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Neuroprotective effects of dried camu-camu (*Myrciaria dubia* HBK McVaugh) residue in *C. elegans*

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ABSTRACT

The effect of hot air dried camu-camu (*Myrciaria dubia* HBK McVaugh) residue on modulating redox response signaling was evaluated in transgenic *Caenorhabditis elegans*. Camu-camu residue was fractionated into low and high molecular weight fractions and used as treatments. Relative fold changes in gene expression in response to camu-camu treatments were quantified using fluorescence microscopy. Also, the neuroprotective effects of camu-camu residue in experimentally induced neurodegeneration were evaluated in *C. elegans* models for Alzheimer's disease (AD) and Parkinson's disease (PD). For AD, time to thermally induced $A\beta_{1-42}$ aggregation mediated paralysis was evaluated in transgenic *C. elegans* (CL4176). For PD, MPP+ induced neurodegeneration was quantified by loss in motility due to paralysis. Results suggest a significant upregulation expression of superoxide dismutase (SOD-3 and SOD-4) and catalases (CTL-1; CTL-2; CTL-3) in response to treatment with camu-camu residue, especially with the low molecular weight fraction. Furthermore, treatment with this fraction significantly extended the life span in *C. elegans* by 20% and delayed $A\beta_{1-42}$ induced paralysis by 21%. Additionally, treatment with camu-camu residue also abrogated MPP+ induced neurodegeneration for PD by 15–21%.

1. Introduction

Camu-camu (*Myrciaria dubia* (H.B.K.) McVaugh) is an exotic tropical fruit native to the Amazon region and it is one of the few Amazon fruits that have been explored for commercial purposes. The camu-camu fruit belongs to the group of the so-called super fruits and it is known by its outstanding content of ascorbic acid and health-relevant flavonoids (Borges, Conceição, & Silveira, 2014; Akter, Oh, Eun, & Ahmed, 2011). They are economically important to the Amazon region, because this exotic fruit grows in low-value areas, which are generally inadequate for the cultivation of other species (Rodrigues, Menezes, Cabral, Dornier, & Reynes, 2001).

Because of its natural acidity, the camu-camu fruit is mainly consumed after processed into juices, concentrates, and for the production of vitamin C capsules. As a result, great volumes of residue, consisting of seeds, peels and residual pulp that represent around 40% of the fruit in weight, are generated (Rodrigues et al., 2001). Despite this, while several studies have focused in investigating the properties of camu-camu pulp (Gonçalves, Lellis-Santos, Curi, Lajolo, & Genovese, 2014; Fujita, Borges, Correia, Franco, & Genovese, 2013), the bioactive potential and biological effects of the residue remain understudied (Azevêdo, Fujita, Oliveira, Genovese, & Correia, 2014; Fracassetti, Costa, Moulay, & Tomás-Barberán, 2013). For example, recent research have described anti-obesogenic, anti-atherogenic anti-inflammatory and anti-genotoxic effects of camu-camu fruit juice (Silva et al., 2012; Langley, Pergolizzi, Taylor, & Ridgway, 2014; Nascimento, Boleti, Yuyama, & Lima, 2013); however, the literature describing the potential health benefits of camu-camu residue are nonexistent. In addition, creating a low-waste agribusiness where environmental-friendly techniques would take full advantage of the entire fruit would be advantageous for the entire productive chain (Moo-Huchin et al., 2015).

The use of invertebrate organisms, especially *Caenorhabditis elegans*, as model systems to study biological processes on molecular and cellular levels in higher organisms has recently become a popular and important research tool. *C. elegans* are attractive models because they are inexpensive and easy to experimentally manipulate (Chakraborty, Bornhorst, Nguyen, & Aschner, 2013). Additionally, they also have a short life span and have a completely sequenced genome possessing 60–80% homology to human genes. Recently, *C. elegans* has been employed as a model to study the effect of bioactive chemicals from natural products on evolutionarily conserved signaling pathways involved in stress response, life span, neurodegeneration and developmental biology (Keith, Amrit, Ratnappan, & Ghazi, 2014). Also, it has been successfully applied to assess the neuroprotective effects of tropical fruit extracts (Bezerra et al., 2014; Bonomo et al., 2014).

We have previously demonstrated that freeze dried and hot air dried camu-camu residue, are rich sources of bioactive compounds, including

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ascorbic acid, proanthocyanidins, carotenoids and important flavonoids (Azevêdo et al., 2014). Based on these results, our objective here was to evaluate for the first time the effects of dried camu-camu on the modulation of stress response in vivo in *C. elegans* model and investigate their potential neuroprotective effects. The experimental results presented here will help to clarify the biological value of the residue obtained from this understudied fruit of the Brazilian biodiversity.

2. Material and methods

2.1. Dried camu-camu production

The fresh camu-camu residue (FR) was submitted hot air drying (HAD) process at 50 °C and air velocity of 4 m/s (HAD50) (Azevêdo et al., 2014). The drying experiments were conducted by spreading the previously thawed camu-camu residue in 10–15 mm thick uniform layers on a perforated tray. The hot air dryer equipment was equipped with centrifugal blower (5.5 hp and 3490 rpm, model 112 M, WEG, Brazil) and the airflow crossed perpendicularly through the tray placed at the center of the drying chamber, where the temperature was monitored by digital thermocouples.

2.2. Low (LMWF) and high (HMWF) molecular weight fractions

The low and high molecular weight fractions of camu-camu powder were prepared using a method described previously (Bezerra et al., 2014). For the low molecular weight fraction, 5 g of camu-camu powder was suspended in 100 ml of water in an Erlenmeyer flask at 60 °C and allowed to mix for 30 min at 250 rpm. The samples were then centrifuged at 4000 rpm at 10 °C for 15 min. The supernatant was filtered under vacuum using a Buchner funnel equipped with Whatman No. 1 filter paper. The filtrate was mixed with acetone in 1:1 (*v*/*v*) ratio and again centrifuged to precipitate soluble gums and fibers in the extract. The acetone in the supernatant was evaporated in a Rotavapor® (Model RII, Buchi, Switzerland) at 60 °C for 5 min under vacuum. The acetone free solution was filter-sterilized (Corning, North Bend, OH) and labeled as LMWF-HAD50.

To prepare the high molecular weight fraction, the residue from the filter paper was scraped with a spatula into an Erlenmeyer flask and combined with the sediment from centrifugation from previous step above. To this, 66.6 ml of 4 N NaOH was added, and the mixture was stirred for 30 min at 250 rpm on a magnetic stirrer. The mixture was then centrifuged and vacuum filtered as described above. The pH of the filtrate was adjusted immediately to 7.0 and filter-sterilized into a sterile bottle labeled as HMWF-HAD50.

2.3. Preparation of polar acid (PA), polar basic (PB) and polar neutral (PN) fractions

The LMWF-HAD50 was solvent fractionated to obtain the PA, PB and PN fractions. The pH of these extracts was adjusted to 9.6 using 5% NaOH and transferred to a separation funnel to which 100 ml of ethyl acetate was added to 100 ml of the extracts. The mixture was vigorously shaken and allowed to rest until a phase separation was achieved. The denser aqueous phase was eluted out, and labeled as PA after adjusting its pH to 7 using 1 N HCl. To the lighter organic phase, 30 ml of HCl 5% (*v*/*v*) was added and the mixture was again vigorously shaken and allowed to rest as described above. The denser aqueous fraction was eluted out, and labeled as PB after adjusting its pH to 7 using 1 N NaOH. The remnant organic fraction was labeled as PN and, along with PA and PB, was filter sterilized and stored at 4 °C until use. Using this procedure, the following fractions PA-HAD50, PB-HAD50 and PN-HAD50 were obtained.

2.4. C. elegans strains and maintenance

Wild type and transgenic *C. elegans* strains (Table 1) carrying green fluorescent protein (gfp) or heat shock protein (hsp) promoter fusions of different genes relevant were obtained from the *Caenorhabditis* Genetics Center (CGC) (University of Minnesota, Minneapolis, MN). Worms were grown and maintained at 20 °C (except CL4176 which was maintained at 16 °C) on 60 mm culture plates with Nematode Growth Medium (NGM) (1.7% agar, 0.3% NaCl, 0.25% Peptone, 1 mM CaCl, 1 mM MgSO4, 5 mg/l cholesterol, 2.5 mM KPO_4 at 16–20 °C, Brenner, 1974). Media was poured aseptically into culture plates (10 ml for 60 mm) using a peristaltic pump and allowed to solidify for 36 h. NGM culture plates were then inoculated with 50 μl of *Escherichia coli* OP50 (CGC, University of Minnesota, Minneapolis, MN) overnight cultures and incubated for 8 h at 37 °C. Strains of *C. elegans* were maintained by picking 2–3 young adult worms onto freshly inoculated NGM plates every 4–7 days.

2.5. Age synchronization

Prior to the beginning of the experiment *C. elegans* were age synchronized. Ten worms at L4 stage (F0) were transferred to single NGM plates and incubated at 20 °C until they progressed to adulthood and laid eggs. Adults were immediately removed from the plates and the eggs were allowed to hatch (F1) and grow to L4 at 20 °C. L4 worms of F1 generation were again transferred to fresh NGM plates and allowed to mature into gravid adults and lay eggs at 20 °C. Adults were quickly removed from the plates and were returned to 20 °C incubator to facilitate egg hatching. L1 worms (F2) generation were collected from the plate by washing with S-basal buffer (0.59% NaCl, 5% 1 M KPO_4,

Table 1
List of *C. elegans* strains included is the gene names with their human homolog.

Strain	Transgene	Gene	Human homolog	Wormbase gene ID[a]
N2	*Wild Type*	N/A	N/A	N/A
GR1352	*xrls87[daf-16alpha::GFP::DAF-16B + rol-6(su1006)].*	*DAF-16α::GFP*	Forkhead Box O (FOXO)	WBGene00000912
CF1553	*muls84 [pAD76(sod-3::GFP)]*	*sod-3::GFP (driven by sod-3)*	Iron/manganese superoxide dismutase	WBGene00004932
LG326	*gels7[skn-1b::GFP].*	*[skn-1b::GFP]*	Nuclear factor-erythroid-2 related factor-2 (Nrf2) transcription factor	WBGene000004802
BC13632	*sEx13632 [rCesF55H2.1::GFP + pCeh361]*	*sod-4::gfp*	Copper/zinc superoxide dismutase	WBGene00004933
GA800	*wuls151[ctl-1 + ctl-2 + ctl-3 + myo-2::GFP]*	*[ctl-1 + ctl-2 + ctl-3 + myo-2::GFP]*	Catalase (ctl-1, ctl-2 e ctl-3)	WBGene00000830, WBGene00000831, WBGene00013220 respectively
CL2166	*dvls19[pAF15(gst-4::GFP::NLS)]*	*gst-4*	Glutathione-requiring prostaglandin D synthase	WBGene00001752
CL4176	*smg-1(cc5460l; dvls27 X. dvls27X[pAF29(myo-3/A-Beta1-42/let UTR) + pRF4(rol-6(su1006))]*	*ABeta42*	Human amyloid β_{1-42}	WBGene00003515

[a] Origination: www.wormbase.org.

5 mg/ml cholesterol in ethanol) (Ching & Hsu, 2011; Vayndorf, Lee, & Liu, 2013) into a sterile 15 ml centrifuge tube. Worms from a minimum of 5 plates were pooled into a single centrifuge tube and centrifuged at 8000 rpm for 10 min at 10 °C. The supernatant was carefully aspirated and the worms were washed again in by S-basal buffer, centrifuged and aspirated to leave approximately 1 ml of S-basal in the centrifuge tube. The tube was gently agitated to disperse the worms and 20 μl was pipetted onto a slide and the number of worms was counted under a stereo microscope. The concentration of the worms was adjusted to be 10–15 worms by diluting with S-complete liquid media (97.7% S-basal, 1% potassium citrate, 1% trace metals, 0.3% $CaCl_2$, 0.3% $MgSO_4$). A 100 mg/ml suspension of *E. coli* OP50 was prepared by centrifuging 100 ml of an overnight *E. coli* OP50 culture in LB at 3500 rpm. Spent LB was aspirated and pellet was washed several times by resuspension and centrifugation in sterile distilled water. The weight of the resultant pellet was determined and adjusted to 100 mg/ml using S-complete medium. *E. coli* OP50 was added to the vial of age synchronized worms to yield a final concentration of 5 mg/ml and used immediately to set up experiments.

2.6. In vivo gene expression studies in transgenic C. elegans

The assay was set up by transferring to each well of a sterile 96-well microplate, 80 μl (around 17–23 worms) of age synchronized L1 worms (item 2.5) in S-Complete containing 5 mg/ml of E. coli OP50 and 1% (*v*/*v*) LMWF-HAD50 or HMWF-HAD50 extracts (or DH_2O for control). A minimum of 8 worms were prepared for each treatment and the plates were sealed with Glad Press'n'Seal ® (Glad, Oakland, CA) and incubated at 20 °C. When the worms reached the L4 stage, they were transferred to a 35-mm culture plate containing a solidified 2 ml layer of 1% Phytagel (Sigma-Aldrich, St. Louis, MO). Worms were immobilized by adding 8 μl of 25 mM sodium azide solution in magnesium buffer and used for direct in vivo fluorescence imaging using a fluorescence microscope (Nikon SMZ1500 with Ri1 CCD camera, Nikon, Japan). 12 replicates with a minimum of 120 worms were used per treatment. L4 worms were randomly selected and the relative fluorescence, with respect to control, was quantified from the corrected total fluorescence using the National Institute of Health's ImageJ software (Iser & Wolkow, 2007).

2.7. Lifespan evaluation

The life span of the wild-type N2 strain was analyzed as described by Solis and Petrascheck (2011). L1 synchronized adult worms in S-complete medium (10–15 worms per 20 μl) containing 5 mg/ml *E. coli* OP50, a mixture of penicillin-streptomycin 1% (*v*/*v*) was added. An aliquot of the worm suspension (120 μl) was transferred to each well of a 96-well plate, homogenized for 2 min and incubated for 45 h at 20 °C until the animals reach the L4 stage. The animals were sterilized by adding 30 μl of a stock solution of 0.6 mM fluorodeoxyuridine (FUDR) to each well. After 8–12 h, LMWF-HAD50 (item 2.2) and their fractions (PA, PB and PN) (item 2.3) were directly added at 1% (*v*/*v*), the plates were sealed (Glad, Oakland, CA) and incubated at 20 °C. Every 5–7 days, 5 μl of the *E.* coli OP50 (100 mg/ml) was added to each well. The life span and survival rates were evaluated by scoring the alive worms (swimming morphology) using a microscope (PARCO, Parco Scientific Company—Westland, MI). Additionally, the mean, median and maximum survival of worms were also calculated. Three replicates were used per experiment with $n > 300$ worms per treatment.

2.8. Alzheimer's disease assay in transgenic C. elegans

The CL4176 strain containing a heat-sensitive mutation developed to express human amyloid β_{1-42} ($A\beta_{1-42}$) present in the muscle tissue was maintained on NGM plates at 16 °C (Dostal, Roberts, & Link, 2010). Previous to the beginning of the experiment, *C. elegans* were age synchronized at 16 °C (item 2.4). In order to stop the growth of *E. coli* OP50, 50 μl of this mixture was spread and exposed to UV light for 2 min (Stratagene UV Stratalinker 2400, La Jolla, CA). After this, the plates were incubated with treatments for 24 h at 23 °C (Sutphin & Kaeberlein, 2009). L1 worms from F2 generation were transferred to control or treatment plates and allowed to mature gravid adult stage to lay eggs. After reaching the L3 stage, the incubation temperature of the plates was increased from 16 °C to 25 °C, in order to induce the expression of $A\beta_{1-42}$. The evaluation of the mobility of worms was started 18–20 h after increasing the incubation temperature in 2-hour increments until all worms were paralyzed. Three replicates per experiment were performed with a minimum of 75 worms were used. The lack of touch response or head movements was used to define them as paralyzed or dead. The time at which 50% of the worms were paralyzed or killed (PT_{50}) was calculated based on the survival curves. Additionally, the mean, median and maximum survival of worms were also calculated post heat shock treatment.

2.9. MPP+ induced Parkinson's disease assay in C. elegans

30 μl of L3 synchronized worms (approximately 17–23 worms) in S-complete containing 5 mg/ml *E. coli* OP50 was mixed to 20 μl of MPP + (1.13 mg/ml DH_2O) and 1% (*v*/*v*) of the treatments LMWF-HAD50 and their fractions (PA, PB and PN) and the control (DH_2O instead of the treatment) were transferred to sterile 96-well plates sealed with Glad Press'n'Seal ® (Glad, Oakland, CA) and incubated at 20 °C. The mobility of the worms was counted in 12-hour intervals and the total number of paralyzed worms was registered after 48 h. At least 8 replicates per experiment with a minimal of 100 worms were used. The lack of touch response and absence of pharyngeal pumping was used to identify the paralyzed or dead worms (Braungart, Gerlach, Riederer, Baumeister, & Hoener, 2004).

2.10. Statistical analyses

Results were expressed as mean ± standard deviation. One-way ANOVA was used to determine statistical significance ($p < 0.05$) for in vivo studies of gene expression and for the assay of MPP + induced Parkinson's disease. For Alzheimer's disease assay, the survival differences were tested for significance ($p < 0.001$) by the Breslow–Gehan–Wilcoxon test. For the life span assay, the Kaplan–Meier method was used to compare the life span survival curves and the survival differences were tested for significance ($p < 0.05$) using the Log rank test (Mantel Cox). Both tests used GraphPad Prism software 6.0 (GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. The effect of dried camu-camu on genes relevant to aging and neurodegeneration

Treatment with LMWF-HAD50 (1% *v*/*v*) resulted in a significant downregulation in the expression of *daf-16* transcription factor (Fig. 1A), whereas treatment with HMWF-HAD50 had no change on the expression of the fork-head transcription factor *daf-16* compared to the control. Expression of the mitochondrial superoxide dismutase *sod-3* gene was upregulated 3-fold relative to control in response to treatment with LMWF-HAD50, but did not have any effect on in response to treatment with HMWF-HAD50 (Fig. 1B). The expression of extra cellular superoxide dismutase *sod-4* gene was also upregulated significantly both in response to treatment with LMWF-HAD50 and HMWF-HAD50 (Fig. 1C). In response to treatment with 1% (*v*/*v*) of camu-camu extracts, the expression of catalases (*ctl-1*, *ctl-2*, *ctl-3*) was upregulated relative to control both in response to treatment with LMWF-HAD50 and HMWF-HAD50 (Fig. 1D).The expression of nuclear factor-erythroid-2 related factor-2 (*Nrf2*) transcription factor or *skn-1* that regulates the expression of several antioxidant response genes was upregulated in response to treatments with both LMWF-HAD50 and HMWF-HAD50 (Fig. 1E). The expression of glutathione-

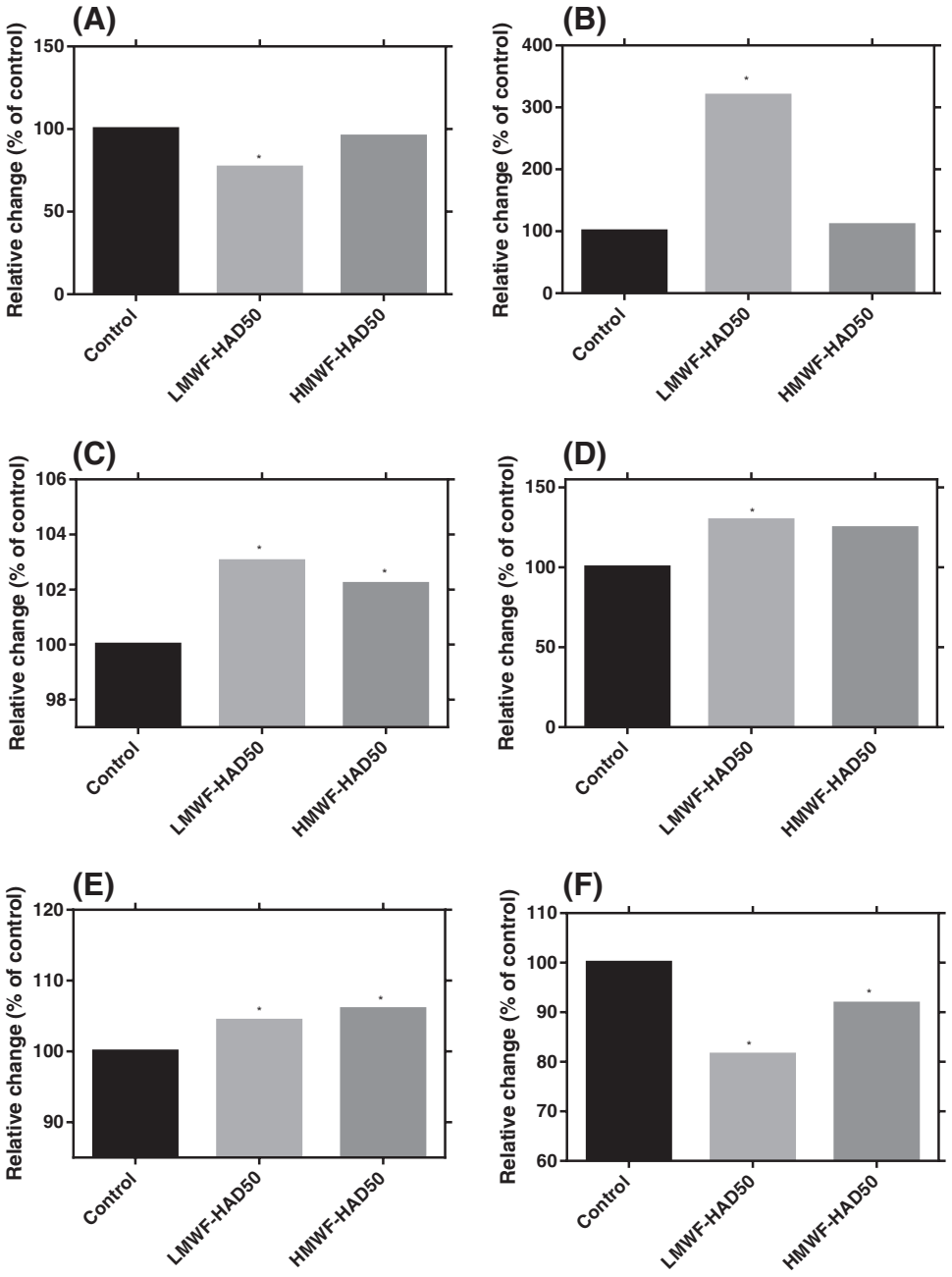

Fig. 1. Relative change (% of control) in expression of (A) *daf-16* (B) *sod-3* (C) *sod-4* (D) *ctl-1 + ctl-2 + ctl-3* (E) *skn-1* (F) *gst-4* in transgenic *C. elegans* worms (GR1352, CF1553, BC13632, GA800, LG326 and CL2166) fed on: control: [NGM]; LMWF-HAD50 [NGM + 1% (*v*/*v*) LMWF-HAD50]; HMWF-HAD50 [NGM + 1% (*v*/*v*) HMWF-HAD50]. *E. coli* OP50 (5 mg/ml); $n > 100$; * $p < 0.05$ (ANOVA).

s-transferase gene (*gst-4)* however, was significantly downregulated in response to treatment with LMWF-HAD50, but again did not change in response to treatment with HMWF-HAD50 (Fig. 1F).

Overall, treatment with LMWF-HAD50 appeared to result in a more robust antioxidant response and enhanced protection against oxidative stress, and therefore, was selected for further fractionation (PA, PB and PN). These fractions were used to evaluate their ability to extend life span (item 3.2) and abrogate experimentally induced neurodegeneration (3.3) in *C. elegans*.

3.2. Effect on the life span of wild type C. elegans

Fig. 2 presents the survival curves of wild type *C. elegans* (N2) treated with the low molecular weight extracts of HAD50 and its PA, PB and PN fractions, respectively. The effect of treatment on the life span of wild type (N2) *C. elegans* was determined using Kaplan–Meier survival analysis with Log-rank significance test. The median life span of *C. elegans* in control group (no treatment) was 15 d (Table 2). The median life span of *C. elegans* treated with LMWF-HAD50 increased significantly by 20% to 19 d ($p < 0.001$) compared to control. Among all the fractions, treatment with PB-HAD-50 (Table 2) also resulted in a significant extension in life span of *C. elegans* to 19 d relative to control. Treatment with other fractions (PA-HAD-50 and PN-HAD-50) only increased the life span of *C. elegans* by 13% relative to control.

3.3. Extracts of dried camu-camu residue ameliorate experimentally induced neurodegeneration in C. elegans

3.3.1. Alzheimer's disease

The mobility curves for the CL4176 strain after the Aß$_{1-42}$ induction of muscular paralysis at 25 °C are shown in Fig. 3. In control worms,

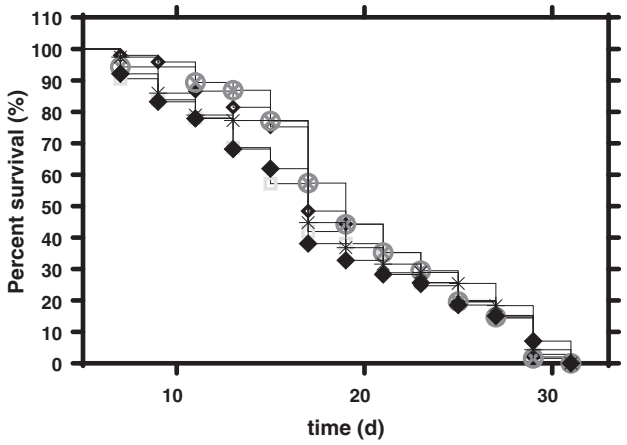

Fig. 2. Kaplan–Meier survival plots of wild type (N2) *C. elegans* worms treated with low molecular weight extract of HAD50 (LMWF-HAD50) and fractions polar acid (PA), polar basic (PB) and polar neutral (PN). (□) Control: [NGM]; (◊) LMWF-HAD50: [NGM + 1% (*v*/*v*) LMWF-HAD50]; (⊗) PA-HAD50: [NGM + 1% (*v*/*v*) PA-HAD50]; (∗) PB-HAD50: [NGM + 1% (*v*/*v*) PB-HAD50]; (♦) PN-HAD50: [NGM + 1% (*v*/*v*) PN-HAD50]. *E. coli* OP50 (5 mg/ml); $n > 300$.

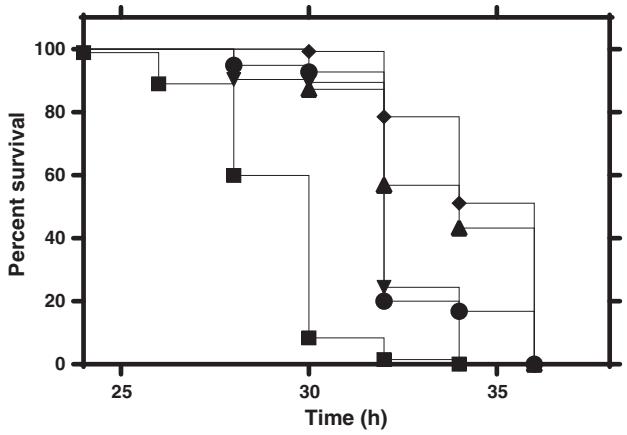

Fig. 3. Kaplan–Meier mobility plots of transgenic *C. elegans* worms (CL4176) treated with low molecular weight extract of HAD50 (LMWF-HAD50) and fractions polar acid (PA), polar basic (PB) and polar neutral (PN) 20 h post $A\beta_{1-42}$ induction of muscular paralysis at 25 °C. (■) Control: [NGM]; (♦) LMWF-HAD50: [NGM + 1% (*v*/*v*) LMWF-HAD50]; (▲) PA-HAD50: [NGM + 1% (*v*/*v*) PA-HAD50]; (●) PB-HAD50: [NGM + 1% (*v*/*v*) PB-HAD50]; (▼) PN-HAD50: [NGM + 1% (*v*/*v*) PN-HAD50]. *E. coli* OP50 (5 mg/ml); $n > 75$.

28.5 h after the temperature upshift, 50% of the worms were paralyzed or dead (PT_{50}) (Table 3). Treatment with LMWF-HAD-50 significantly delayed the $A\beta_{1-42}$ induced paralysis by 20.3% ($PT_{50} = 34.3$ h). Treatment with all fractions also resulted (Table 3) in a significant delay ($p < 0.0001$) in the induction of paralysis by 14.4% for PA-HAD50 ($PT_{50} = 32.6$ h); 8.7% for PB-HAD50 ($PT_{50} = 31.0$ h) and 9.4% for PN-HAD50 ($PT_{50} = 31.2$ h).

3.3.2. Parkinson's disease

The effect of camu-camu extracts on ameliorating Parkinson's disease like symptoms were evaluated in wild type (N2) *C. elegans* by inducing the dopaminergic neurodegeneration using 1-methyl-4-phenylpyridinium (MPP+). MPP+ is a metabolite formed from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and is selectively toxic to dopaminergic neurons (Braungart et al., 2004). In *C. elegans* treated with MPP+ alone, 55.5% of N2 wild type worms were completely paralyzed after 48 h (Fig. 4). In worms that were exposed to MPP+, but co-treated with LMWF-HAD50, only 39.9% of the worms were paralyzed after 48 h. Among the fractions, treatment with PA-HAD50 resulted in only 34.2% worms being paralyzed, treatment with PB-HAD50 and PN-HAD50 did not confer a significant neuroprotection against MPP+ induced neurotoxicity in *C. elegans* relative to control.

4. Discussion

Recent research has shown that camu-camu pulp and residue are promising sources of multifunctional phytochemicals such as natural antioxidants, flavonoids and vitamin C (Azevêdo et al., 2014; Fujita et al., 2013; Chirinos, Galarza, Betalleluz-Pallardel, Pedreschi, & Campos, 2010). However, few studies have investigated the in vivo biological effects of camu-camu and its derivatives (Gonçalves et al., 2014). We have previously shown that HAD50 camu-camu samples are natural sources of important flavonoids, including quercetin (Azevêdo et al., 2014), and therefore, this group was selected to investigate its effect on modulation of gene expression relevant to oxidative stress response signaling pathways and neurodegeneration in *C. elegans*. Additionally, we also looked the effect of the different fractions of HAD50 camu-camu extracts on ameliorating experimentally induced neurodegeneration in *C. elegans* model.

We initially fractionated the HAD50 camu-camu into LMWF and HMWF and investigated their effect on modulation of genes relevant to redox response signaling in transgenic *C. elegans*. Our results suggested that, treatment with LMWF-HAD50 caused a down regulation in the expression of daf-16 in *C. elegans*, an important transcription factor regulating the expression of several genes involved in nutrient, oxidant and stress response including, the iron/manganese superoxide dismutase (SOD-3) in mitochondria and cytoplasmic catalases (CTL-1; CTL-2; CTL-3) (Wen, Gao, & Qin, 2013). On the other hand, treatment with 1% (*v*/*v*) HMWF-HAD50 did not have any effect on the expression of daf-16.

However, the expression of SOD-3 which is under direct transcriptional regulation of daf-16, was significantly upregulated in response to treatment with LMWF-HAD50, but did not change in response to treatment with HMWF-HAD50. Also, the expression of the extracellular copper/zinc superoxide dismutase was significantly upregulated in response to treatment with both LMWF-HAD50 and HMWF-HAD50.

Table 2
Life span (mean, median and maximum number of days) of wild type (N2) *C. elegans* worms treated with low molecular weight extract of HAD50 (LMWF-HAD50) and fractions polar acid (PA), polar basic (PB) and polar neutral (PN).

	Control	LMWF-HAD50	PA-HAD50	PB-HAD50	PN-HAD50
Mean (d)	18.1	19.3	19.8	19.1	17.9
Median (d)	15.0	19.0	19.0	17.0	17.0
Maximum (d)	29.5	29.3	29.3	29.9	30.4
p (relative to control)	–	<0.001	<0.001	<0.001	<0.001

Control: [NGM]; LMWF-HAD50: [NGM + 1% (*v*/*v*) LMWF-HAD50]; PA-HAD50: [NGM + 1% (*v*/*v*) PA-HAD50]; PB-HAD50: [NGM + 1% (*v*/*v*) PB-HAD50]; PN-HAD50: [NGM + 1% (*v*/*v*) PN-HAD50]. *E. coli* OP50 (5 mg/ml); $n > 300$. The data were processed using the Kaplan–Meier survival analysis.

Table 3
Survival (mean, median, maximum number of hours and PT_{50}) of *C. elegans* worms (CL4176) treated with low molecular weight extract of HAD50 (LMWF-HAD50) and fractions polar acid (PA), polar basic (PB) and polar neutral (PN) 20 h post $A\beta_{1-42}$ induction of muscular paralysis at 25 °C.

	Control	LMWF-HAD50	PA-HAD50	PB-HAD50	PN-HAD50
Mean (h)	29.1	34.5	33.7	32.4	32.0
Median (h)	30.1	36.0	34.2	32.1	32.0
Maximum (h)	31.9	36.0	36.0	36.0	34.1
PT_{50} (h)	28.5	34.3	32.6	31.0	31.2
p (relative to control)	–	<0.001	<0.0001	<0.0001	<0.0001

Control: [NGM]; LMWF-HAD50: [NGM + 1% (*v*/*v*) LMWF-HAD50]; PA-HAD50: [NGM + 1% (*v*/*v*) PA-HAD50]; PB-HAD50: [NGM + 1% (*v*/*v*) PB-HAD50]; PN-HAD50: [NGM + 1% (*v*/*v*) PN-HAD50]. [*E. coli* OP50 (5 mg/ml); $n > 75$. The data were processed using the Breslow–Gehan–Wilcoxon test. PT_{50}: The time at which 50% of the worms were paralyzed or killed post temperature upshift.

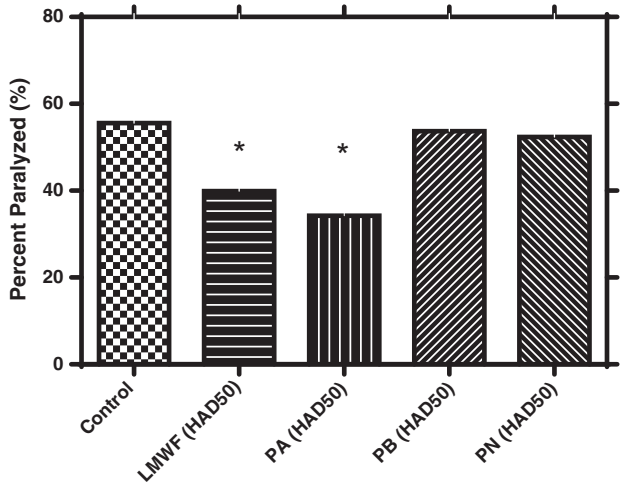

Fig. 4. Mobility scores of wild type (N2) *C. elegans* worms treated with low molecular weight extract of HAD50 (LMWF-HAD50) and fractions polar acid (PA), polar basic (PB) and polar neutral (PN) 48 h post MPP^+ (1.13 mg/ml) induced dopaminergic neurodegeneration and paralysis. Control: [NGM]; LMWF-HAD50: [NGM + 1% (*v*/*v*) LMWF-HAD50]; PA-HAD50: [NGM + 1% (*v*/*v*) PA-HAD50]; PB-HAD50: [NGM + 1% (*v*/*v*) PB-HAD50]; PN-HAD50: [NGM + 1% (*v*/*v*) PN-HAD50]. *E. coli* OP50 (5 mg/ml); $n > 100$; * $p < 0.05$ (ANOVA).

Similarly, expression of cytoplasmic catalases (CTL-1; CTL-2; CTL-3) was also significantly upregulated in response to treatment with LMWF-HAD50 and HMWF-HAD50, suggesting that though the expression of daf-16 was downregulated or did not change in response to treatment with camu-camu extracts, daf-16 was transcriptionally more active relative to control and conferred more resistance against oxidative stress (Kampkötter et al., 2008), more so with LMWF-HAD50 than with HMWF-HAD50.

These results are similar to the observations made in a similar study investigating the anti-aging role of polydatin (a natural resveratrol glycoside) in *C. elegans*, where an DAF-16 expression was found to be delineated from the expression of SOD-3 and CTL (CTL-1; CTL-2; CTL-3) expression (Wen et al., 2013). The SKN-1 is a transcription factor that, when active, translocates into the nucleus and binds to the anti-oxidant responsive element (ARE) to activate the expression of the putative phase-II detoxification enzymes (An et al., 2005), including glutathione-s-transferase enzyme, which was upregulated in response to treatment with both LMWF-HAD50 and HMWF-HAD50. However, the expression of gst-4 was downregulated in response to treatment with LMWF-HAD50 and remained unchanged with HMWF-HAD50 suggesting a possible induction of a normoxic environment in response to treatment with camu-camu extracts (Wang et al., 2006), similar to a recent study where açaí extracts did not affect the expression of *gst*-4 in *C. elegans* (Bonomo et al., 2014). We have recently shown that the experimental group HAD50 contains several bioactive compounds including quercetin, (+)-catechin, myricetin, ellagic and syringic acids (Azevêdo et al., 2014). Quercetin, a low molecular weight flavonoid, was shown to substantially increase *C. elegans* life span (Saul, Pietsch, Menzel, Stürzenbaum, & Steinberg, 2009; Kampkötter et al., 2008; Surco-Laos et al., 2011) that was dependent on FOXO transcription factor DAF-16. Additionally, treatment with quercetin also improved stress resistance and increased nuclear translocation of DAF-16 in *C. elegans* (Kampkötter et al., 2008). Similarly, treatment with the flavonol catechin resulted in a dose-dependent increase in *C. elegans* life span via the modulation of several stress response genes including daf-16, sod-3, and skn-1 (Saul et al., 2009; Zhang, Jie, Zhang, & Zhao, 2009) and may explain some of the results observed here.

Mitochondrial dysfunction, oxidative stress and intra and extracellular accumulation of misfolded or damaged proteins have been linked to the ethiopathology of aging and neurodegenerative diseases (Cohen & Dillin, 2008; Radak, Zhao, Goto, & Koltai, 2011; Jayasena, Poljak, Smythe, Münch, & Sachved, 2013). Since, overall, LMWF-HAD50 had a more robust effect on resistance against oxidative stress in *C. elegans*, we decided to further fractionate this extract and study its effect on extension of life span and on experimentally induced neurodegeneration in *C. elegans*. In response to dietary supplementation with LMWF-HAD50, the life span of the model increased by 20% relative to the control. Moreover, this increase in life span appeared to be due to the bioactive compounds in the polar basic fraction (PB-HAD50) fraction, which also increased the life span of *C. elegans* by 20%, whereas the other fractions were only able to extend the life span by 13% relative to control. Wilson et al. (2006) have shown that the proanthocyanidin-rich fraction of blueberries were especially effective in enhancing the longevity in *C. elegans*. We have recently demonstrated that camu-camu HAD50 extracts have significantly higher levels of proanthocyanidins (Azevêdo et al., 2014) and may be responsible for the significant extension in life span with LMWF-HAD50, and especially with PB-HAD50 fraction. In addition, the heat-shock induced aggregation of $A\beta_{1-42}$ peptide and resultant paralysis was also significantly delayed in *C. elegans* model in response to treatment with LMWF-HAD50, which delayed the induction of paralysis by 20.3% relative to control. Surprisingly, neither one of the fractions (PA-HAD50; PB-HAD50 or PN-HAD50) was as effective as LMWF-HAD50 in delaying the $A\beta_{1-42}$ induced paralysis, suggesting a potential synergistic interaction between the bioactive components in the different fractions.

The transgenic model of *C. elegans* for Alzheimer's disease used in our study specifically measures the intracellular aggregation of the $A\beta_{1-42}$ peptide in muscle tissues. Increased life span and delayed $A\beta_{1-42}$ aggregation induced paralysis in *C. elegans* upon dietary supplementation with camu-camu is similar to observations made with caffeine, ginkgo biloba and with curcumin recently (Dostal et al., 2010; Wu et al., 2006). It was suggested that these bioactive compounds, due to their spatial configuration, may facilitate an increased thermodynamically stable interaction with amyloidogenic proteins to prevent their oligomerization, and thus aggregation (Wu et al., 2006).

The MPP+ induced neurotoxicity is a popular system to study PD in vertebrate and invertebrate models, especially *C. elegans* (Braungart et al., 2004). In our study, treatment with LMWF-HAD50 resulted in a 15.6% reduction in MPP+ induced paralysis relative to the control. It appeared that the neuroprotective effect of LMWF-HAD50 was concentrated mostly in the polar acidic (PA-HAD50) fraction, which reduced the MPP+ induced paralysis by 21.3% relative to control. MPP+ (1-methyl-4-phenylpyridinium) is internalized by the pre-synaptic dopaminergic cell where it inhibits the mitochondrial complex-I protein of the electron transport chain and causes oxidative stress, ATP depletion and mitochondrial dysfunction and dopaminergic neuronal cell death for Parkinson's disease (PD) like symptoms (Chakraborty et al., 2013). It is possible that the protection against MPP+ induced neurotoxicity upon treatment with LMWF-HAD50 and PA-HAD50 could be due to abrogation of oxidative stress and mitochondrial dysfunction via the enhanced activation of oxidative stress response, specifically via the increased expression of SOD-3 and catalase genes as explained above.

5. Conclusion

We have recently shown that hot air dried camu-camu residue is an excellent source of bioactive compounds including ascorbic acid, proanthocyanidins, ellagic acid, myricetin and quercetin (Azevêdo et al., 2014). In this study, we have demonstrated for the first time that the camu-camu residue, especially the low molecular weight fraction was able to increase the expression of critical antioxidant genes and provide increased resistance against oxidative stress in vivo in *C. elegans*. Our results also show that treatment with camu-camu extracts significantly extended the life span of *C. elegans* by 20%, an effect that appeared to be mostly due to the polar basic bioactive compounds in the extracts. The camu-camu residue extracts were also able to significantly reduce the $A\beta_{1-42}$ aggregation induced paralysis

in the Alzheimer's disease model. Our results suggest that this effect was due to a synergistic interaction between the various low molecular bioactive fractions in the camu-camu residue. In the MPP + induced oxidative dopaminergic neurotoxicity model for Parkinson's disease in *C. elegans*, treatment with low molecular weight fraction of camu-camu residue resulted in significant abrogation in neurotoxicity. It is inferred that this effect was mostly due to the polar acidic low molecular weight bioactive fractions. Taken together, here it is shown that dried camu-camu residue is a bioactive-rich product with health-relevant potential to be used as a functional food ingredient and merits further investigation.

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