World J Microbiol Biotechnol (2009) 25:1461–1469
DOI 10.1007/s11274-009-0036-0

ORIGINAL PAPER

Evaluation of the bioactivities of extracts of endophytes isolated from Taiwanese herbal plants

Pei-Wen Hsieh · Li-Chi Hsu · Chern-Hsiung Lai · Chin-Chung Wu · Tsong-Long Hwang · Yin-Ku Lin · Yang-Chang Wu

Received: 31 October 2008 / Accepted: 31 March 2009 / Published online: 14 April 2009

Abstract The endophytic extracts from 19 endophytes, isolated from 13 species of Taiwanese plants, were evaluated for biological activity, including cytotoxicity, anti-platelet aggregation, and anti-inflammatory activity. The extracts of 12 endophytes exhibited inhibitory effects on collagen-induced platelet aggregation with IC_{50} values of 19.85–87.64 μg/ml. Four strains, *Rahnella aquatilis*, *Pantoea agglomerans*, *Rhodotorula* sp., and *Penicillium paxilli*, also showed inhibitory effects on thrombin-induced platelet aggregation with IC_{50} values of 42.80–61.54 μg/ml. Additionally 12 extracts of endophytes exhibited cytotoxicities with IC_{50} values of 0.12–19.83 μg/ml. However, eight extracts revealed inhibitory effects on superoxide anion generation induced by fMLP (*N*-formyl-L-methionyl-L-leucyl-L-phenylalanine) in human neutrophils. The extract of *Rahnella aquatilis* showed anti-platelet aggregation activity, and bioassay-directed fractionation led to the isolation of six compounds, including one isoalloxazine: lumichrome (1); two isoflavones: genistein (2) and daidzein (3); two cyclic peptides: cyclo-Pro-Val (4) and cyclo-Pro-Phe (5); and one benzenoid: methyl 2,4,5-trimethoxybenzoate (6). These results indicated that endophytes from Taiwanese herbal plants could be useful sources for research and development of bioactive lead compounds from nature.

Keywords Endophytes · Cytotoxicity · Anti-platelet aggregation · Neutrophil superoxide anion generation inhibition

P.-W. Hsieh (✉) · T.-L. Hwang
Graduate Institute of Natural Products, College of Medicine, Chang Gung University, Taoyuan 333, Taiwan, ROC
e-mail: pewehs@mail.cgu.edu.tw

P.-W. Hsieh · L.-C. Hsu · C.-C. Wu · Y.-C. Wu
Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung 807, Taiwan, ROC

C.-H. Lai
Graduate Institute of Dental Sciences, Kaohsiung Medical University, Kaohsiung 807, Taiwan, ROC

Y.-K. Lin
Graduate Institute of Clinical Medical Sciences, Chang Gung University, Kweisan, Taoyuan, Taiwan, ROC

Y.-K. Lin
Department of Traditional Chinese Medicine, Chang Gung Memorial Hospital, Keelung, Taiwan, ROC

Introduction

Natural products are a rich source of biologically active compounds. Many of today's medicines are either obtained directly from a natural source or were developed from a lead compound originally from a natural source (Patrick 2005). However, one crucial aspect to be considered for a successful drug discovery program from natural products is selection of the natural source to be studied (Guimaraes et al. 2008). In previous studies, many bioactive natural products have been isolated from endophytes, and have exhibited diverse bioactivities (Tan and Zou 2001; Gunatilaka 2006; Guimaraes et al. 2008; Ryan et al. 2008). Thus, endophytes are recognized as possible useful sources of bioactive secondary metabolites (Phongpaichit et al. 2007). Accordingly, the aim of this study was to isolate, identify, and cultivate endophytes from Taiwanese herbal plants. Furthermore, the endophytes were further used to grow, extract, and evaluate their bioactivities, e.g., anti-platelet aggregation,

anti-inflammation, and cytotoxicity. This is the first report of the isolation and cultivation of endophytes from these plants, and for evaluation of anti-platelet activity of endophytes.

The endophytic chloroform extract of *Rahnella aquatilis* revealed anti-platelet aggregation activity. Using a bioassay-directed fractionation protocol led to the isolation of six compounds, and their structures have been elucidated by spectroscopic methods. This is the first time these compounds were isolated from *Rahnella aquatilis*.

Materials and methods

Isolation of endophytes

Fresh herbal plants (Table 1) were collected from apparently healthy plants in the wild area of Kaohsiung and Tainan, Taiwan. The stems or seeds or leaves or roots were washed in running H_2O for 10 min and surface disinfested by successive soaking in 75% EtOH for 1 min (woody plants for 1.5 min) twice, and sodium hypochlorite solution (0.5%) for 30 s (woody plants for 1.0 min), and then rinsed twice with water. Aseptically, the rods were removed the surface and cut open as 2–5 mm^2 pieces of internal tissues were excised and placed on potato dextrose agar (PDA, Sigma–Aldrich) in Petri plates. During the first 2 weeks of incubation, the cultures were periodically checked for purity and successively subcultured by streak culture and hyphal tipping until pure cultures were obtained.

Identification of the endophytes

All endophytes were identified by Professor Chern-Hsiung Lai, and by the Bioresource Collection and Research Centre in Taiwan. The endophytic bacterial isolates were identified based on Biolog Phenotype MicroArray (PM) technique (Biolog Inc., Hayward, CA). Phenotype MicroArray technology for the screening of chemical sensitivity allows for 95 different chemicals to be tested at four increasing concentrations in a single Phenotype MicroArray plate (96 wells). In each assay, active cellular respiration is probed using the redox chemistry of a patented tetrazolium dye, which forms a purple derivative upon reduction. Strains were grown overnight at 30°C on Biolog universal growth agar plates. The biomass was suspended in 15 ml of inoculation fluid using a sterile cotton swab and the cell density was adjusted to 85% transmittance on a Biolog turbidimeter. The 85% transmittance suspension was diluted 200-fold into 120 ml of inoculation fluid, and 1% tetrazolium dye was added. The mixture was inoculated into the Phenotype MicroArray plates (100 µl per well), which were incubated at 30°C in an Omnilog reader. Quantitative color changes were recorded automatically every 15 min by a charge-coupled device camera for a period of 48 h. The strains were analysed with the Omnilog-PM software. The data were filtered using average height as a parameter (Tremaroli et al. 2009). Furthermore, the endophytic fungi were identified based on their ITS sequences, as well as their 16S rDNA sequences.

Table 1 Thirteen Formosan herbal plants and their bioactive studies

Sources		Bioactivities and references
Scientific name	Use part	
Rhinacanthus nasutus	Stem	Anti-tumor, anti-platelet aggregation and anti-inflammation (Siripong et al. 2006; Wu et al. 1998; Punturee et al. 2004)
Annona squamosa	Seed	Cytotoxicity (Liaw et al. 2008); anti-inflammation (Yang et al. 2008)
Goniothalamus amuyon	Stem and root	Cytotoxicity & anti-cancer (Lan et al. 2003, 2006)
Catharanthus roseus	Stem	Anti-cancer (van der Heijden et al. 2004); antioxidant (Ferreres et al. 2008)
Emilia sonchifolia	Stem	Anti-inflammation and cytotoxicity (Muko and Ohiri 2000; Shylesh and Padikkala 2000)
Artemisia argyi	Stem	Farnesyl protein transferase inhibitor (Lee et al. 2002; Seo et al. 2003)
Ixeris chinensis	Stem	Antifebrile, antidotal, and analgesic effects (Zhang et al. 2006)
Euonymus spraguei	Stem	Not found
Orthosiphon spiralis	Stem	Not found
Prunella vulgaris	Stem	Treatment of scrofula, goiter, dermatosis, and skin allergy diseases (Kim et al. 2007)
Ludwigia octovalvis	Stem	Treatment for edema, nephritis, antihypertension, antidiabetic, and immunosuppressive activities (Chang et al. 2004)
Plantago asiatica	Leave	Antipyretic, antitussive, diuretic, wound healing, antiviral, anticancer and immunomodulation activities (Choi et al. 2008; Chiang et al. 2003)
Cardiospermum halicacabum	Stem	Analgesic, anti-inflammatory and vasodepressant activities (Nadkarni 1976; Sheeba and Asha 2006; Gopalakrishnan et al. 1976; Sadique et al. 1987)

Comparison of sequences from the isolates and GenBank sequences were performed to give the identifications.

Fermentation and extraction

Each strain (Table 2) was cultured on PDA for 7 days at 28°C, respectively, and then to provide the culture broth in 1-l Erlenmeyer flasks each containing sterilized 500 ml of potato dextrose broth (PDB, Sigma–Aldrich). The flasks were incubated at room temperature on a rotary shaker at 150 rev/min for 10–14 days (bacteria) or 28 days (fungus) according to the late exponential phase (optical density at 600 nm > 1.0). Furthermore, the mycelial fraction and culture broth fraction of all endophytes were separated by centrifugation (12,000 rev/min) and filtration. The mycelial part was extracted by EtOH (Merck, ACS grade), to give EtOH extracts. In addition, the culture broth part was subjected to liquid–liquid partition with $CHCl_3$ (3 × 500 ml, Merck, ACS grade), *n*-BuOH (3 × 500 ml, Merck, ACS grade), and followed by removal of the solvent under reduced pressure to yield $CHCl_3$ extract, *n*-BuOH extract, and H_2O extract.

Isolation of compounds

Optical rotations were measured with a JASCO P-1020 digital polarimeter. The UV spectra were obtained on a JASCO V-530 UV/Vis spectrophotometer, and IR spectra were measured on a Perkin Elmer 2000 FT-IR spectrophotometer. 1D (^1H ^3C, DEPT) and 2D (COSY, HMQC, HMBC, NOESY) NMR spectra using C_5D_5N (pentadeuteropyridine, Merck), CD_3OD (tetradeuteromethanol, Merck), and $CDCl_3$ (deuterochloroform, Merck) as solvents were obtained on Varian Unity Plus 400. Chemical shifts are reported in parts per million (δ), and coupling constants (J) are expressed in Hertz and were internally referenced to the solvent signals in C_5D_5N (^1H, δ_H 7.21; ^{13}C, δ_C 123.5), CD_3OD (^1H, δ_H 3.31; ^{13}C, δ_C 48.0) and $CDCl_3$ (^1H, δ_H 7.26; ^{13}C, δ_C 77.0). Low-resolution EIMS were measured on a JEOL JMS-SX/SX 102A mass spectrometer or Quattro GC-MS spectrometer having a direct inlet system. LRESIMS and HRESIMS were obtained on a Bruker Daltonics APEX II 30e spectrometer. HPLC instruments included a Shimadzu LC-10AT pump, a Schimadzu SPM-M10Avp detector,

Table 2 The source and codes of each endophyte

Sources		Identification	Codes
Scientific name	Family name		
Rhinacanthus nasutus	Acanthaceae	*Pantoea agglomerans*	Rn-01
Annona squamosa	Annonaceae	*Penicillium* sp.	As-0A
Goniothalamus amuyon	Annonaceae	*Achromobacter xylosoxidans ss denitrificans*	Gs-01
		Phialemonium sp.	Gs-0A
		Colletotrichum crassipes	Gs-0B
		Curtobacterium citreum	Gr-01
Catharanthus roseus	Apocynaceae	*Actinobacillus porcinus*	B0-061
		Achromobacter xylosoxidans ss denitrificans	B0-062
Emilia sonchifolia	Asteraceae	*Rahnella aquatilis*	Ems-01
Artemisia argyi	Asteraceae	*Rhodotorula* sp.	Al-01
		Fusarium sp.	Al-0A
Ixeris chinensis	Asteraceae	*Bacillus subtilis* subsp.	CC-01
Euonymus spraguei	Celastraceae	*Pseudomonas* sp.	Eus-01
Orthosiphon spiralis	Labiatae	*Bacillus licheniformis*	Os-01
Prunella vulgaris	Labiatae	*Pantoea agglomerans*	Pv-01
		Brochothrix themosphacta	Pv-02
		Penicillium paxilli	Pv-0A
Ludwigia octovalvis	Onagraceae	*Achromobacter xylosoxidans ss denitrificans*	Lo-01
		Sphingomonas sanguinis	Lo-02
Plantago asiatica	Plantaginaceae	*Penicillium citrinum*	Pa-0A
Cardiospermum halicacabum	Spindaceae	*Sphingomonas yabuuchiae*	Ch-01
		Methylobacterium aquaticum	Ch-02

a Shimadzu SCL-10Avp system controller, and a Thermo Hypersil ODS 5 μm (250 × 4 mm i.d.).

The $CHCl_3$ extract (148 mg) was separated on a SPE (Merck) eluting with a gradient of $CHCl_3$/MeOH (Merck, ACS grade) to give 6 fractions (A–F). Fraction C (7.1 mg) was recrystallized from MeOH, and filtered to afford **5** (0.16 mg). Fraction B (23 mg) was chromatographed on Sephadex LH-20 (Pharmacia) with MeOH to give 3 subfractions (B1–B3). The B2 fraction was further purified using preparative TLC ($CHCl_3$/MeOH 20:1) to give **1** (0.5 mg) 2 (0.6 mg), **3** (0.8 mg). Subfraction B3 was recrystallized from MeOH, and filtered to afford **4** (3.3 mg). Fractions A and D–F were combined, and was further separated by ODS HPLC using a gradient of program (40% MeOH/H_2O to 100% MeOH for 75 min) to afford compound **6** (0.5 mg, t_R: 16.9 min).

Compound **1**

Lumichrome, molecular formula $C_{12}H_{10}N_4O_2$, mp 332–333°C, ESI-MS: *m/z* 265 $[M + Na]^+$ ^{1}H-NMR (C_5D_5N, 400 MHz): δ (ppm) 2.31 (3H, s) 2.25 (3H, s), 7.87 (1H, s), 8.05 (1H, s) ^{13}C-NMR (C_5D_5N 100 MHz): δ 19.8 (q) 20.4 (q) 129.6 (d) 131.0 (d) 134.9 (s) 139.1 (s) 139.7 (s) 143.0 (s) 144.8 (s) 147.6 (s) 151.8 (s) 162.0 (s). The spectral data are in full agreement with those of a commercial standard (Acros).

Compound **2**

Genistein, molecular formula $C_{15}H_{10}O_5$, mp 186–187°C, ESI-MS (negativ): *m/z* 269 $[M-H]^+$ ^{1}H-NMR (CD_3OD, 400 MHz): δ (ppm) 6.21 (1H, d, $J = 2.4$ Hz, H-6), 6.33 (1H, d, $J = 2.4$ Hz, H-8), 6.83 (2H, d, $J = 8.4$ Hz, H-3′, H-5′), 7.36 (2H, d, $J = 8.4$ Hz, H-2′, H-6′), 8.04 (1H, s, H-2) ^{13}C-NMR (CD_3OD 100 MHz): δ 94.6 (C-8, d) 100.2 (C-6, d) 106.2 (C-10, s) 116.2 (C-3′, C-5′, d) 123.3 (C-1′, s) 124.7 (C-3, s) 131.3 (C-2′, C-6′, d),154.7 (C-2, d) 158.8 (C-4′, s) 159.7 (C-4, s) 163.8 (C-5, s) 166.2 (C-7, s) 182.2 (C-4, s). The spectral data are in full agreement with those reported (Kinjo et al. 1987).

Compound **3**

Daidzein, molecular formula $C_{15}H_{10}O_4$, mp 285–287°C, ESI-MS: *m/z* 255 $[M + H]^+$ ^{1}H-NMR (CD_3OD, 400 MHz): δ (ppm) 6.83 (2H, d, $J = 8.0$ Hz, H-3′, H-5′), 6.85 (1H, d, $J = 2.8$ Hz, H-8), 6.95 (1H, d, $J = 8.8$ Hz, H-6), 7.38 (2H, d, $J = 8.0$ Hz, H-2′, H-6′), 8.04 (1H, d, $J = 8.8$ Hz, H-5), 8.14 (1H, s, H-2) ^{13}C-NMR (CD_3OD 100 MHz): 103.2 (C-8, d) 116.2 (C-3′, C-5′, d) 116.4 (C-6, d) 116.4 (C-10, d) 124.2 (C-1′, s) 125.7 (C-3, s) 128.5 (C-5, d) 131.4 (C-2′, C-6′, d) 154.6 (C-2, d) 158.6 (C-4′, s) 159.8 (C-9, s) 164.7 (C-7, s) 178.1 (C-4, s). The spectral data are in full agreement with those reported (Kinjo et al. 1987; Kurihara and Kikuchi 1976).

Compound **4**

Cyclo-Pro-Val, molecular formula $C_{10}H_{16}N_2O_2$, mp 99–100°C, ESI-MS: *m/z* 219 $[M + Na]^+$ ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 0.90 (3H, d, $J = 6.4$ Hz) 1.05 (3H, d, $J = 6.4$ Hz) 2.06 (1H, m)/1.88 (1H, m) 2.36 (1H, m)/2.06 (1H, m) 2.63 (1H, d, $J = 2.8$ Hz), 3.53 (2H, m), 3.94 (1H, br. s), 4.06 (1H, br. t, $J = 16$ Hz), 5.82 (1H, br. s) ^{13}C-NMR ($CDCl_3$ 100 MHz): δ16.0 (q) 19.2 (q) 22.3 (t) 28.3 (d) 28.5 (t), 45.1 (t), 58.8 (d), 60.3 (d) 164.8 (s) 169.9 (s). The spectral data are in full agreement with those reported (Pettit et al. 2006).

Compound **5**

Cyclo-Pro-Phe, molecular formula $C_{14}H_{16}N_2O_2$, ESI-MS (negative): *m/z* 244 $[M-H]^+$ ^{1}H-NMR ($CDCl_3$, 400 MHz): δ (ppm) 1.90 (1H, m) 1.98 (1H, m) 2.00 (1H, m) 2.32 (1H, m) 2.76 (1H, dd, $J = 14.6$, 3.8 Hz), 3.58 (1H, d, $J = 3.2$ Hz), 3.62 (1H, m), 3.65 (1H, d, $J = 3.4$ Hz), 4.08 (1H, t, $J = 6.8$ Hz), 4.25 (1H, d, $J = 11.0$ Hz), 5.62 (1H, br. s), 7.32 (2H, m), 7.20 (2H, m), 7.24 (1H, m). The spectral data are in full agreement with those reported (Takaya et al. 2007).

Compound **6**

Methyl 2,4,5-trimethoxybenzoate, molecular formula $C_{11}H_{14}O_5$, mp 91–92°C, ESI-MS: *m/z* 249 $[M + Na]^+$ ^{1}H-NMR (CD_3OD, 400 MHz): δ (ppm) 3.80 (3H, s), 3.83 (3H, s), 3.87 (3H, s), 3.92 (3H, s), 6.72 (1H, s), 7.40 (1H, s) ^{13}C-NMR (CD_3OD 100 MHz): δ 52.2 (q), 56.5 (q), 57.1 (q), 57.1 (q), 99.0 (d) 102.0 (s) 116.1 (d) 143.8 (s) 155.8 (s) 157.5 (s) 143.8 (s) 167.8 (s). The spectral data are in full agreement with those reported (Parker et al. 1987).

Cytotoxicity assay

The extracts were tested against six cancer cell lines: HepG2, Hep3B (human hepatoma cell); A549 (human lung cancer cell); MCF-7, MDA-MB-231 (human breast cancer cell); and Ca9-22 (human oral epithelial cancer cell) was using the MTT method (Chen et al. 2008; Hsieh et al. 2004). Freshly trypsinized cell suspensions were seeded in 96 well microtiter plates at densities of 5,000–10,000 cells per well with tested extracts at 20 10, 5 2 1, 0.5… μg/ml concentration added from DMSO-diluted stock. After 72 h in culture, attached cells were incubated with MTT (0.5 mg/ml 1 h) and subsequently solubilized in DMSO for determining of IC_{50}. The absorbency at 550 nm was then measured using a microplate reader. The cytotoxic activity

of compounds was expressed as the concentration inhibiting cell growth by 50% (IC_{50}) calculated from the survival curves (Chen et al. 2008).

Measurement of platelet aggregation

Platelet aggregation was measured turbidimetrically with a light-transmission aggregometer (Chrono-Log Co., USA). The platelet suspension was incubated with dimethyl sulfoxide (DMSO, vehicle) or test compounds/extracts at 37°C for 3 min under stirring (1,200 rev/min) prior to the addition of the platelet aggregation inducers. The extent of platelet aggregation was measured as the maximal increase of light transmission within 5 min after the addition of inducers (Hsieh et al. 2008).

Preparation of human neutrophils

Blood was taken from healthy human donors (20–32 years old) by venipuncture, using a protocol approved by the institutional review board at Chang Gung Memorial Hospital. Neutrophils were isolated with a standard method of dextran sedimentation prior to centrifugation in a Ficoll Hypaque gradient and hypotonic lysis of erythrocytes. Purified neutrophils that contained >98% viable cells, as determined by the trypan blue exclusion method, were resuspended in a calcium (Ca^{2+})-free HBSS buffer at pH 7.4, and were maintained at 4°C before use (Hsieh et al. 2007).

Measurement of O_2^- generation

The assay of O_2^- generation was based on the SOD-inhibitable reduction of ferricytochrome *c* (Hsieh et al. 2007). In brief, after supplementation with 0.5 mg/ml ferricytochrome *c* and 1 mM Ca^{2+}, neutrophils were equilibrated at 37°C for 2 min and incubated with drugs for 5 min. Cells were activated with 100 nM FMLP for 10 min. When FMLP was used as a stimulant, CB (1 μg/ml) was incubated for 3 min before activation by the peptide (FMLP/CB). Changes in absorbance with the reduction of ferricytochrome *c* at 550 nm were continuously monitored in a double-beam, six-cell positioner spectrophotometer with constant stirring (Hitachi U-3010, Tokyo, Japan). Calculations were based on differences in the reactions with and without SOD (100 U/ml) divided by the extinction coefficient for the reduction of ferricytochrome *c* ($\varepsilon = 21.1$/mM/10 mm; Hsieh et al. 2007).

Results and discussion

A total of 19 endophytic strains were isolated from 13 Taiwanese herbal plants. These included four gram-positive bacteria: *Curtobacterium citreum*, *Bacillus subtilis* subsp. *subtilis*, *Brochothrix themosphacta*, and *Bacillus licheniformis*; eight gram-negative bacteria: *Pseudomonas* sp., *Panotea agglomerans*, *Rahnella aquatilis*, *Actinobacillus porcinus*, *Methylobacterium aquaticum*, *Sphingomonas yabuuchiae*, *Sphingomonas sanguinis*, and *Achromobacter xylosoxidans* ssp. *denitrificans*; four Ascomycota fungi: *Rhodotorula* sp., *Fusarium* sp., *Phialemonium* sp., and *Colletotrichum crassipes*; and three Basidiomycota fungi: *Penicillium citrinum*, *Penicillium paxilli*, and *Penicillium* sp.

In previous studies, *Curtobacterium* spp. have been isolated as endophytes from many crops, including red clover, rice, potato, yam, prairie plants, and citrus (Lacava et al. 2007). Several reports have indicated that *C. flaccumfaciens* can function as a biological control agent against many pathogens, by either triggering of induced systemic resistance or by antibiosis (Lacava et al. 2007). *Bacillus* spp. are common endophytes, and have been isolated from cotton, cucumber root, balloon flower root, and citrus plants (Cho et al. 2007). Additionally, *Bacillus* spp. have been used as biocontrol agents against phytopathogenic microorganisms (Ryan et al. 2008). The endophytic *Pseudomonas* sp. and *Pantoea agglomerans* have been reported to enhance growth and improve the health of plants (Jayatilake et al. 1996; Ryan et al. 2008). *Rahnella aquatilis* was first isolated from drinking and river water, and subsequently from human clinical specimens and the rhizospheres of different plants. Its major outer membrane protein is involved in adhesion of this organism to wheat roots (Achouak et al. 1998). *Methylobacterium* spp. are distributed in a wide variety of environments, including soil, air, dust, fresh and marine water, and sediments (Hiraishi et al. 1995; Trotsenko et al. 2001; Aken et al. 2004). *Methylobacterium* spp. produce phytohormones (cytokinins and auxins) known to stimulate plant growth, enhance their atmospheric nitrogen fixation or help plants fight pathogens (Aken et al. 2004). *Sphingomonas* spp. are often found in association with plants; many strains have been isolated from the rhizosphere and secrete 3-ketolactose (White et al. 1996). Among them, *S. paucirnobilis* has shown antagonism to the phytopathogenic fungus *Verticillium dahliae* (Hashidoko 2005). *Achromobacter* spp. are plant growth-promoting rhizobacteria. They increased the shoot and root dry weight of *Brassica napus* by stimulating the ionic transport system (Bertrand et al. 2000). *Fusarium* spp. are often found in plants and many bioactive components have been isolated from these endophytic fungi, such as pentaketide, cyclic lipopeptides, pyrroles, and indole alkaloids (Brady and Clardy 2000; Tan and Zou 2001; Shiono et al. 2007). *Colletotrichum* spp. are implicated in plant diseases, usually referred to as anthracnose, and include some of the most destructive postharvest pathogens of cereals, legumes, fruits, and vegetables

(Guimaraes et al. 2008). *Penicillium* spp. are a major target of endophytic microorganism research, and many metabolites with cytotoxic and antibacterial effects have been isolated from this genus (Rukachaisirikul et al. 2007; Ge et al. 2008).

In present study, the endophytes were extracted and partitioned to yield EtOH, $CHCl_3$, n-BuOH, and H_2O extracts. The extracts were evaluated in anti-platelet aggregation, cytotoxic, and anti-inflammatory assays as described.

Anti-platelet aggregation activity

Arterial thromboembolitic diseases such as acute coronary syndrome and ischemic stroke are caused by platelet aggregation, and are major causes of death in developed countries. Anti-platelet drugs (e.g., aspirin and ticlopidine) are used to protect against myocardial infarction, stroke, cardiovascular death, and other serious vascular events in patients with a history of previous vascular events or known risk factors for cardiovascular disease (Hsieh et al. 2008). Current anti-platelet drugs have some restrictions in their mode of action and efficacy, and research and development of a new generation of anti-platelet agents is necessary.

The results of anti-platelet aggregation assays are shown in Table 3. Ten $CHCl_3$ extracts (*R. aquatilis*, *M. aquaticum*, two *P. agglomerans*, *Rhodotorula* sp., *Pseudomonas* sp., *C. crassipes*, *Penicillium* sp., *P. citrinum*, and *P. paxilli*) and two EtOH extracts (two *P. agglomerans* from *R. nasutus* and *P. vulgaris*, respectively) exhibited inhibitory effects on platelet aggregation induced by collagen, with IC_{50} values of 19.8–88.0 μg/ml. In addition, four $CHCl_3$ extracts (*Rahnella aquatilis*, *Pantoea agglomerans* from *R. nasutus*, *Rhodotorula* sp., and *P. paxilli*) showed inhibitory effects on platelet aggregation induced by thrombin, with IC_{50} values of 42.0–62.0 μg/ml. This is the first report evaluating anti-platelet activity of endophytes. Interestingly, two *P. agglomerans* were isolated from *R. nasutus* and *P. vulgaris*, respectively, and presented different bioactive profiles. Furthermore, of three *Penicillium* spp., only *P. paxilli* exhibited a dual inhibitory effect on platelet aggregation induced by collagen and thrombin. Therefore, these bioactive endophytes could be sources of new anti-platelet compounds.

The $CHCl_3$ extract of *Rahnella aquatilis* showed anti-platelet aggregation activity, and bioassay-directed fractionation led to the isolation of six compounds: lumichrome (**1**), genistein (**2**), daidzein (**3**), cyclo-Pro-Val (**4**), cyclo-Pro-Phe (**5**), and methyl 2,4,5-trimethoxybenzoate (**6**). These compounds were tested for their anti-platelet aggregation activities. The results showed compounds **1–3** had significant inhibitory effects on platelet aggregation induced by collagen, with IC_{50} values of 65.0 18.8, and 21.1 μg/ml, respectively. The results suggest that lumichrome, genistein, and daidzein were the bioactive components in the $CHCl_3$ extract of *R. aquatilis*.

Cytotoxic activity

The cytotoxic assay results are shown in Table 4. These tests used lung (A-549), breast (MDA-MB-231 and MCF-7), and

Table 3 Anti-platelet aggregation and anti-inflammatory effects of bioactive endophytic extracts

Endophytic codes	Fraction	Anti-platelet aggregation IC_{50} (μg/ml)		Anti-inflammation[a]
		Thrombin (0.05 U/ml)	Collagen (3.00 μg/ml)	
Ems-01	$CHCl_3$	61.54	19.85	−
Ch-01	EtOH	>100	>100	+
Ch-02	$CHCl_3$	>100	47.49	−
Rn-01	$CHCl_3$	42.80	28.40	−
	EtOH	>100	53.33	+
Pv-01	EtOH	>100	48.13	−
Al-01	$CHCl_3$	55.63	34.93	+
	EtOH	>100	87.64	+
Eus-01	$CHCl_3$	>100	72.41	−
Gs-0B	$CHCl_3$	>100	59.80	+
	EtOH	>100	>100	+
Gs-0A	$CHCl_3$	>100	>100	+
	EtOH	>100	>100	+
As-0A	$CHCl_3$	>100	85.40	−
Pa-0A	$CHCl_3$	>100	69.68	−
Pv-0A	$CHCl_3$	49.16	35.32	−

[a] + the percentage inhibition over 50% at least three superoxide anion ($O_2^{\cdot-}$) generation induced by human neutrophils in response to fMLP/CB assays with 10 μg/ml; − the percentage inhibition less than 50% at least three superoxide anion ($O_2^{\cdot-}$) generation induced by human neutrophils in response to fMLP/CB assays with 10 μg/ml

Table 4 Cytotoxic activities of bioactive endophytic extracts

Endophytic codes	Fractions	Cytotoxicity IC_{50} (μg/ml)[a]				
		Hep G2	Hep 3B	A-549	MCF-7	MDA-MB-231
Al-01	$CHCl_3$	19.83	>20	>20	>20	>20
Rn-01	$CHCl_3$	3.67	16.85	>20	>20	>20
Pv-01	$CHCl_3$	0.12	16.21	>20	>20	>20
	EtOH	>20	>20	16.20	>20	>20
Gr-01	$CHCl_3$	14.95	>20	>20	>20	>20
Pv-02	*n*-BuOH	7.46	17.65	>20	>20	>20
Al-0A	$CHCl_3$	0.90	7.65	11.21	7.27	17.10
	H_2O	>20	>20	>20	18.03	>20
Pa-0A	$CHCl_3$	4.06	15.09	>20	>20	>20
Pv-0A	$CHCl_3$	0.50	14.87	>20	11.34	>20
As-0A	$CHCl_3$	1.60	4.58	>20	5.02	>20
Gs-01	*n*-BuOH	4.41	11.92	>20	>20	>20

[a] IC_{50} of crude extract over 20 μg/ml was defined as noncytotoxic following to NCI rule

liver (Hep G2 and Hep 3B) cancer cell lines. Eight $CHCl_3$ extracts (two *Pantoea agglomerans*, *Rhodotorula* sp., *C. citreum*, *Fusarium* sp., *Penicillium* sp., *Penicillium citrinum*, and *Penicillium paxilli*), two BuOH extracts (*B. themosphacta* and *C. crassipes*) and the aqueous extract of *Fusarium* sp. had significant (<20 μg/ml) cytotoxic effects, with IC_{50} values of 0.1–19.8 μg/ml. Among them, the $CHCl_3$ extracts of *Pantoea agglomerans* from *P. vulgaris* were selective against the Hep G2 cancer cell line with IC_{50} of 0.12 μg/ml. Furthermore, the $CHCl_3$ extracts of *Fusarium* sp., *Penicillium paxilli*, and *Penicillium* sp. also exhibited strong cytotoxicity against Hep G2 cancer cell line with IC_{50} values of 0.9, 0.5, and 1.6 μg/ml, respectively. However, only the $CHCl_3$ extracts of *Fusarium* sp. revealed significant cytotoxicity against four cancer cell lines.

In previous studies, some anti-cancer drugs have been found in endophyte cultures (Jiao et al. 2006; Davis et al. 2008). Many cytotoxic constituents were isolated from extracts of the endophytic fungi, *Fusarium* sp. and *Penicillium* sp. (Kour et al. 2008; Ge et al. 2008). However, in the endophytes *Pantoea agglomerans*, *Rhodotorula* sp., *B. licheniformis*, *C. citreum*, *B. themosphacta* and *C. crassipes*, compounds with cytotoxicities were found for the first time. Therefore, these bioactive extracts could be improved as sources for research of new lead compounds with cytotoxic activity.

Anti-inflammatory activity

Human neutrophils are active phagocytes that are crucial components of immunity (Hsieh et al. 2007; Chang et al. 2008). They play important roles in the host defense against microorganisms and in the pathogenesis of various diseases (Malech and Gallin 1987; Witko-Sarsat et al. 2000; Chang et al. 2008). In response to diverse stimuli, activated neutrophils secrete the superoxide anion (O_2^-) and a series of cytotoxins (Malech and Gallin 1987; Roos et al. 2003; Chang et al. 2008). It is essential to control respiratory burst and degranulation in physiological conditions while potentiating these functions in infected tissues and organs (Hsieh et al. 2007; Chang et al. 2008). Since there are only a few agents used to modulate neutrophil pro-inflammatory responses, the research and development of new generation anti-inflammatory agents continues.

The anti-inflammatory assays evaluated the inhibitory effect on O_2^- generation induced by human neutrophils in response to fMLP/CB with the extracts at 10 μg/ml concentration (Table 3). Three $CHCl_3$ extracts (*Rhodotorula* sp., *Phialemonium* sp., and *C. crassipes*), and five EtOH extracts (*Pantoea agglomerans* from *R. nasutus*, *S. yabuuchiae*, *Rhodotorula* sp., *Phialemonium* sp., and *C. crassipes*) showed inhibitory effects on O_2^- generation. This is the first report evaluating anti-inflammatory activity of endophytes.

The potential of endophytes as an effective alternative or novel source of therapeutic compounds has been recognized. In addition, there have been several reports on use of endophytes to produce pacitaxel, camptothecin, and podophyllotoxin isolated from the hosts *Taxus brevifolia* (Stierle et al. 1993), *Nothapodytes foetida* (Puri et al. 2005)/*Camptotheca acuminata* (Kusari et al. 2009), and *Podophyllum peltatum* (Eyberger et al. 2006), respectively. Such results indicate a correlation between bioactivity and chemical constituents of host plants and endophytes. In the present study, the endophytic codes, e.g., Rn-01, As-0A, Gs-01, Gr-01, B0-062, Pa-0A, and Ch-01, showed similar bioactivities with those host plants (Tables 1, 2, 3, 4).

Therefore, these endophytic extracts are worthy of future study.

In conclusion, a high incidence of anti-platelet aggregation, cytotoxicity, and anti-inflammation effects were determined in assays. The tests indicated that bioactive endophytes could be useful sources for research and development of bioactive lead compounds from nature.

Acknowledgments We gratefully acknowledge financial support for this project from the Kaohsiung Medical University (Q-9505) and Chang Gung University (CMRPD-170431). The authors also thank the Bioresource Collection and Research Centre for support in identification of endophytes.

References

Achouak W, Pages JM, De Mot R, Molle G, Heulin T (1998) A major outer membrane protein of *Rahnella aquatilis* functions as a porin and root adhesin. J Bacteriol 180:909–913

Aken BV, Peres CM, Doty SL, Yoon JM, Schnoor JL (2004) *Methylobacterium populi* sp. nov., a novel aerobic, pink-pigmented, facultatively methylotrophic, methane-utilizing bacterium isolated from poplar trees (*Populus deltoids x nigra* DN34). Int J Syst Evol Microbiol 54:1191–1196. doi:10.1099/ijs.0.02796-0

Bertrand H, Plassard C, Pinochet X, Touraine B, Normand P, Cleyet-Marel JC (2000) Stimulation of the ionic transport system in *Brassica napus* by a plant growth-promoting rhizobacterium (*Achromobacter* sp.). Can J Microbiol 46:229–236. doi:10.1139/cjm-46-3-229

Brady SF, Clardy J (2000) CR377, a new pentaketide antifungal agent isolated from an endophytic fungus. J Nat Prod 63:1447–1448. doi:10.1021/np990568p

Chang CI, Kuo CC, Chang JY, Kuo YH (2004) Three new oleanane-type triterpenes from *Ludwigia octovalvis* with cytotoxic activity against two human cancer cell lines. J Nat Prod 67:91–93. doi:10.1021/np030267m

Chang HL, Chang FR, Chen JS, Wang HP, Wu YH, Wang CC, Wu YC, Hwang TL (2008) Inhibitory effects of 16-hydroxycleroda-3, 13(14)E-dien-15-oic acid on superoxide anion and elastase release in human neutrophils through multiple mechanisms. Eur J Pharmacol 586:332–339. doi:10.1016/j.ejphar.2008.02.041

Chen YL, Lan YH, Hsieh PW, Wu CC, Chen SL, Yen CT, Chang FR, Hung WC, Wu YC (2008) Bioactive membrane diterpenoids of *Anisomeles indica*. J Nat Prod 71:1207–1212. doi:10.1021/np800147z

Chiang LC, Ng LT, Chiang W, Chang MY, Lin CC (2003) Immunomodulatory activities of flavonoids, monoterpenoids, triterpenoids, iridoid glycosides and phenolic compounds of *Plantago* species. Planta Med 69:600–604. doi:10.1055/s-2003-41113

Cho KM, Hong SY, Lee SM, Kim YH, Kahng GG, Lim YP, Kim H, Yun HD (2007) Endophytic bacterial communities in ginseng and their antifungal activity against pathogens. Microb Ecol 54:341–351. doi:10.1007/s00248-007-9208-3

Choi SY, Jung SH, Lee HS, Park KW, Yun BS, Lee KW (2008) Glycation inhibitory activity and the identification of an active compound in *Plantago asiatica* extract. Phytother Res 22:323–329. doi:10.1002/ptr.2316

Davis RA, Longden J, Avery VM, Healy PC (2008) The isolation, structure determination and cytotoxicity of the new fungal metabolite, trichodermamide C. Bioorg Med Chem Lett 18:2836–2839. doi:10.1016/j.bmcl.2008.03.090

Eyberger AL, Dondapati R, Porter JR (2006) Endophyte fungal isolates from *Podophyllum peltatum* produce podophyllotoxin. J Nat Prod 69:1121–1124. doi:10.1021/np060174f

Ferreres F, Pereira DM, Valentão P, Andrade PB, Seabra RM, Sottomayor M (2008) New phenolic compounds and antioxidant potential of *Catharanthus roseus*. J Agric Food Chem 56:9967–9974. doi:10.1021/jf8022723

Ge HM, Shen Y, Zhu CH, Tan SH, Ding H, Song YC, Tan RX (2008) Penicidones A-C, three cytotoxic alkaloidal metabolites of an endophytic *Penicillium* sp. Phytochemistry 69:571–576. doi:10.1016/j.phytochem.2007.07.014

Gopalakrishnan C, Dhananjayan R, Kameswaran L (1976) Studies on the pharmacological actions of *Cardiospermum halicacabum*. Indian J Physiol Pharmacol 20:203–206

Guimaraes DO, Borges WS, Kawano CY, Ribeiro PH, Goldman GH, Nomizo A, Thiemann OH, Oliva G, Lopes NP, Pupo MT (2008) Biological activities from extracts of endophytic fugi isolated from *Viguiera arenaria* and *Tithonia diversifolia*. FEMS Immunol Med Microbiol 52:134–144

Gunatilaka AAL (2006) Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. J Nat Prod 69:509–526. doi:10.1021/np058128n

Hashidoko Y (2005) Ecochemical studies of interrelationships between epiphytic bacteria and host plants via secondary metabolites. Biosci Biotechnol Biochem 69:1427–1441. doi:10.1271/bbb.69.1427

Hiraishi A, Furuhata K, Matsumoto A, Koike KA, Fukuyama M, Tabuchi K (1995) Phenotypic and genetic diversity of chlorine-resistant *Methylobacterium* strains isolated from various environments. Appl Environ Microbiol 61:2099–2107

Hsieh PW, Chang FR, Wu CC, Wu KY, Li CM, Chen SL, Wu YC (2004) New cytotoxic cyclic-peptides and dianthramide from *Dianthus superbus*. J Nat Prod 67:1522–1527. doi:10.1021/np040036v

Hsieh PW, Hwang TL, Wu CC, Chiang SZ, Wu CI, Wu YC (2007) The evaluation and structure activities relationships of 2-Benzoylamino-Benzoic esters and their analogues as anti-inflammatory and anti-platelet aggregation agents. Bioorg Med Chem Lett 17:1812–1817. doi:10.1016/j.bmcl.2006.12.038

Hsieh PW, Chiang SZ, Wu CC, Lo YC, Shih YT, Wu YC (2008) Synthesis and anti-platelet evaluation of 2-Benzoylaminobenzoate analogs. Bioorg Med Chem 16:5803–5814. doi:10.1016/j.bmc.2008.03.071

Jayatilake GS, Thornton MP, Leonard AC, Grimwade JE, Baker BJ (1996) Metabolites from an antarctic sponge-associated bacterium, *Pseudomonas aeruginosa*. J Nat Prod 59:293–296. doi:10.1021/np960095b

Jiao RH, Xu S, Liu JY, Ge HM, Ding H, Xu C, Zhu HL, Tan RX (2006) Cheitominine, a cytotoxic alkaloid produced by endophytic *Chaetomium* sp. IFB-E015. Org Lett 8:5709–5712. doi:10.1021/ol062257t

Kim SY, Kim SH, Shin HY, Lim JP, Chae BS, Park JS, Hong SG, Kim MS, Jo DG, Park WH, Shin TY (2007) Effects of *Prunella vulgaris* on mast cell-mediated allergic reaction and inflammatory cytokine production. Exp Biol Med 232:921–926

Kinjo J, Furusawa J, Baba J, Takeshita T, Yamasaki M, Nohara T (1987) Studies on the constituents of *Pueraria lobata*. III. Isoflavonoids and relateed compounds in the roots and the voluble stems. Chem Pharm Bull (Tokyo) 35:4846–4850

Kour A, Shawl AS, Rehman S, Sultan P, Qazi PH, Suden P, Khajuria RK, Verma V (2008) Isolation and identification of an endophytic strain of *Fusarium oxysporum* producing podophyllotoxin from Juniperus recurva. World J Microbiol Biotechnol 24:1115–1121. doi:10.1007/s11274-007-9582-5

Kurihara T, Kikuchi M (1976) Studies on the constituents of flowers. VI. On the components of the flower of *Pueraria thubergiana* Benth. Yakugaku Zasshi 96:1486–1488

Kusari S, Zühlke S, Spiteller M (2009) An endophytic fungus from *Camptotheca acuminata* that produces camptothecin and analogues. J Nat Prod 72:2–7. doi:10.1021/np800455b

Lacava PT, Li W, Araujo WL, Azevedo JL, Hartung JS (2007) The endophyte *Curtobacterium flaccumfaciens* reduces symptoms caused by Xylella fastidiosa in Catharanthus roseus. J Microbiol 45:388–393

Lan YH, Chang FR, Yu JH, Yang YL, Chang YL, Lee SJ, Wu YC (2003) Cytotoxic styrylpyrones from *Goniothalamus amuyon*. J Nat Prod 66:487–490. doi:10.1021/np020441r

Lan YH, Chang FR, Yang YL, Wu YC (2006) New constituents from stem of *Goniothalamus amuyon*. Chem Pharm Bull (Tokyo) 54:1040–1043. doi:10.1248/cpb.54.1040

Lee SH, Kim HK, Seo JM, Kang HM, Kim JH, Son KH, Lee H, Kwon BM (2002) Arteminolides B, C, and D, new inhibitor of farnesyl protein transferase from *Artemisia argyi*. J Org Chem 67:7670–7675. doi:10.1021/jo020299z

Liaw CC, Yang YL, Chen M, Chang FR, Chen SL, Wu SH, Wu YC (2008) Mono-tetrahydrofuran annonaceous acetogenins from *Annona squamosa* as cytotoxic agents and calcium ion chelators. J Nat Prod 71:764–771. doi:10.1021/np0704957

Malech HL, Gallin JI (1987) Current concepts: immunology. Neutrophils in human diseases. N Engl J Med 317:687–694

Muko KN, Ohiri FC (2000) A priliminary study on the anti-inflammatory properties of *Emilia sonchifolia* leaf extracts. Firoterapia 71:65–68. doi:10.1016/S0367-326X(99)00123-9

Nadkarni KM (1976) Indian materia medica. Popular Book Depot, Bombay, p 271

Parker KA, Spero DM, Koziski KA (1987) Evaluation of some preparations of trialkoxyphthalic acid derivatives. J Org Chem 52:183–188. doi:10.1021/jo00378a005

Patrick GL (2005) An introduction to medicinal chemistry, 3rd edn. Oxford University Press Inc, New York

Pettit GR, Du J, Pettit RK, Richert LA, Hogan F, Mukku VJ, Hoard MS (2006) Antineoplastic agents. 554. The manitoba bacterium *Streptomyces* sp. J Nat Prod 69:804–806. doi:10.1021/np058087v

Phongpaichit S, Nikom J, Rungjindamai N, Sakayaroj J, Hutadilok-Towatana N, Rukachaisirikul V, Kirtikara K (2007) Biological activities of extracts from endophytic fungi isolated from *Garcinia* plants. FEMS Immunol Med Microbiol 51:517–525. doi:10.1111/j.1574-695X.2007.00331.x

Punturee K, Wild CP, Vinitketkumneun U (2004) Thai medicinal plants modulate nitric oxide and tumor necrosis factor-α in J774.2 mouse macrophages. J Ethnopharmacol 95:183–189. doi:10.1016/j.jep.2004.06.019

Puri SC, Amna T, Khajuria A, Gupta A, Arora R, Spiteller M, Qazi GN (2005) Immunomodulatory activity of an extract of the novel fungal endophyte Entrophospora infrequens isolated from *Nothapodytes foetida* (Wight) Sleumer. Acta Microbiol Immunol Hung 54:237–260. doi:10.1556/AMicr.54.2007.3.2

Roos D, van Bruggen R, Meischl C (2003) Oxidative killing of microbes by neutrophils. Microbes Infect 5:1307–1315. doi:10.1016/j.micinf.2003.09.009

Rukachaisirikul V, Kaeobamrung J, Panwiriyarat W, Saitai P, Sukpodma Y, Phongpaichit S, Sakayaroj J (2007) A new pyrone derivative from the entophytic fungus *Penicillium paxilli* PSU-A71. Chem Pharm Bull (Tokyo) 55:1383–1384. doi:10.1248/cpb.55.1383

Ryan RP, Germaine K, Franks A, Ryan DJ, Dowling DN (2008) Bacterial endophytes: recent developments and applications. FEMS Microbiol Lett 278:1–9. doi:10.1111/j.1574-6968.2007.00918.x

Sadique J, Chandra T, Thenmozhi V, Elango V (1987) Biochemical modes of action of *Cassia occidentalis* and *Cardiospermum halicacabum* in inflammation. J Ethnopharmacol 19:201–205. doi:10.1016/0378-8741(87)90042-0

Seo JM, Kang HM, Son KH, Kim JH, Lee CW, Kim HM, Chang SI, Kwon BM (2003) Antitumor activity of flavones isolated from *Artemisia argyi*. Planta Med 69:218–222. doi:10.1055/s-2003-38486

Sheeba MS, Asha VV (2006) Effect of *Cardiospermum halicacabum* on ethanol-induced gastric ulcers in rate. J Ethnopharmacol 106:105–110. doi:10.1016/j.jep.2005.12.009

Shiono Y, Tsuchinari M, Shimanuki K, Miyajima T, Murayama T, Koseki T, Laatsch H, Funakoshi T, Takanami K, Suzuki K (2007) Fusaristatins A and B, two new cyclic lipopeptides from an endophytic *Fusarium* sp. J Antibiot 60:309–316. doi:10.1038/ja.2007.39

Shylesh BS, Padikkala J (2000) In vitro cytotoxic and antitumor property of *Emilia sonchifolia* (L.) DC in mice. J Ethnopharmacol 73:496–500. doi:10.1016/S0378-8741(00)00317-2

Siripong P, Yahuafai J, Shimizu K, Ichikawa K, Yonezawa S, Asai T, Kanokmedakul K, Ruchirawat S, Oku Naoto (2006) Antitumor activity of liposomal naphthoquinone esters isolated from Thai medicinal plant: *Rhinacanthus nasutus* Kurz. Biol Pharm Bull 29:2279–2283. doi:10.1248/bpb.29.2279

Stierle A, Strobel G, Stierle D (1993) Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. Science 260:214–216. doi:10.1126/science.8097061

Takaya Y, Furukawa T, Miura S, Akutagawa T, Hotta Y, Ishikawa N, Niwa M (2007) Antioxidant constituents in distillation residue of Awamori spirits. J Agric Food Chem 55:75–79. doi:10.1021/jf062029d

Tan RX, Zou WX (2001) Endophytes: a rich source of functional metabolites. Nat Prod Rep 18:448–459. doi:10.1039/b100918o

Tremaroli V, Workentine ML, Weljie AM, Vogel HJ, Ceri H, Viti C, Tatti E, Zhang P, Hynes AP, Turner RJ, Zannoni D (2009) Metabolomic investigation of the bacterial response to a metal challenge. Appl Environ Microbiol 75:719–728. doi:10.1128/AEM.01771-08

Trotsenko YA, Ivanova EG, Doronina NV (2001) Aerobic methylotrophic bacteria as phytosymbionts. Mikrobiologiya 70:725–736

Van der Heijden R, Jacobs DI, Snoeijer W, Hallard D, Verpoorte R (2004) The *Catharathus* alkaloids: pharmacognosy and biotechnology. Curr Med Chem 11:607–628. doi:10.2174/0929867043455846

White DC, Sutton SD, Ringelberg DB (1996) The genus *Sphingomonas*: physiology and ecology. Curr Opin Biotechnol 7:301–306. doi:10.1016/S0958-1669(96)80034-6

Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L (2000) Neutrophils: molecules, functions and pathophysiological aspects. Lab Invest 80:617–653

Wu TS, Hsu HC, Wu PL, Teng CM, Wu YC (1998) Rhinacathin-Q, a naphthoquinone from *Rhinacanthus nasutus* and its biological activity. Phytochemistry 49:2001–2003. doi:10.1016/S0031-9422(98)00425-7

Yang YL, Hua KF, Chuang PH, Wu SH, Wu KY, Chang FR, Wu YC (2008) New cyclic peptides from the seeds of *Annona squamosa* L. and their anti-inflammatory activities. J Agric Food Chem 56:386–392. doi:10.1021/jf072594w

Zhang S, Zhao M, Bai L, Hasegawa T, Wang J, Wang L, Xue H, Deng Q, Xing F, Bai Y, Sakai J, Bai J, Koyanagi R, Tsukumo Y, Kataoka T, Nagai K, Hirose K, Ando M (2006) Bioactive guainaolides from Siyekucai (*Ixeris chinensis*). J Nat Prod 69:1425–1428. doi:10.1021/np068015j