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A COMPARISON OF PHYTOCHEMICALS PRESENT IN MIDWESTERN MEDICINAL PLANT EXTRACTS

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ABSTRACT. Plants consumed as medicines are thought to exert their physiological effects in part through the activity of their secondary metabolites, which include molecules with antioxidant activities. In this study, the concentrations of total phenolic, flavonoid, and anthocyanin compounds of eight medicinal plant extracts collected from Earlham College in Richmond, Indiana were examined. The collected data showed a wide range of variation in the concentration of antioxidant compounds in the eight examined plant extracts; the locally-collected gingko samples had the highest total phenolic content and plantain had the highest flavonoid content, while the black raspberry and mulberry samples had by far the highest anthocyanin content. In addition to describing the chemical composition of medicinal plants valued in the Midwestern United States, we compared different sources (Earlham College vs. purchased) and preparations (acetone vs. hot water extraction) of gingko leaves and compared the chemical composition of extracts that underwent an unintended additional freeze-thaw cycle. Gingko leaf extracts have the highest phenolic content of all extracts examined, and the purchased gingko teas and powder had higher levels of phenolic contents than all the locally collected, acetone extracts. Additionally, among the Midwestern species tested, no significant changes were observed in the concentrations of compounds measured in the extracts that underwent an additional freeze-thaw cycle. This study not only compares the phenolic compound composition of medicinal plant extracts but also provides pertinent information on the collection, preparation and storage of plant extracts to conserve these phenolic compounds.

Keywords: Midwestern medicinal plants, extracts, phenolic content, flavonoid, anthocyanin

INTRODUCTION

Phenolic compounds are naturally found in plants, including those commonly used as foods and medicines. These compounds are known to have multiple biological effects, including high antioxidant activity. In biochemistry and food industry research, there is increasing interest in the crude extracts of medicinal fruits, herbs, and other plant materials with phenolic compounds for a variety of applications. The abundance of phenolic compounds not only prevents the oxidative degradation of plant-based foods and materials but also improves the quality and nutritional value of such products (Löliger 1991). There is also interest in the role of flavonoid and anthocyanin compounds as "functional foods" in the prevention of coronary heart disease and cancer as well as in the overall maintenance of health (Serafini et al. 1998; Pandey & Rizvi 2009; Reis et al. 2016).

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The following plants were selected for this study because they are commonly used as traditional medicines throughout the world, including Indiana: goldenrod (*Solidago canadensis*), sassafras (*Sassafras albidum*), plantain (*Plantago* sp.), dandelion (*Taraxacum officinale*), ginkgo (*Ginkgo biloba*), black raspberry (*Rubus occidentalis*), and mulberry (*Morus alba*) (Fig. 1).

The leaves of gingko (also known as maidenhair tree) are highly regarded in traditional medicine and by the pharmaceutical industry due to their high concentration of phenolic compounds, specifically flavonoids (Pereira et al. 2013; Isah 2015). There are many narratives, oral traditions, and scientific studies recording the uses and values of this tree. Recently, gingko extracts were demonstrated to improve cerebral and peripheral blood circulation, especially in the lower limbs (Meston et al. 2008; Mashayekh et al. 2011). Moreover, these extracts have been shown to protect the nervous system against the effects of aging such as short-term memory and vertigo

Figure 1.—Local medicinal plants used in this study. Illustration of plants used, listed from left to right with common name, scientific name, parts of the plant used, and known traditional uses of each plant (Mashayekh et al. 2011; Isah 2015): dandelion (*Taraxacum officinale*; root; anti-inflammatory), goldenrod (*Solidago canadensis*; root, leaves; anti-inflammatory), plantain (*Plantago sp.*; leaves; lung related issues), sassafras (*Sassafras albidum*; root; fever, diarrhea), and ginkgo (*Ginkgo biloba*; leaves; memory aid). Figure by research lab member, Evelyn Sanchez.

(Meston et al. 2008; Pandey & Rzivi 2009; Mashayekh et al. 2011).

While multiple freeze-thaw cycles are generally believed to impact the chemical concentration of medicinal extracts, the limited research studies available report varying impacts of this temperature change on the concentrations of phenolic compounds in medicinal plants. One study showed that temperature fluctuations had significant impacts on the change in the anthocyanin concentrations of fruit (Gustafson et al. 2012). However, another study reported that the change in temperature and an additional free-thaw cycle had no significant effects on antioxidant concentration (Ellnain-Wojtaszek et al. 2001).

The current study aimed to describe the chemical differences between valued, medicinal plants, focusing on the phenolic compounds thought to influence the health of humans when consumed. Specifically, we compared the concentrations of total phenolic compounds (containing hydroxyls, -OH, group covalently bonded to an aromatic hydrocarbon ring), total flavonoids (yellow pigment, class of phenolic compound), and anthocyanins (purple/red/blue pigment depending on pH, class of flavonoid compounds) in the samples. Additionally, the role of extract preparation (dried sample with water vs. wet sample with acetone extraction), season of collection (summer vs. fall), and sex of the plant (male vs. female) on gingko leaf extract chemistry were examined.

MATERIALS AND METHODS

Plant collection.—The following plants were collected on the Earlham College back campus (unless otherwise noted) in Richmond, Indiana at the times indicated: goldenrod leaves and root (May 2017), sassafras root (May 2017), ginkgo leaves (mixed male and female trees, May 2017), dandelion root (May 2017), plantain leaves (May 2017), black raspberry (June 2017; Boston, Indiana forest), mulberry (June 2017; Boston, Indiana forest), ginkgo leaves from female tree (September 2017), and ginkgo leaves from male tree (September 2017). Soil was removed from the root samples by gently shaking in the field and rinsing with tap water. All plants were air dried (surface-level) on a counter for several hours before storage. The collected plants were frozen at -20° C until extraction. The two purchased gingko products are as follows: ginkgo tea bag (Budda Organic Herbal Teas – Living Wellness Partner, LLC) and Ginkgo Supplemental Powder (Organic Ginkgo Leaf Powder – Nature Vibe Botanicals). All extracts and the abbreviations used for this in the figures are listed in Table 1.

80% acetone botanical extraction.—The plants collected in Indiana were thawed and

Table 1.—Abbreviations used for each extract and the initial stock concentrations.

Abbreviation	Name of the extracts	Stock concentration (g/mL)
GOL	goldenrod leaves	0.592
GOR	goldenrod roots	0.774
SAR	sassafras roots	0.6001
GIL	ginkgo leaves male & female summer	1.045
DAR	dandelion root	0.296
PIL	plantain leaves	0.34
BLR	black raspberry	1.083
MUL	mulberry	1.614
GIFF	ginkgo leaves female fall	3.73
GIFM	ginkgo leaves male fall	3.837
Tea	ginkgo tea	0.005448
Powder	ginkgo powder	0.00631

extracted using a rotary evaporator and 250 mL 80% acetone in double distilled water. The plant matter and 80% acetone mixture was blended and filtered. Then, 250 mL of filtrate was roto-vaped in a 1L round bottom flask until the acetone was evaporated. The final extract concentration was calculated using the mass of plant used and the final volume (Table 1). Extracts were aliquoted for storage into clearly labeled tubes and stored in -20° C freezer for use in this study and in -80° C freezer for long term storage. Samples used in the extra freeze-thaw comparison of this study underwent the additional thaw from -80° C to room temperature and back to -80° C due to an unplanned ultralow freezer failure in the department in 2017. Not all samples were impacted by this catastrophe.

Water extraction.—The ginkgo tea and supplemental powder were extracted using the directions provided on the containers. One tea bag or 2.5 mL of gingko powder was mixed into 250 mL of boiling DI water, allowed to steep for five min, and filtered. Extracts were aliquoted into clearly labeled tubes and stored in -20° C freezer for use in this study.

Total phenolic content assay (Folin–Coicalteu Method).—The following protocol was based on Herald et al. (2012) with some alterations: 8.5 mL of Folin-Coicalteu (F-C) reagent was freshly prepared for every assay by mixing 2.5 mL F-C reagent with 2.5 mL DI water. Gallic acid standards were prepared using serial dilutions of 10 mL of a 800 μg/mL gallic acid solution and stored at 4° C for reuse for up to three weeks. Next, 100 mL of 75 g/L Na_2CO_3 was prepared and stored in a labeled bottle at room temperature for up to three months. Each 96-well plate included three replicates of each of the following: samples (diluted by the same dilution factor, which was selected such that the absorbance measurements did not max out the instrument and fell within the standard curve); gallic acid standards (five concentrations: 800 g/mL, 400 g/mL, 200 g/mL, 100 g/mL, 50 g/mL); water blank; and one replicate of sample control per extract (sample without F-C reagent and Na_2CO_3). The extracts were thawed and filtered into a clean Eppendorf tube. Sample dilutions were prepared if necessary. According to designed plate layout, 75 μL distilled water was added to each well of the 96-well plate to be used, followed by 25 μL of standard or sample solution. Except for the sample control wells, 25 μL of 1:1 F-C prepared reagent was added to all wells. The plate was then covered with parafilm, mixed by shaking 30 sec, and allowed to sit for six min. One-hundred μL of Na_2CO_3 was added to each well and the plate was mixed again and covered entirely with aluminum foil for 90 min. After 90 min, the plate was shaken for 60 sec and absorbance was measured at 765 nm. Total phenolic concentration was estimated using the gallic acid standard curve, and expressed in terms of mg gallic acid equivalents (GAE) per gram sample to allow for comparisons.

Total flavonoid content assay (Spectrophotometric Method).—The following method was based on Herald et al. (2012) with some alterations: 100 mL each of 50 g/L $NaNO_2$, 100 g/L $AlCl_3$, and 1 mol/L NaOH were prepared in the fume hood and stored at room temperature in clearly labeled containers for up to three months. The catechin standards were stored at 4° C to reuse for up to three weeks. Experiments included three replicates of each of the following on one 96-well plate: samples (with the same dilutions factor for total phenolic content assay); catechin standards (five concentrations: 1.5 mg/mL, 0.75 mg/mL, 0.375 mg/mL, 0.1875 mg/mL, 0.09375 mg/mL); DI water blank. The extracts were thawed and filtered into a clean Eppendorf tube. Sample dilutions were prepared if necessary. All supplies were moved to the fume hood for safety before starting. One-hundred μL distilled water was added to each well of the 96-well

plate to be used, followed by 10 μL of 50 g/L $NaNO_2$ to all of the wells. Then, 25 μL of standard or sample solution was added to the wells, and the plate was incubated for 5 min. Next, 15 μL of 100 g/L $AlCl_3$ was added to all wells, followed by incubation for 6 min. Finally, 50 μL of 1 mol/L NaOH followed by 50 μL of distilled water was added to all of the wells. Before reading the absorbance at 510 nm, the plate was shaken for 30 sec. The total flavonoid concentration was calculated from the catechin standard curve and was expressed in terms of mg catechin equivalent (CE) per gram sample.

Total anthocyanin content assay (pH Differential Method).—The following method was adapted from Zhang et al. (2006) with some alterations. First, 100 mL of 0.025 M KCl/HCl (pH 1.0) and 100 mL of 0.025 M (pH 4.5) CH_3COONa/HCl was prepared and stored at room temperature. All extracts were diluted to the same concentration for easy comparison of the anthocyanin content. Each experiment included three replicates of the following two combinations for each botanical extract: 30 μL sample with 120 μL KCl buffer; and 30 μL sample with 120 μL CH_3COONa buffer. The plate was incubated on the shaker at room temperature for 20 min. The absorbance measurement was determined at 510 nm and 700 nm, and the anthocyanin concentration was estimated using the difference in absorbance measurement at each pH and Beer's Law, using the following formula:

$$\text{mg/L cyanidin 3-glucoside cation (a specific anthocyanin)} = \frac{\text{A*MW*Df*1000}}{(\varepsilon\text{*L})}$$

where:

A (absorbance in nm) = $(A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH\ 4.5}$
MW (molecular weight of cyanidin 3-glucoside cation) = 449 (g/mol)
Df (dilution factor) = 5
ε (molar absorbance of cyanidin 3-glucoside cation) = 26900 (L mol^{-1} cm^{-1})
B (cell path length) = 1 (cm)

Data analysis.—Each analytical experiment was repeated at least three times with freshly thawed samples, each in triplicate in the 96-well plate. The values for each measurement were compared using one-way ANOVA and Tukey post-hoc test using SPSS (Statistical Package for the Social Sciences).

RESULTS

Medicinal plant extracts of Indiana plants contain a range of phenolic compounds.—In the broadest chemical comparison in this study, the total phenolic compound concentration, the extracts collected in Indiana fell into three main statistical groups. The ginkgo leaf extract (mixed sexes, collected at Earlham) showed the highest phenolic content (6800 ± 200 μg GAE/g sample; column c in Fig. 2; one-Way ANOVA, $p < 0.01$). The extracts of goldenrod leaves, goldenrod root, sassafras root, plantain leaves, and black raspberry were all statistically similar (concentration range from 2500 to 5000 μg GAE/g sample; Fig. 2). The mulberry and dandelion root extracts had the lowest phenolic content (from 1000 to 1500 μg GAE/g sample; Fig. 2).

Interestingly, when comparing the flavonoid (a class of phenolic compounds) concentration of extracts prepared from Indiana medicinal plants, different statistical groups appear. Plantain leaf extract has the highest flavonoid concentration with (4 ± 2 mg CE/g; Fig. 3). Most of the other extracts fall into two statistically similar groups regarding flavonoid content: group (b) included goldenrod roots, goldenrod leaves, sassafras root, and ginkgo, ranging from 2.0 to 3.5 mg CE/g sample; and group (c) included dandelion root, black raspberry, and mulberry extracts, ranging from 0.5 to 2.0 mg CE/g sample (Fig. 3).

While the berry extracts (black raspberry and mulberry) had lower flavonoid and total phenolic content than the other extracts, they had the highest anthocyanin content. However, given their dark purple color, this striking result was not surprising. Black raspberry extracts had by far the highest anthocyanin load with 190 ± 11 μg anthocyanin/L sample, followed by mulberry extracts with 40 ± 2 μg (Fig. 4). All other extracts had anthocyanin values much lower than the berries, and separated into three statistical groups: group (a) ranged from 1 to 6 μg anthocyanin/L sample and included goldenrod leaves, goldenrod roots extracts; group (e) included extracts contents below 1 μg anthocyanin/L sample and included sassafras root and ginkgo leaf extracts; and the remaining dandelion root and plantain leaf extract belong to group (b) and contained 6 to 12 μg anthocyanin/L sample (Fig. 4).

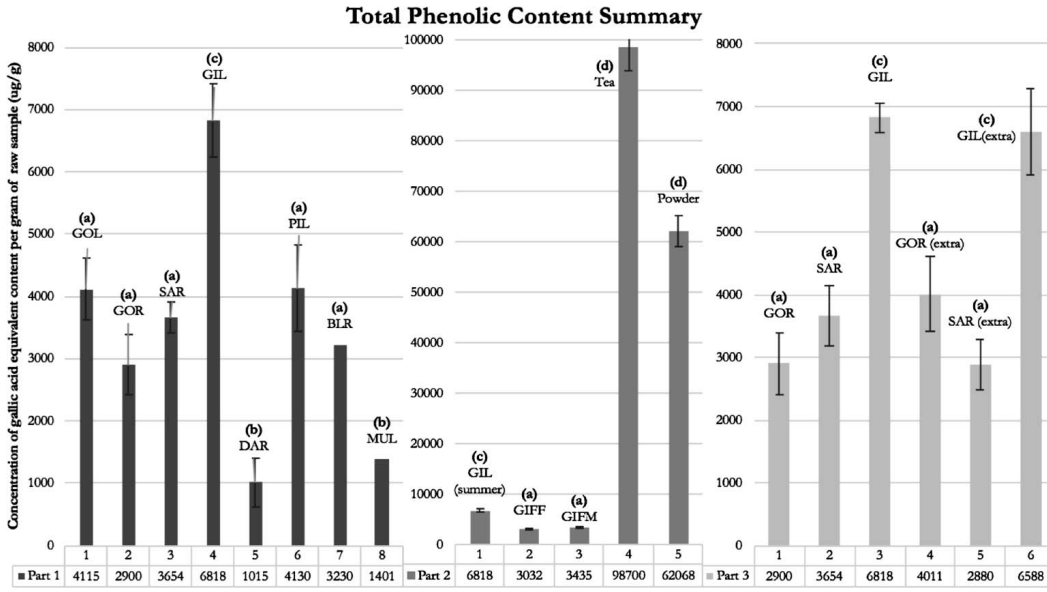

Figure 2.—Total phenolic content of medicinal plant extracts. Total phenolic content measured using Folin-Coicalteu method from Herald et al. (2012). Darkest gray bars represent plants collected in Indiana and extracted with 80% acetone, middle gray bars show comparison of all gingko extracts (note that the GIL bar is the same data as in the darkest gray category), and lightest gray bars show the comparison of original extracts (data not marked "extra" are the same as in darkest gray category) with the same extract preparation that underwent an additional freeze-thaw cycle (labeled "extra"). Bars represent the phenolic content average based on gallic acid equivalence over six independent trials, each with fresh samples. Error bars represent standard deviation between trials. Numbers across the bottom of the graphs are the average value for that extract. Each replicate had a gallic standard curve with $R^2 = 0.9909–0.9955$.

Impact of extract preparation and season of collection on the chemical composition of gingko.—Both purchased gingko products extracted with hot water had higher phenolic and flavonoid content than any acetone-extracted gingko sample in this study. The purchased ginkgo tea had the highest phenolic content (97000 ± 4000 μg GAE/g sample), followed by the purchased ginkgo powder (56000 ± 1000 μg GAE/g sample; Fig. 2). The mixed male and female tree gingko extract collected in the summer had the third highest concentration, followed by the male and female tree extracts collected in the fall, each having much lower phenolic content (concentration range from 2500 to 5000 μg GAE/g sample; Fig. 2). The purchased ginkgo tea and powder also had the two highest flavonoid contents: 9 ± 2 mg CE/g sample and 5 ± 3 mg CE/g sample, respectively (Fig. 3). Interestingly, the acetone extracts of male ginkgo leaves collected in the fall had the highest anthocyanin concentration (2 ± 2 μg anthocyanin/L; Fig. 4). The other gingko extracts, i.e., ginkgo leaves in the summer, ginkgo female leaves in the fall, ginkgo tea, and powder, belonged to group (e) in Fig. 4, which included extracts with contents below 1 μg anthocyanin/L sample.

No difference in extracts experiencing an additional freeze/thaw cycle.—Several acetone-extracts experiencing an unexpected extra freeze/thaw cycle (due to an ultralow freezer failure), provided the opportunity to test the impact of this thaw on the phenolic compounds in the extracts. No significant change was observed in the total phenolic and flavonoid content of goldenrod root, sassafras root, or ginkgo leaves following an additional freeze-thaw cycle (Fig. 2). However, the anthocyanin concentration for these extracts did show a slight difference, although not statistically significant.

DISCUSSION

Many plants are used medicinally throughout the world, and their varying impacts on

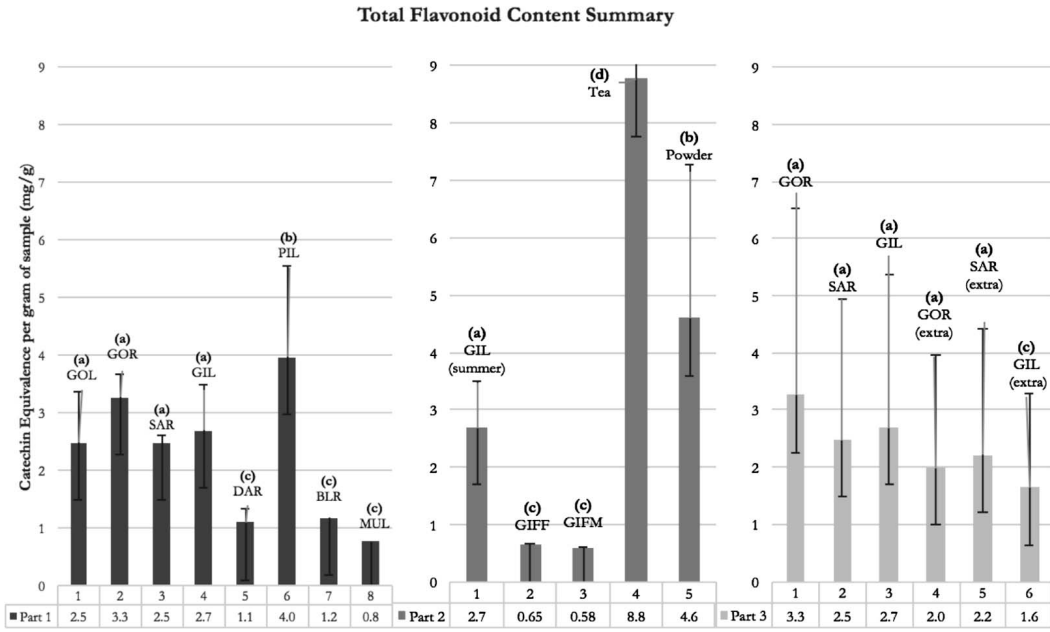

Figure 3.—Total flavonoid content summary. Total flavonoid content measured using spectrophotometric method from Herald et al. (2012). Darkest gray bars represent plants collected in Indiana and extracted with 80% acetone, middle gray bars show comparison of all gingko extracts (note that the GIL bar is the same data as in the darkest gray category), and lightest gray bars show the comparison of original extracts (data not marked "extra" are the same as in darkest gray category) with the same extract preparation that underwent an additional freeze-thaw cycle (labeled "extra"). Bars represent the content average based on catechin equivalence over seven independent trials. Error bars represent standard deviation between trials. Numbers across the bottom of the graphs are the average value for that extract. Each replicate had a catechin standard curve with $R^2 = 0.9641–0.998$.

physiology and health may be described by comparing their chemical components. Here, we describe the similarities and differences in phenolic compound concentrations of eight medicinal plants found in Indiana. The unique chemical mixtures present in medicinal plant extracts likely contribute to their biological action. Thus, due to their varying traditional uses (Gray 2011; Alfs 2013), it is not surprising that we found variation in the total phenol, total flavonoid, and anthocyanin content in acetone extracts of these plants. Among the Indiana plant extracts, we observed different rankings and relationships between plant extracts when measuring total phenol, flavonoid, and anthocyanin concentration, suggesting that other phenolic compounds are involved in the bioactivity of many of these extracts. Future work should relate the concentration of these classes of phenols within extracts to the bioactivity of those phenols (using extract fractionation). Importantly, other phenolic compounds (e.g., tannins) and non-phenolic compounds (e.g., alkaloids such as caffeine) not examined in this study are also known to exert biological effects when consumed.

The comparison between the extracts (sassafras, dandelion, gingko) that underwent an extra freeze-thaw cycle extracts did not show any significant changes in phenolic compound content. It is interesting to note that while the concentration of these compounds did not change, other chemical compounds may be affected by additional freeze-thaw cycles, which may impact bioactivity of these extracts. Future work should explore whether additional freeze-thaw cycles of the (unextracted) fruit or leaf impacts phenolic content and/or bioactivity, mimicking what could happen in a consumer's kitchen (i.e., intended or unintended freezing and thawing of collected or purchased fruit).

The gingko extracts, both the locally-collected and the purchased dried samples, have among the

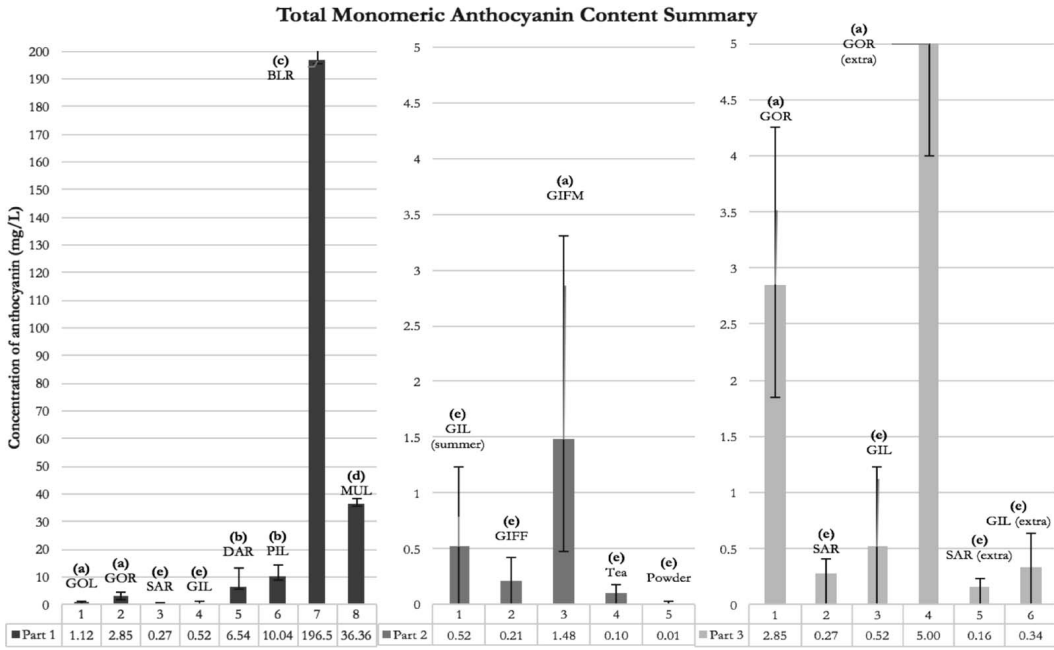

Figure 4.—Total monomeric anthocyanin summary. Total anthocyanin content measured using pH differential method from Zhang et al. (2006). Darkest gray bars represent plants collected in Indiana and extracted with 80% acetone, middle gray bars show comparison of all gingko extracts (note that the GIL bar is the same data as in the darkest gray category), and lightest gray bars show the comparison of original extracts (data not marked "extra" are the same as in darkest gray category) with the same extract preparation that underwent an additional freeze-thaw cycle (labeled "extra"). Bars represent the average concentration of anthocyanin content over four independent trials. Error bars represent standard deviation between trials.

highest values of total phenolic and flavonoid content compared to other extracts. The ginkgo leaves (combined male and female trees) collected in the summer had much higher phenol and flavonoid content than the individual male and female leaf samples collected in the fall. However, there was not a significant difference in phenolic and flavonoid concentration in the ginkgo leaves collected from the female tree and those collected from the male tree. Interestingly, the ginkgo leaves collected from the male tree had a much higher anthocyanin concentration than those from the female tree. This may be due to slightly different environmental exposures (the trees are approximately 3 m apart) or due to biological differences among the two trees; samples from additional pairs of trees should be collected and examined to explore the mechanism behind this difference in anthocyanin content. The purchased ginkgo tea and powder extracts also showed interesting results; both had higher phenolic and flavonoid contents than the other locally-collected ginkgo extracts. However, the anthocyanin content in both ginkgo tea and powder is significantly lower than the concentration in other ginkgo acetone extracts. This may be the result of differences in the acetone and hot water preparations. Moreover, this increase in antioxidant concentration could also be caused by the drying process of the commercial-obtained tea and powder, which removed most of the moisture content to enhance shelf-stability (i.e., the phenolic compounds were more concentrated in the dried, purchased samples than in the fresh, locally-collected samples). Further research should be conducted to evaluate this hypothesis in a more controlled setting, where only one variable is monitored: similar methods of preparation on various samples, or various sample preparation methods on the same sample. In summary, the phenolic content of gingko leaf extracts, and potentially as a consequence, the antioxidant activity and medicinal value of these products, is influenced be season of collection, extract preparation procedures, and appropriate temperature storage.

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