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Antimicrobial and cytotoxic acetogenin from *Polyalthia debilis*

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ABSTRACT

The objective of the study was to isolate bioactive acetogenin compound and to investigate antimicrobial, antioxidant as well as cytotoxic activities of the isolate, fractions and extracts of *Polyalthia debilis*. The *P. debilis* (roots) extracts and isolated compound were tested for their antimicrobial (agar dilution method) against twenty-seven strains of microorganisms (gram positive and gram negative bacteria, and diploid fungus), antioxidant (DPPH assay) and cytotoxic activities. The plant extracts were isolated by column chromatography and structure of compound was confirmed by spectral data. The plant extracts and isolated fractions exhibited antioxidant and cytotoxic activities. The isolated acetogenin **1** (debilisone E) displayed antimicrobial activity against *Morexella catarrhalis* with the MIC of 64 μg/mL, *Corynebacterium diphtheriae* NCTC 10356 and *Streptococcus pyogenes* with partial inhibition (50-75%) at 128 μg/mL. The compound **1** exerted cytotoxic activity against 5 cancer cells (HepG2, A549, HCC-S102,HL-60 and P388) with IC_{50} values 18.4 - 40.3μg/mL. The results demonstrate novel bioactivities of *P. debilis* as antimicrobials and anticancer agents.

INTRODUCTION

Polyalthia debilis (Annonaceae), a Thai medicinal plant, has been used as folk remedy for treatment of abdominal pain and tuberculosis and as a febrigue and a galactogogue (Prachayasittikul *et al.*, 2009). A variety of compounds were found in the *P. debilis* i.e., diterpenes, triterpenes, polyacetylene and alkaloids (Panthama *et al.*, 2010; Kanokmedhakul *et al.*, 2003). Previously, bioactive azafluorenone alkaloids from roots of the plant species were reported (Prachayasittikul *et al.*, 2009). To continue our study on the Thai medicinal plant, herein, antimicrobial and cytotoxic compounds as well as antioxidant activity of the *P. debilis* have been investigated.

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MATERIALS AND METHODS

General

^{1}H - and ^{13}C-NMR spectra were recorded on Bruker AVANCE 300 and 600 NMR spectrometers (operating at 300 and 600 MHz for ^{1}H, and 75 and 125 MHz for ^{13}C, respectively). Infrared spectra (IR) were obtained on a Perkin Elmer System 2000 FTIR. Ultraviolet (UV) spectra were recorded on a Shimadzu UV 240 IPC. Mass spectra were recorded on a Finnigan INCOS 50 and Bruker Daltonics (micro TOF). Column chromatography was carried out using silica gel 60 (0.063–0.200 mm). Analytical thin layer chromatography (TLC) was performed on silica gel 60 PF_{254} aluminum sheets (cat. No. 7747 E., Merck). Melting points were determined on an Electrothermal melting point apparatus (Electrothermal 9100) and are uncorrected. Solvents were distilled prior to use. Reagents for cell culture and assays were of analytical grade: RPMI-1640 (Rosewell Park Memorial Institute medium, Gibco and Hyclone laboratories, USA), Ham's/F12 (Nutrient

mixture F-12), DMEM (Dulbecco's Modified Eagle's Medium) and FBS (fetal bovine serum, Hyclone laboratories, USA) HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), L glutamine, penicillin-streptomycin, sodium pyruvate, glucose, crystal violet, *α*-tocopherol, DPPH (2,2-diphenyl-1-picrylhydrazyl), MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide salt) from Sigma-Aldrich, USA, and gentamicin sulfate (Government Pharmaceutical Organization, Thailand).

Plant materials

P. debilis roots were collected from Ubonratchatanee Province, Thailand. It has been identified (BKF 135063) by The Forest Herbarium, Royal Forestry Department, Bangkok. The plant extracts; hexane (PH), chloroform (PC), ethyl acetate (PE) and methanol (PM) were prepared as previously described (Prachayasittikul *et al.*, 2009).

Isolation

Chloroform extract (PC, 100 g) was isolated by a silica gel column (Prachayasittikul *et al.*, 2009) to give 9 fractions (C1-C9). Fraction C6 (17 g) was subjected to column chromatography to provide seven fractions (C6.1-C6.7) in which C6.6 (15.1g) was rechromatographed on the silica gel (55g) column. Elution with increasing polarity of solvent (hexane : acetone) gave 11 fractions (C6.6.1-C6.6.11) of yellow oil and dark brown gum. The fraction C6.6.7 (350 mg) as an orange oil from hexane : acetone (92:8) was further isolated on the silica gel (20 g) column, eluted with hexane:acetone (9:1) afforded compound **1** (237 mg) as white solid, mp 63-65 °C. Rf = 0.73 (hexane:acetone,8:2). UV(EtOH) λmax (log ε) 214 (4.33), 253 (4.14), 276 (4.30), 283 (4.21) nm. IR (UATR): υ_{max} 3469, 3078, 2919, 2850, 2225, 1769, 1641, 1469, 1169, 1046, 947 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.25-1.49 (m, 14H, H-4-H-10), 1.46 (m, 1H, H-3), 1.53 (quint, *J* = 7.3 Hz, 2H, H-11), 1.83 (m, 1H, H-3), 2.01 (m, 1H, H-23), 2.15 (m, 2H, H-20), 2.22 (m, 2H, H-19), 2.30 (t, *J* = 7.3 Hz, 2H, H-12), 2.31 (m, 1H, H-23), 2.70 (m, 1H, H-2), 3.65 (dd, *J* = 4.8, 12.3 Hz, 1H, H-25), 3.86 (dd, *J* = 2.9, 12.3 Hz, 1H, H-25), 4.58 (m, 1H, H-24), 4.99 (d, *J* = 10.2 Hz, 1H, H-22), 5.03 (d, *J* = 17.1 Hz, 1H, H-22), 5.51 (d, *J* = 15.9 Hz, 1H, H-17), 5.77 (m, 1H, H-21), 6.27 (dt, *J* = 7.0, 15.9 Hz, 1H, H-18). ^{13}C NMR ($CDCl_3$) δ 19.6 (C-12), 27.2-29.4 (C-4-C-10), 28.3 (C-11), 29.6 (C-23), 31.3 (C-3), 32.5 (C-19), 32.6 (C-20), 39.5 (C-2), 64.6 (C-25), 65.2 (C-14), 73.2 (C-15), 73.8 (C-16), 78.3 (C-24), 83.8 (C-13), 109.2 (C-17), 115.3 (C-22), 137.2 (C-21), 146.9 (C-18), 179.4 (C-1). LRMS (EI): *m/z* (%) = 385(6) $[M+H]^+$, 199 (6), 185 (15), 171 (22), 157 (31), 143 (48), 129 (100), 105 (19), 81 (12), 55 (10), 41 (11).HRMS-TOF: *m/z* $[M+H]^+$ 385.2737 (Calcd for $C_{25}H_{37}O_3$: 385.2737).

Bioactivities

Antimicrobial Assay

Antimicrobial activity of the tested compound was assayed using the agar dilution method (Srisung *et al*., 2013). Briefly, the tested compound dissolved in DMSO was mixed with 1 mL Müller Hinton (MH) broth while the MH broth was used as the negative control. The two-fold dilution solution was transferred to the MH agar solution to yield the final concentrations of 32-256 µg/mL. The DMSO was tested in parallel with the compound and showed no effect on the tested organisms. Twenty - seven strains of microorganisms, cultured in the MH broth at 37 °C for 24 h, were diluted with 0.9 % normal saline solution to adjust the cell density of 3×10^9 cell/mL. The organisms were inoculated onto each plate and further incubated at 37 °C for 18-48 h. Efficacy of the compound to inhibit bacterial cell growth was analyzed. The tested microorganisms were gram-negative bacteria: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Salmonella typhimurium* ATCC 13311, *Salmonella choleraesuis* ATCC 10708, *Pseudomonas aeruginosa* ATCC 15442, *Edwardsiella tarda*, *Shigella dysenteriae*, *Citrobacter freundii*, *Morganella morganii*, *Vibrio cholera*, *Vibrio mimicus*, *Aeromonas hydrophila*, *Plesiomonas shigelloides*, *Xanthomonas maltophilia*, *Neisseria mucosa*, *Moraxella catarrhalis*, gram-positive bacteria: *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Micrococcus luteus* ATCC 10240, *Corynebacterium diphtheriae* NCTC 10356, *Bacillus subtilis* ATCC 6633, *Streptococcus pyogenes*, *Listeria monocytogenes*, *Bacillus cereus*, *Micrococcus flavas* and diploid fungus (yeast): *Candida albicans*.

Antimalarial assay

Antimalarial activity of the tested compound was performed against chloroquine resistant *Plasmodium falciparum* (T9.94) using the literature method (Targer and Jensen, 1976; Lambros and Vanderberg, 1979). Human erythrocytes (type O) infected with chloroquine resistant *P. falciparum* (T9.94) were maintained in continuous culture as described previously (Targer and Jensen, 1976). RPMI 1640 culture medium supplemented with HEPES (25 mM), gentamicin sulfate (40 mg/mL) and human serum (10 mL) was used in continuous culture. Before performing the experiment, *P. falciparum* culture was synchronized (Lambros and Vanderberg, 1979) by using sorbitol induced hemolysis to obtain only ring stage-infected red blood cells and then incubated for 48 h prior to the drug testing to avoid effect of sorbitol.

The experiments were carried out using synchronized suspension (0.5% to 1%) of infected red blood cell during ring stage. Parasites were suspended in culture medium supplemented with human serum (15%) to obtain 10% cell suspension. The parasite suspension was added into 96-well microculture plate; 50 µL in each well and then add 50 µL of various tested drug concentrations. The parasite suspensions were incubated for 48 h in the atmosphere of 5% CO2 at 37 °C. Percents parasitemia of the control and tested compound were examined by microscopic technique using methanol-fixed Giemsa stained of thin smear blood preparation. Efficacy of the compound was determined by compound concentration that reduced parasite growth by 50% (IC_{50}).

Antioxidant assay

Antioxidant activity of compound was tested using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The DPPH (a stable purple color radical) reacts with an antioxidant to form a light-yellow colored of diphenylpicrylhydrazine, the reduced product that can be spectrophotometrically detected. The assay (Prachayasittikul *et al.*, 2012) was performed by adding 1 mL solution of DPPH in methanol (0.1 mM) to a sample solution (0.45 mL, 1 mg/mL dissolved in DMSO). The reaction mixture was incubated for 30 min in a dark room and the absorbance at 517 nm was measured using UV-visible spectrophotometer (UV-1610, Shimadzu). Percentage of radical scavenging activity (RSA) was deduced from the following equation:

$$\text{RSA (\%)} = \left[1 - \frac{Abs._{sample}}{Abs._{control}}\right] \times 100$$

where $Abs._{control}$ is the absorbance of the control reaction and $Abs._{sample}$ is the absorbance of the tested compound.

Cytotoxic assay

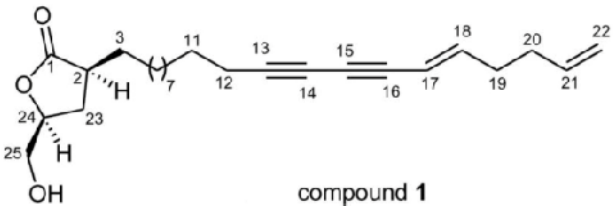
Cytotoxic assays were performed using the method described previously (Prachayasittikul *et al.*, 2009). Cell lines suspended in RPMI-1640 containing 10% FBS were seeded at 1x 10^4cells (100 µL) per well in 96-well plate, and incubated in humidified atmosphere, 95% air, 5% CO2 at 37 °C. After 24 h, additional medium (100 µL) containing the tested compound and vehicle was added to a final concentration of 50 µg/mL, 0.2% DMSO, and further incubated for 3 days.

Cells were subsequently fixed with 95% EtOH, stained with crystal violet solution, then lysed with a solution of 0.1 N HCl in MeOH, and an absorbance was measured at 550 nm. Whreas HuCCA-1, A549 and HepG2 cells were stained by MTT and for MOLT-3 cell was stained by XTT. IC_{50} values were determined as the drug and sample concentration at 50% inhibition of the cell growth. The tested cancer cell lines were human cholangio carcinoma cell line (HuCCA-1), human epidermoid carcinoma of the mouth (KB), human promyelocytic leukemia cell line (HL-60), murine leukemia cell line (P388), cervical adenocarcinoma cell line (HeLa), hormone-independent breast cancer cell line (MDA-MB231), hormone-dependent breast cancer cell line (T47D), multidrug-resistance small cell lung cancer cell line (H69AR), human hepatocellular carcinoma cell line (HepG2), human lung carcinoma cell line (A549), and hepatocellular carcinoma cell line (HCC-S102). Cells were grown in Ham's/F12 medium containing L-glutamine (2 mM) supplemented with 100 U/mL penicillin-streptomycin and FBS (10%), except for, HepG2 cell was grown in DMEM medium.

RESULTS

Isolation

The fraction C6 of chloroform extract (PC) was extensively isolated by the silica gel column to give acetogenin compound **1** (Fig 1). Its structure was determined by comparison of ^{1}H and ^{13}C NMR,MS, IR and UV spectra with the reported debilisone E (Panthama *et al.*, 2010).



compound **1**

Fig. 1: chemical structure of compound1.

Biological activities

Antioxidant activity

The plant extracts and isolated fractions were tested for their radical scavenging activity using DPPH assay. Results (Table 1) showed that the extracts (PC,PE and PM) displayed antioxidant activity with IC_{50} values of 457.0, 154.9 and 128.8 µg/mL, respectively. The isolated fractions (C9, E4 and E5) exhibited the activity with IC_{50} range of 100.0 – 354.8 µg/mL. The fractions (C2,C4,C6,C7 and E3.6) and the extract (PH) were shown to be inactive antioxidants.

Table 1: Radical scavenging activity (IC_{50}) of *P. debilis*

Compound	IC_{50} (µg/mL) [a]
PH	-[b]
PC	457.0
PE	154.9
PM	128.8
C2	-[b]
C4	-[b]
C6	-[b]
C7	-[b]
C9	223.9
E3.6	-[b]
E4	100.0
E5	354.8

[a] α-Tocopherol was used as the control (IC_{50} 5.57 µg/mL)
[b] Compounds exhibited < 50% inhibition at 333.33 µg/mL were indicated as inactive antioxidants

Cytotoxic activity

The extracts (PH,PC,PE and PM) and isolated fractions (C2-C9, E4-E5 and E3.6) were tested at 10 and 50 µg/mL for their cytotoxic activities against 8 cancer cells. Results (Table 2) revealed that PH and PC extracts at 50 µg/mL displayed 80-100 % cytotoxicity toward 7 cell lines (HuCCA-1, KB, HL-60, P388, HeLa, MDA-MB231 and T47D). Most fractions of chloroform extract (C3-C9) exhibited cytotoxic activity (80-100 %) toward 5 cell lines (KB, P388, HeLa, MDA-MB231 and T47D). All fractions (C3-C9) showed 100 % inhibition on MDA-MB231 cell whereas 100 % inhibition of KB cell were observed for fractions C3-C7. Fraction E3.6 of ethyl acetate extract (PE) exerted 100 % inhibition toward the tested cells, except for HL60 and P388 were inhibited by 84 % and 92 % respectively. Fractions C2, E4 and E5 were shown to be inactive cytotoxic compounds (< 50 % inhibition). At 10 mg/mL (Table 3), the extracts and fractions were found to be inacive, except for fractions C3-C6 were active

against MDA-MB231 (68-98 % inhibition) and fractions C6-C9 showed inhibitory effect (73-90 % inhibition) toward P388 cell.

Bioactivity of compound 1

The isolated acetogenin compound **1** was tested for its bioactivities including antimicrobial activity against 27 strains of microorganisms (gram positive and gram negative bacteria and diploid fungus) using the agar dilution method, antimalarial activity against chloroquine resistant *P. falciparum* (T9.94), and cytotoxic activity toward 5 cancer cells (HepG2, A549, HCC-S102, HL 60 and P388). It was found that (Table 4) compound **1** displayed antibacterial activity against gram negative bacteria , *M. catarrhalis* with MIC value of 64 µg/mL; and gram positive bacteria at 128 µg/mL, *C. diphtheriae* NCTC 10356 (50% inhibition) and *S. pyogenes* (75% inhibition) . However, no antimalarial activity was observed for compound **1**. Cytotoxic activity of compound **1** was found in 5 cancer cells including Hep G2, A549,HCC-S102,HL 60 and P388 (Table 5) with IC_{50} range of 18.4 - 40.3 µg/mL.

Table 2: Cytotoxic activity of *P. debilis* at 50 µg/mL

Compound	Cytotoxicity (%)[a]							
	HuCCA-1	KB	HL-60	P388	HeLa	MDA-MB231	T47D	H69AR
PH	100	100	84	91	99	100	98	41
PC	100	100	80	91	99	100	87	46
PE	59	43	0	72	24	55	66	11
PM	17	16	0	0	0	10	24	9
C2	24	6	0	64	0	49	50	21
C3	71	100	55	83	98	100	84	60
C4	100	100	84	92	100	100	100	100
C5	75	100	64	87	95	100	98	42
C6	100	100	84	92	100	100	100	100
C7	81	100	75	91	82	100	90	50
C8	81	95	75	91	88	100	87	55
C9	86	91	75	92	87	100	87	76
E3.6	100	100	84	92	100	100	100	100
E4	44	31	0	65	10	29	67	23
E5	30	10	0	55	0	24	42	11
Compound **1**	100	100	100	100	100	100	100	53
Etoposide[b]	3	0.30	1.29	0.18	0.28	0.20	0.03	25.00

[a] The assays were performed in triplicate.
[b] Cytotoxocity of etoposide was determined as IC_{50} (µg/mL).

Table 3: Cytotoxic activity of *P. debilis* at 10 µg/mL

Compound	Cytotoxicity (%) [a]							
	HuCCA-1	KB	HL-60	P388	HeLa	MDA-MB231	T47D	H69AR
PH	30	13	0	65	0	55	35	0
PC	31	18	0	65	0	55	35	0
PE	8	8	0	0	0	31	22	2
PM	5	7	0	0	0	6	11	0
C2	0	2	0	0	0	31	21	5
C3	5	39	0	0	23	95	57	13
C4	38	26	0	65	0	98	58	0
C5	35	50	0	0	33	92	83	5
C6	49	17	0	73	0	68	67	0
C7	16	11	0	90	0	41	40	10
C8	14	10	0	76	0	40	28	11
C9	12	67	0	83	47	35	70	64
E3.6	36	1	0	67	0	56	48	0
E4	3	7	0	0	0	29	24	0
E5	1	8	0	0	0	23	20	3
Compound **1**	10	8	0	5	0	39	25	6
Etoposide[b]	3	0.30	1.29	0.18	0.28	0.20	0.03	25.00

[a] The assays were performed in triplicate.
[b] Cytotoxocity of etoposide was determined as IC_{50} (µg/mL).

Table 4 : Antimicrobial activity of compound **1.**

Bioactivity	Microorganism	MIC (µg/mL)	IC_{50} (M)
Antibacterial [a,b]	*M. catarrhalis*	64	-
Antimalarial [c]	*P. falciparum* (T9.94)	-	0.26×10^{-3} - 2.6×10^{-3}

[a] Ampicilin at 10 µg/mL showed complete inhibition against *S. aureus* ATCC 25923, *S. epidermidis* ATCC 1228, *B. subtilis* ATCC 6633, *N. Mucosa*, *M. catarrhalis*, *E. tarda* and *S.pyogenes.*
[b] Partial inhibition at 128 µg/mL: *C. diphtheriae* NCTC 10356 (50%) and *S. pyogenes* (75%)
[c] Chloroquine hydrochloride was used as the reference drug.

Table 5: Cytotoxic activity (IC_{50}) of compound **1.**

	IC_{50} (µg/mL) [a,b]	
Cancer cell	Compound **1**	Etoposide
Hep G2	25.0±1.4	0.20
A549	27.5± 0.7	0.34
HCC-S102	30.5±3.5	0.32
HL60	40.3±0.1	1.29
P388	18.4±1.1	0.18

[a] The assays were performed in triplicate.
[b] Etoposide was used as the reference drug.

DISCUSSION

Previously, two bioactive azafluorenone alkaloids, onychine and 7- methoxyonychine together with a mixture of *β*-sitosterol and stigmasterol were isolated from roots chloroform extract (PC) of the *P.debilis* (Prachayasittikul *et al.*, 2009). This study, the fraction C6 of PC extract was isolated by silica gel column to afford a linear acetylenic and olefinic C25 acetogenin (**1**) known as debilisone E (Panthama *et al.*, 2010). Its structure was confirmed by spectral data (H^1 and C^{13} NMR, MS, IR and UV). The acetogenin **1** was reported to be found in methanol extract of roots *P. debilis* (Panthama *et al.*, 2010). Antioxidant potency of the palnt extracts and isolated fractions was investigated (DPPH assay).

The results showed that most of the extracts, except for PH, and some isolated fractions displayed relatively weak radical scarvenging activity. However, antioxidant activity of the *P. debilis* has never been reported elsewhere. The antioxidant activity of the plant extracts and isolated fractions could possibly be derived from the origin of plant triterpenoids i.e., stigmasterol (Prachayasittikul *et al.*, 2009; Prachayasittikul *et al.*, 2009). Stigmasterol is a phytosterol showing antioxidant effect, determined by the thiocyanate mehod (Hung and Yen, 2001), and lipid peroxdation (Ramadan *et al.*, 2007).

In addition, the plant extracts and isolated fractions were assayed for their cytotoxic activity against 8 cancer cells (HuCCA-1, KB, HL-60, P388, HeLa, MDA-MB231, T47D and H69AR) at two concentrations, 10 and 50 µg/mL. Obviously, high cytotoxicity (80-100%) mostly up to 100 % was noted for 50 µg/mL of extracts (PH and PC) toward the tested cells, except for H69AR cell; and of fractions (C3-C9 and E3.6) toward KB, HeLa, MDA-MB231 and T47D cells. Particularly, such extracts and fractions displayed 100% cytotoxicity against MDA-MB231 cell, while inhibition (100%) of KB cell was found in the extracts (PH and PC) and fractions (C3-C7 and E3.6).

At low concentration (10µg/mL), MDA-MB231 cell was inhibited (55-98%) by the extracts (PH and PC) and some fractions (C3-C6 and E 3.6). Similarly, P388 cell was inhibited (65-90%) by the extracts (PH and PC) and fractions (C4, C6-C9 and E3.6). It should be noted that no inhibition at 10 µg/mL was found in HL-60 cells. Improved cytotoxicity (from 0 to 84%) on HL-60 was observed at 50 µg/mL. Mostly, compound **1** displayed 100 % cytotoxicity toward the tested cells. Its IC_{50} values toward Hep G2, A549, HCC-S102, HL 60 and P388 were 25.0, 27.5, 30.5, 40.3 and 18.4 µg/mL, respectively. In addition, the acetogenin compound **1** exerted antibacterial activity against *M. catarrhalis*, *C. diphtheriae* NCTC 10356 and *S. pyogenes*. However, it was shown to be inactive antimalarial agent as indicated by $IC_{50} > 10^{-5}$ M. Previously, compound **1** was reported to display moderate antimycobacterial activity (Panthama *et al.*, 2010). So far, antimicrobial and cytotoxic activities of compound **1** have never been reported. Diverse biological activities of acetogenins were documented such as cytotoxic, antitumoural, antiparasitic and antimalarial activities (Bermejo *et al.*, 2005). Studies on mechanism of action showed that acetogenins are the most potent inhibitors of mitochondrial respiratory chain complex I (Bermejo *et al.*, 2005). Particularly, Annonaceous acetogenins kill cancer cells by inhibition of mitochondrial complex I (NADH-ubiquinone oxidoreductase) (Choo et al.,2014; Zafra-Polo et al., 1998).

In conclusion, the *P. debilis* extracts and isolated fractions exhibited antioxidant activity and cytotoxicity toward a panel of cancer cells. The isolated acetogenin **1** (debilisone E) displayed novel bioactivities as antibacterial and anticancer agents. The finding demonstrates compound **1** as potential lead to be further structurally modified for new bioactive derivatives.

CONFLICT OF INTEREST STATEMENT

Authors declare that we have no conflict of interest.

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