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Cytotoxic Activities of Extracts and Compounds from *Viscum coloratum* and its Transformation Products by *Rhodobacter sphaeroides*

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Abstract The bioassay-oriented fractionation of mistletoe crude extracts (MCEE) using 75% ethanol and culture products of mistletoe transformed by *Rhodobacter sphaeroides*, a photosynthetic bacterium (PSBT), revealed that the high cytotoxic activities were due to the petroleum ether extracts (PEs) and the acid-precipitated proteins from the aqueous extracts (AQs) of MCEE and PSBT. The isolated triterpenes may account for the activities of the PEs of MCEE and PSBT, respectively. Extraction of MCEE using petroleum ether led to the isolation of 3-*epi*-betulinic acid (**1**), betulonic acid (**2**), oleanolic acid (**3**), and β-amyrin acetate (**4**), while petroleum ether extraction of PSBT led to the isolation of 1,3,4,betulinic acid (**5**), erythrodiol (**6**), and (3β)-olean-12-ene-3,23-diol (**7**). The PE of PSBT exerted higher cytotoxicity than the PE of MCEE, which was due to the different triterpene contents of these two extracts. The cytotoxic activities of all compounds were tested, and the results revealed that compounds **1**, **2**, **3**, **5**, **6**, and **7** contributed significantly to the cytotoxicities of both PEs. The AQ of the PSBT exerted almost the same cytotoxic activity and lower toxicity compared to the AQ of the MCEE. These findings indicate that mistletoe products biotransformed by *R. sphaeroides* could be used to treat cancers, since they have lower toxicities and higher antitumor activities compared to standard treatments.

Keywords *Viscum coloratum* (Kom.) Nakai · Mistletoe · Photosynthetic bacteria (PSB) · *Rhodobacter sphaeroides* · Cytotoxic activity · Biotransformation

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Introduction

Mistletoe is a perennial semiparasitic dwarf shrub; and since the 1920s, it has been used for the treatment of cancers, such as breast cancer, gastric cancer, rectal cancer, etc. [1]. With the development of complementary or alternative medicine (CAM) around the world, many countries, including South Korea in Asia [2], Nigeria in Africa [3], and Argentina in South America [4], have begun to carry out studies on their local mistletoe species.

Of the more than 30 species of mistletoe known, 11 grow in various regions of China [5]. *Viscum coloratum* (Kom.) Nakai is the only species found in Northern China, while more than four species are found in the south, such as *V. articulatum* Burm.f., *V. liquidambaricolum* Hayata, *V. nudum* Danser, etc. [5]. Only *V. coloratum* (Kom.) Nakai can be found in the China Pharmacopoeia and is used as a Chinese folk medicine in the form of decoctions to treat cancer. It has been reported that lectins and alkaloids are the main antitumor constituents of mistletoe [6, 7].

Rhodobacter sphaeroides is a type of photosynthetic bacteria (PSB) which has various enzymes and active metabolites [8–11]. There have been a few reports on biotransformation using PSB in the literature [12–14], but none have been conducted on mistletoe. Previous investigations have shown that the culture products of mistletoe transformed by *R. sphaeroides* (PSBT) are more potent inhibitors of human cancer cell growth on the cell lines: BGC-803, MCF7, A2780, and KB in vitro and on the implanted tumors: S_{180} sarcoma, H_{22} liver carcinoma, and Lewis lung carcinoma in vivo. In addition, PSBT have lower toxicities compared to mistletoe extracts [15].

In this study, we used *R. sphaeroides* to convert *V. coloratum* (Kom.) Nakai extract using traditional Chinese fermentation theory and modern biotransformation technology. The bioassay-oriented isolation of mistletoe crude extracts (MCEE) using 75% ethanol and PSBT are discussed in this paper, together with the characterization and evaluation of the cytotoxicities of the isolated compounds.

Materials and Methods

General

Melting points were recorded using a X-4A micromelting instrument with uncorrected temperatures. ^{1}H NMR and ^{13}C NMR spectra were recorded on a JEOL AL-300 NMR spectrometer. Electrospray ionization–mass spectrometry (ESI-MS) was obtained on a ABI Qstar mass spectrometer. Silica gel (200–300 mesh, Qingdao Marine Chemical Factory) and Sephadex LH-20 (Pharmacia) were used for chromatographic separations.

Plant Material

V. coloratum (Kom.) Nakai was purchased from the Chengde Medicinal Company of China National Group Corporation of Traditional and Herbal Medicine in July 2005. The plant was originally collected from Chengde, Hebei Province, China. The specimen was identified by Professor Jianping Gao and deposited in the Department of Traditional Chinese Medicine, School of Pharmaceutical Sciences, Shanxi Medical University.

Microorganism

R. sphaeroides was obtained from the College of Life Science and Technology, Shanxi University, China. It was cultivated in medium containing (per liter): 2 g sodium malate, 0.15 g $MgSO_4 \cdot 7H_2O$, 1.2 g yeast extract, and 1.5 g $(NH_4)_2SO_4$ with the pH adjusted to 6.8–7.0 [16]. Cultures were incubated at 30 °C with illumination under anaerobic conditions. Stock cultures were stored at 4 °C.

Cell Culture and Assay for Cytotoxic Activity

The human cancer cell lines HO-8910, SMMC-7721, T24, HepG2, and SHG were obtained from Shanxi Medical University. All of the cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum and 1% L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. After digestion with 0.25% trypsin solution, the cells were washed and resuspended in the above medium at a concentration of 5×10^4 cells/mL. Next, 180 μL of this cell suspension was placed in each well of a 96-well flat-bottom plate. The cells were incubated for 24 h at 37 °C in 4% CO_2. All test samples were dissolved in dimethyl sulfoxide (DMSO) and the final concentration of DMSO in the culture medium was less than 0.5% (*v*/*v*). The reference compound 5-FU was dissolved in phosphate buffered saline (PBS). After incubation, 20 μL of the sample solution was added to the medium, and the cells were incubated for a further 48 h in the presence of each agent. After termination of cell culture, 25 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in PBS was added to each well, and the plate was again incubated at 37 °C in 4% CO_2 for an additional 4 h. Next, 150 μL of DMSO was added to dissolve the formazan crystals after the supernatant of each well was removed. The plate was shaken on a microshaker for 10 min, then read on a microplate reader (NovapathTM, Japan) at 595 nm. Each sample and corresponding DMSO control was tested in quadruplicate and repeated three times in separate experiments. Growth inhibition was estimated as the reduction of value from a DMSO control and the results are expressed as mean 50% lethal concentrations (IC_{50}) of three different experiments [17, 18].

Extraction and Fractionation Procedure

Preparation of Plant Extracts

A sample of 2 kg of dried powdered leaves and twigs of *V. coloratum* (Kom.) Nakai was extracted with boiling 75% ethanol three times. The extract was evaporated under reduced pressure using a rotary evaporator to give 366 g of MCEE (16.8% yield).

Preparation of PSBT

Half of the MCEE (168 g) was biotransformed using a previously reported method to give PSBT (202 g) [16].

Isolation and Structure Elucidation of Compounds

Half of the MCEE (168 g) was suspended in water, and then consecutively extracted with petroleum ether, chloroform, acetic ether, and *n*-butanol to afford four extracts (PE, CH,

AC, and BU), and the remaining water solution. The PSBT was extracted using the same method as MCEE to afford four extracts (PE, CH, AC, and BU), and the remaining water solution. The two water solutions were each adjusted to pH 8.0 with 5 N NaOH. Each mixture was stirred for 1 h at 30 °C, and then centrifuged at 5,000 rpm for 10 min to obtain the supernatant and precipitate. Each supernatant was adjusted to pH 4.8 with 3.5 $(NH_4)_2SO_4$ to precipitate the proteins. Each suspension was centrifuged at 5,000 rpm for 10 min to yield the acid-precipitated proteins (AQs) of MCEE and PSBT, respectively. Each of the ten extracts (two PEs, two CHs, two ACs, two BUs, and two AQs) were evaporated to dryness. Their cytotoxic activities (IC_{50} in micrograms per milliliter) were evaluated using the human cell lines HO-8910, SMMC-7721, T24, HepG2, and SHG by the MTT assay (Table 1). In order to evaluate the contribution of the PEs and AQs to the activities of MCEE and PSBT, their effects were compared with that of the virtual total extracts (VTE), prepared by pooling PE, CH, AC, BU, and AQ together, according to their respective extraction yields (Fig. 1). The two PEs were separated by silica gel column chromatography (CC), and eluted with petroleum ether–acetone in a gradient manner to give seven fractions (Fr1–Fr7), respectively. Each of the 14 fractions was screened using the bioassay (Table 2). Four compounds were obtained from Fr3 to Fr5 of PE of MCEE and six compounds were obtained from Fr3 to Fr5 of PE of PSBT through further separation by repeated CC on silica gel and Sephadex LH-20. In order to know if all the principal compounds from the PEs of MCEE and PSBT were isolated, thin layer chromatography (TLC) analysis was performed (Fig. 2). All of the compounds were identified by comparison of their spectral data (MS, IR, ^{1}H NMR, and ^{13}C NMR) with literature values and screened using the bioassay (Table 3).

TLC

The solutions were spotted on silica gel plates, which were developed using chloroform–methanol (10:0.05), and visualized by spraying with 10% H_2SO_4 solution, followed by heating at 110 °C for 10 min.

Quantitative Determination of Total Triterpene Content

The ten extracts were analyzed for their total triterpene content according to the spectrophotometry method [32]. The results (total triterpenes) were expressed as the amount of oleanolic acid (milligrams per gram extract, mean±SD of three determinations).

Acute Toxicity Tests

The two PEs and two AQs were submitted to acute toxicity testing according to the intragastric administration method [15]. The results were expressed as the mean 50% inhibition concentrations (LD_{50}). Healthy Kunming mice of both sexes, weighing 20–30 g, were obtained from the Animal Center of Shanxi Medical University.

Protein Estimation

Quantitative protein analysis was performed using the Coomassie Brilliant Blue G-250 method using bovine serum albumin as the standard [33].

Table 1 The contents of total triterpenes and total proteins in the ten extracts from the MCEE and PSBT and the cytotoxic activities of the ten extracts (IC_{50} in micrograms per milliliter) from the MCEE and PSBT.

	MCEE					PSBT				
	PE	CH	AC	BU	AQ	PE	CH	AC	BU	AQ
Weight (g)	21.6	21.9	6.7	5.4	12.6	22.2	30.6	2.5	29.5	10.2
Yield (% *w*/*w* with respect to dry plant material)	2.2	2.2	0.7	0.5	1.3	2.2	3.1	0.3	3.0	1.0
Triterpene content (% *w*/*w*)	25.8±1.1	1.4±0.3	0	20.4±2.3	1.1±0.2	47.3±2.1	6.9±0.9	0	11.9±1.3	3.1±0.6
Protein content (% *w*/*w*)	0	0	0	0	52.4±2.5	0	0	0	0	57.6±2.8
HO-8910	79.8±2.1	340.5±10.9	465.3±12.2	125.3±6.5	18.6±2.4	60.5±1.2	90.6±4.3	400.2±10.2	310.5±9.8	19.2±2.6
SMMC-7721	82.1±2.6	130.5±5.2	140.2±6.5	904.9±7.4	32.1±3.2	71.6±3.2	126.8±6.9	201.2±11.2	>1,000	31.5±4.2
T24	>1,000	–	–	–	506.5±8.2	802.2±16.5	–	–	–	216.5±12.6
HepG2	–	–	–	–	>1,000	–	–	–	–	>1,000
SHG	96.3±3.8	–	203.5±9.4	–	88.6±6.4	99.3±2.6	–	206.8±12.3	–	90.4±6.5

Each value represents the mean±SD of three independent determinations

MCEE mistletoe crude ethanol extract, *PSBT* culture products of mistletoe transformed by *R. sphaeroides*, *PE* petroleum ether extract, *CH* chloroform extract, *AC* acetic ether extract, *BU* *n*-butanol extract, *AQ* aqueous extract, – not active

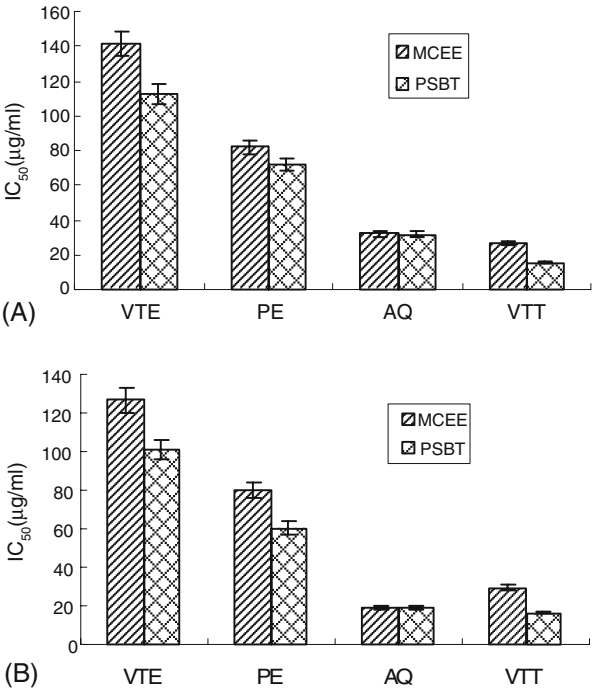

Fig. 1 The cytotoxic activities of VTE, PE, AQ, and VTT in a HO-8910 cells and b SMMC-7721 cells. MCEE mistletoe crude ethanol extract, PSBT culture products of mistletoe transformed by R. sphaeroides, VTE virtual total extracts prepared by pooling the PE, CH, AC, BU, and AQ extracts according to the respective extraction yields, PE petroleum ether extract, CH chloroform extract, AC acetic ether extract, BU n-butanol extract, AQ aqueous extract, VTT virtual total triterpenoids prepared by pooling the compounds according to the compound yields. Each value represents the mean±SD of three independent determinations

SDS-PAGE of the Two AQs

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli, using a 10% SDS-PAGE gel and a discontinuous system as previously described [33]. Protein bands were detected using Coomassie Brilliant Blue R-250 staining.

Table 2 The cytotoxic activities of the 14 fractions (IC_{50} in micrograms per milliliter) from the PEs of MCEE and PSBT.

	PE of MCEE							PE of PSBT						
	Fr1	Fr2	Fr3	Fr4	Fr5	Fr6	Fr7	Fr1	Fr2	Fr3	Fr4	Fr5	Fr6	Fr7
Weight (g)	2.40	0.23	3.06	0.21	0.22	0.21	0.01	0.36	2.90	0.40	1.26	2.39	1.07	0.38
HO-8910	>100	>100	80.2± 3.6	45.5± 5.2	99.6± 3.5	>100	>100	>100	>100	68.8± 6.5	41.2± 3.6	80.1± 5.2	>100	>100
SMMC-7721	>100	>100	85.4± 3.9	57.6± 6.2	87.8± 5.2	>100	>100	>100	>100	86.9± 4.3	45.7± 6.1	76.5± 5.3	>100	>100

Each value represents the mean±SD of three independent determinations

PE petroleum ether extract, *MCEE* mistletoe crude ethanol extract, *PSBT* culture products of mistletoe transformed by *R. sphaeroides*, *Fr3–Fr5* fraction 3 to fraction 5 from the PE of MCEE and the PE of PSBT

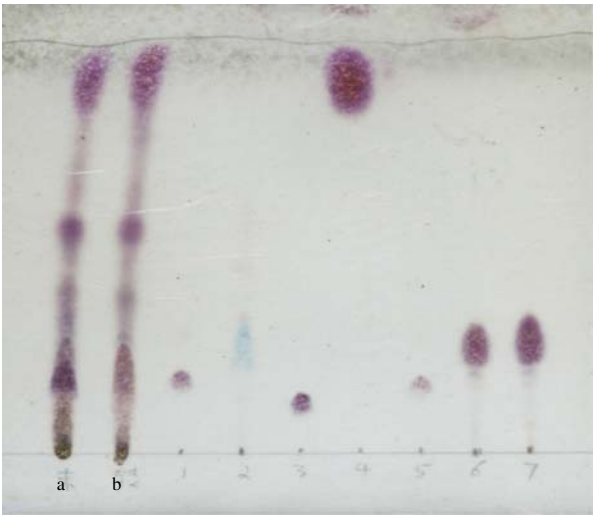

Fig. 2 TLC analysis of the PEs of MCEE and PSBT and the seven triterpenoids. *a* PE of MCEE, *b* PE of PSBT, *1* 3-*epi*-betulinic acid, *2* betulonic acid, *3* oleanolic acid, *4* β-amyrin acetate, *5* betulinic acid, *6* erythrodiol, *7* (3β)-olean-12-ene-3,23-diol

Statistics

Data were expressed as the mean±standard error of the mean. Statistical analysis was performed using SPSS10.0 for analysis of variance followed by Dunnett's test. *P* values lower than 0.05 were considered as statistically significant.

Results and Discussion

Bioassay-Oriented Fractionation and Compound Isolation

The weights and yields of the ten fractions (two PEs, two CHs, two ACs, two BUs, and two AQs) obtained from MCEE and PSBT, as well as the concentrations of total triterpenes and total proteins and their cytotoxic activities (IC_{50} in micrograms per milliliter), are shown in Table 1. The PEs and AQs were the most potent inhibitors of cell growth in HO-8910 and SMMC-7721 cells with IC_{50} values of 79.8 and 82.1 μg/mL and 60.5 and 71.6 μg/mL,

Table 3 The cytotoxic activities of the seven compounds (IC_{50} in micrograms per milliliter) from the PEs of MCEE and PSBT.

	Oleanolic acid	β-Amyrin acetate	3-*epi*-betulinic acid	Betulonic acid	Betulinic acid	Erythrodiol	(3β)-Olean-12-ene-3,23-diol	5-FU
HO-8910	6.7±0.5	–	25.0±0.3	15.1±0.2	>50	25.4±0.3	28.3±0.8	31.6±1.6
SMMC-7721	17.7±0.9	>50	12.1±0.4	>50	>50	31.6±0.5	>50	>50

Each value represents the mean±SD of three independent determinations

PE petroleum ether extract, *MCEE* mistletoe crude ethanol extract, *PSBT* culture products of mistletoe transformed by *R. sphaeroides*

respectively, while the CHs, ACs, and BUs were less cytotoxic. Additionally, the obtained data showed that T24, HepG2, and SHG cell lines were less sensitive to the tested samples. Therefore, further studies to assess the cytotoxic activities of fractions and compounds were performed on HO-8910 and SMMC-7721 cells.

The results (Fig. 1) showed that the PEs and AQs gave the highest contributions to the cytotoxic activities of MCEE and PSBT, which indicated that these two fractions contain the most bioactive constituents. Thus, our research focused on the PEs and AQs.

The bioassay screens of the 14 fractions showed that Fr3–Fr5 from both PEs were moderately cytotoxic against HO-8910 and SMMC-7721 cells, compared to the other fractions (Table 2). Next, these fractions were separated by repeated CC, which led to the isolation of ten compounds in total. Previous literature has reported that 3-*epi*-betulinic acid (**1**) [34], oleanolic acid (**3**) [35, 36], and erythrodiol (**6**) [37] exhibit significant antitumor activities. According to our results, these three compounds exerted high cytotoxicities against both HO-8910 and SMMC-7721 cell lines. These results were consistent with previously published findings (Table 3). In addition, Chiang et al. [38] have reported that betulonic acid (**2**) is a cytotoxic compound; but in this study, it only exhibited cytotoxicity against HO-8910 cells. Furthermore, our data showed that PE of PSBT exerted higher inhibitory activity compared to that of MCEE (Table 1), indicating improved cytotoxicity by using the PSB biotransformation method.

The triterpenes appeared to be largely responsible for the inhibitory effects of MCEE and PSBT with IC_{50} values of 29.5 and 16.5 μg/mL for HO-8910 cells and 26.8 and 15.4 μg/mL for SMMC-7721 cells, respectively (Fig. 1). Moreover, it was shown by TLC that the principal compounds in the PEs of MCEE and PSBT were isolated (Fig. 2). Thus, these findings indicate that compounds **1**–**3** and **5**–**7** contributed significantly to the cytotoxicities of both PEs. Another major compound with R_f value about 0.6 was β-sitosterol, which had also been isolated.

SDS-PAGE of the Two AQ Extracts

When analyzed on an SDS-PAGE gel, the AQ of PSBT showed a multiple protein band pattern with molecular weights ranging from 14 to 90 kDa, which was different from that of the AQ of MCEE (Fig. 3). The differences of protein molecular weights and types suggested that the composition of proteins could be changed by PSB biotransformation. Further investigations on protein separation and identification need to be done in order to specify the bioactive proteins and their biotransformation pathway.

Acute Toxicity Tests

Lectins and toxins are among the bioactive substances in mistletoe [39], which has led to the usage of aqueous or alcoholic extracts from mistletoe as CAM for anticancer treatment in Europe and other countries. However, several papers have reported the danger of using mistletoe products because of their toxicities or side effects [40–42]. Shanker et al. [43] observed that there was a xenobiotics-detoxifying ability in *R. sphaeroides*. According to the acute toxicity test results in this study, PSBT and its AQ were less toxic than MCEE and its AQ (Table 4). Combined with the findings of the SDS-PAGE experiment which showed different protein patterns for the AQs, it may be inferred that the toxicity-lowering effect was due to PSB biotransformation of the toxic proteins. The mechanism is currently under investigation.

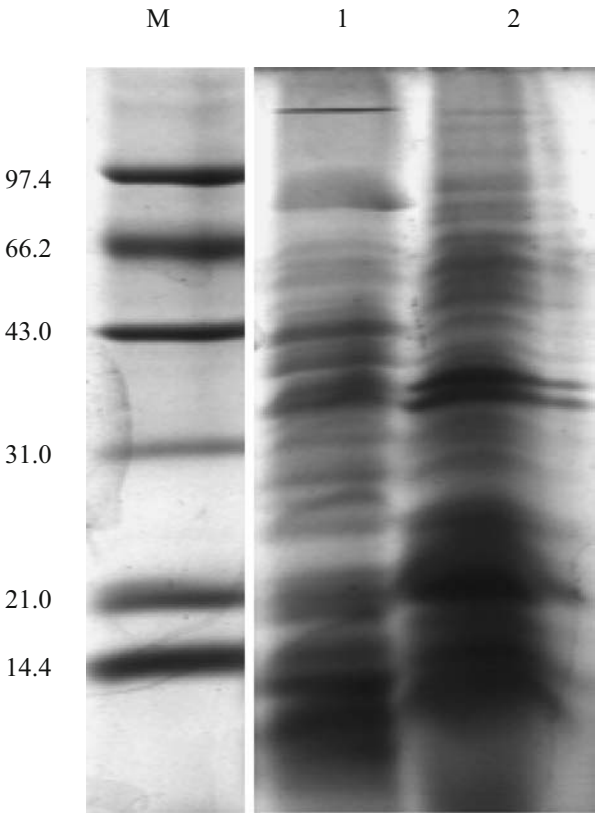

Fig. 3 SDS-PAGE protein patterns from AQ of MCEE (*1*) and AQ of PSBT (*2*). Molecular weight standard indicated (*M*) with the corresponding masses in kilodaltons. *MCEE* mistletoe crude ethanol extract, *PSBT* culture products of mistletoe transformed by *R. sphaeroides*, *AQ* aqueous extract

Conclusion

The cytotoxic activities of extracts and compounds from *V. coloratum* (Kom.) Nakai and its microbial transformation products were investigated in this paper. This was the first report which compared their cytotoxicities. The triterpenes 3-*epi*-betulinic acid (**1**), betulonic acid (**2**), oleanolic acid (**3**), erythrodiol (**6**), and (3β)-olean-12-ene-3,23-diol (**7**) were found to be responsible for this effect.

Table 4 The results of the acute toxicity tests.

	MCEE			PSBT		
	MCEE	PE[a]	AQ	PSBT[b]	PE[c]	AQ[d]
LD_{50} (g/kg)	7.67	–	0.65	–	–	–
95% confidence limits (g/kg)	6.71–8.53	–	0.54–0.75	–	–	–

PE petroleum ether extract, *MCEE* mistletoe crude ethanol extract, *PSBT* culture products of mistletoe transformed by *R. sphaeroides*, *AQ* aqueous extract

[a] No mortality in doses up to 5 g/kg

[b] No mortality in doses up to 10 g/kg

[c] No mortality in doses up to 5 g/kg

[d] No mortality in doses up to 5 g/kg

The results from this study gave scientific support for the use of *V. coloratum* (Kom.) Nakai and its PSB-transformed products in traditional medicine for the treatment of cancer and indicated that the cytotoxicity of mistletoe can be improved by PSB transformation, along with reduced toxicity.

Experimental

The compounds from the PE of MCEE were identified as: 3-*epi*-betulinic acid (**1**, 10.2 mg) [19, 20], betulonic acid (**2**, 12.5 mg) [21, 22], oleanolic acid (**3**, 45.5 mg) [23, 24], and β-amyrin acetate (**4**, 96.4 mg) [25, 26]. The compounds from the PE of PSBT were identified as **1** (10.5 mg), **3** (47.2 mg), **4** (72.5 mg), betulinic acid (**5**, 46.3 mg) [19, 20], erythrodiol (**6**, 30.2 mg) [27, 28], and (3β)-olean-12-ene-3,23-diol (**7**, 19.6 mg) [29–31].

*3-epi-betulinic acid (**1**)* White amorphous powder, $C_{30}H_{48}O_3$, m.p. 280–282 °C, negative ESI-MS *m*/*z* 455 $[M–H]^-$; ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 0.80 (3H, s, H-23), 0.81 (3H, s, H-25), 0.91 (3H, s, H-24, 26), 0.97 (3H, s, H-27), 1.67 (3H, s, H-30), 2.99 (1H, m, H-19), 3.37 (1H, br s, H-3), 4.59, 4.72 (each 1H, s, H-29); ^{13}C NMR (75 MHz, $CDCl_3$, δ, ppm): 33.2 (C-1), 25.4 (C-2), 76.3 (C-3), 37.0 (C-4), 49.0 (C-5), 18.2 (C-6), 34.1 (C-7), 40.8 (C-8), 50.2 (C-9), 37.3 (C-10), 20.7 (C-11), 25.4 (C-12), 38.3 (C-13), 42.5 (C-14), 30.5 (C-15), 32.1 (C-16), 56.4 (C-17), 49.2 (C-18), 46.9 (C-19), 150.4 (C-20), 29.6 (C-21), 37.5 (C-22), 28.3 (C-23), 22.1 (C-24), 16.0 (C-25), 15.9 (C-26), 14.8 (C-27), 181.3 (C-28), 109.7 (C-29), 19.3 (C-30) [19, 20].

*Betulonic acid (**2**)* White powder, $C_{30}H_{46}O_3$, m.p. 244–246 °C, positive ESI-MS *m*/*z* 455 $[M+H]^+$; ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 0.86 (3H, s, H-23), 0.90 (3H, s, H-24), 0.92 (3H, s, H-25), 0.95 (3H, s, H-26), 1.00 (3H, s, H-27), 1.63(3H, s, H-30), 2.95 (1H, m, H-19), 4.55, 4.67 (each 1H, br s, H-29); ^{13}C NMR (75 MHz, $CDCl_3$, δ, ppm): 39.6 (C-1), 34.1 (C-2), 218.3 (C-3), 47.3 (C-4), 54.9 (C-5), 19.6 (C-6), 33.6 (C-7), 40.1 (C-8), 49.8 (C-9), 36.9 (C-10), 21.3 (C-11), 25.4 (C-12), 38.5 (C-13), 42.5 (C-14), 30.5 (C-15), 32.1 (C-16), 56.3 (C-17), 49.1 (C-18), 46.9 (C-19), 150.3 (C-20), 29.7 (C-21), 37.0 (C-22), 26.6 (C-23), 21.0 (C-24), 15.9 (C-25), 15.8 (C-26), 14.6 (C-27), 181.8 (C-28), 109.8 (C-29), 19.3 (C-30) [21, 22].

*Oleanolic acid (**3**)* White powder, $C_{30}H_{48}O_3$, m.p. 288–290 °C, negative ESI-MS *m*/*z*: 455 $[M–H]^-$. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.74 (3H, s, H-23), 0.75 (3H, s, H-24), 0.89 (3H, s, H-25), 0.91 (3H, s, H-26), 0.96 (3H, s, H-29), 0.98 (3H, s, H-30), 1.11 (3H, s, H-27), 3.21 (2H, m, H-3), 5.26 (1H, t, J=3.0 Hz, H-12). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 38.5 (C-1), 27.1 (C-2), 79.0 (C-3), 38.7 (C-4), 55.2 (C-5), 18.3 (C-6), 32.6 (C-7), 3 8.9 (C-8), 47.6 (C-9), 37.0 (C-10), 22.9 (C-11), 122.6 (C-12), 143.5 (C-13), 41.6 (C-14), 27.6 (C-15), 23.4 (C-16), 46.6 (C-17), 41.0 (C-18), 45.8 (C-19), 30.7 (C-20), 33.8 (C-21), 32.4 (C-22), 28.1 (C-23), 15.5 (C-24), 15.3 (C-25), 16.8 (C-26), 26.0 (C-27), 179.1 (C-28), 33.0 (C-29), 23.6 (C-30) [23, 24].

*β-amyrin acetate (**4**)* Colorless needles, $C_{32}H_{52}O_2$, m.p. 230–232 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.83 (3H, s, Me), 0.87 (12H, s, Me), 0.96 (6H, s, 2×Me), 1.13 (3H, s, Me), 2.05 (3H, s, OAc), 4.50 (1H, t, J=7.5Hz, H-3), 5.18 (1H, t, J=3.6 Hz, H-12). ^{13}C NMR (75MHz, $CDCl_3$) δ: 41.0 (C-1), 26.2 (C-2), 83.6 (C-3), 40.4 (C-4), 57.9 (C-5), 21.0 (C-6), 35.2 (C-7), 42.3 (C-8), 50.2 (C-9), 39.8 (C-10), 28.6 (C-11), 124.3 (C-12), 147.8 (C-13),

44.4 (C-14), 31.1 (C-15), 28.8 (C-16), 35.3 (C-17), 49.9 (C-18), 49.5 (C-19), 33.7 (C-20), 37.4 (C-21), 39.5 (C-22), 30.7 (C-23), 19.5 (C-24), 18.2 (C-25), 19.4 (C-26), 26.2 (C-27), 29.6 (C-28), 36.0 (C-29), 26.4 (C-30), 173.5 (–C=O), 24.0 (CH_3COO) [25, 26].

*Betulinic acid (**5**)* White needle crystals, $C_{30}H_{48}O_3$, m.p. 261–262 °C, negative ESI-MS *m/z* 455 $[M–H]^-$;^{1}H NMR (300 MHz, $CDCl_3$) δ:0.74 (3H, s, H-24), 0.80 (3H, s, H-25), 0.91 (3H, s, H-23), 0.95 (6H, s, H-26, 27), 1.67 (3H, s, H-30), 3.01 (1H, m, H-19), 3.16 (1H, dd, J=7.83, 8.21 Hz, H-3α), 4.59, 4.72 (each 1H, s, H-29); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 38.4 (C-1), 26.5 (C-2), 78.7 (C-3), 38.4 (C-4), 55.0 (C-5), 18.0 (C-6), 34.0 (C-7), 40.4 (C-8), 50.3 (C-9), 37.9 (C-10), 20.5 (C-11), 25.2 (C-12), 38.1 (C-13), 42.1 (C-14), 30.2 (C-15), 31.8 (C-16), 56.0 (C-17), 46.6 (C-18), 48.9 (C-19), 150.1 (C-20), 29.4 (C-21), 37.8 (C-22), 27.7 (C-23), 15.0 (C-24), 15.8 (C-25), 15.8 (C-26), 14.3 (C-27), 180.1 (C-28), 109.4 (C-29), 18.8 (C-30) [19, 20].

*Erythrodiol (**6**)* A white raphide, $C_{30}H_{50}O_3$, m.p. 248–250 °C; IR (KBr) ν_{max} 3,460, 1,640 cm^{-1}; positive ESI-MS *m/z* 443 $[M+H]^+$; ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 0.74 (3H, s, H-23), 0.77 (3H, s, H-24), 0.86 (3H, s, H-25), 0.91 (3H, s, H-26), 0.95 (3H, s, H-29), 0.97 (3H, s, H-30), 1.14 (3H, s, H-27), 3.53 (1H, m, H-3), [3.19(1H, d, J=11 Hz), 3.76 (1H, d, J=11 Hz), CH_2OH-28], 5.17 (1H, t, J=3.6 Hz, H-12);^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 38.5 (C-1), 27.2 (C-2), 79.0 (C-3), 38.8 (C-4), 55.1(C-5), 18.3 (C-6), 31.0 (C-7), 39.7 (C-8), 47.5 (C-9), 36.9 (C-10), 23.5 (C-11), 122.3 (C-12), 144.2 (C-13), 41.7 (C-14), 25.5 (C-15), 22.0 (C-16), 34.0(C-17), 42.3(C-18), 46.4 (C-19), 30.9 (C-20), 33.2 (C-21), 28.1 (C-22), 28.0 (C-23), 15.6(C-24), 15.1 (C-25), 16.7 (C-26), 25.9(C-27), 69.7 (C-28), 32.5 (C-29), 23.6 (C-30) [27, 28].

*(3β)-olean-12-ene-3,23-diol (**7**)* White crystals, $C_{30}H_{50}O_3$, m.p. 208–210 °C; IR(KBr) ν_{max} 3,452, 1,630 cm^{-1}; positive ESI-MS *m/z* 443 $[M+H]^+$; ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 0.83 (3H, s, H-28), 0.85 (3H, s, H-29), 0.87 (3H, s, H-30), 0.92 (3H, s, H-24), 0.92(3H, s, H-25), 0.94 (3H, s, H-26), 1.15 (3H, s, H-27), 3.39 (1H, m, H-3), [3.19 (1H, d, J=11 Hz), 3.54 (1H, d, J=11 Hz), CH_2OH-23], 5.17 (1H, t, J=3.6 Hz, H-12);^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 37.0 (C-1), 26.1 (C-2), 76.1 (C-3), 41.7 (C-4), 48.9 (C-5), 21.9 (C-6), 31.0 (C-7), 37.3 (C-8), 47.3 (C-9), 34.1 (C-10), 22.3 (C-11), 122.4 (C-12), 144.1 (C-13), 40.0 (C-14), 25.4 (C-15), 25.2 (C-16), 32.4 (C-17), 46.4 (C-18), 42.3 (C-19), 30.9 (C-20), 33.2 (C-21), 36.9 (C-22), 69.7 (C-23), 15.3 (C-24), 16.7 (C-25), 18.3 (C-26), 23.6 (C-27), 28.3 (C-28), 33.0 (C-29), 23.4 (C-30) [29–43].

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