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Antioxidant Capacity of Polyphenolic Extracts from Leaves of *Crataegus laevigata* and *Crataegus monogyna* (Hawthorn) Subjected to Drought and Cold Stress

ARA KIRAKOSYAN,[†,‡] ELISABETH SEYMOUR,[‡] PETER B. KAUFMAN,*[,†,‡] SARA WARBER,[‡] STEVEN BOLLING,[‡] AND SOO CHUL CHANG[§]

Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, Michigan 48109-1048; Complementary and Alternative Medicine Research Center (CAMRC), University of Michigan, Ann Arbor, Michigan 48104; and Center for Cell Signaling Research, Ewha Woman's University, Seoul 120-750, Korea

Crataegus laevigata and *Crataegus monogyna* (hawthorn) were subjected to drought and cold stress treatments, and polyphenolic extracts from control and stress-treated plants were assayed for antioxidant capacities using a modified version of the Total Antioxidant Status Assay (Randox, San Francisco, CA). In addition, these plants were analyzed for levels of flavanol-type substance [(−)-epicatechin] and flavonoid (vitexin 2″-*O*-rhamnoside, acetylvitexin 2″-*O*-rhamnoside, and hyperoside) constituents that are important metabolites in hawthorn herbal preparations used to treat patients with heart disease. Drought and cold stress treatments caused increases in levels of (−)-epicatechin and hyperoside in both *Crataegus* species. Such treatments also enhanced the antioxidant capacity of the extracts. The results from this study thus indicate that these kinds of stress treatments can enhance the levels of important secondary metabolites and their total antioxidant capacities in leaves of *Crataegus*.

KEYWORDS: ***Crataegus*; hawthorn leaves; stress treatment; flavanols; flavonoids; antioxidant capacity**

INTRODUCTION

Many phenolic compounds in plants possess antioxidant activity and may help protect cells against the oxidative damage caused by free radicals. In addition, some flavonoids and proanthocyanidins are employed to treat several kinds of heart disease or mild and moderate depression (*1*−*4*). Some secondary metabolites synthesized in *Crataegus* (hawthorn) species have recently received more attention, especially due to their vasoactive properties (*5*). Hawthorn extracts have been shown to increase myocardial contractility, reduce reperfusion arrhythmias, dilate peripheral arteries, and mildly decrease blood pressure (*1*, *3*, *5*). Currently, hawthorn (*Crataegus* spp.) leaves, flowers, and both green (unripe) and red (ripe) berries are used to make herbal preparations to treat patients with severe heart disease. The two species of hawthorn used to make such medicinal preparations are *C. laevigata* (native to Europe and North America) and *C. monogyna* (native to Europe, Asia, and North Africa) (*6*). Hawthorn is widely used in Europe, especially Germany, as a cardiotonic in the treatment of chronic heart failure and high blood pressure (*2*, *5*). When standardized extracts of the leaves and flowers have been used for treatment of patients with chronic heart failure, no significant adverse side effects have been reported (*6*, *7*). The mechanisms of action of these protective effects of hawthorn extracts are still unclear and have not been fully characterized. Synergistically or additively, these antioxidants provide bioactive mechanisms to reduce free radical-induced oxidative stress (*3*, *8*).

The phytoactive secondary compounds present in hawthorn leaves, flowers, and fruits are proanthocyanidins and flavonoids (*4*, *9*−*11*). The proanthocyanidins are chains of catechin and/or epicatechin linked by 4 → 8 or 4 → 6 bonds. The primary flavonoids present in hawthorn include vitexin 2″-*O*-rhamnoside, acetylvitexin 2″-*O*-rhamnoside, vitexin, isovitexin, quercetin, hyperoside, and rutin (*6*, *10*, *12*).

Antioxidant capacities of phenolics present in *C. monogyna* flowers and in vitro callus tissue and cell suspension culture extracts have been reported (*8*). These authors showed that all of the extracts were efficient in antioxidant activity and that the free radical scavenging capacity is clearly related to the total phenol content, where, among individual compounds, the flavanol-type derivatives (proanthocyanidins) were the most efficient. Furthermore, antioxidant capacities of extracts from different organs of *C. monogyna* have been reported and compared to the content of total phenolics (*13*). Interestingly,

* Address correspondence to this author at the Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI 48109-1048 [telephone (734) 764-1464; fax (734) 647-0884; e-mail pbk@umich.edu].

† Department of Molecular, Cellular, and Development Biology, University of Michigan.

‡ Complementary and Alternative Medicine Research Center, University of Michigan.

§ Ewha Woman's University.

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the best correlations were established with total phenols, whereas the antioxidant activities in leaves were attributed to flavonoids. Extracts of fresh vegetative and reproductive organs from *C. monogyna* also have been shown to exhibit in vitro antioxidant activities (*14*). Although all of the tested samples showed low IC_{50} values, the most efficient were fresh young leaves, fresh floral buds, and pharmaceutical dried flowers. It was concluded from this study that the antioxidant activities were largely due to the total phenolic proanthocyanidin and flavonoid contents. In another study on *Crataegus sinaica*, antiviral and antioxidant activities of flavonoids and proanthocyanidins have been reported (*15*). However, no information is available regarding the effects of environmental stress treatments on polyphenol levels as they relate to antioxidant capacities in a comparison of the two primary species of *Crataegus* used for the treatments of heart disease, namely, *C. laevigata* and *C. monogyna*.

Because of this lack of information, we report here on the antioxidant capacities of phenolic compounds derived from the leaves of two species of hawthorn (*C. laevigata* and *C. monogyna*) isolated from control plants and plants subjected to drought or cold stress conditions. The primary goal of this study is to determine if drought or cold stress treatments elevate the levels of flavanol-type substance [(−)-epicatechin] and flavonoids (vitexin 2″-*O*-rhamnoside, acetylvitexin 2″-*O*-rhamnoside and hyperoside) in the leaves of these two hawthorn species and, at the same time, elevate their potential antioxidant action.

MATERIALS AND METHODS

Plants and Nature of Stress Treatments. One-year-old dormant plants of *C. laevigata* and *C. monogyna* were obtained from Lawyer Nursery, Plains, MT. Upon arrival of the dormant plants, shipped bare-root, individual plants were potted in 15.24 cm diameter pots for *C. monogyna* and 10.16 cm diameter pots for *C. laevigata*, using Sunshine Mix 4 as a potting medium. Plants were watered daily, kept at 25 °C temperature, and illuminated continuously with P/L Light Systems Canada, Inc. (Grimsby, ON, Canada) lamps with 400 μmol·m^{-2}·s^{-1} light intensity at the tops of the plants.

The following stress treatments were used: drought (water deprivation for 10 days) versus daily watering for 10 days with comparable temperature (25 °C) and light intensity (400 μmol·m^{-2}·s^{-1}) for both regimens; cold stress (4 °C) versus room temperature (25 °C), each for 10 days with comparable light intensity (400 μmol·m^{-2}·s^{-1}) for both temperature regimens. All stress treatments were of 10 days duration, using six plants of each species per treatment.

At the end of 10 days of treatment, 100 fully expanded leaves from each of the respective treatments and hawthorn species were excised, frozen, and then freeze-dried prior to extraction.

Extraction of Total Phenolics. The freeze-dried hawthorn leaves (1 g of dry weight) were powdered using a clean mortar and pestle; 0.5 g of the prepared powder was extracted in 10 mL of dichloromethane overnight in 15 mL screw-capped Pyrex tubes placed on a shaker (150 rpm) inserted in an incubator set at 50 °C. The dichloromethane was poured off, and the residue was extracted with 10 mL of 70% methanol overnight with the tubes placed on a shaker set at 150 rpm in a 50 °C incubator. After removal of the methanol by air-drying, the remaining water layer was separated from the precipitate by centrifugation at 10000*g* for 10 min. The extraction of the precipitates was subsequently performed two times with 5 mL aliquots of diethyl ether and 10 mL of ethyl acetate. The remaining water layer was dissolved in 6 mL of distilled water, the pH was adjusted to 5.4 with orthophosphoric acid, and the mixture was incubated with 0.1 mL of β-glucosidase solution (1 mg·mL^{-1} in water). The mixture was incubated for 1 h in a water bath set at 35 °C in order to release flavonoids from sugar conjugates. Ten milliliters of 100% methanol was then added to the mixture to dissolve the flavonoids more effectively. Following repeated mixing, the tubes were then placed on a shaker at 150 rpm for 60−90 min in an incubator set at 60 °C. The mixture was then centrifuged and the supernatant evaporated using a Büchi flash evaporator. The residues from all steps were combined and dissolved in 10 mL of 80% methanol. After filtration through a 0.22 μm filter, the extracts were ready for HPLC analysis.

HPLC Analysis of Extracts. The following conditions were used for HPLC analysis: a Phenomenex Luna column [5 μm pore size, C-18, 150 mm × 4.60 mm], flow rate of 1 mL·min^{-1}; solvent A [water + 0.1% trifluoroacetic acid (TFA)], solvent B [acetonitrile + 0.1% TFA]; the HPLC running conditions consisted of a gradient of 5% B to 100% B during a 30 min period; the oven temperature was 40 °C. A 10 μL aliquot of sample was injected into a Shimadzu 10 AD HPLC system with an SPDM-10AV photodiode array detector (Shimadzu). The quantitative analysis of each compound in the extracts was analyzed by comparison with the corresponding authentic standards. Each peak was identified by the retention time and the characteristic UV spectrum. Detection was set at 280 nm.

Antioxidant Assays. All standard samples and freeze-dried extracts (1 mg) were dissolved in 10 mL 70% EtOH and filtered through a 0.22 μm filter. Total antioxidant capacity was measured using a modified version of the Total Antioxidant Status Assay (Randox, San Francisco, CA). 2,2′-Azinobis[3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt (ABTS) was incubated with a peroxidase (metmyoglobin) and H_2O_2 to produce a colored radical cation of ABTS, the absorbance of which can be measured at 600 nm. Antioxidants in the sample decrease the intensity of the blue-green color development. This assay was adapted to a microplate method from the manufacturer's cuvette method so as to allow simultaneous analysis, due to strict time dependency of color development. Metmyoglobin and ABTS were combined according to the manufacturer's instructions to yield the functional chromogen. Each microplate well contained either 2 μL of H_2O, 2 μL of standard H_2O_2 as provided with the kit, or 2 μL of test sample from the various hawthorn preparations. Each well received 100 μL of chromogen, and the plate was incubated for 10 min at 37 °C in a water-jacketed 5% CO_2 incubator. All samples, standards, and controls were run in triplicate. After 10 min, initial readings were taken as a baseline for color development. The microplate was read at 600 nm on a Bio-Tek microplate reader (Bio-Tek, Winooski, VT), and data were captured with DeltaSoft3 software (Biometallics, Princeton, NJ). After initial readings, 20 μL of substrate was added to each well using a multichannel pipettor, and the plate was then returned to the incubator for 3 min of color development. After exactly 3 min, a final absorbance reading was obtained at 600 nm. The difference between final and initial absorbance was used to compare samples. The Trolox positive control and the ethanol negative control were used for data transformation into mmol·L^{-1} of Trolox units. Data computations were based on the following:

"factor" = concentration of Trolox standard ÷
(change in absorbance of 70% ethanol blank −
change in absorbance of Trolox standard)

antioxidant status = mmol·L^{-1} of Trolox units = "factor" ×
(change in absorbance of 70% ethanol blank −
change in absorbance of test sample)

Sources of Chemicals. Solvents employed for extraction and HPLC analysis were obtained from Fisher Scientific Co. (Pittsburgh, PA), β-glucosidase was from Sigma Chemical Co. (St. Louis, MO), flavonoid standards were from Indofine Chemical Co. (Somerville, NJ), and (−)-epicatechin and acetylvitexin 2″-*O*-rhamnoside were from Dr. Friederich Lang, Head, Analytical Department, Dr. Willmar Schwabe GmbH and Co. (Karlsruhe, Germany).

Statistical Analysis of Data. Experiments were repeated at least three times, and the data were analyzed statistically. All results are given as mean ± standard deviation (SD). Differences between variables were tested for significance by Student's *t* test. A p value of <0.05 was considered to be significant.

RESULTS

The main hawthorn phenolics are attributed to the presence of proanthocyanidins and flavonoids (*6*, *10*, *12*). No significant

Table 1. Levels of (–)-Epicatechin, Acetylvitexin 2″-*O*-Rhamnoside, Vitexin 2″-*O*-Rhamnoside, and Hyperoside in *C. laevigata* and *C. monogyna* Leaves Following Drought and Cold Stress Treatments[a]

	(–)-epicatechin		acetylvitexin 2″-*O*-rhamnoside		vitexin 2″-*O*-rhamnoside		hyperoside	
treatment	*C. laevigata*	*C. monogyna*	*C. laevigata*	*C. monogyna*	*C. laevigata*	*C. monogyna*	*C. laevigata*	*C. monogyna*
control	0.131 ± 0.014	0.148 ± 0.024	6.525 ± 0.482	4.313 ± 0.372	2.204 ± 0.285	3.095 ± 0.247	0.280 ± 0.019	0.387 ± 0.018
drought stress	2.402 ± 0.191*	0.905 ± 0.071*	2.036 ± 0.128*	3.597 ± 0.208*	1.833 ± 0.094*	0.925 ± 0.068*	0.491 ± 0.039*	0.431 ± 0.031*
cold stress	0.351 ± 0.029*	0.358 ± 0.022*	12.130 ± 0.845*	8.097 ± 0.678*	2.433 ± 0.313	5.198 ± 0.391*	1.475 ± 0.095*	2.292 ± 0.188*

[a] Levels are expressed as milligrams per gram, dry weight biomass. An asterisk (*) indicates significant difference from control ($p < 0.05$).

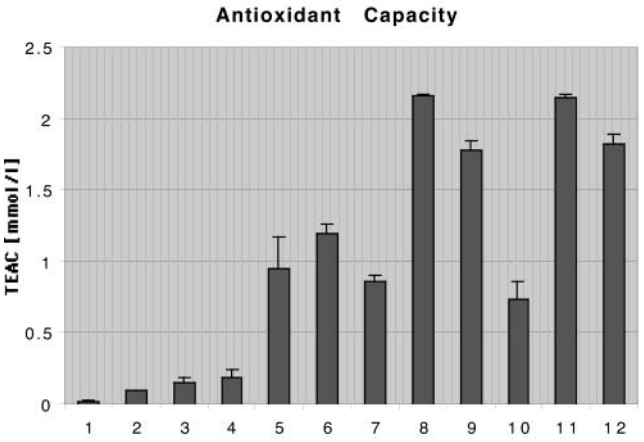

Figure 1. Trolox equivalent antioxidant capacities (TEAC) of crude leaf extracts from *C. laevigata* and *C. monogyna* control, drought- and cold stress-treated plants, compared to those of acetylvitexin 2″-*O*-rhamnoside, vitexin 2″-*O*-rhamnoside, hyperoside, and (–)-epicatechin ($n = 3$, error bars = ±SD; some error bars are not visible due to small standard deviation values). Numbers along the abscissa refer to the following: 1, Trolox positive control; 2, 70% ETOH; 3, acetylvitexin 2″-*O*-rhamnoside; 4, vitexin 2″-*O*-rhamnoside; 5, hyperoside; 6, (–)-epicatechin; 7, *C. laevigata* control; 8, *C. laevigata* drought stress; 9, *C. laevigata* cold stress; 10, *C. monogyna* control; 11, *C. monogyna* drought stress; 12, *C. monogyna* cold stress.

changes in quantities, however, were observed between control and stress-treated plants for flavonoids such as rutin, vitexin, isovitexin, and quercetin and individual compounds, such as catechin and chlorogenic acid, usually present in hawthorn leaves in minor concentrations (data not shown). Thus, we assessed the antioxidant activities of total extracts for both stress-treated and control cultures of the two hawthorn species and for individual standard compounds, which are the predominant phenolic compounds in hawthorn leaves (**Figure 1**). Results of HPLC analysis of the flavanol-type substance (−)-epicatechin and flavonoids in *C. laevigata* and *C. monogyna* are presented in **Table 1**.

Drought Stress Treatment. Drought stress increases the amount of (−)-epicatechin in *C. laevigata* (2.402 mg·g^{-1} dry weight biomass compared to that of control plants with 0.131 mg·g^{-1} dry weight biomass) and in *C. monogyna* (0.905 mg·g^{-1} dry weight biomass compared to that of control plants with 0.148 mg·g^{-1} dry weight biomass) (**Table 1**). On the other hand, flavonoid levels were decreased following drought stress treatment with the exception of hyperoside, which in *C. laevigata* increased by 1.75-fold over the control level and in *C. monogyna* increased slightly by 1.1-fold over the control level (**Table 1**).

Data in **Figure 1** for *C. laevigata* and *C. monogyna* show that the total antioxidant status was changed when plants were subjected to drought stress treatment. For example, antioxidant capacities in extracts of plants subjected to drought stress are almost 2.5-fold higher than in control, non-drought-stressed plant extracts for both species. Taken together, these results indicate that drought stress treatment, which increased the total amounts of (−)-epicatechin, also increased the total antioxidant capacity.

Cold Stress Treatment. There is parallel evidence for enhanced (−)-epicatechin levels following cold stress treatment, but here, also, we observed increases in contents of flavonoids (**Table 1**) and antioxidant capacity (**Figure 1**) for both hawthorn species. Thus, following cold stress treatment, we observed significantly increased levels of hyperoside by 5.3-fold greater than control (1.475 mg·g^{-1} dry weight biomass compared to that of control plants with 0.280 mg·g^{-1} dry weight biomass) and acetylvitexin 2″-*O*-rhamnoside by 1.86-fold greater than control (12.130 mg·g^{-1} dry weight biomass compared to that of control plants with 6.525 mg·g^{-1} dry weight biomass) in *C. laevigata* and of acetylvitexin 2″-*O*-rhamnoside, vitexin 2″-*O*-rhamnoside, and hyperoside by 1.88-, 1.68-, and 5.92-fold greater than control, respectively (8.097, 5.198, and 2.292 mg·g^{-1} dry weight biomass compared to that of control with 4.313, 3.095, and 0.387 mg·g^{-1} dry weight biomass, respectively) in *C. monogyna* (**Table 1**). Taken together, these results indicate that cold stress treatment, which increased the total amounts of (−)-epicatechin and flavonoids, also increased the total antioxidant capacity.

DISCUSSION

In the present study, we compared the antioxidant activities of (−)-epicatechin, acetylvitexin 2″-*O*-rhamnoside, vitexin 2″-*O*-rhamnoside, and hyperoside standards by means of the Randox test (on ABTS radical in vitro), whereas the results of Bahorun and co-workers (*13*) had been performed with the malondialdehyde−thiobarbituric acid test on hepatic microsomal preparations. Each of these compounds could have an antioxidant effect in hawthorn extracts. However, it may be that some minimal level of (−)-epicatechin and hyperoside is needed for these molecules to exhibit their antioxidant effects. Our results show that these two polyphenols have a higher antioxidant capacity than the other ones analyzed (**Figure 1**). *C. laevigata* and *C. monogyna* leaf extracts exhibited substantial antioxidant capacity. The antioxidant values of these plant extracts, compared to values for reference substances, suggest that there may be a possible synergistic action, in terms of expression of antioxidant capacity, between the main polyphenolics in these species. Synergistic action of secondary metabolite molecules at target sites is well-documented for many other medicinal plants by Warber (*16*) and Duke (*17*). However, in the case of hawthorn, further studies are necessary to prove whether this is the case.

Bahorun and co-workers (*13*) have shown that leaf extracts of *C. monogyna* flavonoids account for most of the total antioxidant activities, whereas proanthocyanidins and catechins are primarily responsible in flowers and fruits. In this connection, hawthorn leaves (our study), as compared with hawthorn

flowers (*8*), have similar levels of (−)-epicatechin and hyperoside, in terms of grams per 100 g of dry matter. However, leaves, as compared with flowers, are available at a much earlier stage of plant development, occur in much greater quantity, and can be harvested for a much longer period of time than for *Crataegus* growing under natural conditions. The present study also shows that with greenhouse-grown hawthorn plants, environmental stress treatments (drought stress and cold stress) elevate (−)-epicatechin and some flavonoid levels to much higher levels in the leaves than in leaves of control, nonstressed plants. Such stress treatments are best carried out with greenhouse-grown plants, as used here, because they are not feasible to perform under field conditions.

The present study also provides a workable system for obtaining consistent and high levels of polyphenolics from leaves of *C. laevigata* and *C. monogyna* to test their phytopharmaceutical capacity. We have shown that extracts from hawthorn plants subjected to drought and cold stress not only give higher yields of polyphenolics but also possess higher antioxidant capacity, as compared with control nonstressed plants. Thus, dosages of herbal preparations administered to patients can be reduced. Also, the costs for the preparations can be reduced because of the presence of higher levels of the active metabolites. The primary advantage that accrues from this system for producing medicinal compounds, such as the proanthocyanidins and flavonoids from leaves of greenhouse-grown hawthorn plantlets, is that we can now obtain a good degree of quality control over the levels and kinds of medicinal products produced from hawthorn. This may have considerable significance for quality control that is needed for the preparation of other herbal medicines sold as herbal supplements.

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