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Extension of life-span using a RNAi model and *in vivo* antioxidant effect of *Opuntia* fruit extracts and pure betalains in *Caenorhabditis elegans*

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ABSTRACT

Betalains are nitrogenous plant pigments known for their high antioxidant capacity. For the first time, this antioxidant nature has been studied in an *in vivo* system using the model organism *Caenorhabditis elegans*. The oxidative stress caused in the fluorescent strain TJ375 (*hsp-16.2::GFP*) was reversed by the presence of both natural and semi-synthetic betalains, with an ED_{50} value around 25 μM for betacyanins and up to 10 μM for betaxanthins, with indicaxanthin, the major pigments in prickly pear fruits, as the most effective betalain. The effect of model betalains on the lifespan of the wild-type N2 strain was carefully studied using the automatic platform "Lifespan Machine". In a search for different approaches to suppress progeny, *pop-1* RNAi was used to avoid FUdR use. The presence of betalains in the medium, both as pure compounds and as enriched *Opuntia* extracts significantly increased the lifespan of *C. elegans*.

1. Introduction

Betalains are water-soluble nitrogen pigments responsible for the coloration of plants of the order Caryophyllales, where they replace anthocyanins (Gandía-Herrero & García-Carmona, 2013). Betalains are classified into two groups: yellow betaxanthins and violet betacyanins. Both cases present betalamic acid as the structural unit and differ in the nature of the molecule condensed to it (Gandía-Herrero, Escribano, & García-Carmona, 2010). These betalains confer colour to different parts of Caryophyllales plants (Felker et al., 2008; Gandía-Herrero & García-Carmona, 2013). Betalains are pigments whose stability and safety at high concentrations have led to their being used by the food industry as additives to give colour to food and beverages. In addition, several studies have demonstrated their great antioxidant and free radical scavenging activities and how they are modulated by various structural factors (Gandía-Herrero, Escribano, et al., 2010). Other studies with different cancer cell lines have shown a chemopreventive capacity (Sreekanth et al., 2007) and a reduction of tumours in mice through the administration of these molecules in the diet (Lechner et al., 2010). In addition, an anti-inflammatory activity has been described, which explains the use of plants of the order Caryophyllales in traditional medicine (Vidal, López-Nicolás, Gandía-Herrero, & García-Carmona, 2014). Current evidence suggests a strong health-promoting potential of betalains but studies with purified molecules *in vivo* are needed (Gandía-Herrero, Escribano, & García-Carmona, 2016).

In the present study, the antioxidant and health-promoting effect of the betalains in the *in vivo* model *Caenorhabditis elegans* are investigated. *C. elegans* is a small nematode (approximately 1 mm length) that has a rapid life cycle and a short lifespan. Life extension of *C. elegans* is considered a proof of the health-promoting and anti-aging potential of molecules assayed (Corsi, Wightman, & Chalfie, 2015). Despite its simplicity, *C. elegans* contains a lot of tissues present in higher vertebrates, such as the nervous system, digestive system, musculature, hypodermis and reproductive system, making it a suitable animal model. Previous studies have shown that its genome contains numerous genes and pathways similar to those responsible for human diseases (Shaye & Greenwald, 2011). One of the most important requisites to have significant lifespan data of *C. elegans* is to avoid progeny. Among the few methods used to avoid progeny in *C. elegans*, the most frequent is the daily hand-counting assay to transfer adult worms to fresh plates and leave the progeny in original ones. This manipulation may damage the worms and is only suitable for a small number of specimens and plates. The present study describes a continuous assay of lifespan with a high number of worms and conditions. Thus, a useful method to sterilize the worms is the use of chemical reagents like Floxuridine (FUdR). FUdR is an antineoplastic antimetabolite used as chemotherapeutic agent

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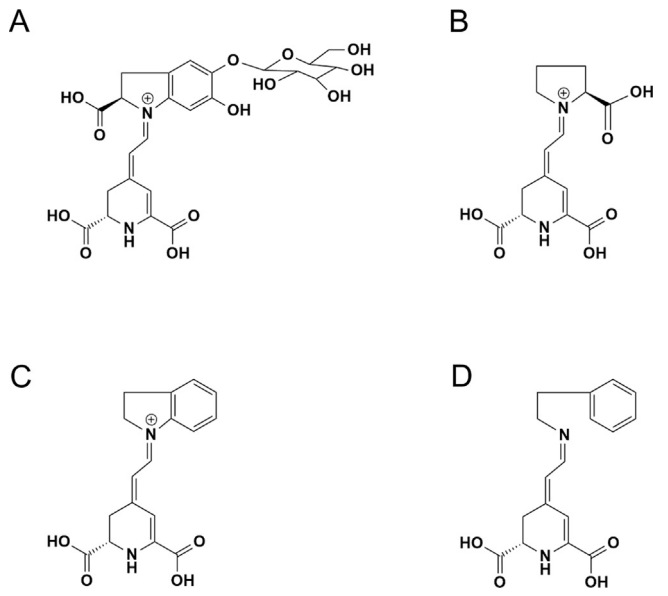

Fig. 1. Structures of betalains used in this study. (A) Betanin. (B) Indicaxanthin. (C) Indoline-betacyanin. (D) Phenylethylamine-betaxanthin.

(Allen-Mersh, Earlam, Fordy, Abrams, & Houghton, 1994) in gastrointestinal tract cancer that spreads to the liver. This compound avoid the presence of progeny in *C. elegans* plates, however, it has been reported that FUdR may expand the *C. elegans* lifespan via thymidylate synthase inhibition, and that may have impacts on *FoxO* transcription factors, sirtuins, and DNA repair pathways (Anderson et al., 2016). It also, increases a resistance of *C. elegans* to acute hypertonic stress, thermal stress, and anoxia. With this in mind, we proposed the use of RNA interference (RNAi) as an alternative method to sterilize the worms and avoid the interference that the use of FUdR may cause in the lifespan of *C. elegans*.

Four different model betalains (Fig. 1) were used for this study: betanin, indicaxanthin, phenylethylamine-betaxanthin and indoline-betacyanin. Betanin (betacyanin), the well-known pigment of beetroots, and indicaxanthin (betaxanthin), the main pigment of *Opuntia* fruits were chosen because they are the most abundant betalains in nature. Phenylethylamine-betaxanthin, naturally present in prickly pear (Castellanos-Santiago & Yahia, 2008), and indoline-betacyanin were chosen for their simple structure and close similarity as pigment models of betaxanthins and betacyanins, respectively, in addition to having a high antioxidant capacity (Gandía-Herrero, Escribano, et al., 2010).

2. Materials and methods

2.1. Chemicals

All chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile was purchased from Fisher Scientific (Dublin, Ireland). Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Extraction and semi-synthesis of betalains

The betalains used were betanin, phenylethylamine-betaxanthin, indicaxanthin and indoline-betacyanin. Betanin was extracted from red roots of *Beta vulgaris*. Phenylethylamine-betaxanthin, indicaxanthin and indoline-betacyanin were obtained as immonium condensation products of betalamic acid with the amines 2-phenylethylamine, proline, and indoline, respectively. Semi-synthesis was carried out following a previously described method (Gandía-Herrero, García-Carmona, & Escribano, 2006). Briefly, betalamic acid was condensed with an excess of individual amines and amino acids to obtain the derived betalains. These were purified to homogeneity by anionic exchange chromatography and solid phase extraction as described below.

2.3. Purification of betalains

Anionic exchange chromatography of betalains was performed in an Äkta purifier apparatus (General Electric Healthcare, Milwaukee, USA). The equipment was operated via PC using Unikorn software version 3.00. Elutions were monitored at 280, 480 and 536 nm. The solvents used were 20 mM sodium phosphate buffer, pH 6.0 (solvent A), and 20 mM sodium phosphate buffer, pH 6.0, with 2 M NaCl (solvent B). A 25 × 16 mm, 20-mL Q-Sepharose Fast Flow column (cross-linked agarose with quaternary ammonium as an exchanger group) purchased from General Electric Healthcare. After sample injection, the elution process was as follows: 0% B from 0.0 to 10 mL; after washing, a linear gradient was performed over 120 mL from 0% B to 26% B, with 3 mL fractions being collected. Injection volume was 25 mL and the flow rate was 2.5 mL/min. Pigment containing fractions were pooled and salts removed through C-18 solid phase extraction by a previously described method (Gandía-Herrero, Escribano, et al., 2010).

2.4. Prickly pears extract

Red and yellow prickly pears from *Opuntia ficus-indica* were purchased from a local distributor. The fruits were peeled, chopped into small pieces and stored at −20 °C in tightly closed containers. Ten grams of red fruit and another ten of yellow fruit were weighed in 50 mL centrifuge tubes and crushed using a spatula to form a soft pulp, which was then passed through a cheesecloth to remove the seeds and tissue debris. The extracts were then diluted with 10 mL of sterile M9 buffer, centrifuged at 5000*g*, filtered through a nylon cloth and finally filtered through a 22 µm sterile filter. The red and yellow extracts prepared were maintained at −20 °C until use.

2.5. C. elegans strains and culture conditions

The wild-type strain N2 and TJ375 (*hsp-16.2/GFP*) strains were obtained from the *Caenorhabditis* Genetic Center (CGC), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). The strains were maintained at 20 °C in solid nematode growth medium (NGM) (Stiernagle, 2006) and the experiments were performed in liquid S medium (Stiernagle, 2006) with animals age-synchronized as described below. *Escherichia coli* OP50 was used as a food source. *E. coli* was grown overnight in Luria-Bertani (LB) medium at 37 °C and was concentrated 10× in sterile M9 buffer.

2.6. Synchronous worm culture

All worms used were age-synchronized. 3-Day old plate worms were collected by washing them from the plate with 2–4 mL of M9 buffer. The worms were transferred to a 1.5 mL Eppendorf and washed by centrifugation for 1 min at 2000 rpm. The supernatant was discarded and 250 µL of M9 buffer and 150 µL of freshly prepared 1:2 10 N NaOH/bleach solution were added. This solution was vortexed at 2-min intervals for 10 s over 10 min. Next, 1 mL of M9 buffer was added to neutralize the reaction and wash the eggs by centrifugation for 1 min at 8000 rpm. The supernatant was discarded and the wash was repeated. 1 mL of M9 buffer was added to the final pellet and the solution was transferred to a sterile 50 mL Erlenmeyer flask. M9 buffer was added for a final volume of 10 mL. The flask was gently shaken overnight at 20 °C for synchronous hatching of the eggs.

2.7. Betalains treatment in C. elegans

The obtained arrested larvae, L1, were collected and transferred to

25 mL sterile flasks containing 250 μL of an *E. coli* OP50 culture 10× concentrated in M9 buffer and 10, 25, 50 and 100 μM of each pure betalain. For a final volume of 5 mL, sterile S medium was added and the flasks were kept under orbital shaking at 20 °C. Incubation time for the wild-type strain N2 was 48 h for the survival assays and 7 days for fluorescence and metabolism assays. The incubation time for the TJ375 strain was 48 h. The prickly pear extracts were used in the same way, but the final concentration of the extracts in the assays was 0.1, 0.5, 1% w/v.

2.8. Quantification of hsp-16.2::GFP expression in C. elegans TJ375

TJ375 (*hsp-16.2::GFP*) worms were cultivated in liquid S medium at 20 °C and the expression of hsp-16.2 was measured by observing the fluorescence of the green fluorescent protein (GFP). The age-synchronized worms were treated for 48 h with different concentrations (10, 25, 50 and 100 μM) of betalains previously purified by ion exchange and C18 reversed phase chromatography, beginning on the day after hatching. To the, worms were then transferred to a new medium, and oxidative stress was exerted by adding a solution of 20 μM juglone to the liquid medium. After 24 h of induction, the worms were washed with M9 buffer and mounted onto glass slides containing 10 mM sodium azide to reduce their mobility. Three independent experiments were performed, and in each of them, the fluorescence of 6–7 individuals was measured at the different concentrations mentioned. Images of fluorescence were taken at constant exposure times using the 20× lens in a Leica DM 2500 LED microscope fitted with a Leica DFC550 camera (Leica Microsystems, Wetzlar, Germany) with incident light beam. The images included the anterior part of the worms from the back of the pharynx. To quantify GFP fluorescence, images were analyzed using ImageJ software. For each raw image, worms were outlined black-to-white inverted and the mean pixel density was measured.

2.9. Platform for the automatic control of the organism C. elegans: Lifespan Machine

A platform for the control of *C. elegans* mobility was built and the lifespan measured following the instructions described by Stroustrup et al. (2013). The Lifespan Machine is a device built to visualize the nematode worm *Caenorhabditis elegans* during its life cycle (around 16–20 days) and to obtain its lifespan automatically. The machine comprises several modified scanners inside an incubator (Fig. S1A), the scanners obtain images of agar plates in which the *C. elegans* are housed. A main computer stores and analyses the digitalized images and records the dead worms. The survival curves and lifespan are obtained with the statistical analysis of the dead worms over time. The ingenious original design was respected in all senses with only two slight modifications. The scanner used was the Epson V800 (Fig. S1B) instead of the Epson V700 in the original paper. The Epson V800 is illuminated with a white LED (light-emitting diodes) lamp (Fig. S1C) while the V700 is illuminated with a cold cathode fluorescent lamp (CCFLs). In addition, worms were placed in closed 50 × 9 mm plates (Falcon) instead of open ones. This reduced contamination and desiccation problems to nil. Scanner lenses were adjusted to focus on the surface of the agar. To assure the best focus, the scanners of the Lifespan Machine were focused as Stroustrup et al. described, but, unlike them, we focused the machine scanners with close lid plates. We decided to maintain the plates closed to avoid cross-contamination and to reduce desiccation of the plates.

2.10. Survival assays

After 48 h in liquid media, the worms were centrifuged at 2.000*g* and washed with M9 buffer three times. 40–50 worms were then transferred to 35 mm analysis plates, containing 8 mL of NGM agar, supplemented with 30 μg/mL of nystatin, 100 μM of ampicillin and 10 μg/mL of FUdR (2′-Deoxy-5-fluorouridine) to avoid progeny. Plates were seeded with 100 μL of *E. coli* from an overnight culture in LB at 37 °C; the culture was concentrated 10× in a sterile M9 buffer and was inactivated using the heat shock method (incubation at 65 °C for 30 min). RNAi model assays were prepared in the same way as the FUdR assays. However, the RNAi analysis plates did not contain FUdR, but were supplemented with 1 mM IPTG and 50 μL of *E. coli* HT115 (10× in M9 from an overnight culture) were seeded in the assay plates and left to induce overnight at 25 °C. All the experiment plates were done in triplicate. Plates were closed and incubated for 20 min at 20 °C. Plates that present condensation were open under sterile conditions and the lids dried with disposable sterile wipers. Closed lid plates were loaded into the scanners of the lifespan machine. The machine acquired an image of each loaded plate every hour for the duration of the experiment and the analysis detected the time of the death for each worm. The experiments were set at 25 °C for 25 days.

2.11. C. elegans sterility via gene knockdown with RNAi

RNAi feeding was used to cause sterility in the N2 strain. The *E. coli* strain HT115 (DE3) with the homologous DNA sequence for the *pop-1* (W10C8.2) gene inside the vector pL4440 DEST was obtained from Source BioScience from the library *C. elegans* ORF-RNAi (Rual et al., 2004). The RNAi feeding was done following the standard protocol (Ahringer, 2006). Briefly, the HT115 strain was cultured in LB supplemented with 100 μg/mL of ampicillin overnight at 37 °C, after which the cells were induced with 1 mM of IPTG at 37 °C for one hour. Cultures were centrifuged to remove LB and concentrated to 10X with M9 buffer. Betalain treatment was done as before, changing the OP50 for HT115 and adding to the reaction medium 100 μg/mL of ampicillin and IPTG to a final concentration of 1 mM.

2.12. Lifespan machine temperature control

C. elegans life cycle is very sensitive to temperature, so in order to avoid differences in lifespan due to temperature, the incubator temperature was adjusted to maintain a temperature of 25 °C inside the scanners, using external temperature probes (USB Reference Thermometers purchased from Thermoworks, Utah, USA). The external probes allowed us to record the temperature inside the scanner for the duration of the experiments.

2.13. Statistical analysis

Mathematical analysis of the obtained data in Lifespan Machine was performed using the online application for survival analysis OASIS 2 (Han et al., 2016), with the Kaplan-Meier estimator, Boschloo's Test, Kolmogorov-Smirnov Test and Survival Time F-Test.

2.14. Protein quantification

The quantity of protein present in the worm's extracts was determined with the Bradford method (Bradford, 1976) using Bradford reagent from Bio-Rad (Bio-Rad Laboratories, Inc).

2.15. Fluorescence assays

For the fluorescence assays, N2 worms were treated with 10, 25, 50 and 100 μM of each betalain. After seven days, worms were centrifuged and washed three times with sterile M9 buffer. Then 10 μL of the worms were loaded in a microscope slide and mixed with 10 μL of sodium azide (10 mM), a coverslip was carefully placed on the sample. The worms were visualized using a Leica fluorescence microscope with the Leica I3 filtercube. Fluorescence intensity was measured with ImageJ as previously described for the TJ375 strain.

2.16. Metabolic assays

On the seventh day of the compound treatment assay, worms were collected, centrifuged and washed three times with sterile M9 buffer. Once cleaned, the worms were resuspended in 20 mM phosphate buffer pH 7.0 supplemented with 10 mM of sodium ascorbate. Then the worms were disrupted by sonication with 3 pulses of 20 s in a Branson Digital sonifier (Branson Ultrasonic Corporation, Connecticut, USA). The debris was precipitated by centrifugation and the supernatant transferred to a clean tube. The samples obtained were stored at −20 °C until analysis.

2.17. HPLC analysis

A Shimadzu LC-10A apparatus (Kyoto, Japan) equipped with a SPD-M10A PDA detector was used for analytical HPLC separations. Reversed phase chromatography was performed with a 250 × 4.6 mm Kinetex 5μ C-18 column (Phenomenex, Torrance, CA, USA). Gradients were formed with the following solvents: solvent A was water with 0.05% TFA, and solvent B was composed of acetonitrile with 0.05% TFA. A linear gradient was performed for 25 min from 0% B to 35% B. The flow rate was 1 mL/min, operated at 25 °C. Injection volume was 20 μL.

3. Results and discussion

3.1. Antioxidant capacity of betalains in vivo in C. elegans strain TJ375

The antioxidant capacity of the betalains was studied measuring the fluorescence intensity emitted by *C. elegans* strain TJ375. This strain contains a reporter transgene that expresses green fluorescence protein (GFP) upregulated by a promoter of small heat shock protein (HSPs), expressed in a stress situation (Feder & Hofmann, 1999). In *C. elegans*, HSPs are a group of 16 kDa proteins and their expression are induced in response to heavy metals (Stringham & Candido, 1994) or quinones (Link, Cypser, Johnson, & Johnson, 1999). The strain TJ375 contain *hsp-16.2::GFP* construction, responsible for the fluorescence emitted in the pharynx (Link et al., 1999) cells under oxidative stress. In this assay, the oxidative stress was produced by the presence of juglone (naphthoquinone) 20 μM. For the four molecules studied, it was observed that as the concentration of the betalain in the medium increases, the expression of *hsp-16.2::GFP* decreases, translating into a decrease of the fluorescence intensity (Fig. 2A–F). At the lowest concentration assayed of 10 μM, the betalains already produce a protection of the worms against the induced oxidative stress. At that concentration betacyanins were less effective with a decrease in fluorescence of 11% for betanin and 27% for indoline-betacyanin in comparison to the untreated control group. The maximum percentages in the decrease of fluorescence due to exposure to betanin or indoline-betacyanin were obtained at 100 μM, reaching values of 76% and 85%, respectively. The values obtained for the betaxanthins indicaxanthin and phenylethylamine-betaxanthin were higher than for the betacyanins. Phenylethylamine-betaxanthin reached a fluorescence decrease of 93% at 100 μM while indicaxanthin reached 94% at the same concentration. The effective dose 50 (ED_{50}) was established as the amount of betalain that produced a reduction of 50% of the initial fluorescence. The values obtained of each betalain were 24.4 μM for betanin, 24.5 μM for indoline-betacyanin, 9.4 μM for indicaxanthin and 13.2 μM for phenylethylamine-betaxanthin. Indicaxanthin was the most protective betalain *in vivo* against the oxidative stress (Fig. 2G) induced in *C. elegans*. The protective effect of some antioxidants had already been studied in the *C. elegans* TJ375 model with positive results, like resveratrol (Chen, Rezaizadehnajafi, & Wink, 2013) or green tea extracts (Abbas & Wink, 2014), rich in catechins, which decreased the fluorescence emission by 40.7% and 68.43%, respectively. However, no antioxidants studied to date have given a 94% decrease, as betaxanthins did.

3.2. The Lifespan Machine use

After making the two modifications on the Lifespan Machine, the temperature control was improved due to the use of scanners with LED lamps, which reduced heat generation. The heat produced by the scanners at different working temperatures (20, 22.5 and 25 °C) is shown in Fig. S2 of the supplementary material. In all case there is a local maximum temperature every 15 min produced by the scanner lamp when passing through the temperature probe while digitalizing the plates. The instant increase in temperature had an amplitude of 0.1 °C and the heat dissipation is fast due to the eight fans mounted in the scanners. The other modification that we proposed is the use of closed plates. In the original paper the authors loaded 16 open plates in a scanner on a glass tray, with the plates sealed using a rubber mat to avoid desiccation. We decided instead to maintain the plates closed with their lids and fix the scanner lens focus on the surface of the agar with closed-lid plates. Stroustrup et al. (2013) reported that approximately 10% of the assay plates was censored (eliminated from the analysis) due to desiccation, contamination or condensation. The use of closed plates meant that the desiccation and contamination were totally avoided. *Caenorhabditis elegans* is very sensitive to temperature and small changes could affect its life cycle and lifespan drastically.

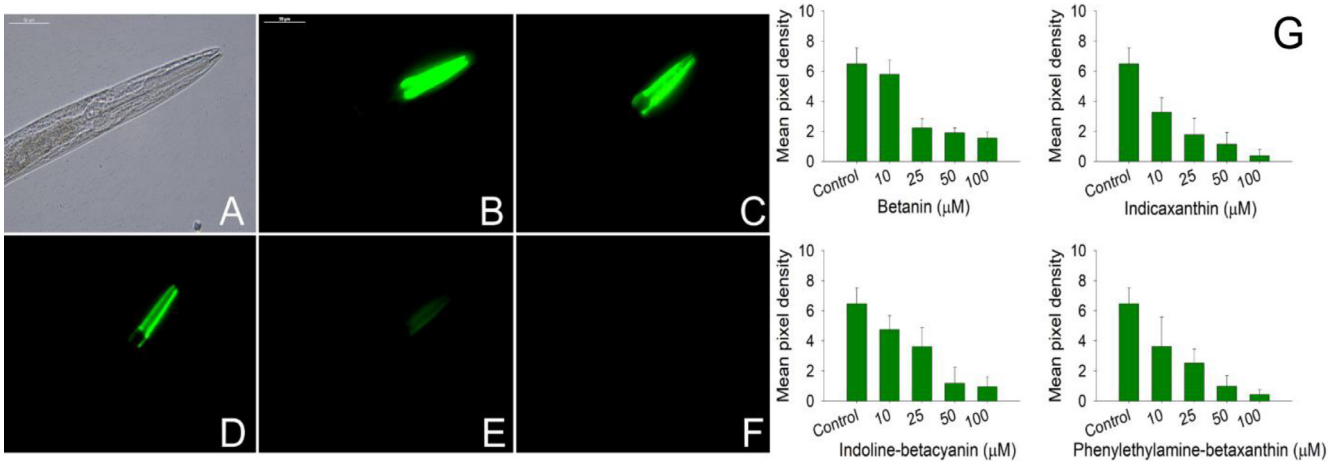

Fig. 2. Antioxidant effect of betalains *in vivo*. (A) Control worm of *C. elegans* strain TJ375 visualized under the bright field technique. (B) The same specimen under fluorescence microscopy using the I3 filtercube. Fluorescence is located in the pharynx after juglone exposure. The diminution of fluorescence was observed in worms pre-treated with 10 (C), 25 (D), 50 (E) and 100 (F) μM of indicaxanthin. (G) Quantification of the effect of pre-treatment with pure betalains measured through fluorescence integration.

Therefore, in order to control the temperature better, several temperature probes were installed inside the machine. These probes recorded the temperature of the scanner every 30 s for the duration of the experiment. The incubator temperature was adjusted so that the temperature of the scanner interior (where the plates are situated) was 25 °C.

3.3. Betalains effect on C. elegans lifespan

The wild-type N2 strain was used to study the health-protective effect of betalains by increasing the lifespan of *C elegans*. Wild-type worms were exposed during 48 h to concentrations of 10, 25, 50 and 100 μM of betalains. The worms were then transferred to fresh NGM supplemented with *E. coli* as a food source and incubated at 25 °C in the Lifespan Machine, where mean and maximum lifespan were measured. Mean lifespan refers to the survival time of 50% of the population. Maximum lifespan is the survival time of the last worm alive (Figs. S3 and S4). As shown in the S1 Table, betanin did not have a significant effect on the lifespan of *C. elegans*. However, the betacyanin derived from indoline increased the average life expectancy by 10% at 25 and 50 μM and its effect reached a maximum value of 21.67% when the highest tested concentration (100 μM) was used. In addition, exposure to indoline-betacyanin increased the maximum lifespan by 0.5 days. These values were even higher in the exposure of the worms to the yellow betalains phenylethylamine-betaxanthin and indicaxanthin (Fig. 3A). Phenylethylamine-betaxanthin achieved an increase in mean survival of 14.29% and an increase of 2.4 days in the maximum lifespan when the worms were exposed to 50 μM dose. The exposure to 50 μM of indicaxanthin was the most efficient, increasing the maximum lifespan by 3.96 days, where mean survival increased by 32.42%. At 25 μM, the increase in mean survival reached 34.34% with a maximum lifespan increase recorded of 3.9 days. Although the maximum duration of life increased significantly ($p < 0.05$) with these betaxanthins around 2–3 days, the use of the highest dose (100 μM) resulted in a decrease in the mean and maximum life expectancy. Therefore, the optimal dose for indicaxanthin was 25 μM, whereas for phenylethylamine-betaxanthin it was 50 μM. Effective concentrations of betalains are much lower than those used for other antioxidant molecules like catechin, which enable an increase of 13% in the maximum lifespan of *C. elegans* at 200 μM concentration (Saul, Pietsch, Menzel, Stürzenbaum, & Steinberg, 2009) or the flavonoids myricetin, quercetin and kaempferol, which at 100 μM provoked an increase in the maximum lifespan (Grünz et al., 2012) of 21,7%, 18.4% and 6.7%, respectively. Thus, betalains are the most active antioxidants assayed in the life extension of *C. elegans*.

3.4. Novel control of progeny based on pop-1 RNAi

The lifespan assay was also realized employing RNAi as an alternative to FUdR use in order to assess sterility. FUdR is an antineoplastic antimetabolite that may expand the *C. elegans* lifespan via thymidylate synthase inhibition (Anderson et al., 2016). The chosen gene to knockdown was *pop-1*, which is involved in the development of the vulva and cell fate (www.wormbase.org). The silencing of this gene causes sterility in L1 worms and embryonic death in L4 worms (Lin, Thompson, & Priess, 1995). Under the RNAi *C. elegans* model, the tested betalains showed the same trend than under the FUdR system. The exposure to betanin did not show any significant effect in the lifespan of worms while the betacyanin derived from indoline at 100 μM increased the mean survival by 11.09% (S2 Table). As shown in Fig. 3B, the lower effective concentration of betalains was obtained with the yellow ones. Phenylethylamine-betaxanthin used at 50 μM was able to increase 7.5% the lifespan and indicaxanthin expanded the mean lifespan up to 18.0% when used at 25 μM. Therefore, with this alternative method the health-protective effect of indicaxanthin was confirmed. As shown in the S1 Video, the use of FUdR or RNAi is efficient to avoid the presence of progeny. The worms sterilized with FUdR and fed with heat shocked *E. coli* lived longer than the RNAi ones where the *E. coli* is active. Overall the effect of FUdR combined with heat shocked bacteria results in around a 40% expansion of lifespan in control worms. These results are in agreement with Anderson et al. (Anderson et al., 2016) research which stated that FUdR treated worms live longer than untreated ones and Stroustrup (Stroustrup et al., 2016), who obtained a longer lifespan when UV inactivated bacteria were used.

3.5. Detection of betalains in worms through fluorescence

Thanks to the thin *C. elegans* epidermis, the natural fluorescence of betaxanthins inside the worm's body could be quantified and their position observed. After seven days of incubation with betalains, worms treated with betacyanins did not give any colour or fluorescence signal in microscopy assays. This may be indicative of a low presence of the compounds and its metabolization. Previous studies demonstrated that betaxanthins maintain their fluorescent properties under physiological conditions and are responsible for visible fluorescence in flowers

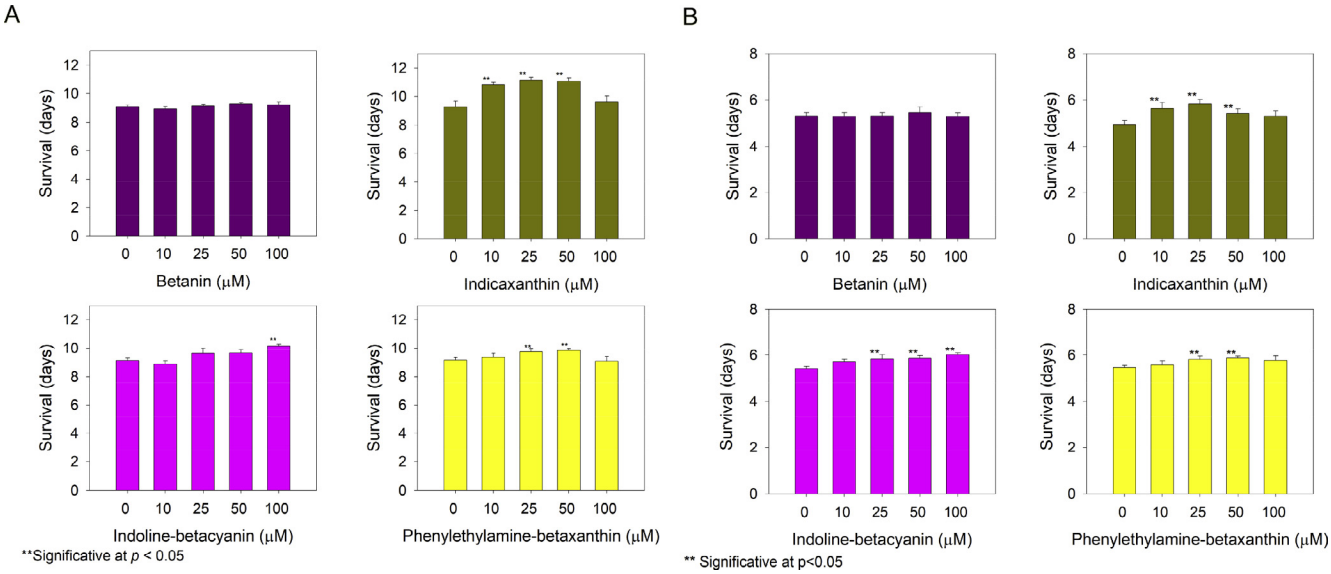

Fig. 3. Mean lifespan of *C. elegans* wild-type strain N2 sterilized by FUdR (A) or sterilized by feed with RNAi (B). Both groups of experiments were pre-treated with betanin, indicaxanthin, indoline-betacyanin and phenylethylamine-betaxanthin.

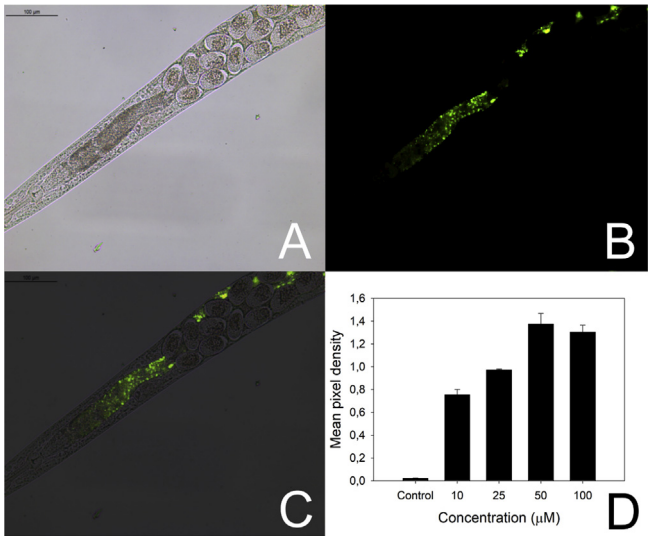

Fig. 4. *C. elegans* wild-type strain N2 after seven days treatment with phenylethylamine-betaxanthin. (A) Brightfield image. (B) Fluorescence emitted followed with Leica I3 filtercube. (C) Merged image showing the location of the fluorescence in the digestive tube of *C. elegans*. (D) Fluorescence emitted by *C. elegans* after treatments with different concentrations of phenylethylamine-betaxanthin.

(Gandía-Herrero, García-Carmona, & Escribano, 2005). Phenylethylamine-betaxanthin fluorescence was observed in *C. elegans* with a maximum value when worms were exposed to a 50 μM dose (Fig. 4D). This result is in agreement with the maximum values obtained for this betalain in the lifespan assay and might to indicative of its prevalence in the worm. With respect to fluorescence localization, Fig. 4A–C shows accumulation of phenylethylamine-betaxanthin in the digestive tube, indicating that betalains are being incorporated into the organism through feeding. No fluorescence was detected for indicaxanthin fed *C. elegans*, which may be indicative of a higher metabolization. Phenylethylamine-betaxanthin is more hydrophobic and might be incorporated more efficiently into the worm tissues in the digestive system. Fluorescence of betaxanthins has previously been employed to visualize petal cells (Gandía-Herrero, Escribano, & García-Carmona, 2005) to detect the presence of *Plasmodium falciparum* (malaria) in erythrocytes (Gonçalves et al., 2013) and to follow the accumulation of betaxanthins in *Celosia argentea* and *Chenopodium quinoa* cell cultures (Guadarrama-Flores, Rodriguez-Monroy, Cruz-Sosa, Garcia-Carmona, & Gandia-Herrero, 2015; Henarejos-Escudero et al., 2018). This is the first time betaxanthins fluorescence is used to visualize animal tissue, thus revealing the potential of the pigments in microscopy applications.

3.6. Quantification of betalains in worms

HPLC was used to follow the presence of betalains and the derived product, betalamic acid, in worms extracts after seven days of exposure to betalains. Detection of supplemented betalains confirmed the accumulation of phenylethylamine-betaxanthin observed by microscopy. The accumulation of indoline-betacyanin was also detected at lower quantities. Indicaxanthin and betanin were not detected in the worm extracts, an indication -as commented above- that the most hydrophobic betalains tended to accumulate, whereas the most hydrophilic ones were rapidly metabolized. Between indoline-betacyanin and phenylethylamine-betaxanthin, the second was accumulated in higher proportions and both responses were dose-dependent (Fig. 5). The lower amount detected for indoline-betacyanin may be responsible for the lack of signal in the microscopy assays commented on above. Worms treated with a dose of 50 μM of phenylethylamine-betaxanthin accumulated 26.35 mol per gram of protein. Worms treated with the same dose of indoline-betacyanin only accumulated 4.4 moles per gram of protein. Betalamic acid, as a possible metabolization product was not detected. In humans, phenylethylamine is a dopamine receptor antagonist rapidly metabolized by the monoamine oxidase B enzyme (MAO-B) (Yang & Neff, 1973).

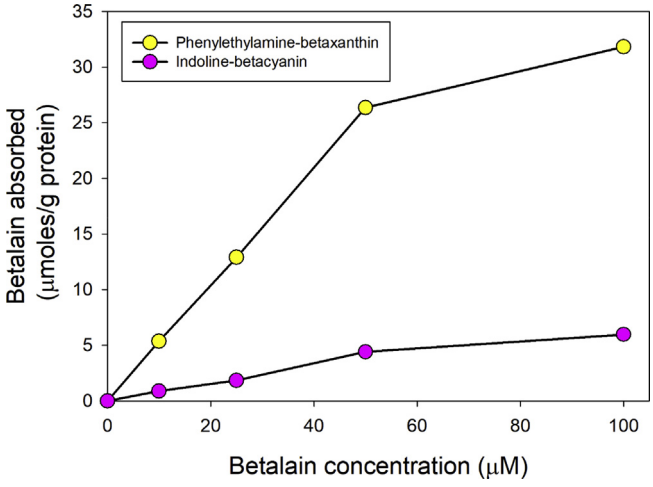

Fig. 5. Quantification of phenylethylamine-betaxanthin and indoline-betacyanin accumulated in worm's soma expressed in μmole betalain/g protein.

3.7. Extract of prickly pear as a source of betalains

In global terms, indicaxanthin was the most effective betalain in increasing the lifespan of *C. elegans*. This betalain is the main pigment present in yellow fruits from *Opuntia ficus-indica* (Gandía-Herrero, Jiménez-Atiénzar, Cabanes, García-Carmona, & Escribano, 2010b), although it is also present in red pears, where betanin is the main betalain (Forni, Polesello, Montefiori, & Maestrelli, 1992). Extracts of red and yellow prickly pears were also used to study their effect on the lifespan of *C. elegans* (Fig. 6). Different weight/volume (w/v) concentrations from extracts were used in the assay and were analyzed by HPLC. Pure indicaxanthin and betanin were used as standards to ascertain the concentration, expressed in μM, in prickly pear extracts. S3 Table shows the relationship between w/v concentration from extracts and the concentration of the major pigment, expressed in μM. The analysis in the Lifespan Machine shows a significant increase in the average lifespan of *C. elegans* in the presence of 0.5% w/v from yellow extract, where the maximum lifespan was increased by up to 14.2 days and the

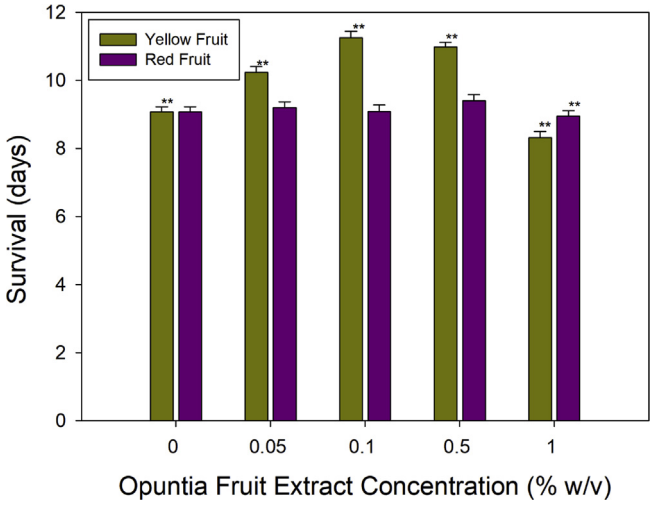

Fig. 6. Lifespan of *C. elegans* wild type strain N2 treated with *Opuntia* extracts.

mean survival increased by 21.06%. The red extract also increased the maximum lifespan from 12.8 to 13.7 days. This supposes an increase of 0.85 days in total lifespan but in this case, the mean survival did not increase significantly in any concentration of red extract, even having a negative effect on lifespan when the highest concentration was used. The antioxidant capacity of prickly pear had been demonstrated in vitro assays (Butera et al., 2002) and the results from our tests in an *in vivo* system are in agreement with this great effect against aging. In addition, our results are comparable with extracts rich in other antioxidant pigments, like purple wheat (Chen, Müller, Richling, & Wink, 2013) or Acai (*Euterpe precatoria*) (Peixoto et al., 2016) extracts, both rich in anthocyanins or Tsai Tai (*Brassica chinensis*) extract (Chen et al., 2016), rich in flavonoids and hydroxycinnamic acid derivatives. The strong effect demonstrated for pure indicaxanthin justified the effect shown for the yellow extract of *Opuntia* fruits in the increase in the lifespan of *C. elegans*.

4. Conclusions

Pure individuals betalains, both natural and semi-synthetic, have proven to exert health-promoting benefits *in vivo* in the *C. elegans* animal model. For the first time, antioxidant activity *in vivo* is demonstrated for betalains and justifies an increase in a life-span of more than 30% for the treated *C. elegans*. The activity is also demonstrated for a natural edible source of the pigments, prickly pears, whose consumption had been previously demonstrated to present health benefits and linked to the presence of indicaxanthin. The results of the present work open new perspectives in the research and application of betalains as phytochemicals of interest for the food, cosmetic and pharmaceutical industries.

Conflicts of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2018.09.067.

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