PAPER

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Amyloid β-but not Tau-induced neurotoxicity is suppressed by Manuka honey *via* HSP-16.2 and SKN-1/Nrf2 pathways in an *in vivo* model of Alzheimer's disease

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Alzheimer's is a chronic degenerative disease of the central nervous system considered the leading cause of dementia in the world. It is characterized by two etiopathological events related to oxidative stress: the aggregation of β-amyloid peptide and the formation of neurofibrillary tangles of hyperphosphorylated Tau protein in the brain. The incidence of this disease increases with age and has been associated with inadequate lifestyles. Some natural compounds have been shown to improve the hallmarks of the disease. However, despite its potential, there is no scientific evidence about Manuka honey (MH) in this regard. In the present work we evaluated the effect of MH on the toxicity induced by Aβ aggregation and Tau in a *Caenorhabditis elegans* model. Our results demonstrated that MH was able to improve indicators of oxidative stress and delayed Aβ-induced paralysis in the AD model CL4176 through HSP-16.2 and SKN-1/ NRF2 pathways. Nevertheless, its sugar content impaired the indicators of locomotion (an indicator of tau neurotoxicity) in both the transgenic strain BR5706 and in the wild-type N2 worms.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease of the central nervous system, characterized by accumulation of amyloid-β (Aβ) peptide and abnormal strands of hyperphosphorylated protein Tau (tangles) in the brain.[1–3] These modifications are accompanied by neuronal loss[2,4]

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along with synaptic and brain tissue damage[2,5] resulting in memory impairment,[1,6] depression, disorientation, poor judgment and, in later stages, speaking and walking difficulties.[5] It is estimated that AD causes between 60–80% of all dementia cases,[5] representing an important economic burden worldwide.[4] Currently, most of the approved drugs for the treatment of the illness only alleviate symptoms, but do not modify the underlying biology of Alzheimer's or alter the course of the disease. In addition, most of these drugs usually are accompanied by side effects including nausea, headache,[5] insomnia and hepatotoxicity.[3] Only one of the FDA-approved drugs, aducanumab, can reduce Aβ plaques in the brain.[5] Hence, the search for new drug candidates to fight AD is still an attractive research area. In the last years, several studies have shown that bioactive compounds from natural products possess neuroprotective properties and are capable of relieving AD symptoms, indicating that natural source-based drugs could be a valid alternative in this therapeutic area.[3,4,7]

In that sense, Manuka honey (MH), a much appreciated honey derived from the *Leptospermum scoparium* tree (Myrtaceae family), typical in New Zealand, eastern Australia and the Mediterranean region, has shown excellent biological activities including antibacterial, antioxidant, wound-healing

and antiproliferative properties as well as a very interesting physicochemical composition.[8,9,10–12] However, to the best of our knowledge, there is no evidence on its neuroprotective potential.

On the other hand, the nematode *Caenorhabditis elegans* (*C. elegans*), due to its short lifespan and its limited number of neurons and tractable genetics, represents an ideal system to study aging and age-related diseases, such as AD.[1,2,13] Likewise, there are multiple transgenic strains, specifically designed to examine the Aβ plaque accumulation and tangle-caused neurotoxicity.[2,6,13]

In the present study, we investigated the effects of MH on Aβ aggregation and tau neurotoxicity in *C. elegans* AD models.

2. Materials & methods

2.1 Honey samples and reagents

MH was bought in a local supermarket in Spain after importation into Europe by the Comptoirs et Compagnies. All reagents were purchased from Sigma-Aldrich (St Louis, Missouri, USA), Merck (Darmstadt, Germany), Thermo Fisher (Waltham, Massachusetts, USA) or Roche (Basel, Switzerland).

2.2 MH characterization

2.2.1 Total phenolic compounds (TPC) and total flavonoids content (TFC) determination. TPC of MH were determined by the Folin-Ciocalteu method,[14] while its flavonoid content was evaluated according to the protocol described by Navarro-Hortal *et al.*[15]

2.2.2 Total antioxidant capacity (TAC). TAC of MH was evaluated by ferric reducing antioxidant power (FRAP), trolox equivalent antioxidant capacity (TEAC) and 2,4-DNP; 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods, as previously reported by our group.[15–17] In all cases, the absorbance was measured in a Neo2 microplate reader (Biotek, Winooski, Vermont, U.S.A.) and the results were expressed as μM of Trolox equivalents (TE) per gram (g) of honey.

2.2.3 Extraction, identification, and quantification of phenolic compounds. The extraction of phenolic compounds was performed according to Afrin *et al.*[11] For their identification a SYNAPT G2 HDMS Q-TOF system (Waters, Mildford, USA) equipped with a dual electrospray ionization probe was used, following the protocol reported by Esteban-Muñoz *et al.*[18] UPLC separation was performed using an ACQUITY UPLC ™ HSS T3 2.1 × 100 mm, 1.8 mm column and the program for chromatography was set with a binary gradient consisting of (A) water with 0.5% acetic acid and (B) acetonitrile, as follows: initial 0.0 min 5% (B), 15.0 min 95% (B); 15.1 min 5% (B); and 18.0 min 5% (B) and the flow rate was 0.4 mL min^{-1}. Phenolic compounds were identified through the MassLynx V4 software (Waters Laboratory Informatics, Mildford, USA) by comparing the ions and molecular fragments obtained with previous data.

The quantification was carried out by an ACQUITY UPLC I-Class System (Waters, Mississauga, ON, Canada) equipped with a mass spectrometer Waters XEVO TQ-XS with ionization performed by UniSpray (US), according to the method described by Sánchez-Hernández *et al.*[19] A gradient consisting of (A) water and (B) methanol with 0.1% [v/v] acetic acid for 25 min at a flow rate of 0.4 mL min^{-1} was set as follows: 0.0 min 5% B, 15–15.10 min 95% B and 15.10–25 min 5% B. An ACQUITY UPLC ™ HSS T3 1.8 mm column was used. Quantification of the phenolic compounds was achieved by comparing the retention times of peaks and fragmentation data obtained, with those of the phenolic compound standards. Measurements in Multiple reaction monitoring was used.

2.3 *C. elegans* strains and maintenance conditions

Wild-type N2 Bristol *C. elegans*, the transgenic strains CL802 [smg-1(cc546) I; rol-6(su1006) II], CL4176 (dvIs27 [myo-3p:: A-Beta (1–42)::let-851 3′ UTR) + rol-6(su1006)] X), CF1553 (mu1s84[pAD76 (sod-3::GFP) + rol-6(su1006)]), CL2166 (dvIs19 [(pAF15)gst-4p:GFP:NLS] III), LD1 (ldIs7 [skn-1b/c::GFP + rol-6 (su1006)]), TJ375 (gpIs1[hsp-16.2::GFP]), OS3062 [myo-2p::hsf-1 + hsp-16.2::GFP + hsp-16.41::GFP + rol-6(su1006)], TJ356 (zIs356[daf-16p::daf-16a/b::GFP + rol-6 (su1006)]), BR5706 (bkIs10 [aex-3p:hTau V337M + myo-2p:GFP]) and *E. coli* OP50 were obtained from the Caenorhabditis Genetics Center (Minneapolis, MI, USA). All worms were grown and maintained on nematode growth medium (NGM)-agar plates seeded with a lawn of *E. coli* OP50 at 20 °C or 16 °C, depending on the strain's characteristics or the experiment design.

To obtain age-synchronized nematodes, the bleaching protocol was used. Briefly, gravid hermaphrodites were exposed to an alkaline bleach solution (0.5 N NaOH in 20% NaOCl) and vigorously stirred for six minutes followed by centrifugation at 1280*g* × 2 minutes. Settled eggs were then resuspended and washed for three times with M9 medium. After that, eggs were seeded on fresh NGM agar plates with a lawn of *E. coli* OP50 and allowed to hatch. Worms were allowed to mature until the desired larval stage, changing the plates every 2–3 days.

2.4 Lethality test

To assess acute lethality, N2 Bristol worms synchronized at L4 larval stage were placed on NGM plates containing 0, 25, 50, 100, 200 or 300 mg mL^{-1} of MH for 24 h at 20 °C in the absence of *E. coli* OP50. After that time, nematodes were scored as live or dead by using a Motic dissecting microscope (Motic Inc., Ltd, Hong Kong, China). Worms that did not respond to repeated stimuli inflicted with a platinum wire were considered dead. At least 30 worms were used per group and the experiment was repeated at least three times. Results were expressed as survival rate. For the subsequent experiments a non-lethal submaximal concentration (100 mg mL^{-1}) of MH was used.

2.5 Pharyngeal pumping assay and growth evaluation

Egg-synchronized N2 worms were placed on NGM plates seeded with *E. coli* OP50 and containing or not 100 mg mL^{-1} MH and then incubated at 20 °C. Ninety-six hours later,

worms were transferred to fresh plates and the number of contractions per minute of the pharynx terminal bulb was counted using a Motic microscope (Motic Inc. Ltd Hong Kong, China). At the end, worms were collected by washing with M9 medium and analyzed through a MultiRange large particle flow cytometer Biosorter (Union Biometrica. Massachusetts, USA) to determine the time of flight (TOF) which is indicative of the length. At least 10 worms were used per group for pharyngeal pump counting and about 100 nematodes to determine the size. Both experiments were performed in triplicate.

2.6 Eggs fertility assay

L4 stage synchronized N2 worms were individually placed in 24 well NGM plates seeded with *E. coli* OP50 and containing or not 100 mg mL^{-1} MH. After 24 h at 20 °C, the laid eggs were counted by using a Motic dissecting microscope (Motic Inc., Ltd, Hong Kong, China) and the worms were moved to a fresh plate to distinguish from the offspring. This procedure was repeated every single day until egg-laying stopped. At least 15 worms were used per group and the experiment was repeated at least three times. Results were expressed as the mean of the total number of eggs per group.

2.7 Oxidative stress resistance assays

To assess oxidative stress resistance, the intracellular reactive oxygen species (ROS) levels in L4 stage-synchronized N2 worms were measured by using the 2′,7′-dichlorofluorescein diacetate (DCFDA) assay, as previously described by Navarro-Hortal *et al.*[15] Briefly, eggs-synchronized embryos were placed on NGM plates seeded with *E. coli* OP50 containing or not 100 mg mL^{-1} MH for 48 h at 20 °C. Next, young-adult worms were collected and washed three times with M9 medium in order to remove bacteria and were exposed (except control group) to 2.5 μM AAPH (2,2′-azobis-2-amidinopropane dihydrochloride) for 15 min to induce oxidative stress. Worms were further washed with M9 medium and directly incubated with the fluorogenic dye (final concentration, 25 μM) for 2 h. The fluorescence was measured by a MultiRange large particle flow cytometer Biosorter (Excitation wavelength 490 nm, emission wavelength 510 nm) (Union Biometrica. Massachusetts, USA). Results were expressed as the mean of the fluorescence intensity normalized by TOF. At least 200 nematodes were used per group and the experiments were repeated in triplicate.

2.8 Paralysis test

Transgenic strain CL4176 and the non-paralyzable control strain CL802 were synchronized as described above in the maintenance conditions section and placed on NGM plates seeded with *E. coli* OP50 containing or not 100 mg mL^{-1} MH. Worms were incubated for 48 h at 16 °C and then transferred to a 25 °C incubator to stimulate the Aβ-induced paralysis. After 20 h, 25 nematodes of each group were picked to fresh NGM plates (maintaining temperature conditions) and scored for paralysis every 2 h until 32 h after the temperature rise. Nematodes were considered paralyzed when they did not respond to repeated stimuli inflicted with a platinum wire or if

they only moved the head without showing a full body wave. Results were expressed as percentage of non-paralyzed worms. The experiment was performed in triplicate.

2.9 β-Amyloid aggregates visualization

Worms of the paralyzable strain CL4176 and the negative control strain CL802 were grown as described above for the paralysis assay. After 26 h of incubation at 16 °C, worms were fixed with a 4% paraformaldehyde/M9 medium solution (pH 7.4) for 24 h and subsequently permeabilized with a solution containing 5% fresh β-mercaptoethanol, 125 mM Tris and 1% Triton X-100 (pH 7.4) for another 24 h at 37 °C. Aβ-aggregates were stained with 0.125% Thioflavin T (Sigma-Aldrich, St Louis, MI, USA) in 50% ethanol for 30 min and were observed by using a Nikon epi-fluorescence microscope (Eclipse Ni, Nikon, Tokyo, Japan). About 20–30 images per group were acquired at 40× magnification using the GFP filter with a Nikon DS-Ri2 camera (Tokyo, Japan).

2.10 RNA Interference (RNAi) Assay

Superoxide dismutase (SOD)-2 and 3, heat shock protein 16.2 (HSP-16.2), transcription factor skinhead-1 (SKN-1) and dauer formation (DAF-16) gene expression was knocked down by feeding CL4176 worms with *E. coli* strain HT115 carrying the specific dsRNA. Briefly, L3/L4 stage-synchronized CL4176 worms were transferred to RNAi plates containing 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 25 μg mL^{-1} carbenicillin and incubated for 48–72 h. Once they reached the fertile age, another synchronization was carried out and the eggs/embryos obtained were placed on fresh RNAi plates containing or not 100 mg mL^{-1} MH and were allowed to mature to L4 young larvae. The resulting adult worms were used for the paralysis assay as previously described.

2.11 Green fluorescence protein (GFP)-reporter transgenic strains

Egg-synchronized worms of the transgenic strains CF1553, CL2166, LD1, OS3062, TJ375, and TJ356 expressing the GFP reporter SOD-3, glutathione *S*-transferase 4 (GST-4), SKN-1, heat-shock transcription factor 1 (HSF-1), HSP-16.2 and DAF-16, respectively, were grown in NGM plates seeded with *E. coli* OP50 containing or not 100 mg mL^{-1} MH for 48 h at 20 °C. Next, 20–30 worms per group were immobilized with sodium azide (1 mM) on a slide and subjected to imaging using a Nikon epi-fluorescence microscope (Eclipse Ni, Nikon, Tokyo, Japan). The total GFP fluorescence was analyzed using the NIS-Elements BR software (Nikon, Tokyo, Japan). The subcellular DAF-16 distribution in the TJ356 strain was analyzed by classifying the expression/location patterns into three categories: cytosolic, intermediate, and nuclear; each of which was assigned a value (1, 2, 3) to carry out a semi-quantification. The experiments were repeated at least three times.

2.12 Locomotion behavior analysis

Egg-synchronized embryos of wild type-N2 and BR5706 strains were placed on NGM plates seeded with *E. coli* OP50 contain-

ing or not different treatments (100 mg mL^{-1} MH, 100 mg mL^{-1} artificial honey (AH) or 232 µg mL^{-1} of MH hydroalcoholic extract (ME) for 72 h at 20 °C. Next, about 20–30 worms per group were forced to swim by placing them in a drop of

M9 medium in the center of a slide. Worms movement was tracked, recorded and analyzed by using a WormLab Imaging System (MBF Bioscience, Williston, Vermont, EE. UU). The wavelength, swimming speed and activity were analyzed as representative parameters of locomotive behavior. The experiment was carried out at least in triplicate.

2.13 Statistical analysis

Statistical analysis was performed by SPSS 24.0 software (IBM, Armonk, NY, USA). Data were subjected to one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) *post hoc* test to compare statistical difference among multiple groups or to *t*-student test for pairwise data sets. A $p < 0.05$ was considered statistically significant. Data are expressed as mean ± SEM from at least three independent experiments.

3. Results

3.1 MH characterization

A total of 31 compounds were identified in MH (Table 1) and despite the limit of quantification for some of them, it was possible to quantify quercetin, quercetin-3-*O*-glucopyranoside, chrysanthemin, epicatechin, apigenin, naringenin, *m*-coumaric acid, ferulic acid, rosmarinic acid, ellagic acid and *o*-vanillin (Table 2). Naringenin (295.3 ± 4.7 ppb) and *o*-vanillin (373.5 ± 5.6 ppb) were the major compounds. Hydroxybenzaldehydes represented 45.14% of the quantified

Table 1 Phenolic compounds identified in MH

Compounds	CAS number	Ion mode	[M]$^{+/-}$ (*m/z*)	Ref.
Flavanones				
Naringenin	67604-48-2	+	153	20–23
		–	151	
Pinocembrin	480-39-7	+	151	23–26
		–	255	
Sakuranetin	2957-21-3	+	133	25 and 26
		–	287	
Flavones				
Apigenin	520-36-5	+	153	22–25 and 27
		–	117	
Chrysin	480-40-0	+	143	21 and 23–28
		–	253	
Luteolin	491-70-3	+	153	20 and 22–28
		–	133	
Nepetin	520-11-6	–	315	29
Flavonols				
Galangin	548-83-4	–	213	23–28
Isorhamnetin	480-19-3	–	199	20, 25, 26 and 28
Kaempferol	520-18-3	+	153	20 and 22–27
		–	151	
Quercetin	117-39-5	–	151	20–27
Quercetin 3-*O*-rutinoside	153-18-4	+	609	20, 22–25 and 27
Rhamnetin	90-19-7	–	315	25, 26 and 28
Isoflavonoids				
Biochanin A	491-80-5	+	283	27
		–	268	
Daidzein	486-66-8	+	253	23 and 27
		–	209	
Formononetin	485-72-3	–	252	27
Genistein	446-72-0	+	269	27
		–	181	
Hydroxybenzaldehydes				
Syringaldehyde	134-96-3	+	181	28 and 30
Vanillin	121-33-5	–	151	30 and 31
Hydroxycoumarins				
Scopoletin	92-61-5	–	190	32
Hydroxybenzoic acids				
Syringic acid	530-57-4	+	197	20, 22, 28, 31 and 33
4-Hydroxybenzoic acid	99-96-7	+	137	22, 23, 26–28, 30 and 33
Vanillic acid	121-34-6	–	152	22, 23, 27, 30 and 33
Hydroxycinnamic acids				
3-Caffeoylquinic acid	906-33-2	–	353	25
5-Caffeoylquinic acid	327-97-9	–	353	23, 31 and 34
Caffeic acid	331-39-5	+	179	20–22, 24–28, 30 and 35
		–	135	
Cinnamic acid	621-82-9	–	147	25 and 33
Ferulic acid	1135-24-6	+	193	20–24, 26, 27, 30, 32, 33 and 35
		–	134	
p-Coumaric acid	501-98-4	+	163	20, 22, 23, 25–27, 30, 32 and 35
		–	119	
Hydroxyphenylacetic acids				
4-Hydroxyphenylacetic acid	156-38-7	–	107	22 and 23
Phenylacetic acid	103-82-2	–	107	27

Table 2 Quantification of the phenolic compounds identified in MH

Phenolic compounds	Mean (ppb) ± SD
Flavonols	
Quercetin	52.6 ± 1.4
Flavonol glycosides	
Rutin	<LOQ
Kaempferol-3-*O*-glucoside	<LOQ
Quercetin-3-*O*-glucopyranoside	0.6 ± 0.2
Anthocyanins	
Chrysanthemin	0.3 ± 0.0
Flavanols	
Epicatechin	4.5 ± 0.4
Flavones	
Apigenin	1.6 ± 0.4
Flavanones	
Naringenin	295.3 ± 4.7
Isoflavones	
Glycitein	<LOQ
Formononetin	<LOQ
Phenolic acids	
m-Coumaric acid	16 ± 1.0
Ferulic acid	6.3 ± 0.1
Rosmarinic acid	3.5 ± 0.2
Ellagic acid	73.2 ± 6.1
Hydroxybenzaldehydes	
o-Vanillin	373.5 ± 5.6
Total phenolic content	**827.4**

LOQ: limit of quantification. Data are means ($n = 3$).

compounds, while flavonoids and phenolic acids represented 42.78% and 11.97%, respectively (Table 2).

According to the colorimetric methods, the TPC of MH was 0.47 ± 0.03 mg GAE g^{-1}, while its TFC was 0.05 ± 0.01 mg CAE g^{-1}. Likewise, its antioxidant capacity was 2.65 ± 0.10 μM Trolox eq. per g, 1.40 ± 0.13 μM Trolox eq. per g and 4.58 ± 0.22 μM Trolox eq. per g according to the FRAP, ABTS and DPPH determination methods, respectively. The yield of the hydroalcoholic extraction of phenolic compounds was 2.32 ± 0.42 mg g^{-1} of honey (Table 3).

3.2 Short term toxicity of MH

The short-term toxicity of MH in *C. elegans* was evaluated through different assays, including 24 h lethality, pharyngeal pumping, egg-laying (fertility) and growth. As shown in Fig. 1A, no statistically significant differences ($p < 0.05$) were found between the control group and worms treated with increasing concentrations (up to 300 mg mL^{-1}) of MH in terms of survival percentage. Likewise, MH supplementation did not affect the number of pumps per minute or the worm's fertility, understood as their spawning capacity. However,

Table 3 Phytochemical characterization, antioxidant capacity and yield of MH

| Parameter	Mean ± SEM
Total phenolic content (mg GAE g^{-1})	0.47 ± 0.03
Total flavonoids content (mg CAE g^{-1})	0.05 ± 0.01
FRAP (μM TE g^{-1})	2.65 ± 0.10
DPPH (μM TE g^{-1})	1.40 ± 0.13
TEAC (μM TE g^{-1})	4.58 ± 0.22
Yield[a]	2.32 ± 0.42

[a] mg of hydroalcoholic extract obtained per g of honey. FRAP: ferric reducing antioxidant power; DPPH: 2,2-diphenyl1-picryl-hydrazyl-hydrate; TEAC: trolox equivalent antioxidant capacity; mg GAE g^{-1}: mg gallic acid equivalent; mg CAE g^{-1}: mg catechin equivalent; TE: trolox equivalent.

worms treated with 100 mg mL^{-1} MH were slightly smaller ($p < 0.05$) (Fig. 1B).

3.3 Effects of MH against AAPH-induced oxidative stress

An escalation in endogenous ROS levels accelerates the aging process and, in turn, the nematodes ability to deal with harmful stimuli decreases drastically over time. To evaluate the effects of MH against AAPH-induced stress, we measured intracellular ROS levels by DCF-DA method. As shown in Fig. 2, nematodes treated with MH presented a greater resistance against these stress conditions. MH completely prevented the increase in ROS observed in the stressed group as no significant difference ($p < 0.05$) was found compared to the control.

3.4 Effects of MH against Aβ-induced paralysis

Aβ aggregation is considered one of the major pathological causes of AD. To evaluate whether MH ameliorated the Aβ-induced toxicity, we used the transgenic *C. elegans* strain CL4176, which expresses the human Aβ peptide in body wall muscle cells following temperature upshift from 16 to 25 °C, resulting in a paralysis phenotype. As shown in Fig. 3A, non-treated CL4176 worms exhibited almost complete paralysis after 34 h of the temperature upshift, while 100 mg mL^{-1} MH treatment significantly ($p < 0.05$) delayed this process. In the MH-treated group, no significant differences ($p < 0.05$) were observed with respect to the non-paralyzable strain CL802 (negative control) up to 30 h after temperature upshift. At the end point of the analysis, the paralyzed worms represented only 25% of the entire population. In addition, MH significantly reduced Aβ deposits in the tail regions of CL4176 worms as evidenced by thioflavin T staining (Fig. 3B).

To further elucidate the molecular mechanisms underlying the protective effects of MH on Aβ toxicity, we repeated the paralysis test in CL4176 worms silencing some genes by RNAi, including *sod-2* and *sod-3*, *hsp-16.2*, *skn-1*, and *daf-16*. 34 h after the temperature upshift, RNAi for *sod-3* and *daf-16* had no effect on the percentage of non-paralyzed worms compared to the

Fig. 1 Short-term toxicity of Manuka honey in the N2 wild type strain. (A) 24 h lethality. (B) Pharyngeal pumping, egg-laying (fertility) and growth. Worms were treated with the indicated concentration of MH for 24 h. Data are expressed as mean ± SEM of three independent experiments ($n = 3$). Asterisk marks (*) indicate statistically significant differences ($p < 0.05$) compared to control (non-treated worms). CTL: control; MH: Manuka honey.
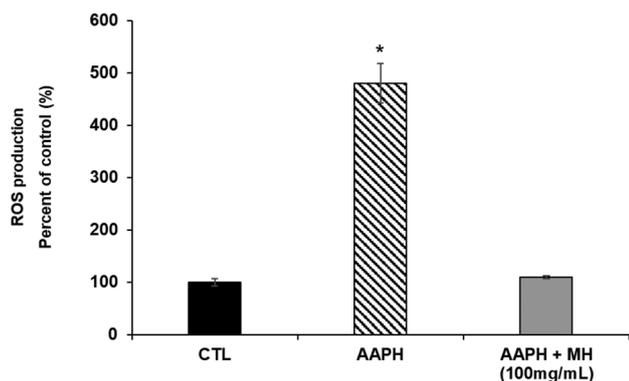
Fig. 2 Effects of Manuka honey on the reactive oxygen species (ROS) production. Worms were pre-treated with MH at 100 mg mL^{-1} for 24 h and then exposed to AAPH (2.5 mM) for 15 min. Data are expressed as mean $\pm$ SEM of three independent experiments (n = 3). Asterisk marks (*) indicate statistically significant differences ($p < 0.05$) compared to control (non-treated worms). CTL: control; MH: Manuka honey; AAPH: 2,20-Azobis (2-methylpropionamidine) dihydrochloride.

MH-treated group. In contrast, RNAi for *hsp-16.2* and *skn-1* showed a significantly lower % of non-paralyzed worms, which indicates that there was a reduction in the protective effects of MH on Aβ-induced paralysis, suggesting that these genes are likely implicated in the protective effects of MH (Fig. 4).

3.5 Effects of MH on SOD-3::GFP, GST-4::GFP, SKN-1::GFP, HSP-16.2::GFP and DAF-16::GFP expression

To deepen the comprehension of the effects of MH on the genes knocked down in the experiments described above, we used several transgenic strains carrying the GFP transcriptional reporters of interest. A marked diminution ($p < 0.05$) in the expression of HSP-16.2::GFP in TJ375 worms treated with MH (by 34.41%) as well as in the expression of SOD-3::GFP in CF1553 nematodes (by 16.30%) was found. No significant effects from MH addition ($p < 0.05$) were observed on the expression of GFP from the promoters for GST-4, SKN-1 and HSF-1 compared to control. Also, MH did not induce the translocation of DAF-16 from the cytoplasm to nuclei (Fig. 5A and B).
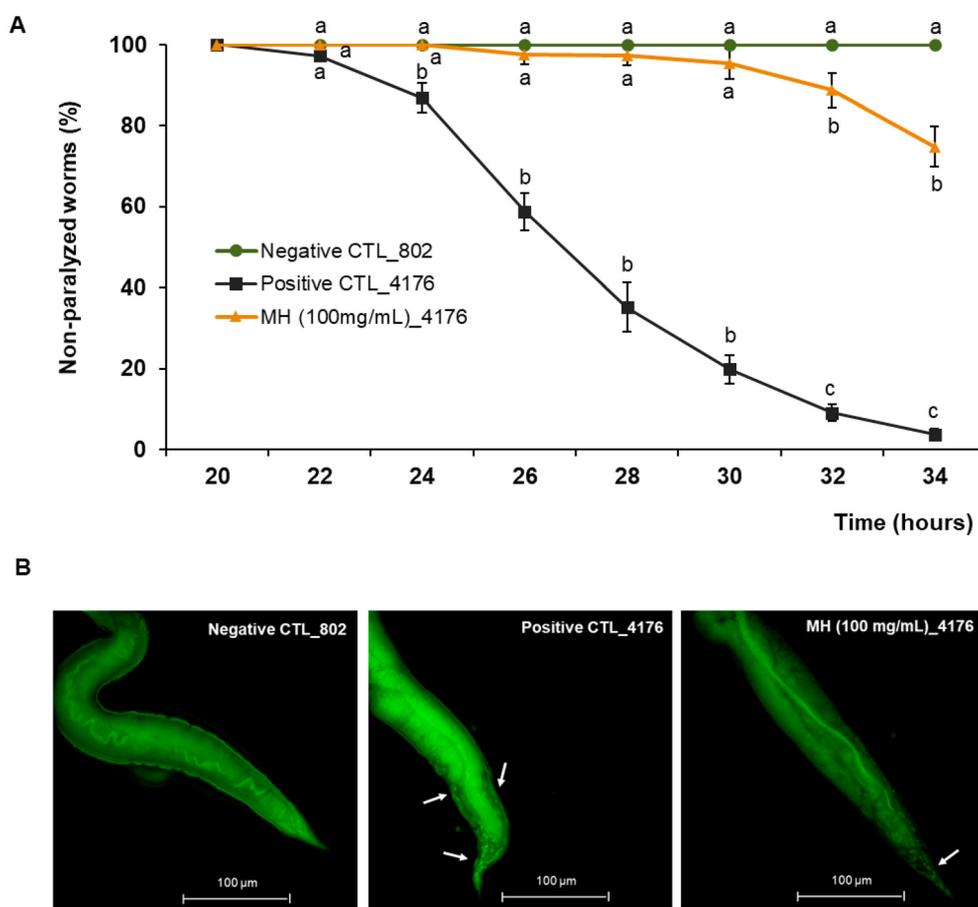
Fig. 3 Effects of Manuka honey on Aβ-induced paralysis. (A) Worm paralysis over time. Data are expressed as mean $\pm$ SEM of three independent experiments (n = 3). For each time, different superscript letters among groups indicate statistically significant differences ($p < 0.05$). (B) Representative images of Thioflavin T aggregates after 30 h of temperature shifting. Aβ aggregates are indicated with white arrows (40× magnification; scale bar, 100 μm). CTL_802: non-paralyzable strain CL802 (negative control); CTL_4176: non-treated worms of the paralyzable strain CL4176 (positive control); MH (100 mg mL^{-1})_4176: worms of the paralyzable strain CL4176 treated with 100 mg mL^{-1} of Manuka honey.
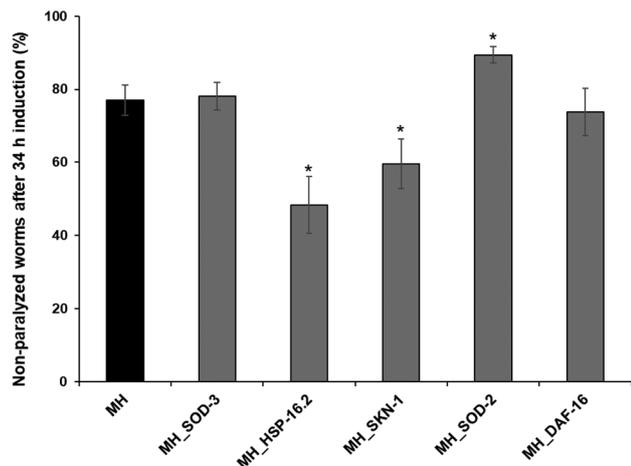
Fig. 4 Effects of different RNAi (*sod-3*, *hsp-16.2*, *skn-1*, *sod-2* and *daf-16*) on the Aβ-induced paralysis after 34 h of induction. Worms of the paralyzable strain CL4176 treated with MH (100 mg mL^{-1}) were used in all groups. Data are expressed as mean ± SEM of three independent experiments (*n* = 3). Asterisk marks (*) indicate statistically significant differences (*p* < 0.05) compared to MH (worms non-exposed to RNAi and treated with MH). MH: Manuka honey.

3.6 Effects of MH on Tau Proteotoxicity

Along with Aβ plaques, the presence of tau protein aggregates is another hallmark of AD. Although the mechanisms of tau-associated neurodegeneration remain unclear, aggregation of this protein is thought to be toxic to neurons. Therefore, the effects of MH on the transgenic strain BR5706 were also evaluated. BR5706 is characterized by accelerated aggregation of insoluble Tau, resulting in severe developmental defects of the nervous system, impaired locomotion, and slowed growth.

Worms treated with 100 mg mL^{-1} MH showed a significant decrease (*p* < 0.05) in swimming speed (by 70.76%), wavelength (by 21.68%) and activity (by 39.32%) compared to the control group (Fig. 6A and B). Trying to find an explanation for these results, the contribution of sugars to the assayed parameters was evaluated. For that, two additional experimental groups were included: (i) worms treated with 232 µg mL^{-1} of a hydroalcoholic extract (ME) only containing the phenolic compounds of MH and (ii) worms treated with 100 mg mL^{-1} of an artificial honey (AH). AH was prepared by dissolving 22.0 g of maltose, 4.4 g of sucrose, 98.5 g glucose and 119.1 g of fructose in 50 mL of de-ionized water.[9] As it can be appreciated in Fig. 6A and B, no significant differences (*p* < 0.05) were found between the ME-treated group and the control group, while AH addition showed the same worsening effects as MH, leading to 39.43-, 71.21- and 70.51-fold diminution of the swimming speed, wavelength, and activity, respectively, compared to the control.

To further determine whether the observed effects were directly related to Tau protein aggregation, the mobility assay was performed in the wild type N2 strain (Fig. 7A and B). Also in this case, MH decreased swimming speed, wavelength, and activity by 54.92, 28.96 and 22.32%, respectively, compared to

non-treated nematodes. Considering these data, it can be suggested that the sugar content of MH affects the general mobility of *C. elegans* and not necessarily the aggregation of the Tau protein. However, future analyzes are needed to corroborate this hypothesis.

4. Discussion

Despite the fact that MH is a natural product with remarkable biological properties both *in vitro*[8–11,36–38] and *in vivo*,[39,40] little is known about its neuroprotective potential. Considering its ability to scavenge free radicals and the implication of oxidative stress in the pathogenesis of age-related diseases such as AD, we decided to investigate the possible effects of MH in alleviating Aβ and Tau-induced toxicity in *C. elegans* models of AD.

The qualitative profile of MH phenolic compounds determined by UPLC-TOF-MS/MS was similar to that found by other authors.[8,9,11,41,42] Nevertheless, we were unable to quantify some of the most representative compounds reported for this honey, such as syringic acid,[11] methyl syringate,[9,41,42] pinocembrin and pinobanksin.[8,42] It is well known that the extraction, conservation, and detection methods may influence these results. Regarding the TPC and TFC, the values obtained (0.47 ± 0.03 mg GAE g^{-1} and 0.05 ± 0.01 mg CAE g^{-1}, respectively) were slightly lower than those reported by Y.-Z. Zhang *et al.*,[43] Gośliński *et al.*,[44] Marshall *et al.*,[45] and Anand *et al.*[46] However, the TAC values (2.65 ± 0.10 µM TE g^{-1} by FRAP; 1.40 ± 0.13 µM TE g^{-1} by TEAC and 4.58 ± 0.22 µM TE g^{-1} by DPPH) were quite similar to those reported by Sadia Afrin *et al.*[10] and Marshall *et al.*[45]

Besides that, 100 mg mL^{-1} MH did not show short term toxicity in the *C. elegans* nematodes as demonstrated by the results of the lethality, pharyngeal pumping and egg-laying (fertility) assays. Next, we also evaluated the effects of MH against AAPH-induced oxidative stress, since multiple lines of evidence indicate that ROS overproduction and consequently, an oxidative stress escalation, not only contributes to the aging process[4,47] but is also one of the main factors involved in the initiation and progression of neurodegenerative diseases.[48] In the particular case of AD, oxidative stress is related to Aβ aggregation[4,15] and the phosphorylation and polymerization of Tau, two hallmarks of the disease.[4] Here, we demonstrated that MH increased resistance to stress conditions as revealed by the DCF-DA method. In similar studies, echinacoside, a phenylethanoid glycoside from *Cistanche deserticola*,[47] carnosic acid,[3] cannabidiol,[48] gennianchun, a Chinese herbal formula[7] and caffeic acid[4] reduced ROS levels in the wild-type *C. elegans* strain.

In addition to the ROS theory of AD, there are multiple hypotheses about AD etiology, including the (i) tau protein hyperphosphorylation hypothesis, (ii) mitochondrial function hypothesis, (iii) cholinergic hypothesis and (iv) amyloid cascade hypothesis.[3] Although the topic is the subject of debate, the amyloid cascade hypothesis, which suggests that
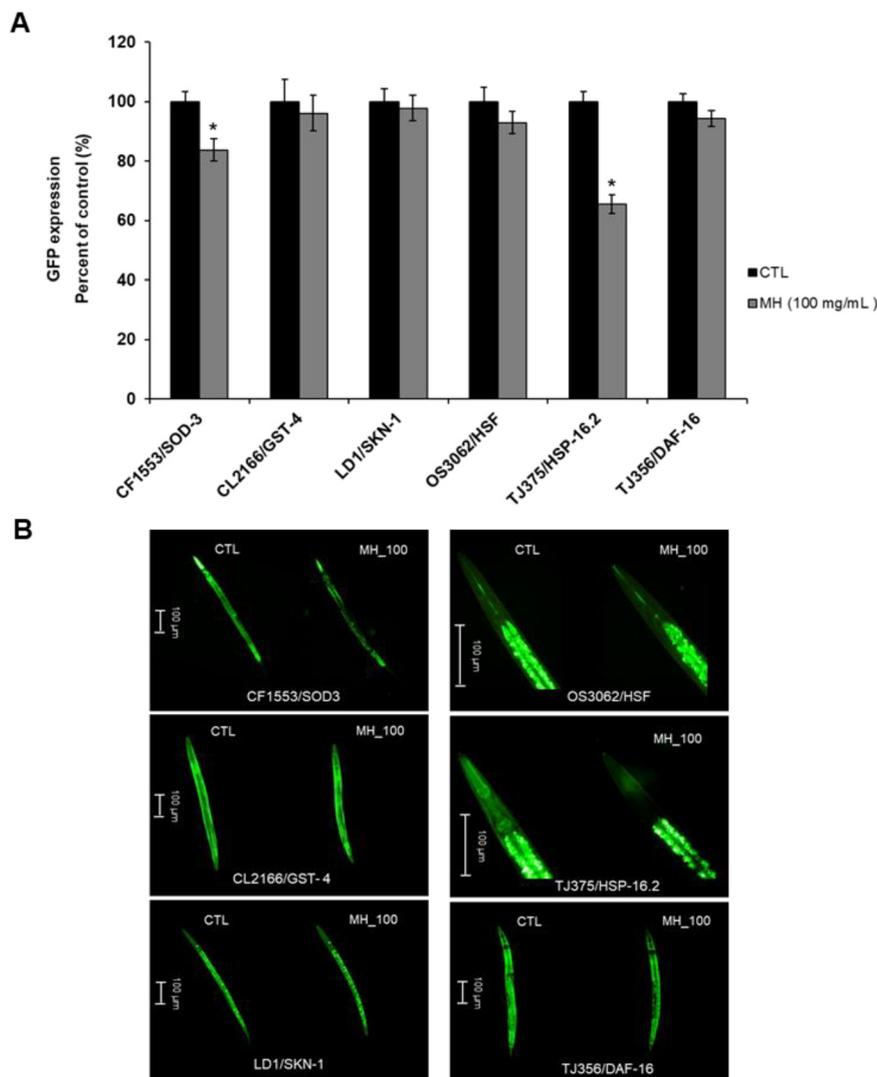
Fig. 5 Effects of Manuka honey on GFP-reporter transgenic strains. (A) Quantification of the GFP expression in the different transgenic transcriptional reporter strains with or without MH treatment (100 mg mL^{-1}). Data are expressed as mean ± SEM of three independent experiments ($n = 3$). Asterisk marks (*) indicate statistically significant differences ($p < 0.05$) compared to control (non-treated worms). (B) Representative images for each transgenic strain (CF1553/SOD-3::GFP strain, CL2166/GST-4::GFP strain, LD1/SKN-1::GFP strain, OS3062/HSF::GFP strain, TJ375/HSP-16.2::GFP strain, TJ356/DAF-16::GFP strain); (40× magnification; scale bar, 100 µm). CTL: control; MH: Manuka honey.

Aβ deposition triggers a series of events (microglial activation, inflammatory response, reactive astrocytosis)[49] that lead to neuronal dysfunction, is the most widely accepted.[3,7,49,50] Consequently, alleviating Aβ accumulation has been considered one of the most promising therapeutic approaches for AD.[48] To evaluate whether MH was able to delay the Aβ-induced neurotoxicity, we conducted a paralysis test in the transgenic strain CL4176, which has been widely used to study this process because of its ability to express human Aβ peptide in nematode body wall muscle and develop a progressive and reproducible paralysis phenotype.[2,6,13,51] Our results showed that MH significantly delayed the paralysis of CL4176 worms caused by Aβ aggregation in response to the temperature upshift and reduced Aβ deposits as evidenced by thioflavin T staining. It would be interesting, for future research, to assess whether MH, in addition to reducing the aggregation of the Aβ peptide, is capable of reducing Aβ oligomers, since it is well known that they are more toxic than Aβ monomers and have been related to memory loss and severity of dementia[13,52] Aβ oligomers may damage neurons by interfering with neuron-to-neuron communication at synapses.[5] Many other natural compounds, such as betaine,[49] Ginkgo biloba extract,[7] genniachun,[7] *Sideritis scardica* extracts,[53] fucoidan,[54] ergothioneine,[55] frondoside A,[56] spermidine,[57] essential Oil of *Acorus tatarinowii* Schott,[58] allophycocyanin,[50] caffeic acid,[4] strawberry extract,[15] carnosic acid,[3] scutellarein,[59] oleuropein,[17] hydroxytyrosol,[16,59] and red-beet betalain pigments,[60] also protect *C. elegans* nematodes against Aβ-induced toxicity.

Most of the studies evaluating the mechanism underlying the effect of natural compounds against Aβ-induced paralysis
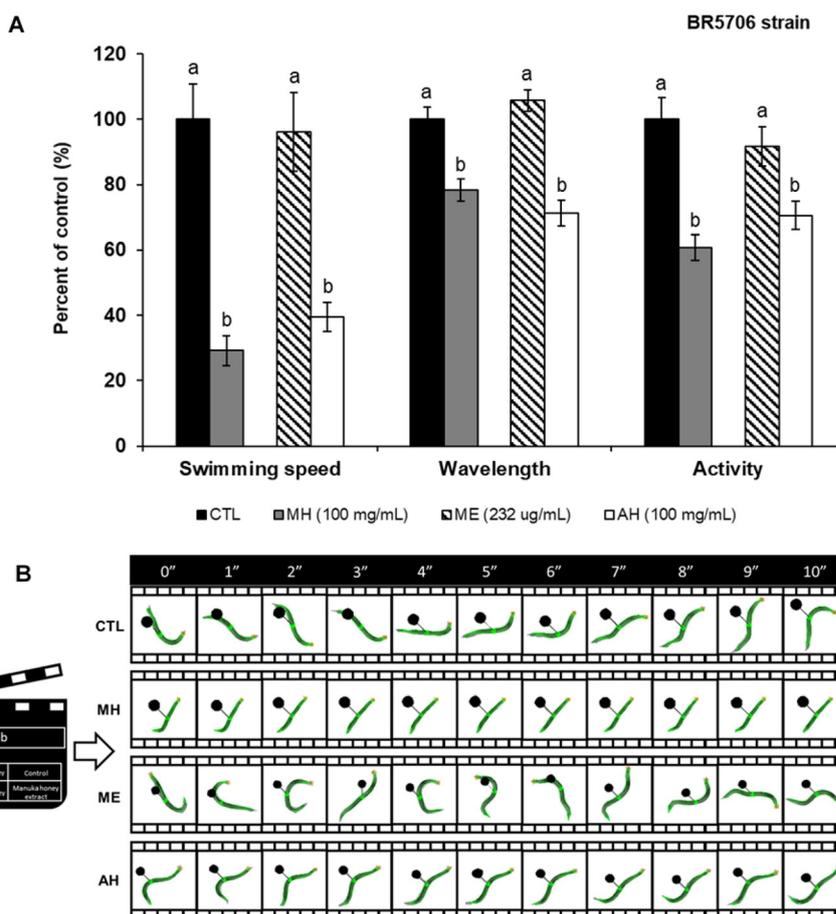
Fig. 6 Effects of Manuka honey on Tau-induced altered locomotive behavioral phenotype. The BR5706 transgenic strain was used in all groups. (A) Swimming speed, wavelength and activity. Data are expressed as mean ± SEM of three independent experiments ($n = 3$). Different superscript letters for the same set of data indicate statistically significant differences ($P < 0.05$) compared to control (non-treated worms). (B) Image sequence illustrating how the nematodes move from left to right in the experimental groups. Images were obtained from frame-by-frame tracking data provided by the Wormlab system/software. CTL: control; MH: Manuka honey, ME: Manuka honey hydroalcoholic extract, AH: artificial honey.

in *C. elegans*, focus on pathways related to oxidative stress, heat shock response, insulin signaling cascade and redox homeostasis.[51] Hence, we knocked down the expression of SOD-2 and SOD-3, HSP-16.2, SKN-1, and DAF-16 genes by RNAi technology and repeated the paralysis test in the CL4176 strain.

DAF-16 is the only homologue of the forkhead box transcription factors class O (FOXO) in *C. elegans* and the main output of the insulin/insulin-like growth factor 1 (IGF-1) signaling (IIS) pathway. Therefore, it plays a fundamental role in the regulation of growth, aging, metabolism, development, reproduction, stress resistance and immunity.[7,52,61,62] DAF-16 activation increases the expression of multiple genes involved in the antioxidant response, including the metallothionein homologue (*mtl-1*), mitochondrial superoxide dismutase (*sod-3*), the catalase genes *ctl-1* and *ctl-2* and some small heat-shock protein (HSPs) genes.[7,62–64] Moreover, the response against oxidative stress is also mediated by the joint action of other factors such as SKN-1, SMK-1 (a regulatory subunit of the PP4 protein phosphatase complex) and HSF-1.[62] Up to the

present, it has been reported that several compounds extend lifespan and reduce Aβ-induced toxicity in *C. elegans* via DAF-16, as for example royal jelly,[65] monascin,[66] dianxianning,[52] gengnianchun,[7] phosphatidylcholine,[64] Zijuan Pu'er tea extract,[67] *Hibiscus sabdariffa* L. extract,[68] strawberry extract,[15] oleuropein,[17] *Moringa oleifera* extract,[69] and hydroxytyrosol.[16] However, under our experimental conditions, DAF-16 and SOD-3 RNAi failed to delay the Aβ-induced paralysis, indicating that MH attenuation of neurotoxicity is not mediated by these pathways. On the contrary, SKN-1 and HSP-16.2 were required for MH protective effects. Zhu *et al.*[70] also demonstrated that a rose essential oil was protective in a *C. elegans* model of Aβ-toxicity through SKN-1 activation but independent of DAF-16. Other compounds that have been shown to ameliorate Aβ-induced toxicity through the SKN-1 pathway are *Hibiscus sabdariffa* L. extract,[68] Panax Notoginseng saponins,[71] strawberry extract,[15] and *Cratoxylum formosum* extract.[72]

SKN-1 is a transcription factor that can act in parallel to DAF-16, that can also inhibit the Aβ-induced toxicity by promoting the expression of stress responsive genes.[52] This tran-
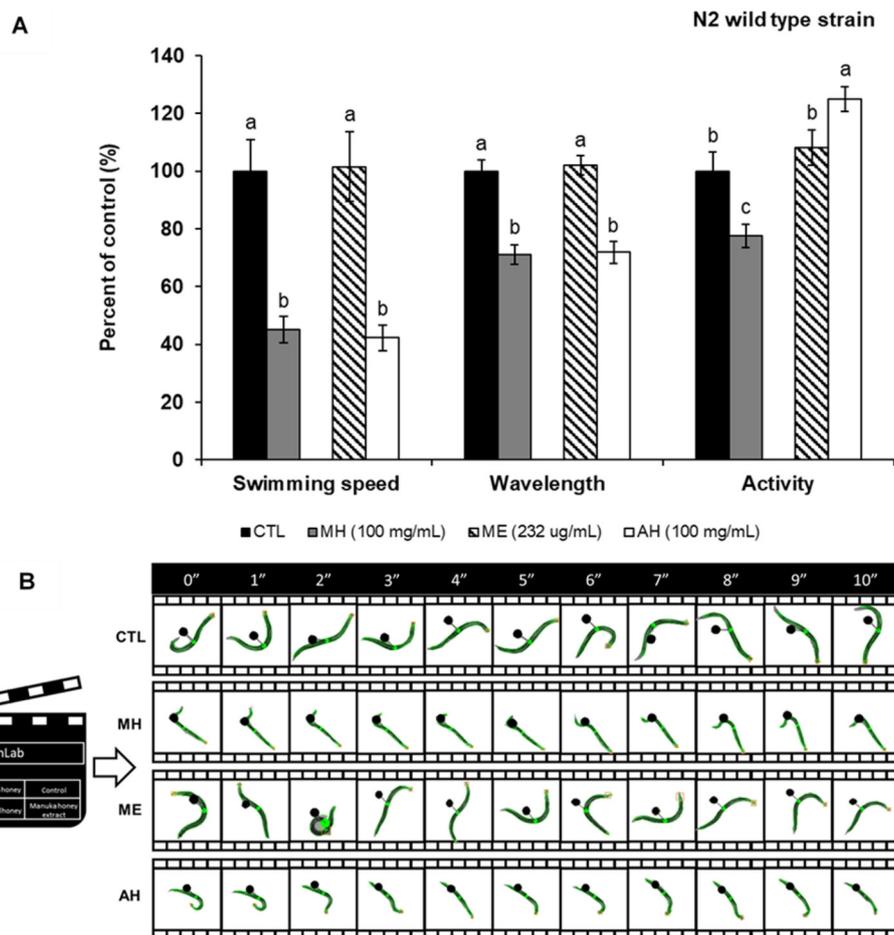
Fig. 7 Effects of Manuka honey on locomotive behavior in wild-type *C. elegans*. Wild type-N2 strain was used in all groups. (A) Swimming speed, wavelength, and activity. Data are expressed as mean $\pm$ SEM of three independent experiments (n = 3). Different superscript letters for the same set of data indicate statistically significant differences (P < 0.05) compared to control (non-treated worms). (B) Image sequence illustrating how the nematodes move from left to right in the experimental groups. Images were obtained from frame-by-frame tracking data provided by the Wormlab system/software. CTL: control; MH: Manuka honey, ME: Manuka honey hydroalcoholic extract, AH: artificial honey.

scriptional factor is analogous to mammalian Nrf2 (nuclear factor erythroid 2-related factor 2) protein, which induce phase 2 detoxification genes regulating both, the xenobiotic and antioxidant response.[50,70–72] For its part, the overexpression of the chaperone HSP-16.2 may improve the Aβ associated toxicity by increasing abnormal protein degradation, sequestration and refolding.[52,71] Guarana (*Paullinia cupana* Mart.)[73] and palmatine[74] also act through this mechanism. In correspondence with the RNAi results, MH did not cause the translocation of DAF-16 from the cytoplasm to nuclei in the transgenic strain TJ356 carrying a GFP-tagged reporter of this gene. In addition, MH did not cause any change in the expression of SKN-1 compared to the control group and it decreased the expression of HSP-16.2 in the transgenic strains LD1 and TJ375, respectively, suggesting that MH effects on Aβ-paralysis mediated by these genes occurs only under stress conditions and not in a basal state. Likewise, MH did not increase the expression of GST-4, SOD-2 and SOD-3, which are target genes of DAF-16 or SKN-1 implicated in ROS elimination, suggesting that the intrinsic

antioxidant capacity of MH is essential for its striking effects on the oxidative stress resistance and attenuation of Aβ-toxicity.

As mentioned before, neurofibrillary tangles represent another distinctive feature of AD. Although the relationship between Aβ-peptides and Tau accumulation is not yet well understood, some studies have indicated that both proteins act synergistically in promoting the pathological progression of AD and neuronal loss.[75–78] Thus, we also evaluated the effect of MH against Tau-induced neurotoxicity, observing that 100 mg mL^{-1} MH was not able to improve the locomotion defects related to Tau protein accumulation in the transgenic strain BR5706 nor in N2 nematodes. In fact, MH-treated worms showed significantly lower swimming speed, wavelength, and activity compared to the control group.

Depending on the growth media, *C. elegans* can swim, burrow and crawl[79] and according to the Wormlab software used in this study, the swimming speed refers to the distance covered by the worm along its central axis over a two-stroke

interval, whereas activity represents the brush stroke normalized by the time taken to perform the two strokes. For its part, the wavelength denotes the distance between negative and positive inflection points. This parameter is approximately twice the body length, generating alternating C-shapes with each ventral or dorsal muscle contraction.[79] Thus, the effects observed in terms of worm mobility are in correspondence with the length diminution observed in the MH-treated groups. Considering that no differences were observed between MH and AH but with the ME (only containing the phenolic fraction of honey), we ascribed the deleterious effects of MH on locomotion to the sugar content of this matrix. Moreover, since the results were similar in both BR5706 and wild-type strain, we assume that the sugar effect is not related to Tau aggregation but directly to mobility. However, further analyzes are needed in this direction.

5. Conclusions

In this report it has been demonstrated that MH improved tolerance to oxidative stress induced by AAPH in the wild-type N2 *C. elegans* strain, and alleviated Aβ-induced paralysis in the AD model CL4176 through HSP-16.2 and SKN-1/Nrf2 pathways. Likewise, it has been shown that the sugar content of MH impaired the indicators of locomotion in both, the transgenic strain BR5706 and in the wild-type N2 worms but did not influence the rest of the favorable effects described above. Results presented here indicated that the antioxidant activity of MH may be responsible for the observed neuroprotective effects.

Abbreviations

AAPH	(2,2′-Azobis-2-amidinopropane dihydrochloride)
AD	Alzheimer's disease
AH	Artificial honey
Aβ	Amyloid-β
C. elegans	*Caenorhabditis elegans*
DAF-16	Dauer formation
DPPH	2,4-DNP; 2,2-diphenyl-1-picrylhydrazyl
FOXO	Forkhead box transcription factors class O
FRAP	Ferric reducing antioxidant power
GFP	Green fluorescence protein
GST-4	Glutathione S-transferase 4
HSF1	Heat-shock transcription factor 1
HSP-16.2	Heat shock protein 16.2
HSPs	Heat-shock protein
IIS	Insulin/insulin-like growth factor 1 (IGF-1) signaling
IPTG	Isopropyl β-ᴅ-1-thiogalactopyranoside
ME	Hydroalcoholic extract
MH	Manuka honey
NGM	Nematode growth medium
Nrf-2	Nuclear factor erythroid 2-related factor 2
ROS	Reactive oxygen species
SKN-1	Transcription factor skinhead-1
SOD	Superoxide dismutase
TE	Trolox equivalents
TEAC	Trolox equivalent antioxidant capacity
TFC	Total flavonoids content
TOF	Time of flight
TPC	Total phenolic compounds

Author contributions

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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