

## Antioxidant Capacity and Phenolic Content in Leaf Extracts of Tree Spinach (*Cnidoscopus* spp.)

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Total phenolic content and antioxidant capacity of two tree spinach species (*Cnidoscopus chayamansa* McVaugh and *C. aconitifolius* Miller.) were determined in raw and cooked leaf extracts. Antioxidant capacity was assessed by the oxygen radical absorbance capacity (ORAC) assay, and flavonoid glycoside composition was quantified by HPLC and identified by GC. Total phenolics and antioxidant capacity were higher in raw than in cooked leaf extracts. The ORAC values were strongly correlated with total phenolic content ( $r = 0.926$ ) in all leaf extracts. The major flavonoids isolated from the leaf extracts were kaempferol-3-*O*-glycosides and quercetin-3-*O*-glycosides. *C. aconitifolius* leaves contained more varieties of the flavonoid glycosides than *C. chayamansa*. Cooking reduced antioxidant activity and phenolic content and resulted in losses of some kaempferol glycoside and quercetin glycoside residues in leaf extracts. The results of this study indicate that tree spinach leaves are a rich source of natural antioxidants for foods.

**KEYWORDS:** Tree spinach; *Cnidoscopus* species; phenolics; flavonoids; antioxidant capacity

### INTRODUCTION

Tree spinach (*Cnidoscopus* spp., Euphorbiaceae), a leafy green vegetable from dry regions of the tropics, is used as a food for its nutritional value and ethnomedicinal properties especially linked to non-insulin-dependent diabetes mellitus (1). The tree spinach is an important green leafy vegetable consumed after cooking in boiled water because the plant is marginally to very poisonous when eaten raw (2). There is little information available on tree spinach (*Cnidoscopus* spp.) flavonoids (3), and the most commonly isolated flavonoids from leaves of *Cnidoscopus* species are *C*-glycosyl flavone and flavonoid glycosides (galactosides, glucosides, rhambosides, and rhamnosylglucosides of quercetin and/or kaempferol) (4). Plant-derived polyphenolic flavonoids exhibit numerous biological and pharmacological properties (5–8) that could potentially afford protection against chronic diseases. Information on flavonoid composition and antioxidant activity of tree spinach leaves is scarce. Recently we reported proximate composition and mineral content of the two edible species of *Cnidoscopus* and showed that tree spinach leaves had significantly higher ascorbic acid and carotenoids than spinach (*Spinach oleraceae*) leaves (9).

It is highly impractical to quantify all of the compounds in plants that exhibit antioxidant activities; a variety of methods have been developed to quantify total antioxidant capacity of plant extracts. Cao et al. (10) used the oxygen radical absorbance capacity (ORAC) assay, which measures the ability of plant extracts to scavenge peroxy radicals, to determine total antioxidant capacity of many fruits and vegetables. Studies have

shown that fruits and vegetable vary greatly in their total antioxidant capacity, and extracts with high levels of total phenolics typically have high ORAC values.

This study was undertaken to determine antioxidant activity using ORAC assay and the total phenolic and flavonoid contents of raw and cooked leaf extracts of two edible species of tree spinach.

### MATERIALS AND METHODS

**Leaf Sampling.** Fully expanded leaves of the two *Cnidoscopus* spp. were sampled in late summer and early fall from field-grown plants at Texas A&M University—Kingsville Horticultural Crops Research plots. Both late summer and early fall leaf samples were pooled for analysis. The two species were identified botanically using Lundell's key (11).

**Chemicals Analyses.** *Chemicals.* *R*-Phycoerythrin (R-PE) from *Porphyridium cruentum* and chlorogenic acid and Folin—Ciocalteu reagent were purchased from Sigma (St. Louis, MO). 2',2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA Inc. (Richmond, VA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and *N,N* dimethylformamide (DMF) were obtained from Aldrich (Milwaukee, WI). Quercetin and kaempferol standards were obtained from Atomergic Chemetals Corp. (Farmingdale, NJ). All chemicals used in the experiments were reagent- or HPLC-grade and were purchased from VWR (West Chester, PA).

*Sample Preparation for Determination of ORAC and Total Phenolics.* Fresh leaf samples were harvested, sorted for green leaves, washed thoroughly in water to remove dirt, and stored in a refrigerator at 5 °C overnight prior to analysis. Refrigerated raw samples (500 g in triplicate) were homogenized for 2 min to the smallest possible particle size using a Black & Decker Handy Chopper and Waring blender. Pooled subsamples were extracted (5 g/20 mL in triplicate) with ethanol/acetone/water/acetic acid (40:40:20:0.1). Samples were placed in screw-

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cap vials to prevent solvent evaporation and heated for 60 min in a 60 °C water bath. Raw leaf samples (500 g in triplicate) to be cooked were cut into smaller even portions and placed in a shallow 1-L volume Corning microwavable container; 10 mL of water was added, and the container was covered with a glass lid to promote steaming. The container was then cooked slowly in 1.3 cu ft Panasonic microwave 1000-W oven with autoturntable for 2 min. The cooked leaf samples were then extracted as previously described. Extracts from both raw and cooked leaf samples were cooled, homogenized for 1 min using a tissue homogenizer Multi-Gen 7 (Pro Scientific Inc., Oxford, CT), filtered through Miracloth (CalBiochem, LaJolla, CA), and kept frozen (−20 °C) until analysis. Prior to ORAC and total phenolic assays, frozen extracts were thawed and diluted 100× with 75 mM phosphate buffer (pH 7.0) and deionized water, respectively.

**Samples Preparation for Determination of Flavonoids.** Flavonoids were extracted according to the method of Merken and Beecher (12). A 25-g raw or cooked sample was homogenized with 75 mL of MeOH/H<sub>2</sub>O (5:95) containing citric acid (0.5 g/L) and EDTA (0.5 g/L). Homogenate was filtered through Miracloth and passed through a 0.45- $\mu$ m-pore filter prior to HPLC and GC analyses.

**ORAC Assay.** Oxygen radical absorbance capacity (ORAC) was measured following procedures previously described (10). This assay measures the ability of antioxidant components in test materials to inhibit the decline in R-PE fluorescence that is induced by a peroxy radical generator, AAPH. The reaction mixture contained 1.7 mL of 75 mM phosphate buffer (pH 7.0), 100  $\mu$ L of R-PE (3.4 mg/L), 100  $\mu$ L of 320 mM AAPH, and 100  $\mu$ L of sample. Phosphate buffer was used as a blank and Trolox (a water soluble  $\alpha$ -tocopherol analogue) as a standard during each run. The final volume of 2 mL was used in a 10 mm wide fluorometer cuvette. R-PE, phosphate buffer, and samples were preincubated at 37 °C for 15 min. The reaction was started by addition of AAPH. Fluorescence was measured and recorded every 5 min at emission of 570 nm and excitation of 540 nm using a Sequoia-Turner model 450 fluorometer (Englewood, NJ) until the fluorescence of the last reading declined to <5% of the first reading. One blank, one standard, and a maximum of 18 samples were analyzed at the same time. Each sample was repeated at least three times. The ORAC value refers to the net protection area under the quenching curve of R-PE in the presence of an antioxidant. The final results (ORAC value) were calculated and expressed using Trolox equivalents per gram of fresh-frozen weight ( $\mu$ M TE/g).

**Total Phenolic Assay.** Total soluble phenolics in the ethanol/acetone/water/acetic acid extracts (three triplicate samples of the two *Cnidioscolus* spp.) were determined with Folin–Ciocalteu reagent according to the method of Singleton et al. (13) using chlorogenic acid as a standard. Results are expressed as chlorogenic acid equivalents per gram of fresh weight.

**Flavonoid Analysis.** Flavonoids were analyzed by HPLC as described by Merken and Beecher (12). Filtered extract (20  $\mu$ L) was injected in triplicate into a Waters HPLC system coupled with an autosampler. A Waters photodiode array detector (model 996) was used to record UV spectra of flavonoids (quercetin and kaempferol). Flavonoid identifications were based on comparisons to TLC *R<sub>f</sub>* values and HPLC retention times with quercetin and kaempferol standards. Flavonoid glycosides extracted from the macerated tree spinach leaf tissues with MeOH by a procedure that gave 98% recovery in experiments with spiked samples were hydrolyzed with 1 N hydrochloric acid (HCl) in 50% methanol (MeOH) at 90 °C for 30 min. Flavonoid aglycones were quantified at 370 nm using a C<sub>18</sub> column with a solvent system of MeOH:water (35:65), pH 2.4, with phosphoric acid at 1 mL/min. Isolated flavonoid aglycones were further hydrolyzed in 2 M aqueous trifluoroacetic acid to isolate sugar residues, and sugars were quantitatively identified by gas chromatography of their alditol acetates (3).

**Statistical Analysis.** All data presented were means of three replicates along with standard errors of means. Treatment mean differences were compared using Waller and Duncan's Bayes least significance difference (LSD) test. Differences at  $p < 0.05$  were considered significant. Correlation coefficients between ORAC values and total phenolic constituents ( $n = 18$ ) were performed using Microsoft Excel Data Analysis.

**Table 1.** Oxygen Radical Absorbing Capacity (ORAC) and Phenolics Content of Raw and Cooked Edible Leaves of Two Tree Spinach (*C. aconitifolius* Miller and *C. chayamansa* McVaugh)

<i>Cnidioscolus</i> species	leaf sample	ORAC <sup>a</sup>	phenolics <sup>b</sup>
<i>C. aconitifolius</i>	raw	15.3 ± 0.3 <sup>c</sup>	2906.2 ± 18.5
	cooked	11.8 ± 1.2	2241.4 ± 37.9
<i>C. chayamansa</i>	raw	15.6 ± 0.7	1217.3 ± 21.4
	cooked	14.8 ± 1.0	733.5 ± 28.2
overall means		14.4 ± 0.8	1774.6 ± 26.5
	LSD (0.05) <sup>d</sup>	1.3 ± 0.2	85.0 ± 11.4

<sup>a</sup> Micromoles of Trolox equivalents per gram of fresh weight. <sup>b</sup> Milligrams of chlorogenic acid equivalents per kilogram of fresh weight. <sup>c</sup> Standard error of the mean ( $N = 3$ ). <sup>d</sup> Means within columns compared by Waller Duncan's Bayes least significant difference (LSD) at  $p < 0.05$  level.

## RESULTS AND DISCUSSION

**ORAC and Total Phenolic Content of Tree Spinach.** In general, total phenolic contents in the tree spinach leaves sampled in early fall were significantly ( $p < 0.05$ ) higher than contents in leaves sampled in late summer (data not presented). The total phenolic content and ORAC varied significantly among the tree spinach (*Cnidioscolus*) species (Table 1). The total phenolics and antioxidant capacity was much higher in raw fresh leaf extracts than cooked leaf extracts. There are significant differences ( $p < 0.05$ ) in ORAC values and phenolic contents among raw and cooked tree spinach leaves. ORAC values ranged from 11.8 to 15.6  $\mu$ M TE/g with an overall mean of 14.4  $\mu$ M TE/g, and total phenolics content ranged from 733.5 to 2906.2 mg of chlorogenic acid/kg with an overall mean of 1774.6 mg/kg in leaves of *Cnidioscolus* species studied. *C. aconitifolius* leaves consistently had higher ORAC values and total phenolic content than *C. chayamansa*. Cooking generally reduced ORAC values and total phenolic contents. There was a reduction in ORAC values (23%) and total phenolic contents (23%) in *C. aconitifolius* when cooked and as much as 5% reduction in ORAC values and 40% phenolic contents in cooked *C. chayamansa*. Cooking is essential prior to consumption of tree spinach leaves in order to inactivate the toxic hydrocyanic glycosides present in *Cnidioscolus* species (2). Higher levels of total phenolic contents and antioxidant capacity in *C. aconitifolius* may be attributed to species differences among the edible tree spinach. *C. chayamansa* leaves, which had higher ORAC values when cooked, are more widely consumed than *C. aconitifolius* leaves (14). Phenolic compounds are commonly found in both edible and inedible plants, and they have been reported to have multiple biological effects, including antioxidant activity (15). Genetic makeup appears to play an important role in phenolic metabolism and antioxidant capacity in fruits and leafy green vegetables (16–17). The ORAC values and total phenolics contents were significantly different among the two tree spinach species (*C. chayamansa* and *C. aconitifolius*), and the wide range of ORAC values (~12–16  $\mu$ M TE/g) observed in *Cnidioscolus* leaves was generally similar to reported ORAC values for spinach (*Spinacia oleracea* L.) leaves, 12–20  $\mu$ M TE/g (17), smaller than reported ORAC values for strawberries, 15–35  $\mu$ M TE/g (18), and higher than reported ORAC values for some common fruits such as orange, 8–10  $\mu$ M TE/g, banana, 3–6  $\mu$ M TE/g, and apple, 3–5  $\mu$ M TE/g, (19). This study is the first report on ORAC value, a measure of water-soluble antioxidant capacity for the tree spinach (*Cnidioscolus* spp.). The data obtained in this study are important information on nutritional and health benefits of the tree spinach leaves because the ORAC assay used here is more accurate than other measures previously used to determine antioxidant capacity of fruits and

**Table 2.** Flavonoid (kaempferol and quercetin) Contents of Raw and Cooked Edible Leaf Extracts of Two Tree Spinach (*C. aconitifolius* Miller and *C. chayamansa* McVaugh)

<i>Cnidoscopus</i> species	leaf sample	flavonol content ( $\mu\text{g/g}$ fresh weight) <sup>a</sup>		
		kaempferol	quercetin	total <sup>b</sup>
<i>C. aconitifolius</i>	raw	58.2 ± 4.4	16.9 ± 0.5	75.1 ± 4.9 A
	cooked	50.0 ± 6.1	12.6 ± 0.7	62.6 ± 6.8 BC
<i>C. chayamansa</i>	raw	22.4 ± 3.5	44.7 ± 1.9	67.1 ± 5.4 B
	cooked	18.1 ± 2.5	40.2 ± 3.3	58.3 ± 5.8 C

<sup>a</sup> Mean ± standard error of mean (SEM),  $N = 3$ . <sup>b</sup> Total flavonol values within the column, with the same letter, are not significantly different ( $P < 0.05$ ).

vegetables (10) and ORAC assay allows for comparison across laboratories of antioxidant capacities of various substances (20).

**Flavonoid Content of Tree Spinach.** Using extraction and HPLC conditions identical to those of Merken and Beecher (12), flavonoid content was quantified (Table 2). Flavonoid aglycones were hydrolyzed and sugar residues isolated and determined by gas chromatographic methods as described by Kolterman and Breckon (4). The gas chromatographic analysis using a Hewlett-Packard GC (model 15830A) revealed individual flavonoid glycosides in raw and cooked tree spinach leaves (Table 3). Total flavonoid glycosides (kaempferol and quercetin) varied significantly among raw and cooked leaf extracts of *Cnidoscopus* species (Table 2). While a greater amount of kaempferol (50–58  $\mu\text{g/g}$ ) was found in cooked and raw *C. aconitifolius* leaves, more quercetin (40–45  $\mu\text{g/g}$ ) was found in cooked and raw leaves of *C. chayamansa*. Cooking reduces total flavonoid content in the tree spinach leaves by 13–17%. In terms of overall flavonoid composition, kaempferol accounted for 77% (58.2 ± 4.4  $\mu\text{g/g}$  fresh weight) and quercetin accounted for 23% (16.9 ± 0.5  $\mu\text{g/g}$  fresh weight) in raw leaf extracts of *C. aconitifolius* and kaempferol accounted for 80% (50.0 ± 6.1  $\mu\text{g/g}$  fresh weight) and quercetin accounted for 20% (12.6 ± 0.7  $\mu\text{g/g}$  fresh weight) in cooked leaf extracts. The total flavonoids in raw leaf extracts of *C. chayamansa* consisted of 67% quercetin (44.7 ± 1.9  $\mu\text{g/g}$  fresh weight) and 33% kaempferol (22.4 ± 3.5  $\mu\text{g/g}$  fresh weight), and in cooked leaf extracts, total flavonoids consisted of 69% quercetin (40.2 ± 3.3  $\mu\text{g/g}$  fresh weight) and 31% kaempferol (18.1 ± 2.5  $\mu\text{g/g}$  fresh weight), respectively. Among the tree spinach species examined in this study, total flavonoids content was as low as ~58.3 ± 5.8  $\mu\text{g/g}$  fresh weight in cooked leaf extracts of *C. chayamansa* and as high as ~75.1 ± 4.9  $\mu\text{g/g}$  fresh weight in raw leaf extracts of *C. aconitifolius* (Table 2).

Eight flavonoid glycosides were identified in leaf extracts of *C. aconitifolius* and three flavonoid glycosides in *C. chayamansa* investigated (Table 3). While raw leaf extracts of *C. aconitifolius* contained six kaempferol glycosides and two quercetin glycosides, *C. chayamansa* contained two kaempferol glycosides and one quercetin glycoside. Cooking of the tree spinach leaves resulted in the loss of three kaempferol glycosides (kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rhamnoside, and kaempferol-3-*O*-rhamnosylglucoside) and two quercetin glycosides (quercetin-3-*O*-rhamnoside and quercetin-3-*O*-rhamnosylglucoside 7-*O*-glucoside) in leaves of *C. aconitifolius* and no loss of flavonoid glycosides in cooked *C. chayamansa* (Table 3). Percentages of kaempferol-3-*O*-galactose, kaempferol-3-*O*-rhamnosylglucoside 7-*O*-glucoside, and kaempferol-3-*O*-rhamnosylgalactoside 7-*O*-rhamnoside slightly increased in cooked leaf extracts of *C. aconitifolius*. In cooked leaf extracts of *C. chayamansa*, while percentages of kaempferol-3-*O*-rhamnosylglucoside and kaempferol-3-*O*-rhamnosylgalactoside 7-*O*-rhamnoside slightly decreased, quercetin-3-*O*-rhamnoside percentage increased slightly.

**Table 3.** Individual Flavonoids in Raw and Cooked Edible Leaf Extracts of Two Tree Spinach (*C. aconitifolius* Miller and *C. chayamansa* McVaugh)

<i>Cnidoscopus</i> species	leaf sample	flavonoid compounds	relative content <sup>a</sup>
			(%)
<i>C. aconitifolius</i>	raw	kaempferol glycosides	
		3- <i>O</i> -galactoside	9
		3- <i>O</i> -glucoside	14
		3- <i>O</i> -rhamnoside	14
		3- <i>O</i> -rhamnosylglucoside	24
		3- <i>O</i> -rhamnosylgalactoside	28
		7- <i>O</i> -glucoside	
		3- <i>O</i> -rhamnosylgalactoside	12
		7- <i>O</i> -rhamnoside	
		quercetin glycosides	
	3- <i>O</i> -rhamnoside	65	
	3- <i>O</i> -rhamnosylglucoside	35	
	cooked	kaempferol glycosides	
		3- <i>O</i> -galactoside	25
		3- <i>O</i> -glucoside	nd <sup>b</sup>
		3- <i>O</i> -rhamnoside	nd
		3- <i>O</i> -rhamnosylglucoside	nd
		3- <i>O</i> -rhamnosylgalactoside	50
		7- <i>O</i> -glucoside	
		3- <i>O</i> -rhamnosylgalactoside	25
7- <i>O</i> -rhamnoside			
quercetin glycosides			
3- <i>O</i> -rhamnoside	nd		
3- <i>O</i> -rhamnosylglucoside	nd		
<i>C. chayamansa</i>	raw	kaempferol glycosides	
		3- <i>O</i> -rhamnosylglucoside	28
		3- <i>O</i> -rhamnosylgalactoside	28
		7- <i>O</i> -rhamnoside	
		quercetin glycosides	
	3- <i>O</i> -rhamnoside	44	
	cooked	kaempferol glycosides	
		3- <i>O</i> -rhamnosylglucoside	24
		3- <i>O</i> -rhamnosylgalactoside	22
		7- <i>O</i> -rhamnoside	
quercetin glycosides			
3- <i>O</i> -rhamnoside	54		

<sup>a</sup> The relative flavonoid contents were calculated from relative sugar residues of each flavonoid using GC analysis. <sup>b</sup> Not detectable in cooked leaf samples of *C. aconitifolius*.

Flavonoid (kaempferol and quercetin) contents in *Cnidoscopus* leaves appear not to correlate well with antioxidant capacity, suggesting that either there are other antioxidant components such as carotenoids and ascorbic acid (9) in the *Cnidoscopus* leaf extracts or possibly that a combination of individual antioxidants are producing synergistic effects. One of the more interesting findings in this study regarding the phytochemical content of tree spinach leaves is that higher flavonols (kaempferol and quercetin glycosides) are consistent with total phenolics. The flavonols have been well studied for their potential health benefits that include antiproliferate, anticarcinogenic, and antioxidant activity (21).

**Relationships between Phenolics and ORAC.** A significant positive linear correlation ( $r = 0.926$ ;  $P < 0.05$ ) was observed between total phenolics and antioxidant capacity in tree spinach leaf extracts (Figure 1). While cooking reduces ORAC value and total phenolic contents in *C. aconitifolius* by 23%, thus validating the correlation between ORAC and total phenolics, no such correlation was observed in cooked *C. chayamansa*, where ORAC value declines by 5% and total phenolics decline by as much as 40%. The appearance of low correlation between total phenolics and ORAC in cooked *C. chayamansa* could be explained by the fact that quercetin-glycoside 3-*O*-rhamnoside

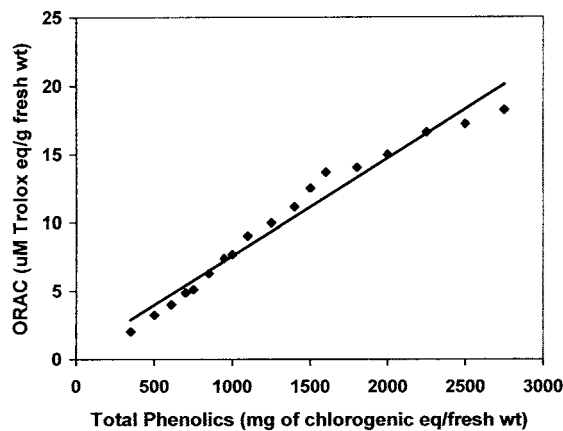


Figure 1. Linear correlation coefficient between total phenolics and antioxidant capacities of tree spinach (*Cnidoscopus* spp.) leaves.

content, the predominant flavonoid, increased by ~23% in the cooked leaf samples when compared to the raw leaf samples (Table 3). Phenolic antioxidant activity is influenced by the structures of binding sugars on the glycoside (22). Quercetin glycosides, conjugated with sugars, have high antioxidant activities (~80–90% at 20  $\mu\text{mol/L}$ ) because of the OH groups that bind to A, B, or C rings; therefore, quercetin glycoside contributes to antioxidant activities through its conformational influences (23). The relationship between total phenolics and ORAC values in the tree spinach leaf extracts was similar to those found in tea and other common vegetables (24–25) but higher than those reported in conventional spinach (*Spinach oleraceae* L.) leaves (17). Phenolic and ORAC levels in fruits and vegetables can be influenced by genetics, environmental growing conditions, maturation, and postharvest storage conditions (26–28).

The results of the present study indicate that the tree spinach leaf extracts are protective against reactive oxygen species, but there is variability in the level of antioxidant capacity among the species of *Cnidoscopus*. On the basis of the data in this study and the phytochemical contents of the tree spinach leaves, there is a high likelihood that tree spinach may provide the types of nutritional and health benefits associated with consumption of leafy green vegetables in general. Further studies into the absorption and effects of tree spinach leaf phytochemicals on antioxidant status in animal models are needed to evaluate their potential health benefits.

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