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Supplementation with *Thymbra spicata* extract ameliorates lifespan, body-weight gain and Paraquat-induced oxidative stress in *Drosophila melanogaster*: An age- and sex-related study

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ABSTRACT

Aging represents a decline in function over time. Polyphenols are dietary antioxidants that could exert beneficial effects on aging. This study investigated the effects of dietary supplementation with two extracts from *Thymbra spicata* L. aerial parts on the lifespan, body-weight control, and antioxidant responses of *Drosophila melanogaster* cohorts distinguished for age and sex. The aqueous extract extended the lifespan of young female flies, and this pro-longevity potential was associated with a reduction in the body-weight gain. Furthermore, the aqueous extract promoted the antioxidant defense when young females were exposed to Paraquat by reducing oxidative stress and mortality. This study provides the first insight into the beneficial effects of *T. spicata* supplementation in an animal model, suggesting that the healthy effect could mainly depend on strengthening the antioxidant defenses which reflect on the lifespan. Further investigations will better clarify the mechanisms and potential of *T. spicata* as nutraceutics in anti-aging strategies.

1. Introduction

Aging is a natural physiological process resulting from an imbalance between stressors and stress buffering mechanisms bringing on accumulation of unrepaired damages (Bektas et al., 2018). The oxidative stress is one of the factors involved in aging processes, and it is mainly derived from an excess of free radicals and oxidants such as the reactive oxygen species (ROS) leading to an imbalance of cell homeostasis (Finkel & Holbrook, 2000). Oxidative stress triggers inflammation processes through the activation of a variety of genes implicated in many pathways (Hussain et al., 2016). On the other hand, a ROS overproduction induces a network of defense responses including the expression of genes coding for antioxidant enzymes such as heme oxygenase (HO) and thioredoxin reductase (TrxR) (Fernandes & Holmgren, 2004; Stocker, 1990), pro- and anti-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and other interleukins (Eleftherianos & Castillo, 2012). An impair in the body weight control seems to be strictly associated to a reduction in lifespan also through triggering the risk of many metabolic disorders (Salvestrini et al., 2019).

Food-based antioxidants are known to boost the antioxidant defense thus they might increase the longevity (Le Bourg, 2001; Luo et al., 2021). Dietary polyphenols are plant-derived bioactive compounds that have emerged as promising anti-aging candidates. In this context, *Thymbra spicata* L., a medicinal plant member of *Lamiaceae* family, has gained popularity for its health-promoting properties that are held accountable for potentially affecting the longevity of the individual. *T. spicata* is traditionally used in flavoring a variety of food either as a single plant e.g. herbal tea and salad, or in a combination of a group of blended plants and spices making the most well-known Lebanese herbal mixture; Za'atar (Khalil et al., 2022). Evidences from cellular and animal studies have highlighted that *T. spicata* possesses potent antioxidant and anti-inflammatory activities that have been described in models of endothelial dysfunction and fatty-liver disease (Akkol et al., 2009; Avcı et al., 2006; Khalil et al., 2019). In particular, a previous study of our

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group showed that the two polyphenol-enriched extracts from *T. spicata* (aqueous-TW and ethanolic-TE) were able to decrease both the lipid accumulation in steatotic hepatocytes, and the release of nitric oxide in dysfunctional endothelial cells (Khalil et al., 2019). Based on these reports, we could hypothesize that *T. spicata* might act also on mechanisms being associated with age-related cellular and molecular dysfunctions.

Drosophila melanogaster, commonly known as "fruit fly" has served as an excellent model for many diseases including obesity, diabetes, and aging (Gáliková & Klepsatel, 2018; He & Jasper, 2014). Indeed, the flies share with humans a panel of highly conserved genes and as a result a large number of key metabolic pathways (Jafari, 2010). Particularly, oxidative stress and antioxidant defense responses are rather conserved from Drosophila to mammals. Moreover, *Drosophila* model is quite relevant for the ability to be genetically manipulated, multiple life stages, and a short lifespan, knowing that lifespan analysis is a rate-limiting step (Yi et al., 2021). The use of *Drosophila* as an *in vivo* model to study the potential health-promoting effects of food-derived polyphenols has been widely noticed (Beghelli et al., 2022; Lee & Min, 2019; Peng et al., 2011).

In the present study, starting from previous *in vitro* studies of our group we translated the investigation to an *in vivo* model, the *Drosophila melanogaster* fly, in an attempt to deepen our knowledge on the molecular pathways sustaining the beneficial effects of dietary supplementation with *T. spicata*. Therefore, both young (1-week old) and early-adult (3-weeks old) flies, being separated by sex, were fed with a diet supplemented with *T. spicata* extracts to simulate the effects of either chronic dietary intake since childhood or nutraceutical effect in adulthood. Using the fly model, we could confirm *in vivo* the beneficial properties of dietary intake of *T. spicata,* considering both the effect of age and sex.

2. Materials and methods

2.1. Chemicals

All chemicals, unless otherwise indicated, were supplied by Sigma-Aldrich Corp (Milan, Italy).

2.2. Plant collection and extraction

The fresh Arieal parts of *Thymbra spicata* were collected from flowering plants growing wild in "*Maarakeh*"- South Lebanon, 280 m above sea level (33° 16′35.59″N and 35° 19′02.89″). After authentication for taxonomic identity by Dr. G. Tohme, President of the National Council for Scientific Research (CNRS) of Lebanon, a voucher specimen was deposited in the Herbarium of the Faculty of Sciences at the Lebanese University, Hadath-Beirut, Lebanon (voucher number L1.125/1).

The leaves were chopped, and shade dried at room temperature for 3 weeks and then grounded to coarse powder to ease the extraction. The plant materials were extracted with two different solvents following a well-standardized procedure (Lupidi et al., 2011). Two different extracts were prepared using either ethanol (100 %) or distilled water as solvent as previously described (Khalil et al., 2019). Both extracts were freeze-dried in Alpha 1–4 LD plus lyophilizer (CHRIST, Osterode am Harz, Germany) and stored at 4 °C until use. Table 1 resumes the major polyphenols identified in the two extracts. The total phenol content (TPC) was assessed previously for both extracts by spectrophotometric assays showing that the TPC of ethanolic extract is higher than to that of aqueous extract (Khalil et al., 2019).

2.3. Fly strains, husbandry, and rearing

The *Drosophila melanogaster* Canton-S wild-type strain was kindly provided by Prof. Daniela Grifoni (University of L'Aquila, Italy). Fly eggs and larvae were maintained at constant temperature (27 °C) and humidity (60 %) under 12/12 h light–dark on a standard Formula 4–24 ® media (Carolina Biological, Burlington, NC, USA). The composition of the diet, listed by the manufacturer is as follows: oat flour, soy flour, wheat flour, other starches, dibasic calcium phosphate, calcium carbonate, citric acid, niocinamide, riboflavin, sodium chloride, sodium iron pyrophosphate, sucrose, thiamine, mononitrate, brewer's yeast, emulsifier preservatives, mold inhibitor, and food coloring. Yeast granules (*Saccharomyces cerevisiae*) were added to each vial after diet hydration. The newly eclosed males and females, which were allowed to mate before being grouped, were collected every 24 hrs, anesthetized by FlyNap (Carolina Biological), and then separated under the stereomicroscope according to the sex. Adult flies were reared in control medium at a density of 30 individuals per vial until the start of the supplementation. At least 10 vials per each experimental group/sex were prepared to obtain the desired numbers of flies (n = 250) in each experimental group.

A stock solution (6 mg/mL) of each extract was prepared in dimethyl sulfoxide (DMSO) or in water, for TE and TW respectively, and kept at 4 °C. For supplementation, the stock solutions were diluted in water obtaining the "supplemented water" containing the final concentration of extract (15 μg/mL) that was used to soak the medium. The extracts were tested on two distinct age cohorts: 1-week (representing the young flies), starting from the first day of 1 week old flies and 3-week (considered early-old flies) starting from the first day of 3 weeks old flies (each group of 250 flies/sex/group). The diet of control groups was lifelong soaked only with water. Fig. 1 summarizes the experimental setup.

Table 1
Most abundant bioactive phenolic compounds in *T. spicata* extracts identified by HPLC-MS/MS as previously reported (Khalil et al., 2019).

ETHANOLIC EXTRACT (TE)	
Bioactive Phenolic Compounds	***Percentage of abundance (%)***
Carvacrol	36.84
Thymusin	20.25
Eriodictyol derivative	9.45
Luteolin	7.95
Eriodictyol	6.8

AQUEOUS EXTRACT (TW)	
Bioactive Phenolic Compounds	***Percentage of abundance (%)***
Rosmarinic acid	38.6
Salvalonic acid I	10.17
Rutin	7.17
Salvalonic acid E/B	5.85
Luteolin-O-diglucuronide	4.81

2.4. Longevity assay

A total of 1500 female and 1500 male fruit flies were randomly divided into 3 groups of study assuming: control diet (CTRL), TE-supplemented diet (TE), and TW-supplemented diet (TW), for each age category: 1-week (1 W) and 3-weeks (3 W). The flies were transferred into fresh medium twice a week and scored for deaths virtually on each day of transfer until all flies were dead.

2.5. Body weight measurement

Body weights of flies were measured once a week starting from the first day of treatment (T0). In details, the weight of a single fly was estimated by calculating the difference between the vial weight before and after flies' transfer, to be divided by the total number of the remaining alive flies.

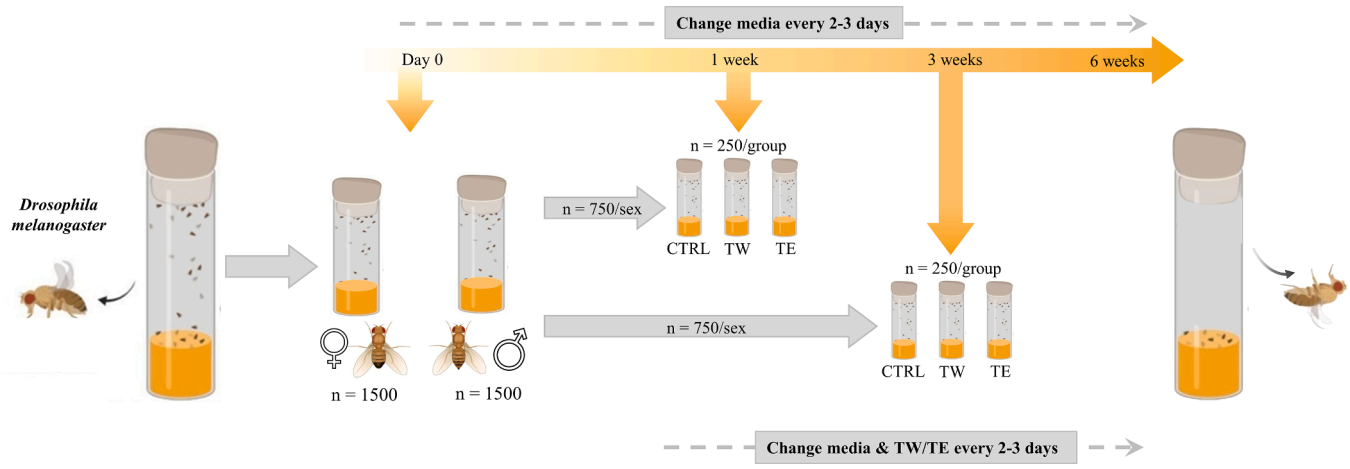

Fig. 1. ***Experimental design.*** A representative scheme summarizing the experimental setup of our *in vivo* study with *Drosophila melanogaster*. The newly eclosed males and females were collected every 24hrs and then separated under the stereomicroscope according to the sex. A total of 1500 female and 1500 male fruit flies were randomly divided into 3 groups of study where at least 10 vials per each experimental group/sex were prepared with a density of 25–30 individuals per vial to obtain the desired numbers of flies (n = 250) in each experimental group, assuming: control diet (CTRL), ethanolic extract-supplemented diet (TE), and aqueous extract-supplemented diet (TW), for each age category: young (1-week old) and early-old (3-weeks old). The flies were transferred into fresh medium twice a week and scored for deaths virtually on each day of transfer until all flies were dead.

2.6. Food intake evaluation

Food rates of consumption were measured using the capillary feeder method (CAFE) as previously described (Beghelli et al., 2022). The assay was performed on flies never supplemented (7 days old male and female flies). On the day of the experiments, flies were separated into CAFE vials (three replicates of ten flies for vial: 30 flies sex/treatment), weighted, and starved for two hours. After the starvation, flies were fed for six hours with 2.5 % sucrose (Ctrl) or 2.5 % sucrose + TE extract (TE) or 2.5 % sucrose + TW extract (TW), respectively. Then flies were transferred back on the regular food medium (Formula 4–24 ® media soaked with water) till the day after. The trial has been repeated three times on three consecutive days. To account for evaporation of the liquid food, three vials were set up with feeding capillaries but without flies. Fly's feeding consumption was evaluated measuring the amount of liquid consumed from the microcapillary tube (in mm) as described by Fiocca et al. (Fiocca et al., 2019) and data were reported as μL/mg of fly weight/hour.

2.7. Paraquat-induced oxidative stress

A stock solution of 2.5 mM Paraquat (1,1′-Dimethyl-4,4′-bipyridinium dichloride), a herbicide triggering mitochondrial oxidative stress and decreasing motor ability (Madabattula et al., 2015), was prepared in 2.5 % sucrose solution. For the experiments, two groups of young (1 W) females (n = 180) were fed with a diet supplemented with the *T. spicata* aqueous extract (TW) for 2 weeks, or without supplementation (CTRL). The early-old flies (3 W) were then subdivided into a double series of new vials utilized for the capillary feeder method (CAFE) prepared as described previously (Beghelli et al., 2022). Briefly, after 2hr of starvation, microcapillary tubes were inserted into the vials through a 200 μL pipette tip in the foam plug. For both TW-supplemented or control-diet flies, four microcapillary tubes were used per vial after filling with 2.5 % sucrose in the presence (TW) or in the absence (CTRL) of the 2.5 mM Paraquat solution. These flies had access to the two different solutions for 40 hr. The number of dead flies was recorded.

2.8. Climbing assay

After the oxidative stress assay, the surviving flies were transferred without anesthesia to 10 cm high tubes (10 flies for tube) for the Climbing assay (Gevedon et al., 2019). Flies were allowed to recover for 5 min and then they were forced to the bottom of the tubes by tapping the tubes on a foam pad resting on a rigid surface. Flies' locomotion was observed for 10 s and the number of flies that reached or passed the five-centimetres line within this period as well as the total number of flies were recorded. The test was repeated ten times with a rest interval of one minute between one test and the next (Gevedon et al., 2019) and the percentages of flies able to reach or pass the five-centimetres line were calculated.

2.9. TBARS assay

Samples were prepared by homogenizing the full-body of each group of flies being collected and frozen during the study following a standard protocol (Adedara et al., 2015). In details, 0.1 M of potassium phosphate buffer pH 7.4 was added to the fly sample in a ratio of 1:5 (mg fly body: μL buffer). The homogenate was obtained using Teflon Potter-Elvehjem homogenizer. The lipid peroxidation end products were quantified using the thiobarbituric acid reactive substances (TBARS) assay, which is based on the reaction of malondialdehyde (MDA; 1,1,3,3-tetramethoxypropane) with thiobarbituric acid (TBA) (Iguchi et al., 1993). Briefly, 1 vol of sample homogenates were incubated at 95 °C for 60 mins with 2 vol of TBA solution (0.75 % TBA, 10 % trichloroacetic acid, 0.1 M HCl) for color development. After cooling, samples were centrifuged (8000 x g for 10 mins) and the absorbance of the supernatant was measured at 532 nm. TBARS tissue levels were expressed as nmol MDA/mg protein.

2.10. Protein content quantification

The protein content was determined by the Bradford assay using serum bovine albumin (BSA) as a standard (Bradford, 1976).

2.11. RNA extraction and quantitative real-time PCR

The total RNA was extracted from flies being collected and frozen at specific time conditions during the study: T0, T1 (2 weeks after supplementation), and T2 (4 weeks after supplementation). The frozen fly samples were mechanically homogenized and then, the mRNA was isolated using RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). NanoVue Spectrophotometer (GE Healthcare, Milano, Italy) was used to measure the yield and purity of the RNA. Only samples with ratios A260/A280 > 1.8 were used.

For each sample, 1 μg of total RNA was reverse transcribed to obtain cDNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instructions. The subsequent polymerase chain reaction (PCR) was performed in a total volume of 10 μL containing 2 μL of dH2O RNAsi free, 2.5 μL (12.5 ng) of cDNA, 5 μL SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories), and 0.5 μL (500 nM) of each primer. The relative quantity of target mRNA was calculated using the comparative Cq (represents the cycle number at which the amount of amplified target reaches the fixed threshold) method and was normalized for the expression of ribosomal protein L32 (*rpl32*). The expression of the target genes was then calculated as relative quantity of mRNA (fold induction) with respect to controls (at T0). The used primer pairs are illustrated in Table 2.

2.12. Statistical analysis

All results were expressed as mean ± SD. GraphPad Prism 8.0.1 software was used for statistical evaluation. Comparisons between different conditions were performed using one-way ANOVA with Tukey's post-test. Difference between two different conditions was calculated by student's *t*-test. All statistical analysis were performed by GraphPad Software Prism 8.0.1, Inc. (San Diego, CA, USA). For lifespan assessment, Kaplan–Meier survival curves were generated by OASIS2 (Han et al., 2016).

3. Results

3.1. T. spicata supplementation ameliorates the D. melanogaster lifespan

The possible anti-aging effect of *T. spicata* extracts was tested on both young and early-old (1 week- and 3 week-old, respectively) male and female flies upon feeding a standard diet supplemented with either TE or TW (15 μg/mL in total polyphenols). The flies that were reared on food containing only water were taken as control (Fig. 2). The results reported different effects on the lifespan depending on both the fly sex (male *vs* female) and the extraction solvent (water *vs* ethanol). In details, we observed an extended lifespan (+5.8 %; $p < 0.05$) in young females feeding TW-supplemented diet with respect to control (Fig. 2A), whereas no effects could be appreciated on the young males (Fig. 2B). For the early-old flies, no effects could be appreciated for both female and male flies. On the other hand, the ethanolic extract TE did not exert any considerable effect on both young and early-old flies.

3.2. T. spicata supplementation reduces the D. melanogaster body-weight gain

Based on the lipid lowering activity previously described *in vitro* for the *T. spicata* extracts (Khalil et al., 2019), here we tested *in vivo* their weight-loss potential on both young and early-old flies (Fig. 3). We observed a slight but significant reduction in the body-weight gain of the TW-supplemented young females at the third week (−15 % *vs* inner Ctrl, and −11 % *vs* 1 W; $p < 0.01$ and $p < 0.05$, respectively), whereas the TE supplementation led to a remarkable increase in the body-weight gain at the fourth week (+24 % *vs* inner Ctrl and +20 % *vs* 1 W; $p < 0.01$ and $p < 0.05$, respectively) (Fig. 3A). An increase in the body-weight gain was observed also in the young males feeding TE-supplemented diet starting from the third week (+29 % *vs* inner Ctrl and +34 % *vs* 1 W; $p < 0.05$ and $p < 0.0001$, respectively). (Fig. 3B).

On the other hand, both TE- and TW-supplementation led to a general increase in the body-weight gain of the early-old females starting from the fifth week compared to the third week (+23 % and +19 % *vs* 3 W; $p < 0.05$, respectively) (Fig. 3C) as well as in the early-old males where the body-weight gain started to increase from the fourth week (+25 % and +17 % *vs* 3 W; $p < 0.01$ and $p < 0.05$, respectively) (Fig. 3D).

Of note, the food consumption evaluated by the CAFE assay was not affected by the supplementation with the TW or TE extracts in both male and female flies (data not shown), thus excluding that the changes in the body-weight gain and in the lifespan of flies might depend on different food consumption (Schriner et al., 2013).

Table 2
List of primers for real-time PCR.

Gene	Primer Name	Primer Sequence 5′-> 3′
HO	Fwd	ATGTCAGCGAGCGAAGAAACA
	Rev	TGGCTTTACGCAACTCCTTTG
TrxR1	Fwd	TGGATCTGCGCGACAAGAAAG
	Rev	GAAGGTCTGGGCGGTGATTG
TotA	Fwd	AACTGCTCTTATGTGCTTTG
	Rev	TCAGCAATTCTAAGGTTGTC
RPL32	Fwd	GCCCACCGGATTCAAGAAGT
	Rev	CTTGCGCTTCTTGGAGGAGA

3.3. T. spicata supplementation protects the D. melanogaster against oxidative stress

Based on the observed beneficial effects of TW supplementation on both the mean lifespan and body-weight gain of young females, we focused on this cohort of flies for the further investigations. We assessed the possible protective effects of TW on young females exposed to Paraquat, which is a strong inducer of oxidative stress *in vivo* (Kojić et al., 2020; Noriega et al., 2002). We observed that TW supplementation improved the fly mortality caused by Paraquat (9.3 % *vs* 22 % of mortality in TW-treated *vs* Ctrl flies, respectively; $p < 0.05$) (Fig. 4A).

In parallel, we measured the lipid peroxidation at the whole-body level of young females by TBARS assay (Fig. 4B). Upon exposure to 2.5 mM Paraquat for 40 hrs, the MDA level increased in Paraquat-insulted flies (+26 % compared to Ctrl flies, $p < 0.05$). When Paraquat-insulted flies were feeding by TW-supplemented diet, the MDA increase was significantly reduced (−30 % with respect to Paraquat-insulted flies as control; $p < 0.01$).

When the motor function was assessed by climbing assay, we observed an impair in locomotor ability after the Paraquat insult in both control (38 % *vs* 22 %, $p < 0.01$) and TW-supplemented (40 % *vs* 23 %, $p < 0.01$) of young females, but the TW supplementation did not rescue significantly the locomotor impair (Fig. 4C).

Therefore, the protective role of TW against the Paraquat-induced oxidative stress could act mainly by reducing the fly mortality through an oxidative stress containment rather than by improving the motor function.

3.4. T. spicata supplementation modulates the expression of defense-related genes

Based on the above results, we tested the expression of genes potentially involved in the antioxidant defense (*Ho* and *Trxr1*), and stress tolerance (*TotA*) by RT-PCR (Fig. 5). The control young females showed an age-related up-regulation of *HO* expression reaching significance at T2 (4.10 fold induction *vs* T0; $p < 0.0001$), while a dramatic decrease occurred in the TW-supplemented flies (0.38 fold induction at T2 *vs* T0; $p < 0.0001$) (Fig. 5A). Similarly, *Trxr1* gene in control females showed an age-related up-regulation with a maximum at T2 (3.75 fold induction *vs* T0; $p < 0.0001$), and a down-regulation in the TW-supplemented flies (0.4 fold induction at T2 *vs* CTRL at T2; $p < 0.0001$) (Fig. 5B).

Also the environmental stress-activated gene *TotA* showed an age-related up-regulation with a maximum at T2 in both control flies (76.70 fold induction *vs* CTRL at T0; $p < 0.0001$), and in TW-fed flies

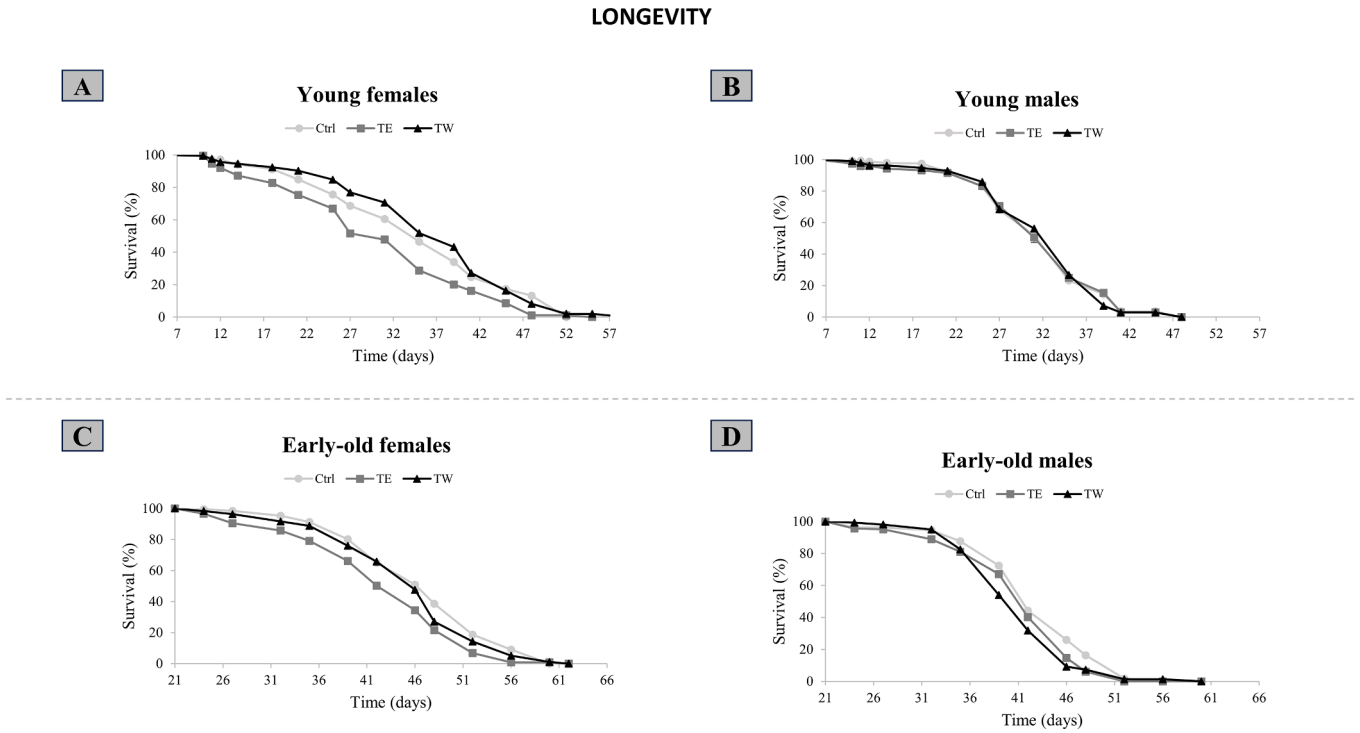

Fig. 2. ***Effects of T. spicata supplementation on the D. melanogaster lifespan.*** Survival curves of young and early-old female and male *Drosophila* reared on different diets. The flies were supplemented with either 15 μg/mL of ethanolic extract (TE) or aqueous extract (TW), or only standard diet. Data are represented as percentage of survival of flies (%) as a function of time (in days). The Kaplan–Meier test was used to detect the significant differences among the three groups of the flies (OASIS2). Lifespan improved significantly in young female flies supplemented with TW (A: $p < 0.05$).

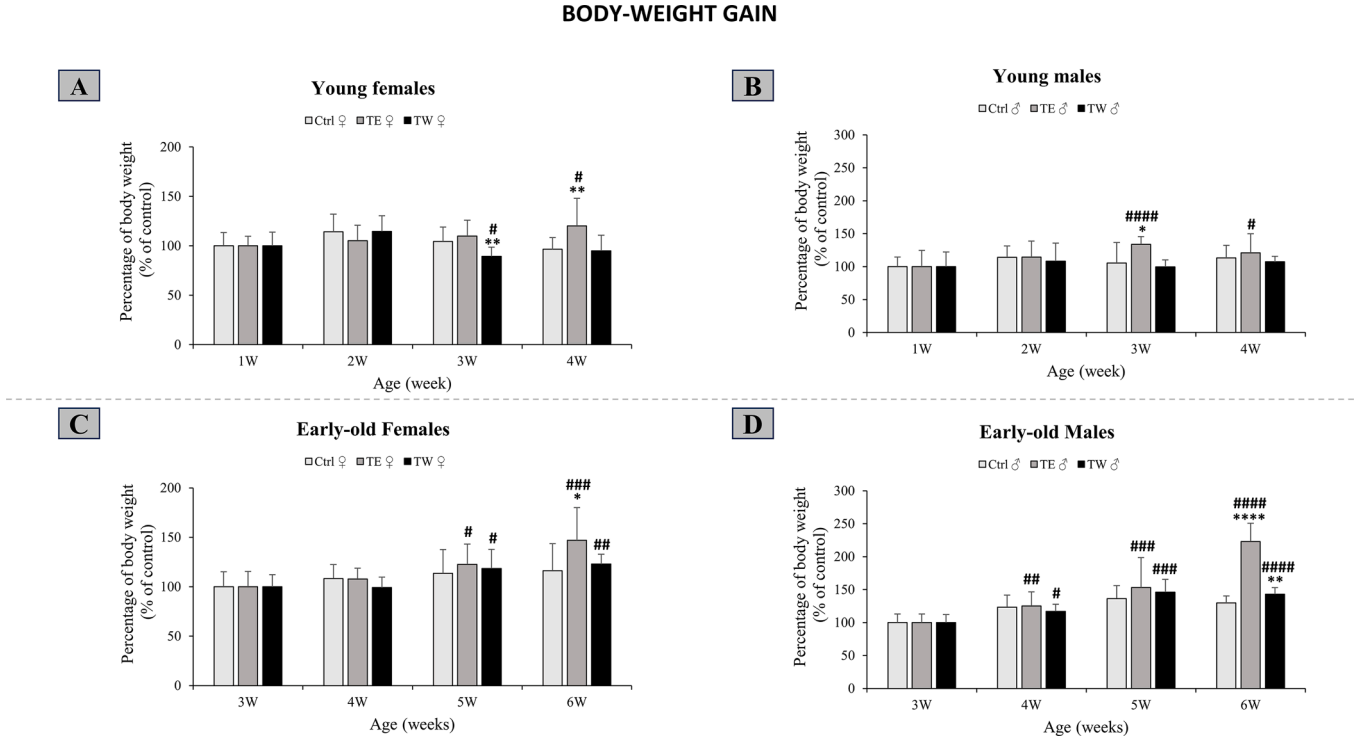

Fig. 3. ***Effects of T. spicata supplementation on the D. melanogaster body-weight gain.*** Effect of TE and TW on the body-weight gain of young and early-old female and male *Drosophila.* Data are reported as percentage of variation of body-weight (%), considering the average of the recorded weights in the CTRL groups as equal to 100 % at the starting day of supplementations. The percentages of variation were studied by calculating the percentage of the average of the recorded weights of each group of flies with respect to the day of supplementation, as a function of time (in weeks). Significant differences are denoted by symbols: Ctrl-1W/Ctrl-3W *vs* different treatments *# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, and #### $p < 0.0001$,* and inner Ctrl *vs* treatments * $p < 0.05$ and ** $p < 0.01$.

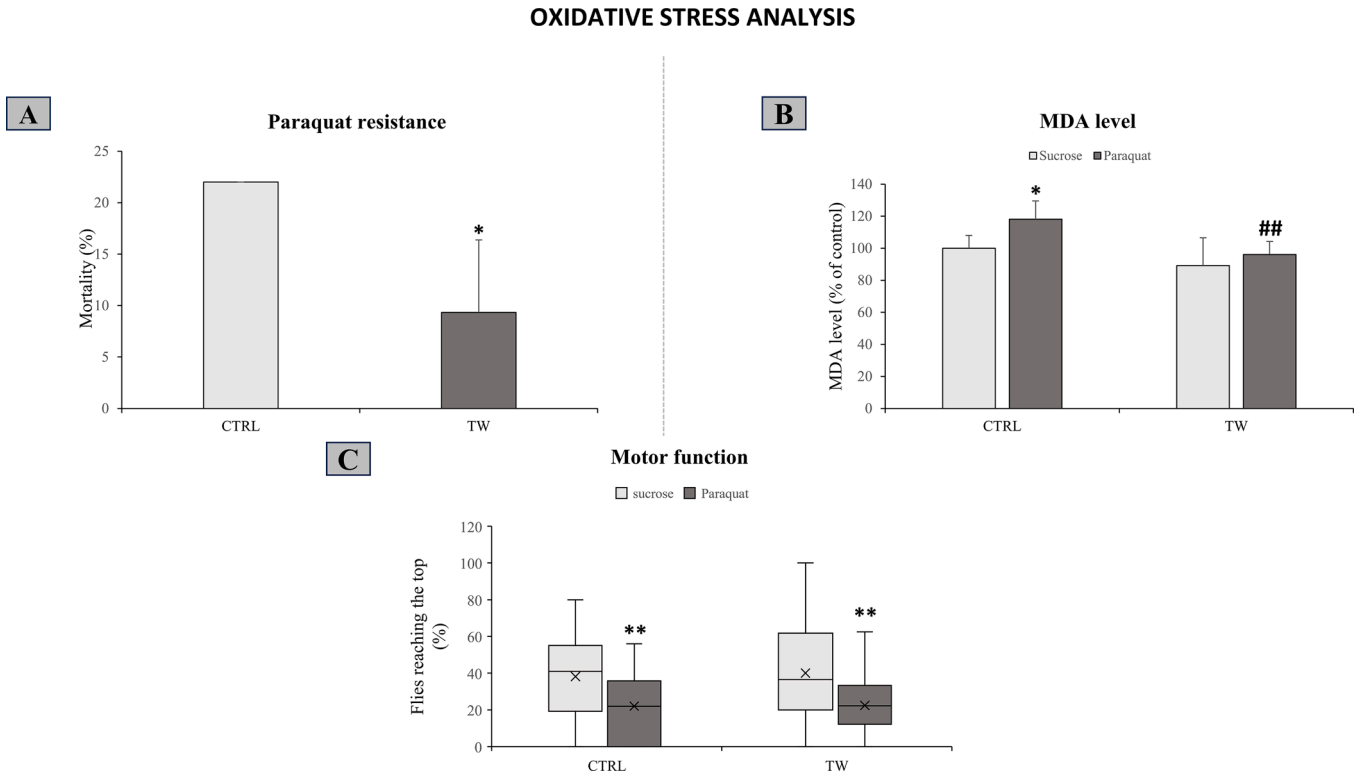

Fig. 4. ***Effects of T. spicata supplementation on the oxidative stress.*** Young female flies fed a TW-supplemented (TW) or standard diet (CTRL) were subjected to Paraquat stress for 40 hrs. **(A)** The mortality was represented in (%) with respect to the CTRL and expressed as mean ± SD. The significant difference is denoted by symbol: CTRL *vs* TW *p ≤ 0.05. **(B)** Lipid peroxidation in Paraquat-insulted flies was studied by measuring the MDA level using TBARS assay as nmol MDA/mg of sample protein. Values are reported as % of control and are mean ± S.D from a least three independent experiments. Significant differences are denoted by symbols: CTRL-sucrose *vs* CTRL-Paraquat *p ≤ 0.05, and CTRL -Paraquat *vs* TW-Paraquat ##p ≤ 0.01. **(C)** The motor function in Paraquat-insulted flies was studied in terms of the climbing ability. Data are expressed as mean ± SD.

(13.92 fold induction *vs* CTRL at T2; $p < 0.0001$) although the up-regulation was markedly lower than in control (Fig. 5C).

4. Discussion

The present study employed the *D. melanogaster* fly as an *in vivo* model to investigate the anti-aging, body-weight control, and antioxidant potentials of two polyphenol-enriched extracts from *T. spicata* leaves. The main findings of this study, taking in consideration both the age (young *vs* early-old) and the sex, demonstrated the potency of *T. spicata* leaves, in particular the aqueous extract, to extend the lifespan of the young female flies, reduce their body-weight gain, and protect them against oxidative stress.

Aging is a complex biological process developed by the progressive deterioration of individuals function and capacity to recover from different inner and external disturbances, leading to increased morbidity and mortality. Although aging cannot be prevented, slowing down the rate of aging is entirely possible to achieve. In this context, medicinal herbs are recognized by the "nourishing of life" and their role as anti-aging phytoterapics is gaining attention (Shen et al., 2013; Valenzano et al., 2006).

T. spicata is a thyme-like plant extremely rich in phenolic compounds and, therefore, represents a promising candidate as phytotherapy and nutraceutical herb. Our previous study reported the phenolome characterization of both the aqueous and ethanolic extract from *T. spicata* aerial parts. These results indicated that the aqueous extract was more effective in reducing hepatic steatosis, and the ethanolic extract had higher antioxidant potential (Khalil et al., 2019). Therefore, based on our previous *in vitro* results, for the *in vivo* study that is rather complex and of long duration we selected the extract concentration (15 μg/ml) which resulted the most effective on the cells (Diab et al., 2022; Khalil et al., 2019, 2022).

The present study demonstrates, for the first time, the beneficial effects *in vivo* of a dietary supplementation with *T. spicata*. First, we found that exclusively the aqueous extract ameliorated the fly lifespan in a sex-dependent manner. Indeed, TW-supplementation significantly increased the mean lifespan of young females (1 W-old) compared to the control group feeding a standard diet. As longevity seems to be related to the control of body-weight and lipid metabolism homeostasis, we assessed in parallel the body-weight gain. Indeed, a recent paper (Johnson, 2020) reported a premature death in lysosomal lipase-deficient mice, while nematodes overexpressing the triacylglycerol lipase were lean and long-lived, and flies lacking the lipase were obese and short-lived. We found also for the body-weight control a sex-dependent effect. The aqueous extract was effective in young females but not in males. By contrast, the ethanolic extract enhanced the body-weight gain in all the cohorts of flies of both sexes and ages, and so did also the TW extract in early-old female and male flies.

Of note, we observed a sex- and age-dependent effect of the *T. spicata* supplementation according to many studies on functional food in flies that described differences with sex. Indeed, the anatomical and physiological characteristics of each sex may influence the response to diet, also depending on the energy demand making the two sexes differentially sensitive to specific nutrients (Davies et al., 2005). Moreover, also the marital-status of the flies plays a role: i.e. virgin females live significantly longer than mated females due to egg production and mating costs (Chapman et al., 1998).

The different biological activity of the two extracts can be explained by the different phenolome profile characterizing them. In details, the aqueous extract is rich in hydrophilic compounds, especially the rosmarinic acid, a phenolic acid deriving from the caffeic acid. Conversely, the ethanolic extract is extremely rich in volatile organic compounds,

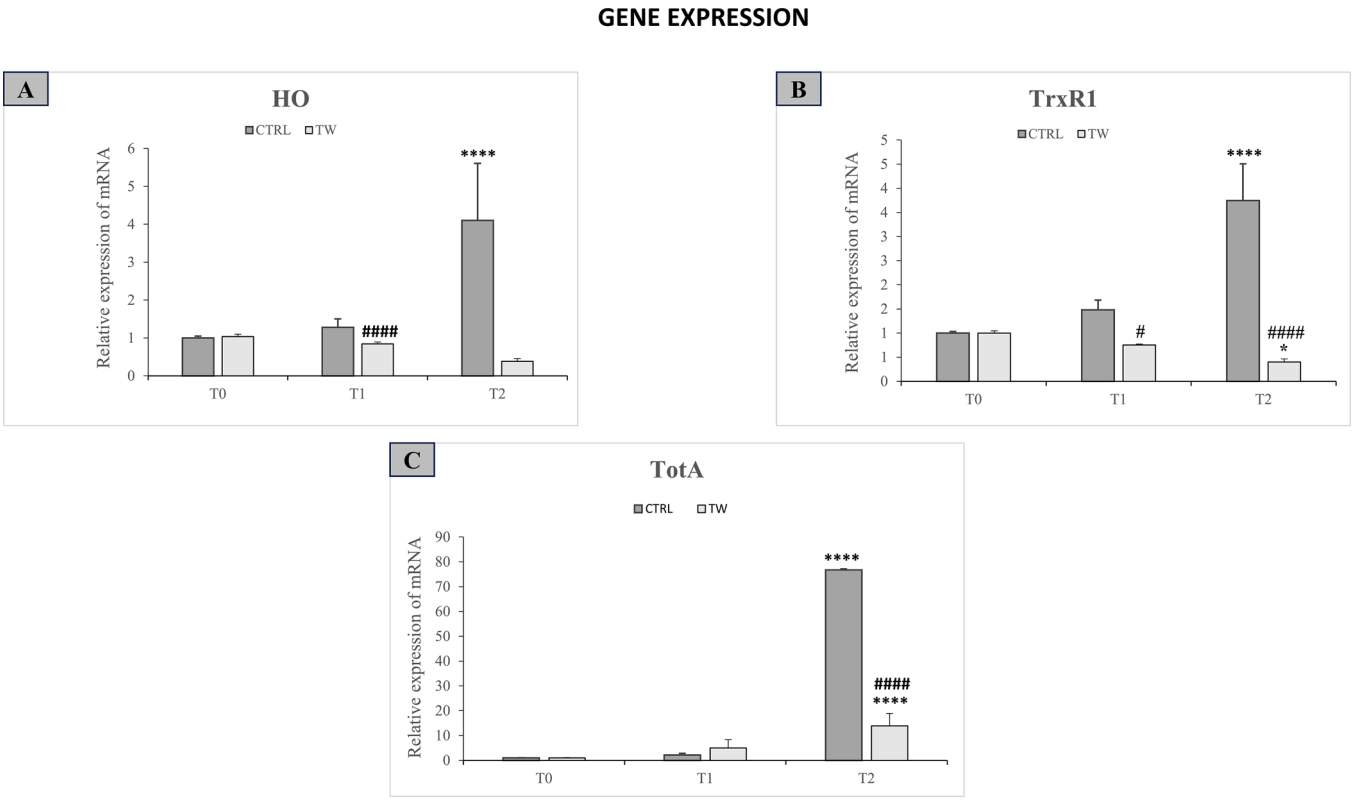

Fig. 5. ***T. spicata supplementation modulates the expression of defense-related genes.*** Two antioxidant genes **(A)** HO and **(B)** TrxR1, and one stress tolerance-related gene **(C)** TotA were analyzed by real-time PCR. Three time intervals were analyzed: T0 (day of supplementation), T1 (2 weeks after supplementation), and T2 (4 weeks after supplementation). The relative quantity of target mRNA was calculated by the comparative Cq method using ribosomal protein L32 (RPL32) as housekeeping gene and expressed as fold induction with respect to controls. Values are mean ± S.D. from at least three independent experiments. Statistical significance between groups is denoted by symbols: CTRL-T0 *vs* CTRL at different times *$p < 0.05$, **** $p \leq 0.0001$, CTRL-T1 *vs* TW-T1 #$p < 0.05$, and CTRL-T2 *vs* TW-T2 #### $p < 0.0001$.

especially carvacrol (2-methyl-5-(1-methylethyl)-phenol), a phenolic monoterpenoid with one hydroxyl group (—OH). We can hypothesize that both the chemical structure of the different polyphenolic compounds, and, most importantly, their different bioavailability can influence their efficacy *in vivo* on the *Drosophila* lifespan. In fact, a published study on quercetin and its derivatives revealed that the quercetin 3-*O*-β-d-glucopyranoside-(4 → 1)-β-d-glucopyranoside was the most potent quercetin derivative in terms of lifespan extension on the *Caenorhabditis elegans* model probably due to its highest hydrophilicity (Xue et al., 2011). We wish to mention that the weight-loss potential of TW could be sustained also by salvianolic acid (SA) which is the most abundant polyphenol detected when TW had been subjected to a simulated *in vitro* digestion (Diab et al., 2022). In fact, a large body of *in vitro* and *in vivo* evidences showed that SA reduces obesity and obesity-related disorders by suppressing adipogenesis and decreasing body-weight in high-fat diet animal model (Wang et al., 2014), and hepatoprotective effects against lipotoxicity (Ding et al., 2016; Li et al., 2020; Meng et al., 2022). The anti-obesity effects of SA could be assigned to its ability in improving lipid and glucose metabolisms (Wang et al., 2022).

A crucial factor sustaining the aging is the over-accumulation of ROS and oxidant species. The idea that ROS might act on the aging process due to the accumulation of ROS-induced damages has been postulated many years ago (Harman, 1956). This theory has been confirmed in many animal models showing reduced oxidative damage and/or increased resistance to oxidative stress in the longer-lived animals (Pérez et al., 2009). In order to better understand the mechanisms through which the aqueous extract improves the mean half-life, we tested its protective effects against Paraquat, which is a strong oxidative stress inducer (Pezzoli & Cereda, 2013; Tanner et al., 2011). We observed that Paraquat increased mortality of the young female flies, and supplementation with TW was protective by decreasing their mortality rate. The mortality could depend on the Paraquat-induced oxidative stress. Indeed, in control young females, the exposure to Paraquat triggered oxidative stress that we verified in terms of increased lipid peroxidation, and the TW supplementation reduced the Paraquat-induced lipid peroxidation. However, when we checked the motor function of young female flies we observed an impair after Paraquat insult, but TW did not rescue the climbing ability. Taken together, our results demonstrated that the protective role of dietary intake of TW occurs mainly by reducing the fly mortality rather than by improving the motor function impair. These findings are in line with a recent report (Wongchum & Dechakhamphu, 2021) demonstrating that xanthohumol, a flavonoid abundant in *Humulus lupulus*, improved the resistance against oxidative stress being induced by both hydrogen peroxide and Paraquat. Another study on *Drosophila* reported that an enhanced resistance against oxidative stress reduced the fly mortality upon ethanol consumption (Deepashree et al., 2019).

In an attempt to better characterize the molecular pathways sustaining the beneficial effects of the extract on lifespan and body-weight control, we investigated the expression of genes directly implicated in antioxidant (*HO* and *TrxR1*), and stress tolerance (*TotA*) processes.

Regarding the antioxidant defense, we tested two genes: heme oxygenase and thioredoxin. *Ho* is known to protect against oxidative stress (Loboda et al., 2008) by acting on apoptotic and autophagic processes in *Drosophila* (Abaquita et al., 2021). Also *Trxr1* in *Drosophila* protects against oxidative stress, being the thioredoxin system a major player in glutathione metabolism (Svensson & Larsson, 2007). We observed an age-related down-regulation in both the *Ho* and *Trxr1* expression in TW-supplemented flies. However, some studies on fruit fly cohorts reported

that lack of glutathione reductase stimulates antioxidant defense (Kanzok et al., 2001), and that the consumption of chili-supplemented food extends lifespan, although the activity of glutathione-S-transferase (GST) was decreased (Semaniuk et al., 2022), suggesting that the extension is not mediated by a strengthening of antioxidant defenses. Therefore, we could suggest that the down-regulation of *Ho* and *Trxr1* genes upon TW supplementation can be a consequence of the lower level of oxidative stress caused by the extract activity.

Regarding the stress tolerance, we tested the Turandot A (TotA) gene belonging to a family of stress-induced genes (Ekengren et al., 2001), which are stimulated by severe stresses (Ekengren & Hultmark, 2001). Therefore, we observed an age-related up-regulation of TotA expression in control flies, while this up-regulation was lower in TW-supplemented flies. This result could be attributed to the antioxidant protection of TW which in turn will keep the flies in a consistent "not severe" stress condition.

In conclusion, our data demonstrate, for the first time, that dietary intake of *T. spicata*, in particular the aqueous extract, is able to extend the lifespan and reduce the body-weight gain of fruit flies in age-related and sex-dependent manners. Our hypothesis is that the extension of lifespan might be mediated by two key processes: (*i)* decreasing the body-weight gain, and (*ii)* boosting the antioxidant defense. The different health-promoting efficacy observed for the two extracts should be attributed to their differences in the panel concentration, bioavailability, and properties of the polyphenols, with the aqueous extracts exhibiting the best nutraceutics potential. Nevertheless, further studies should be carried out to investigate if and how the efficacy of *T. spicata* might affect other parameters including the fertility of the flies.

Author contribution

All authors contributed to this work significantly. FD performed the molecular biology and biochemical experiments, the statistical analysis, and wrote the first draft of the paper. DB designed the methodology of the study, performed the climbing, the CAFE, the Paraquat assays and the statistical analysis, and supervised the experimental activities. AN contributed to the fly maintenance and experiments. GL supervised the biochemical experiments. MK prepared and characterized the *T. spicata* extracts. PP participated in the study design and data interpretation. LV conceived the study, participated in designing the experimental activity and wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Ethical statement

(1) This material is the authors' own original work, which has not been previously published elsewhere.
(2) The paper is not currently being considered for publication elsewhere.
(3) The paper reflects the authors' own research and analysis in a truthful and complete manner.
(4) The paper properly credits the meaningful contributions of co-authors and co-researchers.
(5) The results are appropriately placed in the context of prior and existing research.
(6) All sources used are properly disclosed (correct citation).
(7) All authors have been personally and actively involved in substantial work leading to the paper, and will take public responsibility for its content.

CRediT authorship contribution statement

Farah Diab: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Daniela Beghelli:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Anna Nuccitelli:** Methodology. **Giulio Lupidi:** Methodology. **Mohamad Khalil:** Methodology. **Piero Portincasa:** Investigation, Conceptualization. **Laura Vergani:** Writing – review & editing, Validation, Project administration, Investigation.

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.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.jff.2024.106078.

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