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Antioxidative Activities of Fractions Obtained from Brewed Coffee

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The antioxidative activity of column chromatographic fractions obtained from brewed coffee was investigated to find antioxidants and to assess the benefit of coffee drinking. The dichloromethane extract inhibited hexanal oxidation by 100 and 50% for 15 days and 30 days, respectively, at the level of 5 μg/mL. A GC/MS analysis of fractions, which exhibited oxidative activity, revealed the presence of antioxidative heterocyclic compounds including furans, pyrroles, and maltol. The residual aqueous solution exhibited slight antioxidative activity. The inhibitory activity (%) of the seven fractions from an aqueous solution toward malonaldehde formation from lipid oxidation ranged from 10 to 90 at a level of 300 μg/mL. The results indicate that brewed coffee contains many antioxidants and consumption of antioxidant-rich brewed coffee may inhibit diseases caused by oxidative damages.

KEYWORDS: Antioxidants; brewed coffee; coffee volatiles; heterocyclic compounds

INTRODUCTION

Since the discovery of coffee around the 6th century by Abyssinian/Ethiopian shepherds in the province of Keffa (from which the name *coffee* is derived), it was gradually spread over the world. By the 13th century, coffee's restorative powers were known and spread across the Islamic world, and later on to the rest of the globe. By the late 1500's the first traders were selling coffee in Europe, thus introducing the new beverage into Western life and custom. It was during the period 1740−1805 that coffee growing reached its peak in Central and South America, and now coffee has become one of the most popular beverages in the world.

Most studies on coffee associated with human health have focused on the negative aspects, such as toxicity of caffeine (*1*, *2*). Because coffee has been widely consumed for many centuries, there must be some positive effects, such as an antioxidative effect, on human health. Antioxidants are known to prevent many diseases, including cancer (*3*, *4*), atherosclerosis (*5*−*7*); antioxidants also effect the process of aging (*8*−*10*).

Antioxidants are widely present in natural plants, such as fruits and vegetables, herbs, beans, and tea and coffee. In addition, antioxidative compounds in processed foods and beverages, including coffee, have been reported many times in the literature (*11*−*15*). Formation of these antioxidants in processed foods and beverages is most likely related to the Maillard reaction.

High molecular weight substances, such as melanoidins, produced from a sugar/amino acid model system by the Maillard reaction significantly inhibited oxidation of lipids (*16*). Also, high molecular weight Maillard reaction products obtained from various browning model systems, including a glucose−glycine system (*17*), pregelatinized starch-, glucose-, or soybean oil-lysine systems(*18*), a lactose−lysine system (*19*), and casein−sugar mixture systems (*20*)—exhibited potent antioxidative activities. Higher mass fractions obtained from green and roasted coffee contained certain antioxidants (*21*). Coffee melanoidins exhibited strong antioxidative activity, which was exceeded by low molecular weight phenolcarbonic acids (*22*).

Recently, low molecular weight volatile compounds, particularly heterocyclic compounds, obtained from the Maillard reaction model systems have begun to receive much attention as antioxidants (*23*, *24*). Many heterocyclic compounds, which are major flavor compounds formed by the Maillard reaction, have been reported in various processed foods and beverages (*25*). For example, over 300 heterocyclic compounds, including pyrroles, oxazoles, furans, thiazoles, thiophenes, imidazoles, and pyrazines, were identified and reported in brewed coffee (*26*−*28*). Among the heterocyclic compounds found in coffee volatiles, pyrrols and furans showed the strongest antioxidative activity, which was almost equal to that of α-tocopherol (*29*, *30*).

In the present study, the antioxidative activity of column chromatographic fractions obtained from brewed coffee by silica gel column chromatography was investigated in order to find antioxidants, as well as to assess the benefit of coffee drinking.

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MATERIALS AND METHODS

Chemicals and Coffee Samples. Ground roasted coffee was bought from a local market. Hexanal, undecan, *N*-methylhydrazine (NMH), 2-methylpyrazine, and sodium dodecyl sulfate (SDS) were purchased from Adrich Chemical Co. (Milwaukee, WI). Butylated hydroxytoluene (BHT), trizma hydroxhloride, trizma base, and cod liver oil were bought from Sigma Chemical Co. (St. Louis, MO). All authentic samples were obtained from reliable commercial sources or a gift from TAKATA KORYO Co., Ltd., (Osaka, Japan).

Sample Preparation for Dichloromethane Extract. Ground roasted coffee (25 g) was brewed with 300 mL of distilled water. After 80 mL of a saturated NaCl solution was added to the freshly brewed coffee, it was extracted with 130 mL of dichloromethane using a liquid−liquid continuous extractor for 6 h. The residual aqueous solution was stored at 5 °C. The extract was dried over anhydrous sodium sulfate for 12 h. After removal of the sodium sulfate from the extract, the extract was concentrated using a rotary evaporator, and subsequently further concentrated under a nitrogen stream to 2 mL.

Fractionation of Extract. The concentrated sample was placed in a glass column (15-cm × 1-cm i.d.) packed with 160−200 mesh silica gel (J. T. Baker, Inc., Phillipsburg, NJ) and eluted sequentially with a 100-mL solvent mixture of different ratios of pentane/ethyl acetate−100/0 (Fraction I), 95/5 (II), 80/20 (III), 50/50 (IV), 20/80 (V), 0/100 (VI), and finally with 200 mL of acetone (Fraction VII)−into seven fractions. Each fraction was concentrated to a final volume of 2 mL by distillation and stored at 5 °C for subsequent experiments. The experiment was replicated four times. These experiments were simultaneously performed with controls that contained aldehydes and a GC internal standard only.

Antioxidation Test on Fractions from Extract. Antioxidative activity of the samples was tested using their inhibitory effect toward conversion of aldehyde to acid (aldehyde/carboxylic acid assay) (*31*). Various amounts of whole coffee extract were added to a 2 mL dichloromethane solution of hexanal (10 mg/mL) containing undecan (200 mL) as a gas chromatographic internal standard. The oxidation of the sample solution was initiated by heating at 60 °C for 1 min. The decrease in aldehyde was monitored at 5-day time intervals for 40 days using gas chromatography.

Identification of Volatile Chemicals in the Fractions from Dichlormethane Extract. Volatile chemicals obtained from the fractions from dichloromethane extract of a brewed coffee were identified by comparison with the Kovats gas chromatographic retention index *I* (*32*) and by the MS fragmentation pattern of each component compared with those of authentic chemicals. The concentration of each chemical was calculated using the following equation (*33*):

$$\text{concentration (ppm)} = \frac{\text{weight of fraction (without solvent)} \times \text{GC peak area \%/100 } (\mu\text{g})}{\text{weight of brewed coffee (25 g)}}$$

An HP model 6890 GC interfaced to an HP 5791A mass selective detector (GC/MS) was used for mass spectral identification of the GC components at MS ionization voltage of 70 eV. A 30-m × 0.25-mm i.d. (d_f = 0.25 μm) DB-WAX bonded-phase fused-silica capillary column (J & W Scientific, Folsom, CA) was used for a GC. The linear velocity of the helium carrier gas was 30 cm/sec. The injector and the detector temperatures were 250 °C. The oven temperature was programmed from 50 to 180 °C at 3 °C/min and held for 40 min.

Fractionation of Residual Aqueous Solution from Brewed Coffee. The residual aqueous solution from dichloromethane extraction of brewed coffee was concentrated by a rotary evaporator. A dark brown paste was obtained. The paste was placed in a glass column (40-cm × 4.5-cm i.d.) packed with Amberlite XA-2 resin (Aldrich) and fractionated sequentially with a 1000-mL solvent mixture of different ratios of water/methanol−100/0 (Fraction I), 95/5 (II), 80/20 (III), 50/50 (IV), 20/80 (V), 0/100 VI), and finally with 1000 mL acetone (Fraction VII)−into seven fractions. Each fraction was concentrated to a brown paste using a rotary evaporator. A corresponding ratio of water/methanol was added to each fraction to make up a 10 mg/mL concentration.

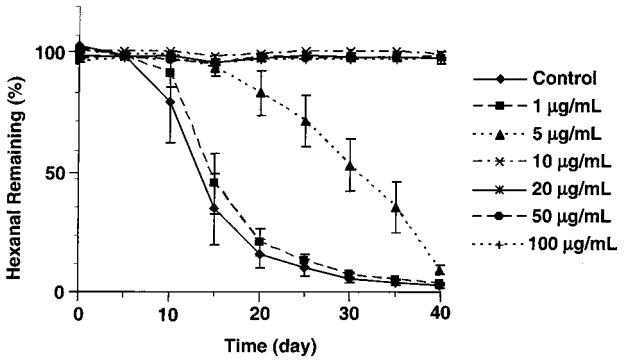

Figure 1. Results of antioxidation tests on dichloromethane extract of brewed coffee.

Antioxidation Tests on Fractions from Residual Aqueous Solutions from Brewed Coffee. The antioxidative activities of Fractions I−VII were evaluated by analyzing malondialdehyde (MA) formed from cod liver oil upon oxidation by the method previously reported (MA/lipid assay) (*34*, *35*). An aqueous solution (5 mL) containing 10 μL of cod liver oil, 0.25 mmol of trizma buffer (pH 7.4), 5 μmol of ferrous chloride, 0.5 μmol of hydrogen peroxide, 0.75 mmol of potassium chloride, and 0.2% of surfactant SDS were incubated with various amounts of each Fraction for 17 h at 37 °C in a 20-mL test tube. The oxidation of samples 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. MA formed in the samples was derivatized to 1-methylpyrazole (1-MP) with *N*-methylhydrazine (NMH). Each reaction solution was extracted with 10 mL of dichloromethane using a liquid−liquid continuous extractor for 3 h. The solution was saturated with NaCl prior to extraction to prevent the formation of an emulsion. The extract was adjusted to exactly 10 mL by adding dichloromethane and 20 μL of a 2-methylpyrazine solution as a GC internal standard. BHT (1 μg/mL) was used for positive control. To avoid damage to the nitrogen−phosphorus detector (NPD), the solvent, dichloromethane, in the adjusted solution was removed under a purified nitrogen stream, and then the volume was adjusted to exactly 10 mL with ethyl acetate. The solution was analyzed for 1-MP by a GC with an NPD.

Quantitative Analysis of Hexanal and MA. The GC internal standard method was used (*36*). A Hewlett-Packard (HP) model 6890 GC equipped with a 30-m × 0.25-mm i.d. DB-1 bonded-phase fused silica capillary column (J & W Scientific, Folsom, CA) and a flame ionization detector was used to monitor the relative amounts of hexanal in the samples. The linear velocity of the helium carrier gas was 34 cm/s. The injector and detector temperatures were 300 and 280 °C, respectively. The oven temperature was programmed from 40 to 180 °C at 8 °C /min.

An HP model 6890 GC equipped with a 30-m × 0.25-mm i.d. (d_f = 0.25 μm) DB-WAX bonded-phase fused-silica capillary column (J & W Scientific, Folsom, CA) and an NPD was used for analysis of MA as 1-MP. The linear velocity of the helium carrier gas was 30 cm/sec. The injector and the detector temperatures were 250 °C. The oven temperature was programmed from 50 to 180 °C at 3 °C/min and held for 40 min.

RESULTS AND DISCUSSION

Antioxidative Activities of Dichloromethane Extract from Brewed Coffee. These samples were water insoluble. Therefore, a testing system in an organic matrix (aldehyde/carboxylic acid asssay) was used. **Figure 1** shows the results of antioxidation tests on dichloromethane extract of brewed coffee. Values are mean ± SD (n = 4). The activity exhibited dose−response. The samples in which the concentrations were higher than 10 μg/mL inhibited hexanal oxidation by 100% over 40 days. The extract from brewed coffee exhibited potent antioxidative activity at the level of 10 μg/mL. The coffee extract inhibited

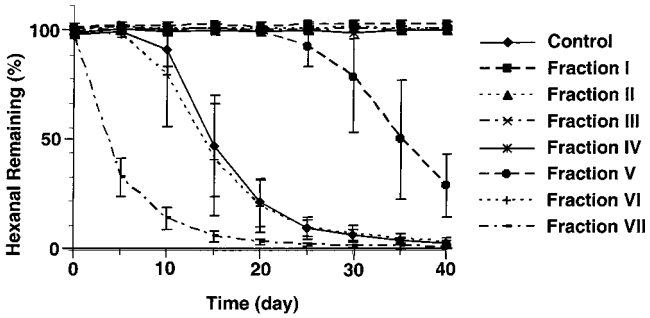

Figure 2. Results of antioxidation tests on fractions obtained from a dichloromethane extract of brewed coffee.

hexanal oxidation by 100 and 50% for 15 days and 30 days, respectively, at the level of 5 μg/mL; however, it did not show appreciable activity at the level of 1 μg/mL.

Antioxidative Activities of Fractions from Dichloromethane Extract. Figure 2 shows the results of the antioxidation tests on fractions obtained from a dichloromethane extract of brewed coffee. Values are mean ± SD ($n = 4$). Each fraction was tested at a level of 100 μg/mL. Fractions I, II, III, and IV inhibited hexanal oxidation by 100% over 40 days at a level of 100 μg/mL. Fraction V inhibited hexanal oxidation by 100% up to 20 days, and then its inhibition activity reduced gradually to 20% at 40 days. Fraction VI exhibited the same activity curve as that of the control, suggesting that this fraction did not have any active chemicals. Fraction VII showed certain pro-oxidative activity. Fraction VII oxidized hexanal by 75% in 5 days at a level of 100 μg/mL, while the hexanal in the control sample remained at 100% for 5 days. Fraction VII oxidized hexanal to hexanoic acid by almost 100% in 20 days at a level of 100 μg/mL. Even though one fraction contained some oxidants, the whole extract still exhibited strong antioxidative activity (**Figure 1**).

Because Fractions I, II, III, and IV exhibited potent antioxidative activities, each fraction was analyzed for volatile constituents by GC/MS to pinpoint antioxidant chemicals. **Table 1** shows that major volatile compounds identified in Fractions I, II, III, and IV exhibited relatively high antioxidative activities. Fraction I contained furan derivatives as major constituents. A previous report indicates that unsubstituted furan inhibited hexanal oxidation by 80% over 40 days at a level of 500 μg/mL, while furfural inhibited hexanal oxidation by 50% over 40 days at the same level (*29*). Addition of electron-withdrawing groups, such as formyl or acetyl groups, to the furan ring decreased antioxidative activity (*30*). Therefore, antioxidative activity of furfuryl acetate may be weaker than that of unsaturated furan.

Furfuryl alcohol composed approximately 40% of the total volatiles of Fraction II. As mentioned above, various furan derivatives exhibited antioxidative activity. Therefore, the antioxidative activity of Fraction II may be due mainly to the presence of furfuryl alcohol. Pyrroles are also known to possess the highest antioxidative activity among heterocyclic compounds (*30*). For example, unsubstituted pyrrole and 1-methylpyrrole inhibited hexanal oxidation by 100% over 40 days at a level of 50 μg/mL. Pyrrole-2-carboxaldehyde, which was also found in Fraction II, inhibited hexnal oxidation by almost 100% over 40 days at a level of 20 μg/mL (*30*). A series of alkylpyrazines were identified in Fraction II; however, pyrazines possessed only slight antioxidative activity. Unsubstituted pyrazine inhibited hexanal oxidation by only 20% over 40 days at a level of 500 μg/mL (*30*). Pyrazines are very important flavor chemicals, which give a pleasant toasted or roasted flavor to heat-treated foods and beverages, including brewed coffee. However, they seem not to contribute antioxidative activity to brewed coffee.

Table 1. Major Volatile Compounds Identified in Each Fraction

compound	Kovats index	concn (ppm)
Fraction I		
p-xylene	1149	0.91
furfural	1469	6.35
furfuryl acetate	1541	8.40
5-methylfurfural	1579	7.18
Fraction II		
methylpyrazine	1276	1.88
3-hydroxyl-2-butanone	1292	0.19
2,5-dimethylpyrazine	1333	1.15
2,6-dimethylpyrazine	1339	1.19
2,3-dimethylpyrazine	1357	0.28
2-ethyl-6-methylpyrazine	1415	0.65
2-ethyl-5-methylpyrazine	1453	0.10
3-ethyl-2,5-dimethylpyrazine	1456	0.41
1-(acetoxy)-2-propanone	1469	4.23
1-(2-furanyl)-ethanone	1511	0.97
1-acetyloxy-2-butanone	1536	0.68
5-methylfurfural	1580	2.85
furfuryl alcohol	1669	40.60
1-(1H-pyrrole-2-yl)-ethanone	1983	2.30
phenol	2012	1.12
1H-pyrrole-2-carboxaldehyde	2036	2.99
1-methyl-1H-pyrrole-2-carboxaldehyde	2112	1.06
Fraction III		
2-butanol	1030	0.80
2-methyl-1-propanol	1094	1.58
methylpyrazine	1276	0.66
1-hydroxy-2-propanone	1307	1.42
γ-butyrolactone	1640	8.84
maltol	1981	10.16
2,5-dimethyl-4-hydroxy-3(2H)-furan	2042	3.99
Fraction IV		
2-methyl-1-propanol	1094	5.92
pyridine	1193	2.43
acetic acid	1455	20.10
maltol	1985	8.68
1-butyl-2-pyrrolidione	2236	7.43

Fraction III contained maltol (10.16 ppm), of which antioxidative activity was previously reported. Maltol inhibited hexanal oxidation by 100% at levels higher than 250 μg/mL (*15*). 2,5-Dimethyl-4-hydroxy-3(2H)-furan (DMHF, 3.99 ppm) was found in Fraction III. DMHF inhibited hexanal oxidation by 20% at a level of 500 μg/mL over 30 days (29). Also, 4 mmol of DMHF inhibited malonaldehyde formation from blood plasma (1 μL/mL) by 60% (37). DMHF was first found in nonenzymatic browning reaction products in the early 1960s (*38*). Later it was found in various Maillard reaction products and characterized as a flavor chemical, which gives a characteristic caramel-like flavor to heat-processed foods and beverages, including coffee (*39*). Acetic acid was the major component of this fraction; however, acetic acid has neither anti- nor pro-oxidative activities.

Fraction IV also contained a high level of maltol (8.68 ppm), which may contribute to the antioxidative activity of this fraction. Pyridine (2.43 ppm) and 1-butyl-2-pyrrolidione (7.43 ppm) were identified in this fraction. Pyridine alone did not show appreciable antioxidative activity at a level of 100 μg/mL. However, 50 μg/mL *N*-methylpyrrole with 50 μg/mL pyridine inhibited oxidation of pentanal for more than 80 days, whereas 100 μg/mL *N*-methylpyrrole alone inhibited it only up to approximately 25 days (*31*). The results suggest a synergistic effect of pyridine with pyrroles. Therefore, this phenomenon may have occurred in this sample.

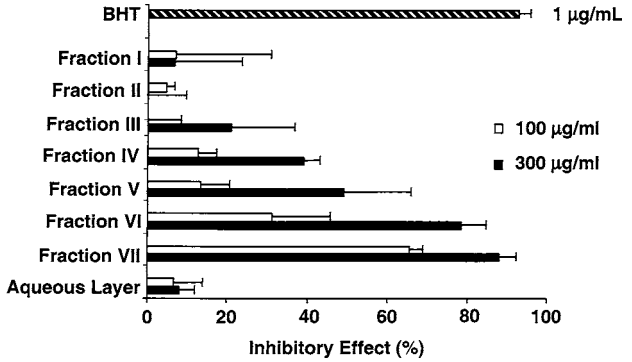

Figure 3. Results of antioxidation tests on residual aqueous solution of brewed coffee and its column chromatographic fractions.

Antioxidative Activities of Fractions Obtained from Residual Aqueous Solutions of Brewed Coffee. The residual aqueous solutions obtained from dichloromethane extraction of brewed coffee were examined for antioxidative activity using an aqueous testing system (MA/lipid assay). **Figure 3** shows the results of antioxidation tests on a residual aqueous solution of brewed coffee and its column chromatographic fractions. The effect of the antioxidant (%) was calculated using the following equation.

Values are mean ± SD ($n = 4$). Standard antioxidant, BHT, inhibited MA formation by over 95% at a level of 1 μg/mL. It is interesting that the fractions eluted with higher methanol concentrations (Fractions VI > V > IV > III > II > I) exhibited higher antioxidative activities. Fractions IV, V, VI, and VII exhibited clear antioxidative activities, but Fraction VII (100% acetone eluate) showed the most potent activity (90%) at a level of 300 μg/mL. Fractions IV, V, VI inhibited MA formation by 40%, 50%, and 75%, respectively, at a level of 300 μg/mL. The results suggest that there are some antioxidants, which were not extracted with dichloromethane, in the residual aqueous solution of brewed coffee. Some less volatile antioxidants, such as polyphenols and isoflavonoids, have been found in coffee (*40*). Polyphenols and some isoflavonoids are water soluble; therefore, these antioxidants must be present in the residual aqueous solution. They may also be more soluble in methanol than in water. A whole aqueous solution before fractionation exhibited only slight antioxidative activity (10% at 300 μg/mL). This is reasonable because antioxidants would be present in higher concentrations in fractions than in a whole aqueous solution.

Antioxidative compounds such as chlorogenic acid, caffeic acid, quinic acid, and furulic acid have been reported in coffee beans (*41*). Those phenolic acids were found in greater amounts in green coffee beans and were present in significantly decreased amounts, up to 50−90%, in roasted coffee (*42*). The overall antioxidative activity of brewed coffee is due not only to the presence of phenolic acids but also, more importantly, to the presence of heterocyclic Maillard reaction products generated during brewing (*43*).

The results from the present study indicate that brewed coffee contains many antioxidants including some heterocyclic compounds. Although the activity of each component is not as strong as the known antioxidant BHT, a total activity of numerous compounds in brewed coffee might be comparable to those of known antioxidants. Therefore, consumption of antioxidant-rich brewed coffee may prevent diseases caused by oxidative damage.

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