Heliyon 11 (2025) e41898

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Black mulberry (*Morus nigra* L.) prevents deleterious effects of excess glucose in obese *C. elegans* decreasing lipofuscin accumulation and ROS production

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ARTICLE INFO

Keywords:
Traditional foods
Flavonoids
Anthocyanins
Functional foods
Obesity
Diabetes
Morus nigra

ABSTRACT

Black mulberries have been traditionally used as antidiabetic agents and are a source of nutrients and phenolic compounds, particularly anthocyanins. The objective of this work is to determine if *Morus nigra* berries could prevent metabolic and obesity-related disorders using *in vitro* systems and *in vivo* alternative models such as *C. elegans*. An aqueous solvent-free extract from *Morus nigra* fruits rich in phenolic compounds like chlorogenic acid, hyperoside, rutin and cyanidin 3-glucoside was evaluated in the *C. elegans* obese model subjected to high glucose concentrations evaluating different parameters such as lipid droplets, lipofuscin accumulation and ROS production. The capacity of the extract to inhibit advance glycation end products and free radicals as well as pancreatic lipase and α-amylase was also evaluated *in vitro*. The black mulberry extract showed a significant capacity to inhibit the accumulation of lipid droplets, reducing by 50.40 % the fat deposits. The extract was able to reverse the deleterious effects of excess glucose in *C. elegans* enhancing stress resistance, preventing the accumulation of lipofuscin, and decreasing the ROS production. The anti-glycation and antioxidant effects *in vitro* were higher than the reference substances aminoguanidine and quercetin respectively. *Morus nigra* was also able to inhibit the pancreatic enzymes α-amylase and lipase and could be considered an interesting traditional food ingredient in the prevention of certain metabolic diseases.

1. Introduction

Morus nigra L. (Moraceae) is a native Asian tree, that is also cultivated nowadays in Europe and other world regions for ornamental or nutritional uses [1]. Its fruits, also known as black mulberries, are edible and very appreciated for food applications such as the elaboration of jams or different types of drinks [2]. Each part of the plant has also been proposed for medicinal uses in traditional medicine; for example, the fruits have been used for minor vascular complaints such as hemorrhoids, the leaves as hypoglucemiant/antioxidant and the tree bark as treatment for pharyngitis or stomatitis [3–5].

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https://doi.org/10.1016/j.heliyon.2025.e41898
Received 2 August 2024; Received in revised form 9 January 2025; Accepted 9 January 2025
Available online 11 January 2025

From a phyto-nutritional point of view, the fruits of *M. nigra* are extensively consumed as edible fruits with an interesting content in phytochemicals such as anthocyanins, phenolic acids, flavonoids but also other antioxidants such as vitamin C [1,2,6–8]

Other mulberry species rich in anthocyanins have shown interesting properties in different experimental models of metabolic disorders of obesity and type-2 diabetes such as golden hamsters [9] or obese mice [10]. However, these studies correspond to other plant species known as *M. alba* and *M. australis*. The body of evidence suggesting antidiabetic and anti-obesogenic properties of black mulberry fruits is very limited. Black mulberry (*M. nigra*) fruit extracts have revealed benefits in nephropathy of diabetic animals [11] and the flavonoids of this fruit seem to be the responsible compounds for the improvement insulin resistance and plasma levels of inflammatory mediators in prediabetic mice. A clinical trial conducted in 100 volunteers has also demonstrated that an hydroalcoholic extract from black mulberries may reduce blood glucose and HbA1c% in the diabetic patients due to the inhibition of α-glucosidase as one of the main mechanisms [12].

Obesity, type-2 diabetes and metabolic disorders are some of the main causes of mortality in developed countries. According to the WHO, approximately 422 million people worldwide have diabetes, type-2 diabetes being the most frequent phenotype [13]. Non-pharmacological treatments like adequate dietary habits, a healthy lifestyle and regular physical activity are the first line treatment for the prevention and treatment of these disorders and their associated complications. Balanced nutrition in terms of macronutrients is essential for the management of metabolic disorders but certain non-nutritive agents like polyphenols have demonstrated beneficial effects in the prevention of non-communicable diseases. A great body of evidence suggests that polyphenols, and in particular anthocyanins, may have an impact on glucose metabolism and therefore they may act as antidiabetic agents with other biological effects acting as pleiotropic agents [14]. Anthocyanins are pigment related flavonoids responsible for the red-violet colors present in many fruits and foods such as berries, plums, pomegranates, or red wines. They contain a flavylium skeleton with various substitutions whose stability and colour is pH dependent. The use of anthocyanins as pigments and colorants in the food industry is very extended but they may also be responsible for the benefits traditionally attributed to raspberries, strawberries, cranberries, and other berry-like fruits.

Black mulberries could be considered a nutraceutical or functional food due to their content in anthocyanins and other phenolic compounds with antioxidant properties. Mustafa and collaborators (2022) have recently optimized a method for the obtention of bioactive polyphenols from *Morus nigra* berries using solvent-free microwave-assisted hydrodiffusion and gravity extraction [15]. The optimized extract resulted to be rich in polyphenols, being the most abundant cyanidin-3-glucoside (6016.72 ± 3.1 mg/kg dry extract) and chlorogenic acid (565.6 ± 1.7 mg/kg dry extract). The objective of this manuscript is to explore the protective effects of a polyphenolic extract from black mulberries on *C. elegans* exposed to a high glucose diet as well as the capacity of this extract to inhibit key enzymes in relation to metabolic disorders such as pancreatic lipase and α-amylase.

2. Materials and methods

2.1. Reagents

Fructose, Nile Red, aminoguanidine bicarbonate (AMG), 2′,7′-Dichlorofluorescein diacetate (DCFH-DA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), pancreatic lipase, α-amylase, starch and p-4-nitrophenyl butyrate (NPB) were from Sigma-Aldrich (St. Louis, MO, USA), whereas bovine serum albumin (BSA) from Santa Cruz Biotechnology, Inc. (Barcelona, Spain). Quercetin was purchased from Fluorochem (Barcelona, Spain). Orlistat was purchased from TCI EUROPE (Zwijndrecht. Belgium).

2.2. Black mulberry processing by microwave hydrodifussion and gravity extraction techniques and chemical composition in bioactive compounds

Black mulberries (*Morus nigra* L.) were acquired from trees in Camerino (Italy) and Prof. Maggi was the scientist responsible for botanical identification and certification. Samples were kept in a freezer at −18 °C until extraction. Extraction of polyphenols and sugars was performed using an advanced microwave solvent-free technique named Microwave Hydrodiffusion and Gravity (MHG) process after its optimization and validation previously published by the authors; briefly, 500 g of fresh black mulberries were extracted in a Pyrex reactor using microwaves and the water from the samples; the crude extract was condensed and cooled at 8 °C outside the microwave and the sample was finally freeze-dried [15].

Phytochemical profile was determined through a HPLC-ESI-MS/MS and published [16]. 36 bioactive compounds were monitored and quantified, resulting in a variety of phenolic acids, anthocyanins, flavanols and flavan-3-ols, being the most abundant molecules identified: cyanidin 3-glucoside (6016.72 ± 3.1 mg/kg dry extract), chlorogenic acid (565.6 ± 1.7 mg/kg dry extract), rutin (222.9 ± 1.2 mg/kg dry extract), hyperoside (207.67 ± 0.6 mg/kg dry extract), pelargonidin 3-glucoside (108.81 ± 4.5 mg/kg dry extract) and and isoquercitrin (94.4 ± 2.1 mg/kg dry extract) [15].

2.3. Caenorhabditis elegans assays

2.3.1. Strains and maintenance conditions

Caenorhabditis elegans strains N2, Bristol (wild type) and SS104 (*glp-4 (bn2)*) were provided by the Caenorhabditis Genetics Center (CGC, University of Minnesota, Minneapolis, MN, USA) and growth on NGM medium at 20 °C with *Escherichia coli* OP50. For all experiments, synchronized worms were obtained by an alkali-bleaching method [17].

2.3.2. Analysis of lipid droplet

N2 worms exposed to 5 % glucose in NGM were used as an *in vivo* alternative model of obesity. *M. nigra* extract was assayed in the obese worms at 500 and 250 μg/mL using also plates with worms exposed only to 5 % glucose and plates without adding glucose as control. Orlistat was used as negative control at 6 μg/mL plus 5 % glucose [18].

Nile Red staining and the obtained value of fluorescence intensity per area of *C. elegans* was used for the quantification of fat storages. 300 nematodes at the L1 stage were grown for 48 h at 20 °C until they reached the L4 stage under the different conditions. Nile Red staining images were analyzed according to a previously described method [19]. 30–40 worms per condition were captured and photographed at 100 × magnification and 20 s of exposure time. Images were analyzed using ImageJ.

2.3.3. Body length measurements

To assess the impact of exposure to excess glucose, the body length of L4 larvae was measured analyzing the same images taken for the quantification of lipid [20].

2.3.4. Lipofuscin accumulation

C. elegans SS104 strain is temperature-sensitive sterile at 25 °C, it was used to analyze the accumulation of lipofuscin in the worms [21,22]. After alkali bleaching synchronization, eggs were exposed to the same treatment conditions as previous assays. Worms were raised at 15 °C till L4 stage, due to temperature sensitivity of mutant phenotypes, and once this phase was reached, animals were transferred into a 25 °C environment.

Worms (20–40 individuals) were photographed at days 7 and 10 using a DAPI filter. Images were analyzed using ImageJ.

2.3.5. Evaluation of reactive oxygen species (ROS) production

To investigate whether the exposure to *M. nigra* extract enhanced stress resistance in the obese model, ROS levels production after exposure to the treatments and non-lethal stress induced by heat were measured. *C. elegans* SS104 strain was raised under the same conditions as lipofuscin assay, and ROS production was quantified at days 7 and 10 after exposure to treatments [23].

Worms were transferred to black 96-well with transparent bottom plates containing 75 μL of PBS and exposed to thermal sub-lethal stress (35 °C) for 2 h. After, 25 μL of DCFH-DA 150 μM solution in ethanol was added and fluorescence from each well was measured every 10 min for 90, using 485 excitation and 535 nm emission wavelengths using the Synergy H1 Hybrid Multi-Mode Reader (Winooski, VT, USA). Three independent experiments were performed per treatment, with at least 15 individual worms.

2.4. Multitarget in vitro mechanistic effects of black mulberries in relation to preventive deleterious effects of glucose

2.4.1. Advanced glycation end products (AGEs) inhibition and antiradical activity

AGEs formation was measured by fluorescence in 96 black well-plates [24]. 50 μL of BSA solution (10 mg/mL), 80 μL of 0.1 M phosphate buffer (containing sodium azide 3 mM and pH = 7.4), 50 μL of fructose solution (0.5 M), and 20 μL of sample extract (serial dilutions in buffer) were mixed. After incubating at 37 °C for 24 h, plates were analyzed (excitation wavelength = 355 nm; emission wavelength of 460 nm). The inhibition of AGEs formation was calculated using Equation (1) (Eq. (1)). Aminoguanidine (AMG) was the positive control [25].

Equation (1):

$$Inhibition\ (\%) = \left[\frac{(ABS\ control - ABS\ sample)}{ABS\ control}\right] x\ 100$$

Scavenging of free radicals was evaluated using the 2,2-diphenyl-1-picryl-hydrazil (DPPH) method as previously described and compared to quercetin, used as positive control substance [26]. The % of DPPH radicals inhibition was calculated using Eq. (1).

2.4.2. Inhibition of pancreatic α-amylase and α-lipase

The inhibition of the two pancreatic enzymes lipase and α-amylase was spectrophotometrically quantified using previous protocols [26,27]. *M. nigra* capacity to inhibit lipase was measured in 96 well plates. Briefly, 40 μL of pancreatic lipase (2.5 mg/mL in 0.1 M phosphate buffer, pH 7.0), previously centrifugated at 2000 g for 7 min were mixed with extract (40 μL) and 20 μL of 10 mM p-nitrophenyl butyrate (p-NPB). Absorbance was recorded (after 10 min at 37 °C) at 405 nm using orlistat as reference substance.

For the α-amylase inhibition assay, reagents were dissolved in buffer (20 mM sodium phosphate with 6 mM sodium chloride, pH 6.9). 100 μL of extract sample and 100 μL of porcine pancreatic α-amylase (2 mg/mL centrifugated) were incubated in microtubes at 37 °C for 5 min 100 μL of 1 % starch solution were added and incubated at 37 °C for 10 min. The reaction was stopped with 200 μL of 1 % 3,5-dinitrosalicyclic acid (DNS) color reagent and 50 μL of NaOH 1M, microtubes were furtherly incubated for 5 min at 100 °C. Once samples had cooled in a cold-water bath, 200 μL were transferred from each microtube to a 96-well microplate. Absorbance was measured at 540 nm. Blanks for each sample without enzyme were performed, and absorbances were compared to the control substance gallic acid. Inhibition for both assays was calculated applying Eq. (1).

2.5. Statistical analysis

All experiments were performed in triplicates. *C. elegans* assays were perfumed as 3 independent experiments in different days and

weeks whereas *in vitro* tests were performed in different days. Results are shown as means ± standard error and GraphPad Prism v. 8 was used for statistical analysis. Lipid droplets and lipofuscin accumulation were quantified using the image processing program ImageJ. Differences with $p \leq 0.05$ were considered statistically significant.

3. Results

3.1. Effects in the obese C. elegans model

3.1.1. Lipid droplet analysis and quantification

The fruit extract of *M. nigra* reduced the fat content in *C. elegans* in a dose-dependent manner. Obese condition worms were exposed to an excess of glucose significantly increasing the lipid content compared to untreated control worms (NGM) as we can observe in Fig. 1. Orlistat was used as anti-obesity reference drug, and the reduction it caused in the lipid content was considered the maximum effect (100 % reduction). The *M. nigra* extract reduced the lipid content by 50.40 % at the highest concentration 500 μg/mL, and by 32.13 % at concentration 250 μg/mL.

To further asses the lipid content reduction, lipid droplet size was studied on every condition [28]. It was expressed as a ratio between droplet size per worm area to relativize the data obtained. The lowest ratio was obtained by untreated worms NGM (0.706 ± 0.037), followed without significant differences by orlistat (0.776 ± 0.038). The medium values for the lipid droplet ratio were obtained by the *Morus* extract without significant differences between the two doses tested (1.426 ± 0.074 at 250 μg/mL and 1.269 ± 0.054 at 500 μg/mL). The highest ratio was obtained by the glucose condition (1.874 ± 0.108).

3.1.2. Body length measurements in C. elegans

The size of the worms was measured after exposure to the different treatments for 48 h (Fig. 2). The excess of glucose in the medium avoided the normal growth and development of *C. elegans*, resulting in lower lengths such as 315.17 ± 4.46 and 320.3 ± 4.31 μm for glucose and orlistat conditions respectively compared to control (442.3 ± 4.36 μm). But those obese worms treated with the *M. nigra* extract, partly reverted that effect ($p < 0.001$) increasing the body length by 13.01 % at 250 μg/mL and by 15.74 % at concentration 500 μg/mL; meaning length values of 356.1 ± 6.02 and 364.8 ± 6.63 μm respectively.

3.1.3. Lipofuscin accumulation

Lipofuscin accumulates naturally within the cells in living organisms as they age, often being used as an aging indicator. SS104 *C. elegans* strain was used for this assay since they become sterile after maintenance at 25 °C. The fluorescence accumulation of lipofuscin per worm area at days 7 and 10 of treatment exposure can be observed in Fig. 3. At day 7, excess glucose shows higher accumulation values than the rest of the conditions ($p < 0.05$), and this tendency remains at day 10. Regarding the dye accumulation increase between both days, untreated worms show the normal rate of accumulation, increasing through the days but without significant differences. The obese conditions excess glucose and orlistat obtained the highest and significant increases ($p < 0.05$),

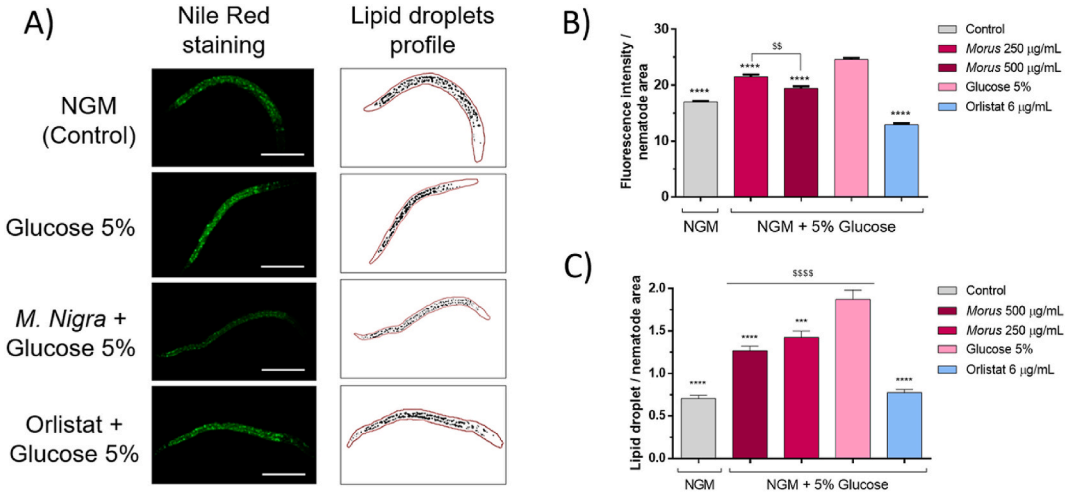

Fig. 1. Fluorescence images, lipid droplet profile and total lipid droplet quantification of *C. elegans* N2 strain. A) Fluorescence images of *C. elegans* taken after exposing the worms to the different conditions and after staining with Nile Red and exposure to ultraviolet light; on the right, lipid droplets are highlighted. For the extract condition, only images at concentration 500 μg/mL are shown. Scale bar = 150 μm. B) Histogram showing the relative values of lipids in *C. elegans* obese model after being exposed to the different conditions and concentrations of *M. nigra* fruit extract (n = 75–100 worms). Results are expressed as mean ± SEM. ****$p < 0.0001$ vs Glucose, $^{\$\$}p < 0.005$. C) Lipid droplet average size per worm area ratio of *C. elegans* obese model after being exposed to the different conditions and concentrations of *M. nigra* fruit extract (n = 75–100 worms). Results are expressed as mean ± SEM. ***$p < 0.001$, ****$p < 0.0001$ vs Glucose; $^{\$\$\$}p < 0.0001$ vs Control.

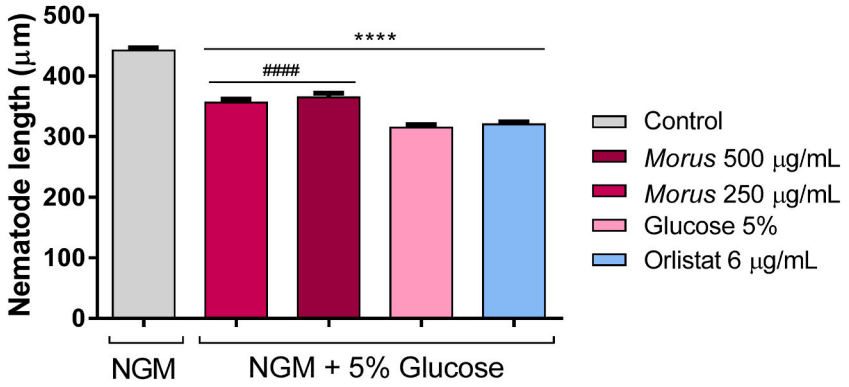

Fig. 2. *C. elegans* N2 strain average length in µm after exposure to different conditions for 48 h. Results are presented as mean ± SEM ($n = 75–100$ worms). $^{****}p < 0.0001$ vs Control, $^{\#\#\#}p < 0.001$ vs Glucose.

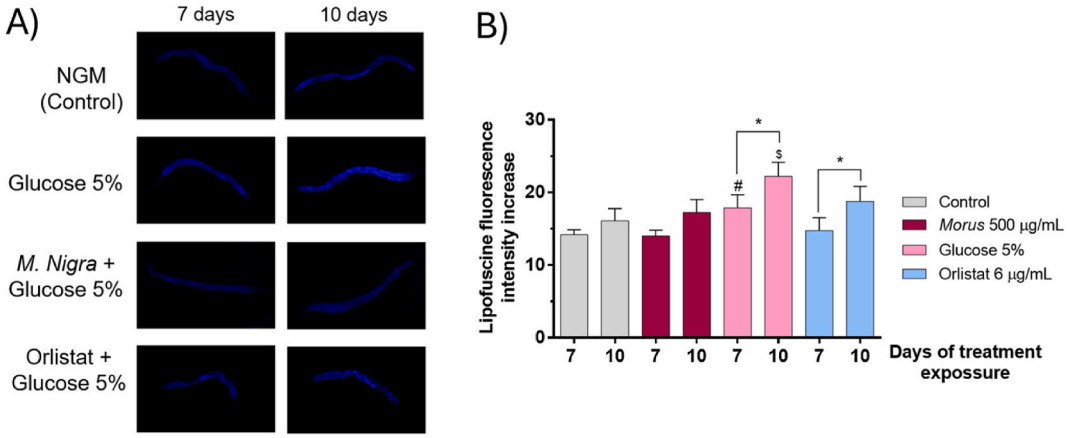

Fig. 3. Accumulation of age pigment lipofuscin in *C. elegans* SS104 strain. A) Images of *C. elegans* autofluorescence age pigment lipofuscin taken at day 7 and day 10 of the worms being exposed to different conditions. B) Histogram with relative values of the fluorescence age pigment lipofuscin accumulation at days 7 and 10 of *C. elegans* being exposed to different conditions. Results are represented as mean ± SEM. $^{*}p < 0.05$, $^{\#}p < 0.05$ vs control day 7, $^{\$}p < 0.05$ vs control day 10.

meanwhile the obese worms treated with *M. nigra* extract at concentration 500 µg/mL behaved similarly to control, increasing the dye accumulation moderately and without significant differences between both days.

3.1.4. Evaluation of reactive oxygen species (ROS) production

Intracellular ROS production was measured for 90 min. As observed in Fig. 4, *C. elegans* SS104 exposed to an excess of glucose produced high levels of ROS compared to control at day 7 and even more so at day 10. But the treatment with *M. nigra* fruit extract at 500 µg/mL partially reversed that effect since the ROS production in this condition showed no differences to the control at day 7 nor at day 10, therefore significantly reducing the production of ROS compared to the glucose condition.

3.2. Multitarget mechanistic effects of black mulberries

3.2.1. In vitro advanced glycation end products (AGEs) inhibition and antiradical activity

Morus nigra MGH extract had the ability to prevent the non-enzymatic formation of advanced glycation end products (AGEs) *in vitro* (Fig. 5A). The calculated IC_{50} for the extract was 8.56 ± 0.53 µg/mL, significantly lower than the control substance used aminoguanidine (AMG) that obtained a value of 73.88 ± 7.71 µg/mL.

Regarding the antiradical activity, *M. nigra* extract inhibited free radicals (Fig. 5B) with greater power than the control substance quercetin, obtaining IC_{50} values of 0.099 ± 0.003 µg/mL for *M. nigra* and 1.67 ± 0.06 µg/mL for quercetin.

3.2.2. Inhibition of pancreatic lipase and α-amylase

As shown in Fig. 6A, black mulberries extract exerted an inhibition of pancreatic lipase in a dose-dependent way obtaining a IC_{50} value of 1.13 ± 0.23 mg/mL, but the activity was not comparable to the reference drug orlistat, which was capable to induce the 100 %

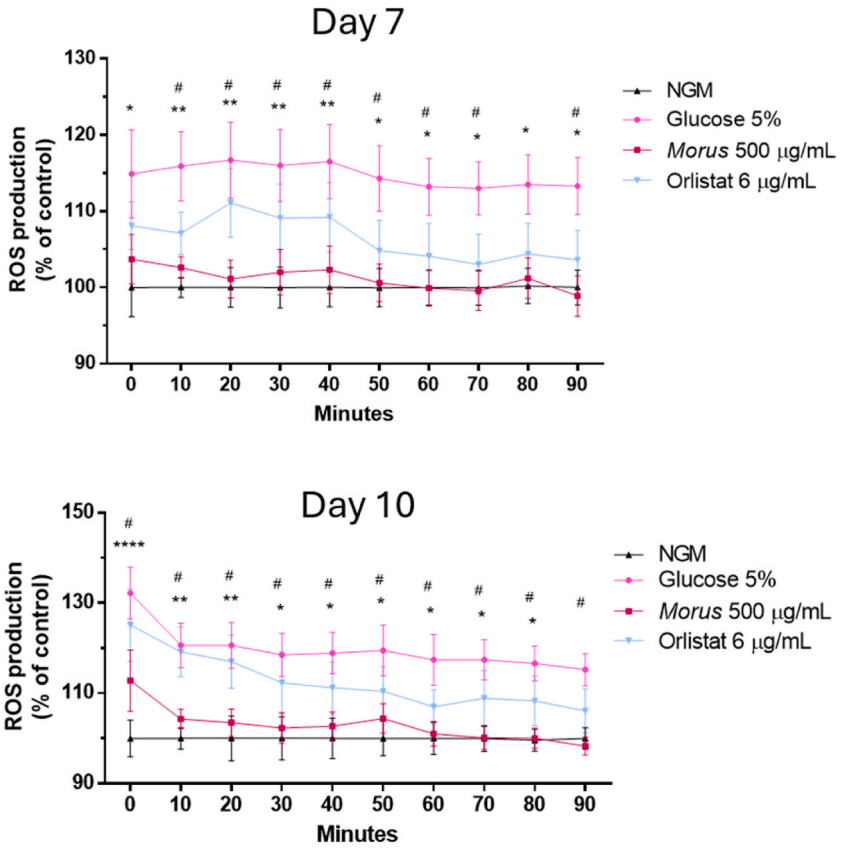

Fig. 4. Production of ROS in *C. elegans* SS104 strain after exposure to the different conditions for 7 and 10 days, and exposition to thermal stress (2h at 35 °C). Data are expressed as % of fluorescence in relation to control untreated worms (NGM). Results are presented as mean values ± SEM (n = 3). $^{****}p < 0.0001$, $^{**}p < 0.01$, $^{*}p < 0.05$ Glucose vs NGM; $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ Glucose vs *Morus* 500 µg/mL. Statistical significance was calculated using two-way analysis of variance ANOVA.

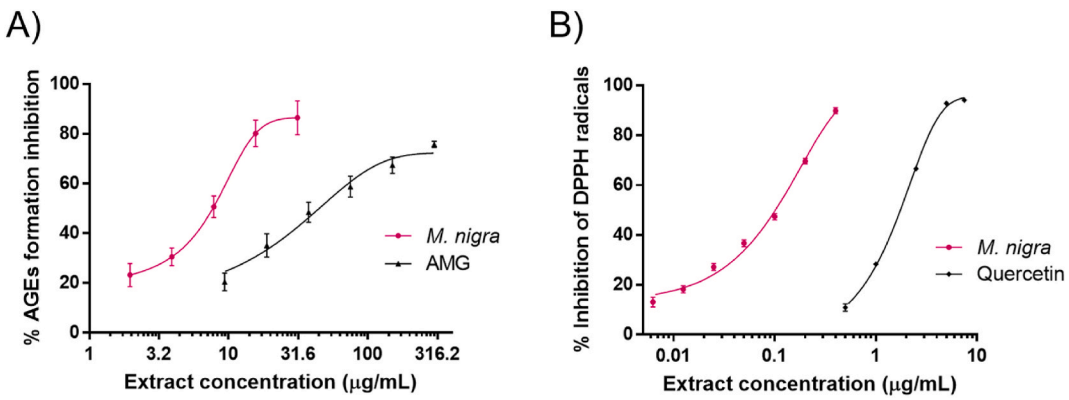

Fig. 5. A) Inhibition of AGEs by the *M. nigra* extract, aminoguanidine (AMG) used as control. B) Inhibition of free radicals by the *M. nigra* extract and quercetin used as control. Data are presented as mean ± SEM. (n = 3).

inhibition at lower concentrations ($IC_{50} = 0.028 \pm 0.02$ mg/mL). The fruit extract was also able to inhibit amylase in a dose-dependent manner, obtaining an IC_{50} value of 2.24 ± 0.13 mg/mL; meanwhile gallic acid, the reference substance used, showed higher inhibitory activity ($IC_{50} = 0.91 \pm 0.01$ mg/mL).

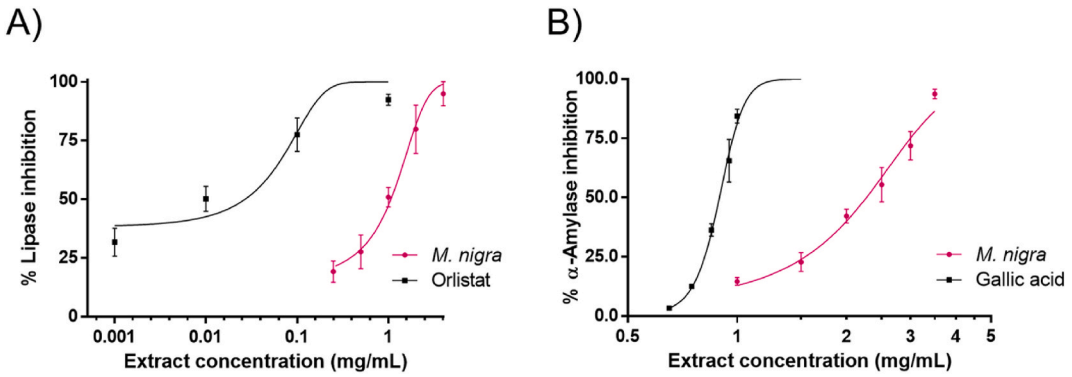

Fig. 6. A) Inhibition of pancreatic lipase by *Morus nigra* fruits, orlistat used as control. B) Inhibition of amylase by *Morus nigra* fruits, gallic acid used as control. Data are presented as mean ± SEM. (n = 3).

4. Discussion

Changes in the food industry together with increased sedentary habits seem to be responsible for the high prevalence of obesity and related disorders [29]. As hypertension, type-2 diabetes, cardiovascular disease, or some types of cancer, obesity belongs to the group of non-communicable diseases (NCDs) involved in diminishing both life expectancy and quality of life. In addition to being a risk factor in frequent pathologies, is a chronic and complex disease whose approach requires the combination of multiple strategies for its prevention and treatment. Obesity is a chronic body's energy imbalance, for this reason a healthy diet along with adequate physical activity is considered the main strategy for its control. Despite the availability of some pharmacological treatments, their limitations and adverse effects have led to the search for alternative and/or adjuvant strategies. Nutraceuticals, functional foods, and bioactive compounds derived from natural sources have been explored as potential alternatives or adjuncts to traditional pharmacological interventions in the management and prevention of metabolic disorders [30]. In the last decade, numerous studies suggest that phenolic compounds have promising potential in managing diabetes and obesity through multiple mechanisms, including regulation of carbohydrate metabolism, glucose uptake, protection of pancreatic β-cells, and enhancement of insulin action [31]. Berries, particularly the popular blueberreies, strawberries or blackberries, are considered healthy fruits for their abundance of diverse phenolic compounds and their wide spectrum of biological activities [32]. Black mulberry fruits are a rich source of ascorbic acid, phenolics and flavonoids, including anthocyanins which could potentially yield positive impacts on health. Recently, Mustafa and collaborators obtained a high-quality extract from *M. nigra* blackberries, enriched in polyphenols free of chemical solvents and biologically active by Microwave hydrodiffusion and gravity (MHG) extraction. This extract showed marked antioxidant and anti-obesity activity *in-vitro* related to its high content of bioactive compounds, especially anthocyanins and phenolic acids. A great body of evidence suggests that anthocyanins exert antidiabetic activity and potential benefits in the field of metabolic disorders [32,33]. *Morus nigra* fruits could be useful in the management of metabolic diseases. Here, it is shown for the first time the biological activity of *Morus nigra* optimized fruit extract in relation with metabolic disorders experimental models.

Caenorhabditis elegans, a small free nematode, offers unique advantages as a model organism for the evaluation of functional foods, nutraceuticals, and bioactive compounds. Its genetic simplicity, short lifespan, and shared biological pathways with humans make it an ideal candidate for natural product bioactivity screening studies. In the field of metabolic disorders and due to the preserved pathways of energy homeostasis, *C. elegans* has become a great model for exploring lipid metabolism [34,35]. This study examines, for the first time, the anti-obesogenic properties of *Morus nigra* in *C. elegans*, as well as its potential to mitigate the harmful impacts of excessive glucose intake.

Morus nigra extract reduced the lipid storages in nematodes in a dose dependent manner as shown in Fig. 1. The inclusion in the diet of the extract rich in polyphenols reduced fat deposits in obese worms by 50.4 % for 500 μg/mL treatment. Our results are in line with those obtained by Yan and collaborators for ethanolic *Morus alba* fruit [36]. This extract, whose main component was also cyanidin-3-glucoside, reduced the accumulation of triglycerides in *C. elegans* subjected to high glucose concentrations. A decrease in fat deposits was corroborated by the modification of the lipid droplet profile that can be observed in Fig. 1A and C. The lipid droplet size per worm's area ratio showed that glucose obese condition had the biggest value, meaning aggregation of fat to form big lipid droplets. Conversely worms in the control and orlistat groups exhibited a similar lipid profile, characterized by smaller droplet size, and reduced aggregation. Similarly, black mulberry extract reduced the size and aggregation of lipid droplets by 32.3 % and 23.9 % for 500 and 250 μg/mL concentrations, respectively. Lipid droplets play a crucial role in fat storage thus modifications in abundance, size or distribution can be connected to whole-animal energy homeostasis, behaviour, and life span [37]. Consistent with earlier studies, the obtained results show that lipid reserves and their profile can be influenced by phenolic compounds that could contribute to energy homeostasis [22,38,39].

Glucose excess ingestion can entail detrimental and toxic effects. *C. elegans* exposure to high levels of glucose triggers the formation of reactive oxygen species (ROS), shortens the worm's lifespan, induces apoptosis, mitochondrial dysfunction, and heightened fat accumulation [40,41]. Our results show that black mulberry extract, in addition to reducing fat deposits, reduces and even reverses the

deleterious effects of excess glucose in this model. As shown in Fig. 2, glucose excess reduced worm's size. This detrimental impact on worm development is well documented. High glucose has been demonstrated to be a key factor in leading to developmental delays and aberrations in morphogenesis as well as reproductive changes by disrupting germ cell development and fertility [42]. Our findings showed that while orlistat had no impact on worm size, *M. nigra* significantly increased the nematode body length suggesting that the extract may mitigate the adverse effects of glucose and obesity on development. The same effect took place over the worm's lipofuscin accumulation levels over time (Fig. 3A and B). The exposure to excess glucose translated into a higher increase in lipofuscin accumulation, but *M. nigra* extract limited that increase. Lipofuscin is a fluorescent pigment generated by the degradation of lipids, proteins and cellular sugars that accumulates inside the cell and is used as a biomarker in the study of aging [43]. The observed reduction in lipofuscin levels implies that blackberries have a beneficial impact on the aging process. The high content of phenolic compounds, especially anthocyanins like cyanidin-3-glucoside and phenolic acids like chlorogenic acid together with its antioxidant activity (Fig. 5B) could be responsible for the observed effects. Thus, as can be seen in Fig. 4, *M. nigra* extract enhanced stress resistance in the obese model after being exposed to sub-lethal thermal stress. In *C. elegans*, thermal stress increases the production of ROS that must be counteracted by antioxidant systems to maintain redox homeostasis. Recent studies have shown that excessive glucose intake disrupts insulin-signalling pathways and downregulates the expression of antioxidant enzymes, such as superoxide dismutase and catalase, exacerbating oxidative stress and compromising the ability to neutralize its adverse effects [44,45]. Yan et al. (2017) showed that an anthocyanin's rich extract of *M. alba* fruit restored superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity in *C. elegans* under hyperglycaemic conditions [46]. Likewise, our results show that *M. nigra* extract significantly reduced total ROS levels to similar values observed in the control group suggesting that the extract could reverse the deleterious effects of glucose on nematode antioxidant systems. The response to oxidative stress due to the extracts could be attributed to cyanidin-3-glucoside, chlorogenic acid and rutin which are abundant in the extract and increased the stress resistance of the nematode [47,48]. In addition, other phenolic compounds present in lower concentration, especially some anthocyanins such as pelargonidin-3-glucoside [49] and flavonoids such as quercetin [50] have been shown to reduce oxidative damage to biomolecules.

Since obesity in *C. elegans* leads to increased levels of oxidative stress markers, recent studies have attempted to elucidate the intricate interaction between natural antioxidants and obesity in this model. There has been reports of polyphenols reducing fat content in *C. elegans* through several biological processes and signalling pathways, including those related to lipid mobilization and fatty acid metabolism, oxidative stress, aging and food intake [51]. One of the main pathways in which polyphenols improve antioxidant defences in *C. elegans* is through daf-16 a gen which mediates the insulin/insulin-like growth factor signalling pathway (IIS). As previously tested, the IIS pathway can be targeted to reduce fat accumulation [52,53]. Fat accumulation in *C. elegans* can be reduced by polyphenols through a series of mechanisms involving the regulation of over 500 genes [54].

Additionally, AGEs production has been related to many metabolic disorders, specially to diabetes, obesity and even to cardiovascular diseases [55,56]. AGEs promote oxidative stress and inflammation processes, key factors for a better understanding of diabetes and obesity complications such as insulin resistance or vascular complications [57,58]. The discovery of AGEs inhibitors from natural origin would offer a potential therapeutic approach for the prevention of diabetes. Thus, in recent years the ability of many polyphenolic compounds to prevent the formation of AGES has been demonstrated in different experimental models. Aminoguanidine, a synthetic inhibitor, interferes with the cross-linked structure and diminishes the production of AGEs and is usually used as a positive control to illustrate the effects of natural products to inhibit AGEs [59]. Our results show important antiglycan properties of *Morus nigra* fruit extract, achieving a IC_{50} more than eight times lower (8.56 $\pm$ 0.53 μg/mL) than aminoguanidine (73.88 $\pm$ 7.71 μg/mL). Similarly, a stronger antiglycation effect was recently obtained by Zahn and collaborators for berries of *Morus alba* [60]. The high chlorogenic acid, cyanidin-3-glucoside and rutin content of our extract could be responsible for this activity previously demonstrated by other authors [61–63].

Pancreatic lipase and α-amylase are digestive enzymes involved in the correct digestion and absorption of nutrients such as fatty acids and carbohydrates. Previous works on mulberries have reported an inhibition on pancreatic lipase but with a higher IC_{50} value and therefore lower potency [64]. The inhibition of α-amylase has been studied with extracts obtained from *M. nigra* leaves [65,66]. According to recent studies, anthocyanins seem to be responsible for the bioactive effects of black mulberries, particularly in relation with antioxidant and enzyme inhibitory properties [67].

This work has shown that the black mulberry polyphenolic extract has beneficial properties in a *C. elegans* obese model by reducing lipid levels in the worms and aging markers such as lipofuscin and ROS production. These results suggest an interesting nutraceutical application of black mulberries and the value of bioactive dietary ingredients in the prevention of metabolic diseases.

CRediT authorship contribution statement

Sonia Núñez: Writing – original draft, Methodology, Investigation, Formal analysis. **Adrián Millán-Laleona:** Methodology, Investigation. **Javier Cano-Lou:** Methodology. **Andrea Corella:** Methodology. **Cristina Moliner:** Methodology. **Guillermo Cásedas:** Methodology. **Filippo Maggi:** Methodology. **Víctor López:** Writing – review & editing, Supervision, Funding acquisition. **Carlota Gómez-Rincón:** Writing – review & editing, Supervision, Conceptualization.

Data and code availability statement

Data is available from the authors on request.

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.

Acknowledgements

Sonia Nuñez and Adrián Millán-Laleona are thanked to Universidad San Jorge, Banco Sabadell and Industrias Químicas del Ebro for the PhD grants. Javier Cano-Lou is thanked to Teva for the PhD grant and Andrea Corella for Programa Investigo by Gobierno de Aragón (subsidies for hiring researchers). Phyto-Pharm group is grateful for the financial support received by Gobierno de Aragón (ref. B44_23R) in the call for the period 2023–2025. Universidad San Jorge is also thanked for providing financial support to the Phyto-Pharm group through Proyecto Interno 2324022 and 232426.

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