Journal of Medicinal Food

Journal of Medicinal Food: http://mc.manuscriptcentral.com/medicinalfood

Antioxidant and anti-aging effects of Licorice on the Caenorhabditis elegans model

Journal:	*Journal of Medicinal Food*
Manuscript ID	JMF-2019-0081
Manuscript Type:	Original Article
Date Submitted by the Author:	10-Apr-2019
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Keyword:	Antioxidant Activity, Bioactivity, Catalase, Dietary supplementation, phenolics
Manuscript Keywords (Search Terms):	ageing, antioxidant, oxidative stress, Glycyrrhiza glabra, catalase, Caenorhabditis elegans

Title: Antioxidant and anti-aging effects of Licorice on the *Caenorhabditis elegans* model.

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ABSTRACT

The causative relationship between oxidative stress and ageing remains controversial, but it is a fact that many of the pathologies of age-related diseases are associated with oxidative stress. Phytochemicals may reduce damage from oxidative stress; the intake of these through diet could represent a strategy to lessen its pathological consequences. The popular and wide consume licorice (*Glycyrrhiza glabra)* is a rich source of potential antioxidants. The aim of this study was to investigate if licorice increases the oxidative stress resistance and lifespan of the animal model *Caenorhabditis elegans*.

Licorice roots ethanolic extract showed *in vitro* antioxidant activity, with an IC_{50} of 51.17 µg/mL using DPPH as free radical. *Caenorhabditis elegans* pretreated with licorice showed an increase of survival rate when exposed to the oxidant juglone, being this increase up to 33.56± 2.97 %. This pretreated population also showed an increase in lifespan of 14.28 % at a concentration of 250 µg/mL.

In conclusion, we suggest that licorice has a high antioxidant capability both *in vitro* and *in vivo* and that this activity may explain the observed extension of lifespan.

Keywords list: Ageing, antioxidant, catalase*, Glycyrrhiza glabra,* oxidative stress, *Caenorhabditis elegans.*

1. INTRODUCTION

Oxidative stress has been correlated with numerous pathological states including cardiovascular diseases, hypertension, diabetes mellitus, cancer and carcinogenesis, neurodegenerative problems and inflammation (1, 2). These conditions are age-related diseases, and are likely to be exacerbate by the decreased ability to survive stress associated with ageing (3). Antioxidants are usually considered as a possible tool to treat or diminish the consequences of these pathologies (4). Phytochemicals are especially interesting as they not only lessen the effects of free radicals but also modulate life prolonging mechanistic pathways (5), and on the other hand, they are frequently in foods commonly consumed by the population. A clear example is licorice (*Glycyrrhiza glabra),* a rich source of antioxidant phytochemicals (6, 7) but which consumption, comes from the fact that its sweet-flavor characteristics makes it very popular in the production of candies (8). This plant has not only been part of diet, but also as part of the Chinese traditional medicine (9), and in fact, licorice has been associated with many pharmacological properties, such as anti-allergic(10), ulcer-healing (11), and anti-inflammatory activities (12). This bioactivity is thought to be due to the presence of triterpenoid saponins, with glycyrrhizin being the most relevant followed by glycyrrhizic acid (13).

Many of the proprieties of licorice have been studied, but its effects on aging have never been reported. *Caenorhabditis elegans* represents a useful tool to study ageing, not only because its short and well-studied life cycle, but also because many manipulations that increase stress resistance also increase *C.elegans* longevity (14). The *C.elegans* genome encodes enzymes of the endogenous antioxidant defense systems such as catalase (CAT),

glutathione peroxidase (GPx), peroxiredoxins (Prxs) and superoxide dismutase (SOD) (15).

The aim of this study was to test if licorice ethanolic extract improves the ability of *C.elegans* to face lethal oxidative stress and to study the influence of this plant in the antioxidant defensive system of the worm, by measuring CAT activity. Lifespan assay was also carried out, to check if the antioxidant capability of licorice could be correlated with a possible increase on the worm lifespan.

2. MATERIALS AND METHODS

2.1 Plant material and extraction

Dry licorice roots were provided by Plantarom®. They were crushed to homogeneous particle size. A soxhlet equipment was used to make the extraction. 50 g of the crushed licorice were extracted with 500 mL of ethanol for 4 h. Solvent was removed from the extract with a rotary flash evaporator and the extract was then preserved at - 20 ºC in order to avoid degradation until its use.

2.2 HPLC analysis of glycyrrhizin

The HPLC analysis was performed with a Waters Alliance e2695 equipped with a 2998 Photodiode Array Detector (PDA) and a 2414 Refractive Index Detector (ri) and chromatographic separations were performed on a C18 (150x4.6mm 2.7um) column. Gradient elution was used with a mobile phase containing water A (H_2O), glacialaceticacid B (CH_3COOH) and acetonitrile C (CH_3CN) in the following manner, 0-10 min (60% A; 30% B; 10% C); 10-18 min (10%A; 30%B; 60% C); 18-25 min (60% A;

30% B; 10% C) at a flow rate of 0.8 mL/min. Separations were carried out at 40ºC with an injection loop of 20 µg/mL. The analysis was performed at 254 nm. Glycyrrhizin content of the licorice extract was identified by comparing its retention times and UV spectra with those of the standard under identical analysis conditions. Solutions containing different concentrations of glycyrrhizin standards were made to calibrate concentration and peak areas, allowing calculation of the glycyrrhizin content of the licorice extract.

2.3 Strains and culture conditions

SS104 and N2 *C.elegans* strains used in this study were obtained from Caenorhabditis Genetics Center (CGC) USA. SS104 glp-4(bn2) I strain is a temperature sensitive defect in germ-line proliferation during larval development. Defect can be reversed by shifting worms from restrictive (25 ºC) to permissive temperature (16 ºC). SS104 was maintained at 16 ºC and N2 was maintained at 20 ºC on Nematode Growth Media (NGM) agar plates with *Escherichia coli* OP50 as the food source. The medium was prepared using: 0.003 g/mL NaCl; 0.005 g/mL peptone; 0.02g/mL high purity agar; 0.1% Cholesterol; 0.1%$MgSO_4$ (1M); 0.1% $CaCl_2$ (1M) and 2.5% potassium phosphate buffer (1M). All chemicals from Sigma-Aldrich.

2.4 Antioxidant activity *in vitro*: DPPH method

To measure the antioxidant activity a method based on the reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was carried out (16). Extracts and DPPH were solved in ethanol. DPPH ethanolic solution was used at 0.04 mg/mL. Absorbance was measured at 517 nm after 30 min of reaction at room temperature in an iEMS Reader Lab systems. Ascorbic

acid was used as a positive control. Each concentration was tested in triplicates. Background interferences from solvents were deducted from the activity values of the corresponding extracts prior to calculating % Radical Scavenging Capacity (%RSC) as follows:

$$\%RSC = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$$

Where $Abs_{control}$ is the absorbance of the control and Abs_{sample} is absorbance of the sample.

The *in vitro* antioxidant activity of the extract is expressed as IC_{50}, which is defined as the concentration of extract (µg/mL) required to scavenge 50% of DPPH radicals.

2.5 Stress- resistance Assays

Surcos-Laos et al. 2011 (17) method was followed with some modifications. Eggs were prepared by bleaching adults, and incubated in M9 buffer until the eggs hatched. L1 larvae were incubated on NGM plates containing *E.coli* OP50 and different concentrations of licorice, from 0 to 500µg/mL. Afterwards, adult worms were subjected to lethal oxidative-stress by means of 5-hydroxy-1,4-naphthalenedione,juglone (150 mM). To assess resistance to oxidative stress, survival was measured after 24 h incubation at 20 ºC by a touch-provoked movement. Worms that reacted to the mechanical stimulus were scored as alive whereas non-responding worms were considered as dead. Survival Rate % (% SR) was calculated as a percentage. Assays were performed three times using about 100 individuals per study group.

$$\%SR = (N^{o} \text{ of worms alive} \times 100)/\text{Total number of worms}$$

2.6 Measurement of catalase (CAT) activity

After oxidative stress induced by juglone, worms were lysed in order to extract their proteins. This lysis was made using a cold buffer (150 mM, NaCl, 50 mMTris-HCl pH 8 y 1% TWEEN 20 at 4 ºC) and breaking worm bodies with ultrasound equipment (JP Selecta 3000683). Catalase quantification was made with Cayman Chemical Catalase assay kit®.

2.7 Lifespan analysis

Virk et al. 2012 (18) method was followed with some modifications. Longevity of temperature-sensitive sterile *C.elegans*glp-4 (bn2) on 4 different concentrations of licorice extract were measured in this analysis: 25, 50, 100 and 250 µg/mL and compared to controls.

Gravid adults were used to lay eggs onto fresh NGM OP50 plates at 15 ºC. Eggs were raised at 15 ºC till L3/L4 stage, due to temperature sensitivity of mutant phenotypes. Once this staged was reached, animals were transferred to 25ºC. After 24 hours at that temperature, 25 worms were put onto each of 5 replicate plates for each condition. Animals were transferred to fresh plates after7 and 14 days and scored for survival every 2 to 3 days. The scoring method was the same used as for the juglone oxidative stressed assays. Results are expressed as Survival Rate % (% SR) and mean lifespan.

2.8 Statictical analysis

Graph Pad Prism® 5.0 was used for statistical analysis. IC_{50} values were estimated by a non-linear regression. The statistical differences between the control and treated worms were determined with the aid of the parametric t-test, in the case of the resistance to

oxidative stress and toxicity experiments and no-parametric, Mann Whitney test, for the measurement of CAT activity (p-value <0.05).

Lifespan data were analyzed by JMP statistical software (SAS Institute Inc., Cary, NC, USA). Relevant, statistical significance was determined using the Log-Rank and Wilcoxon tests of fitting to the Kaplan-Meier survival model (18).

3. RESULTS

3.1 Extract composition

Glycyrrhizin determination on the extract was carried out, as it is the major component of licorice, and one of the main responsible of its bioactivity (19). Figure 1 shows that the retention time (min) for the glycyrrhizin present in Licorice extract used on this essay and glycyrrhizin standard measured at a 254 nm is 9.20 min for both. It can be seen that the presence of glycyrrhizinin licorice dry extract was 1.18 ± 0.18 % (w/w).

3.2 Antioxidant activity *in vitro*: DPPH method

DPPH method was carried out as radical-scavenging activity to scan the hydrogen-donating capacity of licorice extract. The calculated IC_{50} value of licorice was 51.17µg/mL, just one order of magnitude higher than the positive control ascorbic acid IC_{50}=3.20 µg/mL.

3.3 Stress- resistance Assays

Given the rich content of antioxidant phytochemicals, licorice could be expected to increase the resistance of *C.elegans* to oxidative stress. The assessment of the antioxidant capability of licorice was carried out by exposing *C.elegans* to different concentration of

the plant with a posterior exposition to an oxidative stress caused by juglone. Figure 2 shows the % SR of pre-treated worms at different concentrations of licorice after a 24 h exposition to juglone (150 µM). % SR of the pretreated populations at 50, 100, 250 and 500 µg/mL were 17.78 ± 2.27; 24.42 ± 2.97; 24.01 ± 3.21 and 33.56± 2.97 % respectively, being the survival of the control 3.18 ± 0.91%. All of the pretreated population showed significant differences compare to the control ($p < 0.0001$).

3.4 Measurement of catalase (CAT) activity

The modulation of the antioxidant defensive system could have an impact on the protection against oxidative stress. The effects of licorice on one of the enzymes forming part of this defensive system in *C.elegans*, catalase, was measured. Figure 3a shows the catalase activity in worms treated with 100 µg/mL of licorice or untreated (control). On the other hand, figure 3b shows the catalase activity of the worms treated only with juglone (150 mM, control) and worms previously treated with licorice, 100 µg/mL, before exposed to an oxidative stress by juglone (150 mM). Population treated with licorice (Figure 3 a) showed an increase of catalase activity of 1600.0 ± 255.9 %. The pre-treatment with licorice and subsequent exposition to the pro-oxidant juglone (Figure 3 b) produced an increase of catalase activity of 270.5 ± 45.19 %.

3.5 Lifespan analysis

Despite the unclear correlation between oxidation and aging, the effects on lifespan of the proven antioxidant extract were tested, to check its possible effects increasing *C.elegans* lifespan. Figure 4 shows the % SR against time (days) of glp-4 animals (25°C) treated with a concentration of licorice (25µg/mL, 50 µg/mL, 100 µg/mL and 250 µg/mL) compared to control. Mean lifespan was 12.84 ± 0.20; 12.92 ± 0.20; 12.58 ± 0.19 and

13.59 ± 0.19 days for 25µg/mL, 50 µg/mL, 100 µg/mL and 250 µg/mL licorice concentrations respectively, being the control survival 11.87 ± 0.17 days. This effect is stronger at 250 µg/mL, with 14.28% extension of mean lifespan ($p<0.0001$). There were significant differences between 250 µg/mL and the other concentrations ($p<0.05$), but there were not among 25, 50 and 100 µg/mL.

4. DISCUSSION

The chemical composition of the licorice extract mainly includes triterpenoid saponins (glycyrrhizin and glycyrrhizic acid), flavonoids, chalcones and other compounds present in lesser quantities (20). Glycyrrhizin, apart from being present in higher quantity, also presents great bioactivity (19). Licorice capabilities, discussed later, are probably given by its cited content of glycyrrhizin and polyphenols, and their synergic action with other untested components.

As cited before, the IC_{50} value of licorice obtain with the DPPH method was one order of magnitude higher than the ascorbic acid, but, taking into account that licorice extract is a mixture of chemical compounds and vitamin C is a pure substance, licorice ethanolic extract could be considered as a source of antioxidant compounds. This capability is biologically important as the ability to scavenge free radicals of the DPPH type may imply the utility of licorice in preventing the damage of cellular membrane, the oxidation and cross-link of proteins and harmful interactions with DNA, both nuclear and mitochondrial (21).

Due to the complexity of licorice composition, its antioxidant activity could be given by several simultaneous mechanisms of action. Thus, the animal model *C. elegans* was used to test antioxidant capability *in vivo*, and to test the influence of licorice extract on the

endogenous antioxidant defensive system of the worm. This oxidant stress was generated by juglone. Its toxicity is caused by several mechanisms of action, including the ability to undergo oxidation-reduction cycles with concomitant formation of free radicals (22). The pre-treatment of *C.elegans* with licorice has shown an increase of the survival at all tested concentrations (Figure 2). According to our results, the protection is not given in a dose-dependent manner. The increase in survival rates indicates that licorice is an efficient antioxidant *in vivo*.

As far as we know, there are no previous studies of the licorice antioxidant activity on *C.elegans*, but similar results were obtained with green tea, a well-known antioxidant, where the average of survival for a pretreated population pretreated with 100 µg/mL of green tea extract was 32.43 ± 1.71% after 24 h exposure to juglone (80 µM) (23). In this experiment with green tea, the juglone concentration was lower leading to a higher survival of both the control and the treated population. In any case, the increase of survival was in the same order of magnitude of the one obtained with licorice at the same dose.

Flavonoids are one of the most important phytochemical contributors to the antioxidant capability of plants. Epigallocatechin gallate from green tea or quercetin a major flavonoid in human diet have shown their efficacy in increasing *C.elegans* resistance to stress (17, 24). Also, terpenes such as specioside (6-O-coumaroylcatalpol) isolated from the plant *Stereospermum suaveolens* have shown this protection against oxidative stress (25). According to the literature, the rich content of flavonoids and terpenes of licorice (20) could be responsible for its capability to increase the survival of *C.elegans* in response to oxidative stress.

This improvement of the survival against the prooxidant juglone might not be given only because of the radical scavenging capacity of licorice. Formation of free radicals is part

of many physiological processes (1) that, in an ideal situation, are balanced by an elaborated endogenous antioxidant defensive system, composed of enzymes such as superoxide dismutase (SOD), catalase (CAT) or glutathione peroxidase (GPX), among others (26).

As it can be seen in figure 3, catalase activity was enhanced on licorice treated worms in comparison to untreated control group. There was an increase of catalase activity on the untreated population when exposed to oxidative stress. This could be expected, as CAT forms part of the endogenous enzymatic system of protection of *C.elegans* (27). These differences were also observed under oxidative stress conditions, but it was not as strong as the one of treated population against the control group when no oxidative stress was applied. These differences of activity observed under oxidative stress conditions may be due to the fact that licorice enhances CAT activity when facing an oxidative stress, but the catalase activity significantly increases under stress conditions in non-treated *C.elegans*, as it forms part of its defensive system.

Some authors establish a correlation between the enhanced stress resistance and longevity (28, 29). A lifespan essay was carried out with licorice, as its probed antioxidant capability suggested its capability increasing *C.elegans* lifespan. According to our results, licorice treatment increases the life of *C. elegans* in a dose-dependent manner, as even all concentrations extended lifespan, the highest concentration showed the strongest effect.

Despite the fact that there is no background literature on the effects of licorice on *C.elegans* lifespan, there are previous studies about the activity of other phytochemicals. Some examples are polyphenols from blueberry (30)**,** epigallocatechin gallate from green tea (24) and terpenes, such as specioside, from patala (*Stereospermum suaveolens*) (31). Both, this terpene and epigallocatechin gallate, also showed an increase in *C.elegans*

tolerance to oxidative stress induced by juglone, which, in concordance with the results obtained for licorice, agrees with previous studies that show that longevity is closely linked to oxidative stress resistance (24, 32). The way licorice prolongs lifespan by diminishing oxidant damage may be given by its radical scavenging capability itself and the enhancement of the activity of *C. elegans* endogenous enzymatic system.

5. CONCLUSIONS

According to the exposed results, we conclude that licorice has a strong antioxidant capability both *in vitro* and *in vivo*. This antioxidant capability may be given by the radical scavenging capability of licorice and the enhancement of CAT activity provoked by licorice extract, both in basal conditions or when exposed to an oxidative stress. It has been also shown that licorice extends *C.elegans* lifespan. This lifespan extension may be correlated with the previously cited antioxidant capability.

Even the in vivo model used in this essay is still far from the human being, it shows how diet has a huge influence not only in preventing pathologies but also on aging.

Acknowledgement

We thank Dr. Mainar and Carlota Giménez for their collaboration on HPLC analysis. We thank Claire Maynard for the training and carry out of lifespan assays.

This research did not receive any specific grant from funding agencies in the public, commercial, or non-for-profit sectors.

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FIGURES

Figure 1. UA *vs* time (min). HPLC chemical profile of Licorice extract. Detection at 254 nm. (1) glycyrrhizin present in Licorice extract (2) glycyrrhizin standard at 2.5 ppm.

Figure 2. Survival Rate % (% SR) *vs* licorice concentration (µg/mL). Juglone-induced oxidative stress after pretreatment with 50, 100, 250 and 500 µg/mL of licorice compare to untreated control. Results were obtained after 4 different experiments, with 350 worms per condition. Data are presented as percentage of survivals (mean ± S.E.M) *** $p < 0.001$.

Figure 3.a) Catalase activity (nmol/min/mL) of worms treated with 100 µg/mL of licorice compared to a non-treated population **b)** Catalase activity (nmol/min/mL) after an exposure to Juglone-induce oxidative stress of worms pre-treated with 100 µg/mL of licorice compared to a non-treated population. Results were obtained from 3 different experiment, with 300 worms per condition. Data are presented as Ln of catalase activity (nmol/min/mL) (MEAN± S.E.M) *$p < 0.05$ Data are ** $p<0.01$

Figure 4. Survival Rate % (% SR) against time (days) of SS104 glp-4 animals (25°C) treated with a concentration of licorice of (a) 25µg/mL (n=497); (b) 50µg/mL (n=543); (c)100µg/mL (n=471); (d) 250 µg/mL (n=544) compared with the control (n=536) without licorice treatment. Results were obtained from 3 different experiments (n= number of worms per condition).

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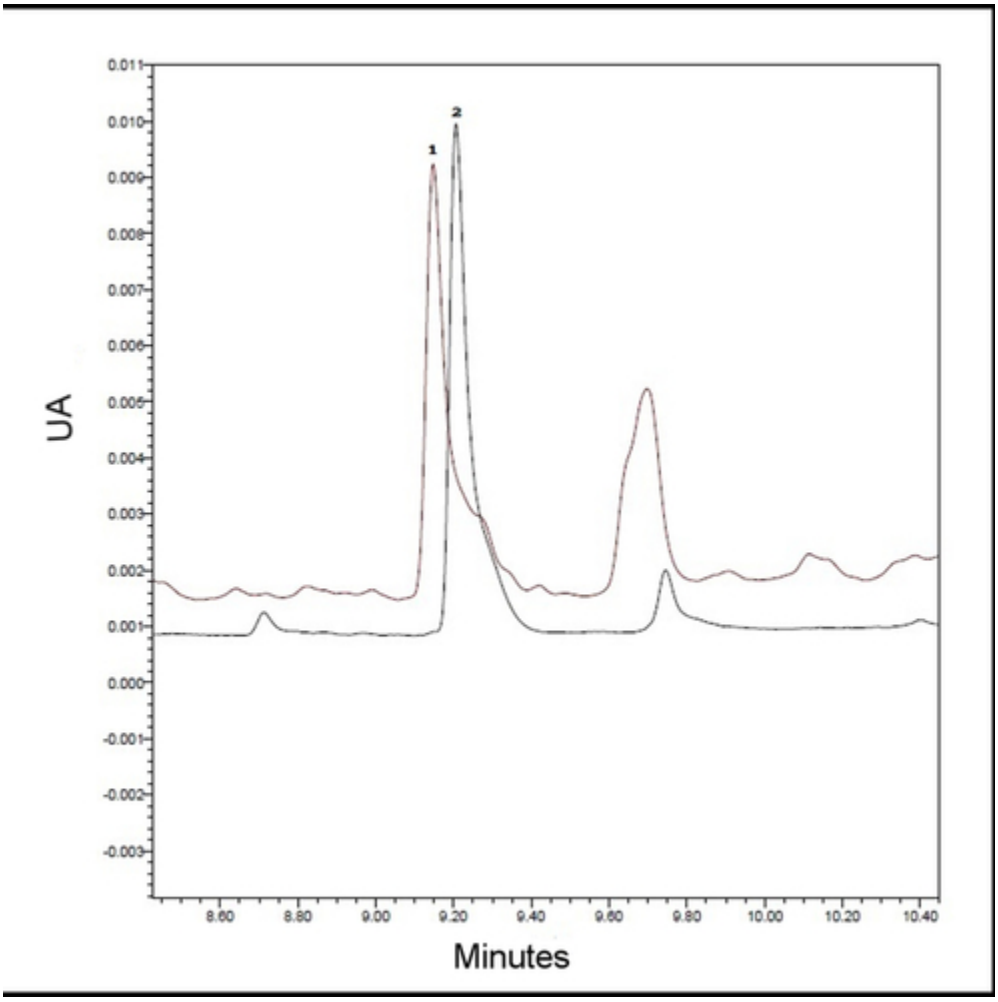

Figure 1. UA vs time (min). HPLC chemical profile of Licorice extract. Detection at 254 nm. (1) glycyrrhizin present in Licorice extract (2) glycyrrhizin standard at 2.5 ppm

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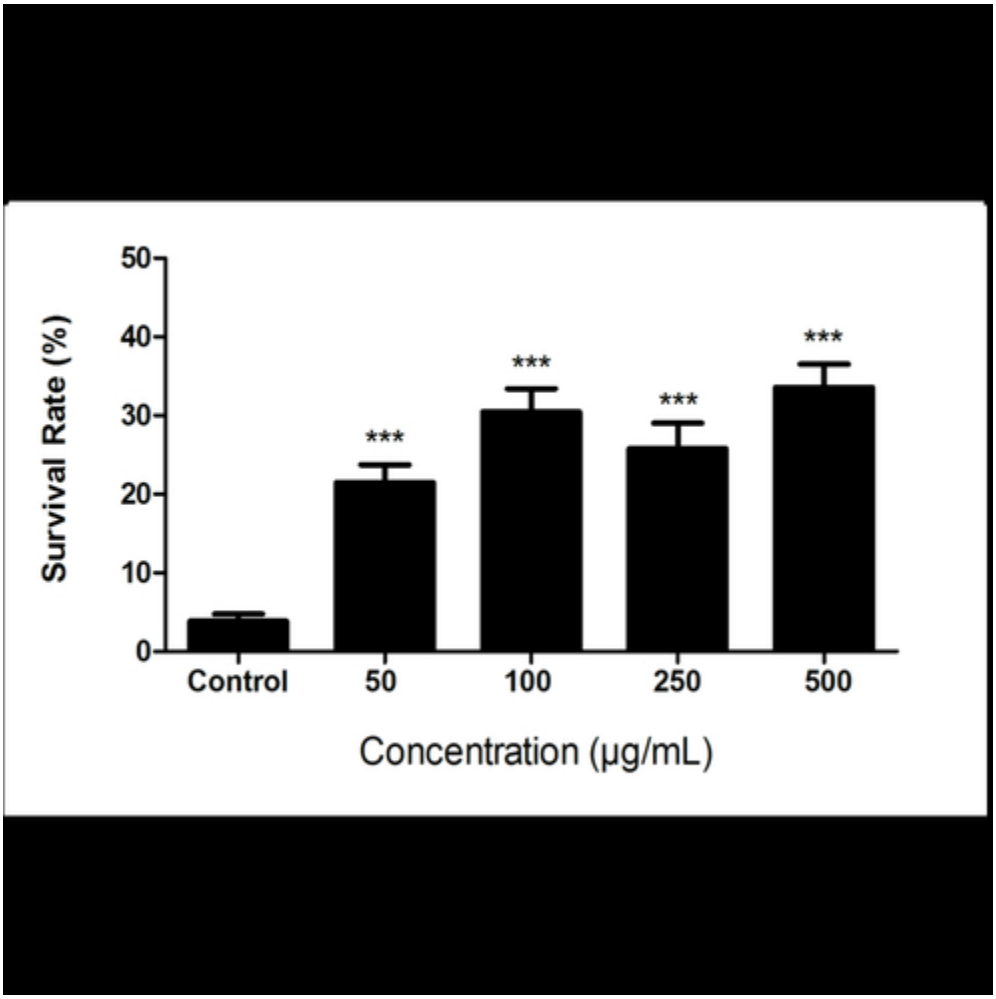

Figure 2. Survival Rate % (% SR) vs licorice concentration (µg/mL). Juglone-induced oxidative stress after pretreatment with 50, 100, 250 and 500 µg/mL of licorice compare to untreated control. Results were obtained after 4 different experiments, with 350 worms per condition. Data are presented as percentage of survivals (mean ± S.E.M) *** $p < 0.001$

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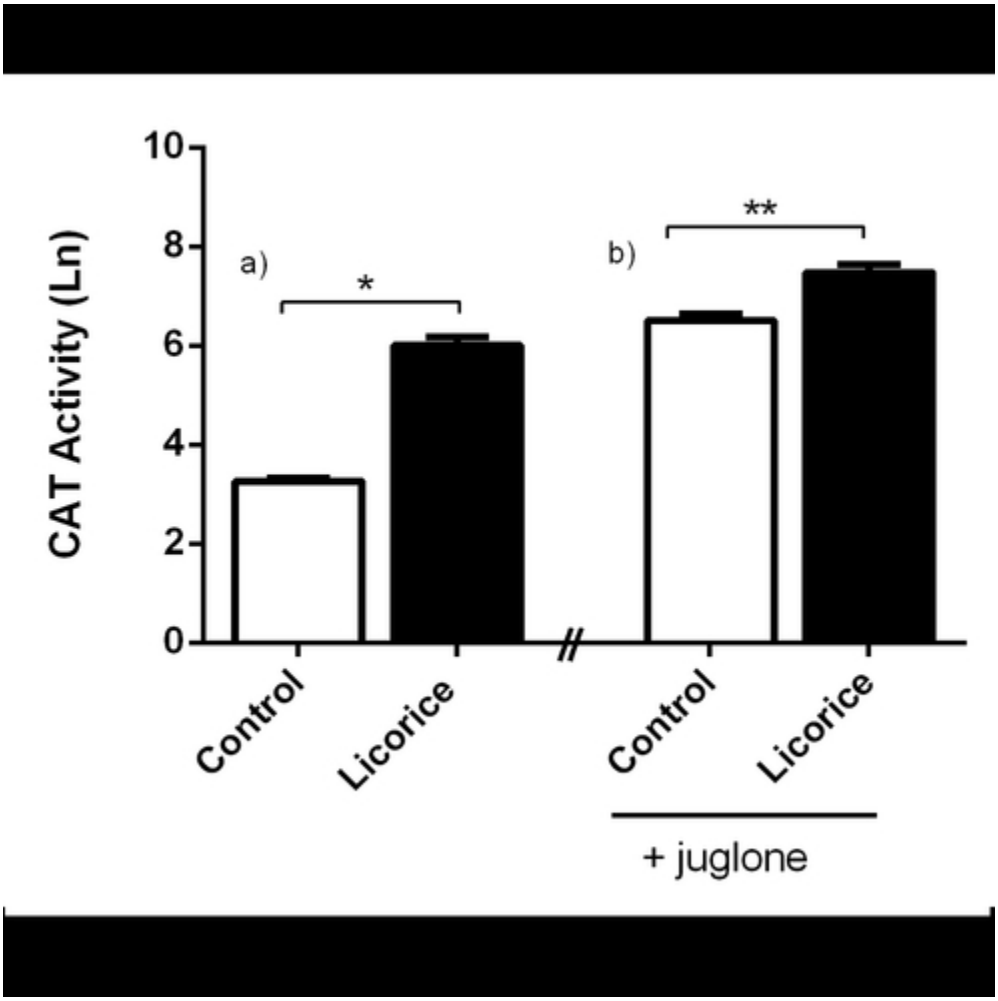

Figure 3.a) Catalase activity (nmol/min/mL) of worms treated with 100 µg/mL of licorice compared to a non-treated population b) Catalase activity (nmol/min/mL) after an exposure to Juglone-induce oxidative stress of worms pre-treated with 100 µg/mL of licorice compared to a non-treated population. Results were obtained from 3 different experiment, with 300 worms per condition. Data are presented as Ln of catalase activity (nmol/min/mL) (MEAN± S.E.M) *p < 0.05 Data are ** p<0.01

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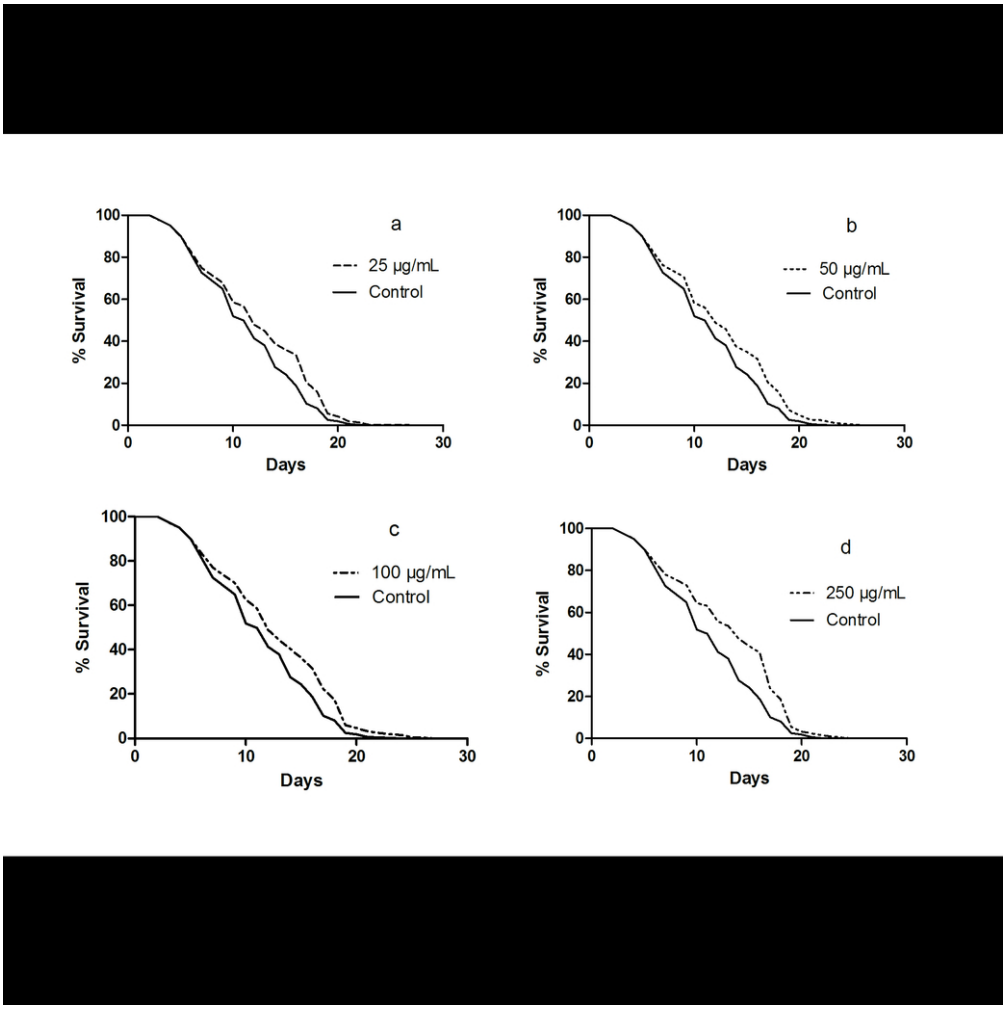

Figure 4. Survival Rate % (% SR) against time (days) of SS104 glp-4 animals (25°C) treated with a concentration of licorice of (a) 25µg/mL (n=497); (b) 50µg/mL (n=543); (c)100µg/mL (n=471); (d) 250 µg/mL (n=544) compared with the control (n=536) without licorice treatment. Results were obtained from 3 different experiments (n= number of worms per condition).

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