Study on the Components of Luobuma with Peroxynitrite-Scavenging Activity

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The origin of the antioxidant activity of Luobuma aqueous extract was examined by measuring the peroxynitrite (ONOO⁻)-eliminating activities of fractions of this extract obtained by Sephadex LH-20 column chromatography. Three of the four fractions obtained, i.e., those excluding the H₂O-eluted fraction, were found to possess ONOO⁻-eliminating activity. These three fractions were combined and fractionated again by Sephadex LH-20 column chromatography, which yielded five fractions. Seven different compounds were isolated from two of these five fractions with high activity. Epigallocatechin-(4 β -8)-epicatechin showed the highest ONOO⁻-eliminating activity.

Key words Luobuma; leaf; peroxynitrite; epigallocatechin-(4 β -8)-epicatechin

It is known that antioxidative enzymes and antioxidants existing in the living body inhibit oxidative injury caused by excessive active oxygen produced under oxidative stress. Excessive oxidative stress may overwhelm the defense mechanisms of the body and, in this context, the role of antioxidants is important.^{1–3)} Therefore, considerable attention has been focused on antioxidants. Synthetic compounds, such as *tert*-butylated hydroxytoluene and *tert*-butylated hydroxyanisole, have been available, while natural antioxidants, such as tocopherol, also have a long history of wide use.⁴⁾ From the viewpoint of safety and efficacy, however, new natural antioxidants have been sought for extensively.

Currently known water-soluble radical scavengers include vitamin C, uric acid and bilirubin, whereas fat-soluble radical scavenger antioxidants include vitamin E, ubiquinol, β -carotene and probucol.^{5–7)} We have been studying the antioxidant activities of medicinal and edible plants^{8–10)} and found previously that Luobuma leaf extract has antioxidant activity.^{11–13)}

Luobuma is a wild plant that grows ubiquitously in the middle to northwestern parts of China. Its leaves have been used as tea leaves for several hundred years in a certain area of China and the tea has been winning popularity among Chinese people as a readily available tea for everyday use. Luobuma leaves are rich in ash elements and minerals, such as Ca, iron, and Na, but differ from green tea leaves in that they contain no caffeine, which has aroused the interest of many researchers regarding their pharmacologic action. In this study, we investigated the origin of the antioxidant activity found in aqueous extract of Luobuma by determining the peroxynitrite (ONOO⁻)-eliminating activities of fractions of this extract.

MATERIALS AND METHODS

General Melting points were measured on a Yanaco micro melting point apparatus and uncorrected values are presented. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. NMR spectra were recorded on a JEOL A-400 spectrometer. Chemical shifts are reported as δ (ppm) with tetramethylsilane (TMS) as the internal standard.

Column chromatography was performed with Sephadex LH-20 (25–100 μ , Pharmacia Fine Chemical Co. Ltd.), MCI-gel CHP 20P (75–150 μ , Mitsubishi Chemical Industries Ltd.) and YMC-gel ODS-A120 (230–400 mesh, YMC Co. Ltd.). TLC was performed on precoated Kieselgel 60 F₂₅₄ plates (0.2 mm, Merck) and spots were detected by spraying with FeCl₃ and anisaldehyde-sulfuric acid reagent.

Reagent ONOO⁻ was synthesized in a quenched flow reactor, as described previously.¹⁴⁾

Extraction and Isolation As shown in Fig. 1, the roasted leaves of *Apocynum venetum* (1.5 kg) were extracted twice with H₂O (15 l) at 80 °C for 2 h. The two extracts were combined and shaken with CHCl₃. The H₂O-layer was concentrated under reduced pressure and subjected to chromatography through a Sephadex LH-20 column. Elution with H₂O yielded a fraction consisting mainly of sugars, fraction 1 (400 g). Further elution successively with 50% MeOH, absolute MeOH, and 50% aqueous Me₂CO yielded three fractions containing polyphenols, fractions 2 (25 g), 3 (16 g), and 4 (12 g). These three fractions were combined and subsequently chromatographed through Sephadex LH-20 with EtOH diluted with increasing amounts of water and finally with 50% aqueous Me₂CO, which yielded five fractions, 2-1 (6.7 g), 2-2 (0.45 g), 2-3 (15.0 g), 2-4 (3.5 g), and 2-5 (24.0 g). Fraction 2-3 was chromatographed repeatedly through MCI-gel CHP 20P [H₂O–MeOH (1:0→0:1)], Sephadex LH-20 (70% MeOH) and YMC-gel ODS-A120 [H₂O–MeOH (1:0→0:1)] to furnish ($\pm$)-gallocatechin (290 mg), (–)-epigallocatechin (210 mg), ($\pm$)-catechin (50 mg) and (–)-epicatechin (200 mg). Repeated chromatography of fraction 2-4 with MCI-gel CHP 20P [H₂O–MeOH (1:0→0:1)], Sephadex LH-20 (70% MeOH) and YMC-gel ODS-A120 [H₂O–MeOH (1:0→0:1)] gave epicatechin-(4 β -8)-gallocatechin (26 mg), epigallocatechin (4 β -8)-epicatechin (30 mg) and procyanidin B-2 (32 mg). The structures of these compounds (Fig. 2) were confirmed by comparing their physical and spectral data with those of authentic samples or with those reported in the literature. Fraction 2-5 contained high-molecular-weight condensed tannins and was fractionated into three fractions by Sephadex LH-20 chromatography with EtOH diluted with increasing amounts of water and fi-

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Fig. 1

Fig. 2

nally with 50% aqueous Me_2CO . Each of these three fractions was subsequently chromatographed through MCI-gel CHP 20P [$\text{H}_2\text{O-MeOH}$ (1:0 $\rightarrow$ 0:1)] yielding fractions 2-5-1 (1.2 g), 2-5-2 (5.7 g), and 2-5-3 (16 g).

Thiolytic Degradation of Fraction 2-5-3 A mixture of fraction 2-5-3 (1.06 g), benzylmercaptan (8 ml), HOAc (8 ml) and EtOH (40 ml) was refluxed for 60 h with stirring. The reaction mixture was concentrated under reduced pressure to give an oily residue, which was chromatographed through Sephadex LH-20. Elution with Me_2CO afforded the thioether, which was subsequently chromatographed through Sephadex LH-20 (80% MeOH) and MCI-gel CHP 20P [$\text{H}_2\text{O-MeOH}$ (3:2 $\rightarrow$ 0:1)] to give epigallocatechin and gallocatechin 4-

thioether (280 mg), and epicatechin and catechin 4-thioether (60 mg). Further elution with Me_2CO gave the corresponding flavan-3-ols, which were subsequently chromatographed through MCI-gel CHP 20P [$\text{H}_2\text{O-MeOH}$ (1:0 $\rightarrow$ 1:1)] to furnish (–)-epigallocatechin (35 mg), (±)-gallocatechin (6 mg), (–)-epicatechin (16 mg) and (±)-catechin (4 mg).

Desulfurization of Thioether Epigallocatechin and gallocatechin 4-thioether (150 mg), and epicatechin and catechin 4-thioether (50 mg) were separately dissolved in 10% HOAc–EtOH (10 and 5 ml, respectively), and treated with an EtOH slurry of Raney-nickel (W-4) for 1.5 h. After removal of the catalyst by filtration, the filtrate was concentrated under reduced pressure and the residue was chromatographed through Sephadex LH-20 (EtOH) and MCI-gel CHP 20P [$\text{H}_2\text{O-MeOH}$ (1:0 $\rightarrow$ 1:1)] to afford (–)-epigallocatechin (63 mg), (±)-gallocatechin (13 mg), (–)-epicatechin (17 mg) and (±)-catechin (5 mg).

Physical and Spectral Data (±)-Gallocatechin: Colorless needles (from H_2O); mp $195\text{--}197^\circ\text{C}$; $[\alpha]_D^{16} 0^\circ$ ($c=0.5$, acetone); $^1\text{H-NMR}$ (acetone- d_6 + D_2O) δ 2.51 (1H, dd, $J=8.5$, 16 Hz, H-4), 2.89 (1H, dd, $J=5.5$, 16 Hz, H-4), 3.96 (1H, ddd, $J=5.5$, 8, 8.5 Hz H-3), 4.47 (1H, d, $J=8$ Hz, H-2), 5.87, 6.02 (each 1H, d, $J=2.5$ Hz, H-6 and 8), 6.47 (2H, s, H-2', 6').

(–)-Epigallocatechin: Colorless needles (from H_2O); mp $215\text{--}218^\circ\text{C}$; $[\alpha]_D^{16} -42.1^\circ$ ($c=0.4$, acetone); $^1\text{H-NMR}$ (acetone- d_6 + D_2O) δ 2.72 (1H, dd, $J=3.5$, 16.5 Hz, H-4), 2.83 (1H, dd, $J=4.5$, 16.5 Hz, H-4), 4.18 (1H, brs, H-3), 4.88 (1H, s, H-2), 5.92, 6.02 (each 1H, d, $J=2.5$ Hz, H-6 and 8), 6.58 (2H, s, H-2', 6').

(±)-Catechin: Colorless needles (from H_2O); mp $199\text{--}201^\circ\text{C}$; $[\alpha]_D^{16} 0^\circ$ ($c=0.6$, acetone); $^1\text{H-NMR}$ (acetone- d_6 + D_2O) δ 2.52 (1H, dd, $J=8.5$, 16.5 Hz, H-4), 2.91 (1H, dd, $J=5.5$, 16 Hz, H-4), 3.99 (1H, ddd, $J=5.5$, 8, 8.5 Hz, H-3), 4.54 (1H, d, $J=8$ Hz, H-2), 5.87, 6.03 (each 1H, d, $J=2.5$ Hz, H-6 and 8), 6.75 (1H, dd, $J=8$, 2 Hz, H-6'), 6.81 (1H, d, $J=8$ Hz, H-5'), 6.91 (1H, d, $J=2$ Hz, H-2').

(–)-Epicatechin: Colorless needles (from H_2O); mp 239--

242 °C; $[\alpha]_D^{16} -44.0^\circ$ ($c=0.4$, acetone); $^1\text{H-NMR}$ (acetone- $d_6 + \text{D}_2\text{O}$) δ 2.72 (1H, dd, $J=3.5$, 16.5 Hz, H-4), 2.85 (1H, dd, $J=4.5$, 16.5 Hz, H-4), 4.20 (1H, br s, H-3), 4.87 (1H, s, H-2), 5.92, 6.02 (each 1H, d, $J=2$ Hz, H-6 and 8), 6.79 (1H, d, $J=8$ Hz, H-5'), 6.83 (1H, dd, $J=8$, 2 Hz, H-6'), 7.05 (1H, d, $J=2$ Hz, H-2').

Epicatechin-(4 β -8)-gallocatechin: A tan amorphous powder, $[\alpha]_D^{16} +25.8^\circ$ ($c=0.25$, acetone); $^1\text{H-NMR}$ (acetone- $d_6 + \text{D}_2\text{O}$) δ 2.58 (1H, dd, $J=7.5$, 16 Hz, H-4'), 2.84 (1H, dd, $J=5$, 16 Hz, H-4'), 3.88 (1H, s, H-3), 4.02 (1H, m, H-3'), 4.62 (1H, d, $J=8$ Hz, H-2'), 4.64 (1H, s, H-2'), 5.07 (1H, s, H-2), 5.87—6.05 (3H in total, m, A-ring H), 6.75 (2H, s, gallocatechin B-ring H), 6.70—7.00 (3H in total, m, epicatechin B-ring H).

Epigallocatechin-(4 β -8)-epicatechin: A tan amorphous powder, $[\alpha]_D^{16} +29.1^\circ$ ($c=0.15$, acetone); $^1\text{H-NMR}$ (acetone- $d_6 + \text{D}_2\text{O}$) δ 2.75 (1H, dd, $J=3$, 16 Hz, H-4'), 2.91 (1H, dd, $J=4$, 16 Hz, H-4'), 3.98 (1H, br s, H-3'), 4.34 (1H, br s, H-3), 4.71 (1H, s, H-4), 4.94 (1H, s, H-2), 5.00 (1H, s, H-2'), 5.96—6.04 (3H in total, m, A-ring H), 6.48 (2H, s, epigallocatechin B-ring H), 6.74—7.00 (3H in total, m, epicatechin B-ring H).

Procyanidin B-2: A tan amorphous powder, $[\alpha]_D^{16} +27.2^\circ$ ($c=0.2$, acetone); $^1\text{H-NMR}$ (acetone- $d_6 + \text{D}_2\text{O}$) δ 2.70 (1H, dd, $J=3.5$, 16 Hz, H-4'), 2.91 (1H, dd, $J=4.5$, 16 Hz, H-4'), 4.00 (1H, br s, H-3'), 4.32 (1H, br s, H-3), 4.72 (1H, s, H-4), 4.88 (1H, s, H-2), 5.06 (1H, s, H-2'), 5.96—6.04 (3H in total, m, A-ring H), 6.65—7.00 (6H in total, m, B-ring H).

Measurement of ONOO⁻ According to the method of Crow,¹⁵⁾ all oxidation reactions were carried out in a stirred 3-ml glass cuvette at 37 °C. Each reaction mixture (total volume of 1 ml) comprising 100 μM diethylenetriaminepentaacetic acid in 100 mM phosphate buffer, pH 7.4, and the required amount of Luobuma sample was incubated for 1 min. Then, 100 μM dichlorodihydrofluorescein and 5 μM ONOO⁻ was added and the reaction mixture was analyzed for 5 min using a spectrophotometer that exposed the sample to the full wavelength range of light to measure absorbance at 500 nm.

RESULTS

When different amounts of Luobuma extract were added to the reaction mixture, ONOO⁻-mediated oxidising activity was inhibited markedly in a concentration-dependent manner, as shown in Table 1. After separation of the extract, significant differences among the inhibitory activities of the various fractions were observed. The inhibitory activity of fraction 1 (eluted with H₂O) was weaker than those of fractions 2, 3 and 4 (the fractions eluted with 50% MeOH, MeOH and aqueous Me₂CO). Fractions 2, 3 and 4 were combined and further separated into 5 fractions. Of these, fractions 2-3, 2-4 and 2-5 possessed high inhibitory activity, whereas fraction 2-1 exhibited lower ONOO⁻-scavenging activity. The yield of fraction 2-2 was too low to enable its activity to be determined. The ONOO⁻-scavenging activities of the seven compounds isolated from fractions 2-3 and 2-4 were also examined. Epigallocatechin-(4 β -8)-epicatechin was found to be the most active compound, followed by epicatechin-(4 β -8)-gallocatechin and procyanidin B-2. ($\pm$)-Gallocatechin, (-)-epigallocatechin, ($\pm$)-catechin and (-)-epicatechin also proved to have the ability to scavenge ONOO⁻, but were

Table 1. Peroxynitrite-Scavenging Activity

Material	Concentration ($\mu\text{g/ml}$)	Inhibition (%)
Extract	0.1	0.78 $\pm$ 0.52
	0.5	16.16 $\pm$ 1.89
	2	44.46 $\pm$ 0.89
Fraction 1	0.1	0.82 $\pm$ 0.15
	0.5	9.13 $\pm$ 0.30
	2	32.00 $\pm$ 0.64
Fraction 2	0.1	4.70 $\pm$ 0.36
	0.5	20.46 $\pm$ 1.04
	2	68.53 $\pm$ 0.17
Fraction 3	0.1	3.91 $\pm$ 0.47
	0.5	24.69 $\pm$ 0.07
	2	67.43 $\pm$ 1.36
Fraction 4	0.1	3.64 $\pm$ 0.52
	0.5	23.40 $\pm$ 0.72
	2	68.54 $\pm$ 0.38
Fraction 2-1	0.1	5.87 $\pm$ 0.81
	0.5	18.88 $\pm$ 0.65
	2	52.57 $\pm$ 0.80
Fraction 2-3	0.1	9.93 $\pm$ 0.94
	0.5	43.27 $\pm$ 2.32
	2	77.59 $\pm$ 0.24
Fraction 2-4	0.1	9.81 $\pm$ 0.84
	0.5	46.38 $\pm$ 0.82
	2	77.57 $\pm$ 0.65
Fraction 2-5	0.1	10.18 $\pm$ 1.29
	0.5	42.26 $\pm$ 2.48
	2	79.85 $\pm$ 0.41
($\pm$)-Gallocatechin	0.1 (0.33)	9.88 $\pm$ 1.11
	0.5 (1.63)	47.74 $\pm$ 1.38
	2 (6.54)	79.19 $\pm$ 1.57
(-)-Epigallocatechin	0.1 (0.33)	11.84 $\pm$ 0.94
	0.5 (1.63)	58.85 $\pm$ 1.26
	2 (6.54)	83.00 $\pm$ 0.48
($\pm$)-Catechin	0.1 (0.34)	10.86 $\pm$ 0.83
	0.5 (1.72)	48.59 $\pm$ 1.18
	2 (6.90)	75.94 $\pm$ 0.31
(-)-Epicatechin	0.1 (0.34)	9.79 $\pm$ 0.76
	0.5 (1.72)	52.29 $\pm$ 0.79
	2 (6.90)	77.07 $\pm$ 0.70
Epicatechin-(4 β -8)-gallocatechin	0.1 (0.17)	16.25 $\pm$ 1.53
	0.5 (0.84)	61.32 $\pm$ 0.65
	2 (3.37)	91.73 $\pm$ 0.23
Epigallocatechin-(4 β -8)-epicatechin	0.1 (0.17)	16.99 $\pm$ 0.55
	0.5 (0.84)	63.97 $\pm$ 2.14
	2 (3.37)	94.51 $\pm$ 0.99
Procyanidin B-2	0.1 (0.17)	13.71 $\pm$ 0.84
	0.5 (0.87)	58.47 $\pm$ 1.89
	2 (3.46)	87.62 $\pm$ 1.80
Fraction 2-5-1	0.1	16.85 $\pm$ 0.98
	0.5	61.56 $\pm$ 1.20
	2	82.87 $\pm$ 0.26
Fraction 2-5-2	0.1	15.17 $\pm$ 1.53
	0.5	63.72 $\pm$ 1.40
	2	91.08 $\pm$ 2.18
Fraction 2-5-3	0.1	13.92 $\pm$ 1.10
	0.5	60.52 $\pm$ 1.87
	2	90.77 $\pm$ 0.98
Penicillamine	0.1	2.81 $\pm$ 0.46
	0.5	58.94 $\pm$ 0.91
	2	76.47 $\pm$ 1.47

Figures in parentheses are μM .

weaker than epigallocatechin-(4 β -8)-epicatechin, epicatechin-(4 β -8)-gallocatechin and procyanidin B-2. These compounds were more effective scavengers than penicillamine, an effective scavenger of ONOO⁻ *in vitro*.¹⁶⁾ Fractions 2-5-2

and 2-5-3, which comprised a mixture of high-molecular-weight condensed tannins, had potent ONOO⁻-scavenging activities, as shown in Table 1. The tannins of these fractions were quite complicated, as demonstrated by their complete thiolytic degradation and desulfurization of their thioethers, which yielded (–)-epigallocatechin, (±)-gallocatechin, (–)-epicatechin and (±)-catechin as their component units. Among these, the racemic flavan-3-ols (±)-gallocatechin and (±)-catechin were considered to have been formed as a result of the roasting procedure for the preparation of Luobuma tea leaves. The results suggest that racemization of the flavan units of the polymeric tannins occurred randomly during the roasting procedure, producing quite complicated polymers. For this reason, it was very difficult to isolate each polymer tannin and we could not compare the activities of these polymers.

DISCUSSION

When nitric oxide (NO) and superoxide (O₂⁻) coexist, such as in vascular endothelial cells and macrophages, NO promptly reacts with O₂⁻ to produce ONOO⁻,¹⁷⁾ which is a potent oxidant of the SH group.¹⁸⁾ This oxidant is weakly acidic (pK_a=6.8) and easily protonated in a near-neutral environment to promptly produce nitrate through isomerization.¹⁹⁾ Therefore, this radical has a short life. During the course of isomerization, ONOO⁻ passes through an activated state, in which it holds a level of reactivity similar to that of the hydroxyl radical, and thus causes lipid peroxidation, as reported by Koppenol *et al.*²⁰⁾ In addition, Beckman *et al.*²¹⁾ reported that ONOO⁻ produces NO₂⁺ in the presence of transition metal ions, including superoxide dismutase, and that the resulting NO₂⁺, which has high nitrating activity, attacks tyrosine and other aromatic amino acid residues. Because of this, the defense system against the production of NO and O₂⁻ and their reactions that produce ONOO⁻, as well as scavenging or eliminating radicals thus produced, is considered to be an important factor in inhibiting progression to a disease or morbid condition. It is believed that capture and removal of ONOO⁻ are essential conditions for correcting oxidative stress.

It seems that numerous factors contribute to the formation of atherosclerosis in a complex manner through interactions of infiltrating macrophages and T lymphocytes as well as vascular endothelial cells and smooth muscle cells.^{22–24)} In particular, the evidence showing the importance of oxidative degeneration of low-density lipoprotein (LDL) in the mechanism of the formation of atherosclerosis has been provided by Steinbrecher *et al.*²⁵⁾ and Steinberg,²⁶⁾ arousing considerable attention to the development of antioxidants capable of inhibiting the oxidation of LDL. In a previous study, we found that Luobuma aqueous extract inhibited foamy changes of macrophages and interfered with their toxicity to endothelial cells.¹²⁾ In the present study, we investigated the components of Luobuma that are active in eliminating ONOO⁻, a type of free radical known to have a variety of cytotoxic properties and to be present in atherosclerotic lesions.²⁷⁾

The highly active fraction 2-3 contained catechin and flavonoids, whereas fraction 2-4 contained condensed forms of tannin, such as dimers and trimers. In contrast, fraction 2-

5 contained no dimers or trimers, but higher polymer forms were predominant. Fraction 2-1 consisted mainly of saccharides. The highly active fractions 2-3 and 2-4 were further fractionated and seven compounds, gallate-free condensed tannins, were isolated from them. Epigallocatechin-(4β-8)-epicatechin had the highest ONOO⁻-eliminating activity, followed by epicatechin-(4β-8)-gallocatechin. Procyanidin B-2 also showed high activity, which was slightly lower than that of the above two compounds, but higher than that of the monomers. All these active compounds were dimers consisting of epigallocatechin, epicatechin or gallocatechin and their activities were higher than those of epigallocatechin, epicatechin or gallocatechin monomers.

Rhubarb and green tea are rich in condensed tannin and generally contain a low proportion of polymers and a high proportion of gallate-bound compounds.^{28,29)} In contrast, fraction 2-5 from Luobuma contained many polymers. However, we could not compare the activity levels of these polymers, because these polymers are difficult to isolate by any currently available technique. Luobuma was found to contain hardly any gallate-bound compounds.

As the results of this study indicate, this peculiar plant possesses ONOO⁻-eliminating activity. The ability of Luobuma extract to inhibit the progression of the atherosclerotic process in which oxidized LDL is involved has previously been demonstrated at the cellular level and in animal experiments.^{11–13)} Thus, these findings suggest that these condensed tannin compounds may play a significant role in defense against oxidative reactions in the body.

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