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The Antioxidation and Antiproliferation Activity of New Flavonoids from the Leaves and Stems of *Cinnamomum reticulatum* Hayate

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Abstract

Cinnamoum reticulatum Hayata (Lauraceae) is an indigenous tree species in Taiwan. In this study, we further isolated the two flavonoids compounds kaempferol-3-*O*-(2",4"-di-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside (**1**) and kaempferol-3-*O*-(3",4"-di-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside (**2**) from the leaves and stems of *C. reticulatum* Hayate. The phytochemical characteristics, antioxidant and cytotoxic activities of the two compounds were evaluated. Kaempferol-3-*O*-(2",4"-di-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside (1) and kaempferol-3-*O*-(3",4"-di-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside (2) have antiproliferation activity in lung cancer cell line (A549 and NCI-H460) and breast cancer cell line (MCF-7 and MDA-MB-231). However, (1) displays better antioxidant activity than (2). Our results showed that the two flavonoids from *C. reticulatum* might have good potential for further development as chemoprevention or antioxidant remedies.

Keywords: *Cinnamoum reticulatum* Hayata; Lauraceae; Flavonoids; Antioxidant

Introduction

The *Cinnamomum* species have used in folk medicine. *Cinnamomum* species have many active chemical compounds with anti-oxidation, anti-cancer, anti-infection and anti-inflammatory activities [1,2]. *Cinnamoum reticulatum* Hayata (Lauraceae) is an evergreen tree, a tree native to Taiwan. Previous studies, we have isolated compounds including reticuol, isoreticulide, 4-hydrox-3-methoxyphenethyl derivate, reticumanone, reticuone, cinnaretamine and isoobtusilactone A from the stems and leaves of *Cinnamomum reticulatum* [3-5].

Flavonoids are a group of nature compounds with phenolic structure and common characteristics C6-C3-C6 skeleton consisting of two benzene rings. Flavonoids possess anti-oxidation, anti-cancer, hepatoprotective, anti-inflammatory activities [6-8]. In the current study, we further isolated reticuol and two new flavonoids compounds kaempferol-3-*O*-(2",4"-di-*E*-*p*-coumaroyl)-α-L- rhamnopyranoside (1) and kaempferol-3-*O*-(3",4"-di-*E*-*p*-coumaroyl)-α-L-rhamno- pyranoside (2) from the leaves of *Cinnamomum reticulatum* (Figure 1). In the current study, we will examine the anti-oxidation and anti-proliferation activity of the two new flavonoids compounds in cancer cells.

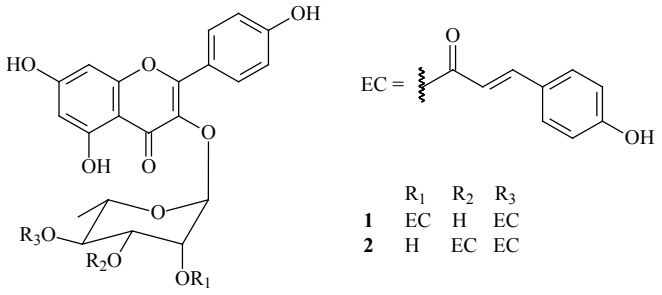
Materials and Methods

Extraction and isolation

The air-dried leaves of *Cinnamomum reticulatum* Hay (3.4 kg) were extracted with *n*-hexane (30L x 5) and $CHCl_3$ (30L x 5) at room temperature and an *n*-hexane extract (43.5 g) and a $CHCl_3$ extract (151.5 g) were obtained upon concentration under reduced pressure. The $CHCl_3$ extract (151.5 g) was chromatographed over silica gel (800 g, 70-230 mesh) using *n*-hexane/$CHCl_3$/MeOH mixtures as eluents to produce five fractions. A part of fraction 4 (10.62 g) was subjected to silica gel chromatography by eluting with $CHCl_3$: MeOH (60:1), enriched with MeOH, to furnish five fraction (4-1~4-5). Fraction 4-3 (1.89 g) was further purified on a silica gel column using $CHCl_3$-MeOH mixtures to obtain reticuol (6 mg), kaempferol-3-*O*-(2",4"-di-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside (**1**) (21 mg) and kaempferol-3-*O*-(3",4"-di-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside (**2**) (15 mg).

Cell culture

Human lung cancer and human breast cancer cell lines A549, NCI-H460 MCF-7 MDA-MB231 were obtained from BCRC (Bioresoure Collection and Research Center, Hsinchu, Taiwan). They were cultured in MEM medium supplemented with 10% FBS, 10 μg/mL of penicillin, 10 μg/mL of streptomycin and 0.25 μg/mL of amphotericin B. The cells were maintained in a humidified incubator at 37 ˚C in 5% CO_2.



kaempferol-3-*O*-(2",4"-di-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside (**1**)
kaempferol-3-*O*-(3",4"-di-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside (**2**)

Figure 1: The chemical structure of two flaonoids

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Cell viability assay—XTT assay

The XTT (2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5- carboxanilide) assay was used to determine cell viability and proliferation. The cell lines were seeded in 96-well culture plates (1×10^4 cells/well). XTT were obtained from Sigma-Aldrich (GmbH, Sternheim, Germany). After seeding cells for 24 h, various different concentrations of compounds were added. After the treatment, the medium was replaced with fresh medium without drugs. XTT reagent was added to each well and cultured for 3 hours. The optical density (OD) values of the supernatant were measured at 492 nm and 690 nm (reference wavelength). All experiments were repeated at least three times.

Determination of DPPH radical scavenging capacity

DPPH is an abbreviation for an organic chemical compound 2,2-diphenyl-1-picrylhydrazyl. It is a dark-colored crystalline powder composed of stable free-radical molecules. The mechanism of its radical scavenging activity is the antioxidant transfer of an electron or a hydrogen atom to DPPH. DPPH were obtained from Sigma-Aldrich (GmbH, Sternheim, Germany).The measurement of radical scavenging properties of compounds was carried out according to the method of Blois [9]. Various concentrations of the three compounds were added to 0.1 M of stable DPPH (60 μmole/L) solution. When DPPH reacts with hydrogen-donating antioxidant, it is reduced, resulting in a decrease in absorbance at 517 nm. The analyzed time interval was 10 min per point, up to 30 min by using UV-Vis spectrophotometer (Jasco V-530, Japan Servo Co.Ltd., and Tokyo, Japan). Vitamin C was used as a positive control. Measurements were taken in triplicate. The DPPH• radical scavenging activity (%) was determined as: $1 - [(A_{control} - A_{sample})/A_{control}] \times 100$.

Reducing power

Briefly, various concentrations of test samples were mixed with 67 mM phosphate buffer (pH 6.8, 0.085 mL) and 20% potassium ferric cyanide [$K_3Fe(CN)_6$, 2.5 μL) The mixture was incubated at 50°C for 20 min, and trichloroacetic acid (10%, 0.16 mL) was then added to the mixture that was then centrifuged for 10 min at 3000 g. The upper layer of the solution (75 μL) was mixed with 2% $FeCl_3$ (25 μL), and the absorbance was measured with a 96-well plate spectrophotometer at 700 nm. 3-tert-butyl-4-hydroxyanisole (BHA) was used as a positive control. A higher absorbance demonstrates a higher reductive capability.

Statistical analysis

All experiments were carried out at three times and at least triplicate. The results were expressed as the average of the mean values ± standard deviation (SD). Analysis of the data was done with SigmaPlot software (Version 8.0, SPSS Scientific, and Chicago, IL, USA) and SigmaStat (Version 2.03, SPSS Scientific) run on an IBM-compatible computer.

Results

Spectra results

The structure of compounds 1 and 2 were elucidated using Nuclear Magnetic Resonance Spectroscopy (NMR), 1-DNMR and 2-DNMR.

Kaempferol-3-*O*-(2",4"-di-*E*-*p*-coumaroyl)-α-L-rhamnopyranosid (1)

Yellow powder; UV λ_{max} (MeOH, log ε) 351 (4.7), 256 (4.8), 208 (5.1) nm; IR (KBr) ν_{max} 3377, 1687, 1655, 1604, 1513 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 0.87 (3H, d, *J*=5.8 Hz), 3.34 (1H, dq, *J*=9.8, 6.2 Hz), 4.19 (1H, dd, *J* =3.5, 9.8 Hz), 4.99 (1H, t, *J*=9.8 Hz), 5.57 (1H, br s), 5.76 (1H, br s), 6.22 (1H, br s), 6.34 (1H, d, *J*=15.9 Hz), 6.40 (1H, d, *J*=15.5 Hz), 6.42 (1H, br s), 6.84 (each 1H, d, *J*=8.2 Hz) , 6.85 (each 1H, d, *J*=8.2 Hz), 7.05 (each 1H, d, *J*=7.4 Hz), 7.53 (each 1H, d, *J*=7.4 Hz), 7.54 (each 1H, d, *J*=7.6 Hz), 7.64 (1H, d, *J*=15.5 Hz), 7.74 (1H, d, *J*=15.9 Hz), 7.85 (each 1H, d, *J*=7.6 Hz); FABMS *m/z* 725 $[M + H]^+$.

Kaempferol-3-*O*-(3",4"-di-*E*-*p*-coumaroyl)- α-L- rhamnopyranoside (2)

Yellow powder; UV λ_{max} (MeOH, log ε) 351 (4.7), 256 (4.8), 208 (5.1) nm; IR (KBr) ν_{max} 3377, 1687, 1655, 1604, 1513 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 0.85 (3H, d, *J*=6.3 Hz), 3.4 (1H, m), 4.44 (1H, m), 5.20 (1H, t, *J*=10.1 Hz), 5.45 (1H, dd, *J*=3.2, 10.1 Hz), 5.60 (1H, d, *J*=1.3 Hz), 6.00 (1H, *J*=6.3 Hz), 6.21 (1H, d, *J*=16.0 Hz), 6.24 (1H, d, *J*=2.0 Hz), 6.33 (1H, d, *J* =16.0 Hz), 6.46 (1H, d, *J*=2.0 Hz), 6.73 (each 1H, d, *J*=8.6 Hz), 6.86 (each 1H, d, *J*=8.6 Hz), 7.09 (each 1H, d, *J*=8.7 Hz), 7.43 (each 1H, d, *J*=8.6 Hz), 7.44 (each 1H, d, *J*=8.6 Hz), 7.56 (1H, d, *J*=16.0 Hz), 7.65 (1H, d, *J*=16.0 Hz), 7.82 (each 1H, d, *J*=8.7 Hz); FABMS *m/z* 725 $[M + H]^+$.

Biological activity

IC_{50} is a measure of the effectiveness of a drug in inhibiting biological or biochemical function. In the present study, the anti-proliferation activity of the two compounds was determined by XTT assay and the results were presented in Table 1. Based on the IC_{50} value of two compounds, kaempferol-3-*O*-(3",4"-di-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside (2) has better anti-proliferation activity than kaempferol-3-*O*-(2",4"-di-*E*-*p*-coumaroyl)-α-L- rhamnopyranoside (1) in cancer cells.

Anti-oxidation activity

Antioxidants are used in cosmetics, food, and medicine against cellular free radicals and reduce metal ions. We examined the two extracts anti-oxidation activity by DPPH and reducing power assays. In the previous study, the vitamin C was used as positive control compared with flavonoids compound [10]. As shown in Table 2, kaempferol-3-*O*-(2",4"-di-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside (1) has better radical scavenging activity (65.21 ± 0.80%) than kaempferol-3-*O*-(3",4"-di-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside (17.40 ± 0.13%) (2) at the same dose. In the ferric reducing antioxidant power (FRAP) assay, the reducing power of the two compounds were examined compared with 3-tert-butyl-4-hydroxyanisole (BHA) and shown in Table 2. Based on the results, kaempferol-3-*O*-(2",4"-di-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside (1) has better reducing power than kaempferol-3-*O*-(3",4"-di- *E*-*p*-coumaroyl)-α-L-rhamnopyranoside (2).

Discussion

Previous studies, we have isolated many compounds from *C. reticulatum* Hayata. Among these compounds, butanolide isoobtusilactone A has many pharmacological activities including cell cycle arrest, apoptosis and reactive oxygen species (ROS) generation in cancer cells [11-15]. Studies have shown that flavonoids possess anti-oxidation, anti-cancer,

Cell Line	1	2
A549	5 ± 0.42 μg / mL	2.1 ± 0.18 μg / mL
NCI-H460	4.6 ± 0.34 μg / mL	1.6 ± 0.09 μg / mL
MCF-7	7.2 ± 0.63 μg / mL	3.9 ± 0.31 μg / mL
MDA-MB-231	8.4 ± 0.82 μg / mL	4.6 ± 0.33 μg / mL

Data were expressed as a mean value of at least three independent experiments.

Table 1: The IC_{50} of kaempferol-3-*O*-(2",4"-di-*E*-*p*-coumaroyl)-α-L-rhamnopyranosid (1) and kaempferol-3-*O*-(3",4"-di-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside (2) in different cancer cell line.

Compound	DPPH (%)	reducing power
1	65.21 ± 0.80	0.136 ± 0.01
2	17.40 ± 0.13	0.071 ± 0.00
Vitamin C	100.00 ± 0.00	-
BHA	-	0.306 ± 0.05

Vitamin C was used as a positive control on DPPH assay at 100 μmole/L; BHA was used as a positive control on reducing power at 100 μmole/L.

Table 2: DPPH radical scavenging activity (%) and reducing power of two extracts kaempferol-3-*O*-(2",4"-di-*E*-*p*-coumaroyl)-α-L-rhamnopyranosid (1) and kaempferol-3-*O*-(3",4"-di-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside (2) of *C. reticulatum*.

hepatoprotective, anti-inflammatory activities [6,7]. In the present study, we demonstrated that two flavonoids kaempferol-3-*O*-(2",4"-di-*E*-*p*-coumaroyl)-α-L-rhamnopyranosid (1) and kaempferol-3-*O*-(3",4"-di-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside (2) have anti-oxidation and cytotoxic activity in cancer cells.

Flavonoids possess anti-oxidative effects as free radical scavengers and metal ion cheaters properties associated with the phenolic hydroxyl groups attached to ring structures. Intensity of the antioxidant activity of a flavonoid strongly depends on its chemical structure. There is a great deal of discussion and contradiction regarding the structure antioxidant activity relationships of flavonoids. However, it is well-accepted that the antioxidant activity of flavonoids is markedly influenced by the number and position of hydroxyl groups on the B and A rings, and by the extent of conjugation between the B and C rings. On the basis of many previous and recent findings, it seems that favorable general structural requirements for effective radical scavenging and/or the antioxidative potential of flavonoids follow the famous three Bors' criteria:

a) The *o*-hydroxy structure in the B ring, which confers high stability to the flavonoid phenoxyl radicals *via* hydrogen bonding or by expanded electron delocalization; b) The C2-C3 double bond (in conjugation with the 4-oxo group), which determines the coplanarity of the heteroring and participates in radical stabilization *via* electron delocalization over all three ring systems; c) The presence of both 3-OH and 5-OH groups for the maximal radical scavenging capacity and the strongest radical absorption (*i.e.,* 1 and 2).

Taken these results together, the two flavonoids possess anti-oxidation and cytotoxic activity in the cancer cells. In the future, the exact mechanisms of anti-cancer activity of the compounds can be further examined.

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