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Antioxidant Activities of Extracts from Teas Prepared from Medicinal Plants, *Morus alba* L., *Camellia sinensis* L., and *Cudrania tricuspidata*, and Their Volatile Components

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ABSTRACT: The antioxidant activity of essences of teas prepared from mulberry (*Morus alba* L.), *Camellia sinensis* L., and *Cudrania tricuspidata* (Carr.) Burea plant was examined using two antioxidant assays. Selected volatile chemicals identified in these plants were also tested for antioxidant activity. All extracts exhibited antioxidant activity with a clear dose response in the aldehyde/carboxylic acid and the malonaldehyde/gas chromatography (MA/GC) assays. Antioxidant activity of extracts at the level of 500 μg/mL ranged from 77.02 ± 0.51% (stems of Burea plant) to 52.57 ± 0.92% (fermented tea of *Camellia* and stems of Mulberry tea) in the aldehydes/carboxylic acid assay. Their antioxidant activity at the level of 160 μg/mL ranged from 76.17 ± 0.27% (roots of Burea plant) to 59.32 ± 0.27% (stems of Mulberry tea) in the MA/GC assay. Among the positively identified compounds (11 terpenes and terpenoids, 15 alkyl compounds, 26 nitrogen containing heterocyclic compounds, 9 oxygen containing heterocyclic compounds, 18 aromatic compounds, 7 lactones, 6 acids, and 4 miscellaneous compounds), eugenol, 2,5-dihydroxyl acetophenone, and isoeugenol exhibited antioxidant activity comparable to that of BHT in both assays. Vanillin and 2-acetylpyrrole showed potent antioxidant activity in the aldehydes/carboxylic acid assay but only moderate activity in the MA/GC assay. These results suggest that consumption of antioxidant-rich beverages prepared from these plants may be beneficial to human health.

KEYWORDS: *antioxidants, volatile constituents, natural plants, tea*

INTRODUCTION

Plant extracts obtained from medicinal plants by steam-distillation or solvent extraction have been used to treat various diseases since ancient times, particularly in Asian countries.[1] They have continued to be used even after the development of modern medicines. To date, over 7000 medicinal plants or herbs have been utilized by 80% of the world's population.[2] Many recent studies demonstrate the medicinal activities, in particular antioxidant activities, of natural plant materials against various diseases: including atherosclerosis, cancer, diabetes, Alzheimer's, HIV, Parkinson's, and cataracts.[3] Therefore, antioxidants found in natural plants, such as vitamin E (α-tocopherol), vitamin C, and polyphenols/flavonoids,[4] have received much attention as alternative medicines preventing these diseases. For example, a recent symposium summarizes the effects of polyphenols, which are relatively less-volatile chemicals, on human health.[5]

In ancient Egypt, plant extracts (essential oils) obtained from aromatic plants were used for disease prevention and treatment. Later, the Greeks and Romans inherited Egyptian practices of using essential oils in aromatherapy and expanded them to improve their life quality.[1] Therefore, in addition to the less-volatile antioxidants, low molecular weight compounds, so-called aroma or volatile chemicals, found in plant extracts have begun to receive much attention as natural antioxidants today.[6] The antioxidant activity of various plant extracts and their constituents has been reported in many articles.[1,7] In the present study, the antioxidant activity of extracts from three medicinal plants—Mulberry (*Morus alba* L.), *Camellia sinensis*, and *Cudrania tricuspidata* (Carr.) Burea—grown in Korea, and their volatile components were measured to search for possible natural antioxidants.

Mulberry (*Morus alba* L.) is a small medium sized mulberry tree, which grows to 10−20 m tall. The species is native to Northern China and is widely cultivated in Asia to use as traditional medicine.[8] *Camellia sinensis* is native to Mainland China, and it is also cultivated across the world in tropical and subtropical regions. Generally, its leaves and stems are used to prepare various teas. *Cudrania tricuspidata* (Carr.) Burea, a deciduous tree belonging to the family Morus, is distributed widely within East Asia, mainly in Korea, Japan, and China. *Cudrania tricuspidata* (Carr.) Burea has been used for the treatment of eczema, mumps, tuberculosis, contusions, and acute arthritis since ancient times.[9]

There have been many reports on the medicinal activities of these plants, such as the antidiabetic,[10] antihyperlipidemia,[11] and antioxidant[12] properties of Mulberry, the antioxidant and antibacterial properties of *Camellia sinensis*,[13] and the antiatherosclerotic and anti-inflammatory properties of *Cudrania tricuspidata*.[14] However, the activities of these plants that have been reported to date are associated with relatively less-

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volatile chemicals such as polyphenols, flavonol glycosides, and catecholic xanthones. Therefore, the present study focused on the antioxidant activity of the volatiles constituents of these plants.

MATERIALS AND METHODS

Chemicals and Plant Samples. Eugenol, 2,5-dimethylpyrazine, 2-phenyl acetaldehyde, pedagogic acid, (*E*)-*β*-ionone, vanillin, 2,5-dihydroxy acetophenone, hexanal, hexanoic acid, undecane, *N*-methylhydrazine (NMH), 2-methylpyrazine, 1-methyl pyrazole (1-MP), sodium dodecyl sulfate (SDS), ferrous chloride, and *α*-tocopherol (vitamin E) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Cod liver oil (approximately 70% *ω*-3 fatty acid methyl esters), butylated hydroxytoluene (BHT), trizma hydrochloride, and trizma base were bought from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide, dichloromethane, and ethyl-acetate were purchased from Fisher Scientific Co., Ltd. (Fair Lawn, NJ). A standard solution of 2-methylpyrazine (2-MP) was prepared by adding 100 mg of 2-methylpyrazine to 10 mL of dichloromethane and was stored at 5 °C. Other authentic volatile chemicals were bought from reliable commercial sources (mainly from Aldrich Chemical Co. and Sigma Chemical Co.) or were gifts from TAKATA Koryo Co., Ltd. (Osaka, Japan).

Mulberry (*Morus alba* L.) teas, tea prepared from the leaves (Sang-Yup teas) and tea prepared from the stems (Sang-Ji teas), were purchased from Cho−Rok−Bit−Ma−Ul, Co. (Sancheong, GN, Korea). Green tea and fermented tea, which was naturally fermented by micro-organisms, of *Camellia sinensis* L. were purchased from Dongdawon, Co. (Hadong, GN, Korea). *Cudrania tricuspidata* (Carr.) Burea plant was purchased from Cho−Rok−Bit−Ma−Ul, Co. (Sancheong, GN, Korea), and its stems and roots were used for the experiments.

Sample Preparations for Analysis of Volatile Chemicals and Antioxidant Assays. A plant sample (20 g) was placed in a 3 L round-bottom flask with 1 L of deionized water. The solution was steam-distilled at 55 °C for 4 h under reduced pressure (95 mmHg). The distillate (900 mL) was extracted with 100 mL of dichloromethane for 6 h using a liquid−liquid continuous extractor. After the extract was dried over anhydrous sodium sulfate, the solvent was evaporated using a rotary flash evaporator. The evaporation was stopped when the volume was reduced to approximately 1 mL, and then, the solvent was further removed under a purified nitrogen stream until the volume was reduced to exactly 0.5 mL. This extract was used for analysis and antioxidant activity assays (standard extract solution). All extracts prepared were stored at 5 °C until use.

Analysis of Volatile Chemicals in the Extracts. The volatile chemicals in each extract were identified by comparison with the Kovats gas chromatographic retention index (KI)[15] and by the mass spectrometry (MS) fragmentation pattern of each component compared with those of authentic chemicals. An Agilent model 6890 GC equipped with a 30 m × 0.25 mm i.d. (d_f = 0.5 μm) DB-WAX bonded-phase fused-silica capillary column (Agilent, Folsom, CA) and a flame ionization detector (FID) was used for measurement of KI and routine analysis of volatile chemicals. A 30 m × 0.25 mm i.d. (d_f = 0.5 μm) DB-1 bonded-phase fused-silica capillary column was also used for measurement of KI. The helium carrier gas flow rate was 1.0 mL/min at a split ratio of 20:1. The injector and detector temperature were 260 and 290 °C, respectively. The oven temperature was programmed to increase from 50 to 210 °C at 2 °C/min and then held for 93 min. The concentration of each chemical was calculated using a previously reported method.[16]

An Agilent model 6890 GC interfaced to an Agilent 5971A mass selective detector (GC/MS) was used for mass spectral identification of the GC components at MS ionization voltage of 70 eV. GC column conditions were exactly the same as the ones used for GC/FID.

Aldehyde/Carboxylic Acid Assay. A detailed description of this assay was previously reported.[3] Various amounts of volatile extracts (prepared by sequentially diluting the standard extract with dichloromethane) and components (prepared by sequentially diluting the standard dichloromethane solution of authentic chemicals) were added to a 2 mL dichloromethane solution of hexanal (3 mg/mL) containing 0.2 mg/mL undecane as a GC internal standard. The oxidation of the sample solution was initiated by heating at 60 °C for 10 min in a sealed vial and then stored at room temperature. The headspace of each vial was purged with pure air (1.5 L/min) for 3 s every 24 h for the first 7 days. The amount of hexanal was analyzed at 7 day time intervals for 4 weeks. The blank value was obtained immediately after the hexanal solution was prepared without the testing sample. The standard antioxidant BHT was also examined to validate the assay. The antioxidant activity (%) was calculated using following equation:

$$\text{Antioxidant activity (\%)} = \left[1 - \frac{\text{Amt of hexanal in blank} - \text{Amt of hexanal in sample}}{\text{Amt of hexanal in blank}}\right] \times 100$$

A Hewlett−Packard (HP) model 6890 GC equipped with a 30 m × 0.25 mm i.d. (d_f = 0.25 μm) DB-1 bonded-phase fused-silica capillary column (J & W Scientific, Folsom, CA) and an FID was used for the analysis of hexanal. The linear velocity of the helium carrier gas was 30 cm/s at a split ratio of 20:1. The injector and the detector temperatures were 300 and 280 °C, respectively. The oven temperature was programmed to increase from 40 to 180 °C at 4 °C/min and held for 10 min. The quantitative analysis of hexanal was conducted with undecane as an internal standard.[16]

Malonaldehyde/Gas Chromatography (MA/GC) Assay. A detailed description of this assay was also previously reported.[3] The antioxidant activity was also determined by analyzing MA formed from cod liver oil upon oxidation after derivatizing to 1-methylpyrazole (1-MP) with *N*-methylhydrazine (NMH). An aqueous solution (5 mL) containing 10 μL of cod oil, 0.25 mmol of trizma buffer (pH 7.4), 5 μmoL of ferrous chloride, 10 μmoL of hydrogen peroxide, 0.75 mmol of potassium chloride, and 0.2% sodium dodecyl sulfate was incubated with various amounts of the volatile extracts (prepared by sequentially diluting the standard extract with ethanol) and their components (authentic chemicals) for 18 h at 37 °C in a 20 mL test tube. The oxidation was stopped by adding 50 μL of a 4% BHT solution. The sample tubes were covered with aluminum foil during incubation to avoid any influence of light on the lipid peroxidation. The control was prepared from the same solution without a testing sample. The known antioxidant BHT was used to validate the assay. The antioxidant activity (%) was calculated using the following equation:

$$\text{Antioxidant activity (\%)} = \frac{\text{Amt of MA in control} - \text{Amt of MA in sample}}{\text{Amt of MA in control}} \times 100$$

NMH (30 μL) was added to the oxidized cod liver oil solutions described above, and the solutions were stirred for 1 h at room temperature. Any 1-MP formed from the solutions was extracted using a solid phase extraction (SPE) cartridge (MEGA BE-C_{18}, 1 g, 6 mL, Varian, USA). 2-Methylpyrazine (20 μL) was added to the extract as a GC internal standard, and the volume of the extract was adjusted to exactly 10 mL with ethyl acetate. The solution was analyzed for 1-MP by an Agilent model 6890 GC equipped with a 30 m × 0.25 mm i.d. (d_f = 0.25 μm) DB-WAX bounded-phase fused-silica capillary column (Agilent, Folsom, CA), and an NPD was used for the analysis of 1-MP. The linear velocity of the helium carrier gas was 30 cm/s at a splitless mode. The injector and the detector temperatures were 250 °C. The oven temperature was programmed from 60 to 140 at 4 °C/min.

Statistical Processing. The results of the present study were averaged, and the comparison between experimental results was made through an ANOVA analysis based on the SAS system. After the ANOVA analysis, the level of significance was computed using Duncan's multiple range test at α = 0.05.

Table 1. Compounds Identified in Extracts from Mulberry teas (Leaves and Stems), *Camellia sinensis* L. Teas (Green and Fermented), and *Cudrania tricuspidata* (Carr.) Burea Teas (Stems and Roots)

			amount (μg/g sample)					
			mulberry tea		*Camellia* tea		Burea tea	
compd	KI[a]	KI[b]	leaves	stems	green	fermented	stems	roots
Terpenes and Terpenoids								
camphene	1085	0952	–[c]	–	–	–	–	0.03
(*E*)-linalool oxide	1422	1082	0.09	–	–	–	–	0.02
(*Z*)-linalool oxide	1451	1094	0.23	–	–	–	–	0.11
linalool	1545	1084	0.90	–	–	–	–	–
(*E*)-α-terpineol	1697	1284	–	0.15	0.19	–	0.30	0.08
dl-borneol	1698	1164	–	0.05	–	–	–	–
(*Z*)-geraniol	1792	1243	–	–	0.13	0.09	–	–
(*E*)-geraniol[d]	1837	1254	–	–	3.98	7.44	–	–
(*E*)-geranyl acetone	1758	1555	1.39	0.23	0.06	0.04	–	0.08
(*E*)-α-ionone	1852	1424	1.38	–	–	–	–	–
(*E*)-β-ionone[d]	1936	1471	9.32	–	0.23	0.31	0.12	0.02
β-ionone-5,6-epoxide[e]	1999	NF	5.63	0.29	1.61	0.62	–	–
terpin hydrat[e]	2083	NF	–	1.23	1.37	0.98	–	0.63
α-cadinol[e]	2225	1630	–	–	–	0.27	–	–
dihydroctinidiolide	2353	1485	9.65	–	8.32	4.15	0.40	–
drimenol[e]	2508	NF	–	–	–	–	–	0.11
Alkylalcohols								
n-butanol	1128	0640	–	–	–	–	–	0.25
1-buten-3-ol	1150	0821	0.81	–	–	–	–	–
n-pentanol	1240	0748	0.73	–	–	–	–	–
(*E*)-2-pentene-1-ol	1312	0711	1.65	–	–	–	–	–
n-hexanol	1368	0838	–	–	–	–	–	0.02
2-ethyl-1-hexanol	1484	0906	0.69	0.03	–	0.16	0.50	0.04
Alkylaldehydes								
n-hexanal	1082	0780	–	–	–	–	–	0.02
n-nonanal	1379	1082	–	–	–	–	0.04	0.04
(*E*,*Z*)-2,4-heptadienal	1462	1109	3.92	–	–	–	–	–
(*E*,*E*)-2,4-heptadienal	1492	1130	4.05	–	–	–	–	–
(*E*,*E*)-decadienal	1811	1281	1.29	–	–	–	–	–
Alkylketones								
2-hydroxy-3-pentanone	1354	0795	0.17	–	–	–	–	–
α-isophorone[e]	1601	NF	0.16	–	–	–	–	–
2,6,6-trimethyl-2-cyclohexene-1,4-dione[e]	1696	NF	0.37	–	–	–	–	–
(*Z*)-jasmone[d]	1914	1378	1.53	–	2.67	1.29	–	–
Alkylesters								
methyldihydrojasmonate	2297	1681	–	–	–	0.58	–	–
methyl jasmonate[e]	2327	NF	–	–	–	0.58	–	–
3-methylbutyl hexadecanoate	2379	1494	7.70	–	–	–	–	–
Nitrogen Containing Heterocyclic Compounds								
pyridine	1184	0691	–	–	–	2.32	–	0.25
2-methylpyrazine	1265	0871	2.19	–	–	–	–	–
2,5-dimethylpyrazine[d]	1325	0885	3.69	–	–	–	–	–
2,6-dimethylpyrazine	1331	0911	1.05	–	–	–	–	–
2-ethylpyrazine	1335	0917	1.71	–	–	–	–	–
2,3-dimethylpyrazine	1349	0903	0.68	–	–	–	–	–
2,3,6-trimethylpyrazine	1387	0915	–	–	–	–	0.27	–
2-ethyl-6-methylpyrazine	1389	0943	0.88	–	–	–	–	–
2-ethyl-5-methylpyrazine	1396	0967	2.32	–	–	–	–	–
2-ethyl-3-methylpyrazine	1409	1015	1.15	–	–	–	–	–
3-ethyl-2,5-dimethylpyrazine[e]	1433	NF	–	–	–	–	0.08	–
2-vinylpyrazine	1439	NF	0.39	–	–	–	–	–
3-ethyl-2,6-dimethylpyrazine	1442	1091	–	–	–	–	0.04	–
2,6-diethylpyrazine	1443	1146	0.31	–	–	–	–	–
3-ethyl-2,3-dimethylpyrazine	1450	1214	2.63	0.02	–	–	–	–
2,5-diethylpyrazine	1457	1150	0.40	–	–	–	–	–
2,3-diethylpyrazine	1464	1169	0.04	–	–	–	–	–

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Table 1. continued

compd	KI[a]	KI[b]	amount (μg/g sample)					
			mulberry tea		*Camellia* tea		Burea tea	
			leaves	stems	green	fermented	stems	roots
Nitrogen Containing Heterocyclic Compounds								
2-ethenyl-6-methylpyrazine	1488	1210	0.35	–	–	–	–	–
4-acetylpyrazole[e]	1506	NF	–	–	–	–	–	0.02
3,5-dimethyl-2-isobutylpyrazine[e]	1532	NF	0.18	–	–	–	–	–
3-*n*-butylpyridine[e]	1559	NF	–	–	–	–	0.02	–
3-methoxypyridine	1582	1401	–	0.15	–	–	–	–
2-*n*-penytlpyrazine	1625	1224	–	–	–	–	0.43	–
2,3-dimethyl-5-isopentylpyrazine[e]	1664	NF	0.45	–	–	–	–	–
2-acetyl-5-methylpyrazine	1684	1210	0.56	–	–	–	–	–
1-methyl-2-pyrrolidone[d]	1764	0710	–	–	–	–	1.10	0.03
1-pyrrolidinecarboxaldehyde	1766	1010	–	1.81	–	–	–	–
1-piperidinecarboxaldehyde	1786	1112	0.71	0.42	–	–	–	–
2-acetylpyrrole[d]	1953	1062	1.73	1.83	1.53	–	1.86	–
pyrrole-2-carboxaldehyde	2013	1167	0.22	1.63	0.28	1.18	0.34	–
2-methyl-4-quinazolinone[e]	2055	NF	0.39	0.36	–	–	–	–
5-methyl 1H-pyrrole-2-carboxaldehyde[e]	2089	NF	0.47	0.77	–	–	0.29	–
6-pentyl-5,6-dihydro-2H-pyran-2-one[e]	2116	NF	–	0.33	–	–	0.53	0.22
3-acetyl-1-methylpyrrole	2124	1128	–	0.47	–	–	–	–
2-ethyl-3-methylmaleimide[e]	2245	NF	1.72	0.72	1.02	0.83	–	–
Oxygen Containing Heterocyclic Compounds								
5-methyl-2(3H)-furanone	1434	0836	1.31	–	–	–	–	–
furfural	1455	0818	0.03	–	–	–	–	–
2-aceylfuran	1496	0892	2.87	–	–	–	–	–
5-methyl-2-furancarboxaldehyde	1564	0940	0.53	0.06	–	–	–	–
5,5-dimethyl-2(5H)-furanone	1608	0951	–	–	–	–	–	0.14
2-furanmethanol	1645	0853	–	0.08	–	–	–	–
3-furanmethanol[e]	1668	NF	–	0.05-	–	–	–	–
3-methyl-2(5H)-furanone	1710	0930	–	–	–	–	–	0.08
5-ethyl-2(5H)-furanone	1754	0984	1.30	0.10	–	–	0.13	0.14
5-(hydroxymethyl)-2-furancarboxaldehyde	2459	1200	–	0.71	–	–	–	–
dihydro-4-hydroxy-2(3H)-furanone[e]	2573	NF	–	0.34	0.38	0.31	–	–
Aromatic Compounds								
benzaldehyde	1512	1023	0.37	0.06	–	–	–	–
2-phenylacetaldehyde[d]	1611	1041	3.37	0.03	–	–	–	–
1-(3-methylphenyl) ethanone	1777	1150	0.70	0.11	–	–	–	–
guaiacol	1850	1056	2.00	0.41	–	–	–	–
benzyl alcohol[d]	1856	1021	1.88	0.07	4.49	0.39	–	–
phenylethyl alcohol[d]	1888	1097	0.36	0.27	6.56	1.81	0.02	0.36
4-methylguaiacol[e]	1951	NF	–	0.21	–	–	–	0.26
phenol	1969	0956	0.62	0.86	–	–	0.41	0.75
4-ethylguaiacol[e]	2030	NF	–	–	0.39	1.39	0.05	0.15
(*E*)-cinnamic aldehyde	2031	1260	–	1.89	–	–	0.13	0.08
anisaldehyde	2039	1240	–	–	–	–	–	0.03
2-phenoxyethanol	2135	1221	–	0.39	–	–	–	–
eugenol[d]	2154	1344	2.26	0.44	–	–	1.70	0.94
p-vinylguaiacol	2169	1325	0.19	–	1.28	1.74	0.57	0.22
2,5-dihydroxyacetophenone[d]	2217	1255	–	2.25	–	–	–	–
2,4-bis(1,1-dimethylethyl)-phenol[d]	2305	1512	–	–	0.35	0.90	0.71	–
isoeugenyl acetate[e]	2322	NF	–	1.12	–	–	–	–
isoeugenol[d]	2322	1430	–	–	–	–	–	1.02
coumarin	2421	1418	–	–	0.60	0.45	–	–
benzophenone[d]	2440	1613	–	0.42	0.70	0.80	0.57	–
vanillin[d]	2525	1392	–	5.33	–	–	–	0.15
Lactones								
4-valerolactone	1613	0920	–	–	–	–	0.04	0.05
γ-butyrolactone	1628	0885	0.86	–	–	–	–	0.08
lavender lactone	1669	0991	0.54	–	–	–	–	0.06
γ-caprolactone	1702	1003	–	0.20	–	–	0.06	0.25

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Table 1. continued

compd	KI[a]	KI[b]	amount (μg/g sample)					
			mulberry tea		*Camellia* tea		Burea tea	
			leaves	stems	green	fermented	stems	roots
Lactones								
γ-crotonolactone[e]	1749	NF	–	0.05	–	–	–	0.07
γ-butylbutyrolactone[e]	1918	1218	0.73	0.32	–	–	0.10	0.11
δ-octalactone	1972	1234	–	–	–	0.27	–	–
γ-amylbutyrolactone[d]	2015	1320	–	0.22	1.12	–	0.13	0.51
γ-decalactone	2194	1426	–	–	–	0.84	–	–
γ-dodecalactone	2294	1521	–	–	–	–	0.22	0.08
γ-palmitolactone[e]	2857	2120	–	–	–	–	–	8.76
Acids								
n-pentanoic acid	1688	0893	–	0.56	–	–	0.20	0.44
n-hexanoic acid	1787	0986	0.73	19.83	–	–	9.89	13.13
n-heptanoic acid	1873	1074	0.28	3.02	–	–	–	2.05
n-octanoic acid	1974	1180	0.74	1.36	–	–	–	1.74
n-nonanoic acid[d]	2089	1275	2.23	3.04	–	–	–	1.48
(*E*)-2-octenoic acid[e]	2164	NF	0.69	1.45	–	–	0.39	0.94
n-decanoic acid	2195	1380	–	1.73	–	–	0.25	0.97
(*E*)-2-decenoic acid[e]	2275	NF	–	–	–	–	–	0.58
Possible Contaminants								
carbitol	1659	0981	–	–	–	–	–	0.30
diethylene glycol	1955	1053	–	–	–	2.45	–	–
p-cresol	2075	1051	–	–	0.83	0.50	–	–
tri-*n*-butylphosphate[e]	2109	NF	–	0.33	–	–	0.13	–
1,2-diethyl phthalate	2353	1549	–	–	–	–	–	0.60
isobutylphtalate[e]	2509	NF	2.52	0.81	0.89	0.73	0.53	0.61
dibutyl phthalate[e]	2671	NF	3.18	1.90	1.47	2.40	3.61	0.46

[a]Kovats index on DB-WAX column. [b]Kovats index on DB-1 column. NF = not found in DB-1 chromatogram. [c]Not detected or less than 0.01 μg/g. [d]Tested for antioxidant activity. [e]Tentatively identified due to lack of authentic compound or not resolved by DB-1 chromatogram.

RESULTS AND DISCUSSION

Volatile Constituents of the Extracts. The yields of volatile chemicals were 0.36 ± 0.03% and 0.25 ± 0.03% from Mulberry leaves and stems, respectively, 0.15 ± 0.02% and 0.24 ± 0.03% from of *Camellia* green and fermented teas, respectively, and 0.15 ± 0.02% and 0.24 ± 0.03% from Burea plant stems and roots, respectively. Table 1 shows the volatile constituents identified in the present study in the extracts from Mulberry tea (leaves and stems), *Camellia* tea (green and fermented), and Burea plant (stems and roots) along with their Kovats index. A total of 101 compounds were positively identified. In addition, 24 chemicals are listed as tentatively identified because authentic samples were not available; of these, however, over 90% matched with the MS library in the NIST AMDIS version 2.1 software. The chemicals that were found by DB-WAX but not by DB-1were also listed as tentatively identified.

The positively identified volatile chemicals were 12 terpenes and terpenoids, 21 alkyl compounds (6 alcohols, 5 aldehydes, 2 ketones, 2 esters, and 6 acids), 26 nitrogen containing heterocyclic compounds (2 pyridines, 20 pyrazines, 4 pyrroles, and 3 others), 9 oxygen containing heterocyclic compounds (4 furanones and 5 furans), 18 aromatic compounds, 7 lactones, and 8 possible contaminants. No duplicate count was made in the case of chemicals with more than two functional groups.

In the case of the Mulberry tea samples, the number of chemicals found in the extracts was 55 in leaves and 36 in stems. The leaves contained dihydroactinidiolide (9.65 μg/g) in the greatest concentration, followed by (*E*)-β-ionone (9.32 μg/g). Dihydroactinidiolide was also found in *Camellia* tea (8.32 μg/g in green and 4.15 μg/g in fermented tea) and in Burea plant tea (0.40 μg/g in stems). β-Ionone is known as an important flavor chemical used to create various imitation berry flavors.[17] One recent study reported that β-ionone possesses antiproliferative and antioxidant activities.[18] Extracts of stems contained *n*-hexanoic acid (19.83 μg/g) in the greatest concentration, followed by vanillin (5.33 μg/g). *n*-Alkylacids, including *n*-hexanoic acid, were also found in relatively high concentrations. These acids may contribute some bitter taste to teas. Vanillin is known as a major constituent of vanilla bean, and it has been used as a flavor ingredient for daily products.[17] It is also reported as one of the volatile components of tea.[19] A recent report demonstrated the antioxidant activity of vanillin.[20]

In the case of *Camellia* tea samples, the number of chemicals present in the extracts was relatively small: 15 and 20 components were identified in green and fermented teas, respectively. Green tea contained dihydroactinidiolide (8.32 μg/g) in the greatest concentration, followed by phenylethyl alcohol (6.56 μg/g) and benzyl alcohol (4.49 μg/g). These aromatic compounds play an important role in the antioxidant activity of the extracts, which will be discussed below. Fermented tea contained (*E*)-geraniol (7.44 μg/g) in the greatest concentration, followed by dihydroactinidiolide (4.15 μg/g). No important differences of constituents between green and fermented teas were observed.

In the case of the Burea plant samples, the number of chemicals identified in the extracts was 26 in stems and 38 in roots. The stem extract contained *n*-hexanoic acid in the greatest concentration (9.89 μg/g) followed by 2-acetylpyrrole (1.86 μg/g). The root extract contained *n*-hexanoic acid (13.13

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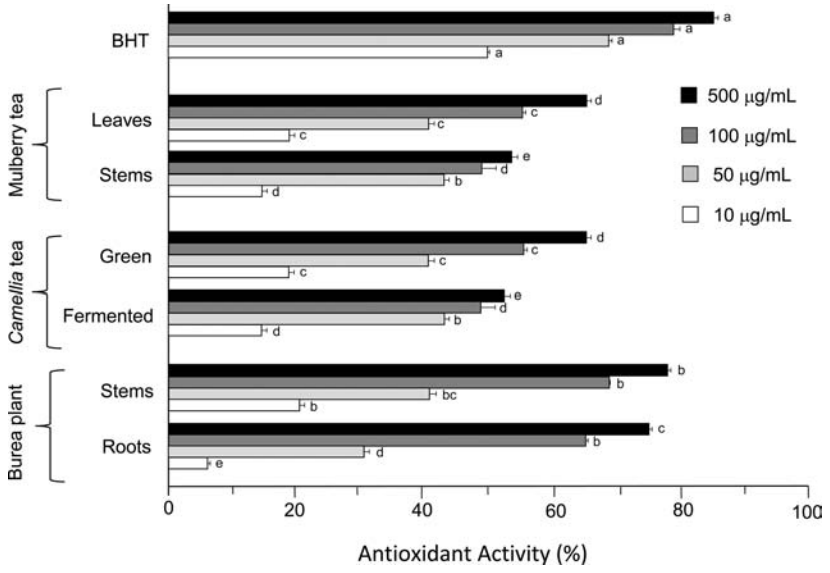

Figure 1. Antioxidant activity of the extracts from the plant samples examined by the aldehydes/carboxylic acid assay. Values are the mean ± SD (n = 3). Letters indicate significant levels computed by Duncan's multiple range test at α = 0.05 after the ANOVA.

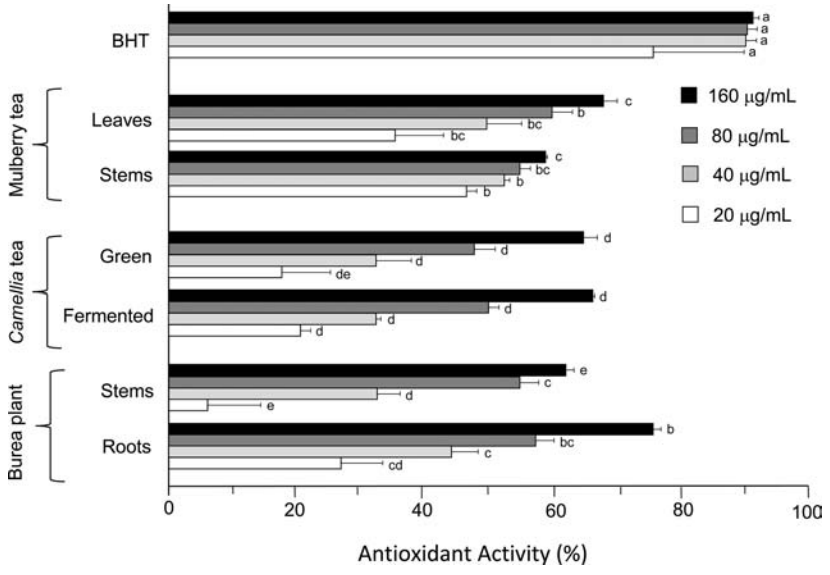

Figure 2. Antioxidant activity of the extracts from the plant samples tested by the MA/GC assay. Letters indicate significant levels computed by Duncan's multiple range test at α = 0.05 after the ANOVA.

μg/g) in the greatest concentration followed by n-heptanoic acid (2.05 μg/g). The largest number of alkylacids was identified in this extract, suggesting that this tea has a relatively bitter taste.

Pyrazines, which possess a toasted or roasted flavor,[17] were present in large numbers, particularly in the extract of tea made from Mulberry leaves. Pyrazines are known to form in foods and beverages by heat treatment via Maillard reaction.[21] However, they are not important constituents from the viewpoint of the antioxidant activity of food or beverage because they do not exhibit appreciable antioxidant activity. On the other hand, pyrrole, such as the 2-acetylpyrrole and pyrrole-2-carboxyaldehyde found in the extracts from samples from Mulberry tea, *Camellia* tea, and Burea plant, exhibited relatively potent antioxidant activity.[22]

Antioxidant Activity of the Extracts and Their Constituents. Figure 1 shows antioxidant activity of the extracts from the plant samples examined by the aldehydes/carboxylic acid assay at various concentrations at the fourth week. Values are mean ± standard deviation (SD) (n = 3). The aldehydes/carboxylic acid assay is convenient for evaluating the effects of antioxidants against slow oxidation phenomena occurring over prolonged periods of time, such as the shelf life of foods.[3] Moreover, the matrix of the assay consists of an organic solvent, dichloromethane, that is easy to apply for water nonsoluble organic compounds, such as volatile chemicals. All extracts exhibited antioxidant activity with dose response. Antioxidant activity at 500 μg/mL ranged from 77.02 ± 0.51% (tea from the stems of Burea plant) to 52.57 ± 0.92% (fermented tea of *Camellia*). The extracts from the stems and roots of the Burea plant showed antioxidant activity comparable to that of BHT at the level of 100 μg/mL. At the level of 50 μg/mL, all extracts exhibited moderate antioxidant activity ranging from 42.85 ± 0.79% stems of Mulberry tea) to 31.13 ± 0.83%

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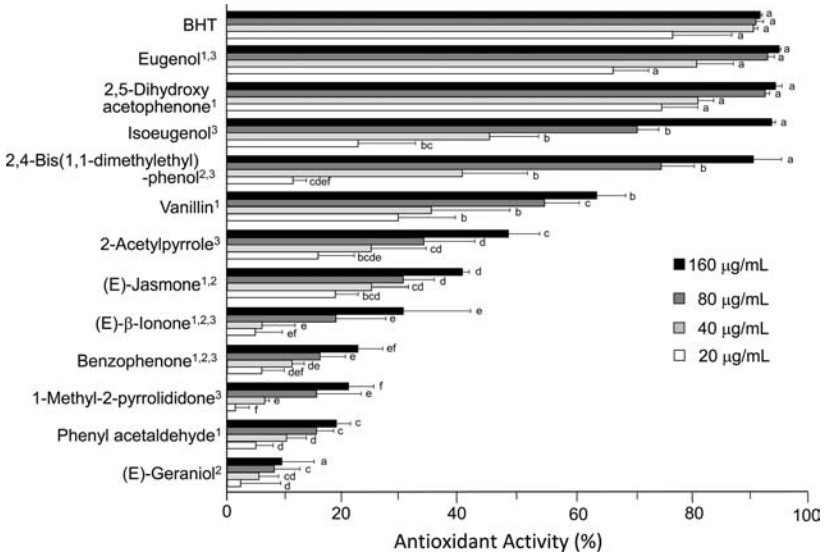

Figure 3. Antioxidant activity of selected chemicals identified in the plant samples examined by the MA/GC assay. Letters indicate significant levels computed by Duncan's multiple range test at $\alpha = 0.05$ after the ANOVA. [1]Found in Mulberry teas. [2]Found in *Camellia* teas. [3]Found in Burea plant teas.

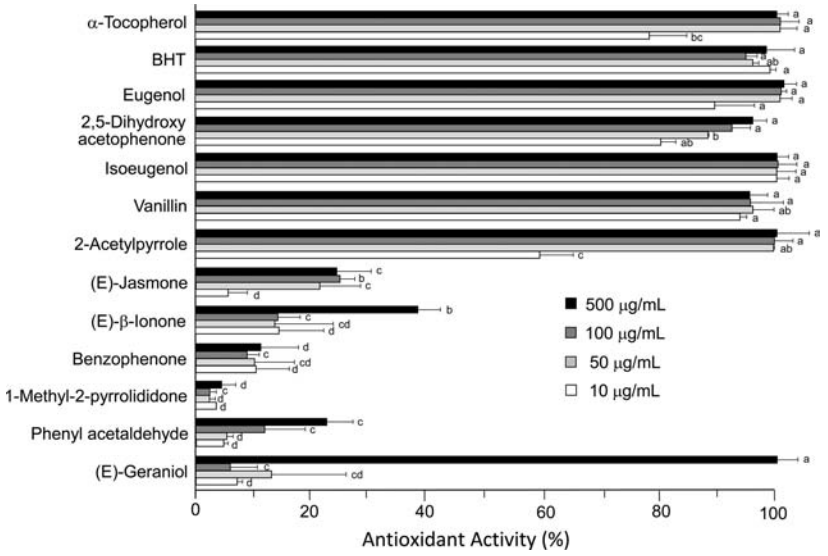

Figure 4. Antioxidant activity of selected chemicals identified in the plant samples examined by the aldehyde/carboxylic acid assay. Letters indicate significant levels computed by Duncan's multiple range test at $\alpha = 0.05$ after the ANOVA.

(roots of Burea plant). Even at the 10 μg/mL level, all extracts showed slight antioxidant activity ranging from 20.05 ± 0.73% (stems of Burea plant) to 5.05 ± 0.49% (roots of Bureau plant).

Figure 2 shows the antioxidant activity of the extracts from the same plant samples tested by the MA/GC assay. Values are mean ± SD ($n = 3$). This assay involves analysis of a secondary lipid peroxidation product, MA, which is formed at the last stage of lipid peroxidation.[3] In addition, this assay can monitor how much antioxidant inhibits the formation of genotoxic MA. All extracts exhibited antioxidant activity with a clear dose response. Antioxidant activity at 160 μg/mL (the highest concentration tested) ranged from 76.17 ± 0.27% (roots of Burea plant) to 59.32 ± 0.27% (stems of Mulberry tea). Even at the level of 20 μg/mL, extract from the Mulberry teas made from stems (47.29 ± 1.59%) and leaves (36.18 ± 7.50%) showed moderate antioxidant activity. Previous reports also indicated the presence of antioxidants in extracts from Mulberry,[23] *Camellia sinensis*,[13] and *Cudrania tricuspidata*.[14] These reports and the results from the present study suggest that some antioxidant constituents were present in these extracts.

Antioxidant Activity of Chemicals Identified in the Extracts. Figure 3 shows the antioxidant activity of chemicals identified in the extracts from plant samples examined by the MA/GC assay. Values are mean ± SD ($n = 3$). All chemicals showed antioxidant activity with a dose response. Eugenol and 2,5-dihydroxy acetophenone showed comparable activities to those of BHT at all levels tested. At the level of 160 μg/mL (the highest level tested), the antioxidant activity of chemicals obtained was 95.17 ± 0.27% from eugenol, 95.03 ± 1.06% from 2,5-dihydroxy acetophenone, 94.00 ± 0.59% from isoeugenol, and 91.22 ± 4.74% from 2,4-bis(1,1-dimethylethyl)phenol. At the same level, the antioxidant activity of the rest of the chemicals tested ranged from 63.89 ± 4.91% (vanillin) to 9.33

± 5.34% [(*E*)-geraniol]. Figure 4 shows the antioxidant activity of those chemicals tested by the aldehydes/carboxylic acid assay. Values are mean ± SD (n = 3). Eugenol, 2,5-dihydroxy acetophenone, isoeugenol, vanillin, and 2-acetylpyrrole exhibited comparable activity to that of α-tocopherol and BHT. All these chemicals showed almost 100% antioxidant activity at the level of 500 μg/mL. Vanillin and 2-acetylpyrrole exhibited over 90% activity at all levels tested except at the level of 10 μg/mL, whereas they exhibited moderate activity (63.89 ± 1.10% and 48.83 ± 5.34%, respectively) in the MA/GC assay. The antioxidant activity of 2-acetylpyrrole, which is a Maillard reaction product, has been reported previously.[22]

Generally phenol derivatives, such as eugenol and vanillin, exhibited strong antioxidant activity. The other nonphenolic compounds, including jasmone, β-ionone, benzophenone, 1-methyl-2-pyrrolidone, and phenyl acetaldehyde showed no appreciable or slight antioxidant activity. It is interesting that geraniol (monoterpene) inhibited hexanal oxidation over 28 days at the level of 500 μg/mL. On the other hand, it did not show appreciable inhibitory activity toward MA formation. This may be due to involvement of various reactive oxygen species in the case of the MA/GC assay, in contrast to only hydroxyl radical involvement in the case of the aldehydes/carboxylic acid assay. Antioxidant activity of terpenes found in natural plants, including α- and β-terpinene and β-terpinolene, has been reported previously.[6] The chemicals tested in the present study are so-called aroma or volatile chemicals present in plant essential oils, including various tea leaves. They are proposed to contribute to the antioxidant activity of essential oils.[7]

The results obtained in the present study demonstrate the wide range of antioxidant activities of the plant extracts. However, it is difficult to pinpoint which constituents give antioxidative activities to the extracts from these plants because over 1000 components are present in plant extracts either as high concentration major components or as minor trace components.[1] Although the activities of these constituents are not as strong as the known natural antioxidants such as α-tocopherol, the total activity of these compounds combined might be comparable to those of known antioxidants. Also, synergism and antagonism phenomena are important in evaluating the antioxidant activities of plant extracts. There are of course many factors involved in the antioxidant activity of plant extracts. Therefore, more investigations of plant extracts with the ultimate goal of maximizing their use as pharmacological products are in order. Determination of the antioxidant activity of medicinal plants' components is one avenue through which we can learn more about their biological activities and increase the range of alternative natural medicines.

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Notes

The authors declare no competing financial interest.

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