Major Phenolic Acids and Total Antioxidant Activity in Mamaki Leaves, *Pipturus albidus*

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ABSTRACT: Three phenolic acids, (+)catechins, chlorogenic acid, and rutin, were identified and quantified in Mamaki leaves using a liquid chromatograph-mass spectrometer technique. Concentrations of (+)catechins, chlorogenic acid, and rutin varied from 1.1 to 5.0 mg/g of Mamaki leaves as determined in the extract using 0.5% acetic acid in 90% aqueous methanol. This study also quantified total antioxidant capacity using the photochemiluminescence method, which was expressed in equivalents to ascorbic acid (AA). Mamaki teas brewed for 30 min contained total antioxidant activity (TAA) between 238 and 259 mg AA/g of tea. Mamaki teas brewed for 1 h and stored at 4 h, 1 d, and 3 d at 4 °C had available TAA 293, 271, 172, and 163 mg AA/g of tea leaves, respectively. The concentrations of (+)catechins and rutin in Mamaki leaves are compared to other types of popular teas. Mamaki teas contained relatively low amounts of TAA compared to green teas and Lipton teas.

Keywords: antioxidant, herbal drink, Mamaki tea, *Pipturus albidus*, polyphenols

Introduction

Phenolic compounds are commonly found in all plants as secondary metabolites. They are derived from phenylalanine and tyrosine in the shikimic acid pathway (Hermann 1995). They are produced in response to environmental stress such as microbial infections, UV radiation, and chemical stressors, rather than involvement in plant growth and development (Dixon and Paiva 1995). Polyphenols in plants include simple phenols, phenolic acids (both benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolyzable and condensed tannins, lignans, and lignins (Dixon and Paiva 1995). These phenolic compounds contribute to color, bitter and astringent taste, flavor, odor, and antioxidant properties.

Recently, there has been a growing interest in the study of nutraceuticals in plants due to their antioxidative, mild estrogenic, and hypolipidemic activity. Research indicates that phytophenolics are potent antioxidants in scavenging free radicals and inhibiting lipid peroxidation in human tissues (Rice-Evans and others 1997; Marja and others 1999; Sugihara and others 1999). They play roles as reducing agents, metal chelators, singlet oxygen quenchers, and hydrogen donors (Rice-Evans and others 1997; Kuo and others 1998; Yoshino & Murakami 1998; Marja and others 1999; Sugihara and others 1999). Furthermore, studies show that these phenolic compounds are linked with lower occurrence of and lower mortality rates from various human diseases (Block and others 1992; Ames and others 1993; Geleinjese and others 2002).

Traditionally, Mamaki plants (*Pipturus albidus)* were used by native Hawaiians to ease childbirth, to discharge blood, and to alleviate listlessness (Chun 1994). Previous studies found that parts of Mamaki plants have antimicrobial, antiviral, and antifungal properties (Locker and others 1995). Recently, Mamaki tea leaves became well known in therapeutic usage for alleviating various preexisting diseases (Chun 1994). According to local folklore, Mamaki leaves are a potential natural therapeutic medicine in regulating blood sugar levels, blood pressure, and cholesterol levels (Chun 1994).

However, due to limited information available on the Mamaki plant and especially the leaves, research on its chemical and antioxidant properties is necessary. Mamaki leaves are commonly prepared as herbal teas, and consumed as fresh-brewed teas or as cold herbal teas. Thus, the purpose of this study was to identify and quantify major polyphenols present in Mamaki leaves and quantify the total antioxidant capacity in Mamaki teas after brewing, steeping, and storing them for 3 d at 4 °C.

Materials and Methods

Identification and quantification of Mamaki leaves

Chemicals. Optima grade solvents including methanol, acetic acid, and formic acid were obtained from Fisher Scientific (Pittsburg, Pa., U.S.A.). (+)Catechins, chlorogenic acid, caffeine, and rutin were purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.). Water was purified and filtered at all times. The water was passed through a Milli-Q water purification system (Millipore Corp., Bedford, Mass., U.S.A.) set at 18 MΩ-cm resistance. HPLC solvents were filtered through Nylon 66, 47-mm i.d., 0.45-μm pore size (Millipore Corp.).

(+)Catechins, chlorogenic acid, and rutin were each dissolved in methanol and sonicated until the powder dissolved into solutions. (+)Catechins were prepared at 200, 400, and 800 μg/mL. Concentrations of chlorogenic acid were 100, 500, and 1000 μg/mL. Rutin was prepared with concentrations of 80, 400, and 800 μg/mL.

Mamaki sample preparation. Four varieties of fresh Mamaki leaves were hand harvested, placed in labeled zip lock bags and immediately chilled in a cooler. They were distinguished by leaf physical characteristics as (1) purple veined and purple leaves, (2) green veined and green leaves, (3) a hybrid plant with green leaves and purple veins, and (4) the so-called "panaewa" by the farmer with green leaves and light pink veins (Figure 1). For each variety, Mamaki leaves were ground in a clean vegetable chopper with dry ice

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until it gave a uniform powder. Ground Mamaki leaves were then lyophilized at 0.2 Pa for more than 48 h and stored in an airtight container in the refrigerator after processing.

Extraction of polyphenols. Mamaki samples (0.5 g) were extracted with 12 mL of 90% aqueous methanol containing 0.5% acetic acid. The solution was sonicated for 3 min followed by centrifugation at 3000 rpm for 15 min. After 3 times of extraction, the supernatants were dried with a speed-vacuum centrifuge (Vacufuge^{TM}, Eppendorf, Germany). The residues were dissolved in methanol and filtered through a PTFE 0.25-μm membrane filter (Millipore Co.). The solution was concentrated to a final volume of 1.5 mL under a gentle nitrogen gas flow.

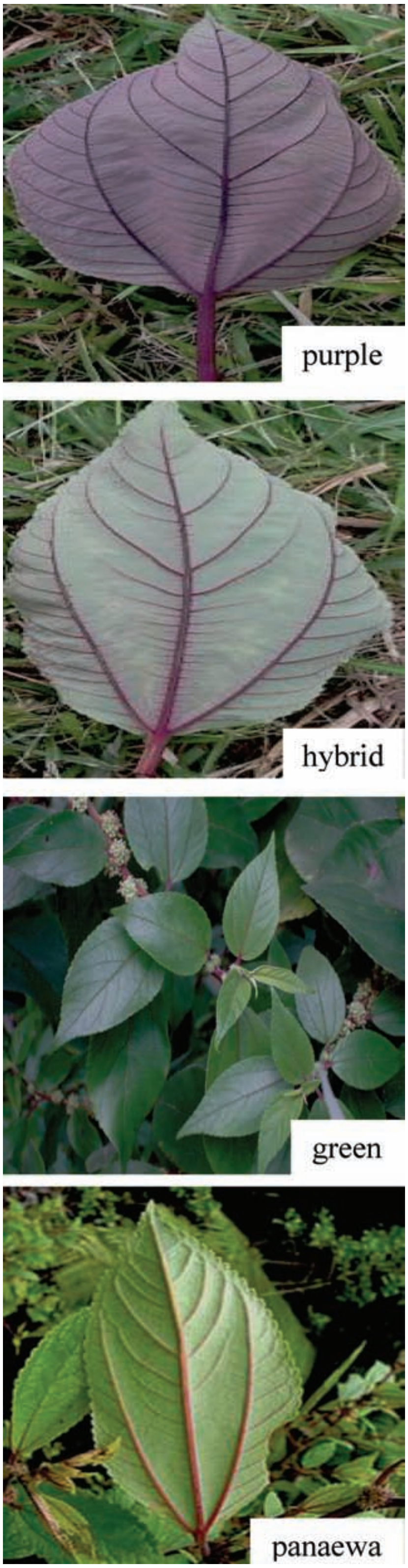

Figure 1 — Four different varieties of Mamaki leaves (*Pipturus alibudus*).

LCMS conditions. The LC–MS system consisted of an Agilent 1100 series liquid chromatograph, a photodiode array (PDA) detector, a single quadruple mass spectrometer, a vacuum degasser, a binary pump, an autosampler, and an electrospray ionization (ESI) source (Agilent Technologies, Wilmington, Del., U.S.A.). Mass spectra were acquired in negative ion mode in a mass range of m/z 110 to 1000. The compounds were monitored at 250, 280, 320, 370, and 510 nm. The drying gas was nitrogen at a flow rate of 10 L/min, creating a nebulizing pressure of 25 psi. The fragmentation voltage was 120 V. The capillary voltage was 4 kV.

LC separations were performed with a Phenomenex Luna, C-18 column (4.6 $\times$ 250 mm, 5-μm i.d.) joined with a guard column (4 $\times$ 3 mm i.d.) at 35 °C. The gradient elution was set up according to the method of Sakakibara and others (2003) with some modification. Gradient elution was carried out with solvent A (0.1% formic acid solution) and solvent B (100% methanol), delivered at a constant rate of 1 mL/min as follows: initially 100% of solution A; for the next 15 min, 70% of solution A; for another 30 min, 65% A; for another 20 min, 60% A; for another 5 min, 50% A; and finally for the last 25 min, 0% A. The injection volume was 10 μL.

Statistical analysis

Concentrations of catechins and rutin in the Mamaki tea leaf samples were compared with other commercial teas using a 1-tailed probability of a Z-test. The Z-test is calculated as $1 - [(x_{\text{bar}} - \mu_0)/(\sigma/\sqrt{n})]$. A 1-tailed probability Z-test was selected to see if the average of the Mamaki samples is greater than other teas.

The total antioxidant activities (TAA) in the Mamaki tea were also compared to other published data using the 1-tailed probability Z-test.

Antioxidant activity of Mamaki tea

Mamaki tea preparation. Three varieties of dried Mamaki leaves, (1) purple veined and purple leaves, (2) green veined and green leaves, and (3) a hybrid plant with green leaves and purple veins, were analyzed for the antioxidant analysis (Figure 1). For tea preparation of each variety, a water extract was made by adding 100 mL of boiling water to 1 g of dried leaves and allowing the tea to stand for 30 min in a covered container. The Mamaki teas were prepared in triplicates and, after steeping, analyzed for their water-soluble antioxidant activities.

In the storage study, teas from the purple variety were prepared by steeping 1 g of leaves in 100 mL of boiling water followed by steeping for 60 min. The Mamaki teas were then stored for 4 h, 1 d, and 3 d at 4 °C. The Mamaki teas were prepared in triplicates and each sample was analyzed 3 times to ensure accuracy of measurement.

Antioxidant study. The antioxidant activities were measured with a Photochem® system (Analytik Jena AG, The Woodlands, Tex., U.S.A.). The system enables the quantification of antioxidant capacity of water-soluble substances based on photochemiluminescence (PCL). This includes photochemical excitation to generate free radicals (superoxide anion radicals) followed by luminescence detection (Popov and Lewin 1994). The free radicals generated by the optical excitation of the photosensitizer substance are partly eliminated by the reaction of antioxidants in the sample to be analyzed. In a measurement cell, the luminescence of the detection substance (luminol) generated by the remaining radicals is measured and thus the quantity of antioxidants present in the sample is determined in equivalents to ascorbic acid. PCL is able to measure antioxidant activity in the nanomolar range, whereas other antioxidant assays determine activity in the micromolar range (Popov and Lewin 1994). The reagent kits used for the analysis were obtained from Analytik Jena AG. A 20 μL aliquot of diluted tea (1:50, 1:100, 1:175) was used for each measurement.

Results and Discussion

Identification of 3 main phenolic acids in Mamaki extracts

Figure 2 represents typical HPLC profiles of extracts of the different varieties of Mamaki leaves monitored at 250, 280, 320, 370, and 510 nm. The main chromatographic difference among the 4 varieties of Mamaki leaves is that the "green" variety does not show a

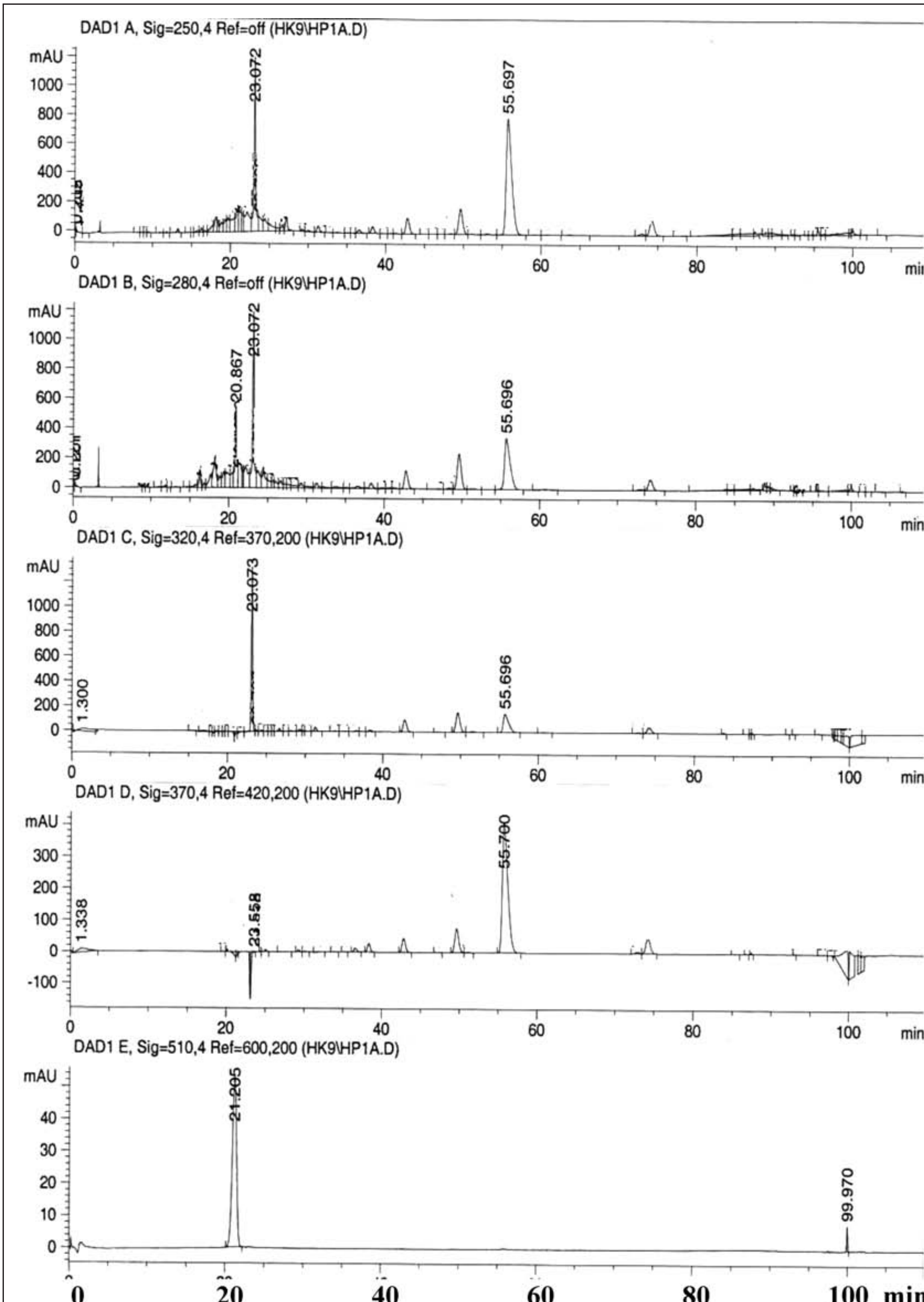

Figure 2—HPLC chromatograms of Mamaki leaf extracts at different wavelengths (250, 280, 320, 370, and 510 nm).

peak at retention time (t_r) 21.2 min with $UV_{\max}$ of 510 nm. Purple, hybrid, and panaewa varieties show the peak at $UV_{\max}$ of 510 nm with strongest intensity in the chromatogram of the purple variety and the weakest intensity in the chromatogram of the panaewa variety.

A compound in teas absorbing at $UV_{\max}$ 510 nm is somewhat rare and usually belongs to the anthocyanins group. This finding relates to the physical appearance of the Mamaki leaves. All of the Mamaki varieties except the green one have a purplish or reddish color either in the leaves, the vein, or both. With the m/z ion of 465, the compound at t_r 21.2 min is possibly cyanidin glucoside (465 = 286 + 180 – 1 H). Cyanidin has a molecular weight (MW) of 286 and glucose has MW of 180. However, further confirmation with the standard is necessary to identify this compound.

Figure 3 represents the chromatograms of the 4 varieties of Mamaki leaves at 280 nm. The 3 strongest and well-defined peaks were selected for chemical identification. These 3 compounds had identical retention times as chlorogenic acid (20 min), (+)catechins

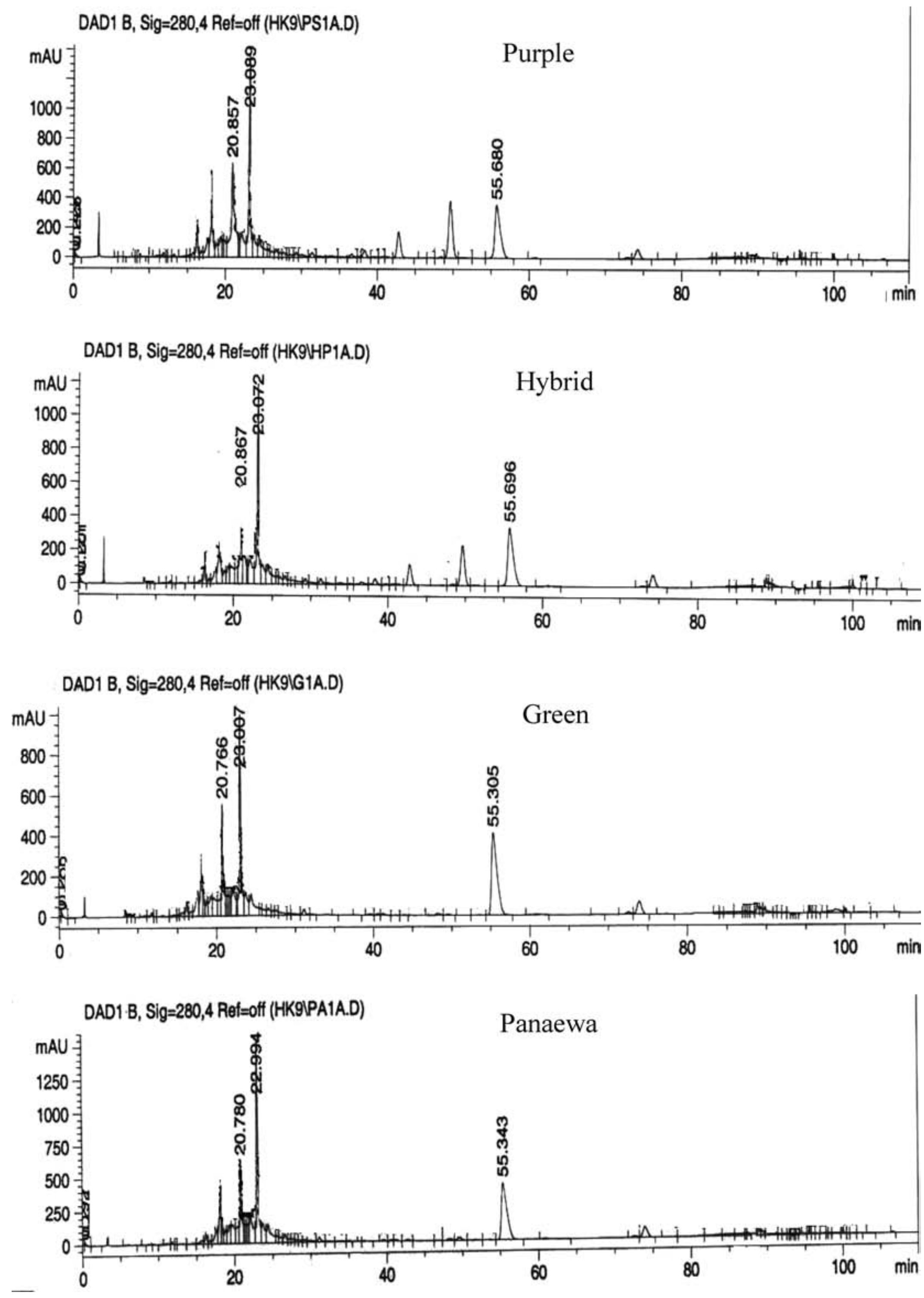

Figure 3—HPLC chromatograms (280 nm) of 4 varieties of Mamaki leaf extracts.

(23 min), and rutin (55 min) and the same corresponding molecular ions. Therefore, they were identified as chlorogenic acid, (+)catechins, and rutin, respectively.

These phenolics could form dimers or sugar conjugates that are commonly found in complex plant extracts. Because of conjugation with different types of sugar molecules, the phenolics–sugar conjugates often become more polar and tend to elute earlier than their corresponding aglycone alone in a reverse-phase HPLC column (Hong and Wrolstad 1990). In a reverse-phase HPLC column, molecules that bind to galactose appear to elute faster than glucose and arabinose due to the degree of glycosylation and the nature of the sugar moieties (Hong and Wrolstad 1990). This was agreed with the phenolic profile from Sakakibara and others (2003) which shows that the retention of rutin is 40.6 min while the retention time of quercetin is 75.5 min. In the Mamaki case, rutin (quercetin-3-O-rutinoside) showed a retention time of approximately 55 min.

Quantification of 3 main phenolic acids in Mamaki extracts

The standards at different concentrations were analyzed to generate calibration curves for quantitation, which showed R^2 equal or near to 1. The concentrations of the phenolics present in Mamaki extracts are shown in Table 1. (+)Catechins were the most abundant phenolic among the 3 identified phenolics. Among the 4 Mamaki varieties, the purple variety contained the highest amount of (+)catechins and the panaewa variety contained the lowest amount. The concentrations of (+)catechins were quantified at $UV_{\max}$ 280 nm and they ranged from 2.1 to 5 mg/g. The concentrations of chlorogenic acid were quantified at $UV_{\max}$ 320 nm and they ranged from 1.1 to 1.7 mg/g. The concentrations of rutin were quantified at $UV_{\max}$ 370 nm and they ranged from 1.1 to 1.8 mg/g.

The beneficial values of these phenolic acids have been reported in the literature. For example, (+)catechins is commonly found in green tea and plays a role as an antioxidant against cancer, obesity, diabetes, cardiovascular disease, aging, and neurodegenerative diseases. (Zaveri and others 2006). Chlorogenic acid is commonly found in root vegetables such as carrot, radish, turnip, and burdock (Sakakibara and others 2003). Chlorogenic acid in hawthorn fruit is found to be beneficial as an antioxidant against low-density liproprotein oxidation. (Zhang and others 2001; Wang and others 2007). Chlorogenic acid is also found in blueberries and plays roles in browning reaction and polymerization (Kader and others 1997). Rutin is commonly found in red wine, buckwheat, citrus, and tomato skin (Heims and others 2002). An animal study showed that rutin plays a role as an antioxidant and is effective in controlling the animal body weight (Gao and others 2003)

The concentrations of (+)catechins in Mamaki leaf are significantly higher ($P < 0.05$) than those reported for other commercial tea leaves, especially the Gyokuro green tea leaves, Chinese oolong tea leaves, and Kenya black tea leaves (Sakakibara and others 2003). The concentrations of rutin in Mamaki leaf are also significantly higher ($P < 0.01$) than those for other tea leaves (Sakakibara and others 2003).

Quantification of antioxidants in Mamaki teas

Three varieties of Mamaki leaves were chosen for total antioxidant activity (TAA) in the brewed extracts and were quantified in equivalents to ascorbic acid (AA). The 3 Mamaki leaves selected for the study were the purple, hybrid, and green varieties.

Table 2 shows that the purple and hybrid Mamaki varieties collected in 2 different seasons (summer and winter) contain similar amounts (mg) of TAA in a gram of brewed tea. The average amount of TAA available in the purple, hybrid, and green Mamaki teas are 238, 244, and 259 mg, respectively. The amount of TAA available in Mamaki teas is also compared to those in some commercial teas. The commercial teas selected for the comparison are green tea, black tea, and oolong tea (Du Toit and others 2001). This comparison is made based on similarity of tea preparation and their unit of measurements. The average TAA available in Mamaki tea (238 to 259 mg AA/g) is similar to those in green teas (246 mg AA/g) (Du Toit and others 2001). However, the average TAA available in Mamaki tea is significantly higher ($P < 0.05$) than oolong tea (217 mg AA/g) and black tea (166 mg AA/g) (Du Toit and others 2001).

Table 1—Concentration of (+)catechins, chlorogenic acid, and rutin in water extracts of Mamaki leaves.

	Concentration (mg/g)[a] ± standard deviation		
Mamaki variety	(+)Catechins	Chlorogenic acid	Rutin
Green	2.4 ± 0.0	1.1 ± 0.1	1.1 ± 0.2
Hybrid purple	2.5 ± 0.1	1.4 ± 0.1	1.6 ± 0.0
Purple	5.0 ± 0.8	1.7 ± 0.1	1.8 ± 0.1

[a]Values are means of 4 replications.

Table 2—Total antioxidant activity in ascorbic acid equivalents (mg AA/g) of Mamaki teas made from summer and winter leaf samples.

Mamaki tea	Winter	Summer	Average ± SD
Purple	254	222	238 ± 23
Hybrid	236	251	244 ± 10
Green	307	211	259 ± 67

Table 3—Total antioxidant activity in purple Mamaki teas after brewed for 1 h and stored for 4 h, 1 d, and 3 d at 4 °C (mg AA/g tea).[a]

Length of storage (h)	Average ± SD
0	293 ± 145
4	271 ± 134
24	172 ± 65
72	163 ± 18

[a]Values are means of triplicate samples and 9 analyses.

Freshly brewed purple Mamaki teas were prepared for the storage study. The highest TAA (293 mg AA/g tea) detected was immediately after Mamaki tea was brewed for an hour while the lowest TAA (163 mg AA/g tea) was present after the tea was stored for 3 d (Table 3).

Conclusions

Three major polyphenols in Mamaki leaves are identified. They are (+)catechins, chlorogenic acid, and rutin with concentrations from 1.1 to 5.0 mg/g of Mamaki leaves. The concentrations of (+)catechins and rutin in Mamaki leaves are higher than those in other commercial tea leaves. The amount of TAA in Mamaki teas varies from 238 to 259 mg of ascorbic acid equivalents. The amount of TAA available in the purple Mamaki tea is the highest immediately after an hour of brewing and gradually decreased over a 3-d period.

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