Antiproliferative Activity of Vietnamese Medicinal Plants

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Methanol, methanol–water (1 : 1) and water extracts were prepared from seventy-seven Vietnamese medicinal plants and tested for their antiproliferative activities against human HT-1080 fibrosarcoma cells. Among them, fifteen extracts including seven methanol extracts of *Caesalpinia sappan*, *Catharanthus roseus*, *Coscinium fenestratum*, *Eurycoma longifolia*, *Hydnophytum formicarum* and *Streptocaulon juventas* (collected at two areas), six methanol–water (1 : 1) extracts of *Cae. sappan*, *Cat. roseus*, *Co. fenestratum*, *H. formicarum* and *S. juventas* (at two areas), and two water extracts of *Cae. sappan* and *S. juventas* exhibited antiproliferative activities in a concentration-dependent manner. Their antiproliferative activities against human cervix HeLa adenocarcinoma, human lung A549 adenocarcinoma, murine colon 26-L5 carcinoma, murine Lewis lung carcinoma (LLC) and murine B16-BL6 melanoma cells were then examined. *Co. fenestratum* showed selective activity against lung carcinoma and/or lung metastatic cell lines, A549, LLC and B16-BL6, while *H. formicarum* and *S. juventas* showed selective activity against human tumor cell lines, HeLa and A549. Characteristic morphological change and DNA fragmentation indicated the antiproliferative activity to be due to the induction of apoptosis.

Key words antiproliferative activity; *Streptocaulon juventas*; Vietnamese plant; apoptosis; human HT-1080 fibrosarcoma; metastasis

Cancer is one of the major causes of death in developed countries, together with cardiac and cerebrovascular diseases.[1)] Cancer is clinically treated by surgery, radiotherapy and chemotherapy. After surgical ablation of progressive cancer, however, metastasized tumor cells continue to progress, and this is one of the causes making cancer treatment difficult.[2)] Radioactive rays and most anticancer drugs damage DNA or suppress DNA duplication to kill tumor cells growing rapidly. At the same time, they also affect normal cells to cause serious adverse effects, such as bone marrow function inhibition, nausea, vomiting and alopecia.[3,4)] Thus, more effective anticancer drugs with high selectivity against only malignant cells and with ability to repress tumor metastasis are desired. As candidates for such drugs, cytotoxic, antitumor or anticancer natural products have been often sought, and plant components such as Vinca alkaloids, taxoids, etoposide and irinotecan are now used in clinical treatments.[4)]

Vietnam has many medicinal resources,[5,6)] but only a few have been examined chemically except for Vietnamese ginseng.[7,8)] In the course of our search for Vietnamese medicinal plants,[9—12)] we collected plants which have been used as tonics, for treatment of inflammation, cancer and other conditions,[13,14)] at Seven-Mountain area, Angiang province and at Lamdong province. From 77 of these plants, 231 extracts were prepared and their antiproliferative activities were screened against highly metastatic human HT-1080 fibrosarcoma cells.

To determine the selectivity of their activities, we examined their antiproliferative activities against five other cell lines including three highly metastatic cell lines, *i.e.*, human cervix HeLa adenocarcinoma, human lung A549 adenocarcinoma, murine colon 26-L5 carcinoma, murine Lewis lung carcinoma (LLC) and murine B16-BL6 melanoma cells. An extract of *Streptocaulon juventas* showed potent antiproliferative activities with selectivity against human tumor cells. This activity was concluded to be due to the induction of apoptosis, based on characteristic morphological changes and DNA fragmentation.

MATERIALS AND METHODS

Plant Materials Vietnamese medicinal plants used in this study were collected at Seven-Mountain area, Tinh Bien district, Angiang province in March 1998, and at Lamdong province in May 1998. The plants collected at Seven-mountain area were identified by Prof. Le Cong Kiet (Department of Botany, University of Hochiminh City, Hochiminh, Vietnam), while these collected at Lamdong province were identified by Mr. Nguyen Duy Chinh (Department of Botany, Faculty of Environment, Dalat University, Dalat, Vietnam). Their voucher specimens are preserved at the Museum of Materia Medica, Research Center for Ethnomedicines, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan.

Chemicals Eagle's minimum essential (EME) and RPMI 1640 media and Dulbecco's phosphate buffered saline (PBS) were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Dulbecco's modified Eagle's medium nutrient mixture Ham's F-12 (1 : 1) (DMEM/F-12) medium and heat inactivated fetal calf serum (FCS) were obtained from Gibco BRL Products (Gaithersburg, MD, U.S.A.). Bovine serum albumin (BSA), penicillin G and streptomycin sulfate were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) and crystal violet were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI, U.S.A.) and Nacalai Tesque, Inc. (Kyoto, Japan), respectively. Cell culture flasks, 6- and 96-well plates were from Corning Inc. (Corning, NY, U.S.A.). 5-Fluorouracil (5-FU), and doxorubicin hydrochloride were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo) and Kyowa Hakko Kogyo Co., Ltd. (Tokyo), respec-

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tively. Ribonuclease (RNase) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The 100 Base-pair ladder was obtained from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ, U.S.A.). Other reagents were of the highest grade available.

Preparation of Samples Each medicinal plant (30—213 g) was cut into small pieces and extracted successively with methanol (200—300 ml, reflux, 2 h, ×2), methanol–water (1 : 1, 200—300 ml, reflux, 2 h), and water (200—300 ml, reflux, 2 h). The methanol solution was evaporated under reduced pressure to give a methanol extract, while methanol–water (1 : 1) and water solutions were concentrated under reduced pressure and lyophilized to give methanol–water (1 : 1) and water extracts, respectively.

Cells A highly metastatic human HT-1080 fibrosarcoma cell line (ATCC # CCL-121)[15] was obtained from American Type Culture Collection (Rockville, MD, U.S.A.). Human cervix HeLa adenocarcinoma (RCB0007)[16] and human lung A549 adenocarcinoma (RCB0098)[17,18] cell lines were purchased from Riken Cell Bank (Tsukuba, Japan). Highly liver metastatic murine colon 26-L5 carcinoma cell line was established by one of the authors (I. Saiki).[19] Highly lung metastatic murine LLC cell line originated spontaneously from murine lung[20] was kindly provided by Dr. K. Takeda (Juntendo University, Tokyo). Highly liver metastatic murine B16-BL6 melanoma cell line, obtained by an *in vivo* selection procedure for invasion,[21] was generously provided by Dr. I. J. Fidler (M.D. Anderson Cancer Center, Houston, TX, U.S.A.).

HT-1080, HeLa, A549, LLC and B16-BL6 cell lines were maintained in 75-cm^2 cell culture flasks in EME medium supplemented with 10% heat inactivated FCS, 2 mM L-(+)-glutamine and 0.1% sodium hydrogen carbonate at 37 °C under a humidified 5% carbon dioxide. 26-L5 cell line was maintained in RPMI 1640 medium containing the same supplement under the same conditions.

Antiproliferative Activity Assay Viability of cells other than LLC, in the presence or absence of experimental extracts, was determined using the standard MTT assay[22] as described.[23] In brief, exponentially growing cells were harvested and 100-μl medium per well with 2×10^3 cells suspended was plated in a 96-well plate. After 24-h incubation at 37 °C under a humidified 5% carbon dioxide to allow cell attachment, the cells were treated with varying concentrations of test specimens in their respective medium (100 μl) and incubated for 72 h under the same conditions. After 2 h of the MTT (0.4—0.5 mg/ml, 100 μl) addition, the formazan formed was extracted with dimethyl sulfoxide (DMSO) and its amount was measured spectrophotometrically at 550 nm with Perkin-Elmer HTS-7000 Bio Assay Reader (Norwalk, CT, U.S.A.).

In the case of LLC cells, standard crystal violet staining assay was used in following the literature procedure.[24] In brief, exponentially growing cells were harvested and 100-μl medium per well with 1×10^3 cells suspended was plated in a 96-well plate. After 24-h incubation at 37 °C under a humidified 5% carbon dioxide atmosphere, 100-μl medium containing various concentrations of test specimen was added to each well and incubated for 72 h under the same conditions. After fixation with 25% glutaraldehyde solution (20 μl), the cells were stained with 0.5% crystal violet in 20% methanol/water for 30 min. After gentle rinsing with water, the retained crystal violet was extracted with 30% acetic acid and measured spectrophotometrically at 590 nm.

Each extract was dissolved in a bit of DMSO and added PBS, and then diluted with the medium; final concentration of DMSO was less than 0.25%. 5-FU and doxorubicin were used as positive controls, and EC_{50} values were calculated from the mean values of data from four wells.

***In Vitro* Growth Inhibition Test** In brief, exponentially growing HT-1080 cells were harvested and 100-μl EME medium per well with 5×10^3 cells suspended was plated in a 96-well plate. After 24-h incubation, the cells were treated with varying concentrations of test specimens in EME medium (100 μl) and incubated for 12 and 24 h. Viability of cells was determined using the standard MTT assay.

Observation of Morphological Changes Morphological changes were observed as described previously.[25,26] Briefly, exponentially growing HT-1080 cells were harvested and plated 1×10^5 cells per well in a 6-well plate. After 24-h incubation, the cells were treated with varying concentrations of test specimens and incubated for 24 h. At the end of incubation, the morphological changes of the cells were recorded by photomicrography using a phase contrast microscope (Olympus Optical Co., Ltd., Tokyo).

Detection of DNA Fragmentation DNA was isolated and detected by the procedure described previously.[25,26] Briefly, HT-1080 or LLC cells ($>2\times10^6$ cells) were preincubated in EME medium for 24 h, and then cultured with various concentrations of test specimen in serum free DMEM/F-12 medium containing 0.1% BSA, 100 IU/ml penicillin G and 80 IU/ml streptomycin for 24 h. At the end of the incubation, cells were pelleted and lysed in 600 μl of lysis buffer (10 mM Tris-HCl buffer, pH 8.0, 10 mM EDTA and 0.2% Triton X-100) for 10 min on ice. After the lysate was centrifuged at 14000 rpm for 10 min, the supernatant was extracted with TE buffer (10 mM Tris–HCl buffer, pH 8.0, 1 mM EDTA)-saturated phenol, and then centrifuged at 14000 rpm for 10 min. The upper layer was then extracted with CIAA solution (chloroform : isoamylalcohol=24 : 1), and DNA in the upper layer (500 μl) was precipitated with 3 M NaCl (50 μl) and cold ethanol (1000 μl) at −20 °C overnight. After drying, DNA was dissolved in TE buffer. Contamination of RNA was eliminated by incubation with 1 mg/ml RNase at 37 °C for 30 min. Following the addition of loading buffer, fragmented DNA was electrophoresed on 1.5% agarose gel in TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) at 100 V for 30 min and visualized by ethidium bromide staining.

RESULTS AND DISCUSSION

From 77 Vietnamese medicinal plants (Table 1), methanol, methanol–water (1 : 1) and water extracts were prepared, and their antiproliferative activities were examined against highly metastatic human HT-1080 fibrosarcoma cells (Table 2). Fifteen of the extracts showed antiproliferative activities in a concentration-dependent manner with EC_{50} values less than 20 μg/ml: methanol extracts of *Caesalpinia sappan* (EC_{50}, 15.8 μg/ml), *Catharanthus roseus* (EC_{50}, 5.88 μg/ml), *Eurycoma longifolia* (EC_{50}, 15.8 μg/ml), *Hydnophytum formicarum* (EC_{50}, 9.97 μg/ml) and *Streptocaulon juventas* (EC_{50},

Table 1. Vietnamese Medicinal Plants Collected at Seven-Mountain Area, Tinh Bien District, Angiang Province and at Lamdong Province, Their Families, Parts Used, Local Names, Therapeutic Applications and Voucher Specimen Numbers (TMPW No.)

Plant name	Family	Part used	Local name	Therapeutic application	TMPW No.
Collected at Seven-Mountain area, Tinh Bien district, Angiang province					
Amomum villosum Lour. (=*A. repeus* Sonn.)	Zingiberaceae	Leaf	Sa nhan	Digestive disease, diarrhoea	20449
Ampelocissus martini Planch	Vitaceae	Root	Sam hong	Tonic	20451
Angelica dahurica (Fisch. ex Hoffm.) Benth. *et* Hook. F.	Umbelliferae	Aerial part	Bach chi	Fever, rheumatism, leucorrhea	20415
Aquilaria crassna Pierre ex Lacomte	Thymelaeaceae	Wood	Tram toc	Asthma	20457
Artemisia vulgaris L.	Compositae	Leaf	Ngai diep	Antibacteria, fever, leucorrhea	20442
Asparagus cochinchinensis (Lour.) Merr.	Liliacea	Root	Thien mon, Thien dong	Tuberculosis, inflammation, diabetes, breast cancer	20421
Barleria lupulina Lindl.	Acanthaceae	Aerial part	Kim vang	Injury, fever, asthma	20435
Borassus flabellifer L.	Arecaceae	Flower	Thot not bong, Thot not, Thot lot	Diuretic, anthelminthic, inflammation	20419
Bupleurum chinense DC.	Umbelliferae	Aerial part	Sai ho	Fever	20458
Caesalpinia sappan L.	Caesalpiniaceae	Wood	To moc, Vang	Diarrhoea, inflammation	20454
Catharanthus roseus (L.) G. Don (=*Vinca rosea* L.)	Apocynaceae	Aerial part	Dua can	Cancer (lung, leukemia), diabetes	20427
Ceiba pentandra (L.) Gaertn.	Bombacaceae	Fruit	Gon	Malaria, inflammation, diarrhoea	20428
Cinnamomum iners Reinw. ex Blume	Lauraceae	Bark	Hau phac, Hau phac nam	Rheumatism, tonic for stomach	20431
Combretum quadrangulare Kurz.	Combretaceae	Aerial part	Tram bau	Anthelminthic, hepatitis, inflammation	20456
Cupressus funebris Endl.	Cupressaceae	Fruit	Hoang dan, Huynh dan	Fever, physical injury	20422
Cyperus rotundus L.	Cyperaceae	Rhizome	Co cu, Co gau	Menstrual disorder, uterus inflammation, diarrhoea	20423
Desmodium heterophyllum (Willd.) DC.	Fabaceae	Aerial part	Hanthe	Fever, inflammation	20430
Drynaria quercifolia (L.) J. Sm.	Polypodiaceae	Rhizome	Rang bay	Tuberculosis, antibacteria	20445
Elsholtzia ciliata (Thunb.) Hyland.	Lamiaceae	Aerial part	Kinh gioi	Fever, inflammation	20436
Euphorbia tirucalli L.	Euphorbiaceae	Stem	Xuong kho	Inflammation, antibacteria	20459
Eurycoma longifolia Jack	Simaroubaceae	Root	Ba benh, Bach benh	Leucorrhea, malaria, fever	20411
Eurycoma longifolia Jack	Simaroubaceae	Aerial part	Ba benh, Bach benh	Tonic, diarrhoea	20412
Ficus sagitta Vahl	Moraceae	Stem	Manh trau	Tonic	20439
Glycyrrhiza uralensis Fisch. (=*G. glabra* L.)	Fabaceae	Root	Cam thao	Tonic, inflammation, diarrhoea, Addison's disease	20420
Hedyotis diffusa Willd.	Rubiaceae	Aerial part	Luoi ran trang, Bach hoa xa thiet thao	Inflammation, hepatitis, antitumor	20416
Hydnophytum formicarum Jack	Rubiaceae	Gall	Bi ky nam	Hepatitis, rheumatism, diarrhoea	20418
Lasia spinosa (L.) Thw.	Araceae	Whole plant	Mop gai, Ray gai	Inflammation, rheumatism	20440
Leonurus heterophyllus Sweet	Lamiaceae	Aerial part	Ich mau	Menstrual disorder, inflammation, tonic for women	20433
Lindernia crustacea (L.) F. Muell.	Scrophulariaceae	Stem	Mau thao, Day luoi dong	Fever, hepatitis, leucorrhea	20437
Luvunga scandens (Roxb.) Buch.-Ham.	Rutaceae	Branchlet	Than xa huong	Rheumatism, fever	20453
Marsilea quadrifolia L.	Marsileaceae	Aerial part	Rau bo, Co bo	Hepatitis, malaria, diabetes	20447
Melaleuca leucadendra (L.) L.	Myrtaceae	Stem and fruit	Tram	Fever, rheumatism, diarrhoea	20455
Miliusa velutina (Dun.) Hook. F. *et* Thoms.	Annonaceae	Wood	Co sen	Inflammation, antibacteria	20424
Nauclea officinalis (Pit.) Merr.	Rubiaceae	Fruit	Huynh ba	Inflammation	20432
Orthosiphon spiralis (Lour.) Merr. (=*O. stamineus* Benth.)	Lamiaceae	Aerial part	Rau meo	Diuretic, inflammation	20448
Panicum repens L.	Poaceae	Aerial part	Cu co ong, Cu gung	Rheumatism, inflammation, leucorrhea	20425
Paraboea treubii (Forbes) Burtt (=*Boea treubii* Forbes)	Gesneriaceae	Aerial part	Bac thau da	Cough, fever	20414
Parameria laevigata (Juss.) Moldenk	Apocynaceae	Stem	Do trong day	Rheumatism, hypertension	20460
Polanisia chelidonii (L. f.) A. DC (=*Cleomo chelidonii* L. f.)	Capparaceae	Aerial part	Man ri tia, Man man tim	Fever, inflammation	20438
Polypodium subauriculatum Blume	Polypodiaceae	Rhizome	Bach xa	Fever	20417
Rhinacanthus nasutus (L.) Kurz	Acanthaceae	Aerial part	Kien co, Bach hac	Tuberculosis, inflammation, rheumatism, hypertension	20434
Sansevieria cylindrica Bojer	Agavaceae	Leaf	Ngai nga, Nanh heo, Nga Voi	Inflammation	20443
Streptocaulon juventas (Lour.) Merr.	Asclepiadaceae	Root	Ha thu o trang	Tonic, malaria, leucorrhea	20429
Tinospora cordifolia (Willd.) Miers	Menispermaceae	Stem	Than thong, Day than thong	Malaria, fever, inflammation	20452
Tinospora crispa Miers	Menispermaceae	Stem	Day coc	Fever, malaria	20426

Table 1. (continued)

Plant name	Family	Part used	Local name	Therapeutic application	TMPW No.
Vernonia cinerea (L.) Less.	Compositae	Aerial part	Bach dau ong	Hepatitis, fever, diarrhoea	20413
Collected at Lamdong province					
Adenosma glutinosum (L.) Druce	Scrophulariaceae	Aerial part	Nhan tran	Hepatitis, tonic for women	20616
Ageratum conyzoides L.	Compositae	Aerial part	Cay cut lon	Inflammation	20462
Aloe vera L.	Aloaceae	Leaf	Lo hoi	Cold, fever	20611
Andrographis paniculata (Burm. f.) Nees	Acanthaceae	Aerial part	Xuyen tam lien	Inflammation, hypertension	20626
Artemisia apiacea Hance ex Walp.	Compositae	Aerial part	Thanh hao	Malaria, inflammation	20621
Cassia tora L.	Caesalpiniaceae	Seed	Thao quyet minh	Hepatitis, hypertension	20622
Codonopsis javanica (Blume) Hook. F.	Campanulaceae	Root	Dang sam	Tonic, leukemia, inflammation, hepatitis	20466
Coscinium fenestratum (Gaertn.) Colber. (=*C. usitatum* Pierre)	Menispermaceae	Stem	Vang dang, Hoang dang	Malaria, diarrhoea, inflammation	20606
Datura metal L.	Solanaceae	Flower	Ca doc duoc	Asthma, inflammation	20463
Eleutherine bulbosa (Mill.) Urb.	Iridaceae	Bulb	Sam dai hanh	Cough, inflammation	20618
Eucommia ulmoides Oliv.	Eucommiaceae	Bark	Do trong	Hypertension, rheumatism	20468
Gymnopetalum cochinchinensis (Lour.) Kurz	Cucurbitaceae	Stem and leaf	Cut qua	Cough, inflammation	20465
Heliotropium indicum L.	Boraginaceae	Aerial part	Voi voi	Inflammation	20625
Kalanchoe pinnata (Lam.) Pers.	Crassulaceae	Aerial part	Truong sinh, Thuoc bong	Antibacteria, inflammation	20624
Launaea pinnatifida Cass.	Compositae	Aerial part	Sa sam, Sa sam nam	Galactopoietic, leucorrhea	20617
Lonicera japonica Thunb.	Caprifoliaceae	Flower	Kim ngan	Inflammation, rheumatism	20609
Luffa cylindrica (L.) Roem.	Cucurbitaceae	Fruit	Muop gai, Muop	Inflammation, cough	20614
Merremia bimbim (Gagnep.) van Ooststr. (=*Ipomea bimbim* Gagnep.)	Convolvulaceae	Seed	Bim bim	Anthelminthic, diuretic	20461
Mimosa pudica L.	Mimosaceae	Aerial part	Mac co	Inflammation, hepatitis, hypertension	20612
Nelumbo nucifera Gaertn.	Nelumbonaceae	Kernel	Tam sen	Hypertension, heart disease	20619
Panax pseudo-ginseng Wall.	Araliaceae	Root	Tam that	Tonic, sterility, cancer	20620
Phyllantus amarus Schum. *et* Thonn.	Euphorbiaceae	Aerial part	Cho de rang cua, Cho de than xanh	Inflammation, hepatitis	20464
Piper lolot C. DC.	Piperaceae	Aerial part	La lot	Rheumatism, diarrhoea	20610
Plantago major L.	Plantaginaceae	Aerial part	Ma de	Inflammation	20613
Polygonum multiflorum Thunb.	Polygonaceae	Root	Ha thu o, Ha thu o do	Tonic, malaria	20469
Polyscias fruticosa (L.) Harms	Araliaceae	Stem and leaf	Dinh lang	Tonic, inflammation	20467
Schefflera octophylla (Lour.) Harms	Araliaceae	Bark	Ngu gia bi chan chim, Chan chim	Tonic, inflammation	20615
Smilax glabra Roxb.	Smilacaceae	Rhizome	Tho phuc linh	Inflammation	20623
Sophora flavescens Ait.	Fabaceae	Root	Kho sam	Inflammation, diarrhoea	20608
Streptocaulon juventas (Lour.) Merr.	Asclepiadaceae	Root	Ha thu o trang	Tonic, malaria, leucorrhea	20470
Xanthium strumarium L.	Compositae	Fruit	Ke dau ngua	Inflammation, malaria	20607

6.04 μg/ml) from Seven-Mountain area; methanol extracts of *Coscinium fenestratum* (EC_{50}, 11.7 μg/ml) and *S. juventas* (EC_{50}, 1.15 μg/ml) from Lamdong province; methanol–water extracts of *Cae. sappan* (EC_{50}, 13.8 μg/ml), *Cat. roseus* (EC_{50}, 8.99 μg/ml), *H. formicarum* (EC_{50}, 11.3 μg/ml) and *S. juventas* (EC_{50}, 12.1 μg/ml) from Seven-Mountain area; methanol–water extracts of *Co. fenestratum* (EC_{50}, 18.1 μg/ml) and *S. juventas* (EC_{50}, 0.886 μg/ml) from Lamdong province; water extracts of *Cae. sappan* (EC_{50}, 17.8 μg/ml) from Seven-Mountain area and *S. juventas* (EC_{50}, 4.96 μg/ml) from Lamdong province.

Next, antiproliferative activities of the 15 extracts were examined against human cervix HeLa adenocarcinoma, human lung A549 adenocarcinoma, murine colon 26-L5 carcinoma, murine Lewis lung carcinoma (LLC) and murine B16-BL6 melanoma cells (Fig. 1, Table 3). These are three human tumor cells (HT-1080, HeLa and A549) and three murine tumor cells (26-L5, LLC and B16-BL6), while the four cell lines (HT-1080, 26-L5, LLC and B16-BL6) are invasive and metastatic and the LLC cell line is drug-resistant.[27]

The methanol and methanol-water extracts of *Co. fenestratum* showed strong and selective antiproliferative activities against two kinds of lung carcinoma cells, A549 and LLC; methanol extract: EC_{50} against LLC cells, 1.65 μg/ml; methanol–water extract: EC_{50} against A549 and LLC cells, 2.88 and 2.84 μg/ml, respectively. Lung cancer is one of the major causes of death by cancer, and lung is one of the tissues where many cancer cells including LLC and B16-BL6 cells metastasize. The methanol and methanol–water extracts also showed antiproliferative activities against B16-BL6 cells, and berberine, a constituent of *Co. fenestratum*,[28] has recently been reported to inhibit metastasis of LLC cells.[29] It is interesting that *Co. fenestratum* and berberine are effective and specific against tumors in lung, in spite of their use as drugs for diseases of the digestive system, *i.e.*, diarrhoea and dysentery.

All extracts of *Cae. sappan* and *Cat. roseus* showed antiproliferative activities against HT-1080 and LLC cells but not against A549 cells, suggesting the presence of components effective for the treatment of drug-resistant tumor. This may be interesting, because the drug-resistant tumor is a problem in long-term chemotherapy. In contrast, all extracts

Table 2. Yields (%) and Antiproliferative Activities against Human HT-1080 Fibrosarcoma Cells (EC_{50} in μg/ml) of Each Extract of Vietnamese Medicinal Plants

Plant name	Yield (%)			EC_{50} (μg/ml)		
	MeOH ext.	MeOH–H_2O ext.	H_2O ext.	MeOH ext.	MeOH–H_2O ext.	H_2O ext.
Collected at Seven-Mountain area, Tinh Bien district, Angiang province						
Amomum villosum	32.6	11.6	10.5	81.5	>100	>100
Ampelocissus martini	2.2	4.7	2.4	>100	>100	>100
Angelica dahurica	9.5	4.9	3.2	60.0	>100	>100
Aquilaria crassna	3.2	1.4	0.9	69.5	73.7	>100
Artemisia vulgaris	17.1	11.4	8.6	38.0	82.2	73.6
Asparagus cochinchinensis	1.1	0.4	0.3	66.9	>100	>100
Barleria lupulina	11.5	7.8	4.4	>100	>100	>100
Borassus flabellifer	3.0	5.4	2.8	73.2	>100	100
Bupleurum chinense	9.7	6.7	4.1	>100	>100	>100
Caesalpinia sappan	10.2	1.9	0.3	15.8	13.8	17.8
Catharanthus roseus	22.7	7.6	4.1	5.88	8.99	95.0
Ceiba pentandra	5.8	4.7	4.1	>100	>100	>100
Cinnamomum iners	9.9	4.4	1.3	>100	>100	>100
Combretum quadrangulare	11.5	5.4	4.2	47.9	49.6	46.6
Cupressus funebris	31.6	5.7	3.9	>100	>100	>100
Cyperus rotundus	2.3	3.1	2.0	70.8	>100	>100
Desmodium heterophyllum	8.6	3.6	1.4	>100	>100	>100
Drynaria quercifolia	4.9	2.2	1.9	>100	>100	>100
Elsholtzia ciliata	3.1	2.2	2.6	>100	>100	>100
Euphorbia tirucalli	8.8	5.7	5.1	>100	>100	>100
Eurycoma longifolia (root)	2.1	1.4	0.7	15.8	53.7	>100
Eurycoma longifolia (arial part)	7.4	4.3	2.5	55.8	55.1	84.5
Ficus sagitta	6.0	4.0	1.7	33.0	26.5	>100
Glycyrrhiza uralensis	8.0	3.5	1.0	>100	88.4	>100
Hedyotis diffusa	12.6	6.2	2.4	63.0	54.7	>100
Hydnophytum formicarum	8.4	2.7	1.2	9.97	11.3	22.3
Lasia spinosa	5.4	3.2	2.3	>100	>100	>100
Leonurus heterophyllus	3.9	2.3	2.1	>100	>100	>100
Lindernia crustacea	10.6	4.7	3.8	>100	>100	>100
Luvunga scandens	2.8	1.3	0.8	>100	>100	>100
Marsilea quadrifolia	4.0	4.3	2.7	>100	>100	>100
Melaleuca leucadendra	16.3	4.8	2.4	66.0	>100	>100
Miliusa velutina	4.8	1.5	0.3	60.7	>100	>100
Nauclea officinalis	9.3	15.7	3.4	23.2	30.5	26.7
Orthosiphon spiralis	6.0	5.5	5.8	>100	>100	>100
Panicum repens	9.1	6.2	1.8	80.1	22.9	>100
Paraboea treubii	16.4	7.8	2.6	58.9	79.3	>100
Parameria laevigata	7.7	4.4	1.6	>100	>100	>100
Polanisia chelidonii	11.5	7.3	4.8	>100	>100	>100
Polypodium subauriculatum	4.2	1.3	1.1	>100	>100	>100
Rhinacanthus nasutus	6.9	7.4	4.3	>100	>100	>100
Sansevieria cylindrica	2.7	0.6	1.2	>100	>100	>100
Streptocaulon juventas	10.9	5.8	3.5	6.04	12.1	47.6
Tinospora cordifolia	6.1	3.1	3.3	68.1	>100	>100
Tinospora crispa	2.6	1.4	1.1	50.4	>100	>100
Vernonia cinerea	8.1	4.1	2.2	86.7	>100	>100
Collected at Lamdong province						
Adenosma glutinosum	4.3	3.5	4.3	>100	>100	>100
Ageratum conyzoides	3.4	2.8	3.1	>100	>100	>100
Aloe vera	57.6	23.8	5.1	>100	>100	>100
Andrographis paniculata	9.3	4.6	3.5	90.0	>100	>100
Artemisia apiacea	6.0	4.7	1.8	97.8	>100	>100
Cassia tora	5.9	1.9	1.4	85.6	>100	>100
Codonopsis javanica	30.5	10.0	12.5	>100	>100	>100
Coscinium fenestratum	9.4	2.8	0.9	11.7	18.1	76.0
Datura metal	9.5	5.8	5.2	51.6	>100	>100
Eleutherine bulbosa	7.4	3.9	0.9	80.2	>100	>100
Eucommia ulmoides	0.6	0.3	0.5	79.7	>100	>100
Gymnopetalum cochinchinensis	6.2	4.6	3.5	93.0	>100	>100
Heliotropium indicum	8.3	5.5	2.6	>100	>100	>100
Kalanchoe pinnata	6.0	1.8	0.9	>100	>100	>100
Launaea pinnatifida	2.6	4.3	3.3	>100	>100	>100
Lonicera japonica	32.8	4.7	1.7	>100	>100	>100
Luffa cylindrica	5.4	5.1	2.1	>100	>100	>100
Merremia bimbim	4.9	1.1	1.3	49.1	71.4	>100

Table 2. (continued)

Plant name	Yield (%)			EC_{50} (μg/ml)		
	MeOH ext.	MeOH–H_2O ext.	H_2O ext.	MeOH ext.	MeOH–H_2O ext.	H_2O ext.
Mimosa pudica	5.5	1.8	1.8	>100	>100	>100
Nelumbo nucifera	12.3	11.0	6.3	61.7	>100	>100
Panax pseudo-ginseng	5.4	5.4	3.4	>100	>100	>100
Phyllantus amarus	7.5	5.2	3.2	>100	36.9	82.8
Piper lolot	7.9	4.3	4.0	85.6	>100	>100
Plantago major	15.0	5.2	4.3	>100	>100	>100
Polygonum multiflorum	17.0	8.7	1.9	70.8	21.9	>100
Polyscias fruticosa	15.8	4.5	5.5	>100	>100	>100
Schefflera octophylla	2.3	1.4	1.7	>100	>100	>100
Smilax glabra	17.5	4.8	1.3	64.9	87.8	>100
Sophora flavescens	11.3	3.6	2.0	>100	>100	>100
Streptocaulon juventas	3.0	1.3	1.9	1.15	0.886	4.96
Xanthium strumarium	3.0	2.8	3.6	88.0	>100	>100

Table 3. Antiproliferative Activities of the Extracts against Human HT-1080 Fibrosarcoma, Human Cervix HeLa Adenocarcinoma, Human Lung A549 Adenocarcinoma, Murine Colon 26-L5 Carcinoma, Murine Lewis Lung Carcinoma (LLC) and Murine B16-BL6 Melanoma Cells (EC_{50} values in μg/ml)

Scientific name	Extract	HT-1080	HeLa	A549	26-L5	LLC	B16-BL6
Caesalpinia sappan	MeOH	15.8	15.5	41.4	23.2	8.20	20.1
	MeOH–H_2O	13.8	27.8	73.8	49.7	16.7	50.6
	H_2O	17.8	23.7	49.8	59.4	16.4	23.0
Catharanthus roseus	MeOH	5.88	20.7	>100	>100	4.36	>100
	MeOH–H_2O	8.99	51.6	>100	>100	12.7	>100
Eurycoma longifolia	MeOH	15.8	14.2	19.1	15.8	2.29	9.16
Hydnophytum formicarum	MeOH	9.97	11.3	1.03	22.6	65.6	50.6
	MeOH–H_2O	11.3	16.3	0.780	87.1	>100	>100
Streptocaulon juventas[a)]	MeOH	6.04	13.6	0.790	48.8	69.8	>100
	MeOH–H_2O	12.1	15.1	0.943	>100	>100	>100
Coscinium fenestratum	MeOH	11.7	30.1	5.32	79.0	1.65	5.85
	MeOH–H_2O	18.1	49.0	2.88	>100	2.84	8.91
Streptocaulon juventas[b)]	MeOH	1.15	4.21	0.121	15.3	48.9	45.3
	MeOH–H_2O	0.886	5.47	0.138	47.0	51.1	66.3
	H_2O	4.96	15.7	0.591	>100	>100	>100
5-FU		0.198	0.0871	0.244	0.0673	0.0276	0.0782
Doxorubicin		0.104	0.195	0.0325	0.0573	0.107	0.0944

a) Collected at Seven-Mountain area. *b*) Collected at Lamdong province.

of *H. formicarum* and *S. juventas* showed the potent activities against A549 cells with EC_{50} values less than 4 μg/ml, but not against LLC cells. The activities against A549 cells of the methanol and methanol–water extracts of *S. juventas* from Lamdong province (EC_{50}, 0.121 and 0.138 μg/ml, respectively) were stronger than a positive control 5-FU (EC_{50}, 0.244 μg/ml). It should be noted here that *H. formicarum* and *S. juventas* selectively suppressed the proliferation of human tumor cells, HT-1080, HeLa and A549. Moreover, the methanol extract of *S. juventas* from Lamdong province showed the activity also against 26-L5 cells (EC_{50}, 15.3 μg/ml), in spite of weaker activities of other extracts. The extract of *E. longifolia* was antiproliferative against all cells.

S. juventas extract, which showed selective and the most potent activity, induced HT-1080 cells in a spindle-shape when the present at more than 1 μg/ml and in a multi-blebbing-shape when the present at 4 μg/ml (Fig. 2), while inhibiting their growth in a time- and concentration-dependent manner (Fig. 3). These are the morphological changes typical of apoptosis.[30] Thus, we examined DNA fragmentation to clarify whether the *S. juventas* extract induced apoptosis or not. As can be seen in Fig. 4, the extract induced ladder-like DNA fragmentation in a concentration-dependent manner against LLC and HT-1080, and this DNA fragmentation was in parallel with the growth inhibition. These ladder fragmentations of DNA and characteristic morphological changes indicate that antiproliferation by *S. juventas* is caused by apoptosis. Many anticancer drugs damage DNA or suppress its duplication, not to kill cells directly but to induce apoptosis.[31—33] The extract of *S. juventas* induced apoptosis with selectivity to tumor cells and thus seems to be a desirable candidate for a clinical drug.

Though there is no report on the antiproliferative activity of *Co. fenestratum*, which shows selective activity against lung-related tumor cells, its constituents, benzylisoquinoline-type alkaloids such as berberine,[28,34] were reported to be cytotoxic.[35] The antiproliferative constituents of *Cae. sappan* also have not been reported, but the major antioxidative component of *Cae. sappan*, brazilin,[36] seemed to inhibit growth of tumor cells, based on a report that antioxidative phenolic compounds were cytotoxic against tumor cells.[37] *Cat.*

roseus, which is used for treatment of cancer and diabetes in Vietnam,[13,14] was reported to contain the anticancer indole alkaloids, vinblastine and vincristine,[38,39] which are commonly called Vinca alkaloids or *Catharanthus* alkaloids. *E. longifolia*, inhibiting proliferation of all cells, was also reported to contain several cytotoxic quassinoids and indole alkaloids.[40,41] *H. formicarum* and *S. juventas*, exhibiting selective activity against human tumor cell lines, are used for treatments of hepatitis, rheumatism and diarrhoea and for treatments of malaria and leucorrhea and as tonic, respectively, in Vietnam.[13,14] But their constituents or biological and pharmacological activities have not been reported scien-

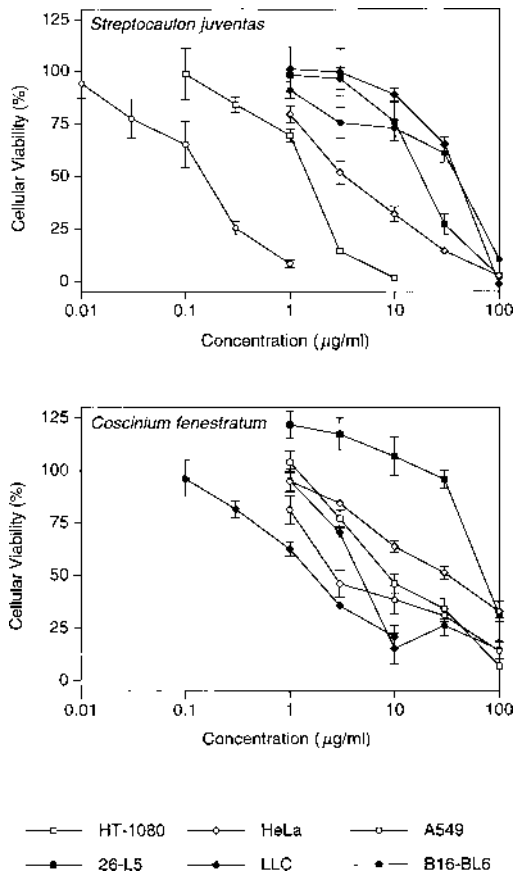

Fig. 1. Cellular Viabilities in the Presence of the Methanol Extract of *Streptocaulon juventas* Collected at Lamdong Province and the Methanol Extract of *Coscinium fenestratum*

After 24-h preincubation at 37 °C, the cells were cultured in medium with each extract for 72 h under the same conditions. Results are expressed as the mean (% of control)±S.D. (n=4).

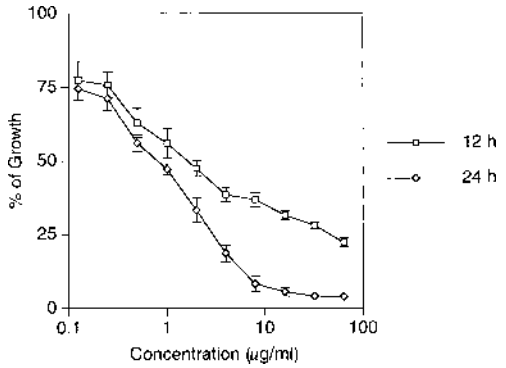

Fig. 3. Growth of Human HT-1080 Fibrosarcoma Cells in the Presence of the Methanol Extract of *Streptocaulon juventas* Collected at Lamdong Province

After 24-h preincubation at 37 °C, the cells were cultured in media with each extract for 12 and 24 h under the same conditions. Results are expressed as the mean (% of control)±S.D. (n=4).

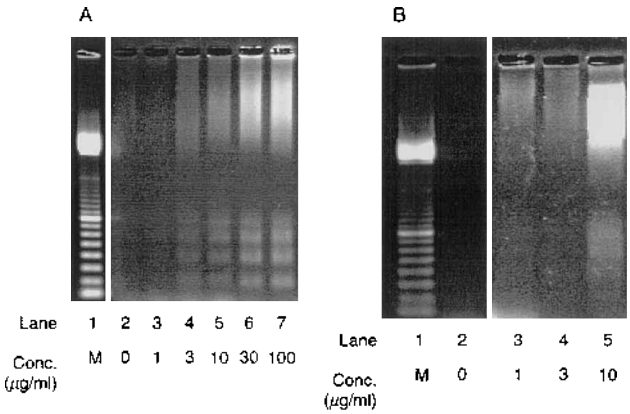

Fig. 4. The Methanol Extract of *Streptocaulon juventas* from Lamdong Province Induced DNA Fragmentation in Murine Lewis Lung Carcinoma (LLC) (A) and Human HT-1080 Fibrosarcoma cells (B)

After the cells were cultured for 24 h with various concentrations of the extract, the fragmented DNA was isolated, electrophoresed on 1.5% agarose gel, and then visualized by ethidium bromide staining. (A) Lane 1: 100 base-pair ladder marker; lane 2: normal; lanes 3—7: treated with 1, 3, 10, 30 and 100 μg/ml of the *S. juventas* extract, (B) Lane 1: 100 base-pair ladder marker; lane 2: normal; lanes 3—5: treated with 1, 3 and 10 μg/ml of the *S. juventas* extract, respectively.

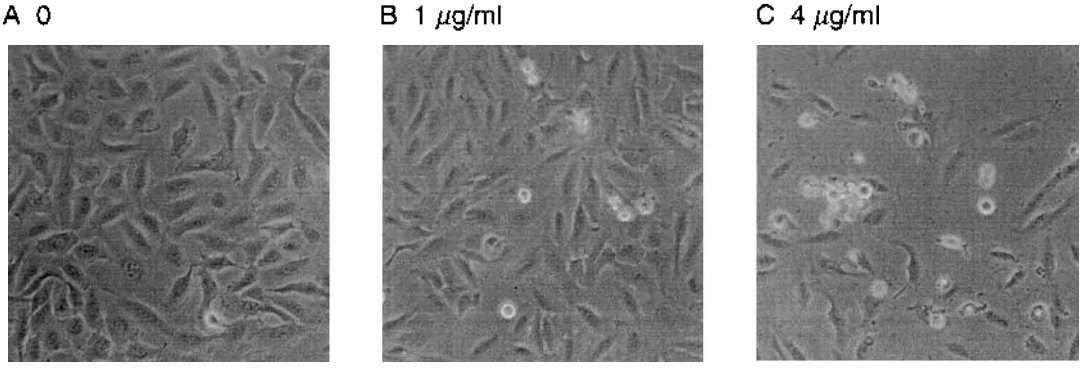

Fig. 2. Morphological Changes in Human HT-1080 Fibrosarcoma Cells Treated with the Methanol Extract of *Streptocaulon juventas* Collected at Lamdong Province

After 24-h preincubation, the cells were cultured for 24 h without (A) or with the extract at 1 (B) and at 4 μg/ml (C). Original magnification: ×100.

tifically. The antiproliferative activities of *Co. fenestratum*, showing selective activity against lung-related tumor cells, of *H. formicarum*, showing selective activity against human tumor cells, and of *S. juventas*, showing selective activity against human tumor cells with induction of apoptosis, are interesting and thus their constituents are under investigation and will be reported elsewhere.

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REFERENCES

1) World Health Organization, "World Health Statistics Annual 1996," World Health Organization, Geneva, 1998.
2) Fidler I. J., Kripke M. L., *Science*, **197**, 893—895 (1977).
3) Kligerman M. M., "Cancer Medicine," ed. by Holland J. F., Frei E. III, Lea & Febiger, Philadelphia, 1973, pp. 541—565.
4) Medical Economic, "Physicians' Desk Reference," 52nd ed., Medical Economic, Montvale, 1998.
5) World Health Organization Regional Office for Western Pacific, Institute of Materia Medica, "Medicinal Plants in Viet Nam," Science and Technology Publishing House, Hanoi, 1998.
6) Ito M., Yamane Y., Nguyen D. C., Tran K. Q., Honda G., *Natural Medicines*, **50**, 420—425 (1996).
7) Cuong N. M., Taylor W. C., Sung T. V., *Phytochemistry*, **52**, 1711—1714 (1999).
8) Kim T. H., Thuy N. T., Shin J. H., Baek H. H., Lee H. J., *J. Agric. Food Chem.*, **48**, 2877—2881 (2000).
9) Banskota A. H., Tezuka Y., Tran K. Q., Tanaka K., Saiki I., Kadota S., *Chem. Pharm. Bull.*, **48**, 496—504 (2000).
10) Tezuka Y., Stampoulis P., Banskota A. H., Awale S., Tran K. Q., Saiki I., Kadota S., *Chem. Pharm. Bull.*, **48**, 1711—1719 (2000).
11) Adnyana I K., Tezuka Y., Banskota A. H., Tran K. Q., Kadota S., *J. Nat. Prod.*, **64**, 360—363 (2001).
12) Tran Q. L., Adnyana I. K., Tezuka Y., Nagaoka T., Tran Q. K., Kadota S., *J. Nat. Prod.*, **64**, 456—461 (2001).
13) Vo V. C., "Dictionary of Vietnamese Medicinal Plants," Medicine Publisher, Hochiminh City, 1996.
14) Do T. L., "Vietnamese Medicinal Plants," Medicine Publisher, Hanoi, 2001.
15) Rasheed S., Nelson-Rees W. A., Toth E. M., Arnstein P., Gardner M. B., *Cancer*, **33**, 1027—1033 (1974).
16) Gey G. O., Coffman W. D., Kubicek M. T., *Cancer Res.*, **12**, 264—265 (1952).
17) Giard D. J., Aaronson S. A., Todaro G. J., Arnstein P., Kersey J. H., Dosik H., Parks W. P., *J. Natl. Cancer Inst.*, **51**, 1417—1423 (1973).
18) Lieber M., Smith B., Szakal A., Nelson-Rees W., Todaro G., *Int. J. Cancer*, **17**, 62—70 (1976).
19) Ohnishi Y., Sakamoto T., Fujii H., Kimura F., Murata J., Tazawa K., Fujimaki M., Sato Y., Kondo M., Une Y., Uchino J., Saiki I., *Tumor Biol.*, **18**, 113—122 (1997).
20) Aptekman P. M., Lewis M. R., *J. Immunol.*, **66**, 361—364 (1951).
21) Hart I. R., *Am. J. Pathol.*, **97**, 587—600 (1979).
22) Rubinstein L. V., Shoemaker R. H., Paull K. D., Simon R. M., Tosini S., Skehan P., Scudiero D. A., Monks A., Boyd M. R., *J. Natl. Cancer Inst.*, **82**, 1113—1118 (1990).
23) Banskota A. H., Tezuka Y., Prasain J. K., Matsushige K., Saiki I., Kadota S., *J. Nat. Prod.*, **61**, 896—900 (1998).
24) Saito K., Oku T., Ata N., Miyashiro H., Hattori M., Saiki I., *Biol. Pharm. Bull.*, **20**, 345—348 (1997).
25) Suda K., Murakami K., Hasegawa H., Saiki I., *J. Trad. Med.*, **17**, 236—244 (2000).
26) Wu W., Murakami K., Koketsu M., Yamada Y., Saiki I., *Anticancer Res.*, **19**, 5375—5382 (1999).
27) Furusawa Eii., Furusawa Eik., Sokugawa L., *Chemotherapy*, **29**, 294—302 (1983).
28) Siwon J., Verpoorte R., van Essen G. F. A., Svendsen A. B., *Planta Med.*, **38**, 24—32 (1980).
29) Mitani N., Murakami K., Yamaura T., Ikeda T., Saiki I., *Cancer Lett.*, **165**, 35—42 (2001).
30) Wyllie A. H., Kerr J. F. R., Currie A. R., *Int. Rev. Cytol.*, **68**, 251—306 (1980).
31) Barry M. A., Behnke C. A., Eastman A., *Biochem. Pharm.*, **40**, 2353—2362 (1990).
32) Ling Y.-H., Priebe W., Perez-Soler R., *Cancer Res.*, **53**, 1845—1852 (1993).
33) Fisher D. E., *Cell*, **78**, 539—542 (1994).
34) Pinho P. M. M., Pinto M. M. M., Kijjoa A., Pharadai K., Diaz J. G., Herz W., *Phytochemistry*, **31**, 1403—1407 (1992).
35) Orfila L., Rodríguez M., Colman T., Hasegawa M., Merentes E., Arvelo F., *J. Ethnopharmacol.*, **71**, 449—456 (2000).
36) Moon C.-K., Park K.-S., Kim S.-G., Won H.-S., Chung J.-H., *Drug Chem. Toxicol.*, **15**, 81—91 (1992).
37) Mahmoud N. N., Carothers A. M., Grunberger D., Bilinski R. T., Churchill M. R., Martucci C., Newmark H. L., Bertagnolli M. M., *Carcinogenesis*, **21**, 921—927 (2000).
38) Noble R. L., Beer C. T., Cutts J. H., *Ann. N.Y. Acad. Sci.*, **76**, 882—894 (1958).
39) Adamson R. H., Dixon R. L., Ben M., Crews L., Shohet S. B., Rall D. P., *Arch. Int. Pharmacodyn. Ther.*, **157**, 299—311 (1956).
40) Morita H., Kishi E., Takeya K., Itokawa H., Tanaka O., *Chem. Lett.*, **1990**, 749—752 (1990).
41) Kardono L. B. S., Angerhofer C. K., Tsauri S., Padmawinata K., Pezzuto J. M., Kinghorn A. D., *J. Nat. Prod.*, **54**, 1360—1367 (1991).