Green coffee extract enhances oxidative stress resistance and delays aging in *Caenorhabditis elegans*

Loredana Amigoni[a], Milda Stuknytė[b], Carlotta Ciaramelli[a], Chiara Magoni[a], Ilaria Bruni[a], Ivano De Noni[b], Cristina Airoldi[a,c], Maria Elena Regonesi[a,c], Alessandro Palmioli[a]

[a] Dipartimento di Biotecnologie e Bioscienze, Università degli Studi di Milano-Bicocca, Piazza della Scienza 2, 20126 Milan, Italy. [b] Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente, Università degli Studi di Milano, via Celoria 2, 20133 Milan, Italy. [c] Milan Center for Neuroscience (NeuroMI), Via Pergolesi 33, 20052, 20126 Monza, Italy.

To whom correspondence should be addressed: Maria Elena Regonesi, Department of Biotechnology and Biosciences, University of Milano-Bicocca, 20126 Milan (Italy); Tel: +39 02 64483437; E-mail: mariaelena.regonesi@unimib.it

Complete mailing addresses: Loredana Amigoni, loredana.amigoni1@unimib.it ; Milda Stuknytė, Milda.Stuknyte@unimi.it, Carlotta Ciaramelli, carlotta.ciaramelli@unimib.it, Chiara Magoni, chiara.magoni90@gmail.com, Ilaria Bruni, ilaria.bruni@unimib.it, Ivano De Noni, ivano.denoni@unimi.it, Cristina Airoldi, cristina.airoldi@unimib.it, Maria Elena Regonesi, mariaelena.regonesi@unimib.it, Alessandro Palmoli, alessandro.palmioli@unimib.it.

Abstract

Nutritional factors play a pivotal role for healthy aging and longevity. This is related to the antioxidant properties of the molecules present in some foods. Due to the high content of polyphenols, Green Coffee Extract (GCE) is a powerful antioxidant. Nevertheless, little is known about its effect on aging. We demonstrated the benefic effects of GCE on stress resistance, fertility and adult mean lifespan using *Caenorhabditis elegans* as a model. The mean and maximum lifespan of worms treated with GCE increased significantly in a dose-dependent manner, and animals pre-treated were more resistant to oxidative stress. NMR and UPLC/ESI-HRMS analyses of GCE confirmed a significant content of chlorogenic acids, being 5-*O*-caffeoylquinic acid (5-CQA) the most abundant isomer. The major activity demonstrated by GCE in comparison to the pure 5-CQA on *C. elegans* phenotypes clearly demonstrated the importance of the employment of a natural extract to develop functional foods and supplements.

Keywords

Green coffee extract, Chlorogenic acids, Antioxidant activity, High resolution mass spectrometry, NMR spectroscopy, *Caenorhabditis elegans*.

Chemical compounds: 5-*O*-Caffeoylquinic acid (PubChem CID: 12310830), 3-*O*-Caffeoylquinic acid (PubChem CID: 5280633), 4-*O*-Caffeoylquinic acid (PubChem CID: 9798666), 5-*O*-Feruloylquinic acid (PubChem CID: 15901362), 3-*O*-Feruloylquinic acid (PubChem CID: 10133609), 4-*O*-Feruloylquinic acid (PubChem CID: 10177048), 3,5 -Di-*O*-caffeoylquinic Acid (PubChem CID: 6474310), 3,4-Di-*O*-caffeoylquinic Acid (PubChem

CID: 71592176), 4,5-Di-*O*-caffeoylquinic Acid (PubChem CID: 6474309), Caffeine (PubChem CID: 2519), Trigonelline (PubChem CID:5570).

1. Introduction

Epidemiological studies suggest that diet rich in fruits and vegetables plays a key role in maintaining health and protecting against diseases (Boeing, 2013). Stress, neuronal damage, cellular degeneration, brain dysfunction and cardiovascular diseases are all significant issues associated with the aging process that are influenced by nutrition (Oguntibeju, Esterhuyse, & Truter, 2013). In the last years, aging research is rapidly gathering increased scientific interest. Genes controlling metabolic activity, antioxidant systems, DNA repair, cellular senescence and cell death affect the lifespan. Several studies led to the general acceptance that oxidative stress and inflammation are major determinants of the aging process (Finkel & Holbrook, 2000; Sikora, Scapagnini, & Barbagallo, 2010). Oxidative stress has been reported to increase in elderly subjects, possibly arising from an uncontrolled production of free radicals by aging mitochondria and decreased antioxidant defenses (Finkel & Holbrook, 2000; Harman, 1998; Knight, 2001). For these reasons, the effect of the dietary supplementation with antioxidants on aging has a growing interest.

Coffee is the first most frequently consumed beverage worldwide. Its status varies from a simple ancient cultural drink to a nutrient component, endowed with potential pharmacological benefits, such as antioxidant (Sato et al., 2011), antitumor (Rocha, Monteiro, & Teodoro, 2012), anti-diabetic (Ong, Hsu, & Tan, 2012) and anti-inflammatory effects (Lee et al., 2013). Most of these properties seem to be linked to the presence in the coffee seed of phenolic compounds, secondary metabolites of plants generally involved in defense against ultraviolet radiation, climatic conditions (Kaur et al., 2015) or aggression of

pathogens (Farah & Donangelo, 2006). In particular, polyphenols are present in coffee predominantly as a family of esters formed between hydroxycinnamic acids and quinic acid, collectively known as chlorogenic acids (CGAs) (Clifford, 1985). Tannins, lignans and anthocyanins are also present in the seed, however in minor amounts. Coffee polyphenols have extensive functions as antioxidants, and they behave as free radical scavengers *in vitro* (Yashin, Yashin, Wang, & Nemzer, 2013). Previous studies on natural polyphenols such as resveratrol (Bass, Weinkove, Houthoofd, Gems, & Partridge, 2007), curcumin (Liao et al., 2011) and blueberry polyphenols (Wilson et al., 2006) clearly support an anti-aging effects in model animals.

To the best of our knowledge, the *in vivo* anti-aging properties of the green coffee extract (GCE) have never been studied in detail. The interest in green coffee is mainly a consequence of its recent increased use as a beverage, in the form of herbal teas or dietary supplements. Its beneficial properties depend on its antioxidant activity, taking in account that the roasting process of the coffee beans reduces the amounts of CGAs (Trugo & Macrae, 1984). However, relevant antioxidant properties were reported also for roasted coffee, as a result of incorporation of phenolic compounds into melanoidins (Perrone, Farah, & Donangelo, 2012).

In this work, we studied the effect of the GCE on aging by employing a model animal, *Caenorhabditis elegans*. During the last three decades the soil nematode *C. elegans* has become a prominent model organism for studying aging. These animals decline behaviorally and physiologically with age in a manner similar to that of higher mammals, including human. The identification of more than 200 genes that modulate lifespan in *C. elegans* revealed evolutionarily conserved pathways involved in aging (Braeckman & Vanfleteren, 2007; Fontana, Partridge, & Longo, 2010; Herndon et al., 2002). Therefore, the short and invariant lifespan (median adult lifespan ranging from 12 to 18 d at 20 °C), ease to maintenance and assays, represent fundamental advantages for the employment

of these animals in research (Fontana et al., 2010). Herein, we reported the effect of the GCE on stress resistance, fertility, adult mean lifespan and delay of aging in *C. elegans*. We also provided an evidence of the importance of the synergistic interactions of the extract components by comparing the effect of the GCE with an equivalent dose of its most abundant antioxidant component, identified as 5-CQA.

2. Materials and methods

All chemicals and solvents were purchased from Sigma-Aldrich (Saint Louis, MO, USA) and were used without further purification. Green coffee beans "El Salvador" SHG EP were received from Co.ind (Casal Maggiore, BO, Italy). Stock solution of green coffee extract (GCE) was prepared in water at 2.0 mg/mL and stored at -20 °C, whereas diluted solution was freshly prepared before the assay.

2.1. Preparation of freeze-dried GCE

Green coffee beans were frozen in liquid nitrogen and ground to a fine powder in a commercial coffee grinder. One gram of grounded sample was extracted with 100 mL of a mixture of acidified (with 0.1M HCl) water (pH 4.5; 70%) and methanol (30%) by sonication at 30 kHz for 15 min in an ultrasound bath (Elmasonic P 30 H, Elma Schmidbauer GmbH, Singen, Germany) at 30 °C. The solutions were filtered through Whatman N°1, Celite® and 0.45 µm PTFE filters (Pall Corporation, Port Washington, NY, USA), concentrated under reduced pressure at 40 °C and freeze-dried. The lyophilized GCE was stored at -20 °C.

2.2. Nuclear Magnetic Resonance spectroscopy

Freeze-dried GCE samples were suspended in 10 mM deuterated phosphate buffer (PB, pH 7.4) at a final concentration of 2 mg/mL, sonicated (37 kHz, 20 min) and centrifuged (3425 xg, 10 min, 20 °C, ScanSpeed 1730R Labogene, Lynge, Sweden). 4,4-dimethyl-4-

silapentane-1-sulfonic acid (DSS, final concentration 0.5 mM) was added to the obtained supernatant as an internal reference for both concentrations and chemical shift. The pH of each sample was verified with a Microelectrode (Mettler Toledo, Columbus, OH, USA) for a 5 mm NMR tubes and adjusted to a value of 7.4 with NaOD or DCl. All pH values were corrected for the isotope effect. The acquisition temperature was 298 K. All spectra were acquired on an AVANCE III 600 MHz NMR spectrometer (Bruker, Billerica, MA, USA) equipped with a QCI (^{1}H, ^{13}C, ^{15}N/^{31}P and ^{2}H lock) cryogenic probe and a Varian Mercury 400 MHz spectrometer. ^{1}H-NMR spectra were recorded with water suppression (cpmgpr1d pulse sequences in Bruker library) and 64 scans, spectral width of 20 ppm, relaxation delay of 30 s. They were processed with a line broadening of 0.3 Hz, automatically phased and baseline corrected. Chemical shift values were internally calibrated to the DSS peak at 0.0 ppm. Compound identification and assignment were performed with the support of 2D NMR experiments, comparison to reported assignments (Wei, Furihata, Hu, Miyakawa, & Tanokura, 2010; Wei et al., 2012) and the SMA analysis tool integrated in MestreNova software (Cobas, Seoane, Domínguez, Sykora, & Davies, 2011). In particular, ^{1}H,^{1}H-TOCSY (Total Correlation SpectroscopY) spectra were acquired with 48 scans and 512 increments, a mixing time of 80 ms and relaxation delay was 2 seconds. ^{1}H,^{13}C-HSQC (Heteronuclear Single Quantum Coherence) spectra were acquired with 48 scans and 256 increments, a relaxation delay of 2 s.

For metabolite quantification, the global spectrum deconvolution (GSD) algorithm, available in the MNova software package (MestReNova v 10.0, 2016, Mestrelab Research, Santiago de Compostela, Spain) was exploited. In this way, overlapping regions were deconvoluted, and absolute quantification was performed also for metabolites with resonances in rare crowded spectral areas. For each compound, the mean value of the different assigned signals was determined.

2.3. Ultra-Performance Liquid Chromatography/Electrospray Ionization-High Resolution Mass Spectrometry (UPLC/ESI-HRMS)

The UPLC/ESI-HRMS analysis was carried by coupling an Acquity UPLC separation module (Waters, Milford, MA, USA) with in-line photodiode array (PDA) eλ detector (Waters) to a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer and an HESI-II probe for electrospray ionisation (Thermo Scientific, San Jose, CA, USA). The ion source and interface conditions were: spray voltage +3.0/-2.5 kV, sheath gas flow 60, auxiliary gas flow 20 and temperature 300 °C, capillary temperature 350 °C. Positive mass calibration was performed with Pierce LTQ ESI Positive Ion Calibration Solution (Thermo Scientific Pierce, Rockford, IL, USA), containing caffeine, the tetrapeptide MRFA and Ultramark 1621. Negative mass calibration was performed with Pierce ESI Negative Ion Calibration Solution (Thermo Scientific Pierce), containing sodium dodecyl sulfate, sodium taurocholate and Ultramark 1621. Four µL of GCE were separated using a Gemini C18 column (150 × 2.0 mm, 3 µm, 110 Å) (Phenomenex, Torrance, CA, USA) kept at 40 °C, and using 0.05 mL 100 mL^{-1} trifluoroacetic acid (TFA) in MilliQ-treated water (solvent A) and 0.2 mL 100 mL^{-1} acetic acid in methanol (solvent B). For the UPLC separation, a linear elution gradient was applied (5% to 50% of solvent B in 34 min) at a flow rate of 0.2 mL min^{-1}. The LC eluate was analyzed by Full MS and data dependent tandem MS analysis (dd-MS^2) of five the most intense ions (Top 5). The resolution was set at 70000 and 17500 and the AGC targets were 1×10^6 and 1×10^5 for Full MS and dd-MS^2 scan types, respectively. The maximum ion injection times were 50 ms. The MS data were processed using Xcalibur software (Thermo Scientific). Metabolites were determined according to their calculated exact mass and absorption spectra. Their structures were confirmed by high resolution tandem MS (HR-MS/MS) and in comparison to reported assignments (Alonso-Salces, Guillou, & Berrueta, 2009).

2.4. Determination of total content of hydroxycinnamic acid derivatives

Total hydroxycinnamic acid content (THAC) was measured by spectrophotometric UV-Vis analysis monitoring the absorbance of the extract solutions at the maximum absorption wavelength of the hydroxycinnamic acid derivatives. Absorbance and fluorescence measurements were performed with Cary 50 Scan UV-Visible Spectrophotometer and Cary Eclipse Fluorescence Spectrophotometer equipped with a multi-well plate reader (Agilent Technologies, Santa Clara, CA, USA), respectively. All measurements were relative to a blank solution. Briefly, 30 µL of diluted sample (1 mg/mL) or standards were added to a quartz cuvette containing 970 µL of MilliQ water, and the absorbance at 325 nm was read against blank. Standard solutions (50–400 µg/mL) of 5-*O*-caffeoylquinic acid (5-CQA) were used for calibration (linear fitting R^2= 0.9999, n=4). Results were expressed as CGA equivalent (µg CGA eq)/mg of freeze-dried extract. Data were reported as means (±SD) of triplicate measures.

2.5. Total reducing capacity – Folin-Ciocalteu assay

Total reducing capacity was measured by Folin-Ciocalteu's phenol assay prior described by Singleton et al. (Singleton, Orthofer, & Lamuela-Raventós, 1999). Briefly, 200 µL of diluted samples or gallic acid standards and 100 µL of Folin's reagent were dispensed in a cuvette containing 1 mL of MilliQ water; then 1.2 mL of 10.75% (w/v) Na_2CO_3 solution was added, and the solution was mixed. After incubation for 30 min at 24 °C, absorbance was read at 760 nm against blank. Extract samples were diluted to 0.4 mg/mL, and standard solution (0–100 µg/mL) of gallic acid was used for calibration (linear fitting R^2= 0.9985, n=6). Results were expressed as µg of Gallic Acid Equivalent (GAE)/mg of freeze-dried extract. Data were reported as means (±SD) of triplicate measures.

2.6. Trolox Equivalent Antioxidant Capacity (TEAC-II/ABTS assay)

TEAC-II/ABTS method (Re et al., 1999) is based on the scavenging ability of antioxidants to the long-life intense colored radical cation $ABTS^{\cdot+}$. A 7 mM stock solution of $ABTS^{\cdot+}$ was produced by mixing an equal amount of a 14 mM ABTS solution and a 4.9 mM $K_2S_2O_8$ solution in MilliQ water (final concentration 7.00 mM and 2.45 mM, respectively). The mixture was left at 24 °C in dark for at least 12–16 h before use and stored at 4 °C for 7 d. A working solution of $ABTS^{\cdot+}$ was daily prepared by diluting the stock solution reaching an absorbance of 0.70 ± 0.05 at 734 nm. Fifty µL of sample or calibration standards were added in a cuvette containing 950 µL of $ABTS^{\cdot+}$ solution, and the absorbance at 734 nm was read after 6 min of incubation. The radical scavenging activity (% RSA) was evaluated by the equation:

$$\%RSA = [(A_0 - A_{sample/std})/ A_0] \times 100 \quad (1)$$

A_0= absorbance of blank; $A_{sample/std}$= Absorbance of sample or calibration standards.

GCE samples were diluted from the stock solution to 0.4 mg/mL, and a standard solution (0–500 µM) of Trolox was used for calibration (linear fitting, R^2= 0.9999, n=6). Results were expressed as µmol Trolox Equivalent (TE)/g of freeze-dried GCE as a mean (±SD) of triplicate measures.

2.7. Oxygen Radical Absorbance Capacity (ORAC-Fluorescein assay)

The oxygen radical antioxidant capacity (ORAC) assay is used to evaluate the capacity of antioxidant compounds that scavenge peroxyl radicals generated by 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), which prevent the degradation of the fluorescent probe (Ou, Hampsch-Woodill, & Prior, 2001). Briefly, 150 µL of a 117 nM fluorescein solution (in 75 mM PBS, pH 7.2) and 25 µL of diluted sample or calibration standards were added into a 96-well black microplate. After an incubation at 37 °C for 30 min, 25 µL of 240

mM AAPH solution were added, and the fluorescence (λ_{Ex} 485nm, λ_{Em} 520 nm) was recorded every 1 min for 240 min. GCE samples were diluted until 20 µg/mL from the stock solution, and standard solution (0–80 µM) of Trolox was used for calibration (linear fitting R^2= 0.9988, n=6). The final ORAC value was calculated from the net area (NET AUC= AUC_x – AUC_0) under the fluorescence decay curve, and the results were expressed as µmol Trolox Equivalent (TE)/g of freeze-dried GCE as a mean (±SD) of duplicate measures.

2.8. DPPH radical scavenging assay

DPPH assay is based on the scavenging of the stable free-radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$), according to the procedure described by Jeszka-Skowron and Zgoła-Grześkowiak (Jeszka-Skowron & Zgoła-Grześkowiak, 2014). Briefly, 950 µL of a diluted solution of DPPH in MeOH (100 µM, Abs 0.70 ± 0.05) and 50 µL of a diluted sample or calibration solution were added into a cuvette. After 30 min of incubation, absorbance at 515 nm was read and the %DPPH quench was evaluated by the equation:

$$\%DPPH_{quench} = [(A_0 - A_{sample/std})/ A_0] \times 100 \quad (2)$$

A_0= absorbance of blank; $A_{sample/std}$= Absorbance of sample or calibration standards.

GCE samples were diluted to 0.4 mg/mL from the stock solution. Standard solution (0–500 µM) of Trolox, obtained by serial dilution from a 500 mM stock solution in MilliQ water, was used as a calibrant (linear fitting R^2= 0.9991, n=8). Results were expressed as µmol Trolox Equivalent (TE)/g of freeze-dried extract. Data were reported as a mean (±SD) of triplicate measures.

2.9. *Carnorhabditis elegans* strain and its handling procedures

C. elegans wild-type N2 Bristol strain was used in this study and was provided by the *Caenorhabditis elegans* Genetics Centre (University of Minnesota, MN, USA). Animals were maintained at 20 °C on plates containing solid nematode growth medium (NGM) seeded with a live *Escherichia coli* strain (OP50) for food according to standard procedures (Brenner, 1974). To avoid the possibility that the treatments with GCE or 5-CQA could directly affect *Escherichia coli* and thus indirectly the nematodes (Liao et al., 2011), all experiments were performed with heat-killed (30 min at 65 °C) *E. coli* according to Gruber et al. (Gruber, Tang, & Halliwell, 2007).

2.10. Stress resistance assay

Ten 3-day-old N2 worms were placed on NGM plates seeded with live *E. coli* strain OP50 and allowed to lay eggs for 12 h at 20 °C to obtain a synchronous population. After removing the adult worms from the plates, newly laid eggs were grown for 3 d at 20 °C. Age-synchronized adult worms ($N \geq 56$) were transferred into freshly prepared NGM/OP50 plates with four different concentrations (0, 500, 1000 and 1500 mg/L) of GCE or 200 mg/L of 5-CQA (Sigma-Aldrich). The next day, the nematodes were moved to fresh NGM plates containing 10 mM paraquat (Sigma-Aldrich) as an oxidative stress inducer, in the presence or in the absence of GCE or 5-CQA. Every day living and dead worms were counted until all were dead. Worms not responding to mechanical stimulation with a platinum wire were scored as dead. Three independent experiments were performed.

2.11. Lifespan assay

Adult worms ($N \geq 41$) synchronized as described above were transferred onto fresh NGM plates seeded with OP50 in the absence or presence of 1000 mg/L GCE or 200 mg/L CGA. Fourty μM 5-Fluoro-2'-deoxyruridine (FuDR, Sigma-Aldrich) were added into the plates to prevent egg hatching. Animals were counted and transferred every other day until

all nematodes were dead. Survival curve and statistical analysis were performed with R software (available at https://www.r-project.org/); *p*-values were obtained using the log-rank test, Kaplan-Meier Survival function. Three independent experiments were performed.

2.12. Fertility assay

Ten 3-day-old N2 worms were allowed to lay eggs on the NGM plate for 12 h at 20 °C in the presence or in the absence of GCE. After three days at 20 °C, a single worm was moved to fresh NGM/OP50 plate with or without 1000 mg/L of GCE. Each plate contained one worm, and five worms were transferred to new plates at the same time every day until they stopped laying eggs. Every plate was examined after 24 h to count new eggs laid, and after 48 h of incubation at 20 °C to determine the number of larvae. Three independent experiments were performed.

3. Results and discussion

3.1. Preparation and characterization of green coffee extract

We prepared green coffee extracts (GCE)s from grounded green coffee beans by ultrasound-assisted H_2O:MeOH extraction, obtaining a 22% wt/wt yield (217.6 ± 16.1 mg out of 1 g). Further, we adopted a combined analytical approach based on NMR spectroscopy and UPLC/ESI-HRMS techniques for the identification of metabolites present in the samples. We performed a PDA detection following the UPLC separation, focusing the attention on polyphenols that show characteristic absorptions at 280 and 320 nm. The complete list of identified compounds, their assignments (obtained by NMR and/or HRMS) and the quantification of the most abundant ones are reported in Table S1. Figure 1 shows

chromatographic UPLC-UV trace at 280 nm (A) and ^{1}H-NMR spectrum of GCE at 2 mg/mL in 10 mM deuterated PBS pH 7.4. In agreement with literature data (Alonso-Salces et al., 2009), we found hydroxycinammic acid derivatives as the major constituents of the mixture (Fig. 2). Among them we clearly detected 5-*O*-caffeoylquinic acid (**3**) and its isomers 3-*O*-caffeoylquinic acid (**1**) and 4-*O*-caffeoylquinic acid (**2**), 5-*O*-feruloylquinic acid (**6**) and its isomers 3-*O*-feruloylquinic acid (**4**) and 4-*O*-feruloylquinic acid (**5**), 3,4- 3,5- and 4,5-di-*O*-caffeoylquinic acid (**7**, **8**, **9**). We also revealed the natural alkaloids caffeine (**12**) and trigonelline (**18**), and other metabolites and nutrients such as acetate (**10**), alanine (**11**), choline (**13**), citrate (**14**), formate (**15**), malate (**16**) and sucrose (**17**). We quantified the three major chlorogenic acids (**1**, **2**, **3**), caffeine (**12**) and trigonelline (**18**) by quantitative ^{1}H-NMR (Kwon et al., 2015). In addition, the total hydroxycinnamic acid derivative content was estimated by UV-VIS spectrophotometric analysis. We found that GCE contained 72 µg/mg of 5-CQA, as the most abundant isomer, 177 µg/mg of total chlorogenic acids (3-, 4- and 5-CQA) and, overall, 274 µg/mg of total hydroxycinnamic derivatives. A discrete amount of trigonelline (28 µg/mg) caffeine (39 µg/mg) and sucrose (331 µg/mg) were also determined (Table S1).

The antioxidant properties of GCE, that have been recently reviewed by (Ningjian & Kitts, 2014), can be ascribed mainly to their content in polyphenols. In general, the antioxidant power of a sample results from the combination of its activity against a number of different oxidant species and free radical sources, as well as different biochemical quenching mechanisms. For this reason, no single assay can efficiently evaluate the antioxidant capacity (AOC) of complex biological matrixes such as plant or food extracts. Therefore, in order to estimate the antioxidant power of GCE and its main component, we selected four different experimental protocols: (1) Folin-Ciocalteu (F-C), (2) Trolox Equivalent Antioxidant Capacity (TEAC-II/ABTS), (3) DPPH and (4) ORAC assay (Prior, Wu, & Schaich, 2005). F-C assay is based on an oxidation/reduction reaction, providing sample

reducing capacity as a result, deriving from total polyphenolic content. TEAC-II and DPPH methods measure the scavenging ability to the long-life $ABTS^{\cdot+}$ and the stable DPPH radicals, respectively, both based mainly on single electron transfer (SET). ORAC assay measures the antioxidant inhibition of peroxyl radical induced oxidation based on hydrogen atom transfer (HAT) mechanism (Schaich, Tian, & Xie, 2015). We reported the results of evaluation in Table 1. GCE showed an amount of total polyphenols equal to 140 µg of GAE (out of mg) and a free radical scavenging equal to 774.7 and 900.1 µmol of TE (out of g) in TEAC and DPPH assay, respectively. In addition, it showed an increased capacity of scavenging of peroxyl radical-related species, equal to of 3291.7 µmol of TE (out of g).

Data clearly indicate that the most relevant GCE anti-oxidant activity is exerted against oxygen free radicals, as demonstrated by the AOX measured trough the ORAC-FL assay. This finding is very interesting, as reactive oxygen species (ROS) are the most involved molecules in physiopathological processes, among which aging.

3.2. Effect of GCE on the stress resistance, fertility and aging of the *wild-type C. elegans*

3.2.1 GCE enhances the stress resistance of the C. elegans

To test the antioxidant activity of the GCE *in vivo*, in particular in a whole living organism, we pretreated synchronized 1-day-adult *C. elegans* worms with different concentrations of GCE (500, 1000 and 1500 mg/L) followed by exposure to 10 mM paraquat, an intracellular free-radical-generating compound. We monitored time-course survival under oxidative stress in the presence of the extract until all animals died. The results showed that GCE extended the lifespan at all tested concentrations (Fig. 3). In particular, the mean survival time of the worms treated with 0, 500 and 1000 mg/L of GCE increased from 55.7±0.2 h (control) to 67.3±0.3 h, 76.6±0.4 h, respectively, suggesting a significant dose-dependent protective effect of GCE against oxidative stress. The maximum effect was obtained at

1000 mg/L concentration (37.5% increase of mean lifespan). In fact, using 1500 mg/L concentration we obtained an extension of the mean lifespan similar to the one induced by a concentration of 1000 mg/L (74.3±0.2 h). For this reason, we performed the following experiments using 1000 mg/L GCE.

3.2.2 GCE extends the lifespan of C. elegans

A strong correlation between aging and oxidative stress caused by the deleterious and cumulative effects of reactive oxygen species (ROS) generated throughout the lifespan of *C. elegans* has been demonstrated (Harman, 1998; Knight, 2001). Since GCE was able to prevent oxidative stress, we further examined its effect on the lifespan of *C. elegans*. We compared the lifespan of untreated (control) and 1000 mg/L GCE-treated nematodes (Fig. 4). We found that the mean and maximum lifespans of the control were 13±0.4 and 23 days in comparison to the 19±0.5 and 29 days obtained in the presence of GCE. Mean lifespan significantly ($p<0.001$) increased by 46.1%.

3.2.3 GCE does not affect brood size of C. elegans but modifies the time-course distribution of fertility

Previous studies have reported that many long-lived *C. elegans* mutants exhibit a reduced reproductive capacity phenotype (Gems et al., 1998; Hughes, Evason, Xiong, & Kornfeld, 2007). Furthermore, chemical compounds with lifespan-extending effects also reduce the reproduction of this nematode *(*Harrington & Harley*,* 1988*)*. For this reason, we determined the effect of GCE on reproduction. We measured egg-laying and progeny production of worms treated with 1000 mg/L of GCE (Fig. 5). The results did not show any significant differences in the total fecundity of egg-laying between the control and treated animals (226.7±18.4 and 212.0±10.6, respectively). Nevertheless, we observed a statistically

significant delay in the distribution of the reproductive capacity in the worms treated with GCE in comparison to the control (1-day later).

3.3. Comparative effects of oxidative stress resistance and longevity of *C. elegans* induced by GCE and 5-CQA treatment

To compare the oxidative stress resistance properties of GCE and its most abundant polyphenolic component the pure 5-CQA, we performed the same experiment described above treating the worms with 1000 mg/L of GCE or 200 mg/L of 5-CQA. We employed the equivalent amount of total CQA present in the extract as estimated by NMR analysis (Table 1). We found that the addition of the GCE enhanced the stress resistance by 27.7% in comparison to the 16.7% of the isolated 5-CQA (Fig. 6a). Subsequently, we analyzed the effect of the above-indicated concentrations of GCE and 5-CQA on the lifespan of *C. elegans*. We observed that the GCE extended the lifespan of 46.1% in comparison to the 23.0% of the 5-CQA (Fig. 6b). The major effect displayed by GCE in both experiments strongly suggests a positive synergy among different components of the whole extract.

4. Conclusions

In this work, we demonstrated for the first time that the green coffee extract (GCE) is capable of extending lifespan and delaying aging using the *in vivo* model organism *Caenorhabditis elegans*. Indeed, we showed that the GCE is able to significantly enhance the oxidative stress resistance and thus prolong the lifespan of *C. elegans.* These capabilities could be ascribed to the antioxidant properties of the polyphenolic component of the extract. We also demonstrated that the lifespan extension of *C. elegans* does not correlate with a fertility reduction, as expected, but it is associated with a delayed

distribution of the reproductive capacity. GCE analysis, performed by NMR and UPLC/ESI-HRMS techniques, clearly confirmed its significant content in polyphenols. Among these, caffeoylquinic acid (CQAs), ferrulolyl quinic acids (FQAs) and di-caffeoyl quinic acids (Di-CQAs) are the most representative, and the 5-CQA is the most abundant. Comparison between the activity of the GCE and pure 5-CQA on the *C. elegans* stress resistance and lifespan clearly demonstrated the major effect of the extract in comparison to the pure 5-CQA. This result strongly supports the employment of a mixture of bioactive compounds, instead of a single molecule, for the development of new nutraceutical and pharmaceutical tools.

Acknowledgements

This work was supported by Fondazione Cariplo, Regione Lombardia and Comune di Milano, grant H42I14000270003 with the project entitled 'Caffè Corretto - la via del caffè tra Guatemala, El Salvador e Italia'.

References

Alonso-Salces, R. M., Guillou, C., & Berrueta, L. A. (2009). Liquid chromatography coupled with ultraviolet absorbance detection, electrospray ionization, collision-induced dissociation and tandem mass spectrometry on a triple quadrupole for the on-line characterization of polyphenols and methylxanthines in green coffee beans. *Rapid Communications in Mass Spectrometry, 23*(3), 363-383.

Bass, T. M., Weinkove, D., Houthoofd, K., Gems, D., & Partridge, L. (2007). Effects of resveratrol on lifespan in Drosophila melanogaster and Caenorhabditis elegans. *Mechanisms of ageing and development, 128*(10), 546-552.

Boeing, H. (2013). Nutritional epidemiology: New perspectives for understanding the diet-disease relationship&quest. *European journal of clinical nutrition, 67*(5), 424-429.

Braeckman, B. P., & Vanfleteren, J. R. (2007). Genetic control of longevity in C. elegans. *Experimental gerontology, 42*(1), 90-98.

Brenner, S. (1974). The Genetics of CAENORHABDITIS ELEGANS. *Genetics, 77*(1), 71-94.

Clifford, M. N. (1985). Coffe Volume 1: Chemistry: Elsevier Applied Science Publication, London, UK.

Cobas, C., Seoane, F., Domínguez, S., Sykora, S., & Davies, A. N. (2011). A new approach to improving automated analysis of proton NMR spectra through Global Spectral Deconvolution (GSD). *Spectroscopy Europe, 23*(1), 26-30.

Farah, A., & Donangelo, C. M. (2006). Phenolic compounds in coffee. *Brazilian Journal of Plant Physiology, 18*(1), 23-36.

Finkel, T., & Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature, 408*(6809), 239-247.

Fontana, L., Partridge, L., & Longo, V. D. (2010). Extending healthy life span—from yeast to humans. *science, 328*(5976), 321-326.

Gems, D., Sutton, A. J., Sundermeyer, M. L., Albert, P. S., King, K. V., Edgley, M. L., . . . Riddle, D. L. (1998). Two pleiotropic classes of daf-2 mutation affect larval arrest, adult behavior, reproduction and longevity in Caenorhabditis elegans. *Genetics, 150*(1), 129-155.

Gruber, J., Tang, S. Y., & Halliwell, B. (2007). Evidence for a Trade-Off between Survival and Fitness Caused by Resveratrol Treatment of Caenorhabditis elegans. *Annals of the New York Academy of Sciences, 1100*(1), 530-542.

Harman, D. (1998). Aging and oxidative stress. *Journal of the International Federation of Clinical Chemistry/IFCC, 10*(1), 24-27.

Harrington, L. A., & Harley, C. B. (1988). Effect of vitamin E on lifespan and reproduction in Caenorhabditis elegans. *Mechanisms of ageing and development, 43*(1), 71-78.

Herndon, L. A., Schmeissner, P. J., Dudaronek, J. M., Brown, P. A., Listner, K. M., Sakano, Y., . . . Driscoll, M. (2002). Stochastic and genetic factors influence tissue-specific decline in ageing C. elegans. *Nature, 419*(6909), 808-814.

Hughes, S. E., Evason, K., Xiong, C., & Kornfeld, K. (2007). Genetic and pharmacological factors that influence reproductive aging in nematodes. *PLoS Genetics, 3*(2), e25.

Jeszka-Skowron, M., & Zgoła-Grześkowiak, A. (2014). Analysis of Antioxidant Activity, Chlorogenic Acid, and Rutin Content of Camellia sinensis Infusions Using Response Surface Methodology Optimization. [journal article]. *Food Analytical Methods, 7*(10), 2033-2041.

Kaur, T., Bhat, H. A., Bhat, R., Kumar, A., Bindu, K., Koul, S., & Vyas, D. (2015). Physio-chemical and antioxidant profiling of Salvia sclarea L. at different climates in north-western Himalayas. *Acta Physiologiae Plantarum, 37*(7), 1-10.

Knight, J. A. (2001). The biochemistry of aging. *Advances in clinical chemistry, 35*, 1-62.

Kwon, D.-J., Jeong, H.-J., Moon, H., Kim, H.-N., Cho, J.-H., Lee, J.-E., . . . Hong, Y.-S. (2015). Assessment of green coffee bean metabolites dependent on coffee quality using a 1H NMR-based metabolomics approach. *Food Research International, 67*, 175-182.

Lee, K.-W., Im, J.-Y., Woo, J.-M., Grosso, H., Kim, Y.-S., Cristovao, A. C., . . . Fernandez, J. R. (2013). Neuroprotective and anti-inflammatory properties of a coffee component in the MPTP model of Parkinson's disease. *Neurotherapeutics, 10*(1), 143-153.

Liao, V. H.-C., Yu, C.-W., Chu, Y.-J., Li, W.-H., Hsieh, Y.-C., & Wang, T.-T. (2011). Curcumin-mediated lifespan extension in Caenorhabditis elegans. *Mechanisms of ageing and development, 132*(10), 480-487.

Ningjian, L., & Kitts, D. D. (2014). Antioxidant Property of Coffee Components: Assessment of Methods that Define Mechanisms of Action. [Article]. *Molecules, 19*(11), 19180-19208.

Oguntibeju, O., Esterhuyse, A., & Truter, E. (2013). *The role of fruit and vegetable consumption in human health and disease prevention*: INTECH Open Access Publisher.

Ong, K. W., Hsu, A., & Tan, B. K. H. (2012). Chlorogenic acid stimulates glucose transport in skeletal muscle via AMPK activation: a contributor to the beneficial effects of coffee on diabetes. *PloS one, 7*(3), e32718.

Ou, B., Hampsch-Woodill, M., & Prior, R. L. (2001). Development and Validation of an Improved Oxygen Radical Absorbance Capacity Assay Using Fluorescein as the Fluorescent Probe. *Journal of Agricultural and Food Chemistry, 49*(10), 4619-4626.

Perrone, D., Farah, A., & Donangelo, C. M. (2012). Influence of Coffee Roasting on the Incorporation of Phenolic Compounds into Melanoidins and Their Relationship with Antioxidant Activity of the Brew. *Journal of Agricultural and Food Chemistry, 60*(17), 4265-4275. doi: 10.1021/jf205388x

Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. *Journal of Agricultural and Food Chemistry, 53*(10), 4290-4302.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine, 26*(9–10), 1231-1237.

Rocha, L. D., Monteiro, M. C., & Teodoro, A. J. (2012). Anticancer properties of hydroxycinnamic acids-A Review. *Cancer and clinical oncology, 1*(2), 109.

Sato, Y., Itagaki, S., Kurokawa, T., Ogura, J., Kobayashi, M., Hirano, T., . . . Iseki, K. (2011). In vitro and in vivo antioxidant properties of chlorogenic acid and caffeic acid. *International Journal of Pharmaceutics, 403*(1), 136-138.

Schaich, K. M., Tian, X., & Xie, J. (2015). Hurdles and pitfalls in measuring antioxidant efficacy: A critical evaluation of ABTS, DPPH, and ORAC assays. *Journal of Functional Foods, 14*(0), 111-125.

Sikora, E., Scapagnini, G., & Barbagallo, M. (2010). Curcumin, inflammation, ageing and age-related diseases. *Immunity & Ageing, 7*(1), 1-4. doi: 10.1186/1742-4933-7-1

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent *Methods in Enzymology* (Vol. 299, pp. 152-178): Academic Press.

Trugo, L. C., & Macrae, R. (1984). A study of the effect of roasting on the chlorogenic acid composition of coffee using HPLC. *Food Chemistry, 15*(3), 219-227.

Wei, F., Furihata, K., Hu, F., Miyakawa, T., & Tanokura, M. (2010). Complex mixture analysis of organic compounds in green coffee bean extract by two-dimensional NMR spectroscopy. *Magnetic Resonance in Chemistry, 48*(11), 857-865.

Wei, F., Furihata, K., Koda, M., Hu, F., Kato, R., Miyakawa, T., & Tanokura, M. (2012). 13C NMR-Based Metabolomics for the Classification of Green Coffee Beans According to Variety and Origin. *Journal of Agricultural and Food Chemistry, 60*(40), 10118-10125.

Wilson, M. A., Shukitt-Hale, B., Kalt, W., Ingram, D. K., Joseph, J. A., & Wolkow, C. A. (2006). Blueberry polyphenols increase lifespan and thermotolerance in Caenorhabditis elegans. *Aging cell, 5*(1), 59-68.

Yashin, A., Yashin, Y., Wang, J. Y., & Nemzer, B. (2013). Antioxidant and antiradical activity of coffee. *Antioxidants, 2*(4), 230-245.

Figure captions

Fig. 1. Panel A: UPLC-PDA chromatogram of GCE. Absorbance at 280 nm. Metabolites were identified according to absorption spectra, calculated exact mass and HR-MS/MS. **Panel B: ^{1}H-NMR spectrum of GCE** at 2 mg/mL in deuterated PB 10 mM pH 7.4 with DSS 0.5 mM as an internal standard.

Fig. 2. Structures of compounds 1-9, **12** and **18** reported in Table S1.

Fig. 3. Effect of GCE on resistance to oxidative stress in *C. elegans*.

1-day-adult animals (N ≥ 56) were treated with different concentration of GCE (0, 500, 1000 or 1500 mg/L) and exposed to 10 mM paraquat on the next day. Paraquat was used as an oxidative stress inducer. Survived and dead worms were counted after 24, 48, 72, 96 and 137 h of chronicle exposure to the drug (panel A). Statistical analysis was reported in the panel B.

Fig. 4. Effect of GCE on the lifespan of *C. elegans.*

On day 0, synchronized worms (N ≥ 55) were transferred in parallel into normal NGM plate and in NGM plate supplemented with 1000 mg/L of GCE. Survived worms were

determined every other day, and the lifespans obtained in absence and presence of the GCE were compared. The survival curve reported in the panel A is representative of one experiment. Statistical significance of the difference between the curves was demonstrated by log-rank test using the Kaplan-Meier survival analysis (panel B).

Fig. 5. Effect of GCE on reproduction of *C. elegans.*

Synchronized 1-day-adult worms were moved to NGM plates in the absence (Control) or presence (1000 mg/L) of GCE and transferred individually to a fresh plate every day until reproduction stopped. Time-course distribution of fertility and total number were determined. Error bars represent the standard error (SE). Significance (*) was determinate with Student's *t*-test ($p<0.05$).

Fig. 6. Comparative effect of resistance to oxidative stress and lifespan induced by GCE and 5-CQA in *C. elegans*.

1-day-adult animals ($N \geq 59$) were moved to plates containing 1000 mg/L GCE or 200 mg/L 5-CQA and exposed to 10 mM paraquat on the next day (panel A). 1-day-adult animals ($N \geq 41$) were moved to plates containing 1000 mg/L GCE or 200 mg/L 5-CQA, and survived worms were counted every other day. The lifespans obtained in absence and presence of the GCE were compared (panel C). Statistical analysis was reported in panel B and D, respectively.

Table 1

Table 1 – Total CGA content and antioxidant properties of GCE.

CGA content	
Total caffeoylquinic acids 3-,4-,5-CQA assessed by NMR analysis (µg/mg)	176.7 ± 12.0
Total Content of hydroxycinammic acids (µg CGA eq/mg)	274.3 ± 10.4
Antioxidant Capacity (AOX) evaluation	
Folin-Ciocalteu (F-C) assay (µg GAE/ mg)	140.7 ± 15.0
ABTS/TEAC assay (µmol TE/g)	774.7 ± 73.5
ORAC-FL (µmol TE/g)	3291.7 ± 16.5
DPPH assay (µmol TE/g)	900.1 ± 99.2
Results are expressed as Trolox equivalent (TE)[a] or Gallic acid equivalent (GAE)[b] for g or mg of freeze-dried GCE (mean ± SD of triplicate measurements of three independent extractions).	

Table S1

Table S1 - Characterization of GCE by UPLC/ESI-HRMS and ^{1}H-NMR analyses.

#	Name	Abbrev	RT (min)	molecular formula	theor *m/z* $[M+H^+]^+$	exp *m/z* $[M+H^+]^+$	error (ppm)	theor *m/z* $[M-H^+]^-$	exp *m/z* $[M-H^+]^-$	error (ppm)	^{1}H chemical shifts (ppm)	Quantity (µg/mg)	StdDev (SD)
1	3-*O*-caffeoyl quinic acid	3-CQA	13.6	$C_{16}H_{18}O_9$	355.1024 377.0843[a] 731.1794[b]	355.1024 377.0845[a] 731.1795[b]	0.0 0.5 0.3	353.0878 707.1829[d]	353.0878 707.1833[d]	0.0 0.6	7.64 (d) - 7.18 (d) - 7.12 (dd) - 6.91 (d) - 6.39 - 5.38 - 4.13 - 3.75 - 2.20 - 2.09 - 1.94		
2	4-*O*-caffeoyl quinic acid	4-CQA	19.7	$C_{16}H_{18}O_9$	355.1024 377.0843[a]	355.1025 377.0845[a]	0.3 0.5	353.0878 707.1829[d]	353.0878 707.1831[d]	0.0 0.3	7.70 (d) - 7.18 (d) - 7.12 (dd) - 6.91 (d) - 6.44 (d) - 4.91 (dd) - 4.33 - 4.23 - 2.15 - 2.14 - 2.03		
3	5-*O*-caffeoyl quinic acid	5-CQA	19.2	$C_{16}H_{18}O_9$	355.1024 377.0843[a] 731.1794[b]	355.1024 377.0844[a] 731.1796[b]	0.0 0.3 0.3	353.0878 707.1829[d]	353.0878 707.1833[d]	0.0 0.6	7.65 (d) - 7.18 (d) - 7.12 (dd) - 6.91 (d) - 6.38 (d) - 5.32 - 4.23 (d) - 3.87 (dd) - 2.19 - 2.13 - 2.04 - 2.03	72.33	6.13
4	3-*O*-feruloyl quinic acid	3-FQA	18.7	$C_{17}H_{20}O_9$	369.118	369.1182	0.5	367.1035	367.1034	-0.3			
5	4-*O*-feruloyl quinic acid	4-FQA	26.2	$C_{17}H_{20}O_9$	369.118 391.1000[a]	369.1182 391.1000[a]	0.5 0.0	367.1035	367.1033	-0.5			
6	5-*O*-feruloyl quinic acid	5-FQA	24.6	$C_{17}H_{20}O_9$	369.118 391.1000[a]	369.1183 391.1001[a]	0.8 0.3	367.1035	367.1034	-0.3			
7	3.4-di-*O*-caffeoyl quinic acid	3.4-diCQA	30.9	$C_{25}H_{24}O_{12}$	517.1341 539.1160[a] 499.1235[c]	517.1346 539.1166[a] 499.1239[c]	1.0 1.1 0.8	515.1195	515.1195	0.0			
8	3.5-di-*O*-caffeoyl quinic acid	3.5-diCQA	31.5	$C_{25}H_{24}O_{12}$	517.1341 539.1160[a] 499.1235[c]	517.1346 539.1165[a] 499.1239[c]	1.0 0.9 0.8	515.1195	515.1195	0.0			
9	4.5-di-*O*-caffeoyl quinic acid	4.5-diCQA	34.4	$C_{25}H_{24}O_{12}$	517.1341 539.1160[a] 499.1235[c]	517.1345 539.1163[a] 499.1237[c]	0.8 0.6 0.4	515.1195	515.1193	-0.4			
10	Acetate										1.91 (s)		
11	Alanine										1.47 (d) - 3.78	1.00	0.00
12	Caffeine	CAFF	18.7	$C_8H_{10}N_4O_2$	195.0877	195.0877	0.0	- - -	- - -	- - -	7.80 (s) - 3.86 (s) - 3.44 (s) - 3.26 (s)	38.67	2.62
13	Choline										4.05 - 3.51 - 3.19 (s)		
14	Citrate										2.69 - 2.54		
15	Formate										8.45 (s)		
16	Malate										4.29 - 2.67 - 2.37		
17	Sucrose										5.40 (d) - 4.20 (d) - 4.04 (t) - 3.88 - 3.84 - 3.81 - 3.80 - 3.75 (t) - 3.67 (s) - 3.55 (dd) - 3.46 (t)	331.67	20.17
18	Trigonelline	TRIG	1.9	$C_7H_7NO_2$	138.0549	138.0549	0.0				9.11 (s) - 8.82 - 8.05 (t) - 4.42 (s)	28.33	0.47
19	Unknown	unkn	23.2	$C_{18}H_{34}O_9N_3$	437.2368	437.2360	1.8	- - -	- - -	- - -			
20	Unknown	unkn	29.8	$C_{27}H_{32}O_6N_4$	509.2395	509.2384	2.2	- - -	- - -	- - -			
21	Unknown	unkn	29.8		- - -	- - -	- - -		621.2168				
22	Unknown	unkn	31.5		- - -	- - -	- - -		621.2168				

[a] $[M+Na^+]^+$; [b] $[2M+Na^+]^+$; [c] $[M-H_2O+H^+]^+$; [d] $[2M-H^+]^-$

Figure 1

Click here to download Figure: Figure 1.pdf

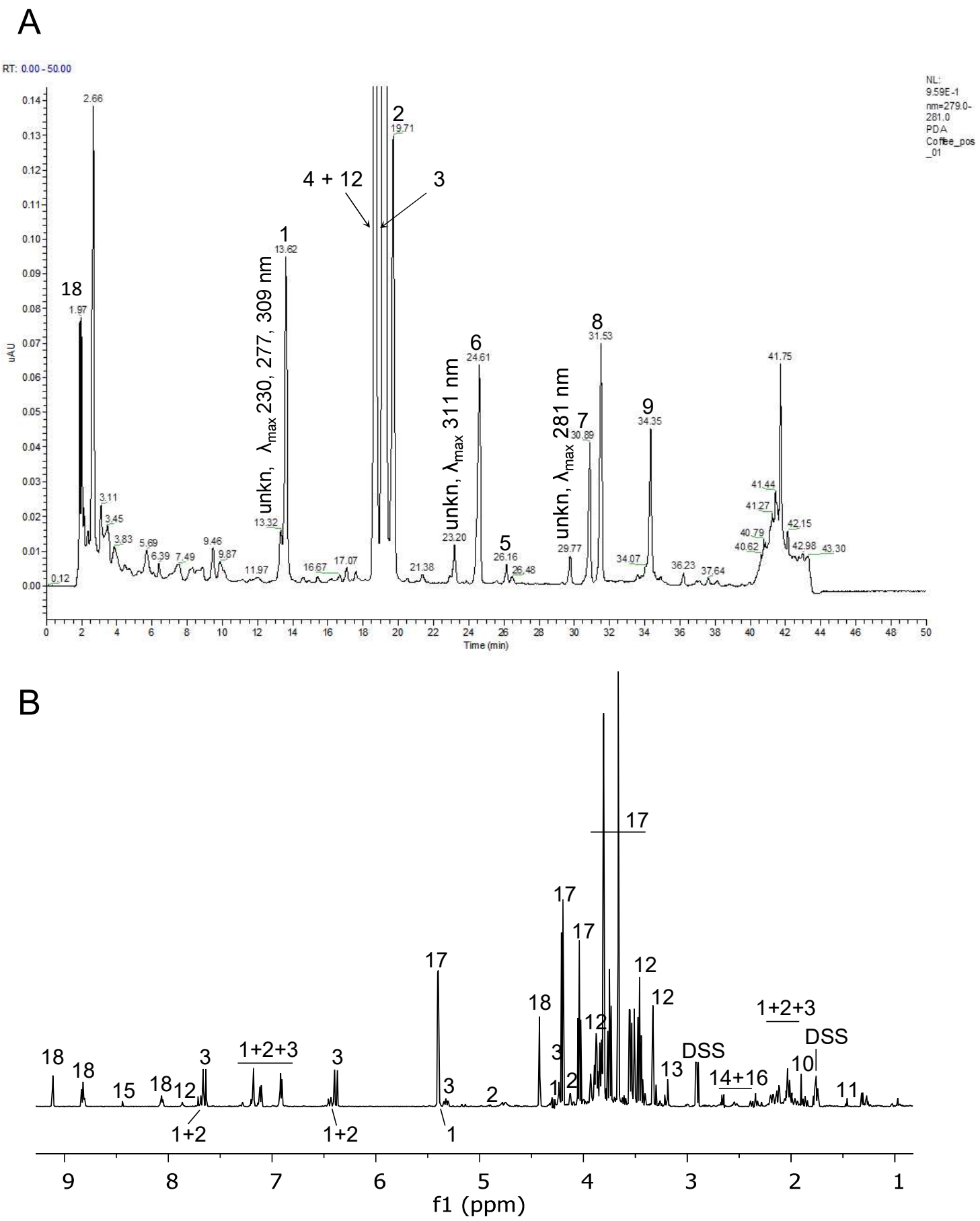


Figure 2

Click here to download Figure: Figure 2.pdf

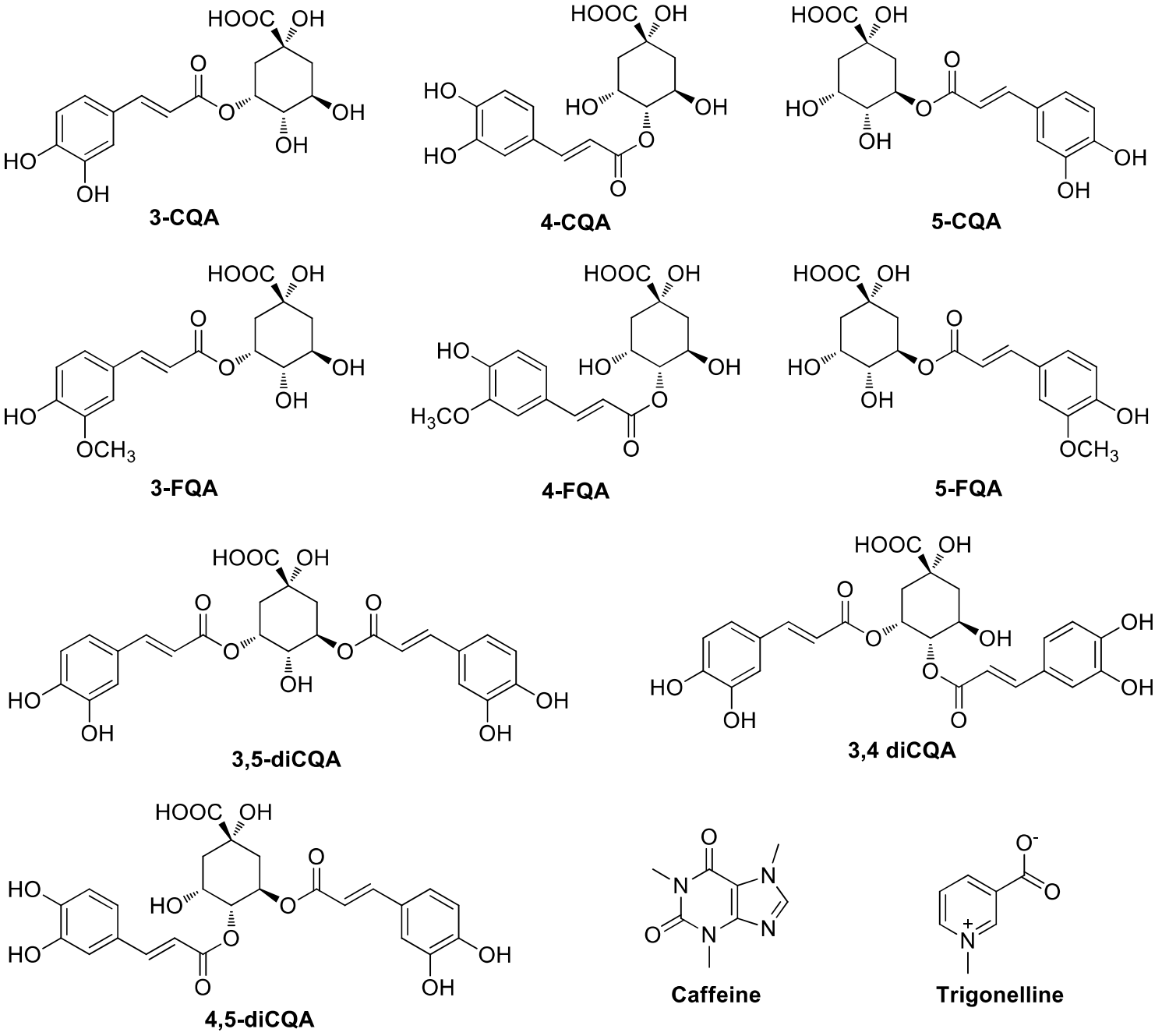
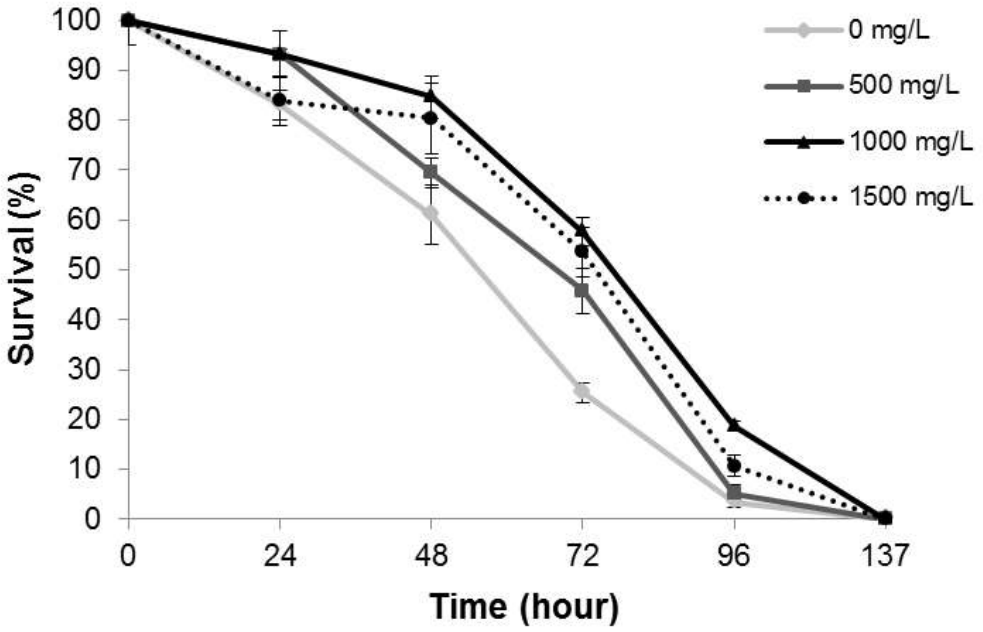


Figure 3
Click here to download Figure: Figure 3.pdf

A.



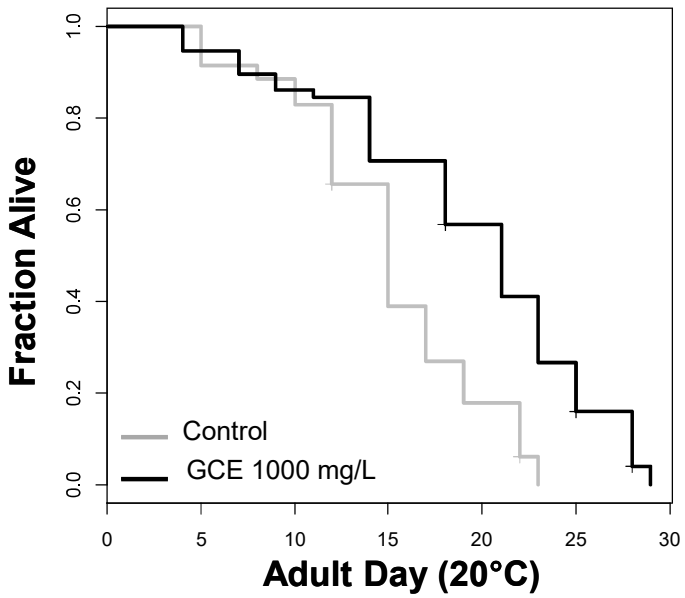
B. Effect of GCE on resistance to chronic challenge with 10 mM paraquat in *C. elegans*

GCE concentration (mg/L)	Number of worms	Mean survival time (h)(1)	*P*-value(2)	% effect(3)
0	59	55.7±0.20		
500	59	67.3±0.32	0.016	20.8
1000	59	76.6±0.35	<0.001	37.5
1500	56	74.3±0.21	0.004	33.3

1) Mean survival time was the time when 50% of nematodes used in the assay survived. Mean ± SEM was reported.
2) *P*-value was calculated using the log-rank test by comparing each concentration of the GCE-treated group with control (0 mg/L of GCE).
3) % effect was calculated by (T-C)/C*100, where T is the mean survival time of *C. elegans* treated with each concentration of GCE and C is the mean survival time of control.

Figure 4
Click here to download Figure: Figure 4.pdf

A.



B. Effect of 1000 mg/L GCE on longevity in *C. elegans*

	Number of worms	Mean lifespan (day)(1)	Maximum lifespan (day)(2)	*P*-value(3)	% effect(4)
Control	55	13±0.4	23		
GCE	58	19±0.5	29	<0.001	46.15

1) Mean lifespan was the day when 50% of nematodes used in the assay survived. Mean ± SEM was reported.
2) Maximum lifespan was the oldest age reached by the last surviving worm in each group.
3) *P*-value was calculated using the log-rank test by comparing the control and GCE treated group.
4) % effect was calculated by (T-C)/C*100, where T is the mean lifespan of *C. elegans* treated with GCE and C is the mean lifespan of control.

Figure 5

Click here to download Figure: Figure 5.pdf

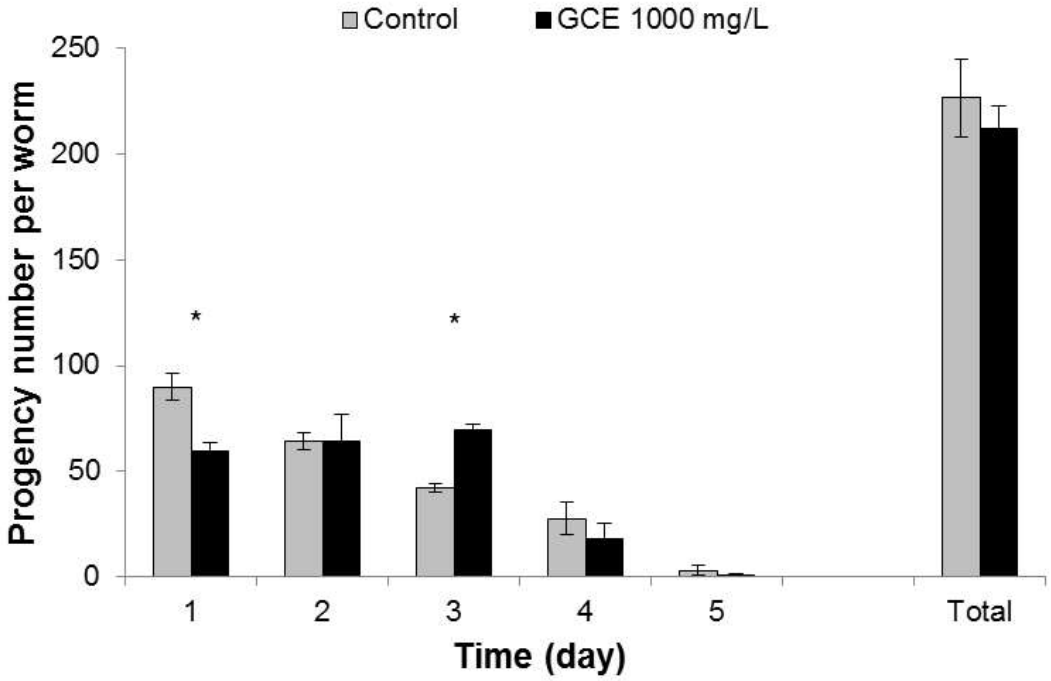
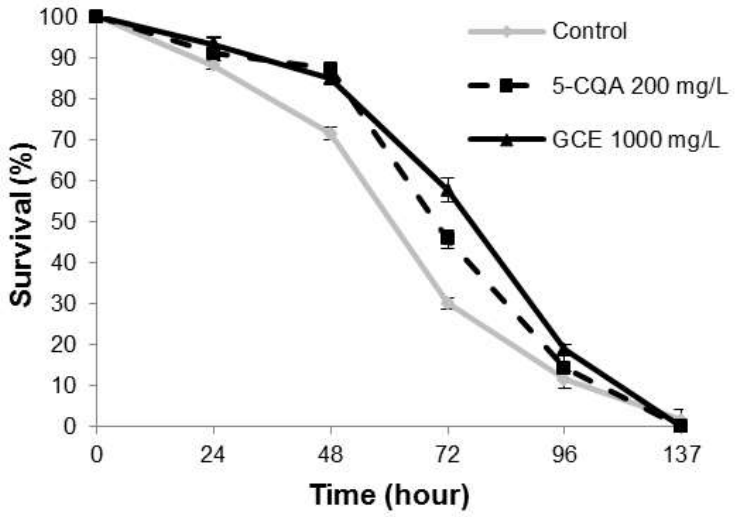


Figure 6

Click here to download Figure: Figure 6.pdf

A.

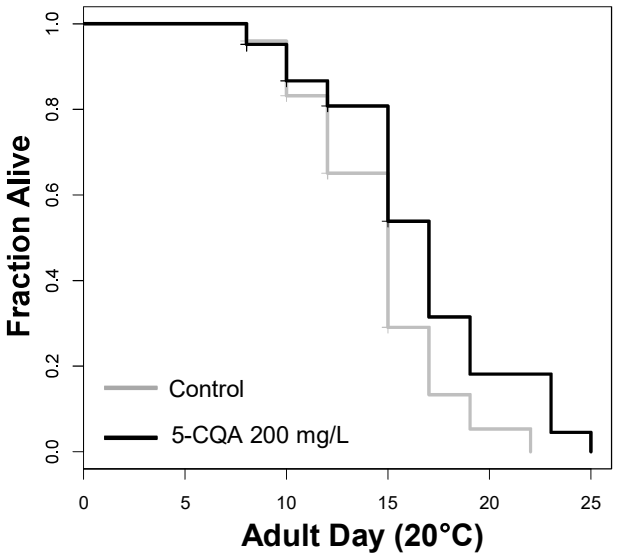


B. Comparison between the effect of GCE and 5-CQA on resistance to chronic challenge with 10mM paraquat in *C. elegans*

	Number of worms	Mean survival time (h)(1)	*P*-value(2)	% effect(3)
Control	70	60.3±0.50		
GCE	59	76.6±0.35	0.0105	27.7
5-CQA	70	70.0±0.25	0.0461	16.7

1) Mean survival time was the time when 50% of nematodes used in the assay survived. Mean ± SEM was reported.
2) *P*-value was calculated using the log-rank test by comparing each treated group with control (not treated).
3) % effect was calculated by (T-C)/C*100, where T is the mean survival time of *C. elegans* treated with GCE or 5-CQA and C is the mean survival time of control.

C.



D. Effect of 200 mg/L 5-CQA on longevity in *C. elegans*

	Number of worms	Mean lifespan (day)(1)	Maximum lifespan (day)(2)	*P*-value(3)	% effect(4)
Control	47	13±0.60	22		
5-CQA	41	16±0.75	25	0.0102	23.07

1) Mean lifespan was the day when 50% of nematodes used in the assay survived. Mean ± SEM was reported.
2) Maximum lifespan was the oldest age reached by the last surviving worm in each group.
3) *P*-value was calculated using the log-rank test by comparing the control and 5-CQA treated group.
4) % effect was calculated by (T-C)/C*100, where T is the mean lifespan of *C. elegans* treated with 5-CQA and C is the mean lifespan of control.