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Commercial extract of the brown seaweed *Ascophyllum nodosum* enhances phenolic antioxidant content of spinach (*Spinacia oleracea* L.) which protects *Caenorhabditis elegans* against oxidative and thermal stress

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ABSTRACT

There is considerable interest to enhance the nutritional quality of fresh produce especially vegetables. The effects of root treatment of spinach with commercial extracts of the brown macro alga, *Ascophyllum nodosum* (ANE) on antioxidant level of spinach were studied. At the concentration of 1.0 g/L, ANE treatment significantly increased the total phenolics and flavonoids content, total antioxidant activity (as measured by DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging capacity) and Fe^{2+} chelating ability in spinach leaves. The ^1H NMR and LC-MS analyses of spinach extract suggests that the increased antioxidant activity is largely associated with flavonoids. The biological effect of ANE-enhanced polyphenols was tested using the *Caenorhabditis elegans* nematode model. The extracts from ANE-treated spinach significantly improved the survival of the animals under oxidative stress by 50% and high temperature stress by 61% as compared to the extracts from untreated plants (0% and 38%, respectively). Taken together, the results suggest that ANE stimulate flavonoid synthesis in spinach leaf thus enhancing its nutritional quality. Furthermore, the increased flavonoid content exerts beneficial effects in *C. elegans* against oxidative and heat stress.

1. Introduction

Oxidative stress results when the rate of generation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxyl radical ($^{\cdot}OH$) and superoxide ($O_2^{\cdot-}$), exceeds the rate of their disposal (Hodges, Lester, Munro, & Toivonen, 2004). ROS can react with various cellular components such as polyunsaturated fatty acids, carbohydrates, proteins, and nucleic acids, leading to oxidative degradation and subsequent senescence, diseases, and accelerated ageing (Hodges & Lester, 2006). Considerable epidemiological studies exist for the role of a wide range of antioxidative constituents, such as vitamin C, vitamin E, β-carotene, and phenolic acids, present in fruits and vegetables in the maintenance of health and reducing the risk of cancer, cardiovascular and Alzheimer's disease (Hung et al., 2004). Thus improving the nutritional quality of fruits and vegetables will be beneficial for human health.

Spinach (*Spinacia oleracea* L.) is one of the most important leafy green vegetables which contains large quantities of bioactive compounds and nutrients such as ascorbate, carotenoids, tocopherols, phenolics, folate, and minerals (Gil, Ferreres, & Tomas-Barberan, 1999). Spinach contains large amounts of *p*-coumaric acid derivatives that exhibit strong antioxidant activity (Bergman, Varshavsky, Gottlieb, & Grossman, 2001). As one of the flavonoid-rich vegetables, spinach is also abundant in glucuronic acid derivatives of flavonoids (e.g. patuletin and spinacetin) that are not common to most other vegetables (Edenharder, Keller, Platt, & Unger, 2001; Pandjaitan, Howard, Morelock, & Gil, 2005).

Ascophyllum nodosum (L.) Le Jol., a perennial brown marine alga the distribution of which extends from the rocky intertidal zone of Nova Scotia and New Brunswick to Norway (Rayorath et al., 2009). It is a widely-researched seaweed species traditionally used as a fertilizer, a soil conditioning agent, animal feed supplement and also as a human nutritional supplement. An extract (ANE) was

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shown to improve stress tolerance in a number of plant species. This was mediated by an increase in the concentration of bioactive molecules including antioxidants in the treated plants (Rayorath et al., 2009; Zhang & Schmidt, 2000). However, the effect of ANE to improve nutritional quality of fresh produce like vegetables and fruits has not been investigated.

The nematode *Caenorhabditis elegans*, a simple yet sophisticated multicellular animal which has become an important model organism for the study of biological processes due to its easy culture conditions, short generation time and lifespan, and experimental flexibility (Kaletta & Hengartner, 2006). Moreover, *C. elegans* and humans share similar aspects of ageing (Herndon et al., 2002), where oxidative stress is considered to be a major limiting factor in lifespan (Finkel & Holbrook, 2000). It is for these reasons that *C. elegans* is highly-valued for identifying compounds, genes, and mechanisms that may extend the longevity of humans (Wilson et al., 2006). Earlier studies suggested that certain extracts of plant origin could be effective in reversing ageing (Joseph et al., 1999), extending lifespan (Peng et al., 2009) and improving stress tolerance (Gruber, Tang, & Halliwell, 2007; Kyung et al., 2007; Siriwardhana, Shahidi, & Jeon, 2006; Wilson et al., 2006) in several biological systems.

In this paper, we report the effects of pre-harvest treatment with ANE on antioxidant capacity, phenolic compound profiles, ascorbic acid content, and the activity of the related enzymes of spinach. We also determined the effects of spinach extracts (with and without ANE treatment) on lifespan and stress tolerance of *C. elegans*.

2. Materials and methods

2.1. Chemicals, seeds and C. elegans

All chemicals and reagents were of analytical grade and purchased from Sigma Chemical Company (Oakville, ON, Canada) unless otherwise stated. Spinach seeds (*S. oleracea* L., var. Unipack 12) were purchased from Stokes Seeds Company (Thorold, ON, Canada). The wild type *C. elegans* strain Bristol N2 and the OP50 *Escherichia coli* strain were obtained from the *Caenorhabditis* Genetics Center, University of Minnesota, St. Paul, MN, USA. The *C. elegans* population was maintained at 20 °C on 3.5-cm nematode growth medium (NGM) agar plates seeded with live OP50 *E. coli* (50 μL of 10^{10} cells/mL *E. coli* stock per plate) as a food source.

2.2. Plant culture and treatment

Spinach seeds were sown 1.5 cm deep in plastic pots (5″ diameter; 3 seeds per pot) containing potting mix. Pots were placed in a growth chamber maintained at 18 °C with a photoperiod of 10 h light (350–400 μmol m^{-2} s^{-1})/14 h dark. Fertilizer (N:P:K, 20:20:20, 200 ppm) was initiated 3 weeks after planting and then applied every 4 days at the rate of 200 mL per pot. Plants were grown for 7 weeks.

The sample of soluble extract powder from the commercial extraction of *A. nodosum* (Acadian®) was provided by Acadian Seaplants Limited, Dartmouth, NS, Canada. The total organic and inorganic composition and elemental analysis of this alkaline extract and the lipophilic fraction of the ANE have been published (Rayorath et al., 2009). Solutions of ANE were prepared by dissolving 0.1, 1.0, or 5.0 g soluble powder in 1.0 L distilled water with continuous stirring for 10 min. Spinach plants were irrigated with various ANE solutions (i.e. 0, 0.1, 1.0, or 5.0 g/L) at the rate of 50 mL per plant 14 and 7 days prior to harvest. Earlier we had established that 1 g/L elicited the most beneficial effect on plant growth and development (Rayorath et al., 2009).

2.3. Preparation of spinach leaves and their extracts

Leaves from the 5–8th positions counted from the bottom of the stem were manually cut at the petiole using a pair of sharp scissors and placed in perforated plastic bags in a refrigerated cooler (4 °C) within 1 h of harvest. Leaf samples were de-ribbed and chopped into small pieces (≈0.5–1 cm^2). These were either thoroughly mixed and sub-samples immediately analysed (for total antioxidant capacity and ascorbic acid) or bagged, flash-frozen in liquid nitrogen, and stored at −80 °C (for total phenolics and flavonoids). The frozen spinach leaf tissues were extracted twice with 70% aqueous methanol (v/v) at 40 °C for 2 h; the extracts were centrifuged and combined (Edenharder et al., 2001). These extracts were either directly used in the determination of total phenolics and total flavonoids, or freeze-dried for later use.

2.4. Measurement of total phenolic content

The total phenolic content was analysed using the Folin–Ciocalteu method as described previously (Singh, Muthy, & Jayaprakasha, 2002) with slight modifications. Spinach extracts, or standard solutions, were pipetted into 2 mL tubes and to each 1.58 mL water and 100 μL Folin–Ciocalteu reagent (Sigma Chemical Company, Oakville, ON, Canada) was added. After 8 min incubation, 300 μL 20% (w/v) sodium carbonate solution was added to stop the reaction. The vortexed mixture was left at room temperature in the dark for 2 h and the absorbance was read at 760 nm using a Hitachi U1100 Spectrophotometer versus a blank (70% methanol). A standard calibration curve (20–500 mg/L) was prepared using the same procedure as above. Total phenolics were expressed as gallic acid equivalents (GAE, mg gallic acid/100 g FW).

2.5. DPPH radical scavenging assay

The total antioxidant capacities of extracts were measured using the $DPPH^{\cdot}$ assay according to the procedure of Brand-Williams, Cuvelier, and Berset (1995) with some modifications. Fresh spinach leaf tissue (1.5 g) was homogenised in 15 mL methanol using a mortar and pestle. After centrifugation at 10,000*g* for 10 min, the supernatant was recovered and the pellet re-extracted with 10 mL methanol. The supernatants were combined and the total volume was made up to 25 mL. The pellet was re-dissolved in 5 mL dichloromethane (DCM), re-homogenised and centrifuged again at 10,000*g* for 10 min. Pellet was re-extracted with 5 mL DCM, and the supernatant was re-centrifuged, combined, and made up to 10 mL. Each extract was added to 2850 μL DPPH solution (0.11 mM), and then incubated for 6 h at 22 °C. Absorbance was then read at 515 nm against a methanol or DCM blank. The scavenging activity was calculated according to the equation: Inhibition % = $[(A_b - A_s)/A_b] \times 100$, where A_b is the absorption of blank sample and A_s is the absorption in the presence of the test sample. The results were expressed in μM Trolox equivalents (TE, μM Trolox)/100 g FW through comparison against a standard curve (25–800 μM Trolox).

2.6. Ferrous ion chelating ability

The chelating activity of spinach samples on Fe^{2+} was determined according to Heimler, Isolani, Vignolini, Tombelli, and Romani (2007). Each sample was diluted in 1.4 mL 250 mM acetate buffer (pH 4.75) and mixed with 25 μL of 2 mM $FeCl_2$ and 1 mL of 70% MeOH. After 20-min incubation at room temperature, 100 μL of 5 mM ferrozine were added to initiate the reaction. The mixture was shaken vigorously, and the absorbance was read after 10 min at 562 nm against a blank (70% MeOH) using a Hitachi U1100 Spectrophotometer (Hitachi, Tokyo, Japan). The ability of

disrupting the formation of the ferrozine–Fe^{2+} complex was calculated as follows: chelating activity % = $[(A_b - A_s)/A_b] \times 100$, where A_b is the absorbance of the blank sample and A_s is the absorbance of the test sample.

2.7. Determination of ascorbic acid

Total ascorbate was analysed based on the method of Hodges and Lester (2006) using L-ascorbic acid as a standard. Fresh chopped leaves (5 g) were immediately ground in a mortar and pestle with sand and 15 mL ice-cold freshly prepared 5% (w/v) *m*-phosphoric acid. The homogenate was centrifuged at 10,000*g* for 15 min at 4 °C. Total ascorbate was determined by incubating 100 μL supernatant, 500 μL 150 mM KH_2PO_4 buffer (pH 7.4) containing 5 mM Ethylene diamine tetraacetic acid (EDTA), and 100 μL 10 mM Dithiothreitol (DTT) at room temperature for 50 min. Following that, 100 μL of 0.5% (w/v) *N*-ethylmaleimide (NEM) was added to remove excess DTT. In order to develop colour in the reaction mixtures, reagent solutions were added in the order of 400 μL 10% (w/v) trichloroacetic acid (TCA), 400 μL 44% *o*-phosphoric acid, 400 μL 4% (w/v) α-α^1-dipyridyl, and 200 μL 30 g/L $FeCl_3$. The reaction mixtures were incubated at 40 °C for 60 min in a shaking water bath, and absorbance was recorded at 525 nm using a Hitachi U1100 Spectrophotometer. The results were expressed as μmol/g FW.

2.8. Determination of flavonoid content

The total flavonoid content in spinach leaves was quantified following a colorimetric method described by Liu and Zhu (2007).

2.9. NMR spectroscopy analysis of spinach extracts

Spinach leaf extracts from freeze-dried tissues extracted in 70% MeOH were dissolved to a concentration at 100 mg DW/mL, and 750 μL were transferred to 5 mm NMR tube for both 1D and 2D analysis. NMR spectra were acquired on a Bruker Avance III 600 MHz NMR spectrometer (Bruker Biospin Ltd., Milton, ON, Canada) operating at 600.284 MHz ^{1}H observation frequency and a temperature of 25.00 ± 0.01 °C. Spectra were obtained with a 5 mm gradient inverse probe, autotuned and matched and shimmed on each sample, at a flip angle of 30° ($\pi/2$ = 7.3 μs) followed by a 2.66 s acquisition time and 2 s relaxation delay. Each spectrum consisted of 32 scans of 64 k data points with a spectral width of 12335.52 Hz. ^{1}H NMR chemical shifts in the spectra were referenced to δ 2.50 ppm solvent signal. The signals were acquired, processed and analysed using TopSpin® NMR data acquisition and processing Software (Bruker Biospin Ltd., East Milton, ON).

2.10. LC-MS analysis of spinach extracts

Samples used for NMR profiling were subjected to LC-MS analysis. Of the 100 μL DMSO-d_6 solution was diluted with MeOH (10×), and then filtered through a 0.45 μm filter before loading to HPLC. The samples were analysed on both Agilent 1100 and 1200 LC systems with Agilent Zorbax Eclipse XDB-C18 (PN 993967-902) 4.6 × 150 mm, 5 micron column (Agilent Technologies Canada Inc., Mississauga, ON, Canada), 30 °C, eluted with solvents A (water with 2.7% formic acid) and B (MeOH with 2.7% formic acid) with a linear gradient of 5–100% solvent B over 26 min and then held at 100% B until 45 min, at a low rate of 0.25 mL/min. Both diode-array detector (DAD) (monitored at 210, 254, and 320 nm with continuous recording of UV spectrum from 190 to 600 nm) and mass selective detector (MSD) (API-ES source with positive ion scan range of 100–1000 with a step size of 0.25 amu. The drying gas was set at 10 L/min and 350 °C; the nebulizer pressure was 25 PSIG and the capillary was set at 4000 V) were used for the Agilent 1100 LC system, and the injection volume was 5 μL.

For LC-MS/MS analysis, an AB/Sciex 4000 QTRAP (AB Sciex, ON, Canada) was used coupled to the Agilent 1200 LC system under the same chromatography conditions. The Turbo V IonSpray source was used in either positive or negative ion mode. Q1 scans were performed over the mass range 100–1200 amu with a 3 s scan period. The curtain gas was set at 10 a.u. (arbitrary units); the source temperature was 450 °C, ion source gases 1 and 2 were both 40. The declustering potential was set at 100 V. The source voltage was 5500 V for positive ion and −4500 V for negative ion experiments. Positive and negative ion MS/MS experiments were performed using Information Dependent Acquisition (IDA) mode with the same source parameters as the scan runs. An enhanced MS scan was followed with enhanced resolution to determine charge state. Using collision assisted dissociation (CAD), enhanced product ion spectra were generated for the three most intense peaks in each scan using rolling collision energies.

2.11. Effect of spinach extract on lifespan of C. elegans

The stock solutions (10 mg/mL) of spinach extracts were prepared by dissolving freeze-dried spinach extracts in 70% ethanol and storing at −20 °C until use. To prepare plates supplemented with the spinach extracts, the stock solution of extract was added to freshly autoclaved nematode growth medium (NGM) (55 °C) to a final concentration of 0, 5, 10, 50, 100, or 200 μg/mL. To achieve synchronised animals, reproductive nematodes were placed onto NGM plates and allowed to lay eggs for 6 h. Eggs were permitted to hatch at 20 °C and develop into adults 3 days later. These were transferred to treatment plates with heat-killed OP50 (Gruber et al., 2007) as nematode food and 400 μM 5-fluorodeoxyuridine (FUdR) which was used to prevent progeny development (Hosono, 1978). Nematodes were transferred to fresh treatment plates every 3 days for the first 9 days of the lifespan assay. Survival of the worms was evaluated each day and animals were scored as dead if they failed to respond to gentle repeated taps with a platinum pick. The first day of adulthood was considered as day 1.

2.12. Effect of spinach extract on oxidative and heat stresses of C. elegans

The oxidative resistance assay was performed at 20 °C with nematode hermaphrodites (2 days old). Juglone was dissolved in ethanol and added to a final concentration of 500 μM in melted NGM (55 °C). Plates were allowed to dry for 30 min and then 50 μL of OP50 *E. coli* (at 10^{10} cells/mL) was spotted per 3.5 cm NGM agar plate. After drying for 45 min in a laminar hood, the worms which were treated on plates containing spinach extracts for 48 h were transferred to the freshly prepared juglone plates. Viability was scored every 2 h until all of the control worms had died.

For the thermal tolerance assay, young adults were incubated on treatment plates for 2 days at 20 °C and then exposed to heat shock (35 °C) for 16 h. The number of dead worms was counted every hour. Nematode worms that crawled up the walls of the plates and died from desiccation were considered lost and excluded from the analyses.

2.13. Statistical analysis

Experimental data were analysed by one-way analysis of variance (ANOVA) and differences between control and ANE-treated plants were considered statistically significant at $P \leqslant 0.05$ using Tukeys Honestly Significant Differences (HSD) test of the COSTAT®

statistical software. In growth chamber experiments, 20 plants constituted a replicate. This experiment was repeated four times.

Results from lifespan and stress resistance experiments were processed using Kaplan–Meier survival analysis of SPSS 15.0 software and compared amongst the control and the spinach extract-treated groups for significance by means of a log-rank pairwise comparison test.

3. Results and discussion

3.1. Antioxidant content and the Fe^{2+} chelating ability of spinach

Pre-harvest root treatment of spinach plant with 1.0 g/L ANE resulted in a 1.21-fold increase in the DPPH radical scavenging activity in the leaves (Fig. 1A). Similarly, the Fe^{2+} chelating ability of extract of leaves from 1.0 and 5.0 g/L ANE treatments were significantly higher than those of extracts of 0.1 g/L treated leaves or untreated controls. At 100 mg FW/mL, 1.0 and 5.0 g/L treated leaves chelated 32 ± 1.6% and 29 ± 1.7% of ferrous ions whereas control and leaves from 0.1 g/L treatment chelated 18 ± 0.8% and 21 ± 1.9%, respectively. In order to determine the extent to which phenolic compounds contribute to total antioxidant capacity in spinach, linear regression was performed with data from both control and treated plants. A positive, linear relationship ($R^2 = 0.933$) between total phenolic content and DPPH measurements was observed. The higher phenolic content was correlated with a stronger antioxidant capacity. Few studies have shown that exogenous application of ANE increased endogenous antioxidant activity in plants, such as increased amounts of non-enzymatic antioxidant compounds (α-tocopherol, ascorbate and β-carotene) and enhanced activities of antioxidant enzymes including ascorbate peroxidase, glutathione reductase, and superoxide dismutase (Allen et al., 2001). High antioxidant amounts/activities following ANE treatment are important for improving the nutritional value of fruit and vegetables as well as prolonging their shelf life, thus enhancing the overall quality and marketable value of fresh produce. In this study, we found an increase in the phenolic content and total antioxidant capacity of spinach treated with 1.0 g/L ANE.

The significant increase in concentrations of phenolics and total antioxidant capacity and Fe^{2+} chelating ability specifically obtained with a 1.0 g/L ANE treatment may partly be attributed to the increase of endogenous plant hormone activities stimulated by ANE. For example, elicitors causing cytokinin-like responses which are present in ANE have been shown to increase endogenous antioxidant activity and subsequent stress tolerance of creeping bentgrass (Zhang & Schmidt, 2000). Interestingly, there was no effect with respect to total ascorbate concentration in all ANE treatments. This suggests that ANE treatment through root drench may induce specific systemic physiological responses that specifically alter the concentration of phenolic antioxidants in spinach leaves.

3.2. Effect of ANE treatment on flavonoid content of spinach leaves

To examine the quality of phenolics that was increased due to ANE treatment we estimated the concentration of flavonoids in spinach leaves. Pre-harvest treatment of spinach with 1.0 g/L ANE significantly enhanced the concentration of flavonoids. The total amounts of flavonoids in spinach leaves increased in all ANE treatments, the 1.0 and 5.0 g/L treated leaves showed 1.5- and 1.2-fold higher concentrations of flavonoids as compared to those of the controls (Fig. 1B). We observed that ANE treatment significantly increased the activity of chalcone isomerase (CHI, EC 5.5.1.6), one of the key enzymes for the biosynthesis of flavanone precursors and phenylpropanoid plant defence compounds (data not shown). Thus it appears that the increase in total flavonoids was largely due to an increase in the biosynthesis mediated by chalcone isomerase.

In order to further investigate the quantitative changes of major flavonoids induced by the ANE treatment, the 12 representative spinach samples were analysed by HPLC-MS/MS. The HPLC profiles of these samples were very much the same (data not shown). Based on our MS/MS data and the spinach flavonoids MS data provided in literature (Bergquist, Gertsson, Knuthsen, & Olsson, 2005; Cho, Howard, Prior, & Morelock, 2008), the major flavonoids in the spinach samples were tentatively identified as: peak 1, patuletin-3-glucosyl-(1 → 6)[apiosyl(1 → 2)]-glucoside; peak 2, spinacetin-3-glucosyl-(1 → 6)[apiosyl(1 → 2)]-glucoside and patuletin-3-(2″-feruloylglucosyl)-(1 → 6)[apiosyl(1 → 2)]-glucoside; peak 3, spinacetin-3-(2″-feruloylglucosyl)-(1 → 6)[apiosyl(1 → 2)]-glucoside glucoside and spinacetin-3-(2″-coumaroylglucosyl)-(1 → 6)[apiosyl(1 → 2)]-glucoside; peak 4, spinatoside; peak 5, jaceidin-4′-glucuronide; peak 6, 5,3′,4′-trihydroxy-3-methoxy-6:7-methylendioxyflavone-4′-glucuronide; and peak 7, 5,4′-dihydroxy-3,3′-dimethoxy-6:7-methylendioxyflavone-4′-glucuronide (Fig. 2A and Table 1). The changes of these flavonoids from the control and ANE-treated samples were based on the peak areas of chromato-

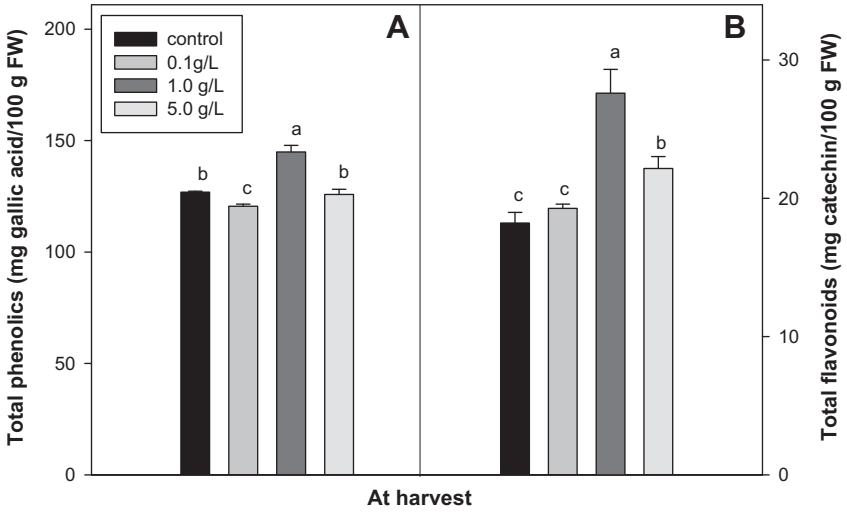

Fig. 1. Effect of root treatment of spinach (*Spinacia oleracea* L.) with *Ascophyllum nodosum* extract (ANE) on total phenolics content (A) and total flavonoids content of spinach leaves. Values with different letters are significantly different ($P \leqslant 0.05$).

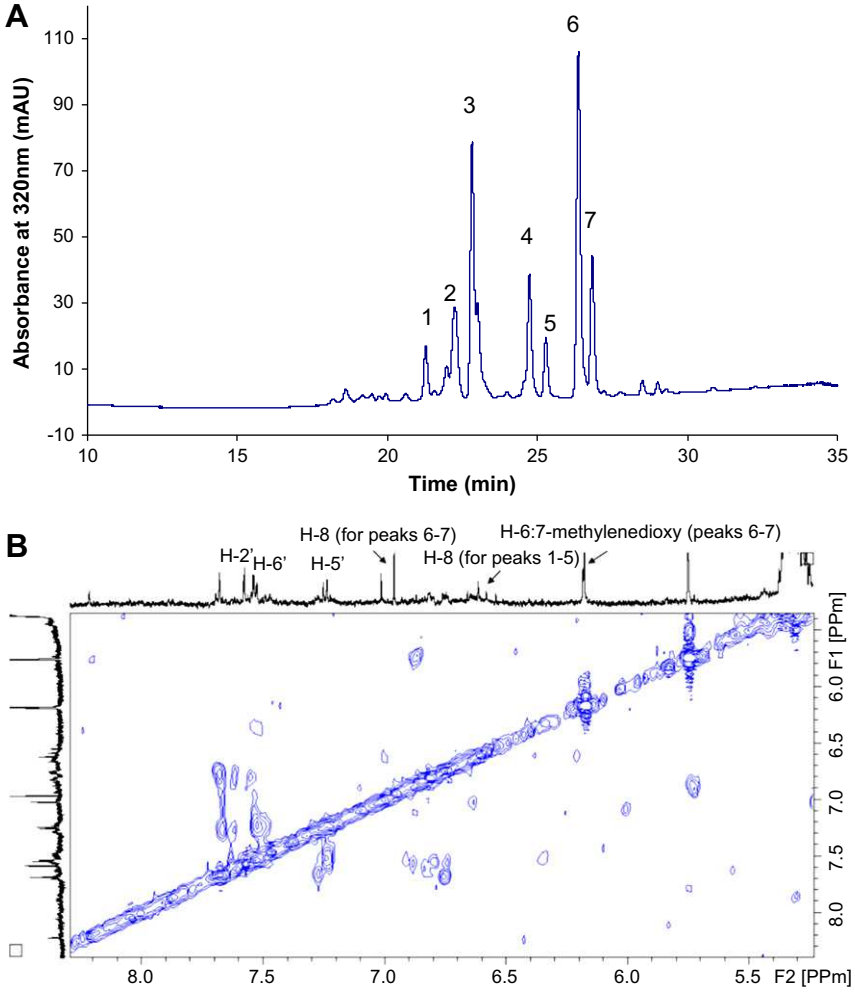

Fig. 2. High Pressure Liquid Chromatography (HPLC) and ^{1}H NMR analysis of spinach extracts. HPLC chromatogram of spinach flavonoids at 340 nm (A) and ^{1}H–^{1}H COSY spectrum (B) of spinach extracts. See Table 1 for peak identification.

grams. The results indicated an overall increase from 35–51% (Table 1). As for 0.1 and 5.0 g/L ANE treatments, the changes of the nine major flavonoids, as compared to control samples, were minor. At 0.1 g/L ANE, the concentration of spinacetin-3-glucosyl-(1 → 6)[apiosyl(1 → 2)]-glucoside and patuletin-3-(2″-feruloylglucosyl)-(1 → 6)[apiosyl(1 → 2)]-glucoside (peak 2), spinatoside (peak 4) and jaceidin-4′-glucuronide (peak 5) were decreased by 12%, 22%, and 15%, respectively. While 5.0 g/L ANE treatment showed no differences when compared to controls in regard to the nine major flavonoids.

The proton NMR spectra of six representative samples of spinach extracts (1–3: control; 4–6 ANE-treated) were compared. In general, no major differences were observed (data not shown). As phenolics have been documented as the main bioactive components in spinach extracts (Bergman et al., 2001), the aromatic proton region of the spectra was expanded (data not shown). It appeared that the samples have very similar phenolic component profiles. Clearly, the signals within the aromatic proton region indicated the presence of flavonoids as the dominant phenolics. With the assistance of the ^{1}H–^{1}H COSY spectrum (Fig. 2B) and the NMR data reported previously (Ferreres, Castaner, & Tomas-Barberan, 1997; Edenharder et al., 2001), the peaks at δ 7.58 ppm (d, J = 2.2 Hz), 7.53 ppm (dd, J = 8.7; 2.2 Hz), and 7.24 (d, J = 8.7 Hz) can be assigned to the protons of H-2′, H-6′, and H-5′ in B-ring. H-8 proton in A-ring for 6:7-methylenedioxy flavonoids appeared at δ 7.01 or 6.96 ppm as singlet; while in other 5,6,7-oxygenated substituted flavonol derivatives the H-8 singlet is at δ 6.62–6.54 ppm. The two protons in methylenedioxy moiety are shown at δ 6.18 ppm.

3.3. Spinach extracts extend the lifespan of *C. elegans*

In order to determine the longevity-extending effects of spinach extract, *C. elegans* were exposed to different concentrations of extracts from control plants (Spi) or those treated with 1.0 g/L ANE (Spi 1.0). There were no differences in the lifespan of nematodes following treatment with either 2% ethanol or water, thus distilled water controls were used in all experiments.

A concentration-dependent effect on longevity was observed. We did not find any difference in longevity between Spi and Spi 1.0 treatments (data not shown). A low concentration (5 μg/mL) did not affect longevity notably, while all the other treatments led to a significantly higher percentage of living worms in the population than that of the control treatment. The most effective beneficial concentration of extract (100 μg/mL) from both control and treated spinach extended the mean lifespan from 23 to 31 days, which is an extension of the mean lifespan of approximately 35%. Treatment with this concentration also led to an increase in the maximum lifespan of the nematodes by approximately 36%. In

Table 1
Peak assignment, retention time (RT), UV spectra, mass spectral data and changes in the quantity of flavonoids in spinach leaf.

Peak	RT (min)	Identification	Spectral characterisation (nm)	*m/z*	Fragments	Increase (compared with control) (%)
1	21.277	Patuletin-3-glucosyl-(1 → 6)[apiosyl(1 → 2)]-glucoside	353, 257	789 ([M+H]$^+$): 811 ([M+Na]$^+$) 787 ([M−H]$^-$)	657 ([M−apiose+H]$^+$), 495 ([M−apiose−glucose+H]$^+$), 332 (patuletin)	38
2	22.247	Spinacetin-3-glucosyl-(1 → 6)[apiosyl(1 → 2)]-glucoside and Patuletin-3-(2″-feruloylglucosyl)-(1 → 6)[apiosyl(1 → 2)]-glucoside	345, 245	803 ([M+H]$^+$): 825 ([M+Na]$^+$) 801 ([M−H]$^-$) 965 ([M+H]$^+$): 987 ([M+Na]$^+$) 963 ([M−H]$^-$)	693 ([M−apiose+Na]$^+$), 347 ([spinacetin+H]$^+$) 333 ([patuletin+H]+)	51
3	22.826	Spinacetin-3-(2″-feruloylglucosyl)-(1 → 6)[apiosyl(1 → 2)]-glucoside and Spinacetin-3-(2″-coumaroylglucosyl)-(1 → 6)[apiosyl(1 → 2)]-glucoside	313, 242, 230	979 ([M+H]$^+$): 1001 ([M+Na]$^+$) 977 ([M−H]$^-$) 949 ([M+H]$^+$): 971 ([M+Na]$^+$) 947 ([M−H]$^-$)	869 ([M−apiose+Na]$^+$), 531 ([M−apiose−glucose−feruloyl+Na]$^+$), 346 (apinacetin)	41
4	24.737	Spinatoside	341, 271, 252	523 ([M+H]$^+$) 521 ([M−H]$^-$)	346 ([M−glucuronide])	36
5	25.282	Jaceidin-4′-glucuronide	344, 272, 246	537 ([M+H]$^+$) 535 ([M−H]$^-$)	361 ([M−glucuronide+H]$^+$)	47
6	26.370	5,3′,4′-Trihydroxy-3-methoxy-6:7-methylendioxyflavone-4′-glucuronide	341, 277, 250	521 ([M+H]$^+$): 543 ([M+Na]$^+$) 519 ([M−H]$^-$)	344 ([M−glucuronide])	44
7	26.831	5,4′-Dihydroxy-3,3′-dimethoxy-6:7-methylendioxyflavone-4′-glucuronide	342, 279	535 ([M+H]$^+$): 557 ([M+Na]$^+$) 533 ([M−H]$^-$)	359 ([M−glucuronide+H]$^+$)	44

addition, this treatment almost doubled the time before the first dead worm was observed, from day 9 to day 19.

3.4. ANE-treated spinach imparts increased resistance to stresses of *C. elegans*

To investigate whether spinach extracts are protective against thermal and oxidative stress in *C. elegans*, young adults were pre-treated with Spi or Spi 1.0 for 48 h before being exposed to 35 °C or 500 μM juglone (a pro-oxidant that is able to convert oxygen to the superoxide anion, thus generating intracellular oxidative stress (Kampkotter et al., 2007). Our results showed that pre-treatment enhanced the worms' resistance to heat shock and juglone, and consequently increased survival rates. Under heat stress, when all control worms died, there were still 63% and 51% alive in the Spi 1.0 5 μg/mL and Spi 10 μg/mL groups, respectively (Fig. 3A). The mean survival rate was significantly increased by 29% with Spi 1.0 5 μg/mL, and 27% with Spi 10 μg/mL versus control (Table 2). All concentrations except Spi 50 μg/mL showed strong protective effects for resistance to oxidative stress induced by juglone. Though the optimum effective concentration from both extracts was the same (200 μg/ml), Spi 1.0 200 μg/ml showed a much higher mean survival rate ($P < 0.05$) (Fig. 3B and Table 2).

Phenolic compounds in plants have been shown to function as free radical quenchers due to their high reactivity as electron donors (Podsędek, 2007). Recent studies demonstrated that blueberry polyphenols (Wilson et al., 2006), resveratrol from wine grape (Gruber et al., 2007), *Ginkgo biloba* (Kampkotter et al., 2007), and epigallocatechin gallate (EGCG) from green tea (Zhang, Jie, Zhang, & Zhao, 2009) were able to prolong the lifespan and/or increase resistance to stressors in *C. elegans*. Spinach extracts have been studied in both *in vitro* and *in vivo* systems, and are widely shown to possess anti-inflammatory effects, anti-mutagenic potential, anti-neoplastic effects, as well as chemo-preventive activities (Boivin et al., 2009; Hait-Darshan, Grossman, Bergman, Deutsch, & Zurgil, 2009). These activities in spinach were found to be superior to those of the well-documented antioxidants such as EGCG, ascorbic acid, and vitamin E, probably due to the broad free radical scavenging capacity of the natural antioxidants, especially the novel antioxidant glucuronated flavonoids (Bergman, Perelman, Dubinsky, & Grossman, 2003; Cho et al., 2008) in spinach. Here for the first time, we demonstrated that spinach extracts are capable of modulating the normal lifespan of wild type *C. elegans* Bristol N2. Exposure of *C. elegans* to spinach extracts resulted in a dose-dependent increase in their longevity. In addition, significant increases in survival of *C. elegans* under heat and oxidative stress were observed when they were pre-incubated in the presence of spinach extracts. These results were in agreement with the total antioxidant capacity, total phenolics and flavonoids in spinach. Higher anti-radical capacity and higher phenolic and flavonoid content in the spinach leaf extract led to a greater ability in reducing mortality of *C. elegans* under either normal or stress conditions.

Wilson et al. (2006) reported that blueberry polyphenols prolong lifespan and increase thermal tolerance, but not oxidative resistance in *C. elegans*. In this study, we observed that *C. elegans* treated with spinach extract besides extending longevity also increased resistance to both thermal and oxidative stresses. Though spinach extract prolonged the lifespan of *C. elegans* in a concentration-dependent manner, higher concentrations of spinach extract conferred an increase in oxidative stress resistance whereas lower concentrations exerted higher survival under thermal stress. Apart from the possible direct roles as radical scavengers, spinach extract may act as moderate stimuli to trigger adaptive stress responses or cellular defence mechanisms resulting in an increased stress tolerance in nematodes (Wiegant et al., 2009). Further detailed study might concentrate on these aspects to clarify the underlying mechanisms.

There are no studies which have, as yet, discovered the specific chemical compounds within ANE that elicit the phenylpropanoid and flavonoid pathways in plants. The use of natural, environmentally friendly, safe extracts to increase antioxidant level of vegetables would be ideal, thus it would be of great interest to elucidate the mechanism(s) by which ANE may exert such effects on spinach. Furthermore, the bioactive compounds in spinach extract deserve to be further studied. The physiological and molecular signalling mechanisms of spinach extract on elongated lifespan and increased

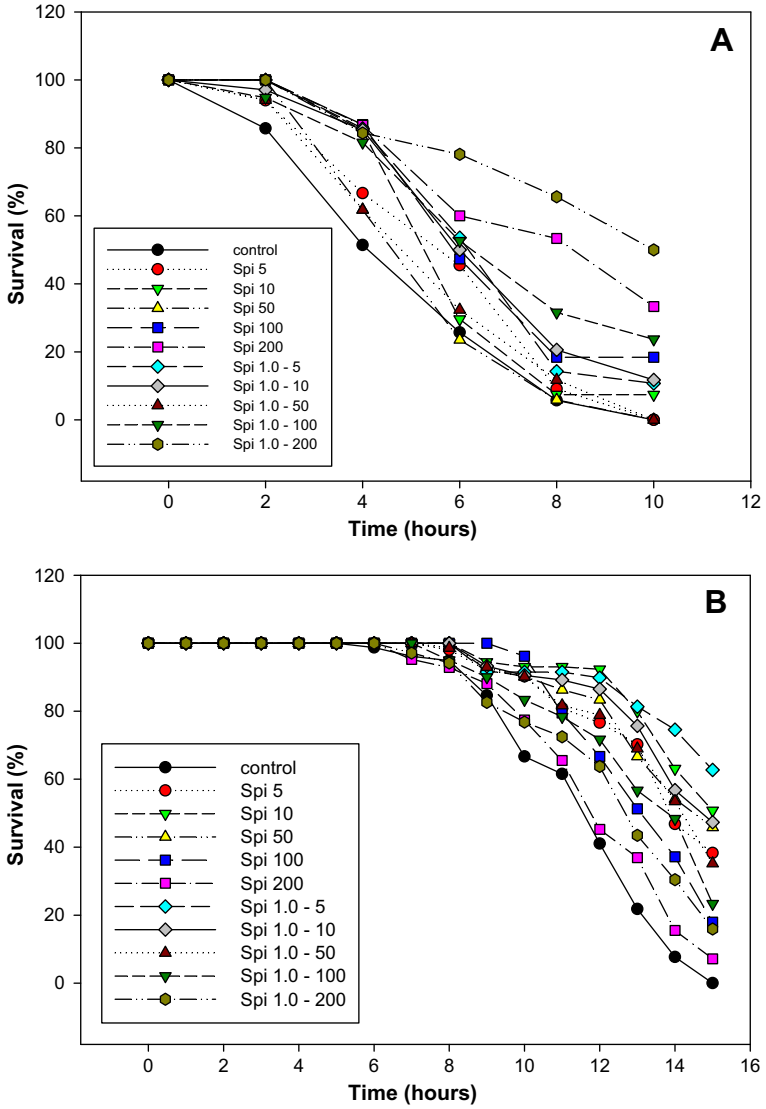

Fig. 3. Protective effects of spinach extracts on *Caenorhabiditis elegans* wild type Bristol N2 under heat stress (A) and oxidative stress (B).

Table 2
Protective effects of spinach extracts on *Caenorhabditis elegans* wild type Bristol N2 under thermal and oxidative stresses.

Stress	Treatment (20 °C)	Total (N)	Censored (N)	Adult lifespan hours (Mean, SE)	*P* vs. control (log-rank)
35 °C	Control	156	0	10.8, 0.21	
	Spi 5	192	73	12.9, 0.23	<0.0001
	Spi 1.0–5	144	91	13.9, 0.20	<0.0001
	Spi 10	156	80	13.7, 0.19	<0.0001
	Spi 1.0–10	186	87	13.4, 0.21	<0.0001
Juglone	Control	141	0	3.4, 0.23	
	Spi 10	149	10	4.6, 0.20	0.00038
	Spi 1.0–10	165	20	5.3, 0.25	<0.0001
	Spi 200	112	37	6.7, 0.30	<0.0001
	Spi 1.0–200	199	100	7.6, 0.30	<0.0001

stress resistance in *C. elegans* should be thoroughly investigated, and synergy among individual compounds of spinach extract requires elucidation.

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References

Allen, V. G., Pond, K. R., Saker, K. E., Fontenot, J. P., Bagley, C. P., Ivy, R. L., et al. (2001). Tasco: Influence of a brown seaweed on antioxidants in forages and livestock—A review. *Journal of Animal Science, 79*, E21–E31.

Bergman, M., Varshavsky, L., Gottlieb, H. E., & Grossman, S. (2001). The antioxidant activity of aqueous spinach extract: chemical identification of active fractions. *Phytochemisty, 58*, 143–152.

Bergman, M., Perelman, A., Dubinsky, Z., & Grossman, S. (2003). Scavenging of reactive oxygen species by a novel glucuronated flavonoid antioxidant isolated and purified from spinach. *Phytochemistry, 62*, 753–762.

Bergquist, S. M., Gertsson, U. E., Knuthsen, P., & Olsson, M. E. (2005). Flavonoids in baby spinach (*Spinacia oleracea* L.): Changes during plant growth and storage. *Journal of Agricultural and Food Chemistry, 53*, 9459–9464.

Boivin, D., Lamy, S., Lord-Dufour, S., Jackson, J., Beaulieu, E., Côté, M., et al. (2009). Antiproliferative and antioxidant activities of common vegetables: A comparative study. *Food Chemistry, 112*, 374–380.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *Lebensmittel-Wissenschaft und-Technologie/Food Science and Technology, 28*, 25–30.

Cho, J. M., Howard, L. R., Prior, R. L., & Morelock, T. (2008). Flavonoid content and antioxidant capacity of spinach genotypes determined by high-performance liquid chromatography/mass spectrometry. *Journal of the Science of Food and Agriculture, 88*, 1099–1106.

Edenharder, R., Keller, G., Platt, K. L., & Unger, K. K. (2001). Isolation and characterization of structurally novel antimutagenic flavonoids from spinach (*Spinacia oleracea*). *Journal of Agricultural and Food Chemistry, 49*, 2767–2773.

Ferreres, F., Castaner, M., & Tomas-Barberan, F. A. (1997). Acylated flavonol glycosides from spinach leaves (*Spinacia oleracea*). *Phytochemistry, 45*, 1701–1705.

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

Gil, M. I., Ferreres, F., & Tomas-Barberan, F. A. (1999). Effect of postharvest storage and processing on the antioxidant constituents (flavonoids and vitamin C) of fresh-cut spinach. *Journal of Agricultural and Food Chemistry, 47*, 2213–2217.

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*, 530–542.

Hait-Darshan, R., Grossman, S., Bergman, M., Deutsch, M., & Zurgil, N. (2009). Synergistic activity between a spinach-derived natural antioxidant (NAO) and commercial antioxidants in a variety of oxidation systems. *Food Research International, 42*, 246–253.

Heimler, D., Isolani, L., Vignolini, P., Tombelli, S., & Romani, A. (2007). Polyphenol content and antioxidative activity in some species of freshly consumed salads. *Agriculture and Food Chemistry, 55*, 1724–1729.

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

Hodges, D. M., & Lester, G. E. (2006). Comparisons between orange- and green-fleshed non-netted and orange-fleshed netted muskmelons: Antioxidant changes following different harvest and storage periods. *Journal of the American Society for Horticultural Science, 131*, 110–117.

Hodges, D. M., Lester, G. E., Munro, K. D., & Toivonen, P. M. A. (2004). Oxidative stress: Importance for postharvest quality. *Hort Science, 39*, 924–929.

Hosono, R. (1978). Sterilization and growth inhibition of *Caenorhabditis elegans* by 5-fluorodeoxyuridine (FdUrd). *Experimental Gerontology, 13*, 369–374.

Hung, H.-C., Joshipura, K. J., Jiang, R., Hu, F. B., Hunter, D., Smith-Warner, S. A., et al. (2004). Fruit and vegetables intake and risk of major chronic disease. *Journal of National Cancer Institute, 96*, 1577–1584.

Joseph, J. A., Shukitt-Hale, B., Denisove, N. A., Bielinski, D., Martin, A., McEwen, J. J., et al. (1999). Reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. *The Journal of Neuroscience, 19*, 8114–8121.

Kaletta, T., & Hengartner, M. O. (2006). Finding function in novel targets: *C. elegans* as a model organism. *Nature Reviews Drug Discovery, 5*, 387–399.

Kampkotter, A., Pielarski, T., Rohrig, R., Timpel, C., Chovolou, Y., & Watjen, W. (2007). The *Ginkgo biloba* extract EGb761 reduces stress sensitivity, ROS accumulation and expression of catalase and glutathione S-transferase 4 in *Caenorhabditis elegans*. *Pharmacological Research, 55*, 139–147.

Kyung, K. E., Lee, S. J., Jeon, Y. J., Ahn, C. B., Song, M. D., Park, T. K., et al. (2007). Antioxidant effect and DNA protective effect of various enzymatic extracts from *Perilla frutescens* var *crispa*. *Journal of Food Lipids, 14*, 335–349.

Liu, B., & Zhu, Y. (2007). Extraction of flavonoids from flavonoid-rich parts in tartay buckwheat and identification of the main flavonoids. *Journal of Food Engineering, 78*, 584–587.

Pandjaitan, N., Howard, L. R., Morelock, T., & Gil, M. I. (2005). Antioxidant capacity and phenolic content of spinach as affected by genetics and maturation. *Journal of Agricultural and Food Chemistry, 53*, 8618–8623.

Podsędek, A. (2007). Natural antioxidants and antioxidant capacity of *Brassica* vegetables: A review. *Lebensmittel-Wissenschaft und-Technologie/Food Science and Technology, 40*, 1–11.

Rayorath, P., Benkel, B., Hodges, D. M., Allan-Wojtas, P., Mackinnon, S., Critchley, A. T., et al. (2009). Lipophilic components of the brown seaweed, *Ascophyllum nodosum*, enhance freezing tolerance in *Arabidopsis thaliana*. *Planta, 230*, 135–147.

Singh, R. P., Muthy, K. N. C., & Jayaprakasha, G. K. (2002). Studies on the antioxidant activity of pomegranate (*Pinica granatum*) peel and seed extracts using *in vitro* models. *Journal of Agricultural and Food Chemistry, 50*, 81–86.

Siriwardhana, N., Shahidi, F., & Jeon, Y. J. (2006). Potential antioxidative effects of cactus pear fruit (*Opuntia ficus-indica*) extract on radical scavenging and DNA damage reduction in human peripheral lymphocytes. *Journal of Food Lipids, 13*, 445–458.

Wiegant, F. A. C., Surinove, S., Ytsma, E., Langelaar-Makkinje, M., Wikman, G., & Post, J. A. (2009). Plant adaptogens increase lifespan and stress resistance in *C. elegans*. *Biogeronology, 10*, 27–42.

Wilson, M. A., Shukit-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*, 59–68.

Zhang, L. Z., Jie, G. L., Zhang, J. J., & Zhao, B. L. (2009). Significant longevity-extending effects of EGCG on *Caenorhabditis elegans* under stress. *Free Radical Biology & Medicine, 46*, 414–421.

Zhang, X., & Schmidt, R. E. (2000). Hormone-containing products' impact on antioxidant status of tall fescue and creeping bentgrass subjected to drought. *Crop Science, 40*, 1344–1349.