Antioxidant Activities of Three Dihydrochalcone Glucosides from Leaves of *Lithocarpus pachyphyllus*

Wei-Min Yang[a,b], Ji-Kai Liu[a,*], Xiang-Dong Qin[a], Wan-Lin Wu[b], and Zhi-He Chen[b]

[a] Kunming Institute of Botany, The Chinese Academy of Sciences, Kunming 650204, P. R. China. Fax: +86-871-5150227. E-mail: jkliu@mail.kib.ac.cn

[b] Yunnan Pharmacological Laboratories of Natural Products, Kunming Medical College, Kunming 650031, P. R. China

* Author for correspondence and reprint requests

Z. Naturforsch. **59 c**, 481–484 (2004); received December 9, 2003/January 15, 2004

In vitro antioxidant activities of three sweet dihydrochalcone glucosides from the leaves of *Lithocarpus pachyphyllus* (Kurz) Rehd. (Fagaceae), trilobatin 2″-acetate (**1**), phloridzin (**2**) and trilobatin (**3**), were investigated. The IC_{50} (50% inhibitory concentration) values for compounds **1**–**3** of lipid peroxidation in rat liver homogenate were 261, 28, 88 μM, respectively. Compounds **1**–**3** increased superoxide dismutase (SOD) activity with EC_{50} (50% effective concentration) values of 575, 167, 128 μM, and glutathione peroxidase (GSH-Px) activity with EC_{50} values of 717, 347, 129 μM, respectively, and showed only weak DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity.

Key words: Antioxidant Activities, *Lithocarpus pachyphyllus*, Sweet Dihydrochalcone Glucosides

Introduction

Free radicals such as the superoxide and hydroxyl radicals play an important role in tissue damage involved in many pathological processes including aging, cardiovascular diseases, cancer, and inflammatory (Finkel and Holbrook, 2000; Bauerova and Bezek, 1999; Halliwell, 1994). Consequently, antioxidants may be used as protective or therapeutic agents. In the past few years, addition of synthetic antioxidants has begun to be restricted because of their health risks and toxicity (Buxiang and Fukuhara, 1997). The importance of exploiting natural antioxidants from various sources and replacing synthetic antioxidants with natural ingredients has attracted increasing attention.

Results and Discussion

The leaves of *Lithocarpus pachyphyllus* (Kurz) Rehd. (Fagaceae) are known to taste sweet and used as a kind of sweet tea for the anti-hypertensive treatment in the folk of Yunnan, China. Three sweet dihydrochalcone glucosides, trilobatin 2″-acetate (**1**), phloridzin (**2**) and trilobatin (**3**), were isolated from the leaves of *L. pachyphyllus* (Kurz) Rehd. (Qin and Liu, 2003). The voucher specimen was deposited at the herbarium of the Kunming Institute of Botany, The Chinese Academy of Sciences. Compounds **1**–**3** (Fig. 1) inhibited lipid peroxidation induced by a non-enzymic Fe(II)-ascorbic acid system in rat liver homogenate with IC_{50} values of 261, 28, 88 μM, respectively, in a dose-dependent manner. The controls with vitamin E (VE, α-tocopherol) and butylated hydroxyanisole (BHA) yielded IC_{50} values of 296 and 224 μM (Table I). They scavenged superoxide radicals generated by the xanthine/xanthine oxidase system with EC_{50} values of 575, 167, 128 μM, respectively, and BHA as control with 424 μM. Compounds **1**–**3** also elevated GSH-Px activity with EC_{50} values of 717, 347, 129 μM, respectively (GSH-Px activity VE, 262 μM) (Table II), but only exhibited lower DPPH radical scavenging activity with EC_{50} values of 519, 2179, 432 μM, respectively (EC_{50} for BHA, 111 μM).

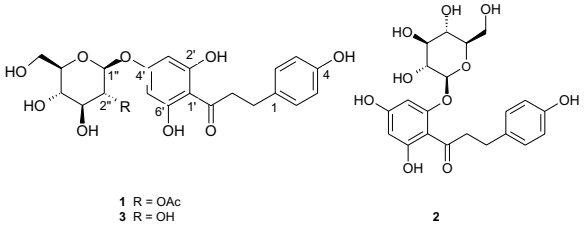

Fig. 1. Structures of trilobatin 2″-acetate (**1**), phloridzin (**2**) and trilobatin (**3**).

Table I. Lipid peroxidation inhibitory activity and SOD activity in rat liver homogenate for trilobatin 2″-acetate, phloridzin, and trilobatin.

Sample	Lipid peroxidation activity		SOD activity	
	Concentration [μg/ml]	Inhibitory rate (%)	Concentration [μg/ml]	Activity rate (%)
VE	10	16.1 ± 1.5	–	–
	30	26.1 ± 2.6	–	–
	90	41.5 ± 3.7	–	–
	270	85.6 ± 5.1	–	–
BHA	10	19.2 ± 2.6	10	5.6 ± 1.2
	30	34.9 ± 5.7	30	19.8 ± 2.5
	90	66.5 ± 6.9	90	52.1 ± 4.6
	270	95.5 ± 5.6	270	87.3 ± 6.2
Trilobatin 2″-acetate	1.1	8.0 ± 1.5	30	6.5 ± 1.3
	10	14.7 ± 3.6	90	29.1 ± 2.1
	90	33.2 ± 4.9	270	44.3 ± 3.6
	810	70.6 ± 5.5	810	77.4 ± 5.1
Phloridzin	0.1	17.8 ± 1.7	10	6.6 ± 2.2
	1.1	29.0 ± 2.6	30	22.3 ± 3.8
	10	43.0 ± 5.4	90	49.3 ± 5.4
	90	70.6 ± 5.4	270	88.6 ± 5.2
Trilobatin	1.1	15.2 ± 1.3	10	10.2 ± 2.3
	10	29.4 ± 2.9	30	29.6 ± 3.6
	90	54.6 ± 3.4	90	60.1 ± 5.1
	810	86.8 ± 5.3	270	90.5 ± 6.1

The data are given as mean ± S. E. M, n = 6 for each group. IC_{50} values of lipid peroxidation inhibitory activity for trilobatin 2″-acetate, phloridzin, trilobatin, VE and BHA were 261, 28, 88, 296, 224 μM respectively. EC_{50} values of SOD activity for trilobatin 2″-acetate, phloridzin, trilobatin and BHA were 575, 167, 128, 424 μM, respectively. In the test of lipid peroxidation inhibitory activity, the activity rate of the vehicle (100% control) was 3 nmol MDA/mg protein (MDA: methane dicarboxylic aldehyde). In the test of SOD activity, 100% control was 135×10^{-3} IU/mg protein.

Experimental

Male SD (Sprague Dawley) rats, weighing 200–250 g, were used for *in vitro* studies in accordance with the Ethics Committee of Kunming Medical College (Grade II, Certificate No. 000208).

Trilobatin 2″-acetate (**1**, purity > 99%, HPLC analysis), phloridzin (**2**, > 99%), and trilobatin (**3**, > 99%) were isolated from the leaves of *Lithocarpus pachyphyllus* (Qin and Liu, 2003) and dissolved in DMSO. Thiobarbituric acid (TBA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), xanthine, xanthine oxidase, 5,5′-dithiobis-nitrobenzoic acid (DTNB) and glutathione (GSH) were obtained from Sigma Chemical Co. (St. Louis). All chemicals and solvents were analytical grade. The preparation of rat liver homogenate was carried out as described and prepared in a ratio of 1 g of wet tissue to 9 ml of 0.86% NaCl (Yue *et al.,* 1995).

Lipid peroxidation activity assay

Lipid peroxidation inhibitory activity in rat liver homogenate was evaluated by the thiobarbituric acid (TBA) method with minor modification (Yue *et al.*, 1995; Ohkawa *et al.*, 1979). The reaction was initiated by addition of 4 mM $FeCl_2$ (30 μl) into a mixture of 6 mM ascorbic acid (60 μl), 5% liver homogenate (4.0 mg protein/ml, 600 μl) and 30 μl of sample solution. The reaction mixture was incubated at 37 °C for 30 min. After incubation, the reaction was stopped by addition of 20% trifluoroacetic acid (TFA, 500 μl) and the mixture was centrifuged at $3500 \times g$ for 10 min. The reaction supernatant (1 ml) was mixed with 0.67% (w/v) TBA (500 μl), and then heated in boiling water bath for 10 min. After cooling down, the absorbance of the solution was determined at 532 nm by an UV-visible spectrometer. Lipid peroxidation inhibition was calculated as follows: $[1-(T-B)/(C-B)] \times 100\%$, in which T, C and B are absorbance values at 532 nm of the sample treatment (T), the vehicle control (C) and the reaction zero time control (B), respectively.

Table II. GSH-Px activity in rat liver homogenate and DPPH radical scavenging activity for trilobatin 2″-acetate, phloridzin, and trilobatin.

Sample	GSH-Px activity		DPPH radical scavenging activity	
	Concentration [μg/ml]	Inhibitory rate (%)	Concentration [μg/ml]	Activity rate (%)
VE	10	6.6 ± 1.3	–	–
	30	15.1 ± 2.2	–	–
	90	35.8 ± 3.6	–	–
	270	78.6 ± 4.9	–	–
BHA	–	–	3.3	8.4 ± 1.3
	–	–	10	23.4 ± 3.5
	–	–	30	41.0 ± 4.6
	–	–	90	86.8 ± 6.7
Trilobatin 2″-acetate	30	5.5 ± 1.3	10	9.1 ± 1.9
	90	20.9 ± 2.3	30	23.2 ± 3.6
	270	43.1 ± 4.5	90	41.2 ± 4.7
	810	71.4 ± 5.6	270	79.6 ± 5.9
Phloridzin	10	3.1 ± 1.9	90	3.7 ± 1.1
	30	9.4 ± 2.3	270	25.4 ± 2.5
	90	30.3 ± 3.9	810	44.1 ± 5.1
	270	71.2 ± 4.8	2430	74.2 ± 5.4
Trilobatin	10	11.1 ± 2.3	30	8.6 ± 2.3
	30	31.3 ± 2.9	90	30.8 ± 3.6
	90	62.2 ± 5.2	270	67.5 ± 4.5
	270	87.9 ± 6.5	810	82.2 ± 5.7

The data are given as mean ± S. E. M, n = 6 for each group. EC_{50} values of GSH-Px activity for trilobatin 2″-acetate, phloridzin, trilobatin and VE were 717, 347, 129, 262 μM, respectively. EC_{50} values of DPPH radical scavenging activity for trilobatin 2″-acetate, phloridzin, trilobatin and BHA were 519, 2179, 432, 111 μM, respectively. In the test of GSH-Px activity and DPPH radical scavenging activity, 100% control was 340 IU/mg protein and 147 μM DPPH, respectively.

SOD activity assay

The method chosen for SOD activity assay in rat liver homogenate was a modified direct nitrite method (Oyanagui, 1984; Shen and Chen, 2002). The xanthine/xanthine oxidase system was utilized to generate superoxide flux which oxidizes hydroxylamine to nitrite, and nitrite can be measured by the color developing reagent. The reaction mixture contained the following reagents: 50 mM potassium phosphate buffer (pH 7.8, 250 μl), purified water (100 μl), 10 mM hydroxylamine hydrochloride (25 μl), 7.5 mM xanthine (25 μl), 23.4 mU/ml xanthine oxidase (25 μl), 1% liver homogenate (0.8 mg protein/ml, 10 μl) and 25 μl of sample solution. After incubation at 37 °C for 30 min, 3.3 g/l sulfanilic acid (250 μl) and 10 g/l naphthamide (250 μl) were added to the mixture. SOD activity was assessed by measuring the absorbance of the reaction solution at 550 nm. The inhibitory ratio of each compound to the formation of nitrite was calculated as follows: $[1-(T-B)/(C-B)] \times 100\%$, in which T, C and B are absorbance values at 550 nm of the sample treatment (T), the vehicle control (C) and the reaction zero time control (B), respectively.

GSH-Px activity assay

GSH-Px activity in rat liver homogenate was evaluated by the DTNB method with minor modification (Sun *et al.*, 2002). GSH-Px catalyzes the reaction of GSH and H_2O_2, and through determining the consumption of GSH the GSH-Px activity can be assessed. The reaction system contained: 0.1 M sodium phosphate buffer (7.0, 50 μl), 2 mM GSH (25 μl), 0.25% liver homogenate (0.2 mg protein/ml, 25 μl) and 25 μl of sample solution. After incubation at 37 °C for 30 min, the reaction was initiated by addition of 1.5 mM H_2O_2 (50 μl), and the mixture immediately re-incubated at 37 °C for 5 min. Then 1.67% metaphosphoric acid (500 μl), 0.32 mM Na_2HPO_4 (250 μl) and 0.04% DTNB (50 μl) were added, and the DTNB reduction by GSH was measured at 412 nm. The GSH-Px activity of each compound was calculated as follows: $[1-(T-B)/(C-B)] \times 100\%$, in which T, C and B

are absorbance values at 412 nm of the sample treatment (T), the vehicle control (C) and the reaction zero time control (B), respectively.

DPPH radical scavenging activity assay

DPPH radical scavenging activity was assessed after Blois (1958) and Yun *et al.* (2000). Each concentration of sample solution in DMSO (20 μl) was added to 980 μl of 150 μM ethanolic DPPH solution. The mixture was incubated for 30 min at room temperature and the absorbance at 517 nm was measured. The DPPH radical scavenging activity of each compound was calculated as follows: $[1-(Ai-Aj)/Ac] \times 100\%$, in which Ai, Aj and Ac are absorbance values at 517 nm of the sample treatment (Ai), the sample control (Aj, without DPPH, containing sample and the vehicle ethanol) and the DPPH control (Ac, without sample, containing DPPH and the vehicle DMSO), respectively. All data were expressed as means ± S. E. M. IC_{50} and EC_{50} values were calculated by nonlinear regression analysis after logarithmic transformation of the sample concentrations.

Acknowledgement

We acknowledge the support from National Natural Science Foundation of China (30225048) and the support of the project (2001CCC00600).

Bauerova K. and Bezek A. (1999), A role of reactive oxygen and nitrogen species in pathogenesis of rheumatioid disease. Gen. Physiol. Biophys. **18**, 15–20.

Blois M. S. (1958), Antioxidant determination by the use of a stable free radical. Nature **181**, 1199–1200.

Buxiang S. and Fukuhara M. (1997), Effects of co-administration of butylated hydroxytoluene, butylated hydroxyanisole and flavonoids on the activation of mutagens and drug-metabolizing enzymes in mice. Toxicology **122**, 61–72.

Finkel T. and Holbrook N. J. (2000), Oxidants, oxidative stress and biology of aging. Nature **408**, 239–247.

Halliwell B. (1994), Free-radicals, antioxidants and human diseases: curiosity, cause and consequences. Lancet **322**, 721–724.

Ohkawa H., Ohishi N., and Yagi K. (1979), Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. **95**, 351–358.

Oyanagui Y. (1984), Reevaluation of assay methods and establishment of kit for superoxide dismutase activity. Anal. Biochem. **142**, 290–301.

Qin X. D. and Liu J. K. (2003), A new sweet dihydrochalcone glucoside from leaves of *Lithocarpus pachyphyllus*. Z. Naturforsch. **58c**, 759–761.

Shen N. and Chen X. (2002), Assay methods for superoxide dismutase activity. In: Pharmacological Methodology (Xu S. Y., Bian R. L., and Chen X., eds.). The People's Public Health Publishing House, Beijing, pp. 529–537.

Sun Y., Wu X. M., and Zhang H. Q. (2002), Assay methods for gluthathione peroxidase activity. In: Pharmacological Methodology (Xu S. Y., Bian R. L., and Chen X., eds.). The People's Public Health Publishing House, Beijing, pp. 549–554.

Yue T. L., Gu J. L., Lysko P. G., Sponer G., Cheng H. Y., and Ruffolo R. J. (1995), Antioxidant activity of a β-adrenoceptor antagonist, carazolol, caparison with propranolol and carvedilol. Pharmacol. Commun. **7**, 11–19.

Yun B. S., Lee I. K., Kim J. P., and Yoo I. D. (2000), Curtisians A~D, new free radical scavengers from the mushroom *Paxillus curtislii*. J. Antibiot. **53**, 114–122.