Antioxidative and antihepatotoxic principles of Tuocha

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Abstract

Tuocha is one of the special varieties of fermented compressed tea leaves, praised for its important health benefits, such as anti-aging, lowering cholesterol, enhancing immune function, lowering of blood pressure, reducing heart attacks *etc*. In the present study, we carried out fractionation and isolation of the active constituents, guided by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. In addition, antioxidative and antihepatotoxic potency on D-galactosamine (D-GalN)/tumor necrosis factor-alpha (TNF-α)-induced cell death in primary cultured mouse hepatocytes was also examined. Our study revealed that, tuocha is rich in antioxidants such as phenolics, lignans, flavanoides and flavan-3-ols, and showed a good understanding between structure and activity relationship.

Key words Tuocha, DPPH free radical scavenging activity, hepatoprotective activity.

Introduction

Tea, the dried leaves of the plant *Camellia sinensis var. assamica*, is the most popular beverage next to water and is consumed by over two-thirds of the worlds population. It was first discovered in China in 2737 BC by the Second Emperor, Shen Nung, known as the Divine Healer. There are three main types of tea, black tea, green tea, and oolong tea. Black tea is made via a post-harvest fermentation, and green tea is steamed to inactivate polyphenol oxidase prior to drying. On the other hand, oolong tea is the intermediate between green tea and black tea and is produced by a partial oxidation of the tea leaf. Tuocha is one of a special variety of fermented and compressed tea leaves grown in the misty mountain region of Yunnan province in southwest China. It is processed according to an ancient fermentation technique, which used to be a state secret that involves the aging of leaves, sometimes as old as 40-50 years. Tuocha has been praised for its flavor and important health benefits such as enhancing immune function, lowering blood pressure, lowering cholesterol levels, reducing the risk of a heart attack, stroke and boosting longevity. Although many epidemiological studies on green, black and oolong tea have been reported,[1-7] no previous study on this special variety of tea has been reported until now. Thus, we conducted a bio-assay guided study and isolated 20 compounds. They were identified based on their spectroscopic data and in comparison with literature data and were evaluated for their antioxidative and hepatoprotective activites.

Materials and Methods

Tuocha : Imported commercial Tuocha from Yunnan Province, China was obtained from Tanabe Seiyaku Trading Co., Ltd. (Osaka, Japan). A voucher sample (TMPW 21960) is preserved in the Museum for Materia Medica, Analytical Research Center for Ethnomedicines, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan.

Extraction and isolation : Commercial Tuocha (900 g) was successively extracted with water (7 L, reflux, 1 h×3), MeOH-H_2O (1:1, 7 L, 1 h×3) and MeOH (7 L, 1 h×3) to give water (282 g, yield 31.4%), MeOH-H_2O (31.4 g, yield 3.5%) and MeOH (23.6 g, yield 2.6%) extracts, respectively. The water extract (250 g) was

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suspended in MeOH and refluxed (1.5 L, 1 h×3) to give MeOH soluble (38.0 g) and MeOH insoluble (212 g) fractions.

The MeOH soluble fraction (38.0 g) was chromatographed (8×46 cm) over ODS (Cosmosil 75C_{18}-OPN, Nacalai Tesque, Kyoto, Japan) with the MeOH-H_2O (0%→100%) solvent system to give five fractions [fraction 1, 10.0 g; fraction 2, 11.8 g; fraction 3, 0.50 g; fraction 4, 0.50 g; and fraction 5, 21 g].

Fraction 1 (10 g) was rechromatographed on ODS (5 × 45 cm) with H_2O-MeOH (3:1) to afford three subfractions (fractions 1-1, 4.0 g; 1-2, 7.4 g; 1-3, 4.2 g). A part of each subfraction (100 mg) was subjected to reversed-phase preparative TLC (0.25 mm, RP-18F_{254S}, Merk) with H_2O-CH_3CN-MeOH (2:1:1) to give gallic acid[8] (**1**, 16.0 mg); **1** (15.0 mg) and 3,5-dihydroxybenzoic acid[8] (**2**, 3.0 mg); and **1** (8.0 mg), 4-hydroxybenzoic acid[8] (**3**, 10.0 mg), pyrogallol[8] (**4**, 15.0 mg), 3,4-dihydroxybenzoic acid[8] (**5**, 15.0 mg) and methyl-3,4,5-trihydroxybenzoate[8] (**6**, 8.0 mg), respectively.

Fraction 2 (11.8 g) was rechromatographed on ODS (5 × 45 cm) with H_2O-MeOH (3:1) to afford three subfractions (fractions 2-1, 5.4 g; 2-2, 4.3 g; 2-3, 2.1 g). A part of each subfraction (200 mg) was subjected to reversed-phase preparative TLC with H_2O-CH_3CN-MeOH (2:1:1) to give **1** (35.0 mg); **1** (40.0 mg) and **5** (2.3 mg); and (+)-catechin[9] (**13**, 12.0 mg), (-)-epicatechin (**14**, 6.0 mg), (-)-epigallocatechin[9] (**15**, 13.0 mg) and epigallocatechin gallate[9] (**16**, 2.0 mg), respectively.

A part of fraction 3 and fraction 4 (each 100 mg) was subjected to reversed-phase preparative TLC with H_2O-CH_3CN-MeOH (2:1:1) to give 2,5-dihydroxybenzoic acid[8] (**7**, 3.7 mg) and isolariciresinol[10] (**9**, 10.5 mg), and quercetin-3-*O*-glucoside[11] (**11**, 14.0 mg), respectively.

Fraction 5 (21 g) was rechromatographed on ODS (5 × 45 cm) with H_2O-MeOH (1:1) to afford three subfractions (fractions 5-1, 4.7 g; 5-2, 4.9 g; 5-3, 11 g). A part of each subfraction (200 mg) was subjected to reversed-phase preparative TLC with H_2O-CH_3CN-MeOH (2:1:1) to give kaempferol[12] (**10**, 35.0 mg); 1-(4-hydroxy-3-methoxy)phenyl-1,2,3-propanetriol[13] (**8**, 7.0 mg), epicatechin-[8,7-e]-4β-(4-hydroxyphenyl)-dihydro-2-(3*H*)-pyranone[14] (**18**, 5.6 mg); and kaempferol-3-*O*-glucoside[12] (**12**, 5.7 mg), epicatechin-[8,7-e]-4β-(3,4-dihydroxyphenyl)-dihydro-2-(3*H*)-pyranone[15] (**17**, 4.6 mg), epicatechin-[8,7-e]-4α-(3,4-dihydroxyphenyl)-dihydro-2-(3*H*)-pyranone[15] (**19**, 6.0 mg) and epicatechin-[8,7-e]-4α-(4-hydroxyphenyl)-dihydro-2-(3*H*)-pyranone[14] (**20**, 3.0 mg), respectively.

Chemicals: 1,1-Diphenyl-2-picrylhydrazyl (DPPH), sodium bicarbonate, glutamine, D-galactosamine (D-GalN) and collagenase were purchased from Wako Pure Chemicals Industry (Osaka, Japan). Lipopolysaccharide (LPS, Escherichia coli 055:B5) was from Difco

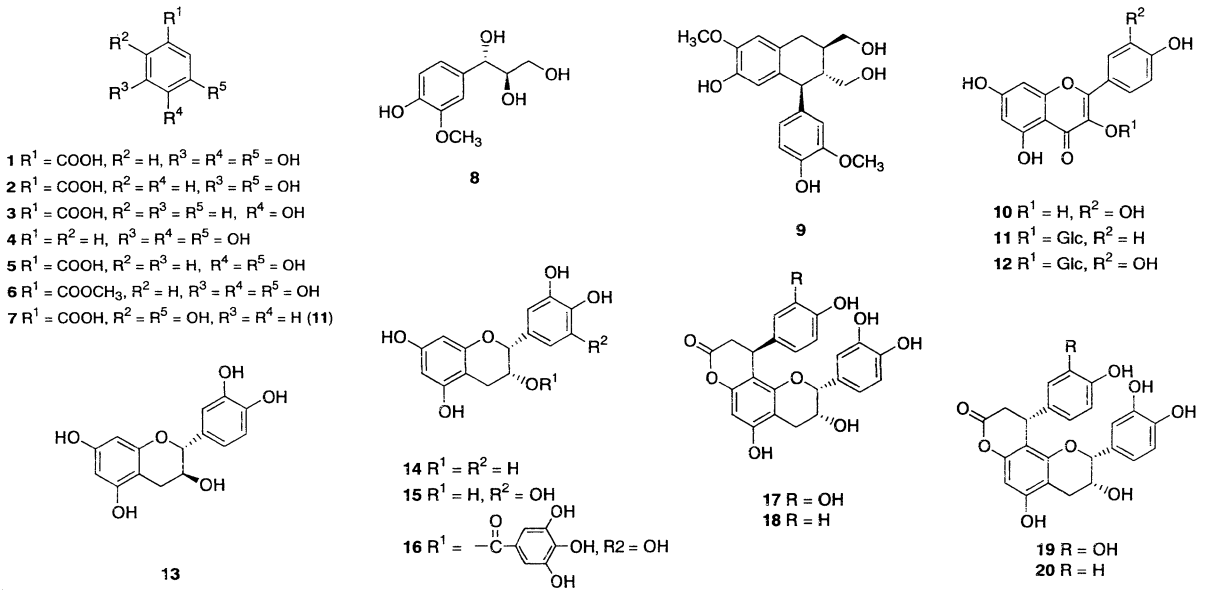

Chart 1 Structures of the compounds isolated from Tuocha

Laboratories (Detroit, MI, USA). RPMI and Eagle's minimum essential medium (EMEM) were from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Mouse recombinant tumor necrosis factor-alpha (TNF-α), William's E medium, bovine serum albumin (BSA), insulin, dexamethasone, penicillin G, streptomycin and 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) were purchased from Sigma Chemicals (St. Louis, MO, USA). Ethyleneglycol-*O,O*-bis(2-aminoethyl)-*N,N,N',N'*-tetraacetic acid (EGTA) was purchased from Fluca Chemie (Switzerland). Heat-inactivated fetal calf serum (FCS) and Hanks' balanced salt solution (HBSS) were from Gibco BRL Products (Gaitherburg, MD, USA). Falcon primaria surface-modified polystyrene culture plates with 96 wells were from Becton Dickinson (Lincoln Park, NJ, USA).

DPPH radical scavenging activity : DPPH radical scavenging activity was measured according to the procedure described by Hatano *et al.*[16] In brief, the different extract dissolved in EtOH or in water (500 μL) was mixed with equal volume of DPPH solution (60 mM). The resulting solution was thoroughly mixed by vortex and absorbance was measured at 520 nm after 30 min. The scavenging activity was determined by comparing the absorbance with that of the blank (100%) containing only DPPH and solvent.

D-GalN/TNF-α-induced cell death in primary cultured mouse hepatocytes : Mouse liver parenchymal cells were isolated according to the procedure described previously by Seglen.[17] In brief, the liver was perfused with Ca^{2+}-free HBSS containing 0.5% BSA and 5 mM EGTA and then recirculated with collagenase solution composed of Ca^{2+}-free HBSS, 0.075% collagenase, 4 mM $CaCl_2$ and 0.005% trypsin inhibitor. Isolated hepatocytes were cultured in William's E medium supplemented with 10% FCS, 100 IU/mL penicillin G, 100 mg/mL streptomycin, 100 mM dexamethasone and 50 ng/ml insulin and incubated in a 96-well plastic plate (1.5×10^4 cells/well). After 2 hrs pre-incubation, the medium was replaced with fresh medium containing D-GalN (0.5 mM) and test specimens at various concentrations. Thirty minutes later, TNF-α (100 ng/ml) was added to each well, and the hepatocytes viability was assessed 18 h thereafter by MTT method. The cell death protection (%) was calculated using the following formula and IC_{50} was determined graphically. Protection (%) = $[A-B/C-B]\times 100$, *A*: cells treated D-GalN/TNF-α and sample; *B*: cells treated with D-GalN/TNF-α only; *C*: Normal.

Results

DPPH radical scavenging activity

The MeOH soluble and MeOH insoluble fractions of water extract, MeOH-H_2O extract and MeOH extract of tuocha were subjected for DPPH radical scavenging activity. They all exhibited concentration-dependent scavenging activity: IC_{50} value of the MeOH soluble fraction of water extract, 4.5 μg/mL; MeOH insoluble fraction of the water extract, 5.2 μg/mL; MeOH-H_2O extract, 5.0 μg/mL; MeOH extract, 23.6 μg/mL. Thus, the MeOH soluble fraction was subjected to ODS column chromatograpy, which led to the isolation of 20 compounds (Chart 1). All the isolated compounds were tested for DPPH radical scavenging activity (Table I). Among them, epigallocatechin (**15**) and epigallocatechin gallate (**16**) possessed the most potent scavenging activities with IC_{50} values of 2.42 μM and 2.46 μM, respectively, which were comparable to that of caffeic acid (2.1 μM), a well known antioxidant.[18] The activities of other flavan-3-ols, **13** (IC_{50}, 6.34 μM) and **14** (IC_{50}, 6.34 μM), and phenylpropanoid-substituted flavan-3-ols, **17** (IC_{50}, 3.33 μM), **18** (IC_{50}, 8.31 μM), **19** (IC_{50}, 6.7 μM) and **20** (IC_{50}, 5.5 μM), were slightly weaker than those of **15** and **16**. On the other hand, gallic acid (**1**) possessed the most potent activity with an IC_{50} value of 6.34 μM among the simple phenolics. These data may suggest the importance of the pyrogallol moiety for the enhancement of antioxidative activity of the flavan-3-ols. Among flavanoids, quercetin-3-*O*-glucoside (**11**) displayed the most potent activity with an IC_{50} value of 4.18 μM.

Table I DPPH free radical scavenging effects of the compounds isolated from Tuocha

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
1	4.57	**11**	4.18
2	8.51	**12**	16.3
3	>50	**13**	6.34
4	5.06	**14**	5.75
5	19.3	**15**	2.42
6	>50	**16**	2.46
7	11.4	**17**	3.33
8	37.2	**18**	8.31
9	17.4	**19**	6.70
10	14.0	**20**	5.50
		Caffeic acid	2.10

Hepatoprotective activity

Hepatoprotective activity of all the isolated compounds were tested on D-GalN/TNF-α-induced cell death in primary cultured mouse hepatocytes at concentrations of 100, 50 and 10 μM (Table II). Silibinin, a clinically used drug, was taken as a positive control in this experiment. The majority of the compounds displayed concentration-dependent protective activities, and the results are summarized in Table II. Simple phenolic compounds, in general, possessed only mild protecting activities. Flavanoids and flavanoid glucosides, on the other hand, displayed potent activities. Quercetin-3-*O*-glucoside (**11**; IC_{50}, 31.2 μM) and kaempferol-3-*O*-glucoside (**12**; IC_{50}, 30.6 μM) were twice stronger than kaempferol (**10**; IC_{50}, 65.7 μM), suggesting that the presence of a glucose moiety may enhance the activity in the flavanoids. Flavan-3-ols displayed weak activities (IC_{50} >100 μM), but phenylpropanoid-substituted flavan-3-ols dramatically enhanced the hepatocytoprotective activity and epicatechin-[8,7-e]-4β-(3,4-dihydroxyphenyl)-dihydro-2-(3*H*)-pyranone (**17**) exhibited the most potent activity with an IC_{50} value of 23.1 μM, comparable to that of the positive control.

Discussion

Active oxygen species and free radical play an important role in various disease states, and constant oxida-

Table II Hepatocytoprotective effects of the compounds isolated from Tuocha on D-GalN/TNF-α-induced cell death in primary cultured mouse hepatocytes.

Compounds	Conc. (μM)	Cell survival rate ± S.D. (% of Normal)	% protection	IC_{50} (μM)
Normal[c]		100 ± 5.5		
Control[d]		32.8 ± 5.7		
Silibinin	100	42.0 ± 5.5	9.4 ± 8.6	20.8
	50	106.1 ± 6.3*	109.6 ± 9.8	
	10	53.7 ± 14.6**	27.7 ± 22.8	
1	100	40.9 ± 4.0*	7.3 ± 6.2	>100
	50	29.7 ± 6.9*	-10.2 ± 10.9	
	10	34.9 ± 1.9	-2.1 ± 3	
2	100	41.1 ± 4.1*	7.6 ± 6.4	>100
	50	39.0 ± 4.3	4.4 ± 6.7	
	10	35.2 ± 7.6	-1.6 ± 11.9	
3	100	40.4 ± 2.8**	6.6 ± 4.4	>100
	50	34.2 ± 7.1	-3.1 ± 11.2	
	10	35.9 ± 6.4	-0.4 ± 10	
4	100	45.8 ± 4.1***	15.0 ± 6.5	>100
	50	37.1 ± 6.0	1.5 ± 9.4	
	10	40.6 ± 5.1*	6.9 ± 8	
5	100	36.0 ± 4.5	-0.3 ± 7	>100
	50	39.6 ± 9.3	5.3 ± 14.5	
	10	34.1 ± 7.6	-3.3 ± 12	
6	100	39.9 ± 4.7*	5.8 ± 7.4	>100
	50	46.1 ± 9.3**	15.6 ± 14.5	
	10	31.2 ± 5.3*	-7.9 ± 8.3	
7	100	34.2 ± 2.5	-7.4 ± 4	>100
	50	34.3 ± 4.4	-7.4 ± 7.2	
	10	38.3 ± 5.2	-0.7 ± 8.6	
8	100	63.4 ± 2.1***	42.9 ± 3.2	> 100
	50	43.6 ± 7.2*	12.0 ± 11.2	
	10	44.2 ± 2.5*	12.9 ± 3.9	
9	100	60.5 ± 11.8***	41.2 ± 17.6	>100
	50	38.1 ± 4.1	7.8 ± 6.1	
	10	36.6 ± 2.4	5.6 ± 3.6	
10	100	83.8 ± 7.5***	74.8 ± 11.6	65.7
	50	60.6 ± 4.8***	38.5 ± 7.6	
	10	45.4 ± 3.7*	14.8 ± 5.7	

Results are expressed as mean ± S.D. (n = 4; for normal and control, n = 8). ***p<0.001, **p<0.01, *p<0.05, significantly different from control.

Table II *Cont.*

Compounds	Conc. (μM)	Cell survival rate± S.D. (% of Normal)	% protection	IC_{50} (μM)
11	100	85.9 ± 3.8***	79.0 ± 5.6	31.2
	50	80.1 ± 5.9***	70.3 ± 8.7	
	10	51.1 ± 7.8***	27.1 ± 11.7	
12	100	99.3 ± 4.6***	98.9 ± 6.8	30.6
	50	82.0 ± 3.2*	73.1 ± 4.7	
	10	49.9 ± 15.4**	25.4 ± 22.9	
13	100	46.1 ± 5.0	11.9 ± 8.2	>100
	50	35.4 ± 2.0	-5.5 ± 3.3	
	10	32.0 ± 8.1	-11.1 ± 13.2	
14	100	43.1 ± 2.8	7.1 ± 4.6	>100
	50	31.8 ± 5.4	-11.5 ± 8.8	
	10	32.7 ± 4.5	-9.9 ± 7.4	
15	100	31.4 ± 4.3	-12.0 ± 7	>100
	50	30.4 ± 4.2	-13.3 ± 8.4	
	10	35.0 ± 1.0	-6.1 ± 1.6	
16	100	47.7 ± 7.4*	14.6 ± 12.1	>100
	50	38.3 ± 3.5	-0.7 ± 5.7	
	10	35.5 ± 1.7	-5.3 ± 2.7	
17	100	93.8 ± 18.7***	90.8 ± 27.8	23.1
	50	85.2 ± 12.0***	78.0 ± 17.8	
	10	57.3 ± 8.0***	36.4 ± 12	
18	100	78.2 ± 3.4***	64.4 ± 5.6	85.5
	50	47.7 ± 7.5*	14.5 ± 12.2	
	10	42.4 ± 4.3	5.9 ± 7	
19	100	74.9 ± 11.8***	62.6 ± 17.5	80.6
	50	53.0 ± 8.4***	30.0 ± 12.5	
	10	36.8 ± 4.3	5.9 ± 6.4	
20	100	79.4 ± 7.6***	69.3 ± 11.3	64.1
	50	61.3 ± 7.3***	42.4 ± 10.9	
	10	42.4 ± 5.8*	14.2 ± 8.7	

Results are expressed as mean ± S.D. (n = 4; for normal and control, n = 8). ***p<0.001, **p<0.01, *p<0.05, significantly different from control.

tive stress is suspected to be a major causes of aging.[19] These radicals and the reactive species derived from the radicals react with cell membrane and induce lipid peroxidation, which are responsible for various deleterious effects in cells and progressive degeneration of tissues.[20] Antioxidants present in consumable fruits, vegetables and beverages have received considerable attention not only as anti-aging agents but also as cancer chemopreventive agents. The inhibition of free radical generation could also exert a beneficial effect against hepatotoxicity by scavenging ROS and affecting the signal transduction triggered by TNF-α.[21] In this regard, we investigated the constituents from tuocha and evaluated their DPPH radical scavenging activity and hepatocytoprotective activity. Our study revealed that tuocha is rich in simple phenolics and lignans. Although these simple phenolics displayed potent free radical scavenging activity, they displayed only a mild hepatocytoprotective activity, even at higher concentrations. Flavanoids displayed both potent free radical scavenging and hepatocytoprotective activity. All the isolated flavan-3-ol derivatives (**15-19**) also exhibited the potent antioxidative activity, but only those having phenylpropanoid-unit in their structure (**17-20**) displayed significant hepatocytotprotective activity in a concentration-dependent manner (Table II). These results suggested the importance of the phenylpropanoid-unit in flavan-3-ols for the enhancement of activity and also indicated that although antioxidative activity of compounds may play an important role in their hepatoprotective action, the real mechanism of protection by flavan-3-ols might have been different. Since (-)-epicatechin was shown to inhibit TNF-α production,[22] one of the possible mechanisms involved with phenylpropanoid-substituted flavan-3-ols could be due to its ability to inhibit TNF-α production. The present study led to a good understanding between

structure and activity relationship and concluded that a presence of glucose moiety in flavanoids and phenylpropanoid-unit in flavan-3-ols highly enhances the hepatocytoprotective activity. Tuocha is rich in antioxidants, and phenylpropanoid-substituted flavan-3-ols, which contributes to both potent antioxidative activity and potent hepatocytoprotective activity, were isolated for the first time from Tuocha, making this highly praised traditional health drink unique from other varieties of tea.

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和文抄録

沱茶は中国雲南省のアッサム茶の葉を蒸して作られる緊圧茶で，抗老化，コレステロール低下，免疫増強，血圧降下等の作用があると言われている。我々は，DPPHラジカル捕捉活性を指標として成分検索を行ない，得られた成分についてDPPHラジカル捕捉活性と共にD-GalN/TNF-α誘発マウス肝細胞死阻害活性を測定した。その結果，沱茶がフェノール類，リグナン，フラボノイド，フラバン3オール類などの抗酸化成分を多く含む事を明らかにし，それら成分の構造と活性との関係について考察した。

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