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Strawberry (*Fragaria* × *ananassa* cv. *Romina*) methanolic extract attenuates Alzheimer's beta amyloid production and oxidative stress by SKN-1/NRF and DAF-16/FOXO mediated mechanisms in *C. elegans*

María D. Navarro-Hortal [a,1], Jose M. Romero-Márquez [a,1], Adelaida Esteban-Muñoz [b], Cristina Sánchez-González [a,c], Lorenzo Rivas-García [a,c], Juan Llopis [a,c], Danila Cianciosi [d], Francesca Giampieri [d,e], Sandra Sumalla-Cano [f], Maurizio Battino [e,g], José L. Quiles [a,f,*]

[a] Department of Physiology, Institute of Nutrition and Food Technology "José Mataix", Biomedical Research Centre, University of Granada, Armilla, Avda. del Conocimiento s.n., 18100 Armilla, Spain
[b] Department of Nutrition and Bromatology, University of Granada, 18071 Granada, Spain
[c] Sport and Health Research Centre, University of Granada, C/. Menéndez Pelayo 32. 18016, Armilla, Granada, Spain
[d] Department of Biochemistry, Faculty of Sciences, King Abdulaziz University, Jeddah, Saudi Arabia
[e] Department of Clinical Sciences, Polytechnic University of Marche, Ancona 60131, Italy
[f] Research Group on Foods, Nutritional Biochemistry and Health, Universidad Europea del Atlántico, Isabel Torres, 21, 39011 Santander, Spain
[g] International Research Center for Food Nutrition and Safety, Jiangsu University, Zhenjiang 212013, China

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ABSTRACT

Bioactive compounds from strawberries have been associated with multiple healthy benefits. The present study aimed to assess chemical characterization of a methanolic extract of the Romina strawberry variety in terms of antioxidant capacity, polyphenols profile and chemical elements content. Additionally, potential toxicity, the effect on amyloid-β production and oxidative stress of the extract was *in vivo* evaluated in the experimental model *Caenorhabditis elegans*. Results revealed an important content in phenolic compounds (mainly ellagic acid and pelargonidin-3-glucoside) and minerals (K, Mg, P and Ca). The treatment with 100, 500 or 1000 μg/mL of strawberry extract did not show toxicity. On the contrary, the extract was able to delay amyloid β-protein induced paralysis, reduced amyloid-β aggregation and prevented oxidative stress. The potential molecular mechanisms present behind the observed results explored by RNAi technology revealed that DAF-16/FOXO and SKN-1/NRF2 signaling pathways were, at least partially, involved.

1. Introduction

Many studies have evidenced the benefits on health of the intake of vegetables and fruits, and the strong correlation between this intake and the decreased risk for many chronic diseases (Wallace et al., 2020). Strawberry (*Fragaria* × *ananassa*) is a member of the berries, which overall are very rich in bioactive compounds and phytochemicals that have been associated with multiple healthy properties (Battino et al., 2021; Giampieri et al., 2017). Strawberries are rich in a wide variety of phenolic compounds, including flavonoids (particularly anthocyanins) and phenolic acids. But they are also rich in vitamins (C and folate) and minerals, which lead to consider this fruit a powerful resource in human health. In fact, there is multiple evidence supporting the role of strawberries against inflammatory- and oxidative-based diseases, like cardiovascular diseases and several types of cancer (Battino et al., 2021; Cianciosi et al., 2019; Giampieri et al., 2018).

The *Romina* strawberry variety (AN99.78.51) is agronomically of interest because of its high adaptability to non-fumigated soil and to open field cultivation in climatic conditions from the mid-Adriatic to the center-north of Europe and for its resistance to diseases (Forbes-Hernández et al., 2017). It is also of interest for producers and consumers for its early ripening time and its nutritional quality (Forbes-

* Corresponding author at: Department of Physiology, Institute of Nutrition and Food Technology "José Mataix", Biomedical Research Centre, University of Granada, Armilla, Avda. del Conocimiento s.n., 18100 Armilla, Spain.
E-mail address: jlquiles@ugr.es (J.L. Quiles).
[1] These authors contributed equally to this manuscript.

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Hernández et al., 2017). *Romina* fruit also combines a high content of anthocyanins and an elevated antioxidant capacity. In addition, its content of vitamin C, folic acid and flavonols is good, so it is expected that its health benefits are high. In this sense, it has been described that this variety improved lipid metabolism and redox status (Forbes-Hernández et al., 2017), promoted the conversion of white adipocytes to brown-like adipocytes (Forbes-Hernández et al., 2020) and had preventive and/or therapeutic actions against uterine leiomyomas (Giampieri et al., 2019) *in vitro*. Despite the wide evidence supporting healthy effects of strawberry intake, there is a paucity in the information about the role of this fruit in relation to cognitive-related diseases like dementia, among which Alzheimer disease (AD) is included. AD is responsible for around 60–80% cases of dementia and it is estimated that more than 35 million persons are affected by AD globally (Robinson et al., 2017). Alzheimer's dementia is considered an age-related disease and it is expected that as the world population becomes older, the number of people with AD will also increase. AD is associated with several health problems including motor decline, loss of memory and others. Mechanisms linked to these conditions have been often associated with inflammation and oxidative stress in the brain (Querfurth & LaFerla, 2010).

Many studies have demonstrated the usefulness of *Caenorhabditis elegans* to screen AD drug candidates and to investigate its molecular mechanisms (Paul et al., 2020). In the same way, the use of this nematode to analyze food compounds for its potential toxicity or biomedical applications, including AD, is well known (Shen et al., 2018). As far as we know, no studies on Alzheimer in *C. elegans* have been published investigating potential use of strawberries. Available pharmacological treatments for AD are very limited and there is strong evidence concerning the role of strawberries against diseases related to inflammation and oxidative stress. According to that, the present study had the aim to investigate a methanolic extract from strawberries of the variety *Romina* from the point of view of its characterization in terms of antioxidant capacity, polyphenols profile and chemical elements content. Moreover, by using *C. elegans* as an experimental model, the putative toxicity of the extract as well as the amyloid beta peptide production and oxidative stress, two key features of AD, have been assayed.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, Missouri, USA), Thermo Fisher (Waltham, Massachusetts, USA), Roche (Basel, Switzerland) or Merck (Darmstadt, Germany). Double distilled deionized water was obtained from a Milli-Q purification system from Millipore (Milford, MA, USA).

2.2. Plant material and methanolic extraction

Plant material collection and methanolic extraction was performed as previously described (Forbes-Hernández et al., 2017). Briefly, the strawberry fruits *Fragaria* × *Ananassa (cv. Romina)* were collected in the experimental fields of the Agricultural Faculty of the Università Politecnica delle Marche, Italy. The extraction procedure began with homogenization of 50 g of fruit with 100 mL of the solution composed of methanol/Milli-Q water/concentrated formic acid (80:20:0.1, v/v/v) using an Ultraturrax T25 homogenizer (Janke & Kunkel, IKA Labortechnik, Staufen, Germany) during 2 min. Extraction was maximized by stirring the suspension (ARE Magnetic stirrer, VELP Scientifica, Usmate, Italy) for 2 h in the dark at room temperature. After that, the mixture was centrifuged at 2400g for 15 min for two sequential times, in order to sediment solids. Supernatants were filtered through a 0.45 μm Minisart filter (PBI International, Milan, Italy). The methanolic extract was concentrated and dried through a rotary evaporator and the sample was stored in aliquots at −80 °C. The dry extract was conveniently diluted in water for its use. One-hundred, 500 and 1000 μg/mL of extract were used as dosages for all experiments with worms.

2.3. Evaluation of the total antioxidant capacity (TAC)

The antioxidant properties of the strawberry methanolic extract were investigated by a 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) and a 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) scavenging assays, and by the ferric reducing antioxidant power (FRAP) method (Forbes-Hernández et al., 2017). FRAP assay was carried out following the protocol previous published (Deighton et al., 2000). The test evaluates the capacity of the samples to reduce the complex formed by ferric iron and 2,4,6-tripyridyl-s-triazine (TPTZ). The reaction takes place in an acid pH and the absorbance is measured at 593 nm. The DPPH method was performed according to the protocol proposed by Kumaran and Karunakaran (2007). It is based on the spectrophotometric measurement of the free radical DPPH reduction. The radical has a purple coloration that is progressively lost when reacting with antioxidant compounds. That discoloration is determined at 517 nm. Finally, the protocol described by Re et al. (1999) was followed for the ABTS test, which is based on the ability of antioxidant compounds to deactivate the fluorescence of the ABTS radical. Thus, the absorbance values measured to 734 nm are inversely related with the antioxidant capacity. All determinations were carried at least in triplicate. Synergy Neo2 microplate reader (Biotek, Winooski, Vermont, U.S.A.) were used to measure the absorbance. Results are expressed as μmol trolox equivalent/g of dry extract (DE).

2.4. Total phenolic and flavonoids content measurement

Total phenolic content was assessed using the Folin-Ciocalteu method (Singleton et al., 1999). Briefly, the extract was reacted with the Folin-Ciocalteu solution for five minutes. Next, the mixture was incubated with sodium carbonate (Na_2CO_3) for 2 h at room temperature in the dark and the absorbance was read at 760 nm. The determination of flavonoids content was performed as previously published (Forbes-Hernández et al., 2017; Zhishen et al., 1999). The sample was reacted with $NaNO_2$ for six minutes and then, the $AlCl_3{\cdot}6H_2O$ was added. After five minutes, NaOH was added to the reaction and the absorbance was measured at 510 nm. Synergy Neo2 microplate reader (Biotek, Winooski, Vermont, U.S.A.) was used to measure the absorbance. Results are expressed as mg of gallic acid equivalent/g of DE, and mg of catechin equivalent/g of DE, respectively. All determinations were carried at least in triplicate.

2.5. Chromatographic operating conditions

2.5.1. Identification of individual phenolic compounds

An UPLC-QTOF-MS / MS method was used to identify the different phenolic compounds. The UPLC and mass spectrometer conditions were the same previously used by Esteban-Muñoz et al. (2020). The experiments were performed on a UPLC with mass spectrometer high resolution SYNAPT G2 HDMS Q-TOF model (Waters, Mildford, USA). Conditions consisted of a full MS, and data-dependent scanning was performed in negative and positive mode with electrospray ionization (ESI). The UPLC separation was performed using an ACQUITY UPLC ™ HSS T3 2.1 × 100 mm, 1.8 mm column. 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. The phenolic compounds were identified by comparing the molecular ions and fragments obtained with previous investigations carried out in strawberry with the help of the MassLynx V4 software (Waters Laboratory Informatics, Mildford, USA).

2.5.2. Quantification of individual phenolic compounds

UPLC analysis was performed using a Waters ACQUITY I CLASS model chromatography instrument (Waters, Mississauga, ON, Canada) equipped with a mass spectrometer Waters XEVO TQ-XS, with ionization performed by UniSpray (US). The same method described in Sánchez-Hernández et al. (2021) was used. A gradient of solvent A (water) and solvent B (methanol with 0.1% [v/v] acetic acid) for 25 min at a flow rate of 0.4 mL/min was followed. The gradient was programmed as follows: at 0 min 5% B, 15–15.10 min 95% B and 15.10–25 min 5% B. An Acquity UPLC HSS T3 1.8 μm column was used. Quantification of the phenolic compounds was accomplished by comparing the retention times of peaks and fragmentation data in samples, with those of the phenolic compound standards and measurements in Multiple Reaction Monitoring (MRM) was used.

2.6. Chemical elements analysis

Strawberry methanolic extract was lyophilized in a vacuum pump (Telstar, Madrid, Spain) and prepared by attack with nitric acid and hydrogen peroxide of supra-pure quality in a microwave digester (Milestone, Sorisole, Italy) for the quantitative determination of metals. Determination of Na, Mg, Al, P, K, Ca, Cr, Mn, Fe, Co, Ni, Cu and Zn total content in the sample was performed using an ICP-MS instrument (Agilent 7500, Agilent Technologies, Tokyo, Japan) coupled with a Meinhard type nebulizer (Glass Expansion, Romainmotier, Switzerland) and equipped with a He collision cell. A Milli-Q system (Millipore, Bedford, MA, USA) was used to obtain deionized water (18 MΩ). All reagents used were of the highest available purity. Hydrogen peroxide and nitric acid were of supra-pure quality (Merck, Darmstadt, Germany). A standard solution of 100 μg/L of Li, Mg, Sc, Co, Y, In, Ce, Ba, Pb, Bi, and U in 1% (v/v) of HNO_3 was prepared from a 1000 mg/L multi-element stock standard solution (Merck) and used to optimize the ICP parameters daily. Single-element standard solutions for ICP-MS containing 1000 μg/mL of each analyte were also purchased from Merck. Calibration curves were prepared using Ga as an internal standard and by the dilution of stock solutions of 1000 mg/L in 1% HNO_3. The accuracy of this method was evaluated by recovery studies after complete digestion of spiked samples with multi-element standards. The calculated recoveries for each element were between 95% and 105% in all cases. All determinations were carried at least in triplicate.

2.7. Caenorhabditis elegans strains and maintenance

The *Caenorhabditis elegans* strains used in this work were N2 Bristol (wild type), CL4176 (dvIs27 [myo-3p::A-Beta (1–42)::let-851 3′UTR) + rol-6(su1006)] X), CL802 (smg-1(cc546) I; rol-6(su1006) II), TJ375 (gpIs1[hsp-16.2::GFP]), TJ356 (zIs356[daf-16p::daf-16a/b::GFP + rol-6 (su1006)]), LD1 (ldIs7 [skn-1b/c::GFP + rol-6(su1006)]) and CF1553 (mu1s84[pAD76(sod-3::GFP) + rol-6(su1006)]). CL4176 and CL802 were routinely maintained in an incubator (VELP Scientifica FOC 120 E, Usmate, Italy) at 16 °C and, the rest of them, at 20 °C. All the worms were maintained on plates with nematode growth media (NGM) containing an *Escherichia coli* OP50 lawn as source of food, as previously described (Corsi et al., 2015). Worms and bacteria were obtained from the Caenorhabditis Genetics Center (CGC) (Minneapolis, MI, USA). All the experiments were conducted starting with age-synchronized nematodes from gravid hermaphrodite adults treated with bleaching solution (0.5 N NaOH in 20% bleach).

2.8. Lethality test

To determine the acute toxicity and to evaluate the death rate of the strawberry methanolic extract, a concentration–response curve was performed. Age-synchronized N2 Bristol worms in L4 larvae were exposed to increasing concentrations of extract (0, 0.1, 1, 10, 100, 500 and 1000 μg/mL) for 24 h at 20 °C in absence of food. After that time, the animals were scored as live or dead using a Motic dissecting microscope (Motic Inc., LTD., Hong Kong, China). Death was assumed when there was no response to mechanical stimulus generated with a platinum wire. Three independent assays for each concentration were made, and each independent assay included three NGM plates with, at least, ten worms in each one. Lethality was expressed as a total percentage of worm survival.

2.9. Pharyngeal pumping and growth assays

The pharyngeal pumping test was carried out in order to assess the worm metabolism, and the body length measurement was employed to evaluate the treatment effect on the worm development, both in N2 strain. Egg-synchronized worms were placed in plates containing NGM with or without the strawberry methanolic extract (0, 100, 500 and 1000 μg/mL) and a lawn of bacteria, and incubated at 20 °C. Four days after the synchronization, worms were moved to fresh plates to count the terminal pharynx bulb contractions number per minute with a microscope (Motic Inc., LTD., Hong Kong, China). Those adults were washed from the plates and they were made to pass through the Multi-Range Large Particle Flow Cytometer Biosorter (Union Biometrica. Massachusetts, USA) to assess the time of flight (TOF) which is indicative of the length. Ten worms per group were studied for pharyngeal pumping and at least one hundred animals per treatment were measured for growth assay. The experiments were performed in triplicate.

2.10. Reproduction and fertility evaluation

The effect of the strawberry methanolic extract on the brood size and egg-laying capability was evaluated by the reproduction and fertility assays, respectively. L4 stage synchronized worms belonging to the N2 Bristol strain were placed on NGM plates containing 0, 100, 500 or 1000 μg/mL of the extract and a lawn of *E. coli*. After 24 h of exposition at 20 °C, each worm was moved to an untreated NGM well with bacteria as a source of food. This step was repeated daily until the hermaphrodite stopped laying eggs. Eggs per worm were counted the same day of adult removal and the larvae number were counted the day after, using a microscope dissection (Motic Inc., LTD. Hong Kong, China). At least ten worms were used per treatment. The results are expressed as the mean of total number of eggs or larvae.

2.11. Lifespan curves

The strain N2 was used to evaluate the extract effect in a long-term point of view. Egg-synchronized worms were deposited in plates with or without the strawberry methanolic extract (0, 100, 500, 1000 μg/mL) and maintained at 20 °C. On day 1 of experiment, about 72 h after synchronization (when the animals had reached young adulthood), 120 worms were transferred to fresh plates containing the treatments or control and seeded with *E. coli* OP50. From there, worms were moved to fresh plates and scored of survival every day. Death was assumed when there was no response to mechanical stimulus. The worms dragged out of the dish, with extruded internal organs, dead from progeny in utero, or with a bag of worms were not counted in the number of deaths (censored). Results were represented as the Kaplan-Meier curve.

2.12. Measurement of intracellular ROS

Aged-synchronized eggs of wild type N2 strain were placed in plates with the three dosages of strawberry methanolic extract or without them. When the worms reach the L4 larvae stage (approximately 48 h after synchronization), all the groups except one control plate, were exposed to 2.5 mM of 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) for 15 min in order to induce oxidative stress. After that, three washes with M9 were made to remove de AAPH. Then, the worms were incubated for 2 h with 25 μM of 2',7'-Dichlorofluorescin

Diacetate (DCFDA) at 20 °C. The DCFDA allows the intracellular reactive oxygen species (ROS) quantification since this dye can penetrate through the cell membrane and emits fluorescence when it reacts with free radicals. The fluorescence intensity of at least 350 worms per group was measured using a Multi-Range Large Particle Flow Cytometer Biosorter (Union Biometrica. Massachusetts, USA). The results are expressed as the mean of the fluorescence intensity, which is related to the ROS content (AU).

2.13. Paralysis assay and RNAi experiments

The strain CL4176 contains a temperature sensitive mutation that expresses human amyloid β1–42 peptide in muscle cells, which leads to paralysis in the nematode. Egg-synchronized CL4176 were placed onto plates with different dosages of strawberry extract (100, 500 or 1000 μg/ml) or without it, and seeded with *E. coli* OP50. The strain CL802 was used as non-paralyzable control for the test. Worms were incubated at 16 °C during 48 h. After that, the plates were transferred to another incubator at 25 °C to induce the Aβ expression. The paralysis was scored from 20 h until 32 h after the temperature upshift. Worms were considered as paralyzed when there was no response to mechanical stimulus generated with a platinum wire. At least three different experiments were carried out, with 25 worms per each one. The test results are expressed as non-paralyzed worm percentage (%).

For the RNAi experiments, *E. coli* HT115 expressing transcription factor skinhead (SKN)-1, heat shock protein 16.2 (HSP 16.2) (Sources BioScience, Notthingham, UK), dauer formation (DAF)-16 or superoxide dismutase (SOD)-3 (Cultek SL, Madrid, Spain) dsRNA were spread on NGM plates containing 25 μg/mL carbenicillin and 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and L3-L4 synchronized CL4176 worms were placed on those plates. When the nematodes reached the second day of adulthood (fertile age), a few of them were placed in similar new plates but, this time, with the strawberry treatment (0, 100, 500, 1000 μg/mL). After four hours, the adults were removed, and the remaining eggs were used for the paralysis experiment. Paralysis was tested according to the standard experiment, explained above.

2.14. β-amyloid plaques staining

Synchronized CL4176 eggs were placed on plates with the treatments and the control seeded with *E. coli* OP50. After the incubation at 16 °C during 48 h, all the plates, except one of CL4176 control, were moved to another incubator at 25 °C for 26 h. Then, the nematodes were collected by washes with M9 buffer and fixed with 4% paraformaldehyde (pH 7.4) at 4 °C for 24 h. After fixation, worms were permeabilized in 5% β-mercaptoethanol, 1% Triton X-100, and 125 mM Tris, pH 7.4 at 37 °C for 24 h. After washing 2 times with M9 buffer to remove the permeabilization buffer, the samples were stained with 0.125% thioflavin T in 50% ethanol for 30 min and destained with sequential ethanol washes (50%, 75%, 90%, 75%, and 50% v/v), each one for 2 min (Du et al., 2019). Thioflavin-T stained worms were observed under a Nikon *epi*-fluorescence microscope (Eclipse Ni, Nikon, Tokyo, Japan) and images

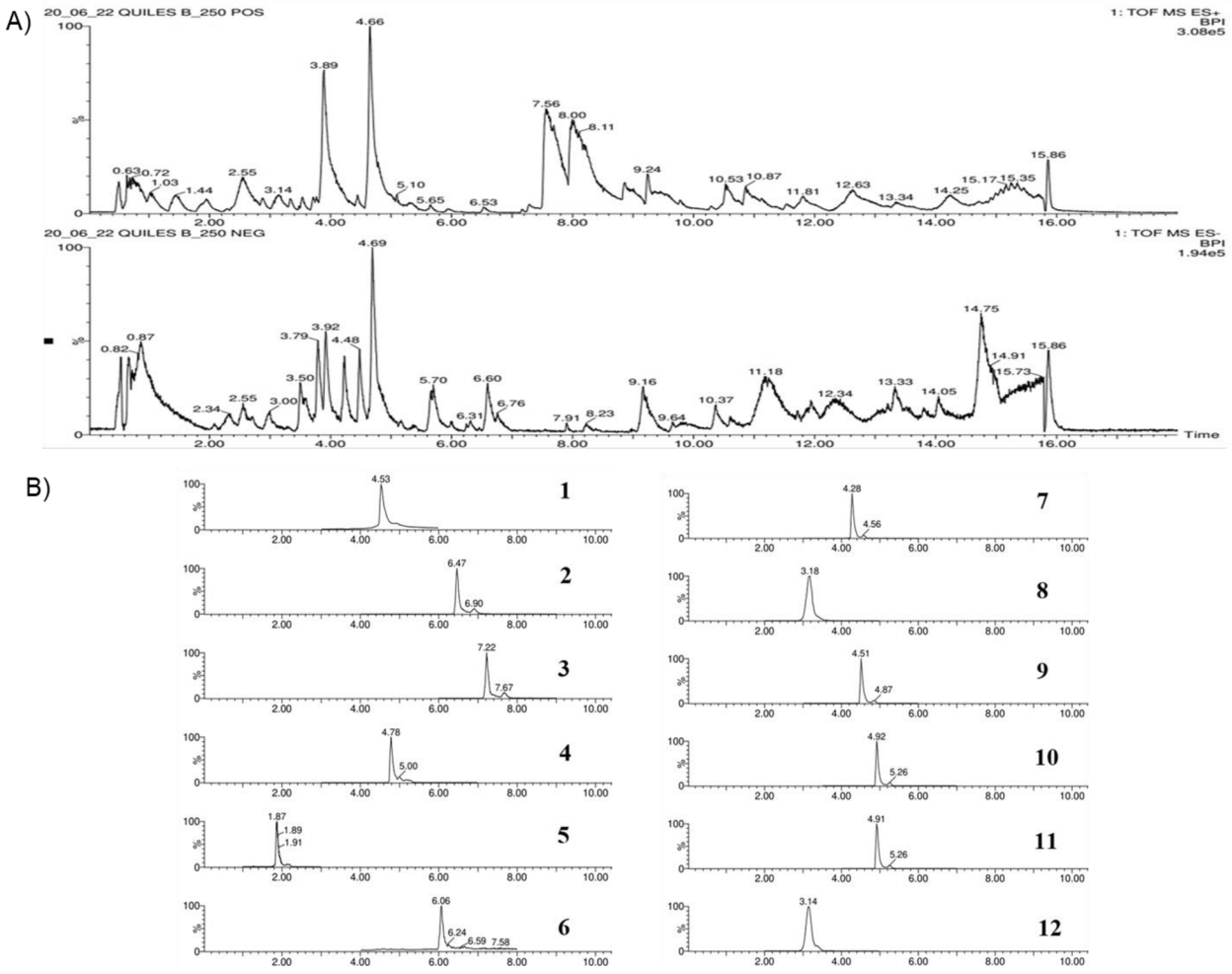

Fig. 1. A) UPLC-QTOF-MS/MS chromatogram of Romina strawberry methanolic extract. B) Ultra-performance liquid chromatographic profile of 12 standard phenolic acids. 1: Ellagic acid; 2: Quercetin; 3: Apigenin; 4: Ferulic acid; 5: Gallic acid; 6: o-Vanillin; 7: Rutin; 8: Pelargonidin-3-O-rutinoside; 9: Quercetin-3-O-glucopyranoside; 10: Kaempferol 3-O-D-glucoside; 11: Chrysanthemin; 12: Pelargonidin-3-glucoside.

were acquired at 40X magnification using the GFP filter with a Nikon DS-Ri2 camera (Tokyo, Japan). CL4176 untreated worms incubated at 16 °C were considered as negative control and the CL4176 untreated incubated at 25 °C were the positive control.

2.15. GFP-reporter transgenic strains for DAF-16, SKN-1, HSP16.2 and SOD-3

Strains LD1, TJ356, TJ375 and CF1553 were used in order to deepen the molecular mechanisms of the assessed fruit extract. In transgenic LD1 and TJ356 worms, the gene for SKN-1 and DAF-16, respectively, are fused to the green fluorescent protein (GFP). Thus, the translocation of those genes to the nucleus can be studied by fluorescence microscopy. SKN-1 is present in ASI chemosensory neurons in a constitutive way and accumulates in intestinal nuclei in response to oxidative stress. TJ375 worms express the HSP 16.2 as a fusion protein with the GFP, mainly in the anterior pharynx bulb, which becomes activated under oxidative stress. The SOD-3 protein is expressed in the transgenic CF1553, also fused with the GFP.

For all strains, synchronized eggs were placed on plates containing the treatments (0, 100, 500 or 1000 μg/mL of the strawberry extract). After 48 h, the experiments were carried out. Nematodes were mounted on glass slides which contained sodium azide to reduce their mobility. Images of worms were acquired at 10X or 40X magnification using the GFP filter in a Nikon *epi*-fluorescence microscope (Eclipse Ni, Nikon, Tokyo, Japan) fitted with a Nikon DS-Ri2 camera (Tokyo, Japan). The analysis of the images was performed using the software NIS-Elements BR (Nikon, Tokyo, Japan). A semi-quantitative scale was used for the TJ356 strain, assigning the value '1′ to worms with cytosolic expression of DAF-16::GFP, '2′ to the intermediate status, and '3′ to the nuclear location. The fluorescence intensity of the gut area below the pharynx was measured for the individuals LD1 worms. The HSP16.2::GFP expression was quantified in the area anterior of the pharyngeal bulb in individual TJ375 worms. For CF1553, the fluorescence intensity of the whole worm was measured.

2.16. Verification of the RNAi effect in GFP-reporter transgenic strains

The strains LD1, TJ356, CF1553 and TJ375 were used to verify whether RNAi treatments were effective for the particular gene inhibition in the paralysis assay. For all strains, L3-L4 synchronized worms (F0) were placed on plates containing or not the RNAi for the specific gene, prepared as mentioned in section 2.12. When the worms reached adulthood, they were synchronized and the eggs (F1) were placed on new plates, again, with or without the specific RNAi. After 48 h, the experiments were carried out as already explained in the section 2.15.

2.17. Statistical analysis

Variables were studied for normality (Kolmogorov-Smirnov) and homogeneity of variance (Levene). Non-normally distributed variables were analyzed by non-parametric tests. For normally distributed variables, one-way ANOVA and Bonferroni as *post hoc* tests were employed. For non-normally distributed variables, Kruskal-Wallis and Mann-Whitney-U tests were performed. Data are expressed as mean ± SEM from at least 3 independent experiments unless otherwise stated. Significance was considered for $P < 0.05$. For lifespan curves, differences among survival distributions of cohorts were evaluated using the log-rank test. Statistical analysis was performed by SPSS 24.0 (IBM, Armonk, NY, USA).

Table 1
Individual phenolic compounds identified in the Romina strawberry methanolic extract.

Tentative identification	Molecular formula	Mode + or −	*m/z* $[M-H]^-$ or $[M-H]^+$	Reference used for identification
Anthocyanins				
Cyanidin 3-O-glucoside	$C_{21}H_{21}O_{11}$	−	448.1006	(Fernández-Lara et al., 2015; Forbes-Hernández et al., 2017; Seeram et al., 2006)
Cyanidin 3-O-rutinoside	$C_{27}H_{31}O_{15}$	− +	594.1585 596.1741	(Fernández-Lara et al., 2015)
Cyanidin 3-O-(6″-acetyl-glucoside)	$C_{23}H_{23}O_{12}$	−	490.1111	(Fernández-Lara et al., 2015)
Pelargonidin 3,5-diglucoside	$C_{27}H_{31}O_{15}$	+	596.1741	(Fernández-Lara et al., 2015; Forbes-Hernández et al., 2017; Seeram et al., 2006)
Pelargonidin 3-O-glucoside	$C_{21}H_{21}O_{10}$	−	432.1056	(Fernández-Lara et al., 2015; Forbes-Hernández et al., 2017; Seeram et al., 2006)
Pelargonidin 3-O-galactoside	$C_{21}H_{21}O_{10}$	−	432.1056	(Fernández-Lara et al., 2015)
Flavanols				
(+)-Catechin	$C_{15}H_{14}O_6$	− +	289.0712 291.0869	(Fernández-Lara et al., 2015; Seeram et al., 2006; Yildirim & Turker, 2014)
(-)-Epicatechin	$C_{15}H_{14}O_6$	− +	289.0712 291.0869	(Yildirim & Turker, 2014)
Procyanidin dimer B1 and B2	$C_{30}H_{26}O_{12}$	− +	577.1346 579.1503	(Yildirim & Turker, 2014)
Flavonols				
Kaempferol 3-O-acetyl-glucoside	$C_{23}H_{22}O_{12}$	−	489.1033	(Forbes-Hernández et al., 2017)
Kaempferol 3-O-glucoside	$C_{21}H_{20}O_{11}$	− +	447.0927 449.1084	(Seeram et al., 2006)
Quercetin	$C_{15}H_{10}O_7$	− +	301.0348 303.0505	(Yildirim & Turker, 2014)
Quercetin 3-O-glucoside	$C_{21}H_{20}O_{12}$	−	463.0877	(Fernández-Lara et al., 2015; Seeram et al., 2006)
Quercetin 3-O-rutinoside	$C_{27}H_{30}O_{16}$	− +	609.1456 611.1612	(Seeram et al., 2006)
Phenolic acids				
Ellagic acid	$C_{14}H_6O_8$	− +	300.9984 303.0141	(Fernández-Lara et al., 2015; Forbes-Hernández et al., 2017; Seeram et al., 2006)
p-Coumaric acid	$C_9H_8O_3$	− +	163.0395 165.0552	(Fernández-Lara et al., 2015; Yildirim & Turker, 2014)
Isoflavonoids				
Daidzein	$C_{21}H_{20}O_9$	−	253.0501	(Yildirim & Turker, 2014)
Genistein	$C_{15}H_{10}O_5$	−	269.0450	(Yildirim & Turker, 2014)
Flavones				
Apigenin	$C_{15}H_{10}O_5$	−	269.0450	(Yildirim & Turker, 2014)

Table 2
Quantification of the phenolic compounds present in the methanolic extract of Romina strawberry.

Phenolic compounds	Mean (μg/g) ± SD
o-Vanillin	12.54 ± 0.21
Gallic acid	68.74 ± 0.60
Ferulic acid	7.50 ± 0.28
Apigenin	4.95 ± 0,16
Quercetin	5.10 ± 0.05
Ellagic acid	10360.91 ± 26.84
Pelargonidin-3-glucoside	1153.71 ± 20.22
Chrysanthemin	571.37 ± 6.62
Kaempferol-3-O-D-glucoside	529.43 ± 3.90
Quercetin-3-O-glucopyranoside	63.67 ± 0.39
Pelargonidin-3-rutinoside	173.77 ± 0.49
Rutin	8.11 ± 0.04

3. Results

3.1. Antioxidant capacity, total flavonoid and total phenolic content of the strawberry methanolic extract

The extract presented 228.187 ± 12.385 μmol trolox/g of DE for DPPH, 279.895 ± 18.988 μmol trolox/g of DE for ABTS, and 271.401 ± 19.340 μmol trolox/g of DE for FRAP. Total flavonoid content was 4.733 ± 0,324 mg catechin eq/g of DE and the amount of total phenolic content was 15.321 ± 1.148 mg GA eq/g of DE.

3.2. Identification and quantification of phenolic compounds

Both positive and negative chromatograms were performed which led to the identification of a total of 19 molecules (Fig. 1). Table 1 shows the tentative identification of the compounds together with the molecular formula and the *m/z* $[M-H]^-$ or $[M-H]^+$. Anthocyanins, a subclass that provides the bright red color of strawberry fruit, was the main type of phenolic compounds found in the strawberry methanolic extract. Besides that, other compounds belonging to flavanols, flavonols (kaempferol, quercetin and derivatives), phenolic acids, isoflavonoids and flavones families were also present in the samples. Regarding the phenolic compound quantification (Table 2), highlighted the content of ellagic acid (10,360.91 μg/g) and, to a lesser extent, the amount of pelargonidin-3-glucoside (1153.71 ± 20.22 μg/g), chrysanthemin (571.37 ± 6.62 μg/g), kaempferol-3-O-D-glucoside (529.43 ± 3.90 μg/g) and pelargonidin-3-rutinoside (173.77 ± 0.49 μg/g) found in the extract.

3.3. Chemical elements content

The chemical elements found in the sample and the mean concentration ± standard deviation (SD) (in μg/g) was: Na (166.67 ± 19.99), Mg (1481.82 ± 107.24), P (1423.50 ± 124.80), K (22643.38 ±

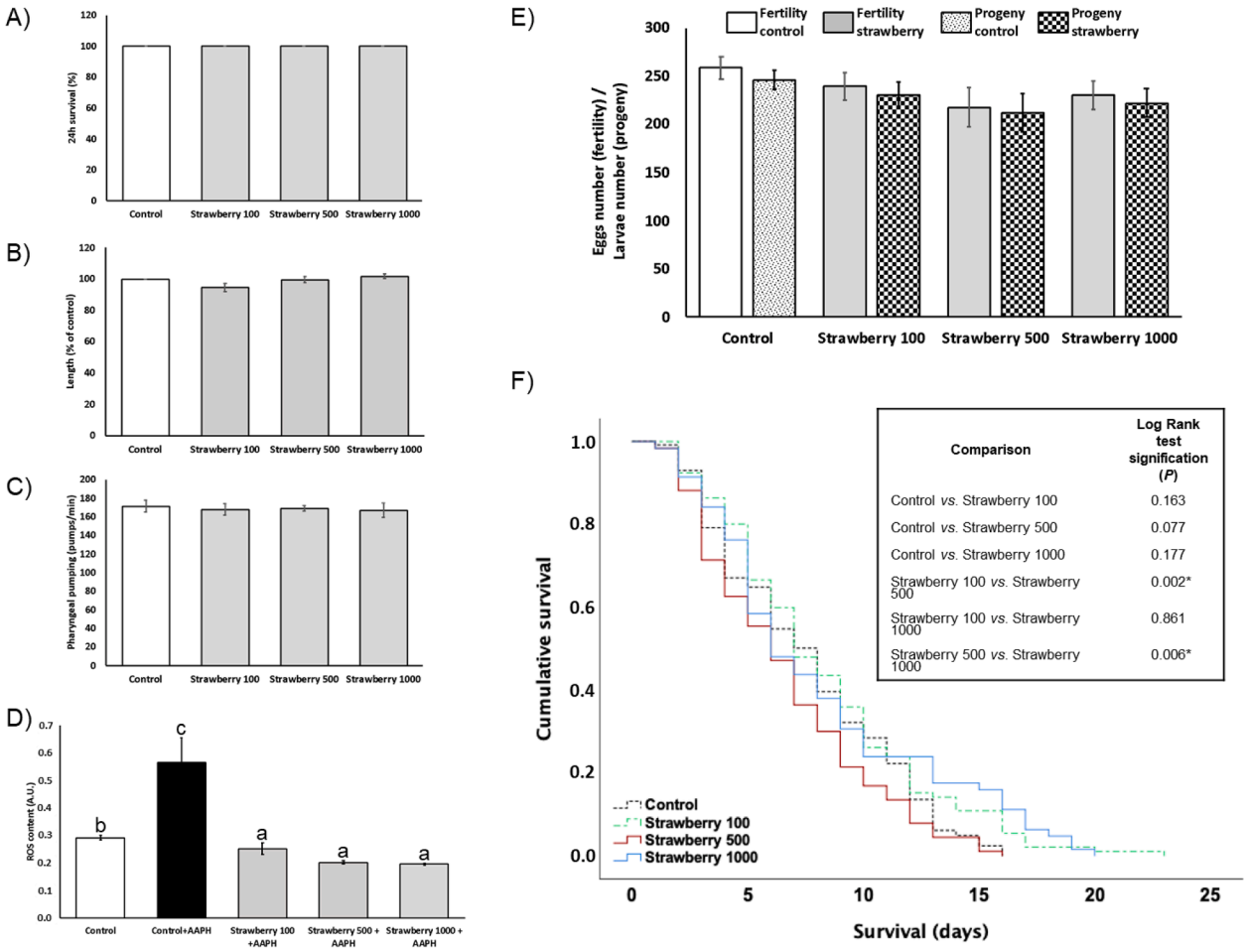

Fig. 2. *In vivo* toxicity assay of the Romina strawberry methanolic extract at 100, 500 and 1000 μg/mL in the *Caenorhabditis elegans* wild type N2 strain. A) Lethality; B) Growth; C) Pharyngeal pumping; D) Reactive oxygen species (ROS) content. Lower-case letters, when different, represent statistically significant differences ($P < 0.05$); E) Reproduction and fertility; F) Kaplan-Meier survival curves. * means statistically significant differences ($P < 0.05$). Results are expressed as mean ± SEM.

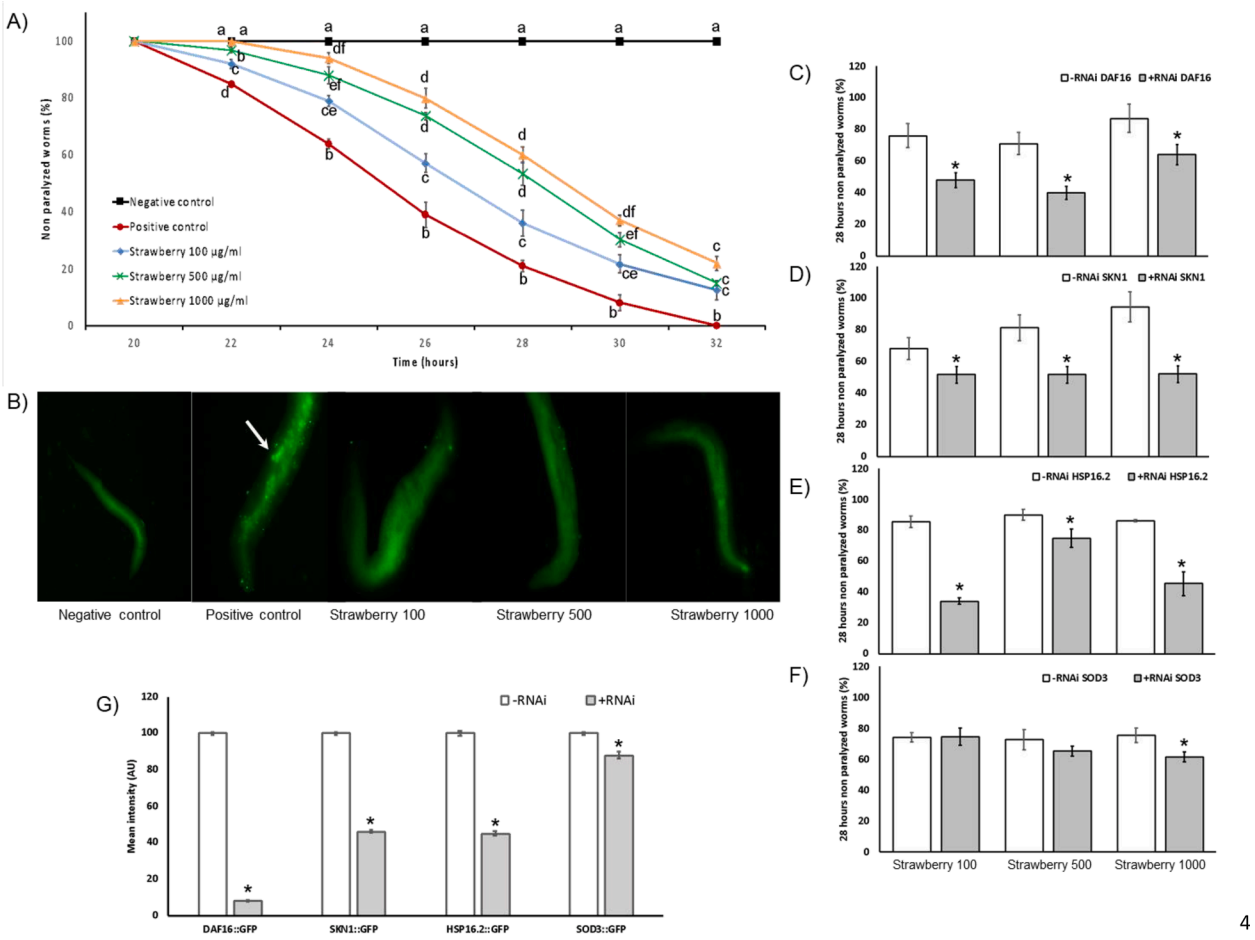

Fig. 3. Effects of the Romina strawberry methanolic extract at 100, 500 and 1000 µg/mL on the paralysis phenotype in the transgenic strain CL4176, the effect of the different RNAi in CL4176, and the verification of RNAi working in transgenic strains. A) Paralysis curve represented as non-paralyzed CL4176 worms (%) from 20 h until 32 h after the temperature upshift. Lower-case letters, when different, means statistically significant differences ($p < 0.05$) between treatments for the specific time. B) Representative images of the thioflavin T staining in CL4176 worms collected 26 h after the temperature upshift (40X magnification). White arrow shows the Aβ aggregated. C-F) Non-paralyzed CL4176 worms (%) at 28 h after the temperature upshift with and without RNAi. * Means statistically significant differences ($P < 0.05$) between the treatment with and without the RNAi: C) DAF-16/FOXO D) SKN-1/NRF2 E) HSP16.2F) SOD-3. Results are mean ± SEM. **G)** Effect of RNAi in the transgenic strains TJ356 (DAF16::GFP), LD1 (SKN1::GFP), TJ375 (HSP16.2::GFP) and CF1553 (SOD3::GFP). Results are expressed as percentage to control without the RNAi for the specific gene. * Means statistically significant differences ($P < 0.05$) with respect to the control group of the same strain without the RNAi.

2041.75), Ca (1362.96 ± 119.36), Al (1.68 ± 0.42), Cr (0.96 ± 0.09), Mn (11.93 ± 1.12), Fe (2.18 ± 0.47), Co (0.03 ± 0.02), Ni (0.85 ± 0.13), Cu (1.12 ± 0.21), and Zn (31.79 ± 2.32).

3.4. *In vivo toxicity study of the strawberry extract*

As a first approach to the *in vivo* effects of the strawberry methanolic extract, toxicity was evaluated in *C. elegans* as an experimental model by several tests, including lethality, pharyngeal pumping, growth, reproduction and fertility, as well as survival curves. Acute toxicity was firstly assessed by the lethality test (Fig. 2A). Results of this assay showed non-lethal effects for all tested concentrations, with a 100% of survival. Fig. 2A presents only 0, 100, 500 and 1000 µg/mL, which were the selected dosages for the subsequent experiments. The pharyngeal pumping experiment was performed with the goal of evaluating the extract effect on worms' metabolism, and the growth assay intended to elucidate the influence of strawberry extract on the nematode development. There were no differences neither in the number of pumps per minute nor in the body length between the nematodes treated with the different concentrations of the extract and the control group, as shown in Fig. 2B and C. Regarding fertility and reproduction (Fig. 2E), the egg-laying ability of the worms (fertility) or the viability of the eggs measured by the larvae number (progeny) were not affected by the treatments. Finally, the potential long-term toxic effects of the strawberry methanolic extract on the worms was evaluated by analyzing longevity through the use of survival curves. Results showed an absence of toxicity of the used concentrations (100, 500, 1000 µg/mL) in the wild type N2 worms, as reflected in Fig. 2F. On the contrary, 100 and 1000 µg/mL treatments prolonged longevity compared to the dosage of 500 µg/mL, according to the statistical analysis by Log-Rank test. These results open the door for further experiments on the putative ability of Romina strawberries to modulate lifespan *in vivo*.

3.5. *Effects of strawberry methanolic extract on oxidative stress*

To test the effect of strawberry extract on reactive oxygen species (ROS) content, DCFDA was measured, using AAPH as an oxidative stress inductor. As expected, AAPH increased the ROS content in the live worms compared to the control baseline (without the oxidant agent). According to results, shown in Fig. 2D, the three concentrations of the strawberry extract conferred protections to the N2 worms from oxidant agent. Indeed, the ROS content was even lower in the treated nematodes than in the negative control group.

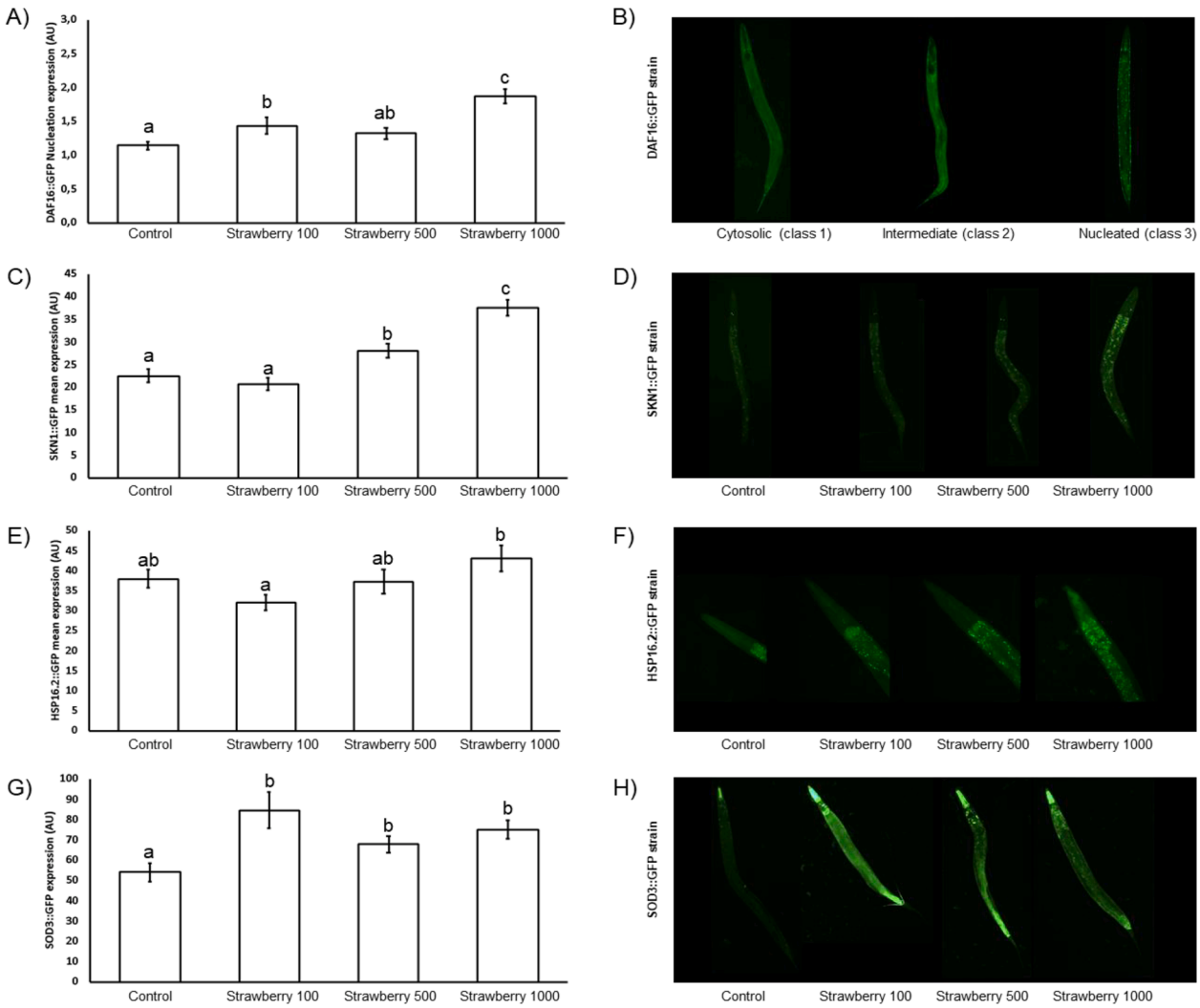

Fig. 4. Effects of the Romina strawberry methanolic extract at 100, 500 and 1000 μg/mL on GFP-reporter transgenic strains. A) DAF-16::GFP nucleation in TJ356. B) Representative images of cytosolic, intermediate or nuclear status of DAF-16::GFP in the strain TJ356 (10X magnification). C) SKN-1::GFP expression in LD1. D) Representative images of SKN1::GFP expression for each treatment in the strain LD1 (10X magnification). E) HSP16.2::GFP expression in TJ375. F) Representative images of HSP16.2::GFP expression for each treatment in the strain TJ375 (40X magnification). G) SOD-3::GFP expression in CF1553. H) Representative images of SOD3::GFP expression for each treatment in the strain CF1553 (10X magnification). Results are mean ± SEM. Lower-case letters, when different, means statistically significant differences between groups ($P < 0.05$).

3.6. *Effects of strawberry methanolic extract on Aβ peptide production*

To assess the effect of strawberry methanolic extract on Aβ toxicity, the paralysis test was performed using the transgenic strain CL4176, which expresses the human Aβ peptide on muscle cells leading to paralysis phenotype. Results, represented in Fig. 3A, showed an undoubted delay of the paralysis in treated worms regardless of the extract dosage. Notwithstanding, the highest concentrations (500 and 1000 μg/mL) exhibited the best results, especially 26 and 28 h after the temperature upshift. Those results were correlated with the findings revealed by the thioflavin T staining (Fig. 3B). Thioflavin T binds specifically to Aβ aggregation, as it displays the panel of positive control in which we can observe the large amount of Aβ deposits. The adjacent images reflect the clear effect of the strawberry extract, with less thioflavin *T*-positive aggregates than positive control. Thus, treatments were able to minimize the Aβ production and accumulation in the transgenic model CL4176. To continue exploring the molecular mechanisms underlying the effects found in the paralysis assay several tests were conducted using the RNAi technology. Paralysis assay was repeated in CL4176 strain treated with the strawberry extracts and subjected to RNAi for different genes. Fig. 3C shows RNAi paralysis assay for the DAF-16 (abnormal DAuer Formation-16) gene 28 h after the increase in temperature. It can be observed how, for the three extract concentrations, DAF-16 RNAi significantly reduced the percentage of non-paralyzed worms. Similar findings are shown in Fig. 3D for RNAi for the SKN-1 (skinhead-1) gene. The putative action of HSP16.2 (Heat Shock Protein 16.2) on the paralysis prevention was also assayed through RNAi (Fig. 3E). Results showed an implication of this protein as well, since the percentage of non-paralyzed worms 28 h after the temperature upshift was lower than in the groups without RNAi for all the dosages. The involvement of SOD-3 enzyme in the protective effect of the extract is represented in Fig. 3F. Here, no effects of RNAi were observed for 100 and 500 μg/mL of strawberry extract. However, for the highest extract dosage, a reduction in the percentage of non-paralyzed worms was found after SOD-3 RNAi was applied.

To verify that the feeding technique of RNAi was effective, the four RNAi assessed in CL4176 were tested in specific transgenic strains. Those strains express the protein that we are silencing, but fused with GFP. As shown Fig. 3G, the RNAi decreased the expression of the respective protein, namely DAF-16::GFP, SKN-1::GFP, SOD-3::GFP and HSP16.2::GFP. These results prove that the RNAi inhibition was effective.

3.7. Effects of strawberry methanolic extract on SKN-1::GFP, DAF-16::GFP, SOD-3::GFP and HSP16.2::GFP expression

Individual worms belonging to the TJ356 strain were classified in cytosolic, intermediate or nuclear according to the presence of DAF-16::GFP nucleation (Fig. 4B). Cytosolic was considered when the fluorescence was diffusely distributed along the body, the intermediate situation presented diffuse fluorescence in the background with some bright nuclei especially in the anterior or posterior part, and the worm was catalogued as nuclear when the bright nuclei appeared along the worm. The bright nuclei indicate that the transcription factor DAF-16::GFP is translocated inside the nucleus from the cytosol. Results, represented in Fig. 4A, showed that both 100 and 1000 μg/mL of extract induced the nuclear translocation of the transcription factor, being more patent at the highest dose. The highest doses (500 and 1000 μg/mL) increased the expression of SKN-1 in the LD1 strain (activation of this transcription factor is also mediated by its nucleation), as reflect the Fig. 4C and D. However, none of the three assessed dosages modified the expression of the heat shock protein (HSP 16.2) compared to control in the anterior pharyngeal bulb, which is also fused with GFP in the TJ375 transgenic worms (Fig. 4E and F). The three tested concentrations increased the fluorescence intensity of CF1553 transgenic strain, which expresses the SOD-3 protein fused with GFP (Fig. 4G and H). Wherefore, the extract assayed in the present study promoted the translocation of the transcription factor DAF-16 and SKN-1 to the nucleus, and increased the SOD-3, which is a downstream target of DAF-16 and SKN-1.

4. Discussion

Strawberry consumption has been associated with numerous health benefits and with the protection against many chronic degenerative diseases. Those effects are attributed to their great content in micronutrients and phytochemicals (Battino et al., 2019; Mazzoni et al., 2019). Concerning total flavonoids content, our results were similar to those found by Forbes-Hernández et al., in *Romina* strawberry methanolic extract (Forbes-Hernández et al., 2017). However, our extract showed higher values for antioxidant capacity by the three methods (DPPH, FRAP and ABTS) and lower numbers in total phenolic content. Those differences could be due to the variations in growth, stage of ripening or storage conditions (Giampieri et al., 2012).

Storage positively affects the antioxidant capacity of strawberries because the formation of compounds with enhanced antioxidant capacity may be facilitated by reactions during the post-harvest period (Giampieri et al., 2012). Different extraction procedures (for example, ethanolic extract) in different strawberry varieties (e.g. Clery strawberries) showed values of total phenolic content and antioxidant capacity in the same line as those presented here (Garzoli et al., 2020). The UPLC-QTOF-MS/MS method was used for the identification of the phenolic compounds present in the methanolic extract. Compared with our analysis, similar qualitative profile was found for *Romina* (Forbes-Hernández et al., 2017) and other strawberry varieties (Milosavljević et al., 2020), standing out the pelargonidin-3-glucoside as the predominant anthocyanin in the strawberry fruit (Forbes-Hernández et al., 2017; Milosavljević et al., 2020). Milosavljević and co-workers also found catechin, kaempferol, quercetin and derivatives in different strawberry cultivars. Moreover, as in our extract, phenolic acids were also present, especially the ellagic acid (Milosavljević et al., 2020). The quantification of phenolic compounds in the present study was slightly different compared to other *Romina* strawberry methanolic extracts, in which authors found a higher presence of pelargonidin-3-glucoside and a lower amount of ellagic acid, the opposite than was found here (Forbes-Hernández et al., 2017). That fact could be related, as the antioxidant capacity mentioned above, to differences in fruit storage conditions, growth or stage of ripening (Giampieri et al., 2012). Regarding chemical element content, other studies focused on the whole fruit also revealed the wealth of the strawberry in elements such as K, Mg and P. Those were also the most abundant elements in our samples. Besides, the presence of Ca, Na, Mn, Zn, Cu and Fe were described as well, same as our extract (Hossain et al., 2016). Hence, strawberry, and as presented here strawberry methanolic extract, has a remarkable mineral dietary contribution that must be taken into account due to the biological implications of these elements. For instance, Cu, Zn and Mn are involved in the antioxidant status since they are necessary for the normal functioning of superoxide dismutases (Zelko et al., 2002). Conversely, dysregulation of Cu, Zn or Fe balance due to failure of endogenous regulatory mechanisms could lead to functional deprivation in some cell compartments, and toxic excess in others. That alteration, when it takes place in the brain, is associated with AD-neurodegeneration and cognitive decline (Sensi et al., 2018).

C. elegans was used in the present study as an *in vivo* model to assess the toxicity of the tested material. There were no differences between the control group and the three dosages (100, 500, 1000 μg/mL) in all the experiments carried out, including lethality, pharyngeal pumping, growth, reproduction and fertility, as well as survival curves. Therefore, the strawberry methanolic extract used in the present research had no toxicity *in vivo*. Similar results have been found for the Romina variety of strawberries *in vitro,* but only for low concentrations (Forbes-Hernández et al., 2017; Forbes-Hernández et al., 2018; Giampieri et al., 2019). Specifically, authors found that the treatment with a methanolic extract of the Romina variety at 250 μg/mL for 48 h did not affect myometrial cell viability (Giampieri et al., 2019). Furthermore, concentration higher than 100 μg/mL of the strawberry methanolic extract for 24 h decreased the HepG2 (Forbes-Hernández et al., 2017) and 3 T3-L1 (Forbes-Hernández et al., 2018) cell viability. Likewise, other natural extracts from fruits belonging to the berry family tested in *C. elegans* have been reported to exhibit toxicity. For instance, a blueberry extract decreased the total offspring in a dose-dependent manner, affecting the nematode fertility (Wang et al., 2018). Moreover, lethality, lifespan, pharyngeal pumping and body length were negatively affected by goji berry juice (de Freitas Rodrigues et al., 2021). Therefore, the fact that the methanolic extract of strawberries of the Romina variety used in the present study has exhibited an absence of toxicity is extremely interesting for using the extract in the search for biomedical applications.

Higher strawberry intake, as well as vitamin C, pelargonidin, total anthocyanidins, and total flavonoids, have been associated with reduced risk of Alzheimer's dementia in old people (Agarwal et al., 2019). Therefore, once the toxicity of the extract had been ruled out in the nematode, the next step was to elucidate the strawberry effect on a *C. elegans* model of AD. AD is a neurodegenerative disorder whose main features are the amyloid-β (Aβ) peptide aggregation and tau protein hyperphosphorylation. Amyloid cascade hypothesis posits that the deposition of β-amyloid peptide initiates a sequence of events that lead to neuronal dysfunction and dementia. That process is also related with apoptosis, metal ion imbalance, mitochondrial dysfunction and inflammation (Ataie et al., 2016). The toxicity of Aβ involves the formation of hydrogen peroxide, accumulation of ROS as well as oxidative stress (Ataie et al., 2016). Thus, oxidative stress is considered as an early and critical event on the AD pathogenesis. In this context, all the tested doses of our extract decreased the ROS content in the wild strain N2 damaged with AAPH, exhibiting levels even lower than the negative control, as shown in the classic detection test with DCFDA. Romina methanolic extract also reduced the ROS content *in vitro* in myometrial cells (Giampieri et al., 2019), HepG2 cells (Forbes-Hernández et al., 2017), and in both mature and pre-adipocytes (Forbes-Hernández et al., 2018). In the last case, oxidative damage was evaluated by TBARS and it was reduced by the treatment too (Forbes-Hernández et al., 2018). Methanolic extract from other strawberry varieties exerted properties in the same line: San Andreas strawberry reduced ROS production in the cell line RAW 264.7 damaged with LPS (Van de Velde et al., 2019) and Alba cultivar was efficient against DNA damage in peripheral blood lymphocytes of Alzheimer's disease patients (Zivković et al., 2021). Thus, according to our results, strawberry extract might display positive

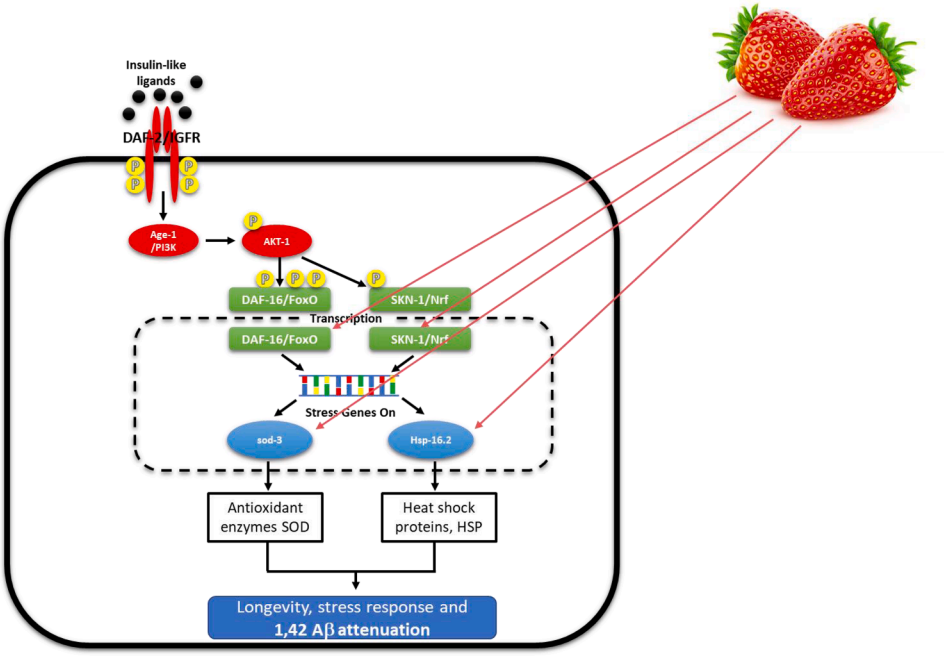

Fig. 5. Summary of the mechanisms of action of the Romina strawberry methanolic extract against the amyloid toxicity and oxidative stress *in vivo* in the experimental model *Caenorhabditis elegans*. The extract improved markers of AD, namely oxidative stress and Aβ aggregation in the nematode. Those effects were mediated, at least in part, by the two master regulators DAF-16/FOXO and SKN-1/NRF2 signaling pathways.

effects on neurodegenerative diseases by its influence on oxidative stress.

In *C. elegans,* the increase of oxidative stress precedes the Aβ accumulation in muscle cells in the transgenic strain CL4176 (Drake et al., 2003), so the effect of Romina extract on that strain was explored. To the best of our knowledge, this is the first study carried out in *C. elegans* in which the effect of a strawberry extract on the expression of amyloid peptide is investigated. The strawberry methanolic extract decreased the Aβ expression in the nematode muscle cells, delaying the paralysis phenotype and the thioflavin-T stain confirmed the reduction of Aβ aggregation. This effect was partially dose-dependent. Other berries such as cranberry have also exerted protective effects in the paralysis assay in *C. elegans* (Guo et al., 2016). Those effects could be attributed to specific compounds that are present in our extract, such as anthocyanins or gallic acid. Indeed, anthocyanin-enriched extract from some berries such as strawberry, blackberry, cranberry or raspberry inhibited Aβ fibrillation *in vitro* (Ma et al., 2018) and the mulberry extract rich in anthocyanins prevented Aβ-induced toxicity in PC12 cells (Suttisansanee et al., 2020). *In vivo*, using the *Drosophila* model of AD, treatment was able to reduce Aβ peptide formation and to improve locomotor dysfunctions by inhibiting beta-secretase 1 (BACE-1) activity (Suttisansanee et al., 2020). In the same line, gallic acid significantly reduced Aβ1-42 aggregation both *in vitro* and *in vivo* (Yu et al., 2019).

To elucidate other putative mechanisms underlying positive effects of the methanolic extract of the Romina strawberries found concerning the arrest in Aβ peptide formation in the present study, RNAi technology was used. It has been reported that insulin/insulin-like growth factor 1 (IGF-1) signaling (IIS) pathway plays an important role in regulating Aβ aggregation through the DAF-16 transcription factor. DAF-16 is the forkhead box transcription factor class O (FoxO) homolog in *C. elegans* and it acts as the canonical transcriptional target of IIS (Zečić & Braeckman, 2020). The IIS pathway is an evolutionarily conserved phosphorylation cascade that is involved in growth, metabolism and reproduction in response to nutrient availability in all eukaryotes. Furthermore, this pathway has implications in other aspects of *C. elegans* physiology such as autophagy or proteostasis, which have been linked to aging and diseases (Zečić & Braeckman, 2020). DAF-16 acts as a central regulator within a gene network in which is included SKN-1 transcription factor, the ortholog of mammalian Nrf-2 (nuclear factor erythroid 2–related factor 2) protein, which is involved in longevity and in xenobiotic and oxidative stress responses (Tullet et al., 2017). It has been described that DAF-16 promotes the expression of SKN-1 (Tullet et al., 2017) and reduced IIS results in an increase of oxidative stress and in activation of SKN-1. Among the downstream target genes are the SOD-3 and HSP16.2, involved in the antioxidant system (Zečić & Braeckman, 2020). According to that, CL4176 worms treated with the strawberry methanolic extract and exposed to 25 °C to stimulate the production of Aβ amyloid were in parallel exposed through separate experiments to RNAi clones for DAF-16, SKN-1, SOD-3 and HSP16.2. The knock-down worms for DAF-16 or SKN-1 genes exhibited a greater paralysis degree than the counterparts without RNAi for the three assessed doses, which means that those transcription factors are involved in the protective action of the strawberry extract on Aβ-induced paralysis. The downstream gene HSP16.2 was also implicated in the positive action of the extract in all the doses. However, SOD-3 inhibition only exerted an increase in the paralysis degree at the 1000 μg/mL treatment. Ellagic acid, the predominant compound quantified in our extract, have been demonstrated to exert effects on Nrf2 translocation in the hypothalamus of rodents, improving learning and memory and reducing Aβ deposition (Zhong et al., 2018). As previously mentioned, those pathways and oxidative stress, considered as a key factor in AD, are closely related. Oxidative stress was ameliorated by raspberry extract via SKN-1 in *C. elegans* (Song et al., 2020b). Other specific compounds found in our extract as kaempferol (Kampkötter et al., 2007) or mulberry anthocyanin extract (in which predominates the presence of pelargonidin-3-glucoside) (Yan et al., 2017), have been shown to activate in the nematode DAF-16, and both DAF-16 and SKN-1, respectively. In a mouse model of AD, similar results were found for anthocyanin, where treatment attenuated the increase of ROS level and oxidative stress by Aβ oligomers via Nrf2/HO-1 pathways (Ali et al., 2018).

GFP-tagged reporter strains were used for further understanding the mechanism of action of the Romina extract regardless of the paralysis phenotype. We also used them to verifying the effectiveness of different RNAi, applied to the paralysis strain, in inhibiting the specific genes. In

other words, the transgenic strain that expresses a given gene fussed with GFP, namely SOD-3, HSP16.2, SKN-1 and DAF-16, was tested in pairs with the RNAi that inhibits that gene. The knock-down worms exhibited lower fluorescence intensity than the control groups, meaning that the expression of those genes was reduced by the RNAi treatment, even in basal conditions. Therefore, it is confirmed that RNAi is affecting the matching gene. The localization of DAF-16 and the expression of SKN-1, SOD-3 and HSP-16 were evaluated through the green fluorescence related to the GFP. The treatments induced the translocation of DAF-16 from the cytosol to the nucleus and the expression of SKN-1, especially noticeable at the highest dose, which is in concordance with the results found in the paralysis assay. We also identified an upregulation of the SOD-3 expression, mitochondrial enzyme that catalyzes the conversion of superoxide radicals to hydrogen peroxide and oxygen, for all the concentrations. However, the RNAi test in CL4176 exhibited a need of that enzyme only in the highest dose, which means that the SOD-3 is only partially required for the protection against Aβ toxicity. On the other hand, HSP16.2 expression was not increased by the treatments in TJ375, which could be due to the absence of the stimuli for that fact (the oxidative stress). The inhibitions of those genes could lead to a redox imbalance with negative effects. However, the extract not only does not inhibit but also increases the expression of some of them. Those genes, particularly the master regulators DAF-16 and SKN-1, are involved, at least in part, in the protection of Romina strawberry methanolic extract from oxidative stress and participate in suppressing the Aβ toxicity.

5. Conclusions

Strawberry methanolic extract from Romina variety shows a noteworthy composition in micronutrients and phenolic compounds, highlighting the presence of minerals such as K, Mg, P or Ca and phenols as pelargonidin-3-glucoside and ellagic acid. Specially, the presence of compounds from anthocyanin family stands out. The phenolic compound content is related with the high antioxidant capacity assessed *in vitro*. The extract has no toxicity in the *in vivo* model *Caenorhabditis elegans,* demonstrated by the lethality, pharyngeal pumping, growth, reproduction and fertility tests, and survival curves. On other hand, the extract was able to improve markers of AD, namely oxidative stress and Aβ aggregation in the nematode, showing promising results. The mechanisms under the observed effects regarding Aβ accumulation and oxidation were mediated, at least in part, by the two master regulators DAF-16/FOXO and SKN-1/NRF2 signaling pathways. Fig. 5 summaries the mechanisms of action of the extract against the amyloid toxicity and oxidative stress. Overall, these results open the door for the design and testing of nutraceuticals based on strawberry extracts aimed at preventing or mitigating various aspects related to Alzheimer's disease.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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