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Antioxidant and Cytotoxic Activities of Isolated Compounds from Ethyl Acetate Fraction of *Alpinia oxyphylla* Fruits

WEI FAN, CHENG-ZHONG WANG, XIAO-LI BAO, HUI-HUI YUAN* and MIN-BO LAN*

Shanghai Key Laboratory of Functional Materials Chemistry and Research Center of Analysis and Test, East China University of Science and Technology, Shanghai 200237, P.R. China

*Corresponding authors: Fax: +86 21 64252947; Tel: +86 21 64253574; E-mail: yuanhuihui@ecust.edu.cn; minbolan@ecust.edu.cn

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A bioactivity-guided isolation and purification process was used to identify the antioxidant and cytotoxic components from the ethyl acetate fraction of *Alpinia oxyphylla* fruits. Four subfractions of the ethyl acetate fraction were obtained by a silica gel column chromatography eluted with petroleum ether-ethyl acetate, of which subfraction A showed the highest antioxidant and cytotoxic activities was selected for the isolation and identification of major active compounds. Seven compounds including nootkatone (**1**), tectochrysin (**2**), β-sitosterol (**3**), chrysin (**4**), protocatechuic acid (**5**), (4S*, 5E, 10R*)-7-oxo-tri-nor-eudesm-5-en-4β-ol (**6**) and (4R, 6R, 10S)-4,10-dimethyl-6-(1'-hydroxyisopropyl)-1-en-3,4,5,6,7,8-hexahydronaphthalen-2-one (**7**) were isolated. Compounds **6** and **7** were found firstly from this plant. Biological tests revealed that compounds **5**, **6**, **7** were the major antioxidant compounds and compounds **1**, **3**, **4**, **5** exhibited potent cytotoxicity on different cancer cells, which probably responsible for the antioxidant and anticancer activities of the ethyl acetate fraction of *Alpinia oxyphylla* fruits.

Keywords: *Alpinia oxyphylla*, Antioxidant activity, Cytotoxicity, Bioactive compounds.

INTRODUCTION

Oxidation is an essential biological process to many organisms for the production of energy. However, the uncontrolled production of oxygen derived free radicals can induce DNA damage, protein carbonylation and lipid peroxidation[1] leading to a variety of diseases, such as cancer, ageing, Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis[2]. Many evidences have indicated that reactive oxygen species (ROS) could also promote tumor heterogeneity, invasion and metastasis, through inhibiting antiprotease and injuring local tissues[3,4]. Therefore, it is important to develop naturally occurring products with antioxidant activity to protect the human body from free radicals and retard the progress of many chronic diseases[5,6].

The plant of *Alpinia oxyphylla* Miq. is widely cultivated in South China. The fruits of this plant have been used to treat diseases such as intestinal disorders, ulceration, dementia and diuresis in tradition Chinese Medicine (TCM) and were coded in Chinese Pharmacopeia as an aromatic stomachic[7]. Previous pharmacological studies have indicated that the extracts of *Alpinia oxyphylla* fruits possess antianaphylactic[8], antiinflammatory[9], neuroprotective[10], antioxidant[11,12], antitumor[9,13] and insecticidal[14] effects. The fruits are rich in sesquiterpenes, diterpenes, flavonoids and diaryl-heptanoids[15-18]. To date, more than 32 sesquiterpenoids have been isolated from the extracts of *Alpinia oxyphylla* fruits, amongst which eight compounds[19-21] showed significant antiinflammatory effects. However previous reports concerning the antioxidant and anticancer effects of *Alpinia oxyphylla* mainly focused on its crude extracts, the active components responsible for these activities remain to be further elucidated. Our previous work has demonstrated that the ethanol extract of *Alpinia oxyphylla* fruits, especially its ethyl acetate fraction, possessed potent antioxidant and anticancer activities[22]. Therefore, as a continuous effort to discover biologically active secondary metabolites from this plant, the aim of this work was to find the active compounds from the ethyl acetate fraction responsible for its antioxidant and anticancer activities through a bioactivity-guided isolation process.

EXPERIMENTAL

The air-dried fruits of *Alpinia oxyphylla* collected from Guangxi province were purchased from Leiyunshang Medicine Corporation (Shanghai, China). A voucher specimen (No. 11010) was authenticated by Prof. Yongchuan Zhou from East China University of Science and Technology and deposited at the herbarium of Research Center of Analysis and Test, East

China University of Science and Technology, China. The dried fruits of *Alpinia oxyphylla* were finely powdered with an electric mill and were kept at -20 ºC in the dark until use.

1,1-Diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$), 2,2-azo-*bis*(3-ethylbenzo-thiazoline-6-sulphoic acid) diammonium salt (ABTS$^{\bullet+}$), ascorbic acid, 5-fluorouracil (5-FU), standard samples of β-sitosterol and sulforhodamine B (SRB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other solvents and chemicals were analytical grade and were obtained from Sinopharm Chemical Reagent Co. (Shanghai, China).

The following instruments were used to obtain physical data: UV spectra, Thermo, Evolution 220 spectrometer; IR (KBr disks) spectra, Nicolet 6700 spectrometer; EI-MS or ESI-MS, micromass LCT mass spectrometer; ^{1}H NMR spectra, AVANCE III (400 MHz) spectrometer; ^{13}C NMR spectra, AVANCE III (101 MHz) spectrometer with tetramethylsilane as an internal standard; HPLC detector, Agilent 1200 Series ultraviolet-visible detector.

Following experimental conditions were used for chromatography: Silica gel (Qingdao Haiyang Chemical Co., Ltd., China, 200-300 mesh) and RP-C_{18} (30 um, High Tech Chromatography Co., Ltd, China) were used for column chromatography. Preparative HPLC was carried out using an Elite P270 instrument with a Elite reversed phase C_{18} column (20 × 250 mm, 5 μm) and a UV 230 II ultraviolet-visible detector.

Isolation and identification of active constituents: The ethyl acetate fraction (110 g) was obtained from the 95 % ethanol extract of *Alpinia oxyphylla* fruits by liquid-liquid partition and then subjected to a silica gel column chromatography (CC) eluted with petroleum ether-ethyl acetate (4:1, 1:1, 1:3, 0:1, v/v) to afford four pooled fractions (A, B, C and D), respectively. The fraction A (63.8 g) eluted with a gradient of petroleum ether-ethyl acetate (1:0 to 4:1, v/v) on a silica gel CC to give four fractions (A1-A4). A1 was chromatographed continuously into five fractions (A1.1-A1.5) using reversed phase C_{18} (RP-C_{18}) CC with a gradient of MeOH-H_2O (1:1 to 19:1, v/v). A1.3 was further separated by a silica gel CC [petroleum ether-ethyl acetate (4:1 to 1:4, v/v)] to give 1 (107 mg). A2 was separated by RP-C_{18} CC [MeOH-H_2O (2:3 to 9:1, v/v)] to obtain four fractions (A2.1- A2.4). A2.3 was separated by a silica gel CC [petroleum ether-CH_2Cl_2-Me_2CO, (3:1:0.1, v/v)] to furnish six fractions (A2.3.1- A2.3.6). A2.3.4 was further purified by preparative HPLC using MeOH-H_2O (7:3, v/v, 10 mL/min) as the mobile phase to give 2 (20 mg). A3 was separated by RP-C_{18} CC [MeOH-H_2O (3:7 to 4:1, v/v)] to furnish six fractions (A3.1-A3.6). A3.4 was separated by a silica gel CC [CH_2Cl_2-Me_2CO (8:1, v/v)] to afford four fractions (A3.4.1-A3.4.4). A3.4.4 was further purified by crystallization in $(CH_3)_2CO$ to afford 3 (220 mg). A4 was separated by a silica gel CC eluting with MeOH-H_2O (2:8 to 7:3, v/v) to yield ten fractions (A4.1-A4.10). A4.3 was subjected to a silica gel CC [petroleum ether-ethyl acetate (10:1 to 0:1, v/v)] to afford four fractions (A4.3.1-A4.3.4). A4.3.1 was further purified by a silica gel CC [petroleum ether-ethyl acetate (4:1 to 1:4, v/v)] to yield 4 (88 mg). A4.3.3 was sequentially extracted with petroleum ether and methanol, using liquid-liquid partition, to obtain two extracts, then the methanol extract was concentrated under reduced pressure to give 5 (65 mg). A 4.4 was subjected to a silica gel CC [petroleum ether-ethyl acetate (17:1 to 1:1, v/v)] to afford four fractions (A4.4.1-A4.4.4). A4.4.2 was further purified by preparative HPLC using MeOH-H_2O (5:5, v/v, 10 mL/min) as the mobile phase to yield 6 (9 mg). A4.6 was subjected to a silica gel CC [petroleum ether-ethyl acetate (12:1 to 1:1,v/v)] to afford six fractions (A4.6.1-A4.6.6). A4.6.3 was further separated by preparative HPLC [MeOH-H_2O (6:4, v/v, 10 mL/min)] to give 7 (37 mg).

The structure of β-sitosterol (**3**) was identified by its identical chromatographic behavior with authentic samples. The structure of other isolated compounds **1**, **2**, **4**, **5**, **6** and **7** were identified by comparison of their spectral data (^{1}H NMR, ^{13}C NMR and EI-MS or ESI-MS) with those from the corresponding values in the literatures as nootkatone[23], tectochrysin[24], chrysin[15], protocatechuic acid[25], (4S*, 5E, 10R*)-7-oxo-trinor-eudesm-5-en-4β-ol[26] and (4R,6R,10S)-4,10-dimethyl-6-(1'-hydroxyis-opropyl)-1-en-3,4,5,6,7,8-hexahydronaphthalen-2-one[27], respectively.

Antioxidant activity: The ability of the samples to scavenge DPPH$^{\bullet}$ was determined by the described method[28]. Briefly, the samples were dissolved in ethanol solution to form sample solutions in final concentrations of 12.5 to 200 μg/mL for fractions and 25 to 400 μg/mL for compounds. Then 2 mL of the sample solution at different concentrations was mixed with 2 mL of 0.1 mmol/L DPPH ethanol solution. The solution was well mixed and then left at room temperature for 0.5 h in the dark. The absorbance of the resulting solution was read at 517 nm. The radical scavenging activity was calculated as a percentage of DPPH discoloration using the equation:

$$\text{DPPH}^{\bullet}\text{ scavenging activity (\%)} = \left(\frac{(A_{control} - A_{sample})}{A_{control}}\right) \times 100$$

where $A_{control}$ is the absorbance of the blank and A_{sample} is the absorbance in the presence of the sample at different concentrations. Ascorbic acid is used as the positive control. All analyses were run in triplicate. IC_{50} values calculated denote the concentration of a sample required to decrease the absorbance at 517 nm by 50 %.

The ABTS$^{\bullet+}$ radical scavenging activities of the samples were determined according to the method of the literature[29]. ABTS$^{\bullet+}$ radical solution was produced by mixing ABTS$^{\bullet+}$ aqueous solution (final concentration 7 mmol/L) with potassium persulphate (final concentration 2.45 mmol/L) and the mixture was incubated in the dark at room temperature for 12-16 h. After incubation, the ABTS$^{\bullet+}$ radical solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. The samples were dissolved in ethanol solution to form sample solutions in final concentrations of 12.5 to 200 μg/mL for fractions and 25 to 400 μg/mL for compounds. Then 0.1 mL of the sample solution at different concentrations was mixed with 0.9 mL of the ABTS$^{\bullet+}$ radical solution and the mixture was allowed to stand at room temperature for 10 min in the dark. The absorbance was measured at 734 nm. The scavenging activity on ABTS$^{\bullet+}$ radical was calculated by the following formula:

$$\text{ABTS}^{\bullet+}\text{ radical scavenging activity (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

where $A_{control}$ is the absorbance of the blank and A_{sample} is the absorbance in the presence of the sample at different concentrations. Ascorbic acid is used as the positive control. All analyses were run in triplicate. IC_{50} values calculated denote the concentration of a sample required to decrease the absorbance at 734 nm by 50 %.

Cytotoxic activity: Human liver carcinoma cell line (HepG2), human lung adenocarcinoma cell line (A549), human cervix carcinoma cell line (Hela), human breast cancer cell line (MCF-7), human gastric cancer cell line (MNK-45), human colon cancer cell line (SW480) and mouse embryonic fibroblast cell line (NIH/3T3) were obtained from the Committee of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). These cell lines were cultured in a high glucose concentration (4.5 g/L) DMEM medium (Biochrom AG, Germany) with 10 % fetal bovine serum (Biochrom AG, Germany), 1 % penicillin-streptomycin (100 IU-100 µg/mL, Hyclone, USA) and adjusted to a concentration of 1×10^6 cells/mL in a humidified incubator at 37 °C and in 5 % CO_2 atmosphere. The effects of fractions and compounds on cell growths were according to the SRB assay procedures[30]. In brief, After being harvested from culture flasks, the cells (1×10^4) were seeded in each well of a 96-well plate containing 100 µL fresh growth medium and permitted to adhere for 24 h. The samples were dissovled in DMSO and further diluted with culture medium to form sample solutions in final concentrations of 12.5 to 200 µg/mL. Then add 200 µL of each concentration of test sample in culture medium per well. The plates were incubated at 37 °C for 48 h, then cells were fixed by layering 100 µL of ice-cold 10 % trichloroacetic acid (TCA, Aldrich Chemical) on top of the growth medium. Cells were incubated at 4 °C for 1 h, after which plates were washed five times with distilled water, the excess water drained off and the plate left to dry in air. SRB stain (100 µL) was added to each well and left in contact with the cells for 0.5 h, after which they were washed with 1 % acetic acid, rinsed five times until only dye adhering to the cells was left. The plate was dried and 100 µL of 10 mM *tris* base (pH 10.5) were added to each well to solubilise the dye. The optical density (OD) of each well was read on a 96-well microtiter plate reader at 492 nm. 5-FU was used as the positive control. Cytotoxicity was expressed as the concentration of the samples inhibiting cell growth by 50 % (IC_{50}). The IC_{50} values were obtained using a non-linear dose-response curve fitting analysis *via* OriginPro v.8.0 software.

RESULTS AND DISCUSSION

Bioassay-guided fractionation: Since previous study[13] has demonstrated that the ethyl acetate fraction (EF) possessed potent antioxidant and anticancer activities, a silica gel column chromatography was performed to obtain four pooled subfractions (A, B, C and D) in this work. Antioxidant and cytotoxic activities of the fractions were tested by DPPH$^\bullet$ assay, ABTS$^{\bullet+}$ assay and SRB assay. As summarized in Table-1, the most potent DPPH$^\bullet$ scavenging activity was achieved with fraction A with IC_{50} value of 25.8 µg/mL, followed by B (65.4 µg/mL), ethyl acetate fraction (EF) (68.7 µg/mL), C (75.2 µg/mL) and D (110.5 µg/mL). The ABTS$^{\bullet+}$ radical scavenging activity of fractions had the same trend (A > B > EF > C > D) as their DPPH$^\bullet$ scavenging activity. Among the fractions, fraction A had the highest activity with IC_{50} value of 30.9 µg/mL which was comparable to that of ascorbic acid, the positive control (31.3 µg/mL). In SRB assay, the IC_{50} values of the fractions and 5-FU (positive control) on A549 and SW480 cells were provided in Table-2. Fraction A exhibited the strongest growth inhibition effect on A549 and SW480 cells with IC_{50} values of 39.3 and 55.1 µg/mL, respectively. The cytotoxicity of different fractions decreased with the following order: A > B ≥ C > EF > D. In a word, fraction A showed the highest antioxidant and cytotoxic activities, indicating that the antioxidant and anticancer compounds were probably concentrated in this fraction.

TABLE-1
DPPH$^\bullet$ AND ABTS$^{\bullet+}$ RADICAL SCAVENGING ACTIVITIES (IC_{50}) OF THE ETHYL ACETATE FRACTION AND ITS SUBFRACTIONS (A, B, C AND D)

Samples	IC_{50} (µg/mL)	
	DPPH$^\bullet$	ABTS$^{\bullet+}$
EF[a]	68.7 ± 1.5	82.2 ± 2.0
A	25.8 ± 2.5	30.9 ± 0.8
B	65.4 ± 2.5	77.2 ± 2.4
C	75.2 ± 1.2	89.7 ± 2.1
D	110.5 ± 1.4	136.2 ± 2.0
Ascorbic acid[b]	27.7 ± 0.7	31.3 ± 0.7

Data are means ± SD of three independent experiments;
[a]EF was the ethyl acetate fraction; [b]Positive control

TABLE-2
CYTOTOXICITY (IC_{50}) OF THE ETHYL ACETATE FRACTION AND ITS SUBFRACTIONSON (A, B, C AND D) ON A549 AND SW480 CELL LINES

Cell lines	IC_{50} (µg/mL)					
	EF[a]	A	B	C	D	5-FU[b]
A549	69.4 ± 1.6	39.3 ± 2.4	55.7 ± 2.6	55.4 ± 0.7	>150	7.2 ± 4.3
SW480	95.4 ± 2.0	55.1 ± 1.1	61.3 ± 1.7	64.3 ± 0.9	>100	0.8 ± 2.6

Data are means ± SD of three independent experiments;
[a]EF was the ethyl acetate fraction; [b]positive control

Consequently, further detailed phytochemical analysis of fraction A using chromatography technique led to the isolation of seven major compounds presented in Fig. 1: Nootkatone (**1**), tectochrysin (**2**), β-sitosterol (**3**), chrysin (**4**), protocatechuic acid (**5**), (4S*, 5E, 10R*)-7-oxo-tri-nor-eudesm-5-en-4β-ol (**6**) and (4R, 6R, 10S)-4,10-dimethyl-6-(1'-hydroxy-isopropyl)-1-en-3,4,5,6,7,8-hexahydronaphthalen-2-one (**7**), of which compounds **6** and **7** were isolated firstly from this plant.

Antioxidant activity of isolated compounds: The antioxidant activities of seven isolated compounds and ascorbic acid (positive control) were evaluated using two methods based on the free radical scavenging capacity, namely the DPPH$^\bullet$ scavenging assay and the ABTS$^{\bullet+}$ radical scavenging assay. The results showed that compounds **5**, **6** and **7** presented antioxidant activity, while other compounds were nearly inactive on free radical scavenging activities at the indicated concentrations. As shown in Fig. 2a, the DPPH$^\bullet$ scavenging activities of compounds **6** and **7** were found to be weak. However, protocatechuic acid (**5**) exhibited potent free radical scavenging

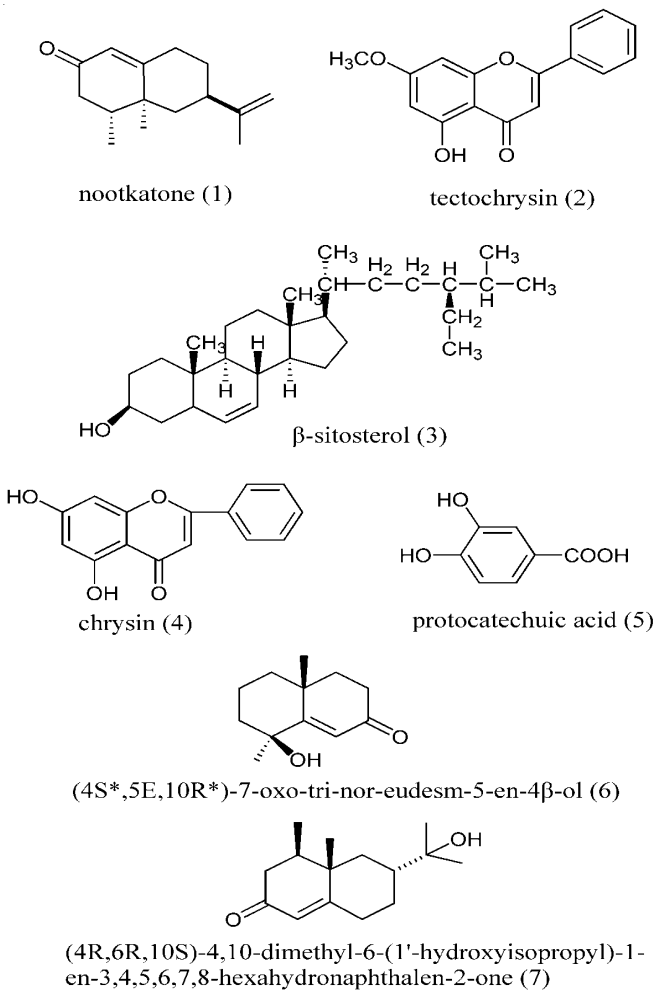

Fig. 1. Molecular structures of the isolated compounds

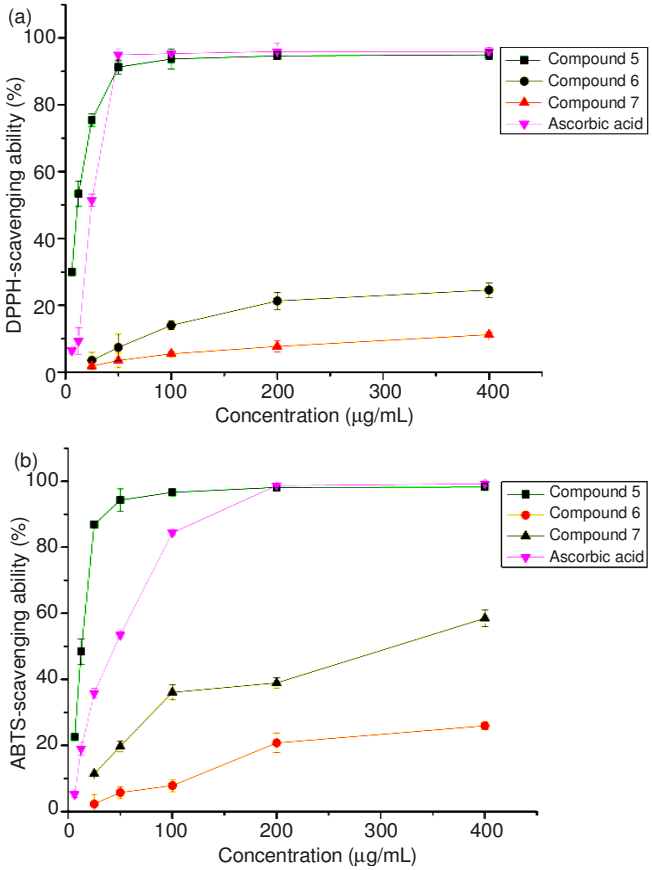

Fig. 2. (a) DPPH and (b) $ABTS^{\bullet+}$ radical scavenging activities of compounds **5**, **6**, **7** and ascorbic acid. Compounds **5**, **6** and **7** were protocatechuic acid, (4S*, 5E, 10R*)-7-oxo-tri-nor-eudesm-5-en-4β-ol and (4R, 6R, 10S)-4,10-dimethyl-6-(1'-hydroxyisopropyl)-1-en-3,4,5,6,7,8-hexahydronaphthalen-2-one, respectively

activity on $DPPH^{\bullet}$ with scavenging rate was 91.30 % at 50 μg/mL, which approximately had the same activity as ascorbic acid. As summarized in Fig. 2b, the $ABTS^{\bullet+}$ radical was scavenged by compounds **5**, **6** and **7** in a dose dependent manner and the relatively low scavenging rates of compounds **6** and **7** were observed. Whereas the scavenging rate of protocatechuic acid (**5**) was 94.32 % at 50 μg/mL, which was higher than that of ascorbic acid. These results suggested that compounds **5**, **6** and **7** might be the primary active chemical components in the ethyl acetate fraction responsible for its potent antioxidant activity.

Cytotoxicity of isolated compounds: The cytotoxicity of the isolated compounds and 5-FU (positive control) on six human cancer cell lines (HepG2, A549, HeLa, MCF-7, MNK-45 and SW480) and mouse embryonic fibroblast cell line (NIH/3T3) were tested using SRB assay. As shown in Table-3, compounds **1**, **3**, **4** and **5** were found to possess potent cytotoxicity on different cancer cells. However, compounds **2**, **6** and **7** did not exhibit significant inhibitory effects on cells growth at the indicated concentrations. Compared with 5-FU, chrysin (**4**) exhibited very higher cytotoxicity against six human cancer cell lines (HepG2, A549, HeLa, MCF-7, MNK- 45 and SW480) with IC_{50} values of 19.6, 9.2, 5.9, 16.6, 6.9 and 11.3 μg/mL, respectively. Nootkatone (**1**) had stronger cytotoxicity towards HepG2 and MNK-45 cells and weaker cytotoxicity against SW480, MCF-7, HeLa and A549 cells than 5-FU. β-Sitosterol (**3**) was only sensitive to HepG2 and SW480 cells and it was hardly cytotoxic to other four human cancer cell lines at the indicated concentrations. Protocatechuic acid (**5**) displayed very pronounced cytotoxicity on two cancer cell lines (HeLa and MCF-7) with IC_{50} values of 4.3 and 1.8 μg/mL, respectively. However, it was not sensitive to the other four cancer cell lines. Though the above four compounds revealed different cytotoxicity on six human cancer cell lines, they had superiority to 5-FU against some kinds of cancer cells. Table-3 showed that all these four compounds exhibited very weak activity against NIH/3T3 cell (IC_{50} > 100 μg/mL) and calculation of the IC_{50} values for nootkatone, β-sitosterol, chrysin, protocatechuic acid confirmed that they have ability to kill cancer cells but exert little damage as possible to normal cell. This effect indicated that nootkatone, β-sitosterol, chrysin, protocatechuic acid were probably the active chemical componentents in the ethyl acetate fraction responsible for its anticancer effects.

Conclusion

In the present study, a bioactivity-guided fractionation process led us to identify seven compounds from the ethyl acetate fraction of *Alpinia oxyphylla* fruits. Among them compounds **6** and **7** were isolated firstly from this plant. Furthermore, we provided evidence that compounds **5**, **6** and **7** were isolated as the major antioxidant components and compounds **1**, **3**, **4** and **5** were responsible for the anticancer

TABLE-3
CYTOTOXICITY (IC_{50}) OF THE ISOLATED COMPOUNDS[a] AGAINST SIX HUMAN CANCER CELL LINES AND NIH/3T3 CELL LINE

Cell lines	IC_{50} (μg/mL)				
	1	3	4	5	5-FU[b]
HepG2	8.9 ± 1.7	72.0 ± 2.0	19.6 ± 1.0	>100	25.6 ± 3.3
A549	26.6 ± 2.2	>100	9.2 ± 0.8	>100	7.2 ± 1.6
HeLa	28.3 ± 0.6	>100	5.9 ± 0.9	4.3 ± 1.5	12.2 ± 1.9
MCF-7	32.7 ± 1.3	>100	16.6 ± 2.6	1.8 ± 2.1	5.8 ± 1.7
MNK-45	10.5 ± 0.8	>100	6.9 ± 1.9	>100	19.7 ± 2.8
SW480	41.3 ± 3.1	50.5 ± 2.2	11.3 ± 2.5	>100	0.8 ± 1.2
NIH/3T3	>100	>100	>100	>100	>100

[a]All isolated compounds were examined in a set of experiments repeated three times, and compounds **1**, **3**, **4** and **5** were nootkatone; β-sitosterol, chrysin, protocatechuic acid, respectively; [b]5-FU was used as the positive control

properties. Therefore, the above active components from the ethyl acetate fraction of *Alpinia oxyphylla* fruits might be used in the pharmaceutical products and functional foods. However, the precise molecular mechanism underlying the antioxidant and anticancer effects of these compounds have still to be further elucidated.

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