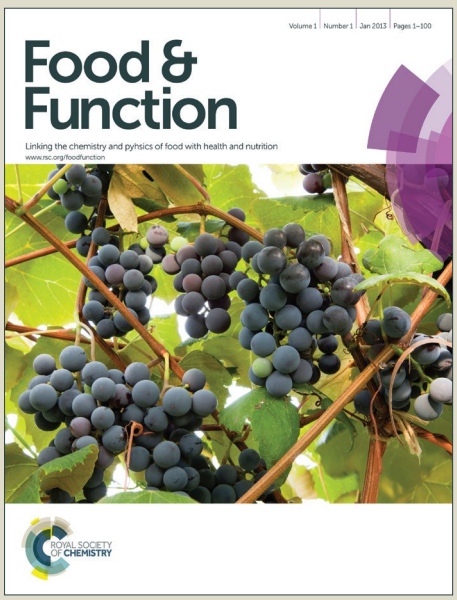
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Extract of Tsai Tai (*Brassica chinensis*): Enhanced Antioxidant Activity and Anti-aging Effects both *in vitro* and in *Caenorhabditis elegans*

Jing Chen[a], Ju Zhang[a], Yanxia Xiang[a], Limin Xiang[a], Yongmei Liu[a], Xiangjiu He[a], Xiaoju Zhou[a], Liu Xin[a]* and Zebo Huang[a, b]*

[a] *Key Laboratory of Combinatorial Biosynthesis and Drug Discovery, Ministry of Education, and School of Pharmaceutical Sciences, Wuhan University, Wuhan, 430071, China*

[b] *Guangdong Province Key Laboratory for Biotechnology Drug Candidates, School of Biosciences and Biopharmaceutics, Guangdong Pharmaceutical University, Guangzhou 510006, China*

✉Correspondence to: Dr. Xin Liu, School of Pharmaceutical Sciences, Wuhan University, 185 East Lake Road, Wuhan 430071, China. Phone: 86-27-68759006. Fax: 86-27-68759850. E-mail: lx@whu.edu.cn (X. Liu); Dr. Zebo Huang, zebohuang@gdpu.edu.cn (Z. Huang)

Running title: Antioxidant Activity and Anti-aging Effects of Tsai Tai Extract (*Brassica chinensis*)

Abstract

Tsai Tai is one of the most widely consumed *Brassica* vegetables in Asian countries because of its good taste and its nutritional benefits. This study evaluated the antioxidant capacity and possible associated health benefits of 3 Tsai Tai (*Brassica chinensis*) varieties, namely, Hon Tsai Tai, Pak Choi and Choi Sum. The DPPH radical scavenging ability and reducing power assays were performed to evaluate the *in vitro* activities of the extracts. *Caenorhabditis elegans* was used as an *in vivo* model for evaluation of beneficial health effects, including antioxidant activity and delayed aging. *In vitro*, the Hon Tsai Tai extract exhibited higher antioxidant activities than that of Pak Choi and Choi Sum, and the total phenolic contents were significantly correlated with the DPPH and RP values. *In vivo*, the three assayed Tsai Tai extracts significantly increased resistance against paraquat-induced oxidative stress with an increase in survival rates from 15% to 28% compared with controls. However, only the extract from Hon Tsai Tai significantly prolonged the lifespan of *Caenorhabditis elegans,* with an 8% increase in the mean lifespan with respect to controls. Further evidence of antioxidant protection was obtained by assessing ROS production via DCF assay. Analyses of intracellular SOD activity and MDA content confirmed the existence of an antioxidant protective effect. These results suggest that Tsai Tai might serve as a good source of natural antioxidants, and in particular, Hon Tsai Tai could be explored as a potential dietary supplement to retard aging.

Keywords: antioxidant capacity; *Brassica chinensis*; *Caenorhabditis elegans*; radical scavenging ability; reducing power; superoxide dismutase; reactive oxygen species

Introduction

Epidemiological evidence suggests that consumption of a diet rich in vegetables and fruits has positive implications for human health, and interest inedible plants is increasing, especially for those that are rich in phytochemicals. [1] Studies have indicated that phytochemicals have high antioxidant activity, which helps to

decrease the risk of developing chronic diseases such as cancer, cardiovascular disease, diabetes, and age-related neuronal degeneration. [2-5] Generally, natural antioxidants assist the body in neutralizing free radicals or inhibiting generation of reactive species during the course of normal cell metabolism, thus preventing damage to lipids, proteins, and nucleic acids and eventual cellular damage and death. [6] An appropriate intake of dietary antioxidants has been suggested to play an important role in enhancing the body's defense systems and preventing reactive oxygen species (ROS)-related diseases. [7-9]

The *brassicas* are one of the most widely cultivated vegetable groups in the world due to their economic significance and nutritional value. Recent reports suggest that *Brassica* vegetables act as a good source of natural antioxidants due to their high levels of carotenoids, tocopherols, ascorbic acid and phenolic compounds. [10] Tsai Tai (*Brassica chinensis*) is an important leafy *brassicas* in east and southeast Asia, e.g., Hon Tsai Tai (*Brassica rapa* L. ssp. *Chinensis* (L.) Hanelt var. *purpurea*), Pak Choi (*Brassica rapa* L. ssp. *chinensis* (L.) Hanelt var. *chinensis*) and Choi Sum (*Brassica rapa* L. ssp. *chinensis* (L.) Hanelt var. *parachinensis*). Hon Tsai Tai, also known as Purple Tsai Tai, displays stalks with a distinct purple color that differs from the others, and it is also known as Hongshan Tsai Tai because it originated from Hongshan Temple in Wuhan, China. Hon Tsai Tai was an important tribute paid to the emperor in ancient times and is acclaimed as a jade dish in a golden palace because it was one of the Empress Dowager's favorite dishes. Pak Choi, also known as Chinese cabbage (non-heading group), is heavily consumed in eastern and southern China, whereas Choi Sum (also known as Chinese flowering cabbage) is produced in south China. However, shifts in consumer trends toward healthier lifestyles over the past decade have resulted in increased consumption of sprouts in salads or in Asian-style cooking worldwide.

Tsai Tai plants develop leaves and fleshy stems that are 0.5-2 cm in diameter, are 15-30 cm tall, and produce yellow flower buds. This crop is commercially harvested when young flower buds have formed but have not opened and the stems are tender. [11] The stem of the plant at the flowering stage is the main edible portion, and

Tsai Tai varieties differ in stem length, thickness and proportion of leaves to stem. Hon Tsai Tai is particularly different from the others because of its purple color. *Brassica* vegetables are a significant source of polyphenols. Several studies have investigated the phenolic composition of members of the Brassicaceae family, including Pak Choi and Choi Sum. [11-13] The Harbaum research group confirmed that Pak Choi contains a significantly higher amount of flavonoids than many other vegetables,[14] and the potent antioxidative and various free-radical scavenging activities of Pak Choi and Choi Sum have been reported by several research groups. [15, 16] However, most of this research has focused only on qualitative and quantitative analysis of active components, and information on the Hon Tsai Tai is scarce. Furthermore, *in vivo* studies on the bioactivities of Tsai Tai are limited. Therefore, the potential biological properties of Tsai Tai extracts remain to be further exploited.

Increasing evidence indicates that oxidative stress plays a crucial role in the process of aging. [17, 18] Many believe that antioxidant intake is beneficial to prolonging lifespan. Studies on model organisms of the effects of antioxidant supplementation on aging and longevity have been extensively reviewed. [19] The model organism *Caenorhabditis elegans* has been frequently used to study stress resistance and longevity because it shares several key genes with vertebrates, and the mechanisms of aging are apparently similar for both *C. elegans* and mammals. [20, 21] This similarity allows findings from *C. elegans* to be extrapolated and further confirmed in humans.

Although Tsai Tai is usually consumed as a constituent of diet and several studies have reported its antioxidant activities, the effect of Tsai Tai in experimental animals is still unknown. In this work, for the first time, we used *C. elegans* as model organism to explore the potential beneficial health effects of extracts from 3 varieties of Tsai Tai, namely, Hon Tsai Tai, Pak Choi and Choi Sum. Spectrophotometric methods were used to determine the total contents of phenolics and anthocyanins in the 3 varieties of Tsai Tai. DPPH radical scavenging activity and reducing power assays were performed to evaluate the *in vitro* activities of the extracts. *C. elegans*

was used as an *in vivo* model to evaluate the beneficial health effects, including antioxidant activity and anti-aging properties. Additionally, the possible correlation of phenolic content with antioxidant and/or anti-aging effects was investigated.

Materials and methods

Plant Materials. For all experiments, reagents were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. The three varieties of *Brassica* vegetables selected for the study were Hon Tsai Tai (*Brassica rapa* L. ssp. *Chinensis* (L.) Hanelt var. *purpurea*), Pak Choi (*Brassica rapa* L. ssp. *chinensis* (L.) Hanelt var. *chinensis*) and Choi Sum (*Brassica rapa* L. ssp. *chinensis* (L.) Hanelt var. *parachinensis*). Stems of *Brassica* vegetables were collected in December 2012 in a rural district of Wuhan City, China, at the stage when young flower buds are formed but not opened and the stems are tender. Fresh stems were washed and cut into small pieces.

Sample Extraction. The solvent extracts were prepared according to the method described in previous studies. [22-24] In brief, 30 g of sample was dispersed in 60 mL of extraction solvent, i.e., distilled water, methanol/water (80:20, v/v), Methanol/HCl (99.9:0.1, v/v), and acetone/water (80:20, v/v). The mixture was protected from light and stirred at 100 rpm for 2 h on a rotary shaker (THZ-C; Suzhou Pui Ying Experimental Equipment Co., Ltd., Jiangsu, China) at ambient temperature. The solutions were separated from the solid matrix by filtration through a sheet of qualitative filter paper (Hangzhou Special Paper Industry, Zhejiang, China). The solvent was removed by rotary evaporation. The residue was lyophilized, weighed, and stored at -20 °C until further analysis.

Measurement of Total Phenolics. Total phenolics were determined colorimetrically using Folin-Ciocalteau reagent, as described by Conde-Hernandez et al. [25] with slight modification.

A properly diluted amount of 200 μL sample or a standard solution of varying concentration was mixed with

400 μL of Folin-Ciocalteu reagent. Deionized water was used for dilution and control. The solution was diluted to a total volume of 4.6 mL using deionized water and thoroughly mixed. After incubation for 10 min at room temperature, 1 mL of 20% Na_2CO_3 solution was added, immediately mixed, and incubated for 2 h. The absorbances were measured at 725 nm using a Multiskan MK3 microplate reader (Thermo Fisher, USA). Measurements were recorded in triplicate. Total phenolics were quantified using a calibration curve obtained by measuring the absorbance of known concentrations of a gallic acid standard. The concentrations are expressed in terms of milligrams of gallic acid equivalents per 100 g of fresh weight. (mg GAE/100 g)

Measurement of Total Anthocyanins. Total anthocyanins were determined according to the pH differential method using two buffer systems, i.e., potassium chloride buffer, pH 1.0 (0.025 M) and sodium acetate buffer, pH 4.5 (0.4 M), as described by Golmohamadi et al. [26] and adapted to microplate volumes in our laboratory. In brief, 20 μL of a standard or sample solution was pipetted into two different 96-well plates and mixed with the respective buffers (180 μL). The absorbances were measured at 510 and 700 nm. Wells containing buffer without the sample solution were used as blanks. The absorbance (A) was calculated according to the following formula:

$$A = (A_{510} - A_{700})pH1.0 - (A_{510} - A_{700})pH4.5.$$

Cyanidin-3-glucoside was used as a standard, and the results were calculated using the following equation and expressed as milligrams of cyaniding-3-glucoside equivalents per 100 g of fresh weight (mg CYE/100 g).

$$\text{total anthocyanins (mg/100 g)} = \frac{A}{eL} \times MW \times D \times \frac{V}{G} \times 100$$

where A is the absorbance, *e* is the cyaniding-3-glucoside molar absorbance (26900), L is the cell path length (1 cm), MW is the molecular weight of anthocyanins (449.2), D is the dilution factor, V is the final volume (mL), and G is the sample weight (mg).

DPPH Radical Scavenging Activity (RSA). The DPPH RSA of samples were determined according to the method of Chao et al. [27] An amount of 0.1 mL of extract was mixed with 0.9 mL of 0.041 mM DPPH• in ethanol.

The mixture was vortexed vigorously and allowed to stand for 30 min in the dark. The absorbance of the sample was measured at 517 nm against a blank. Ascorbic acid, a well-known antioxidant, was used as the positive control, and all tests were performed in triplicate. A calibration curve constructed with DPPH• between 0.1 and 2.0 mg/L was used to calculate the remaining concentration of DPPH• in the reaction medium. RSA was expressed as a percentage of inhibition and was calculated using the following formula:

DPPH RSA (%) = $[(A_0 – A_1)/A_0]\times100$,

where A_0 is the absorbance of the control reaction, and A_1 is the absorbance in the presence of samples.

Reducing Power (RP). The reducing power was measured as described by Romero-de et al. [28] A 50 µL volume of extract solution (0.1 mg/mL) was mixed with 250 µL of phosphate buffer (0.2 M, pH 6.6) and 250 µL of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. After 30 min of incubation at 50 °C, 250 µL of 10% trichloroacetic acid was added, and the mixture was centrifuged at 4500 g for 10 min. The supernatant (200 µL) was mixed with 40 µL of ferric chloride solution (0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates increased reducing power. Ascorbic acid was used for comparison.

***C. elegans* Strains and Culture Conditions.** The *C. elegans* strains used in this study were Bristol N2 (wild-type). *C. elegans* was maintained and assayed (unless otherwise stated) at 20 °C on nematode growth medium (NGM) agar plates with *Escherichia coli* strain OP50 as a food source, as previously described. [21]

Semi-synchronized worm cultures were obtained by treating gravid hermaphrodites with bleach [50% sodium hypochlorite (12% Cl), 2.5 M sodium hydroxide] and recovery of hatched L1 larvae on NGM/OP50 plates.

Three days after synchronization, the worms (L4-young adults) were treated with 5-fluoro-2'-deoxyuridine to block reproduction for *in vivo* antioxidant assays. All chemicals in NGM plates and liquid are expressed as the final concentrations.

Food Clearance Test. To determine the impact of extracts from Tsai Tai on *C. elegans* physiology, a food

clearance test was conducted as described by Fu et al. [29] A culture of *E. coli* was grown overnight and resuspended at a final optical density (OD) of 6.6 in nematode S-medium. Extracts from Tsai Tai were diluted into the *E. coli* suspension to the desired concentrations. Forty microliters of the final mixture was loaded per well in a 96-well plate. Approximately 20-30 synchronized L1 animals in 10 mL of S-medium were added to an *E. coli* suspension containing a series of concentrations of extracts from Tsai Tai and incubated in a 96-well microtiter plate at 25 °C. The absorbance (OD 595 nm) of the culture was determined every day for 4 days.

***C. elegans* Oxidative Stress Resistance Assays.** To evaluate the *in vivo* antioxidant effect of the Tsai Tai extracts, the paraquat assay was performed according to Lima et al. [30] Synchronized wild-type L1 larvae were incubated in S-medium in the presence or absence of Tsai Tai extracts (2 mg/mL) for 2 days at 20 °C. The worms were transferred to 96-well plates (～10 worms/well; >100 worms for each treatment) and exposed to 0.1 mol/L of paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride). The numbers of live and dead worms were scored based on movement every 12 h until all animals were dead.

***C. elegans* Intracellular Reactive Oxygen Species (ROS) Measurement.** *C. elegans* were pretreated with 2 mg/mL Tsai Tai extracts for the ROS assays. After 3 days at 20 °C, nematodes were harvested and washed three times in M9 buffer. Approximately 500 animals were homogenized in 400 μL PBS with 0.1% Tween-20 at 4 °C. After centrifugation, 200 μg/mL of supernatant was transferred into a 96-well plate and incubated with 50 μM of DCFH-DA. The fluorescence was measured every 20 min for 2 h using a fluorescence microplate reader with an excitation of 485 nm and an emission of 535 nm.

***C. elegans* Intracellular Superoxide Dismutase (SOD) and MDA Content Measurement.** Age-synchronized worms were treated with 2 mg/mL Tsai Tai extracts using the same procedure described above. After homogenization and centrifugation as described, the supernatant was collected and used for determination of SOD activity and MDA content using an assay kit (Beyotime, China).

***C. elegans* Lifespan Assays.** Lifespan analyses were performed in the same manner for all treatments at 20 °C. Synchronized adult hermaphrodite worms (3 days old) were placed in populations of 20 individuals into 2 mL of S-medium in 35-mm-diameter tissue culture dishes (CELLSTAR; Greiner Bio-One GmbH) in the absence or presence of 2 mg/mL Tsai Tai extracts. Live and dead worms were scored daily until all nematodes had died. Nematodes that did not move when gently prodded (with a platinum wire) were judged to be dead. During the reproductive period, adult nematodes were transferred to fresh NGM plates to keep them separated from their progeny every day and every other day thereafter. The test was performed on at least three independent biological replicates.

Live subject statement. All experiments were performed in compliance with the relevant laws and institutional guidelines, and approved by the Committee of Experimental Animal Administration in School of Pharmaceutical Sciences, Wuhan University.

Statistical analysis. Statistical analysis was performed using SPSS Statistics 17.0 software. The results are presented as the mean ± standard errors of mean (SEM). Three replicates were performed for each experiment. A one-way ANOVA was performed on the mean values to determine whether they differed significantly. Linear regression analyses were conducted to determine the correlation between phenolic content and antioxidant activity. The relative determination coefficients (R^2 adjusted) were reported with R^2 close to 0 indicating no linear relationship and R^2 values close to 1 suggesting a strong linear relationship. Two-way ANOVA was used to analyze the treatment effects of varieties and solvent types on TPC and DPPH IC_{50} and to determine the effects of varieties and concentrations on DPPH radical scavenging activity and reducing power. Statistical significance was determined at the $p< 0.05$ level using the appropriate *post hoc* test. For lifespan, animal survival was plotted using Kaplan-Meier survival curves and analyzed by the *log-rank* test using GraphPad Prism.

Results

Effect of Solvent Type on the Extraction of Antioxidants from Tsai Tai. Direct extraction using various types of solvents is the most common technique used to obtain extracts with high antioxidant activity from plants. However, no satisfactory solvent extraction methods are suitable for isolation of all classes of food antioxidants or even for a specific class of these components. Therefore, the focus of the current study was to compare the extraction efficiency of various solvent systems, including distilled water, methanol/water (80:20, v/v), Methanol/HCl (99.9:0.1, v/v), and acetone/water (80:20, v/v).

Phenolic compounds are the most effective antioxidants in fruits and vegetables, [31] and in this work, the total phenolic contents (TPC) were determined to evaluate the influence of the solvent type on the extraction efficiency. As shown in Figure 1A, the TPC values of both 80% methanol and Methanol/HCl extracts were much higher than those of distilled water and 80% acetone extracts, indicating that 80% methanol and Methanol/HCl solvent might recover more phenolic components from Tsai Tai. Furthermore, the DPPH radical scavenging activity assay (RSA) was applied to evaluate the *in vitro* antioxidant activities of extracts from different solvents. Figure 1B shows that the type of solvent had no impact on the DPPH RSA of extracted substances from Hon Tsai Tai ($p > 0.05$), whereas significant differences were found in the DPPH RSA of extracted substances from Pak Choi and Choi Sum in the order of 80% acetone > 80% methanol > Methanol/HCl > distilled water.

Anthocyanins are known as the pigments responsible for colors from orange to blue in plants and possess great potential in free-radical-scavenging activity. [32] Because Methanol/HCl also showed considerable ability to extract phlorotannins from Tsai Tai and Hon Tsai Tai has a purple color, the use of Methanol/HCl is preferred for extraction of antioxidants because anthocyanins are more stable and extractable at pH<2. The Methanol/HCl extract was therefore chosen for further investigation.

Total Phenolic and Anthocyanin Contents. Phenolic and polyphenolic compounds such as flavonoids, phenolic acids, and tannins are well known as effective free radical scavengers and antioxidants. TPC values in Tsai

Tai extracted by Methanol/HCl were determined with a linear gallic acid standard curve (y = 0.0035x + 0.0098; R^2 = 0.9987). As shown in Figure 2, Hon Tsai Tai had the highest total phenolic contents (92.6 mg GAE/100 g), and the lowest content was measured in Choi Sum (57.3 mg GAE/100 g). Khanam et al. [33] reported that the phenolic content of Pak choi was 0.317 mg ferulic acid equivalents per gram of dry weight, a value similar to that found in this study. The discrepancy might be due to differences in living environment, extraction method, and analytical method, etc. Moreover, anthocyanins were detected only in Hon Tsai Tai (0.72 mg CYE/100 g).

Antioxidant Activities *in vitro*. The antioxidant activity of food, plant extracts and beverages has been tested using a wide variety of assays. Schlesier et al. strongly suggested the application of at least two methods for determination of antioxidant activity due to differences between the test systems. [34] One portion of our research investigated the antioxidant activity of Tsai Tai extracts using two assays, namely, DPPH radical scavenging activity and reducing power.

The DPPH radical scavenging activity (RSA) assay has been used extensively to predict antioxidant activities because this method is easy to perform and highly reproducible. In the DPPH assay, the antioxidants were able to reduce the stable radical DPPH to the yellow-colored diphenyl-picrylhydrazine. The DPPH radical scavenging activities (RSA) of Tsai Tai extracts are shown in Figure 3A. The DPPH RSA of Tsai Tai extracts increased as the concentration increased from 0.2 to 5.0 mg/mL. Hon Tsai Tai showed higher activities than the other varieties at concentrations from 0.5 to 5.0 mg/mL, and these differences were statistically significant ($p < 0.05$). The results suggest that the DPPH RSA of the extracts was depended on varieties and concentration.

The IC_{50} values of Tsai Tai extracts, as determined based on the DPPH radical scavenging activity, are presented in Table 1. A lower IC_{50} indicates a higher DPPH free radical scavenging activity. The DPPH free radical scavenging effects of extracts and Ascorbic acid based on the IC_{50} values decreased in the order of Ascorbic acid > Hon Tsai Tai > Pak Choi > Choi Sum, and the values were 0.007, 0.968, 1.331 and 1.608 mg/mL, respectively.

Different studies have indicated that the electron donation capacity, which reflects the reducing power of bioactive compounds, is associated with antioxidant activity. [36] The presence of compounds such as antioxidants reduces the ferric-ferricyanide complex to the ferrous-ferricyanide complex of Perl's Prussian blue. In this assay system, the presence of antioxidants causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}) monitored at 700 nm. Figure 3B depicts the reducing power of the Tsai Tai extracts using the potassium ferricyanide reduction method. Similar to the DPPH radical scavenging activity, the reducing power of Tsai Tai extracts increased with increasing concentration. At different concentrations, the reducing power of Hon Tsai Tai was significantly (P<0.05) stronger than the other species at concentrations from 0.4 to 1.2 mg/mL.

The IC_{50} values, which were determined based on the reducing power of Tsai Tai extracts, are shown in Table 1. The RP values of the extracts and Ascorbic acid based on the IC_{50} values decreased in the order of Ascorbic acid > Hon Tsai Tai > Pak Choi > Choi Sum, and the values were 0.018, 0.846, 1.946 and 2.029 mg/mL, respectively.

Determination of the concentration of Extracts from Tsai Tai for Treatment of *C. elegans* by Food Clearance Test. To assess the *in vivo* antioxidant activity of Tsai Tai, we first determined the optimal concentrations of extracts from Tsai Tai for evaluation in *C. elegans* models via the food clearance test. Given the advantages of the ability of *C. elegans* to grow in a liquid culture of *E.coli* and their short lifecycle, the Tsai Tai extracts were tested at the rate at which the food source (*E. coli* suspension) was consumed. Each adult animal can generate hundreds of offspring that rapidly consume the limited *E. coli* supply. Therefore, the OD of the wells without extracts of Tsai Tai was significantly reduced in 3 days in N2 strains (Figure 4). The addition of 1 mg/mL or 2 mg/mL of all types of Tsai Tai extracts to the varieties containing the N2 strains showed no effect on food clearance compared with that of the control animals, whereas animals treated with 3 mg/mL Choi Sum extracts displayed significantly delayed food clearance (Figure 4C). In the experiments following this test, animals were exposed to extracts of Tsai Tai at concentrations of 2 mg/mL.

Tsai Tai Extract Enhancement of the Survival of Worms against Lethal Oxidative Stress. Paraquat, an herbicide that is highly toxic to animals and humans, is known to increase intracellular superoxide anion levels, which might result in generation of additional toxic hydrogen peroxide and hydroxyl radicals. To test the effect of Tsai Tai extracts on oxidative stress-mediated toxicity in general, we examined the survival rate of paraquat-challenged *C. elegans*. As shown in Figure 5, the rates of survival significantly ($p < 0.05$) increased in the Tsai Tai-pretreated worms when they were exposed to paraquat compared with the controls. The results shown in Table 2 revealed that the protection provided by Tsai Tai Extracts decreased in the order of Choi Sum > Pak Choi > Hon Tsai Tai, and the increases in survival rate were approximately 28%, 24% and 16%, respectively.

Tsai Tai Extract Reduction of Intracellular ROS Production in *C. elegans*. The overproduction of reactive oxygen species (ROS) is directly associated with oxidative stress, and therefore, to gain insight into how the Tsai Tai extracts suppress the paraquat-induced oxidative stress in *C. elegans*, intracellular ROS production in *C. elegans* after Tsai Tai extract pretreatment was measured using the DCF assay. As shown in Figure 6, the Tsai Tai extracts significantly delayed the increasing trend of ROS levels *in vivo* compared with the untreated control ($p < 0.0001$). A significantly greater ROS clearance capacity was provided by Pak Choi (decrease of approximately 50% in relative fluorescence intensity) *vs.* that of Hon Tsai Tai and Choi Sum (19%).

Tsai Tai Extract Increase in Intracellular SOD Activities and Inhibition of Lipid Peroxidation in *C. elegans*. It is known that oxidative stress caused by excessive ROS can be ameliorated by cellular antioxidant defense systems such as antioxidant enzymes superoxide dismutase (SOD), [39] which maintains ROS at an optimal level either through prevention of formation of oxidants or by removal of them. To further explore the protective mechanism of Tsai Tai extracts against oxidative damage, the intercellular SOD activity and the content of lipid peroxidation product malondialdehyde (MDA) in *C. elegans* after Tsai Tai extract pretreatment were measured. As shown in Figure 7A and B, the activities of SOD were significantly enhanced in N2 when the nematodes were treated with 2

mg/mL of Tsai Tai extracts for 3 days, whereas the MDA content was significantly decreased compared with the untreated animals ($p < 0.05$).

Hon Tsai Tai Extract Prolongation of *C. elegans* Lifespan. Aging has been correlated with oxidative stress in *C. elegans*. [41] Therefore, we evaluated whether the Tsai Tai extracts have effects on the lifespan of *C. elegans*.

Figure 8 shows the curves of survival of wild-type nematodes grown at 20 °C in media containing Tsai Tai extracts compared with untreated worms. As shown in Table 3, The Hon Tsai Tai extract-treated worms showed an increase of 8% in lifespan with respect to the control (32.5 days *vs*. 30.1 days), whereas the other extracts had no significant effects in *C. elegans*. The *long-rank* test revealed that the difference in treatment with Hon Tsai Tai extract was significantly different with respect to the control (p =0.017).

Discussion

Brassica vegetables are a good source of dietary antioxidants. However, little information is available on Tsai Tai varieties. In our study, Hon Tsai Tai, Pak Choi and Choi Sum were analyzed for total contents of phenolics, DPPH radical scavenging activity (RSA) and reducing power (RP). The DPPH RSA of Hon Tsai Tai was significantly higher than the other varieties at concentrations from 0.5 to 2.0 mg/mL (Figure 3A), probably due to pigment compounds such as cyanidins that were detected only in Hon Tsai Tai and are known to have antioxidant activities, including antimutagenic activities[35]. Similar studies [24] reported that the DPPH RSA of the violet-colored *Magnolia denudate* flower was higher than that of the white-colored version, probably due to the cyanindins compounds.

Previous studies suggested that the TPC of crude extracts from dietary plants and medicinal herbs could significantly contribute to their total antioxidant capacities. [37] In the current study, the correlation test showed that the DPPH radical scavenging activities of Tsai Tai extracts were well correlated with their TPC (Hon Tsai Tai, R^2=0.906; Pak Choi, R^2=0.973; Choi Sum, R^2=0.953), indicating that the phenolic compounds in Methanol/HCl extracts of Tsai Tai were significantly responsible for their antioxidant activities.

In addition, a significant linear correlation was observed between RP and TPC (Hon Tsai Tai, R^2=0.998; Pak Choi, R^2=0.961; Choi Sum, R^2=0.975). These results are in agreement with those of a previous study [38], which reported that the reducing power and DPPH radical-scavenging activity of *Magnolia denudata* flower extracts were strongly correlated with its phenol contents. The data presented in this work indicate that the reducing power of Tsai Tai extracts appears to be attributed to their antioxidant activity. A desirable outcome of reducing reactions is termination of free radical chain reactions that might otherwise damage cell structure and function.

Positive correlations were observed among total phenolic contents, DPPH scavenging activity, and reducing power, suggesting that phlorotannins are the major antioxidant components in Tsai Tai. However, a significant difference was noted among the varieties with respect to *in vivo* antioxidant activity, and the TPC of Tsai Tai did not correlate with *in vivo* antioxidant activity. As shown in Figure 5, the rates of survival significantly ($p < 0.05$) increased in the Tsai Tai-pretreated worms when they were exposed to paraquat compared with the controls. These data indicate that Tsai Tai extracts proved efficient in protecting the nematodes against paraquat-induced oxidative stress. Significantly greater protection was provided by Choi Sum (increase of approximately 28% in the survival rate) and Pak Choi (24%) than Hon Tsai Tai (16%).

To explore the protective mechanism of Tsai Tai extracts against oxidative damage, intracellular ROS production was measured in *C. elegans* after Tsai Tai extract pretreatment. Figure 6 shows that Tsai Tai extracts significantly delay the increasing trend of ROS levels *in vivo* compared with the untreated control ($p < 0.0001$). Pak Choi exhibits significantly greater ROS clearance capacity than the other varieties. This result is consistent with the antioxidant protective effect, as observed in Figure 5. To confirm the existence of an antioxidant protective effect, intracellular SOD activity and the content of lipid peroxidation product malondialdehyde (MDA) were measured to check the oxidation status in worms treated with the different Tsai Tai extracts and controls. The results (Figure 7) confirmed that exposure to Tsai Tai extracts induced markedly increased SOD activities and decreased MDA

content, confirming the antioxidant effect of the studied compounds, which is particularly higher in the case of Choi Sum extract.

The protective effects against oxidative stress provided by Tsai Tai extracts might be due to their ability to decrease intracellular ROS accumulation together with parallel upregulation of SOD activities. Similar studies reported that extracts of *Uncaria tomentosa* have neuroprotective effects in *C. elegans* through attenuation of oxidative stress. [40]

Notably, all extracts from Tsai Tai extracts proved efficient in protecting the nematode against oxidative stress, but only the extract from Hon Tsai Tai showed the effect of prolonging lifespan. These data are consistent with the notion that superoxide dismutases protect against oxidative stress but have little or no effect on lifespan in *Caenorhabditis elegans.* [42]

In conclusion, to the best of our knowledge, this is the first report to demonstrate that the extracts from Tsai Tai exert multiple beneficial health effects in an intact organism. The Tsai Tai extracts significantly decrease the intracellular ROS level, and extract from Hon Tsai Tai prolongs the *C. elegans* lifespan. Oxidative stress has been correlated with aging, and therefore, the observed effect of prolonged lifespan via Hon Tsai Tai extract might be due to its antioxidant properties. Although the extracts from Pak Choi and Choi Sum provide significant antioxidant activity, there was no effect of prolonged lifespan in *C. elegans,* probably because not all varieties contain the same antioxidative component profile or relative proportions of compounds within the profile; differences in these profiles might subsequently result in complex changes in antioxidant activity or other bioactivities. Our results showed that Tsai Tai extracts have multiple beneficial health effects, and Hon Tsai Tai extract prolongs *C. elegans* lifespan, suggesting that Hon Tsai Tai could be explored as a potential dietary supplement for anti-aging and warrants further investigations.

Acknowledgements

This study was supported by the National Program on Key Basic Research Project (973 Program, No. 2010CB529804) and the National Natural Science Foundation of China (Grant No. 30971456, 31400155, 81472550, 81274048 and 81273540).

Conflicts of interest: The authors declare no conflict of interest.

References

1. G.-F. Deng, X. Lin, X.-R. Xu, L.-L. Gao, J.-F. Xie and H.-B. Li, *Journal of Functional Foods*, 2013, **5**, 260-266.
2. D. C. Schwenke, *Current opinion in lipidology*, 2013, **24**, 526-527.
3. H. Boeing, A. Bechthold, A. Bub, S. Ellinger, D. Haller, A. Kroke, E. Leschik-Bonnet, M. J. Muller, H. Oberritter, M. Schulze, P. Stehle and B. Watzl, *European journal of nutrition*, 2012, **51**, 637-663.
4. D. A. Freedman, N. Pena-Purcell, D. B. Friedman, M. Ory, S. Flocke, M. T. Barni and J. R. Hebert, *Journal of cancer education : the official journal of the American Association for Cancer Education*, 2014, **29**, 790-795.
5. S. L. Navarro, Y. Schwarz, X. Song, C. Y. Wang, C. Chen, S. P. Trudo, A. R. Kristal, M. Kratz, D. L. Eaton and J. W. Lampe, *The Journal of nutrition*, 2014, **144**, 1850-1857.
6. N. Yanai, S. Shiotani, S. Hagiwara, H. Nabetani and M. Nakajima, *Bioscience, biotechnology, and biochemistry*, 2008, **72**, 3100-3106.
7. A. Vlassopoulos, M. E. Lean and E. Combet, *The Proceedings of the Nutrition Society*, 2014, **73**, 430-438.
8. E. Fasano, S. Serini, N. Mondella, S. Trombino, L. Celleno, P. Lanza, A. Cittadini and G. Calviello, *BioMed research international*, 2014, **2014**, 327452.
9. X. J. Wu and X. Hua, *Cancer biology & therapy*, 2007, **6**, 646-647.
10. S. R. Bhandari and J. H. Kwak, *Molecules*, 2015, **20**, 1228-1243.
11. P. Hanson, R.-y. Yang, L.-c. Chang, L. Ledesma and D. Ledesma, *Journal of Food Composition and Analysis*, 2011, **24**, 950-962.
12. B. Harbaum, E. M. Hubbermann, C. Wolff, R. Herges, Z. Zhu and K. Schwarz, *Journal of agricultural and food chemistry*, 2007, **55**, 8251-8260.
13. W. Thosaikham, K. Jitmanee, R. Sittipout, S. Maneetong, A. Chantiratikul and P. Chantiratikul, *Food chemistry*, 2014, **145**, 736-742.
14. B. Harbaum, E. M. Hubbermann, Z. Zhu and K. Schwarz, *Food chemistry*, 2008, **110**, 838-846.

15. M. Roy, M. Takenaka, S. Isobe and T. Tsushida, *Food chemistry*, 2007, **103**, 106-114.
16. S. Wachtel-Galor, K. Wong and I. Benzie, *Food chemistry*, 2008, **110**, 706-710.
17. M. Abdollahi, M. Y. Moridani, O. I. Aruoma and S. Mostafalou, *Oxidative medicine and cellular longevity*, 2014, **2014**, 876834.
18. F. Bonomini, L. F. Rodella and R. Rezzani, *Aging and disease*, 2015, **6**, 109-120.
19. I. Sadowska-Bartosz and G. Bartosz, *BioMed research international*, 2014, **2014**, 404680.
20. S. K. Kim, J. Lund, M. Kiraly, K. Duke, M. Jiang, J. M. Stuart, A. Eizinger, B. N. Wylie and G. S. Davidson, *Science*, 2001, **293**, 2087-2092.
21. Y. Volovik, F. C. Marques and E. Cohen, *Methods*, 2014, **68**, 458-464.
22. L. Zhang, A. S. Ravipati, S. R. Koyyalamudi, S. C. Jeong, N. Reddy, P. T. Smith, J. Bartlett, K. Shanmugam, G. Munch and M. J. Wu, *Journal of agricultural and food chemistry*, 2011, **59**, 12361-12367.
23. B. J. Xu and S. K. C. Chang, *Journal of agricultural and food chemistry*, 2008, **56**, 8365-8373.
24. Y. H. Jo, G. U. Seo, H. G. Yuk and S. C. Lee, *Food Res Int*, 2012, **47**, 197-200.
25. L. A. Conde-Hernandez and J. A. Guerrero-Beltran, *Food chemistry*, 2014, **142**, 455-460.
26. A. Golmohamadi, G. Moller, J. Powers and C. Nindo, *Ultrasonics sonochemistry*, 2013, **20**, 1316-1323.
27. P. Y. Chao, S. Y. Lin, K. H. Lin, Y. F. Liu, J. I. Hsu, C. M. Yang and J. Y. Lai, *Nutrients*, 2014, **6**, 2115-2130.
28. M. D. Romero-de Soto, P. Garcia-Salas, S. Fernandez-Arroyo, A. Segura-Carretero, F. Fernandez-Campos and B. Clares-Naveros, *Plant foods for human nutrition*, 2013, **68**, 200-206.
29. R. H. Fu, H. J. Harn, S. P. Liu, C. S. Chen, W. L. Chang, Y. M. Chen, J. E. Huang, R. J. Li, S. Y. Tsai, H. S. Hung, W. C. Shyu, S. Z. Lin and Y. C. Wang, *PloS one*, 2014, **9**, e85305.
30. M. E. Lima, A. C. Colpo, W. G. Salgueiro, G. E. Sardinha, D. S. Avila and V. Folmer, *International journal of environmental research and public health*, 2014, **11**, 10091-10104.
31. A. K. Jaiswal, G. Rajauria, N. Abu-Ghannam and S. Gupta, *Natural product communications*, 2011, **6**, 1299-1304.
32. A. Nems, A. Peksa, A. Z. Kucharska, A. Sokol-Letowska, A. Kita, W. Drozdz and K. Hamouz, *Food chemistry*, 2015, **172**, 175-182.
33. U. K. S. Khanam, S. Oba, E. Yanase and Y. Murakami, *Journal of Functional Foods*, 2012, **4**, 979-987.
34. K. Schlesier, M. Harwat, V. Bohm and R. Bitsch, *Free radical research*, 2002, **36**, 177-187.
35. F. Galvano, L. La Fauci, G. Lazzarino, V. Fogliano, A. Ritieni, S. Ciappellano, N. C. Battistini, B. Tavazzi and G. Galvano, *J Nutr Biochem*, 2004, **15**, 2-11.
36. S. Stajcic, G. Cetkovic, J. Canadanovic-Brunet, S. Djilas, A. Mandic and D. Cetojevic-Simin, *Food chemistry*, 2015, **172**, 225-232.
37. M. Drakou, A. Birmpa, A. E. Koutelidakis, M. Komaitis, E. Z. Panagou and M. Kapsokefalou, *International journal of food sciences and nutrition*, 2015, **66**, 197-202.
38. Y.-H. Jo, G.-U. Seo, H.-G. Yuk and S.-C. Lee, *Food Res Int*, 2012, **47**, 197-200.
39. J. N. Sampayo, A. Olsen and G. J. Lithgow, *Aging cell*, 2003, **2**, 319-326.
40. Z. Shi, Z. Lu, Y. Zhao, Y. Wang, X. Zhao-Wilson, P. Guan, X. Duan, Y. Z. Chang and B. Zhao, *Neurochemistry international*, 2013, **62**, 940-947.
41. T. Moriwaki, S. Kato, Y. Kato, A. Hosoki and Q. M. Zhang-Akiyama, *Journal of clinical biochemistry and nutrition*, 2013, **53**, 81-88.

42. R. Doonan, J. J. McElwee, F. Matthijssens, G. A. Walker, K. Houthoofd, P. Back, A. Matscheski, J. R. Vanfleteren and D. Gems, *Genes & development*, 2008, **22**, 3236-3241.

FIGURE LEGENDS

Figure 1. Comparison of extraction solvents for TPC (A) and DPPH IC_{50} (B) in Tsai Tai.

All data are shown as the means ± SD for triplicate determination in the same sample.

GAE, Gallic acid equivalents.

IC_{50} (mg/mL), concentration for scavenging 50% of DPPH radicals.

Figure 2. Total phenolic content of the 3 Tsai Tai varieties (mean ± SD, n=3).

Figure 3. DPPH radical scavenging activity (A) and reducing power (B) of Tsai Tai extracts (mean ± SD, n=3).

Figure 4. Concentration of Tsai Tai extracts for the experiments was determined using a food clearance assay. Newly hatched L1 synchronized N2 animals were incubated in *E. coli* (OD A_{595} = 0.6) in a 96-well plate at 25 °C containing different Tsai Tai extract concentrations. The OD of *E. coli* was recorded daily for each concentration of extracts from Hon Tsai Tai (A), Pak Choi (B) and Choi Sum (C).

Figure 5. Tsai Tai extracts increased the survival rates of paraquat-intoxicated wild-type N2 nematodes. After treatment with Tsai Tai extracts at 20 °C for 3 days, the nematodes were exposed to 0.1 mol/L paraquat and scored every 12 h for survival rate. Representative Kaplan–Meier survival curves are shown from three independent experiments.

Figure 6. Tsai Tai extracts significantly delayed the increasing trend of ROS levels *in vivo* compared with that in the untreated control ($p < 0.0001$). The nematodes were treated with Tsai Tai extracts at 20 °C for 3 days and lysed for determination of ROS level via DCF assay every 20 min for 120 min. Data represent three independent experiments and are presented as the mean ± SD.

Figure 7. Effects of Tsai Tai extracts on SOD activity and MDA content in *C. elegans*. (A) Tsai Tai extracts increased the activity of SOD in N2. (B) Tsai Tai extracts decreased MDA content in N2. Synchronized wild-type L1 larvae were pretreated with Tsai Tai extracts for 3 days at 20 °C and lysed for determination of SOD activity and MDA content. Data represent three independent experiments and are presented as the mean ± SD.

Figure 8. Effects of Tsai Tai extracts on the lifespan of wild-type *C. elegans* N2. Synchronized adult hermaphrodite worms were placed in S-medium in the absence (control) or presence of 2 mg/mL Tsai Tai extracts. Surviving and dead animals were counted daily until all nematodes had died. Survival curves show the untreated control compared with Tsai Tai extract-treated worms. At least three independent biological replicates were performed. Statistical significance of the difference between the curves (treated *vs.* untreated control) was demonstrated by *log-rank* test using Kaplan-Meier survival analysis. Differences at the $p < 0.05$ level were considered significant.

Table 1. IC_{50} (mg/mL) values of Tsai Tai extracts and control compounds in the DPPH and RP assays.

DPPH IC_{50} (mg/mL), concentration for scavenging 50% of DPPH radicals.

RP IC_{50} (mg/mL), concentration for increasing 0.500 value in optical density.

Table 2. Influence of Tsai Tai extracts (2 mg/mL) on lifespan of paraquat-intoxicated wild-type N2 nematodes.

Mean±standard deviation (n=3). Statistical significance was calculated by *long-rank* testing, changes in mean lifespan are considered significant at $p < 0.05$.

Table 3. Influence of Tsai Tai extracts (2 mg/mL) on adult lifespan of *C. elegans*.

Mean±standard deviation (n=3). Statistical significance was calculated by *long-rank* testing, changes in mean lifespan are considered significant at $p < 0.05$.

Abbreviations Used

C. elegans = Caenorhabditis elegans;

mg GAE/100 g = milligrams of gallic acid equivalents per 100 g of fresh weight;

mg CYEs/100 g = milligrams of cyanidin-3-glucoside equivalents per 100 g of fresh weight;

RSA = Radical Scavenging Activity;

RP = Reducing Power;

SOD = Superoxide Dismutase;

MDA = malondialdehyde;

TPC = Total Phenolic Contents.

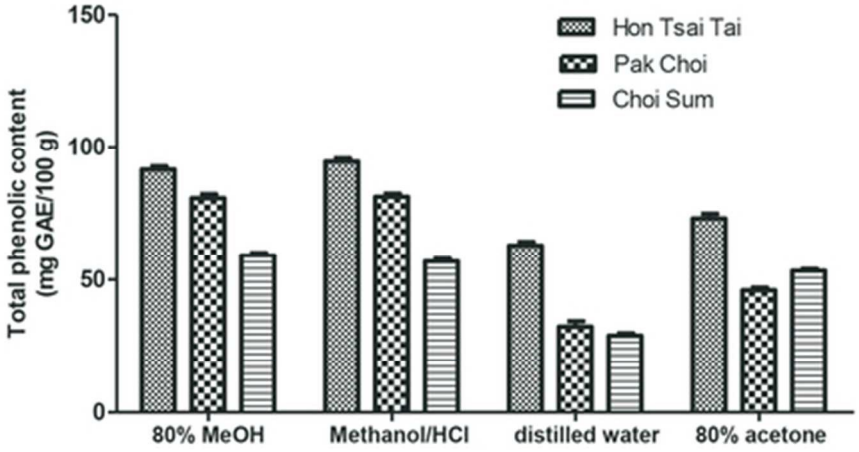


Figure 1A

40x26mm (300 x 300 DPI)

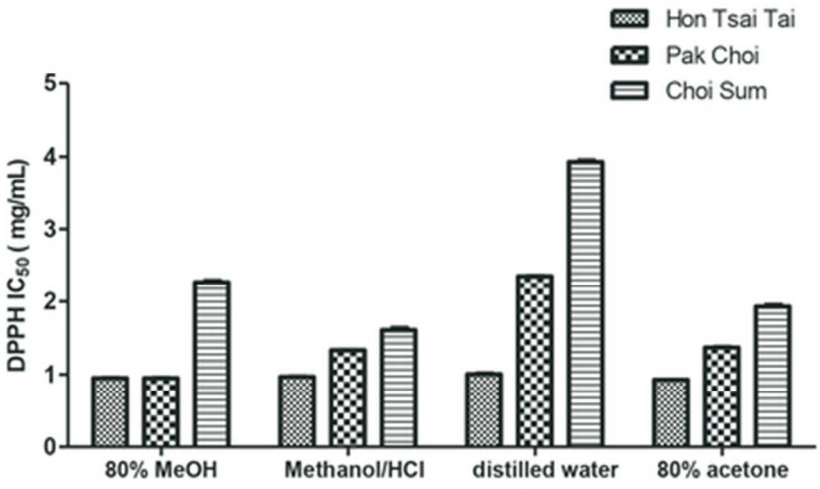


Figure 1B

40x26mm (300 x 300 DPI)

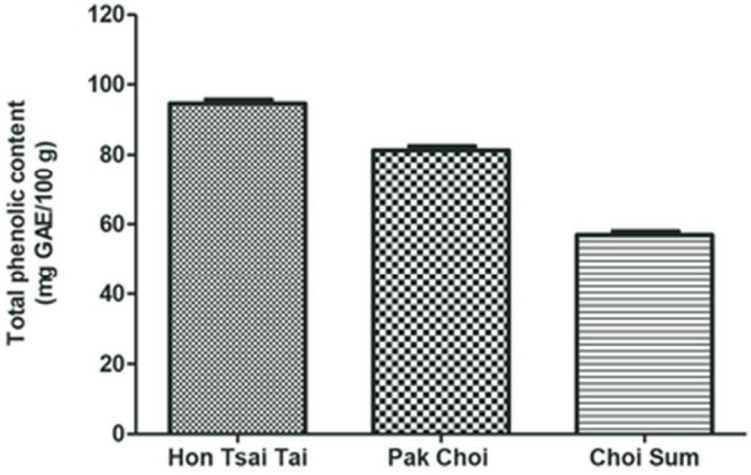


Figure 2

39x26mm (300 x 300 DPI)

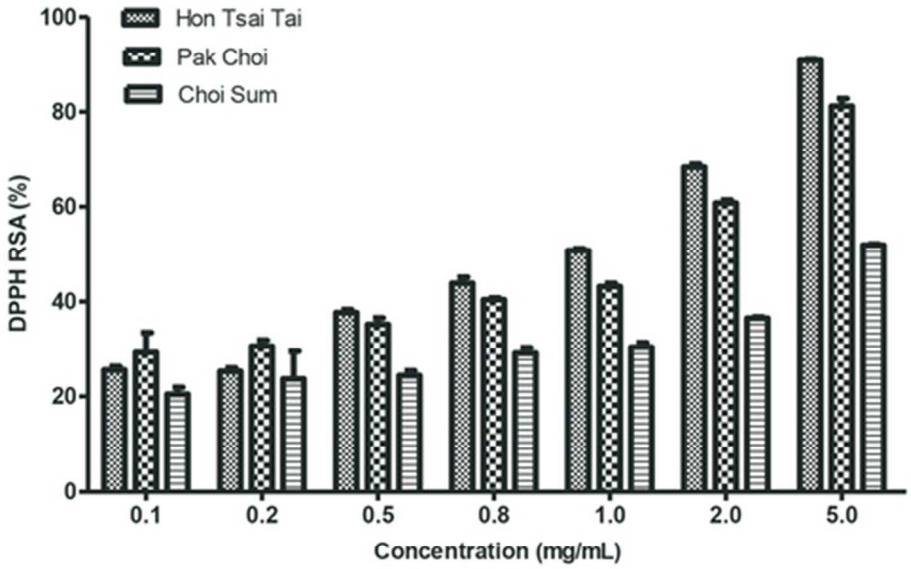


Figure 3A

44x30mm (300 x 300 DPI)

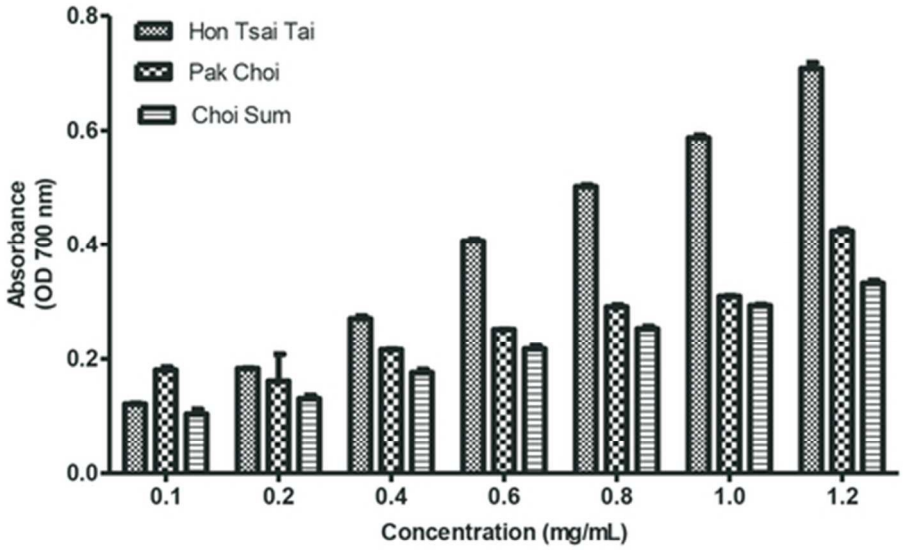


Figure 3B

44x31mm (300 x 300 DPI)

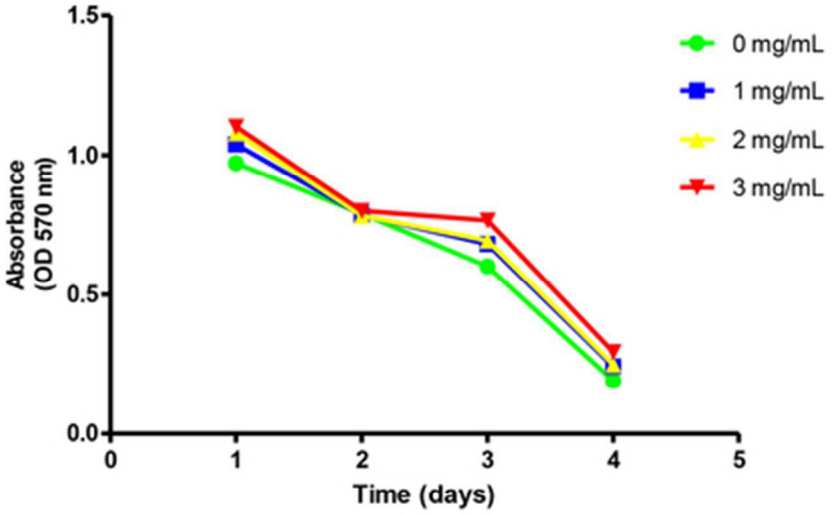


Figure 4A

40x26mm (300 x 300 DPI)

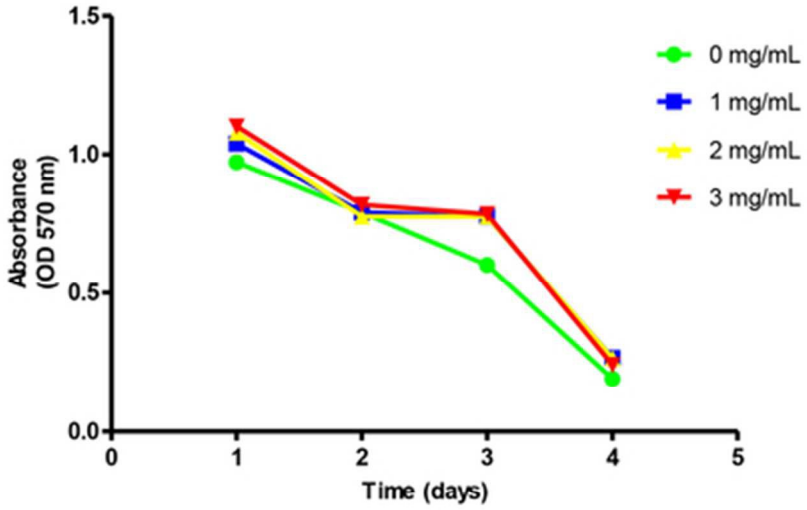


Figure 4B

39x26mm (300 x 300 DPI)

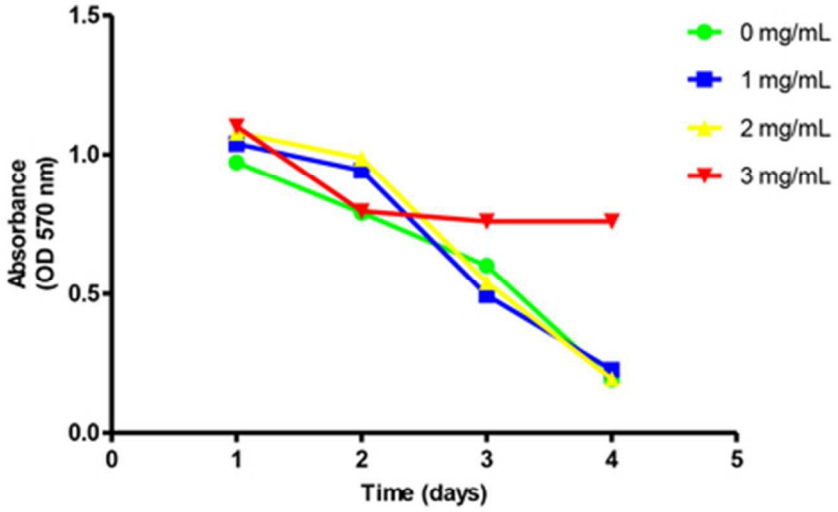


Figure 4C

39x26mm (300 x 300 DPI)

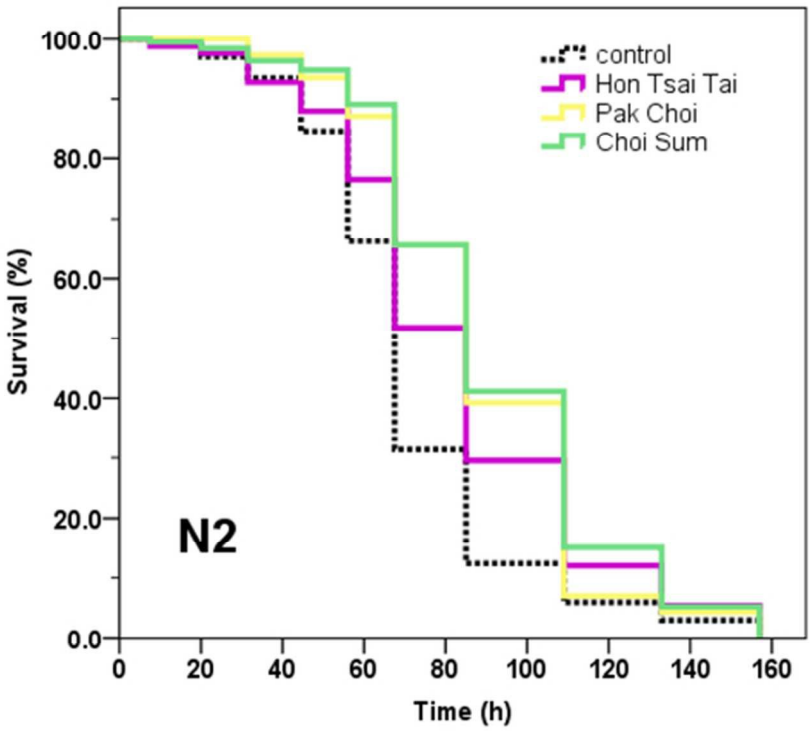


Figure 5

55x50mm (300 x 300 DPI)

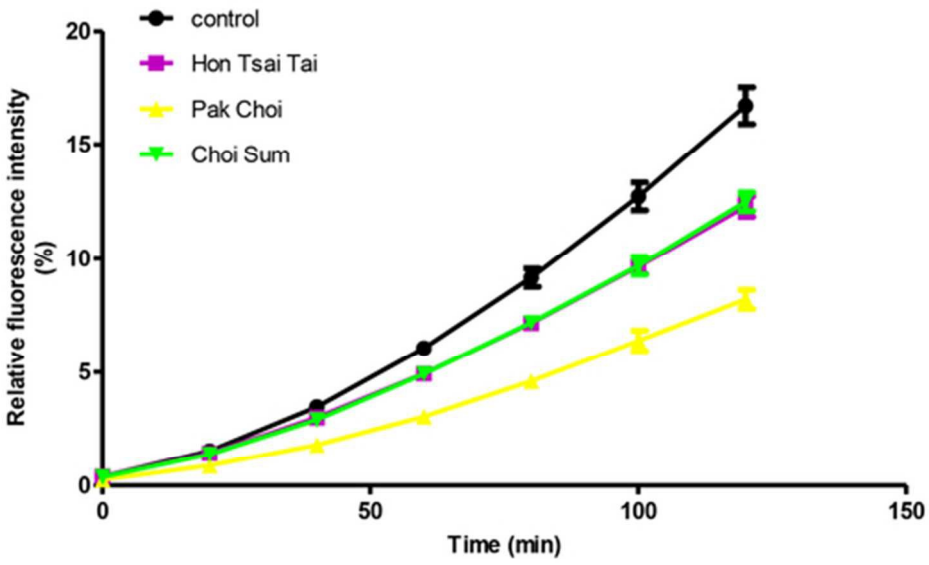


Figure 6

46x32mm (300 x 300 DPI)

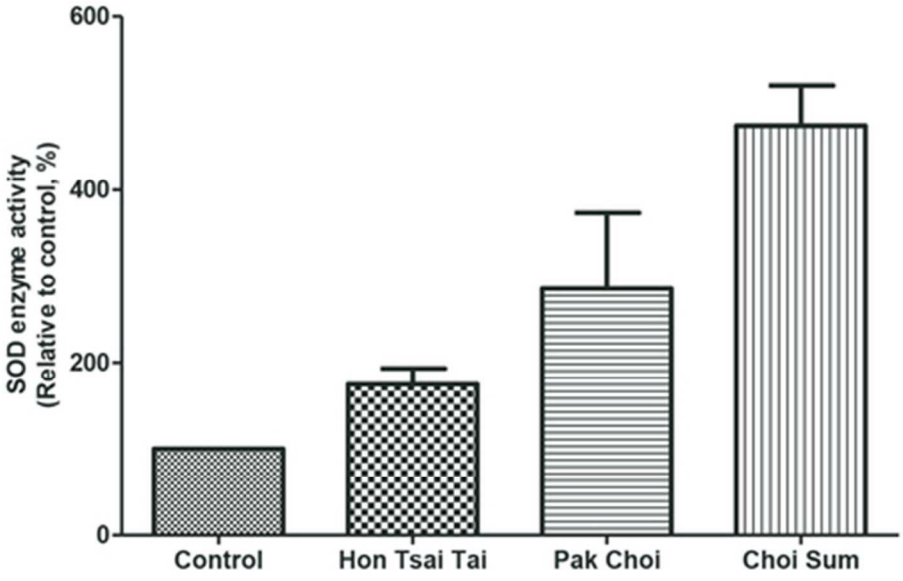


Figure 7A

41x29mm (300 x 300 DPI)

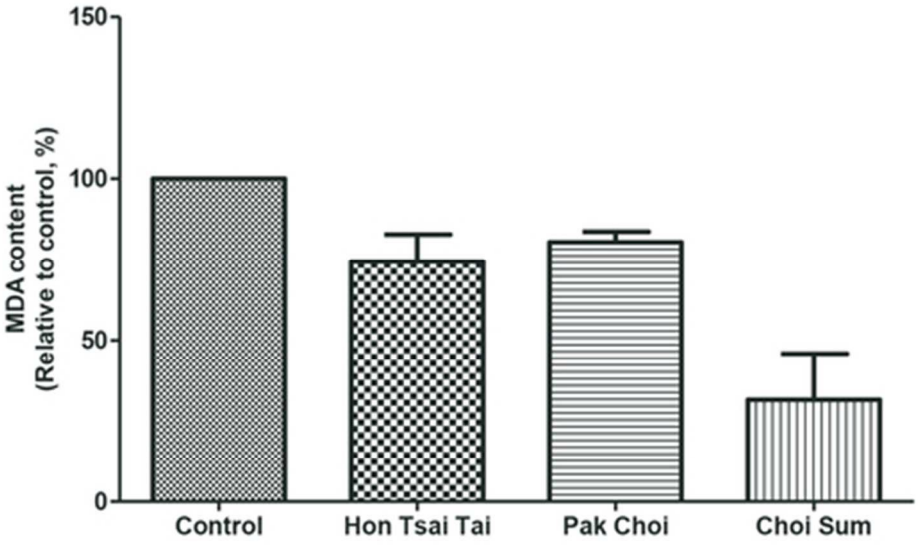


Figure 7B

41x29mm (300 x 300 DPI)

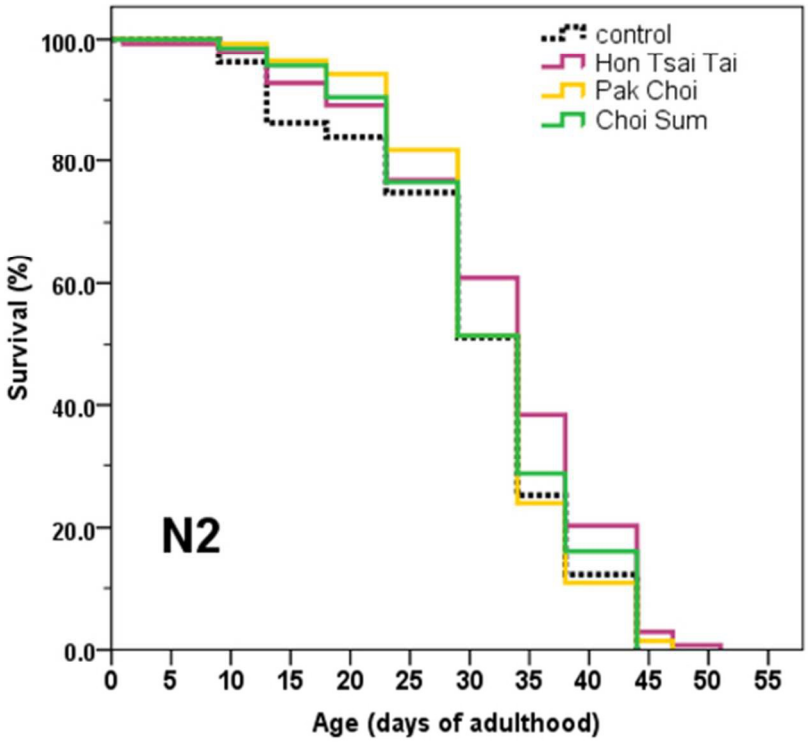


Figure 8

54x50mm (300 x 300 DPI)

Table 1. IC_{50} (mg/mL) values of Tsai Tai extracts and control compounds in the DPPH and RP assays.

Extracts and controls	DPPH	RP
Hon Tsai Tai	0.968±0.003	0.846±0.002
Pak Choi	1.331±0.003	1.946±0.004
Choi Sum	1.608±0.004	2.029±0.004
Ascorbic acid	0.007	0.018

Table 2. Influence of Tsai Tai extracts (2 mg/mL) on lifespan of paraquat-intoxicated wild-type N2 nematodes.

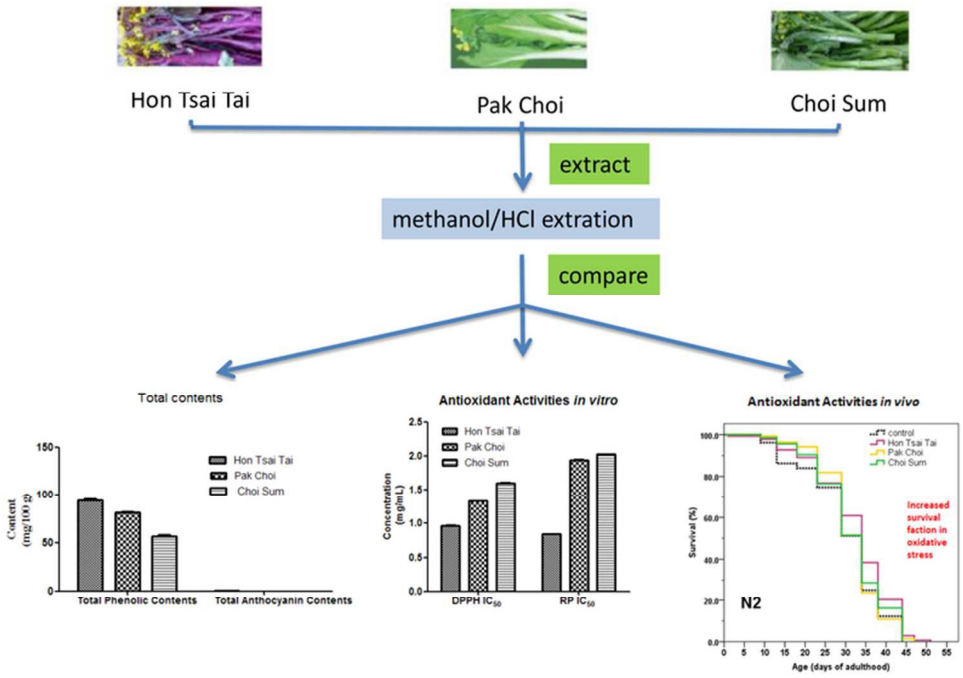
Treatment	Mean (h)	*n*	*p vs.* control
Control	71.2±2.1	169	
Hon Tsai Tai	82.4±2.5	166	0.000
Pak Choi	88.5±2.0	186	0.000
Choi Sum	91.2±2.2	192	0.000

Mean±standard deviation (n=3). Statistical significance was calculated by *long-rank* testing, changes in mean lifespan are considered significant at $p < 0.05$.

Table 3. Influence of Tsai Tai extracts (2 mg/mL) on adult lifespan of *C. elegans*.

Treatment	Mean (days)	*n*	*p vs.* control
Control	30.1±0.8	131	
Hon Tsai Tai	32.5±0.8	138	0.017
Pak Choi	31.6±0.6	138	0.680
Choi Sum	31.5±0.7	136	0.411

Mean±standard deviation (n=3). Statistical significance was calculated by *long-rank* testing, changes in mean lifespan are considered significant at $p < 0.05$.



69x49mm (300 x 300 DPI)