Original communication

Pomegranate juice and extract extended lifespan and reduced intestinal fat deposition in Caenorhabditis elegans

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Abstract: Pomegranate juice with a high content of polyphenols, pomegranate extract, ellagic acid, and urolithin A, have anti-oxidant and anti-obesity effects in humans. Pomegranate juice extends lifespan of *Drosophila melanogaster*. *Caenorhabditis elegans* (*C. elegans*) (n = 6) compared to the control group in each treatment, lifespan was increased by pomegranate juice in wild type (N2, 56 %, P < 0.001) and *daf-16* mutant (*daf-16(mgDf50)I*) (18 %, P = 0.00012), by pomegranate extract in N2 (28 %, P = 0.00004) and in *daf-16(mgDf50)I* (10 %, P < 0.05), or by ellagic acid (11 %, P < 0.05). Pomegranate juice reduced intestinal fat deposition (IFD) in *C. elegans* (n = 10) N2 (–68 %, P = 0.0003) or in the *daf-16(mgDf50)I* (–33 %, P = 0.0034). The intestinal fat deposition was increased by pomegranate extract in N2 (137 %, P < 0.0138) and in *daf-16(mgDf50)I* (26 %, P = 0.0225), by ellagic acid in N2 (66 %, P < 0.0001) and in *daf-16(mgDf50)I* (74 %, P < 0.0001), or by urolithin A in N2 (57 %, P = 0.0039) and in *daf-16(mgDf50)I* (43 %, P = 0.0001). These effects were partially mediated by the *daf-16* pathway. The data may offer insights to human aging and obesity due to homology with *C. elegans*.

Keywords: Pomegranate, ellagic acid, urolithin acid, lifespan, fat, C. elegans

Introduction

Health determines human lifespan as well as optimal quality of life [1, 2]. Aging at the cellular level involves complex interacting mechanisms that lead to functional decline which become manifest following birth and proceed through life. A U.S. population-based study demonstrated that mortality risk was directly correlated with body mass index (BMI). Thus, above 25 kg/m2, BMI inversely correlated with lifespan [3]. The increasing age of western societies creates a greater burden of chronic diseases including cancer, cardiovascular disease, diabetes, and neurodegenerative disorders including Parkinson's disease and Alzheimer's disease [4, 5], leading to a high cost of health care and a great financial burden to the public health system and families [6]. Among complex factors, both obesity and aging decrease insulin sensitivity, impair the immune response, increase inflammation, impair the gut-bloodstream barrier, and decrease physical mobility [7]. Many dietary interventions provide noninvasive approaches to reinforcing optimal nutrition, fighting metabolic dysfunction, enhancing physiological function, and promoting a healthy lifespan [8].

Dietary polyphenol antioxidants play important roles in health [9]. Polyphenols in blueberries extends lifespan in C. elegans [10]. Pomegranates (Punica granatum L.) juice have a high content of polyphenols. Anthocyanins (387 mg/L), punicalagins (1561 mg/L), ellagic acids

(121 mg/L), and other hydrolyzable tannins (417 mg/L) are the major polyphenols in PJ (Pom Wonderful) [11]. Pomegranates consist of about 80 % of juice and 20 % of seeds with water (85 %), and 10 % sugars [12, 13] (Table I). Pomegranate juice (PJ) has been shown to extend lifespan in mice [14, 15] and Drosophila melanogaster [16], and fructose demonstrated the similar effects in C. elegans in one of our recent studies [17]. PJ has shown effects in controlling the condition of type 2 diabetic patients [18]. Consumption of PJ (1.5 ml per kilograms body weight) reduces the fasting serum glucose and insulin resistance in type 2 diabetic patients significantly [19]. Pomegranate extract (POMx) potentiates lifespan extension with dietary restriction, a finding attributed by polyphenols [14, 15]. POMx and PJ can also act as prebiotics, having demonstrated antibacterial properties *in vitro*, and can block DNA repair and inhibit proliferation of breast cancer cells (MCF-7) *in vitro*, as well as modulate the IGF-IGFBP axis [20, 21]. POMx and PJ down-regulate androgen-synthesizing genes and induce apoptosis in human prostate cells (kappaB-dependent) *in vitro* and in mice *in vivo* [22, 23]. POMx and PJ decrease prostate specific antigen in humans after surgery or radiation [24, 25], inhibit tumor-associated angiogenesis *in vitro* and *in vivo* [26], suppress inflammatory cell signaling in colon cancer cells (50 mg/L PJ, *in vitro*) [27], improve memory [28] and improve fecundity in humans [29].

As the main bioactive component, ellagic acid (EA) is a measure of the quality of products [30]. EA in humans reaches a maximum plasma level in 1 hour (31.9ng/ml), is eliminated within 4 hours [31] and exerts an antioxidant effect [32]. The colonic microflora forms the main active metabolite of EA, urolithin A (UA). UA lasts longer in the body than EA, other EA metabolites [11, 33], and has better bioavailability [34]. UA also suppresses colorectal, hepatic, and prostate cancers synergistically with EA *in vitro* and in mice *in vivo* [35, 36].

The C. elegans model-organism is the first animal model to have its genome completely sequenced. C. elegans conserves 65 % of the genes associated with human disease, and has more than 300 transgenic and mutant strains available for use in functional biomedical research [37–45]. The C. elegans *daf-16* gene, a homologue of the human gene FOXO, regulates C. elegans lifespan and mediates both lipid metabolism and insulin signaling pathways [46, 47]. Transcriptional factor *skn-1* regulates lifespan in parallel to *daf-16*/FOXO in the *daf-2*-mediated insulin/IGF-1-like signaling pathway and mutation of *skn-1* is sensitive to oxidative stress and has shorter lifespan [48]. Heat shock factor (*hsf-1*) also played important role in the regulation of lifespan by regulating stress-inducible gene expression [49].

We hypothesized that pomegranate would increase lifespan while reducing intestinal fat deposition (IFD). We evaluated four PJ products using the C. elegans wild type (N2) to assess their effect on lifespan, IFD using Nile red and to predict their potential effect on aging and obesity in humans. In addition, we also used a *daf-16* deficient mutant to assess the role of the FOXO signaling pathway in mediating the effects of botanicals evaluated.

Table I. Phenolic content of pomegranate juice.

Phytochemical Bioactive	PJ (µg/ml)	POMX (µg/ml)	
		Ambient	Control
Ellagic acid (EA)	96.2	6.44+0.06	6.50+0.02
Punicalagins A&B (PA&B)	418.7	7.32 + 0.10	7.47+ 0.13

PJ : Pomegranate juice; POMX :pomegranate extract.

Materials and Methods

The *Caenorhabditis elegans* model organism is a low cost and convenient model for testing dietary substances. C. elegans strains and their standard lab food, Escherichia coli (E. coli, OP50, Uracil auxotroph), were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN). PJ was obtained from the first-press squeezing of the whole pomegranate fruit (POM Wonderful® LLC, Los Angeles, CA, USA) (http://www.pomwonderful.com/pomegranate-products/juice/100-pomegranate-juice/) and was purchased from a local grocery store. Extraction of the remaining fruit residue obtained after the first pressing for PJ, POMx powder was produced from a solid-phase extraction with a high concentration of polyphenols, a gift from POM Wonderful® LLC (Los Angeles, CA, USA). We obtained EA from Sigma (St. Louis, MO, USA). Dr. David Heber's laboratory synthesized the UA [11, 50].

Culture of Escherichia coli (E. coli, OP50)

We cultured the standard laboratory food of C. elegans, E. coli strain (OP50, uracil auxotroph) by the standard method described elsewhere [44, 51, 52]. Briefly, we added approximately 10 µL of stock E. coli solution to media and incubated at 37 °C for 24h. The OP50 were then plated in PetrifilmTM (3M Corporate, St. Paul, MN) at 37 °C for 24h until densities of 5×108 to 5×1011 *colony forming units (*cfu/ml) were reached and then were fed to the C. elegans ad libitum [44, 53]. We added additional concentrated OP50

to the plate every two weeks to maintain the concentration of OP50. The OP50 stock feeding solution was enriched to 2×109cfu/ml by centrifuging at 2,200 g for 10 Minutes and washed with S-complete buffer [54] twice.

Culture of C. elegans

Mature gravid wild type C. elegans (N2, Bristol) and *daf-16* deficient mutants [*daf-16(mgDf50)I*] were treated with NaOH (1M) and sodium hypochlorite solution (5.25%, 5:2 ratio) to dissolve the body and release viable eggs [44]. The eggs hatched the next day after washing with S-complete solution 3 times. The age-synchronized C. elegans were diluted to 100 animals/ml, plated in liquid culture in a 96 well plate (120μl/well, 10–15 animals) [54] with OP50 (109cfu/ml), and incubated in 20 °C (N2) or 15 °C (*daf-16* mutant) low temperature incubators (Revco Tech., Nashville, NC, USA). We added thirty microliters of 5-Fluoro-2′-deoxyuridine (FUDR, 0.6mM) stock solution to each well at L4 stage. The C. elegans model does not require regulation of the Institutional Animal Care and Use Committee (IACUC).

Lifespan assays

We added fifty microliters of the treatments to each well three days after egg-synchronization. The control group received OP50 only. The experimental groups received additional PJ (0.01%, 0.1%, 1%, 3%, 5%, 10% or 25%, v/v), POMx (5, 10, 20, 40, 80, 160, and 320μg/ml), EA (1, 2, 5, 10, 25, and 50μM in dimethyl sulfoxide (DMSO 0.05%), or UA (1, 2, 5, 10, 25, and 50μM in DMSO) (n = 10–15/well/6well). We used a second control (DMSO, 0.05%) in the EA and UA groups. We manually recorded the numbers of live animals every other day under a microscope (Nikon, Eclipse Ti –S, Japan).

Fluorescence microscopy

We used the lipophilic dye, Nile red, to stain for intestinal fat deposition (IFD), and fluorescent intensity was evaluated [44]. C. elegans in each group were collected after 3 days of treatments, washed with S-Basal twice, fixed with paraformaldehyde (4%) over 2h at 4 °C and washed with PBS for 5 min x 3. Nile red (50 μL) was applied to the specimens for 10 min. Ten microliters of Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) was applied to a glass slide followed by 10 μL of the medium containing Nile red stained C. elegans. A cover glass was mounted on the glass slide, and the slides were viewed with an epifluorescence microscope (Nikon Eclipse, Ti) equipped with a Texas Red filter. We randomly selected ten animal/slides, and took fluorescent micrographs with a digital camera (Andor, DU-885k) and analyzed using Nikon-Elements (version 3.22.11). Optical densities (arbitrary units) of Nile red stained IFD were determined for adult animals (larvae 4).

Statistical Analysis

Analyses were carried out using SAS/STAT® software, Version 9.4 of the SAS System for Windows (Cary, NC, USA). All results were expressed as mean ± S.E.M. Normality and homogeneity of variance of data was checked by Shapiro-Wilk test and HOVTEST. Survival curves were displayed by binomial probabilities obtained from logistic regression models as surrogates for survival probabilities and mean lifespan was estimated via Kaplan-Meier (log-rank). ANOVA models were used to analyze fluorescence intensity data and pairwise differences of least squares means for different doses were compared via two-sample t-tests. Statistical significance was defined as $P < 0.05$.

Results

PJ treatment dose-dependently extended lifespan of N2 up to 56% or *daf-16* mutant up to 30% in an A-shape curve, and decreased the magnitude of the lifespan extension at higher doses. A similar trend with half the magnitude was seen in POMx treated animals in N2 (28%). EA or UA did not significantly affect lifespan overall, however, lifespan extension was observed in several days of the experiment in each treatment (ESM 2). The fluorescent intensity of IFD in C. elegans was reduced by PJ in N2 (–68%) or in *daf-16* (–33%). In contrast, IFD was increased in N2 more than the *daf-16* mutant by POMx (137% at 320μg/ml vs. 26% at 20μg/ml), or UA (57% at 10μM vs. 43% at 50μM). IFD was increased by EA in N2 (66% at 5μM) and in *daf-16* mutant (74% at 25μM).

PJ dose-dependently extended then reduced lifespan in N2 and the *daf-16* mutant

In a dose-dependent manner, PJ added to the cultures significantly increased mean lifespan in N2 up to the 1% dose but produced reduced lifespans at higher doses, an A-shape curve relationship (Figure 1a & b). The mean lifespan was increased at lower doses (0.1% – 1%) from 21 to 33 days (42%, $P = 0.00642$ & 56%, $P < 0.001$) while it was decreased at higher doses (10% & 25%) from 33 to 4 days

(–67%, P < 0.001 & –81%, P < 0.001) (Figure 1b). Similarly, in the *daf-16* mutant, PJ also significantly increased mean lifespan in a dose-dependent manner up to 5%, but lifespan was significantly reduced at higher doses. The dose-response curve shifted to the right (Figure 1c). The mean lifespan of the *daf-16* mutant was increased from 27 to 32 days at a dose (5%) that reduced lifespan in N2 (18%, P = 0.00012) and decreased at the higher doses (–61% at 10%, P < 0.001 & –49% at 25%, P < 0.001, Figure 1b). Additionally, we observed specific days during the experiment at which significant differences in survival probabilities, which were provided (ESM 2).

PJ dose-dependently reduced the fluorescent intensity of IFD in N2 and the *daf-16* mutant (L4). In N2, the IFD was reduced from 6971 ± 607 to 2372 ± 245 (–30% at 1%, P = 0.0459, –44% at 3%, P = 0.0052, –66% at 5%, P = 0.0005, –68% at 10%, P = 0.0003 & –55% at 25%, P = 0.0028, n = 10, P < 0.05). The IFD was increased at lower doses in the *daf-16* mutant from 4677 ± 136 to 7222 ± 240 (54% at 0.01%, P < 0.0001 & 28% at 0.1%, P < 0.05, n = 10), followed by reduction at higher doses to 3134 ± 252 (–24% at 1%, –21% at 5%, P < 0.05; & –33% at 25%, n = 10, P = 0.0034) (Figure 1d).

POMx dose-dependently increased

A dose-response curve for lifespan extension was also present in the N2 group treated with POMx, but was absent in the *daf-16* mutant (Figure 2a). The mean lifespan was elevated from 18 to 22 days (18% at 10µg/ml, P = 0.00622 & 28% at 20µg/ml, P = 0.00004, Figure 2b & 2c). The mean

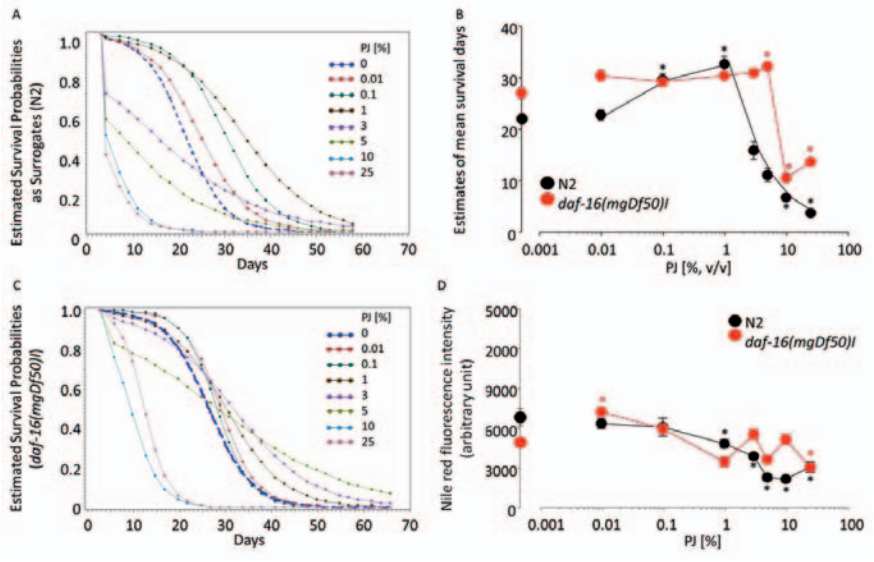

Figure 1. PJ dose-dependently increased estimated survival probabilities (displayed by binomial probabilities obtained from logistic regression models as surrogates for survival probabilities, binomial) and mean lifespan (estimated via Kaplan-Meier, log-rank, A-shape) in both N2 (a & b) and *daf-16* mutant (c & b) at lower doses, and dose-dependently reduced it at higher doses (n = 6). PJ dose-dependently reduced the fluorescent intensity of IFD (arbitrary unit) in N2 and *daf-16* mutant (d, n = 10). *P < 0.05 All results were expressed as mean ± S.E.M.

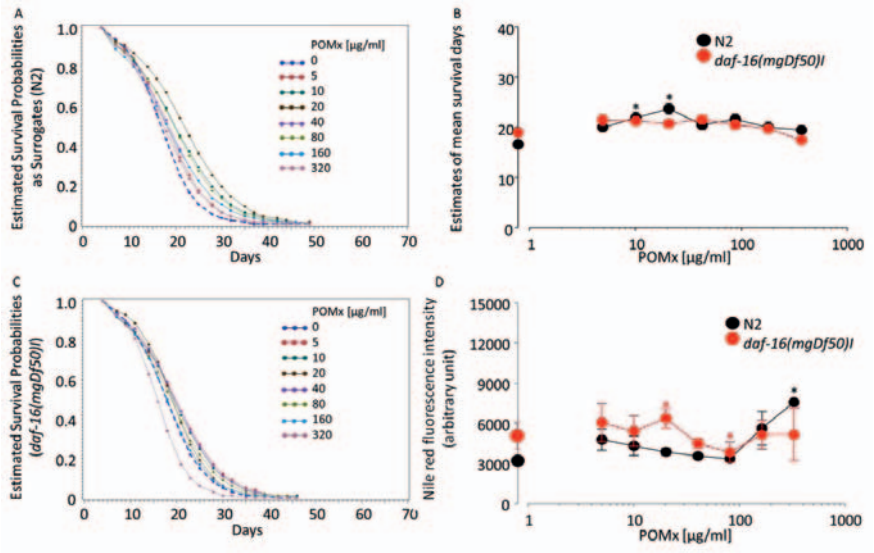

Figure 2. An A-shape dose-responses of lifespan extension was present in POMx (extract of pomegranate juice) treated group in both N2 (a & b) and the *daf-16* mutant (c & b), however, POMx was less potent than PJ. In N2, had an initial elevation of life span in the presence of lower doses of POMx which also reduced the IFD (d, n = 6). IFD was increase at the highest dose. In the *daf-16* mutant, POMx increased IFD at mid-dose followed by a reduction at a higher dose (n = 10). *P < 0.05 All results were expressed as mean ± S.E.M.

lifespan of the *daf-16* mutant was not altered (Figure 2b). Additionally, specific days during the experiment at which significant differences in survival probabilities were observed are provided (ESM 2).

In N2, lower doses of POMx slightly reduced the IFD ($P < 0.05$) followed by an increase from 3323 ± 63 to 7515 ± 125 at the highest dose (137% at 320µg/ml, n = 10, $P < 0.0138$). Similarly in the *daf-16* mutant, POMx increased the IFD from 5012 ± 1032 to 6327 ± 716 at mid-dose (26% at 20µg/ml, n = 10, P = 0.0225) followed by a reduction to 3815 ± 736 at a higher dose (23.9% at 80µg/ml, n = 10, P = 0.0429) (Figure 2d).

EA did not alter lifespan in N2 or in the *daf-16* mutant

Unlike PJ and POMx that increased lifespan dose-dependently, EA did not alter lifespan in N2 or the *daf-16* mutant and the mean lifespan also being unchanged ($P < 0.05$, Figure 3a & 3b). The mean lifespan of the *daf-16* mutant was not significant ($P < 0.05$, Figure 3b & 3c). The specific days during the experiment at which significant differences in survival probabilities were observed are provided (ESM 2). The IFD elevation by EA was dose-dependent, similar in both N2 and the *daf-16* mutant, and in parallel with a 2-fold elevation in the *daf-16* mutant. In N2, EA increased IFD from 5190 ± 158 to 8608 ± 323 at higher doses (66% at 5µM, 59% at 10µM, 58% at 25µM, & 47% at 50µM, n = 10, $P < 0.0001$). Similarly in the *daf-16* mutant, EA increased IFD at the higher doses from 8237 ± 227 to 14324 ± 113 (60% at 10µM, 74% at 25µM, & 49% at 50µM, n = 10, $P < 0.0001$) (Figure 3d).

UA did not alter lifespan in N2 or in the *daf-16* mutant

As with EA, UA did not alter the lifespan in N2 or the *daf-16* mutant. In N2, the mean lifespan was not changed ($P < 0.05$, Figure 4a & 4b). The mean lifespan of the *daf-16* mutant seemed reduced, but the change was not significant ($P < 0.05$, Figure 4b & 4c). Additionally, during the experiment, significant differences in survival probabilities were observed within two days (ESM 2). IFD was increased in N2, from 1362 ± 61 to 6278 ± 991 in the DMSO-control group (the increase was 361% at 0.05% DMSO, n = 10, $P < 0.05$). There was a greater increase to 9860 ± 216 at higher doses (the increase was 57% at 10µM, P = 0.0039, was 47% at 25µM, P = 0.0177 and was 46% at 50µM, P = 0.0216, n = 10). The IFD in the *daf-16* mutant was increased from 3021 ± 127 to 4324 ± 177 dose-dependently (34% at 10µM, P = 0.0273 & 43% at 50µM, P = 0.0001, n = 10) (Figure 4d).

Discussion

Our findings reveal that the lifespan extension has the same trend, on a larger or on a smaller scale, in N2 and the *daf-16* mutant by treatment with PJ or POMx, and has minimal effects on lifespan in cultures treated with EA or UA. These results indicated that the *daf-16* pathway was partially required for lifespan extension in the present study. We approximated the survival curves displayed by estimated probabilities of survival across the lifespan (Binomial). Although some treatment groups show statis-

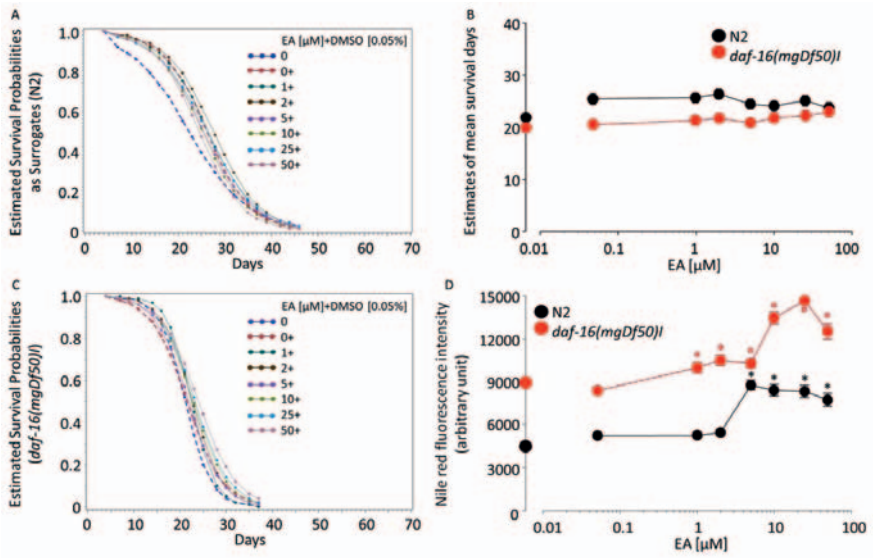

Figure 3. EA did not alter lifespan in N2 (a & b) but increased the lifespan in the *daf-16* mutant (c & b) at the highest dose (n = 6). The IFD (d) was dose-dependently elevated by EA in both N2 and *daf-16* mutant, and the responses were in parallel with a greater elevation (by 2-fold) in the *daf-16* mutant (n = 10). *$P < 0.05$ All results were expressed as mean ± S.E.M.

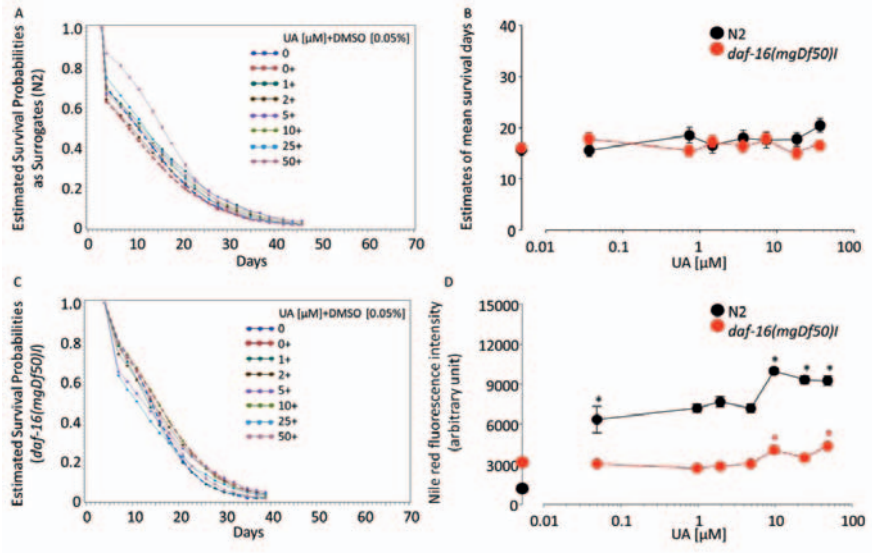

Figure 4. The mean lifespan was increased in N2 (a & b) but decreased in *daf-16* mutant (c & b) in UA treated group (n = 6). Animals treated with UA had an increased IFD (d) in both N2 and the *daf-16* mutant with a parallel pattern of response with N2 showing a greater increase than the *daf-16* mutant (n = 10). *P < 0.05 All results were expressed as mean ± S.E.M.

tically significant differences, one cannot use comparisons based on these probabilities to draw conclusions regarding lifespan.

The maximum lifespan of N2 could be affected by the concentration of OP50 and FUDR and as long as 58 days of lifespan in N2 is observed [55]. In a similar condition, our study recorded the maximum 44 days of lifespan in N2 in control group, while PJ extended the maximum lifespan of N2 to 60 days (Figure 1a).

As energy sources, appropriate amount of sugar should have beneficial effects on lifespan extension. However, high sugar (<2%) in the diet is detrimental to the C. elegans and reduces lifespan [45, 56]. We saw dose-response curves with an A-shape in PJ and POMx treated animals, but were puzzled by the fact that the lower doses of PJ increased lifespan, while the higher doses decreased the lifespan. We wondered if the unusual dose-response relationship might be due to PJ's sugar content, since fructose increases lifespan in yeast [58], mosquitoes [59], and nematodes, while decreasing fat deposition (in nematodes) at lower concentrations [17]. We found a reversal of the lifespan extension by PJ at a dose that contained 0.4% sugar. PJ has a high content of sugars in which the fructose is 1.25-fold greater than that of glucose which, in turn, is 4.7-fold higher than that of mannitol [60]. In agar dish culture, extra glucose (<0.001% or 2%) reduces lifespan mainly due to reducing signals of the *DAF-16*/FOXO and heat shock transcription factor (*HSF-1*) gene signaling [61, 62]. The effect of fructose, however, is controversial. Supplemental fructose reduces the plasma glucose level, glycohemoglobin, serum cholesterol, triglycerides, lactate, and body weight in type 2 diabetes [63, 64]. Many epidemiological studies link the consumption of high dietary fructose usage to an increased prevalence of obesity. Since associations do not show cause and effect, we need clinical trials to support this hypothesized cause for obesity [65, 66]. It appears that the A-shape dose-response relationship with the lifespan in the present study relates to the increasing sugar content. On the other hand, the level of sugar tolerance may be specie-dependent, since a diet emented with PJ (10%) increased the lifespan of flies [16]. Since the reduced lifespan was observed only at the highest dose in the *daf-16* mutant group, the data implies involvement of the *daf-16* pathway. The A-shape curve of PJ showed the longest lifespan to be at a sugar content of 0.13% in N2 and 0.66% in the *daf-16* mutant. The fact that PJ increased lifespan of the *daf-16* mutant in our study may indicate alternative mechanism. The lifespan curve shifting rightward might indicate an "independent or compensatory effect of the *daf-16* pathway".

Fat storage is one of the outcomes of energy consumption and energy expenditure in living organisms. In C. elegans, the fluorescent intensity of IFD of the PJ group was reduced in both N2 and the *daf-16* mutant. This inverse relationship of lifespan and fat content (PJ 1%) is in agreement with studies that PJ is effective in reducing cardiovascular risk factors in overweight humans [67].

POMx is characterized by its rich polyphenol content and extended the lifespan in a similar manner to PJ with an A-shape curve in N2. POMx in the *daf-16* mutant extended lifespan by several days (ESM 2). Unlike PJ, POMx did not reduce lifespan at higher doses. One can attribute the results to either an absent or reduced sugar content and/or other unknown factors. The effect of POMx on lifespan extension was only half the magnitude of the lifespan extension induced by PJ, which suggests that

multiple factors in the PJ products extend lifespan as was suggested by the Heber's studies [68]. Also unlike PJ, a V-shape dose-response curve was detected for the IFD in POMx treated animals at the doses that were used in this study, and only a minimal reduction of IFD was observed ($P < 0.05$). The reversal of the pattern of increase in lifespan extension and decrease in IFD occurred at 80 mg/ml, which seems to relate to the sugar content as it did in PJ treated group. Elevating IFD in the *daf-16* mutant suggests the involvement of this pathway controlling body fat accumulation.

Unlike either PJ or POMx, EA, at the doses used, did not alter lifespan in N2 or in the *daf-16* mutant. We observed a lifespan extension on several days of the experiment, which was similar to a study of N2 in agar culture in which the EA (50μM at 0.3% DMSO) prolonged mean lifespan [51].

DMSO is a solvent that extends lifespan dose-dependently in N2 with an "A-shape" curve, in agar culture (24% at 0.05% to 5%, v/v) [69] and in a liquid culture (20% at 0.9%, v/v) [70]. In the present study, DMSO doses were limited to the minimum. The lower DMSO dose (0.05%) extended lifespan up to 12.7%, while the higher amount (0.1%) decreased the lifespan which is different from the observations of the Wang group [69], which observed a 30% reduction in lifespan in the *daf-16* mutant (9.4%). The effect of EA on extending lifespan suggested complex mechanisms involving *daf-16*, a gene known for lifespan in C. elegans. EA also dose-dependently increased IFD at higher doses in N2 with parallel findings in the *daf-16* mutant. The enhanced IFD in the *daf-16* mutant that was not seen in the PJ treated groups uncovered an important role of the *daf-16* in lipid/energy metabolism.

Like all polyphenols, EA's large molecular weight and hydrophilic properties could have limited the absorption in the intestine as well as the bioavailability to the hosts. Since the main metabolites of EA have many beneficial effects, the effects of UA which is a metabolite of the colonic microbiota that has a higher absorption index has attracted extensive research in recent years [71]. UA has anti-inflammatory, anti-carcinogenic, anti-glycative, anti-oxidant, and anti-microbial effects [71–73]. In our study, some of the treatment groups showed a statistically significant increase in mean survival probabilities in the presence of UA. Although one cannot draw conclusions from these observations, it is possible that higher doses of UA might alter lifespan, and the *daf-16* mutant pathway may mediate the effect of UA on lifespan. UA also dose-dependently increased IFD at higher doses in both N2 and in the *daf-16* mutant, in parallel, with the latter having half the increase in IFD.

The antioxidant potency of PJ is higher (by at least 20%) compared to other polyphenol-rich products including red wine, Concord grape juice, blueberry juice, black cherry juice, cranberry juice, orange juice, iced tea beverages, and apple juice *in vitro* [74]. Thus, PJ has superior antioxidant bioactivity compared to its purified polyphenols, EA, punicalagin, or total pomegranate tannin. The "A-shaped" dose-response curves demonstrated in this study with PJ seemed similar to a hormesis reaction [57]. The mechanism by which a certain dosage-range achieved an extended lifespan but reversal at a lower or higher dosage was incompletely understood [17]. Ellagitannin comprised 90% of the tannin polyphenols in the POM products used in this study, but elagitannin did not extend lifespan suggesting multifactorial effects and chemical synergy of the action of multiple compounds in PJ compared to single purified active ingredients [68].

EA content varies by variety of the fruit, and many commercial pomegranate extracts were found not to contain the same amount of EA [75]. We used a commercially available PJ product , described in a full analysis of 477 commercial PJ in the market across North America and Asia [76]. Although studies in humans the pharmacokinetics of EA and UA that are equivalent [50], our study showed, as previously reported, that PJ was the most potent in extending lifespan and reducing IFD with POMx being only half as potent. The total phenolic content, by organic phenolic acid gallic acid equivalent (GAE), are 4.7-fold higher in PJ (2,825μg/ml GAE) than in that of POMx (606μg/mg GAE) [20]. In the present study, the estimated GAE (mg/ml) by calculation was also directly related to the lifespan extension at lower doses by PJ or POMx, which was reduced by higher doses (ESM 1, ESM 3, ESM 4). Thus, the fact that PJ was more potent than POMx may be beyond differences amount of polyphenols but the synergy of various components rather than, for example, EA only [77]. The lower or lack of effect of other tested substances compared to PJ suggest that some effective compounds may be reduced or absent, and multifactorial effects were present.

Our data from the C. elegans model indicate that the extension of lifespan by pomegranate is partially dependent on the *daf-16* pathway, but mechanisms other than those involving polyphenols such as EA and its metabolites including UA may play a role as well. The unique effects of PJ in reducing IFD suggest that the effects are due to more than one component. The results of the C. elegans studies provide useful information and suggest that, at the proper dilution, PJ may have optimal health benefits including control of fat storage, prevent obesity and offer a solution to delay aging in humans.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Electronic supplementary materials

The electronic supplementary material is available with the online version of the article at http://dx.doi.org/10.1024/0300-9831/a000570.

ESM 1. Figure.
The estimated GAE (gallic acid equivalent, mg/ml) by calculation related to the lifespan extension at lower doses.

ESM 2. Table.
Additional statistics for lifespan ($P < 0.05$).

ESM 3. Table.
Calculated GAE (gallic acid equivalent) content in the doses of the PJ and POMx (extract of pomegranate juice) used in this study.

ESM 4. Table.
Correlation of estimated GAE with the dose-responses (A-shape).

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