1200. **d** Effect of ellagic acid on resistance to oxidative stress in flies. Mean lifespan was calculated by the Kaplan–Meier lifespan analysis in the OASIS2 software. Mean values of individual replicate of control, and two treatments of male and female flies were fed into the GraphPad Prism v.6 software. Data represented as mean lifespan (in hours) following exposure to oxidative stress in flies, with error bars denoting SD. Data were subjected to two-way ANOVA followed by Dunnett’s multiple comparisons test. Symbols represented as ** $p < 0.01$ and **** $p < 0.0001$. For each treatment and sex along with their respective control, ten vials containing 20 flies/vial were counted. The total number of flies used was 1200

change), dFOXO (11.2-fold change), ATG1 (3.7-fold change), and SOD2 (1.85-fold change) compared with the control (Fig. 4a). Male flies treated with 200 μ M ellagic acid showed upregulated expressions of CAT (5.8-fold change), ATG1 (4.1-fold change), and dTSC1 (2.95-fold change) compared with the control (Fig. 4a). In female flies, addition of 200 μ M ellagic acid enhanced the expression of dFOXO (3-fold change) and

anti-oxidant genes CAT (2.8-fold) and SOD2 (1.5-fold change) compared with the control (Fig. 4b).

Discussion

Ellagic acid–treated female flies had shown significant gain in body weight over the control. There are genes

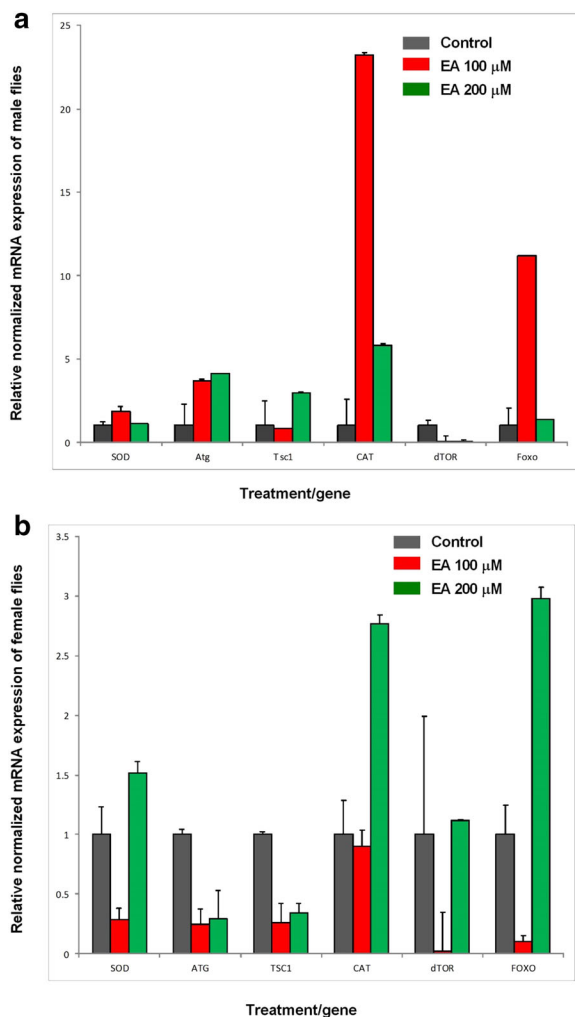


Fig. 4 **a** Gene expression in male flies. Three vials were set up, with 30 flies/vial. Experiments were performed two times ($n = 3 + 3$). mRNA of manganese-containing superoxide dismutase (SOD2), autophagy gene (ATG1), tuberous sclerosis (TSC1), catalase (CAT), mammalian target of rapamycin (TOR), and forkhead box transcription factor (FOXO) in wild-type Canton-S male fruit flies fed with ellagic acid at the concentration of 100 μ M and 200 μ M. Rpl32 is used as internal control. Levels of gene expression in all groups were normalized with the control group for each gene. Error bars show standard deviation. **b** Gene expression in female flies. Three vials were set up, with 30 flies/vial. Experiments were performed two times ($n = 3 + 3$). mRNA of manganese-containing superoxide dismutase (SOD2), autophagy gene (ATG1), tuberous sclerosis (TSC1), catalase (CAT), mammalian target of rapamycin (TOR), and forkhead box transcription factor (FOXO) in wild-type Canton-S female fruit flies fed with ellagic acid at the concentration of 100 μ M and 200 μ M. RPL32 is used as internal control. Levels of gene expression in all groups were normalized with the control group for each gene. Error bars show standard deviation

TSC1 and TOR that regulate growth and size in *Drosophila* (Kapahi et al. 2004). In our study, control flies

had shown a shorter lifespan. This might be due to several reasons: sensitivity of the flies to subtle variations in environment (temperature, humidity, and lighting); vial conditions (bubbles, cracks, bacterial accumulation, and dryness in the food); concentration of the diet (Linford et al. 2013). Adult flies reared at low temperature live longer than flies reared at high temperature (Pletcher et al. 2000). Female flies live shorter on more concentrated food (15% SY) compared with those on less concentrated food (5% SY) (Linford et al. 2013). Fly lifespan reduces when flies are reared on yeast extract instead of lyophilized whole brewer's yeast (Bass et al. 2007a). Male fruit flies treated with ginger extract at 2 mg/ml increased the mean lifespan from 37 to 40 days compared with control flies (Zhou et al. 2018). Male fruit flies fed with cranberry anthocyanin extract at 20 mg/ml for 81 days increased the mean lifespan from 48 to 53 days compared with control flies (Wang et al. 2015). There is an increase (19%) in longevity of male and female *Canton-S* flies fed with pomegranate juice (10%) for 20 days with a median survival of 24.8 days compared with control (20.8 days) (Balasubramani et al. 2014). Male and female *Canton-S* flies when treated with resveratrol (200 μ M) for 20 days had shown 11% increase in longevity with a median survival of 23.1 days compared with control (20.8 days) (Balasubramani et al. 2014). Male flies treated with lamotrigine (6 mg/ml) for 28 days showed a mean lifespan of 24.3 days as compared with control flies (21.6 days) (Avanesian et al. 2010). Female flies treated with lamotrigine (6 mg/ml) for 28 days showed a mean lifespan of 27.4 days as compared with control flies (24.3 days) (Avanesian et al. 2010). Intake of low-sugar high-protein diet supplemented with cranberry extract extended lifespan in male flies, whereas female flies fed with high-sugar low-protein diet supplemented with cranberry extract had shown prolonged lifespan (Wang et al. 2014). Longevity is related to sex type and diet composition. Incorporation of resveratrol at 200 μ M to low-sugar high-protein diet increased the mean lifespan of female flies by 15% ($p < 0.001$), whereas male flies were not influenced (Wang et al. 2013). In our work, hormetic effect has been observed at lower doses and not at higher doses. Korean mistletoe extract had shown a similar hormetic effect on flies at lower doses (Lee et al. 2014). Male flies treated with lutein at 0.1 mg/ml for 30 days increased the mean lifespan from 50 to 55 days compared with control flies (Zhang et al. 2014). In a biological system, moderate

hormesis observed at the initial period amplified to a wider extent affecting many parameters related to healthy aging (Rattan 2008). Male flies fed with blueberry extract at 5 mg/ml for 76 days extended the mean lifespan from 48 to 53 days compared with control flies (Peng et al. 2012). Wild-type flies treated with apple polyphenol at 10 mg/ml for 74 days extended the mean lifespan to 55 days compared with control flies (50 days) (Peng et al. 2011).

Female flies treated with pomegranate juice (10%) for 20 days showed a significant increase in the number of offspring produced (Balasubramani et al. 2014). Female flies treated with 1 mg/ml of extract from *Senecio brasiliensis* leaves significantly reduced the rate of eclosion without changing the number of larvae and pupae (Macedo et al. 2017). Lifespan extension observed at 200 μ M of ellagic acid may be due to reduced fecundity in female flies. This suggests the energy conserved in reproductive endeavor may be channeled to prolong the lifespan (Bass et al. 2007b). Reduced fecundity and increased longevity may have a positive connection to climbing efficiency in flies. Recent study by Staats et al. (2018b) did not find any significant change in locomotion ability of resveratrol-treated flies from control flies. Wang et al. observed a reduced climbing ability of fruit flies with age (Wang et al. 2015). Climbing ability of male and female flies treated with pomegranate juice (10%) for 20 days did not show any significant change in climbing ability, whereas the aged flies (treated for 40 days) showed a significant change (Balasubramani et al. 2014). Lamotrigine-treated (6 mg/ml) 4-week-old male flies showed 32% reduction in locomotion compared with 1-week-old flies which displayed 7% reduction. Four-week-old female flies when treated with lamotrigine (6 mg/ml) showed 27% reduced locomotion compared with 15% reduction observed in 1-week-old flies. This indicates performance of flies decline with aging.

Compared with male flies, female flies had shown better tolerance for heat shock. Knockdown of insulin-like receptor (InR) reduced the heat stress resistance in female flies than in male flies (Gruntenko et al. 2016). This result shows stress resistance of flies depends on sex and such context-dependent lifespan extension was reported earlier in our group (Chattopadhyay et al. 2016; Chattopadhyay et al. 2017). Female flies treated with luteolin at 0.3- μ M concentration showed increased starvation resistance while it did not affect the male flies (Lashmanova et al. 2017). Rat embryonic fibroblast

cells pre-treated with ellagic acid and then treated with hydrogen peroxide showed a protective effect (Baeri et al. 2018). When female flies were pre-treated at an adaptive concentration of H₂O₂ (10 μ M or 100 μ M) for 8 h and then challenged with 4.4 M H₂O₂ showed a longer survival than male flies treated similarly (Pomatto and Davies 2017). Male and female flies treated with pomegranate juice (10%) for 20 days, starved for 2 h, and then treated with 5% H₂O₂ showed an increase in the median survival of male flies to 28 days; female flies to 29.5 days, compared with control flies (18 days) (Balasubramani et al. 2014). These flies when treated with resveratrol (200 μ M) for 20 days, starved for 2 h, and then treated with 5% H₂O₂ showed a median survival of 24.5 days in male flies and 26.5 days in female flies, as compared with 19 days in control flies (Balasubramani et al. 2014). Male flies treated with lutein at 0.1 mg/ml for 30 days, starved for 3 h, and then treated with 9% H₂O₂ showed an increase in the mean lifespan of 18.54 h compared with control flies (15.83 h) (Zhang et al. 2014).

Reduced activity of TOR pathway through modulation of genes dTSC1, 2, dTOR, and dS6K extends lifespan (Kapahi et al. 2004). Addition of ginger extract (1.0 mg/ml) to the basic diet significantly increased the expression level of CAT in male flies (Zhou et al. 2018). Black tea extract (BTE) supplementation (10 mg/ml diet) to male flies had displayed a significant expression ($p < 0.01$) of CAT gene (Peng et al. 2011). Male flies treated with blueberry extract at 5 mg/ml upregulated the gene expressions of SOD1, SOD2, and CAT ($p < 0.01$) (Peng et al. 2012). Wild-type Oregon-R flies fed with lutein at 0.1 mg/ml for 30 days displayed increased gene expressions of SOD2 and CAT compared with the control group (Zhang et al. 2014). Transcriptomic analysis of male *Drosophila* flies treated with black rice anthocyanin extract expressed aging-related pathways like mTOR signaling pathway and FoxO signaling pathway (Li et al. 2019). Aging organisms have reduced ability to defend stressful conditions. As first line of defense, production of endogenous anti-oxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase protects the organism from the oxidative damage caused by reactive oxygen species. In addition, there are exogenous intake of flavonoids and anti-oxidants that act as second line of defense in terminating the free radical cascade (Wang et al. 2015). TSC1 and TSC2 physically associate with dTOR to inhibit its activity, and upregulation of TSC promotes longevity in

drosophila (Kapahi et al. 2004). Overexpression of forkhead transcription factor dFOXO temporally and spatially extends lifespan (Giannakou et al. 2004). Overexpressed dFOXO in *Drosophila* fat body increased the lifespan of female flies by 20% and reduced fecundity by 50%, and there is no effect on male lifespan (Giannakou et al. 2004). FOXO expression activates SOD, heat shock proteins (HSP), and DNA repair proteins to protect the organism from various age assaulting stress factors (Lashmanova et al. 2015).

Conclusion

This study displayed the ability of ellagic acid to extend the mean lifespan of *Drosophila melanogaster*. Ellagic acid given at a lower dose of 100 μM and 200 μM prolonged fly longevity, reduced fecundity, and increased resistance to heat shock, cold shock, starvation stress, and oxidative stress in flies. For stress assays, female flies respond better than male flies. Ellagic acid beyond 200- μM concentration is not promoting the longevity of male and female flies. Gene expression studies displayed upregulated expressions of CAT, dFOXO, ATG1, and SOD2 in male flies, and upregulated expressions of dFOXO, CAT, and SOD2 in female flies. Ellagic acid can be useful as an anti-aging compound to extend the lifespan of *Drosophila melanogaster*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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