The anti-aging effects of *Ludwigia octovalvis* on *Drosophila melanogaster* and SAMP8 mice

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Abstract We investigated the anti-aging effects of *Ludwigia octovalvis* (Jacq.) P. H. Raven (Onagraceae), an extract of which is widely consumed as a healthful drink in a number of countries. Using the fruit fly, *Drosophila melanogaster*, as a model organism, we demonstrated that *L. octovalvis* extract (LOE) significantly extended fly lifespan on a high, but not a low, calorie diet, indicating that LOE may regulate lifespan through a dietary restriction (DR)-related pathway. LOE also attenuated age-related cognitive decline in both flies and in the senescence-accelerated-prone 8 (SAMP8) mouse, without causing any discernable negative trade-offs, including water intake, food intake, fecundity, or spontaneous motor activity. LOE contained high levels of polyphenols and

flavonoids, which possess strong DPPH radical scavenging activity, and was shown to attenuate paraquat-induced oxidative damage and lethality in flies. Gas chromatography–mass spectrometry (GC-MS) analyses identified 17 known molecules, of which β -sitosterol and squalene were the two most abundant. We further demonstrated that β -sitosterol was capable of extending lifespan, likely through activating AMP-activated protein kinase (AMPK) in the fat body of adult flies. Taken together, our data suggest that LOE is a potent anti-aging intervention with potential for treating age-related disorders.

Keywords *Ludwigia octovalvis* · Dietary restriction · Lifespan · AMPK

Wei-Sheng Lin and Jun-Yi Chen contributed equally to this work.

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Introduction

Understanding aging is a major challenge of our time, given demographic trends and the predicted burdens of an elder society. Studies investigating age-related diseases such as neurodegenerative disease, cancers, and cardiovascular disease have already yielded significant insights into human senescence. Dietary restriction (DR), by reduction of calorie intake without malnutrition or alteration in the balance of dietary components, is known to be the most effective intervention for prolonging healthy lifespan across species, from yeast to flies to mammals (Lin et al. 2000; Wood et al. 2004; Bonkowski et al. 2006; Wu et al. 2013). In rodents, DR can significantly increase lifespan in normal mice and in short-lived senescence-accelerated-prone 8 (SAMP8) mice (Weindruch and Walford 1982; Choi and Kim 2000; Simons et al. 2013). In flies, a striking lifespan extension can also be observed (Zheng et al. 2005; Soh et al. 2013). Although studies in primates (rhesus monkey) have not yet generated definitive results regarding the effect of DR on mortality, it is clear that DR in monkeys produces beneficial physiological effects similar to those observed in rodents and flies (Colman et al. 2009; Mattison et al. 2012). It has been shown that lifespan extension by DR is likely mediated through a number of genetic pathways involving nutrient sensing and metabolism, such as insulin/IGF, sirtuins (Sir), SKN-1, target of rapamycin, and AMP-activated protein kinase (AMPK) pathways (Lin et al. 2000; Kaeberlein et al. 2005; Bishop and Guarente 2007; Alcedo and Kenyon 2004; Stenesen et al. 2013). It appears that Sir2/Sirt1 (Lin et al. 2004) and AMPK (Xiao et al. 2011) can be activated by increased AMP/ATP, ADP/ATP, or NAD/NADH ratios, reflective of a lower cellular energy status. These, in turn, induce a series of downstream signaling events that transform the metabolic state of cells from energy consumption to energy storage and generation.

Given the practical limits for humans in practicing DR in normal daily life, the development of DR mimetics has emerged as an exciting field for anti-aging research. Dietary supplements containing nutraceutical compounds or plant extracts may be able to provide safe and effective interventions for prolonging healthy life. Fruit extracts from nectarines and apples, as well as herbal extracts from *Rhodiola rosea* and *Curcuma longa*, have already been shown to extend lifespan in fruit flies, through reduction of oxidative damage (Boyd et al. 2011; Peng et al. 2011;

Schriner et al. 2009; Lee et al. 2010; Schriner et al. 2013). The major components identified in these health-promoting plants as likely candidates for the lifespan-extending effects are polyphenols, which have the potential to act as free radical scavengers or redox-active metal chelators (Galleano et al. 2010). Resveratrol, a polyphenolic compound found largely in the skin of red grapes, is the best-known example of this class of molecules and has been shown to extend the lifespan of species as evolutionarily distant as *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Nothobranchius furzeri* (Howitz et al. 2003; Wood et al. 2004; Valenzano et al. 2006). Although the anti-oxidative properties of resveratrol have been demonstrated, accumulating evidence suggests that this compound may also act as a strong activator for several longevity-associated genes, including Sir2/Sirt1 mentioned above (Hubbard et al. 2013).

Ludwigia octovalvis (Jacq.) P. H. Raven (Onagraceae) has a wide distribution in tropical areas of many countries around the world. Although the complete chemical composition of *L. octovalvis* has not been fully described, this plant has a long history of use as a herbal medicine for treating various conditions, including edema, nephritis, and hypotension. Large populations in Asia consume *L. octovalvis* extract (LOE) as a healthful drink on a daily basis. Toxicological evaluation in mice has suggested that chronic LOE treatment may provide immunoregulatory, hepatoprotective, and cardiovascular-protective properties, without observable side effects (Kadum Yakob et al. 2012). In addition, LOE has been reported to have anti-bacterial and anti-cancer activities (Chen et al. 1989; Chang et al. 2004). In this study, we investigated the anti-aging effects of LOE, using both fruit flies and SAMP8 mice as model organisms. Our findings suggest that LOE has potential for development as an anti-aging intervention.

Methods

LOE preparation

Whole plants of *L. octovalvis* were usually collected during the summer season, and dried plants were obtained from the local Agriculture Improvement Station and Taiwan Herbal Biopharma Company Ltd. in Taiwan. Quality control for *L. octovalvis* was monitored by Taiwan Herbal Biopharma Company Ltd. and a

voucher specimen (No. 034) was deposited at the herbarium of Hsiehyu Biotech Company Ltd. For LOE preparation, 100 g of *L. octovalvis* powder was soaked in 400 ml of 95 % ethanol overnight at 4 °C and then extracted twice at 55 °C for 30 min. The supernatant was centrifuged (at 8,000×g for 5 min) and filtered (TOYO No. 1) to remove debris. LOE was obtained following a decompression process.

Gas chromatography–mass spectrometry (GC-MS)

GC-MS analysis of LOE was performed using an HP-6890 gas chromatograph and HP-5973 mass-selective detector (MSD, Agilent Technologies), equipped with a capillary gas chromatography column HP-5MS (12.5 m×0.2 mm I.D., 0.33 µm film thickness) (J&W Scientific). Injection volume was 2 µl in splitless mode, and inlet temperature was 290 °C. Helium was used as the carrier gas (flow rate of 0.6 ml/min), and oven temperature was programmed as follows: initial temperature: 35 °C, increased by 5 °C/min to 90 °C, then by 10 °C/min to 200 °C, and finally by 20 °C/min to 290 °C, where it was held for 8 min. Analysis was performed in the EI mode, with ionization voltage fixed at 70 eV. Scan acquisition (*m/z* 45–550) of MSD was carried out with HP Chemstation software.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and total content of polyphenols and flavonoids

DPPH radical scavenging activity was measured using a modified method described previously (Brand-Williams et al. 1995). Briefly, 100 µl of different concentrations of LOE, ascorbic acid, resveratrol, squalene, or β-sitosterol (Sigma) was added to 200 µl of 100 µM DPPH in 96-microwell plates to reach final concentrations of 0, 1, 5, 10, and 50 µg/ml. The reaction mixture was incubated in the dark at room temperature for 30 min. Decrease in absorbance was measured at 520 nm in a microplate reader (AccuReader 965). Total phenolic constituents of LOE were determined with the Folin–Ciocalteu reagent, using gallic acid as a standard (Slinkard and Singleton 1977). Total flavonoid contents were measured with an aluminum chloride colorimetric assay, with rutin used as a standard (Shih et al. 2010).

Cell culture and Western blot analyses

Drosophila Schneider (S2) cells were grown at 25 °C in Schneider's *Drosophila* medium (Invitrogen), supplemented with 10 % heat-inactivated fetal bovine serum (Gibco). Cells were treated with different concentrations of LOE and β-sitosterol (Sigma) under normal (1× medium, as above) growth conditions or under DR conditions (0.5× medium, 1:1 dilution of the 1× medium in phosphate-buffered saline (PBS)) for 12 h. Protein levels of phospho-AMPK and α-tubulin (antibodies from Cell Signaling Technology) were analyzed by Western blot as described previously (Wang et al. 2005).

Flies and lifespan assays

Canton-S (CS), *w¹¹¹⁸*, fat body *GeneSwitch* driver S106 (Bloomington Stock Center #8151), and UAS-AMPK.RNAi (Bloomington Stock Center #32371) stocks were raised on standard sucrose/yeast/cornmeal food. S106 virgins were crossed with UAS-AMPK.RNAi males in AMPK knockdown experiments. For lifespan assays, flies that had eclosed within 48 h (approximately 100 males and 100 females) were transferred to a 1-l population cage and maintained in a humidified, temperature-controlled incubator with 12-h on/off light cycle at 25 °C (Wang et al. 2009). Fresh food was provided every other day, and the number and gender of dead flies were scored. High-calorie and low-calorie foods contained 15 or 5 % both dextrose and yeast, respectively. Two percent agar and 0.23 % Tegosept (Apex) were added to all foods. Different concentrations of LOE, squalene, β-sitosterol, RU486 (Mifepristone, Sigma), or vehicle control were added to the foods described in each experiment.

Feeding, fecundity, and spontaneous motor activity of flies

Ten-day-old flies maintained on high-calorie food containing different concentrations of LOE were transferred to fresh vials of the same food, with addition of 0.5 % FD&C no. 1 blue food dye. After 24 h, 10 flies were homogenized in a single tube containing PBS, and the amount of ingested dye was determined by spectrophotometer for dye absorbance at 625 nm. Female fecundity was determined by daily counting of eggs produced by three mating pairs. Flies were passed daily to new vials containing appropriate food, and the number of eggs

laid was counted and recorded for the first 20 days of adult life. For monitoring activity levels, glass vials containing a single male fly on the appropriate food source were placed in locomotor recording chambers that have circular rings of infrared light sources; beam breaks are recorded as activity (TriKinetics). The data were recorded every 20 s over a 72-h period. Activity monitors were housed in the incubator described above.

Pavlovian olfactory associative short-term memory of flies

Flies at different ages were trained by exposure to electroshock (70 V) that was paired with one odor for 60 s. Subsequently, flies were exposed to a second odor without electroshock for 60 s. Flies were allowed to rest for 45 s and then were subjected to a T-maze test for 2 min, in which they were required to choose between the two odors. The odors used were methylcyclohexanol (1:1,000 dilution in mineral oil) and octanol (1.5:1,000 dilution in mineral oil). For all experiments, at least five sessions were performed for each of the odor–electroshock pairing. All data were averaged, and they are shown as percentage of memory index for each group. Performance index was calculated by subtracting the number of flies entering the arm in which the odor was paired with the electroshock from the number entering the opposite arm, where the odor was not paired with electroshock, and dividing by the total number of flies.

Paraquat-induced oxidative stress and 4-hydroxy-2-nonenal (HNE) determination

Ten-day-old flies maintained on high-calorie food containing different concentrations of LOE were transferred to vials with filter paper soaked in a solution of 0 or 20 mM paraquat and 6 % dextrose. The number of dead flies was counted every 3 to 4 h. At 12 h, some of the paraquat-treated flies were collected, and HNE was measured by the OxiSelect™ HNE Adduct ELISA Kit (Cell Biolabs), following the protocol provided by the manufacturer.

Animals

All experimental protocols followed the local animal ethics regulations. SAMP8 mice were bred and maintained in the Modular Animal Caging System®

(Alternative Design), and their food was sterilized by gamma irradiation. The room had a 12-h on/off light cycle, with the dark phase beginning at 8 p.m. For mice, LOE was added to the drinking water at final concentrations of 0, 50, and 100 µg/ml. Food intake, water consumption, and change in body weight were monitored regularly.

RNA preparation, cDNA synthesis, and quantitative polymerase chain reaction (qPCR)

Total RNA was prepared from at least 30 flies using the RNeasy Mini Kit (Qiagen). The RNA was treated with DNase and converted to cDNA using oligo-d(T)₁₅ (Invitrogen) and SuperScript III reverse transcriptase (Invitrogen) as described previously (Chou et al. 2013). qPCR reactions were performed using a StepOnePlus Real-Time PCR Systems (Applied Biosystems), SYBR Green Master Mix (Applied Biosystems), and gene-specific primers were CAAG AGCTATCTGCTCGACTTCAA and CACATTTCTGA AAAACTCCATGGT for AMPK-α, and GACGAAAT CAAGGCTAAGGTCG and AATGGGTGTCGCTG AAGAAGTC for GAPDH. A two-step PCR reaction was carried out with denaturation at 95 °C for 15 s, annealing and extension combined at 60 °C for 1 min in a total of 40 cycles. The mRNA expression level of each target gene compared to GAPDH was quantified by subtraction: $C_{t_{\text{target}}} - C_{t_{\text{GAPDH}}} = \Delta C_t$. A difference of one PCR cycle equates to a twofold change in mRNA expression level. The uniqueness of amplicons was confirmed using dissociation curves.

Novel object recognition, elevated-T maze, and open field tests

For the novel object recognition test, mice were first habituated to an open box (50 cm×25 cm×50 cm) for 10 min per day for two consecutive days, with no objects present. On the third day, mice were placed in the box for 2 min, and two similar objects were then presented to each mouse. The time spent exploring each object was recorded during the subsequent 5-min period (defined as the training session). The mice were then returned to their home cage. One, two, or three hours later, the same animals were re-tested for 5 min in the box with a familiar and a novel object (defined as the memory test session). The object discrimination index was calculated by subtracting the time spent exploring a familiar object

from the time spent exploring a novel object and dividing by the total time spent exploring both objects.

The elevated-T maze was modified from previous studies (Cruz-Morales et al. 2008; De-Mello and Carobrez 2002; Maia et al. 2010; Santos et al. 2006; Takahashi et al. 2005). It consisted of one arm enclosed by walls 15 cm high, perpendicular to two opposing open arms. The three arms had equal dimensions (30 cm×5 cm), and the whole apparatus was elevated 50 cm above the floor. On the training day, each mouse was placed at the distal end of the enclosed arm, facing the intersection of the arms, and was allowed to explore the enclosed arm. The trial ended when the mouse either entered the open arms (all four paws were in the open arm area) or had remained in the enclosed arm for 300 s. The number of trials required to reach the avoidance criterion was used to assess the learning ability of the mice during the training sessions. Twenty-four hours following the training sessions, the mice were re-exposed to the enclosed arm, and the time that they remained in the enclosed arm was recorded as an assessment of memory retention.

The open field test was performed in an open box (50 cm×25 cm×50 cm). Each mouse was placed at the center of the open field and was allowed to freely explore the apparatus for 1 h. Direction and travel speed for each mouse were recorded by automated observation (Diagnostic and Research Instruments).

Results

LOE-induced lifespan extension is associated with DR

To test whether LOE has an anti-aging effect *in vivo*, we first examined the lifespan of *CS* flies raised on foods containing different concentrations of LOE. Male *CS* flies on high-calorie food had a mean lifespan of 41.5 days, and addition of 50, 100, and 1,000 µg/ml of LOE to the fly foods extended the lifespan of male flies by 8.9, 16.1, and 9.6 %, respectively (Fig. 1a and Table 1). Female *CS* flies on high-calorie food had mean lifespan of only 32.2 days, short in comparison to their male counterparts. However, LOE induced even larger lifespan extension in female *CS* flies, where 100 µg/ml of LOE had the largest effect (24.2 % extension; Fig. 1b and Table 1), and 100 µg/ml LOE also extended lifespan in both male and female *w¹¹¹⁸* flies, suggesting that

LOE-extended lifespan is not limited to the *CS* genetic background (Fig. 1c, d and Table 1).

Since DR is a well-documented intervention for extending lifespan, we wanted to test whether the longevity effects of LOE would be altered by DR. Male and female *CS* flies raised on low-calorie foods showed 17.8 and 49.4 % increases in mean lifespan, respectively, compared to *CS* flies reared on high-calorie foods (Fig. 1e, f and Table 1). LOE, however, did not induce lifespan extension above that already seen for control flies on low-calorie food (Fig. 1e, f and Table 1), which suggests a relationship between LOE and DR-induced lifespan extension, implying that these interventions may act, at least in part, through the same pathways.

Chronic LOE treatment does not induce negative tradeoffs

Since the LOE-induced lifespan extension is potentially coupled with the DR pathway, we examined whether LOE might reduce the food intake of flies and result in a DR-like status. We monitored the food intake of flies using a standard system of adding dye to their food (Wang et al. 2009). On average, female *CS* flies ingested much more food compared to age-matched males, but no difference in food intake was observed between control and LOE-treated flies for either gender (Fig. 2a).

In addition to reduced food intake, interventions that extend lifespan can involve negative tradeoffs, such as decreased fecundity and physical activity (Flatt et al. 2008; Wang et al. 2009; Sohal and Buchan 1981). We found that LOE-treated *CS* flies did not show any difference in egg production for the first 20 days of adult life (Fig. 2b). Both LOE-treated and control *CS* flies also showed normal circadian rhythm and spontaneous motor activity (Fig. 2c), as noted previously (Wang et al. 2009).

LOE attenuates age-related memory deficits in fruit flies and in SAMP8 mice

Functional decline of neurons is a common hallmark of the aging process. We examined the functional memory of flies, using a Pavlovian olfactory associative short-term memory test. *CS* flies on high-calorie food showed a gradual cognitive decline in the first 20 days of adult life (Fig. 3a). This memory decline was attenuated by DR, with a statistically significant difference seen by day 20 (Fig. 3a, b). LOE also reduced the age-related memory decline in a dose-dependent manner, with flies

Fig. 1 LOE-induced lifespan extension of flies is associated with DR. **a, b** Survivorship curves of male (**a**) and female (**b**) CS flies on high-calorie food containing 0 µg/ml (control, red), 50 µg/ml (green), 100 µg/ml (blue), and 1,000 µg/ml (black) of LOE. **c, d** Survivorship curves of male (**c**) and female (**d**) *w¹¹¹⁸* flies on high-

calorie food containing 0 µg/ml (control, red) and 100 µg/ml (blue) of LOE. **e, f** Survivorship curves of male (**e**) and female (**f**) CS flies on low-calorie food containing 0 µg/ml (control, red) and 100 µg/ml (blue) of LOE. Survival curves were analyzed by log-rank test (Table 1)

on 100 µg/ml LOE attaining a plateau level similar to that seen for DR flies (Fig. 3b).

We further investigated the pro-cognitive effect of LOE in SAMP8 mice, which are known to develop early onset of deficits in learning and memory and have been demonstrated to be a suitable model for studying fundamental mechanisms of Alzheimer's disease (Flood and Morley 1992, 1993; Chan et al. 2006; Takeda 2009). In the novel object recognition tests, 3-month-old SAMP8 mice showed sustained recognition memory up to 3 h after the training session, spending more time on the novel object compared to the familiar object (Fig. 3c, d). This memory retention, however, was not

observed in 8-month-old SAMP8 mice (Fig. 3c, d). Addition of LOE (100 µg/ml) to the drinking water for 5 months (starting from 3 month old) significantly enhanced the recognition memory retention of SAMP8 mice, even at 3 h after the training session (Fig. 3d). Water supplementation of 50 µg/ml of LOE had fewer beneficial effects, but was able to improve memory retention for up to 2 h (Fig. 3d).

In the elevated-T maze tests, all groups of mice required similar numbers of training trials to reach the avoidance criterion, suggesting that all mice had similar learning ability (Fig. 3e). In the 24-h memory retrieval tests, only 8-month-old, but not 3-month-old, SAMP8

Table 1 The effect of LOE on *Drosophila* lifespan (LS)

Gender	Strain	Diet	LOE (μg/ml)	Number	Mean LS	Extension (%)
Male	CS	High calorie	0	268	41.5	
			50	179	45.2	8.9 ^a
			100	168	48.2	16.1 ^b
			1,000	171	45.5	9.6 ^a
		Low calorie	0	168	48.9	17.8 ^b
			100	180	49.8	1.8 ^c
			0	199	32.2	
			50	180	36.4	13.0 ^b
Female	CS	High calorie	100	181	40.0	24.2 ^b
			1,000	173	35.9	11.5 ^b
			0	197	48.1	49.4 ^b
			100	195	47.6	-1.0 ^c
		Low calorie	0	339	41.1	
			100	319	44.2	7.5 ^b
			0	279	32.5	
			100	295	36.2	11.4 ^b

^aP value<0.05 compared to high calorie control of LOE by log-rank test

^bP value<0.01 compared to high calorie control of LOE by log-rank test

^cNot significant compared to low calorie control of LOE by log-rank test

mice showed a significant deficit in memory retention (Fig. 3f). This age-related deficit of fear memory in SAMP8 mice could also be rescued by a 5-month LOE supplementation in drinking water (Fig. 3f). The pro-cognitive effects of LOE observed above cannot be ascribed to any change in the general motor activity of the mice, as no difference in travel speed or distance was observed in the open field tests (Fig. 3g and data not shown). SAMP8 mice receiving a 5-month LOE treatment showed no effects on food intake or water consumption and no change in body weight (Fig. 3h–j).

Chemical composition of LOE

In order to identify the mechanisms by which LOE induces lifespan extension in flies, the chemical composition of LOE was analyzed by GC-MS. Seventeen major peaks were seen in the total ion chromatogram (Fig. 4) and were identified as the known compounds listed in Table 2.

LOE attenuates acute oxidative damage

Since oxidative stress is the most popular theory for explaining how aging occurs at the molecular level, we decided to examine the DPPH radical scavenging activity of LOE as a critical indicator of its antioxidant capacity. In our assays, LOE had strong DPPH radical scavenging

activity (IC₅₀ 3.15 μg/ml), in between the activity of ascorbic acid (IC₅₀ 1.31 μg/ml) and that of resveratrol (IC₅₀ 7.86 μg/ml), which were used as controls (Fig. 5a). However, DPPH radical scavenging activities of the two most abundant compounds identified in LOE, β-sitosterol, and squalene were detected only at minimal levels (Fig. 5a). The antioxidant capacity of LOE could be attributable to its antioxidant-rich composition, since a high level of polyphenols (146.3±3.1 mg of gallic acid equivalent per g of dry weight; Table 3) was present in our LOE preparation, and flavonoids were a major component among the polyphenols (90.2±2.6 mg of rutin equivalent per g of dry weight).

We therefore further tested the anti-oxidative properties of LOE by exposing 10-day-old female LOE-treated CS flies to paraquat (methyl viologen, a chemical known to generate reactive oxygen species). We found that flies receiving LOE supplementation in their food for 10 days showed increased resistance to paraquat-induced lethality (Fig. 5b). This result was further confirmed by analyzing the levels of oxidative damage in flies that had been treated with paraquat for 12 h. We used 4-hydroxynonenal (HNE), a protein adduct generated from products of lipid oxidation, as an indicator of oxidative damage. Although LOE supplementation showed no effect on the basal level of HNE in 10-day-old flies, it significantly reduced HNE accumulation in flies treated with 20 mM paraquat (Fig. 5c).

Fig. 2 LOE does not affect feeding, fecundity, or spontaneous motor activity of flies. **a** CS flies were maintained on high-calorie food containing different concentrations of LOE [0 µg/ml (control, red), 50 µg/ml (green), 100 µg/ml (blue), and 1,000 µg/ml (black)] for 20 days. Feeding rates of flies were examined by measuring the amount of food dye (0.5 % FD & C no. 1 blue) ingested over a 24-h period. Data were collected from more than 50 flies for each group and presented as mean $\pm$ SEM. **b** Average number of eggs laid per day for individual females was determined from daily counts of eggs. Data were collected from more than 30 flies for each group. **c** CS flies were maintained on high-calorie food containing different concentrations of LOE for 20 days. Spontaneous motor activity of flies was recorded over a 72-h period. Data were collected from more than 10 flies for each group

β -Sitosterol as a major component in LOE that contributes to the lifespan extension

We further examined the effects of β -sitosterol and squalene on the lifespan of flies. Intriguingly, β -

Fig. 3 LOE attenuates age-related memory decline in both flies and SAMP8 mice. **a** The memory performance of CS flies on high-calorie (red) and low-calorie (orange) foods at 5 day ($n=506$ for high calorie, $n=491$ for low calorie), 10 day ($n=633$ for high calorie, $n=632$ for low calorie), and 20-day ($n=555$ for high calorie, $n=498$ for low calorie) of age. **b** The memory performance of 20-day-old CS flies raised on low-calorie (orange, $n=1,251$) or high-calorie foods containing different concentrations of LOE [0 µg/ml (control, red, $n=1,279$), 50 µg/ml (green, $n=1,119$), 100 µg/ml (blue, $n=1,220$), and 1,000 µg/ml (black, $n=1,320$)]. Data were collected from 10 sessions of Pavlovian olfactory associative short-term memory test for each time point. Data are presented as mean $\pm$ SEM. $*P<0.05$, compared to high calorie vehicle control group of flies using two-way ANOVA and Student's t test. **c, d** The memory performance of 3-month-old (3 m) or 8-month-old (8 m) SAMP8 mice receiving different concentrations of LOE in their drinking water from 3 months of age was tested using the novel object recognition test. The average time that each group of mice spent on a familiar object (red) and a novel object (blue) at different points after the training session is shown in **c**. The discrimination index between familiar object and novel object for each group is shown in **d**. **e, f** Learning performance (**e**) and memory performance (**f**) of each group of SAMP8 mice were tested using the elevated-T maze tests. **g** The motor activity of each group of SAMP8 mice was tested in the open field test. **h–j** Food intake (**h**), water consumption (**i**), and body weight change (**j**) of SAMP8 mice receiving different concentrations of LOE for 5 months were recorded. Data are presented as mean $\pm$ SEM ($n=7–16$ for each group; $*P<0.05$, compared to 8 m vehicle group of SAMP8 mice using two-way ANOVA and Student's t test)

sitosterol induced lifespan extension in female CS flies (Fig. 6a) in a dose-dependent manner. This pro-longevity effect, however, was not seen in female CS flies receiving the same doses of squalene (Fig. 6b).

The AMPK pathway has been shown to mediate lifespan extension by DR in both *C. elegans* (Apfeld et al. 2004; Greer et al. 2007) and in flies (Stenesen et al. 2013). We therefore examined whether LOE and β -sitosterol could regulate lifespan through modulating the AMPK pathway. Using *Drosophila* S2 cells, we found that LOE and β -sitosterol could significantly enhance AMPK phosphorylation (Fig. 6c). Similar observations were made when cells were maintained under DR conditions (0.5 $\times$ medium) (Fig. 6c). If AMPK had a major role in β -sitosterol-induced lifespan extension in flies, loss of AMPK should blunt the longevity effect by β -sitosterol. We tested this hypothesis by knocking-down AMPK expression, specifically in fat body of adult flies (Fig. S1). *Drosophila* fat body is the fly equivalent of mammalian liver and adipose tissue and is responsible for metabolism. AMPK overexpression or knockdown in fat body cells has been associated with increased or

decreased fly lifespan, respectively (Stenesen et al. 2013). We combined a UAS-AMPK.RNAi allele with a RU486-inducible *GeneSwitch* GAL4 for fat body (S106-GAL4). Adult flies carrying appropriate genetic elements were

randomized to foods containing RU486 (the activating agent for the *GeneSwitch*-GAL4 drivers) and/or β -sitosterol. We found that the β -sitosterol-induced lifespan extension was not seen when AMPK expression was

Fig. 4 The chemical composition of LOE. Total ion chromatogram obtained by GC-MS analysis of LOE. Peak numbers correlate to compounds listed in Table 2

knocked down specifically in the fat body of adult flies (Fig. 6d). Together, these data imply that LOE-derived β -sitosterol may modulate lifespan, at least in part, through activating AMPK in the fat body of adult flies.

Discussion

Fruit flies have a relatively short lifespan, ranging from a few weeks to several months, depending upon

Table 2 Known compounds identified from LOE by GC-MS analyses

Peak No.	Compound	Area (%)
1	Dehydro-desoxyindolin codeine	0.8
2	Trihydroxy-isopropyl-methoxy anthraquinone	2.2
3	Neophytadiene	9.8
4	Trimethyl pentadecanone	1.2
5	Methyl octadecyne	1.4
6	Phytol	4.5
7	Ethyl hexadecanoic acid ester	4.9
8	2-N-Butyldecalin	0.9
9	Ethyl linoleic acid ester	7.5
10	Distearyl phosphate	0.8
11	Squalene	12.4
12	Spiro acid	1.1
13	Stigmastan-triene	4.0
14	Campesterol	4.2
15	Stigmasterol	5.8
16	Beta-sitosterol	38.1
17	Beta-sitosterol ether	0.4

Peak No. correlates to GC traces in Fig. 4

Fig. 5 The anti-oxidative properties of LOE. **a** DPPH radical scavenging activity of ascorbic acid, LOE, resveratrol, β -sitosterol, and squalene. Data are presented as mean $\pm$ SEM ($n=3$). **b**, **c** 10-day-old female CS flies, raised on food with/without 100 $\mu\text{g/ml}$ LOE, were challenged with different concentrations of paraquat. **b** The survival rates of flies were monitored every 3 to 4 h. Data were collected from more than 50 flies for each group. $*P<0.01$ by log-rank test. **c** Levels of NHE were measured from flies receiving different concentrations of paraquat for 12 h. Data were collected from more than 50 flies for each group and presented as mean $\pm$ SEM. $*P<0.05$, compared to vehicle group using Student's *t* test

nutritional and other environmental conditions. For instance, altered calorie intake, mating conditions, and ambient temperature can all significantly change the fly lifespan (Bauer et al. 2004). In well-defined conditions, we were able to demonstrate that food supplementation by LOE could extend lifespan in flies without any obvious negative tradeoff.

Development of DR mimetics has become an emerging field for anti-aging research. 2-Deoxyglucose, an

Table 3 Total contents of polyphenols and flavonoids in LOE

Components	Content (mg/g)
Polyphenol	146.3±3.1 ^a
Flavonoid	90.2±2.6 ^b

Data are shown as mean ± SEM

^a Milligram of gallic acid equivalents per g of dry weight

^b Milligram of rutin equivalents per g of dry weight

inhibitor of glycolysis (Lane et al. 1998), and metformin, which stimulates AMPK (Anisimov et al. 2003), are two molecules currently being explored as DR mimetics. However, long-term administration of 2-deoxyglucose or metformin may produce unwanted side effects, including heart failure and lactic acidosis, making them unlikely candidates for use as DR mimetics (Ingram et al. 2004; Sinclair 2005). Flies receiving metformin also showed no beneficial effect on lifespan extension (Slack et al. 2012). Thus, plant-derived

polyphenols such as resveratrol may provide an alternative option. Resveratrol has been shown to extend the lifespan of yeast, worms, and flies by mimicking DR through a Sir2-dependent pathway (Wood et al. 2004). Although resveratrol did not extend the lifespan of mice fed ad libitum, it improved the health and survival of obese mice fed a high-calorie diet (Baur et al. 2006; Pearson et al. 2008). These results suggest that resveratrol may enhance resistance to high-calorie-induced stress, which is consistent with results seen in studies demonstrating that resveratrol conferred cellular protection from stresses including oxidative damage (Araki et al. 2004) and gamma radiation (Howitz et al. 2003).

Our data suggest that LOE may act in part as a DR mimetic. We found that LOE significantly extended the lifespan of flies fed a high-calorie diet, but showed no additive effect under DR conditions (Fig. 1). LOE also attenuated age-related cognitive decline, keeping it to a level similar to that seen in flies under DR conditions. We showed that these DR-like effects of LOE were not due to

Fig. 6 β-sitosterol as a major component in LOE that may contribute to lifespan extension through activating AMPK in fat body of adult flies. **a** Survivorship curves of female CS flies on high-calorie food containing 0 μg/ml (control, $n=181$, red), 10 μg/ml ($n=186$, green), and 100 μg/ml ($n=186$, blue) of β-sitosterol. * $P<0.01$ by log-rank test. **b** Survivorship curves of female CS flies on high-calorie food containing 0 μg/ml (control, $n=188$, red), 10 μg/ml ($n=191$, green), and 100 μg/ml ($n=190$, blue) of squalene. **c** Western blots of phospho-AMPK and α-tubulin in *Drosophila* S2 cells supplemented with different concentrations of

LOE and β-sitosterol under a normal (1× medium) condition for 12 h. A DR condition (0.5× medium) is shown as a positive control. **d** Survivorship curves of female flies carrying fat body *GeneSwitch* driver S106 and UAS-AMPK.RNAi on food containing 0 μg/ml RU486 and 0 μg/ml β-sitosterol (control, $n=175$, red line), 0 μg/ml RU486 and 100 μg/ml β-sitosterol (β-sitosterol, $n=205$, blue line), 86 μg/ml RU486 and 0 μg/ml β-sitosterol (RU, $n=176$, red oval), and 86 μg/ml RU486 and 100 μg/ml β-sitosterol (RU+β-sitosterol, $n=196$, blue oval). * $P<0.01$ by log-rank test

lower food consumption induced by LOE (Figs. 2 and 3). Neither were other typical DR-coupled physiological changes, such as reduced female fecundity or enhanced motor activity, observed in flies treated with LOE (Fig. 2). DR has been shown to increase mitochondrial activity (Nisoli et al. 2005; Lopez-Lluch et al. 2006), but to delay or reduce oxidative stress and damage in flies (Zheng et al. 2005), mice (Qiu et al. 2010), monkeys (Zainal et al. 2000), and women (Buchowski et al. 2012). This observed effect has been suggested to be a “mitohormesis” effect, a hypothesis that increased mitochondrial activity and ROS production can trigger an intrinsic defense program, which results in improved stress resistance and possibly lifespan extension (Schulz et al. 2007). We found that LOE had strong DPPH radical scavenging activity and that it reduced paraquat-induced oxidative damage and lethality in flies (Fig. 5), further supporting our hypothesis that LOE may act, at least in part, as a DR mimetic.

Our chemical analyses confirmed that LOE was a rich source of antioxidants, including polyphenolic compounds, phytosterols, and squalene (Fig. 4 and Table 2). It has been reported that β -sitosterol may up-regulate protein expression and antioxidant activity of manganese superoxide dismutase through the estrogen/phosphatidylinositol 3-kinase pathway in macrophages (Vivancos and Moreno 2005). Dietary squalene supplementation was also shown to attenuate age-related oxidative damage and to maintain the energy status of liver mitochondria in aged rats (Buddhan et al. 2007). Although β -sitosterol and squalene were the two most abundant compounds identified in LOE by GC-MS analyses, neither showed obvious DPPH radical scavenging activity in our assay system (Fig. 5). It is possible that the anti-oxidative activities of LOE could include free radical scavengers such as polyphenolic compounds, while β -sitosterol and squalene could act through other pathways, as discussed above. Other traditional and integrative medical practices such as *R. rosea* and *C. longa* have been shown to extend lifespan of flies through pathways associated with anti-oxidation and regulation of known aging-related genes (Lee et al. 2010; Schriner et al. 2013, 2009). However, herbal extracts usually contain many compounds that require systematic biochemical analyses for identification. A mechanistic interpretation is therefore difficult without identification of a pure compound. In the present study, we identified β -sitosterol as a major component that contributed to the lifespan-extending activity of LOE. Our data suggest that β -sitosterol-

induced lifespan extension requires AMPK activation in fat body of adult flies. This is largely in agreement with previous reports demonstrating that AMPK is the major nutrient sensor and mediates lifespan extension by DR in *C. elegans* (Apfeld et al. 2004; Greer et al. 2007) and in flies (Stenesen et al. 2013).

It is desirable that longevity interventions not only extend lifespan, but also delay the onset and morbidity of age-related diseases, that is, they increase both lifespan and health-span. The precognitive effects of LOE that we describe in both flies and SAMP8 mice may also be of great interest to the aging field. It has been shown that SAMP8 mice exhibit age-related deficits in memory retention from 2 months of age, when tested on passive avoidance tasks (Miyamoto et al. 1986). In a footshock avoidance test, SAMP8 mice also show significant impairment in acquisition and memory retention from 6 to 9 months of age, compared to young SAMP8 mice (Flood and Morley 1993). Using novel object recognition and elevated-T maze tasks, we could observe clear age-related deficits in memory retention between 3- and 8-month-old SAMP8 mice, although there were no differences during the acquisition period of the elevated-T maze task (Fig. 3). These observations are further evidence that SAMP8 mice display an early onset of age-related cognitive deficits and that the impairment becomes worse with age. However, long-term LOE treatment (more than 5 months) in SAMP8 mice attenuated the age-related decline in memory retention without affecting physical activity, food intake, water consumption, or body weight (Fig. 3). We did not observe any side effects in mice receiving 5 months of LOE treatment (unpublished observations). These findings are largely in agreement with a recent report demonstrating that repeated administration of *L. octovalvis* for 28 days in mice (800 mg/kg $\approx$ 2,400 μ g/day vs. 400 μ g/day used in our study) did not cause lethality or toxicological effects (Kadum Yakob et al. 2012).

In summary, we have identified LOE as a novel anti-aging intervention, capable of extending the lifespan of flies and attenuating age-related cognitive decline in both flies and SAMP8 mice. GC-MS and other biochemical analyses suggest that LOE may exert its anti-aging effects by attenuating oxidative damage and activating AMPK-related pathways.

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