J Food Sci Nutr
Vol 14, p 49~53 (2009)
DOI: 10.3746/jfn.2009.14.1.049

J Food Science and Nutrition

Isolation of Pentacyclic Triterpenoids from Semi-fermented Tea and Its Effects on Oxidative Stress

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Abstract

Antioxidative activities of major pentacyclic terpenoids from the semi-fermented tea of *Camellia sinensis* L. were investigated. The free radical scavenging activities of triterpenoids 1~3 were examined with of DPPH and superoxide anion radical scavenging activity. The IC_{50} of compounds 1 and 2 for DPPH radical scavenging activities were 23.1 and 37.2 μg/mL, respectively, and for superoxide anion radical scavenging activities were 37.2 and 35.2 μg/mL, respectively. According to this result, compounds 1 or 2 in semi-fermented tea could be the candidates for bioactive material having antioxidant activity.

Key words: *Camellia sinensis* L., semi-fermented tea, pentacyclic triterpenoids, RDA-MS fragment, 2D-NMR, antioxidant, chung tea, oolong tea

INTRODUCTION

Phytochemicals in natural sources with biological activity, are considered to be important for human health. Many plants have proven to be important sources of a number of secondary metabolites (1).

In aerobic metabolism, the oxygen is reduced to give the superoxide anion radicals. They are generally small molecules and are highly reactive due to the presence of unpaired valence shell electrons. Therefore, superoxide radicals need an additional electron to make them more stable, so they take an electron from the nearest source, non enzymatic antioxidants and vitamins C or E. A high level of radicals may cause damage to the mitochondria thus, a cell undergoes apoptosis or programmed cell death. Reactive oxygen species (ROS) such as superoxide anion radical, hydroxyl radicals and H_2O_2 are unwanted and toxic substances produced as a by-product during aerobic metabolism. Mammals constantly form ROS, by oxidative and reductive processes in the mitochondria, from oxygen derived from respiration or by the immune system exposed to foreign antigen, and externally by radiation or various chemicals. Oxidative stress impacts upon almost all acute and chronic progressive disorders in human physiology. Interest in oxidative stress and cellular longevity continues to grow at an exponential pace (2-5).

Camellia sinensis L. had been used as a daily beverage and as a folk medicine in Asia for thousands of years. In previous paper, we reported that antibacterial activities of the major phenolic components in *Camellia sinensis* L. Among them, (-)-epicatechin was a predominant antibacterial components (6). It has been reported cytotoxicity against prostate cancer, antimutagenicity and inhibition of off-odor (7-9). As a part of our ongoing study on the identification of phytochemicals, we used open column chromatography to isolate bioactive compounds from chung tea, semi-fermented tea. This samples were chosen since *n*-hexane soluble fraction exhibited a potent antioxidant activity by scavenging activity of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals (IC_{50}: 28.1 μg/mL) and superoxide anion radical (IC_{50}: 21.7 μg/mL). Bioassay-monitored fractionation of the active *n*-hexane soluble fraction led to the isolation of three pentacyclic triterpenoids, which were then evaluated for their individual biological activities. The structures of compounds **1~3** were elucidated on the basis of spectroscopic evidences, particularly withthe results of EI-MS spectrometry and NMR spectroscopy (^{1}H-NMR, ^{13}C-NMR, DEPT, HMQC, and HMBC).

MATERIALS AND METHODS

Chemicals

DPPH was obtained from Sigma Chemical Co. (St. Louis, MO). The pure compounds were dissolved in deuterium chloroform ($CDCl_3$) and stored at 4°C. All chemicals were purchased from commercial sources and were of the highest purity available.

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Preparation of samples

Commercial semi-fermented tea, chung tea (10%-fermented tea) and oolong tea (45%-fermented tea) were purchased by traditional tea market in Insa Dong, Seoul, Korea in 2007.

Instrumental analyses

Melting points (mp) were determined using a Mitamura-Riken melting point apparatus and are uncorrected. A Hewelett Packard Model 5985B gas chromatography (GC)/MS system was used for electron impact mass spectrometry (EI-MS). A Bruker Avance DRX 300 spectrometer (300 MHz for ^{1}H and 75 MHz for ^{13}C), respectively, was used to record nuclear magnetic resonance (NMR) spectra with tetramethylsilane (TMS) as an internal standard and $CDCl_3$ as a NMR solvent. Two-dimensional NMR spectroscophic techniques were used for ^{1}H-^{1}H correlation spectroscopy (COSY) and for experiments on heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC). Thin-layer chromatographic (TLC) analysis was performed on silica gel (Kieselgel 60 F_{254} plates; 0.25 mm layer thickness; Merck, Darmstadt, Germany). Silica gel (Merck 60 A, 230～400 mesh ASTM) was used for open column and vacuum column for chromatographic separations.

Extraction and solvent fractionation of samples

The dried and fully ground chung and oolong tea (each 10 g) were extracted using 90% ethyl alcohol (EtOH, 100 mL) for 24 hours at room temperature three times. The ethanolic solutes were filtered and concentrated to yield dark green for the chung tea and dark yellow extracts for oolong tea. The ethanolic extracts were dissolved in H_2O followed sequential fractionation ly with *n*-hexane, chloroform ($CHCl_3$), and ethyl acetate (EtOAc). The concentrated solvent extracts were dissolved in dimethylsulfoxide (DMSO) for the biological tests.

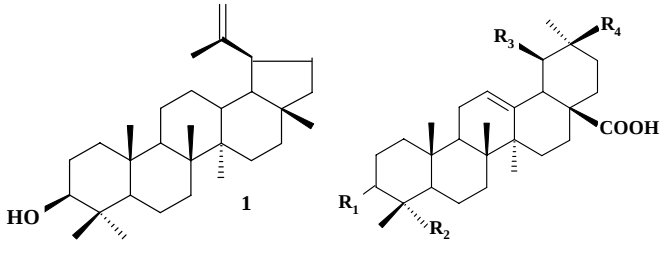
Isolation and structure identification of compounds

The dried and fully ground chung tea (450 g) was extracted using 90% (v/v) EtOH for three days at room temperature. The extraction was repeated three times with same sample. The combined ethanolic extracts were concentrated under reduced pressure to obtain EtOH extracts (28.1 g). The extracts were suspended in H_2O (2 L) and then partitioned sequentially with *n*-hexane, $CHCl_3$, and water-saturated EtOAc for three times with each solvent. The *n*-hexane soluble fraction (3.1 g) was subjected to a silica gel open column chromatography by elution with a gradient of *n*-hexane-EtOAc to give twenty-six fractions (Frs 1-26). Fraction 5 was further chromatographed on a silica gel column using *n*-hexane-methylene chloride (CH_2Cl_2) (98:2→95:5, v/v) to give compound **1**. Fractions 7～12 was re-chromatographed into twelve subfractions (Subfrs 1～12) eluted with CH_2Cl_2-MeOH (99:1→94:6, v/v). Subfractions 4 and 5 were combined and further chromatographed on a silica gel vacuum column by elution with *n*-hexane-EtOAc (95:8, v/v) to isolate compounds **2** and **3**. TLC patterns of the compounds were examined by UV lamp (254 nm) and with 20% $SbCl_3$-$CHCl_3$ (Carr-Price reagent) spray method (10). Complete identification of the pure compound carried out with various physical and chemical methods including EI-MS, ^{1}H-NMR and ^{13}C-NMR spectroscopy. According to the color reaction, compounds **1**～**3** were terpenoid (purplish to red with Liebermann-Bürchard reagent). Further identification of compounds was done by comparing its spectral data with literatures (Fig. 1).

Scavenging capacity of extracts and compounds

DPPH radical-scavenging activity: Reaction mixtures containing 5 mL of test samples dissolved in DMSO and 95 mL of 300 mM DPPH in ethanol solution (final DPPH concentration) were incubated at 37°C for 30 min in 96-well micro filter plates. Absorbance was then measured at 512 nm. IC_{50} values denote the concentration of sample required to scavenge 50% of DPPH radicals. Ascorbic acid and 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) were also examined for their DPPH radical-scavenging activity (11).

Superoxide anion radical scavenging by NBT method: The superoxide anion radical scavenging ability of compounds was studied by xanthine/xanthine oxidase/NBT method according to Ibrahim et al (12). The reaction mixture contained 0.5 mL of 1.6 mM xanthine, 0.48 mM



	R_1	R_2	R_3	R_4
2	OH	H	H	CH_3
3	OH	CH_2OH	CH_3	H

Fig. 1. Chemical structure of compounds **1**～**3**.

Table 1. Antioxidant activities of solvent fractions of semi-fermented teas

Solvent fractions	DPPH[1)]		X/XO NBT[2)]	
	Chung tea	Oolong tea	Chung tea	Oolong tea
n-Hexane fraction	28.1	43.1	21.7	20.5
$CHCl_3$ fraction	51.6	61.5	78.0	54.2
EtOAc fraction	18.5	34.2	35.1	43.2
H_2O fraction	45.1	67.2	89.3	62.9

[1)]DPPH radical-scavenging activity (IC_{50}: μg/mL).
[2)]Superoxide scavenging activity (IC_{50}: μg/mL).

NBT in 10.0 mM phosphate buffer (pH 8.0). After incubation at 37°C for 5 min, the reaction was initiated by adding 1.0 mL of xanthine oxidase and incubation at 37°C for 20 min. The reaction was supported by adding 1.0 mL of 69.0 mM SDS, and the optical absorbance was measured at 570 nm.

RESULTS AND DISCUSSION

Antioxidant activities of the ethanolic extracts and solvent soluble fractions of semi-fermented tea varieties, chung tea and oolong tea were evaluated at a final concentration of 100 mg/mL using a DPPH radical-scavenging and superoxide anion. In the present study, *n*-hexane soluble fraction of chung tea showed significant inhibitory effects on DPPH radicals and formazan formation from NBT react with superoxide anion radical generated by xanthine oxidase system (Table 1).

Isolation and structure identification of compounds 1～3

The dried and fully ground chung tea was extracted and partitioned by successive extraction with *n*-hexane, $CHCl_3$, EtOAc and H_2O. The *n*-hexane soluble fraction of chung tea showed a potent free radical scavenging activity. Therefore this fraction was subjected to a silica gel open and vacuum column chromatography by elution with gradient of *n*-hexane-EtOAc, *n*-hexane-CH_2Cl_2 and CH_2Cl_2-MeOH, to give compounds **1** (32.7 mg), **2** (21.2 mg), and **3** (28.1 mg). TLC patterns of the compounds were monitored using a UV lamp (254 nm) and Carr-Price reagent spray method. Further identification of the isolated compound was carried out with various physical and chemical methods, including EI-MS fragmentation pattern, ^{1}H-NMR and ^{13}C-NMR spectroscopy (Figs. 2, 3). All compounds exhibited typical color reaction for steroid skeleton with Liebermann-Bürchard reagent.

The structures of the compounds were identified by comparing the spectral data with the data from related publications (13-17). As a result, the isolated compounds

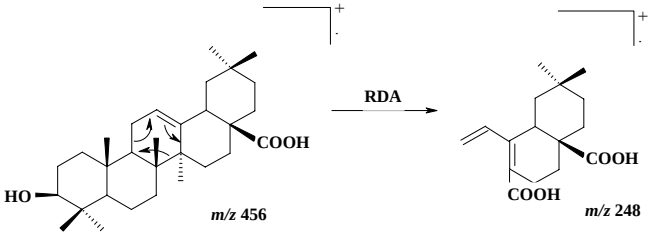


Fig. 2. Retro Diels-Alder fragmentation of compound **2**.

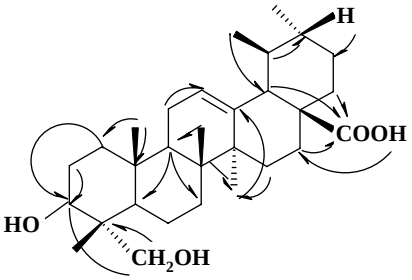


Fig. 3. Selective HMBC correlations of compound **3**.

were determined as triterpenoids, lupeol (**1**), oleanolic acid (**2**) and 23-hydroxyursolic acid (**3**) (Fig. 1). The C_{30} terpenes are based on six isoprene units and are biosynthetically derived from squalene. These compounds have relatively high-melting point and are widely distributed among plant resins, cork, and cutin as a form of colorless solids. Oleanolic acid and ursolic acid are found in e waxy coatings of leaves or in protective coating of certain fruits (18).

Spectral data of compounds 1～3

Lupeol (**1**). White amorphous powder (MeOH); mp 215～217°C; EI-MS (70 eV) *m/z* (rel. int., %): 426 $[M]^+$ (14.2), 411 $[M\text{-}CH_3]^+$ (11.8), 393 $[M\text{-}CH_3\text{-}H_2O]^+$ (4.3), 189 [a (ring D/E)]$^+$ (100.0); ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.82 (3H, *s*, CH_3-25), 1,01 (3H, *s*, CH_3-24), 1.03 (3H, *s*, CH_3-26), 1.06 (3H, *s*, CH_3-27), 1.22 (3H, *s*, CH_3-23), 1.76 (3H, *s*, CH_3-30), 3.43 (1H, *t*, *J*=7.8 Hz, H-3), 5.02 (1H, *m*, H-12); ^{13}C-NMR (75 MHz, $CDCl_3$): see Table 2.

Oleanolic acid (**2**). White amorphous powder (MeOH); mp 231°C; EI-MS (70 eV) *m/z* (rel. int., %): 456 $[m]^+$ (10.0), 410 $[M\text{-}COOH\text{-}H]^+$ (12.2), 248 [a (ring D/E)]$^+$ (32.0), 233 [a (ring D/E)-CH_3]$^+$ (12.4); ^{1}H-NMR (300 MHz, $CDCl_3$) *δ* 0.89 (3H, *d*, *J*=6.2 Hz, CH_3-30), 0.94 (3H, *s*, CH_3-25), 1.01 (3H, *d*, *J*=6.2 Hz, CH_3-29), 1.02 (3H, *s*, CH_3-26), 1.03 (3H, *s*, CH_3-24), 1.24 (3H, *s*, CH_3-27), 1.28 (3H, *s*, CH_3-27), 3.31 (1H, *dd*, *J*=4.2, 13.5 Hz, H-18), 3.44 (1H, *dd*, *J*=6.2, 9.3 Hz, H-3), 5.50 (1H, *t*, *J*=3.3 Hz, H-12); ^{13}C-NMR (75 MHz, $CDCl_3$): see Table 2.

Table 2. ^{13}C-NMR spectral data of compounds **1**～**3**[1)]

Position	1	2	3
1	39.2	38.9	39.0
2	28.2	28.1	27.7
3	78.1	78.0	73.5
4	39.8	39.4	48.6
5	55.9	55.6	48.8
6	18.7	18.8	18.6
7	34.8	33.2	33.1
8	41.1	39.6	40.2
9	51.9	48.1	48.3
10	37.5	37.7	37.2
11	21.1	23.7	23.7
12	26.1	121.6	123.5
13	38.6	144.8	139.4
14	42.8	42.2	42.6
15	31.7	28.5	28.8
16	32.8	23.8	25.0
17	56.8	48.7	48.1
18	47.8	46.5	53.6
19	49.7	42.2	39.6
20	151.3	31.2	39.5
21	30.2	34.3	31.2
22	37.6	33.3	37.5
23	28.6	28.8	67.9
24	16.3	16.5	13.2
25	16.4	15.5	16.2
26	16.4	17.4	17.6
27	14.8	26.2	23.9
28	178.8	180.2	180.7
29	19.4	33.2	17.6
30	109.6	23.6	21.3

[1)]Measured at 75 MHz in $CDCl_3$.

23-Hydroxyursolic acid (**3**). White amorphous powder (MeOH); mp 284°C; EI-MS (70 eV) *m/z* (rel. int., %): 472 $[M]^+$ (10.0), 410 $[M\text{-}COOH\text{-}H]^+$ (12.2), 248 [a (ring D/E)]$^+$ (12.0), 233 [a (ring D/E)-CH_3]$^+$ (10.1); ^{1}H-NMR (300 MHz, $CDCl_3$) δ 0.94 (3H, *d*, *J*=6.5 Hz, CH_3-30), 0.98 (3H, *s*, CH_3-25), 1.01 (3H, *d*, *J*=6.4 Hz, CH_3-29), 1.02 (3H, *s*, CH_3-26), 1.06 (3H, *s*, CH_3-24), 1.19 (3H, *s*, CH_3-27), 2.64 (1H, *d*, *J*=11.0 Hz, H-18), 3.72 (1H, *d*, *J*=10.4 Hz, H-23a), 4.10 (1H, *d*, *J*=10.4 Hz, H-23b), 4.21 (1H, *dd*, *J*=4.9, 10.4 Hz), 5.42 (1H, *br s*, H-12); ^{13}C-NMR (75 MHz, $CDCl_3$) : see Table 2.

Biological activity of compounds 1～3

The free radical scavenging activity of pentacyclic triterpenoids **1**～**3** have been evaluated from the reduction of absorbance at 512 nm due to scavenging of DPPH radical. The IC_{50} of compounds **1**, **2**, and **3** were 23.1, 37.2, and 84.8 μg/mL, respectively. We also investigated superoxide anion radical scavenging activity of compounds using xanthine/xanthine oxidase/NBT biological system. In the present assay, superoxide anion radical scavenging activities of compounds **1** and **2** were found to be moderate (Table 3). It seems that compounds

Table 3. Antioxidant activities of compounds **1**～**3**

Compounds	DPPH[1)]	X/XO NBT[2)]
1	23.1	31.0
2	37.2	35.2
3	84.8	108.1
Ascorbic acid[3)]	22.3	19.8
BHA[3)]	24.1	20.9

[1)]DPPH radical-scavenging activity (IC_{50}: μg/mL).
[2)]Superoxide scavenging activity (IC_{50}: μg/mL).
[3)]Control.

1 and **2** could be used as natural ROS scavenger. The antioxidant property of compounds in semi-fermented tea has been reported extensively (19-21). Dental caries and postprandial hypertriglyceridemia were significantly reduced by semi-fermented tea (22,23). The further research on the differentiation of bioactive components through processing methods of *Camellia sinensis* L. and mechanism of compounds should be conducted *in vivo* experimental system.

ACKNOWLEDGEMENT

This research was supported by Institute of Natural Sciences, Duksung Women's University, Seoul, Korea (2007).

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(Received January 28, 2009; Accepted March 9, 2009)