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Original Article

Bioactive interruptins A and B from *Cyclosorus terminans*: antibacterial, anticancer, stem cell proliferation and ROS scavenging activities

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Abstract

The fern *Cyclosorus terminans* has long been consumed as a vegetable in northern Thailand. Nevertheless there has been no definitive investigation on its biological properties. Here we have isolated three coumarin derivatives, interruptins A, B and C from *C. terminans*. Interruptin A exhibited antibacterial activity against four Gram-positive bacteria including methicillin-sensitive *Staphylococcus aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), *S. epidermidis* and *Bacillus subtilis* with MIC values as low as 2 μg/ml. Interruptins A and B inhibited the growth of MCF-7 human breast and HT-29 human colon cancer cells with IC_{50} values as low as 0.13 ng/ml yet stimulated proliferation of normal ASC stem cells with no signs of toxicity. Moreover, both interruptins A and B showed a powerful capacity for scavenging intracellular ROS and performed an anti-apoptotic effect against extracellular oxidative damage by H_2O_2. As a result, it is suggested that this lower plant could find a use in natural diets for treatment of infection with a special reference to MRSA, controlling breast and colon cancers, and reducing oxidative stress induced by ROS.

Keywords: interruptins A-C, *Cyclosorus terminans*, antibacterial, anticancer, stem cell proliferation, ROS scavenging

1. Introduction

Staphylococcus aureus is among the most widespread pathogens of bacterial infections involving the blood stream, lower respiratory tract, skin and soft tissues (Diekema *et al*., 2001; de Kraker *et al*., 2011). Apart from its ability to cause a diverse range of life-threatening infections, *S. aureus* has extraordinary potential to develop antimicrobial resistance. *S. aureus* that is resistant to an antibiotic methicillin is called as methicillin-resistant *S. aureus* (MRSA). In fact, MRSA is not more dangerous or virulent than methicillin-sensitive *S. aureus* (MSSA), but it is more difficult to treat because many commonly prescribed antibiotics are not effective against MRSA (Burnie *et al*., 2000; Lowry, 2003; Rybak and LaPlante, 2005; Schito, 2006). Therefore they cause increased pain, discomfort, inconvenience, cost of hospitalization and mortality. Additionally, MRSA infections are associated with a worse prognosis than MSSA infections (Athanassa *et al*., 2008; de Kraker *et al*., 2011). The prevalence of MRSA has therefore facilitated the search for alternative antibacterial agents from alternative natural sources.

Breast and colon cancers are also responsible for substantial mortality and morbidity in both industrialized and developing countries (Ferlay *et al*., 2010). Cancer can be

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caused by inherited mutation, hormone, stress and depression, immune system, chemical, radiation, infection, and reactive oxygen species (ROS) (Sieber *et al*., 2003; Reiche *et al*., 2004; Waris and, Ahsan 2006; Stanley *et al*., 2007; Ghaffari, 2008; Tan *et al*., 2008; Narayanan *et al*., 2010). Of note, ROS have been known as the most important mutagens in stem cells since their elevation blocks self-renewal and serves as a signal to induce stem cell differentiation (Dayem *et al*., 2010). The treatments of cancer are surgery, chemotherapy, radiotherapy that may be used alone or in combination. Although chemotherapy is one of the treatments that can cure cancer, most anticancer agents also frequently kill normal cells resulting in some unpleasant side effects. The exploration for cancer-cell-specific anticancer agents without normal cell destruction would therefore be expected to assist cancer management and overcome adverse problems.

Natural products are now recognized as a potential alternative source for discovering and developing new bioactive compounds. A number of lower plants have long been used in folk medicines. For example, the leaves of *Cyclosorus interruptus* are used in Papua New Guinea for the treatment of cough, burn, malaria and general sickness (Webb, 1959; Holdsworth, 1974; Holdsworth and Rali, 1989). Subsequently, the antimicrobial activity against *S. epidermidis*, *Bacillus cereus* and *Micrococcus luteus* and anticancer activity toward nasopharylgeal carcinoma of interruptin derivatives isolated from *C. interruptus* were reported about 14 years ago (Quadri-Spinelli *et al*., 2000). In China, *C. acuminatus* is consumed as a vegetable and also used in traditional medicine for the treatment of blood circulation stasis, oedema, inflammation and external bleeding. Nevertheless, the renoprotective properties in diabetic mice by the flavanone-rich extract from *C. acuminatus* have also been documented, whereas the certain active compounds are not discovered yet (Chen *et al*., 2011).

Another related fern *C. terminans* is widely distributed in the Tropics of Asia to Australias. In addition to the availability all over Thailand (Tagawa and Iwatsuki, 1988), it has usually been consumed as a vegetable in northern Thailand (Kumboonruang, 2009). Nevertheless, its biological study has not been fully explored. We are therefore interested in the exploration of *C. terminans* biological properties. The present study investigates the effects of purified interruptins from *C. terminans* on antibacterial, anticancer, normal stem cell proliferation, ROS scavenging and protective effects against oxidative stress.

2. Meterials and Methods

2.1 Plant material

The fresh aerial parts (8 kg) of *C. terminans* (J. Sm. ex Hook.) Panigrahi were collected from the forest area at the Prince of Songkla University (PSU), Songkhla province, Thailand, on April 2010 by Miss Arpaporn Kaewchoothong. The plant was identified by Prof. Dr. Thaweesakdi Boonkerd (Chulalongkorn University, Thailand) and the voucher specimen with the identification number (SKP 208 03 20 001) is now kept in the herbarium of Faculty of Pharmaceutical Sciences, PSU, Thailand.

2.2 Extract preparation and purification of interruptins A-C from *C. terminans*

A dried powder (850 g) of the aerial parts of *C. terminans* was sequentially macerated at room temperature with *n*-hexane, dichloromethane, ethyl acetate and methanol three times, each time for 3 days. Each extract was evaporated to dryness at less than 50°C under reduced pressure. The active ethyl acetate extract (9.71 g) was chosen for purification with flash chromatography on silica gel, and eluted using a step gradient of *n*-hexane with increasing concentrations of dichloromethane followed by dichloromethane with increasing concentrations of ethyl acetate then ethyl acetate with increasing concentrations of methanol to give 15 fractions (A-O). Fractions D (36 mg), F (52.5 mg) and H (152.5 mg) were further purified by open column chromatography over silica gel using the same step gradient and this provided the compounds 3 (10.9 mg), 2 (21.7 mg), and 1 (17.5 mg), respectively.

For structural identifications, spectroscopic data were obtained as follows; UV spectra were obtained from a Hewlette Packard® 8452A diode array spectrophotometer (France); IR spectra were performed on a Jasco® IR-810 infared spectrophotometer (Japan); NMR spectra, both ^{1}H and ^{13}C, were measured on an FT-NMR Varian® Unity Inova 500 spectrophotometer (500 MHz for ^{1}H and 125 MHz for ^{13}C) (Germany); and mass spectra were recorded on a MAT 95 XL mass spectrophotometer (Germany).

The purity of isolated compounds was determined by Water 1525 binary HPLC pump with a photodiode array detector (PDA) and autosampler (Water, Ireland). Separation was achieved at 25°C on a 250 mm ´ 4.6 mm i.d. TSK-gel ODS-80Ts column (Tosho Bioscience, Japan). The mobile phase consisted of 85% MeOH and 15% of 1% acetic acid in water with a flow rate of 1 ml/min. The injection volume was 20 ml.

2.3 Bacterial strains and media

The four Gram-positive test bacteria, including methicillin-sensitive *Staphylococcus aureus* (MSSA) 2673I-01, methicillin-resistant *S. aureus* (MRSA) US-002-SA, *S. epidermidis* TISTR 518 and *Bacillus subtilis* NCTC10073, and one Gram-negative bacterium *Escherichia coli* ATCC 25922 were kindly provided by Assist. Prof. Dr. Narisara Chantratita, Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University; Department of Pathology, Faculty of Medicine, Prince of Songkla University; and the Thailand Institute of Scientific and Technological Research, Thailand. All bacteria were grown with Mueller Hinton agar (MHA, Merck, Germany).

2.4 Disc diffusion test

Antimicrobial activity was tested using the standard disc diffusion method (Jorgensen and Ferraro, 2009). A swab of the test microorganism (approximately 1 to 2×10^8 CFU/ml based on a McFarland turbidity standard of 0.5) was spread onto Petri dishes containing MHA. Each extract was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 100 mg/ml. Sterile filter paper discs (6 mm in diameter) impregnated with 1 mg of the plant extracts or 100 μg of the isolated interruptins A-C were placed on the culture dishes. The dishes were inverted and incubated at 37°C for 24 h. DMSO served as a negative control, while commercially standard vancomycin (30 μg) and norfloxacin (10 μg) were used as the positive controls for Gram-positive and Gram-negative bacteria, respectively. The antimicrobial activity was indicated by the presence of a clear inhibition zone around the disc. Each test was replicated three times.

2.5 Determination of the minimum inhibitory/bactericidal concentrations (MICs/MBCs)

The MIC and MBC values of plant extracts and isolated interruptins were determined based on the broth microdilution method (CLSI, 2012) with some modifications. Stock solutions of extracts and isolated interruptins were freshly prepared in DMSO and other agents were made in sterile deionized water. DMSO constituted less than 1% of the total test volume. The highest concentrations of extracts and compounds to be tested were 1024 and 256 μg/ml, respectively. Serial 2-fold dilutions in 100 ml of MHB were prepared in 96-well microtiter plates. The 0.5 McFarland microbial suspension (1×10^8 CFU/ml) was diluted 1: 10 to yield 1×10^7 CFU/ml. Then 5 ml of diluted microbial suspension was added to each well (5×10^4 CFU/well). For each strain, the growth conditions and sterility of the medium was checked and the plates were incubated at 37°C for 24 h. The MIC was defined as the lowest antibacterial concentration that resulted in $\geq$ 99% inhibition of growth compared to the drug-free control. For determination of the MBC, a loop of broth from each well that showed growth inhibition was streaked onto MHA and further incubated at 37°C for 24 h. The lowest concentration that exhibited no growth was taken as the MBC. Experiments were performed in triplicate and repeated twice.

2.6 Cytotoxicity against cancer cells

The human breast adenocarcinoma MCF-7 (CLS No. 300273) and colonic adenocarcinoma HT-29 (CLS No. 300215) were obtained from the Cell Line Service GmbH (Eppelheim, Germany). The cells were cultured in an incubator at 37°C under humidified 5% CO_2 atmosphere. Dulbecco's minimal essential medium (DMEM) (Thermo Scientific, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY. USA), 1% (v/v) penicillin/streptomycin (Gibco, Grand Island, NY. USA) was used as the culture medium. Cytotoxicity was measured using the sulforhodamine B (SRB) assay (Vichai and Kirtikara, 2006). Briefly, 96-well plates were seeded with 4×10^3 cells/well and the cells were allowed to adhere at 37°C for 24 h. Various concentrations (0.00006, 0.00032, 0.0016, 0.008, 0.04, 0.2, 1, 5 μg/ml) of isolated interruptins A-C and camptothecin (Sigma, St. Louis, MO, USA) were added to each well. DMSO was used as the negative control. Each test was replicated three times. After incubation for 72 h, sample media were removed and the bound cells were further cultured for 72 h. Then, the cells were fixed with 10% (w/v) trichloroacetic acid (TCA) and stained by 1% (v/v) sulforhodamine B for 30 min, after which the excess dye was removed by washing repeatedly with 1% (v/v) acetic acid. The protein-bound dye was dissolved in 10 mM Tris base solution for absorbance measurement at 492 nm using a microplate reader (BioTek, Winooski, VT, USA). The IC50 values were calculated using probit analysis.

2.7 Cell proliferation of human adipose-derived stem cells (ASCs)

Normal cell proliferation was assessed by the Countess™ Automated Cell counter (Invitrogen, Carlsbad, CA, USA). Briefly, human ASCs were plated in 6-well plate (4×10^4) and grown with an a-minimum essential medium (a-MEM) (Thermo Scientific, South Logan, UT, USA) supplemented with 10% FBS (Gibco, Grand Island, NY. USA), 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA) overnight at 37°C in a humidified 5% CO_2 atmosphere. Then, cells were treated with 0 and 1 μg/ml of the interruptins A-C in the complete medium. After further incubation for 2 days, the cells were detached from the wells by trypsinization, stained with trypan blue to reveal dead cells, and viable cells counted in a Countess™ cell counting chamber slide using the Countess™ Automated Cell Counter (Invitrogen, Carlsbad, CA).

2.8 Intracellular reactive oxygen species (ROS) scavenging assay

The level of ROS in the cells was assayed according to Eruslanov and Kusmartsev (2010). The antioxidative property was determined through intracellular ROS using 2′,7′-dichlorofluorescin diacetate (DCF-DA) and flow cytometry (Eruslanov and Kusmartsev, 2010). Human ASCs (3×10^5) were seeded in a 100 mm culture dish with the complete a-MEM medium and cultured at 37°C under a humidified 5% CO_2 atmosphere overnight, then changed to a 0.5% FBS containing medium and further cultured for 24 h. Cells were pretreated with DCF-DA (20 μM) for 10 min at 37°C in the dark and subsequently incubated with 10 μg/ml interruptins A-C for 20 min. The incubated cells were then harvested by trypsin-EDTA and the ROS fluorescence was measured using a flow cytometer.

2.9 Anti-apoptosis against H_2O_2

The effect of interruptins A and B on the survival of ASCs against H_2O_2 exposure was achieved by fractioning cells with the sub-G1 phase through flow cytometry. ASCs were seeded in 100 mm dishes (4×10^5) with the complete a-MEM medium and incubated overnight at 37°C in a humidified 5% CO_2 atmosphere until they reached 80% confluence. Cells were then pretreated with 1 μg/ml interruptins in the same media for 24 h, then the media were changed to 0.5 mM H_2O_2 with the same concentration of interruptins in 0.5% FBS medium. The cells were incubated for 5 h before analysis of the cell cycle. In brief, ASCs were washed twice with cold phosphate-buffered saline (PBS), harvested and permeabilized with 70% cold EtOH. The cells were then washed with PBS containing 0.1% BSA and 0.2% Tween 20. Cellular DNA was stained with 50 μg/ml of propidium iodide (PI)-treated RNase (100 μg/ml). The distribution of the cell cycle phases with different DNA contents was examined in a FACScan flow cytometer (Becton-Dickinson, Frankinson Lakes, NJ, USA).

2.10 Statistical analysis

Data are expressed as a mean ± standard error. The statistical analysis was performed by Duncan's multiple range test to determine significant difference at the $p<0.01$ level.

3. Results and Discussion

3.1 Isolation of interruptins A-C from *C. terminans*

The antibacterial-active EtOAc extract was fractionated chromatographically using silica gel to yield compounds 1, 2 and 3 with 0.002, 0.003, and 0.001 % w/w, respectively (Table 1). The structures were elucidated based on the complete spectroscopic analysis of UV, IR, NMR, and MS spectra and confirmed by the comparisons with the published data (Quadri-Spinelli *et al*., 2000). Compounds 1, 2 and 3 were therefore identified as interruptins A, B and C, respectively (Figure 1). Their purity was shown to be ³96% by HPLC-PDA analysis. This finding is the first time to report interruptin derivatives isolated from the fern *C. terminans*. To the best of our knowledge, these compounds have not so far discovered from any other natural sources except the fern *C. interruptus* (Quadri-Spinelli *et al*., 2000).

3.2 Antibacterial activity

In vitro screening of antibacterial activity from the four *C. terminans* extracts included the *n*-hexane, CH_2Cl_2, EtOAc and MeOH extracts at a dose of 1 mg/disc against the four Gram-positive bacteria. All extracts inhibited the tested bacteria, except that *B. subtilis* was resistant to the MeOH extract. It was noted that the CH_2Cl_2 and EtOAc extracts were the most potent of the tested plant extracts against Gram-positive bacteria. In contrast, it is apparent that *E. coli* was resistant to all extracts (Table 1). These findings were similar to the previous reports of antibacterial activity from various fern extracts which have been shown to inhibit only Gram-positive strains (Lai *et al*., 2009). The insensitivity of Gram-negative bacteria to the fern extracts was presumably because of the impermeable characteristics of the bacterial outer membrane.

However, further work on the MICs/MBCs against the four Gram-positive bacteria showed that the EtOAc extract provided the strongest inhibition or killing of MSSA (128/128 μg/ml), MRSA (128/512 μg/ml) and *B. subtilis* (64/>1024 μg/ml), whilst the CH_2Cl_2 extract was the most active against *S. epidermidis* (64/128 μg/ml) (Table 2). As a result, the

Table 1. Percentage yields and inhibition zone diameters of *Cyclosorus terminans* extracts, its constituents, compared with standard antibiotics.

Extract/Sample (μg/disc)	% Yield (w/w)		Zone of inhibition (mm)				
			MSSA (2673I-01)	MRSA (US-002-SA)	*S. epidermidis* (TISTR 518)	*B. subtilis* (NCTC10073)	*E. coli* (ATCC 25922)
Hexane (1000)	0.72	8.3 ± 0.6	7.5 ± 0.0	9.5 ± 0.5	12.2 ± 0.3	-	
CH_2Cl_2 (1000)	2.30	9.8 ± 1.4	7.3 ± 0.3	12.1 ± 0.3	16.3 ± 0.3	-	
EtOAc (1000)	1.15	9.0 ± 1.0	8.2 ± 0.2	12.0 ± 1.6	12.5 ± 0.5	-	
MeOH (1000)	4.39	7.0 ± 0.3	7.2 ± 0.5	9.2 ± 0.3	-	-	
Interruptin A (100)	0.002	13.3 ± 0.5	13.6 ± 0.4	13.7 ± 0.4	19.8 ± 0.5	-	
Interruptin B (100)	0.003	-	-	-	-	-	
Interruptin C (100)	0.001	-	-	-	-	-	
Vancomycin (30)		17.0 ± 0.4	22.5 ± 0.9	22.0 ± 0.4	20.9 ± 0.2	nt.	
Norfloxacin (10)		nt.	nt.	nt.	nt.	30.7 ± 0.3	

nt : not tested.
- : no activity.

Table 2. Minimum Inhibition Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) of *Cyclosorus terminans* extracts, and its constituents, compared with standard antibiotics.

Extract/ Sample	MIC/MBC (μg/ml)			
	MSSA (2673I-01)	MRSA (US-002-SA)	*S. epidermidis* (TISTR 518)	*B. subtilis* (NCTC10073)
Hexane	512/1024	256/512	256/>1024	256/>1024
CH_2Cl_2	256/512	128/>1024	64/128	256/>1024
EtOAc	128/128	128/512	128/128	64/>1024
MeOH	1024/>1024	1024/>1024	256/256	>1024
Interruptin A	4/32	4/8	2/16	16/64
Interruptin B	>256/>256	>256/>256	>256/>256	>256/>256
Interruptin C	>256/>256	>256/>256	>256/>256	>256/>256
Vancomycin	1/4	1/4	2/4	1/4

EtOAc extract was chosen for further chromatographic purification. The three coumarins were then isolated and identified as interruptins A, B and C (Figure 1) among which interruptin B was the major ingredient. Interestingly, 100 μg interruptin A produced a 13.3-19.8 mm inhibition zone, against Gram positive bacteria, while the commercially prepared antibiotic vancomycin (30 μg) demonstrated zone of 17.0-22.5 mm. However, interruptins B and C were inactive against any tested bacteria (Tables 1-2). There has been a similar report that interruptin A isolated from *C. interruptus* displayed antibacterial activity against Gram-positive bacteria *S. epidermidis*, *B. cereus* and *Micrococcus luteus* with MICs of 1, 2 and 2 μg/ml, respectively, compared with chloramphenicol (MICs 8, 4 and 2 μg/ml, respectively), while interrupin B was inactive towards those bacteria (Quadri-Spinelli *et al*., 2000). Nevertheless, we found that interruptin A displayed MICs against *S. epidermidis*, and *B. subtilis* of 2 and 16 μg/ml, respectively, compared with vancomycin (MICs 2 and 1 μg/ml, respectively). Although the broth doubling dilution method was used similar to this study, the somewhat diverse results may due to different inoculum size and bacterial strains used. Moreover, our results have been the first to demonstrate a strong activity of interruptin A against MSSA and MRSA with MIC values of 4 μg/ml, while vancomycin showed an MIC of 1 μg/ml. Although these values are higher than that of vancomycin (MIC 1-2 μg/ml), they do indicate that interruptin A is an efficient natural antibacterial agent isolated from the fern and could be developed into various pharmaceutical or cosmeceutical products such as antibiotic sprays, toothpaste, handwash, body foam bath or shampoo, etc. for protecting our environment from undesirable microbials, particularly for MRSA that is highly prevalent and prone to resist many of the currently available antibiotics.

Various studies have demonstrated the antibacterial activity of the coumarins. For example, osthenol isolated from *Zanthoxylum monophyllum* showed significant activity against *S. aureus* and *B. cereus* (Cuca-Suarez *et al*., 1998; de Souza *et al*., 2005). Mammea A/AA and mammea A/BA, isolated from *Mammea americana* and *Calophyllum brasiliense*, respectively, are other compounds with antibacterial activity against Gram-positive MSSA and MRSA, but were not active against the Gram-negative *E. coli* (Yasunaka *et al*., 2005). Daphnetin from *Daphne gnidium* inhibited *Pseudomonas aeruginosa* growth (Cottiglia *et al*., 2001). These reported active antibacterial coumarins as well as interruptin A present a free-OH at position 7 which has

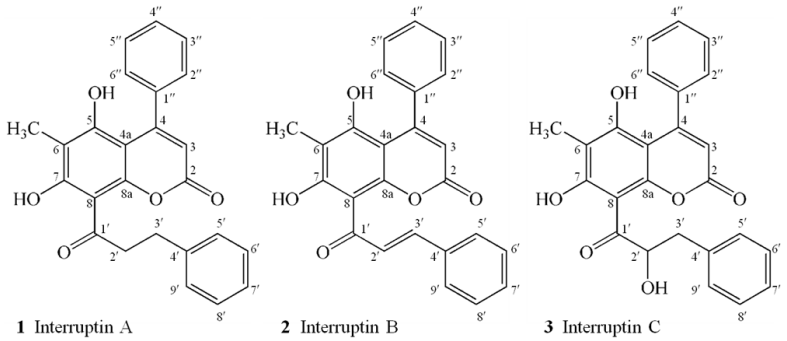

Figure 1. Structures of isolated interruptins A-C.

been implicated as an important group required for antibacterial activity (Sardari *et al*., 1999; de Souza *et al*., 2005). In addition, Kayser and Kolodziej (1999) suggested that the relatively high antibacterial activity of coumarin is due to its lipophillic character and planar molecular structure which contribute to an ability to penetrate through the bacterial cell membrane or cell walls (Kayser and Kolodziej, 1999). Interruptins A-C also belong to the coumarin derivatives that carry very similar structures, while only a double bond and a hydroxyl in the 8-substituted group was detected from interruptins B and C, respectively. Only the pharmaceutically active interruptin A was able to inhibit and kill Gram-positive bacteria including MSSA, MRSA, *S. epidermidis* and *B. subtilis* whereas interruptins B and C showed no activity. This might indicate that the absence of a double bond and a hydroxyl group in the propionyl chain at 8-substitution is essential for the antibacterial activity of the free 7-OH containing coumarins.

3.3 Cytotoxicity against cancer cells

The ability of interruptins A-C to inhibit cancer cell proliferation was determined and only interruptins A and B were cytotoxic towards human breast adenocarcinoma MCF-7 and colonic adenocarcinoma HT-29 cell lines. At 5 µg/ml the interruptins A, B, and C exhibited 97, 93, and 52%, inhibition of the growth of MCF-7 respectively, as well as having 86, 91, and 20%, growth inhibition of HT-29 respectively. Interruptins A and B demonstrated a significant cytotoxic effect against MCF-7 (IC_{50} 0.35 and 0.16 ng/ml) and HT-29 (IC_{50} 0.15 and 0.13 ng/ml, respectively) which are 1.7-7.2 times stronger than the standard camptothecin drug now in use (IC_{50} 1.16 and 0.26 ng/ml, respectively). This is the first report that interruptins A and B, but not interruptin C, contributed a significant cytotoxicity to MCF-7 and HT-29 cancer cells. Likewise, it has been shown that interruptin B extracted from *C. interruptus* inhibited another cancer nasopharylgeal carcinoma (KB) cell growth (IC_{50} 3.8 µg/ml) better than interruptin A (IC_{50} 5.1 µg/ml) (Quadri-Spinelli *et al*., 2000). Interruptin A resembles interruptin B in its primary skeletal chemical structure of coumarins with 8-subtitution of phenyl-propionyl and phenyl-*trans*-acryloyl chains, respectively, while interruptin C does carry a hydroxyl group on the propionyl chain. As a result, it is reasonable to conclude that 8-propionyl and -acryloyl substitutions on interruptins A and B are necessary for anticancer activity, by which 8-acryloyl substitution may facilitate greater cytotoxicity towards cancer cells. In contrast, the presence of a hydroxyl function such as on interruptin C was not sufficient for producing anticancer activity. A complete understanding of the mechanisms of their reaction remains to be further elucidated. Although there have been a number of studies on the *in vitro* anticancer activity of coumarins the mechanism of their action has not been exhaustively examined (Quadri-Spinelli *et al*., 2000; Gacche and Jadhav, 2012; Luo *et al*., 2012).

3.4 Cell proliferation and anti-apoptosis of human adipose derived stem cells (ASCs)

Since we have shown that interruptins A and B exhibited cytoxicity against cancer cells, we therefore investigated their effects on normal cell using human ASCs. After the treatments of ASCs with 1 µg/ml of interruptins A-C for 2 days, cell proliferation was examined using the Countess™ Automated Cell Counter (Figure 2A). All the tested chemicals

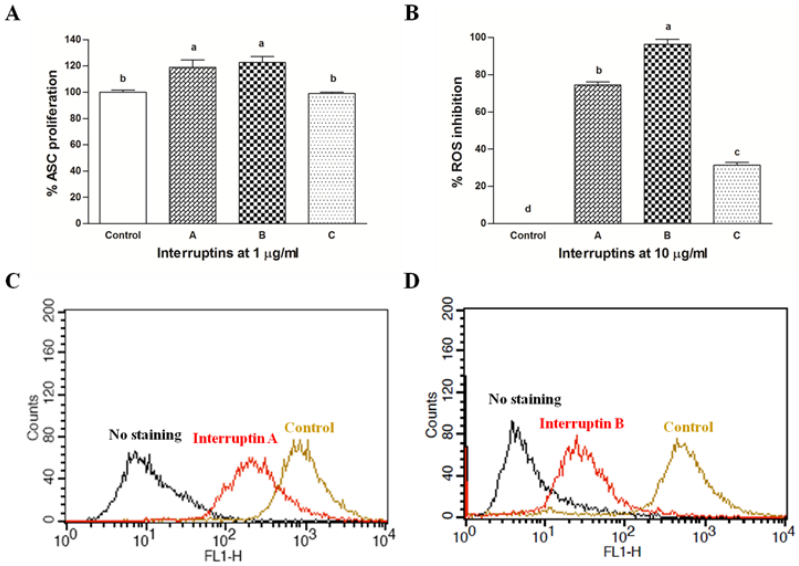

Figure 2. Proliferating and ROS scavenging effects of isolated interruptins A-C. Proliferation of ASCs was determined at 48 h after 1 µg/ml interruptins A-C treatment (A). ROS scavenging was measured at 20 min after 10 µg/ml interruptins interruptins A-C treatment by DCF-DA staining using flow cytrometry (B-C). The values are presented as a mean ± SEM (n=3). Means with the same letter are not significantly different at $p<0.01$ level by Duncan's multiple range test.

were non-toxic towards normal ASCs compared with the control without treatment. In contrast, cell proliferation (118.9 and 122.5%) of ASCs was significantly improved after treatment with 1 μg/ml of interruptins A and B, respectively. Furthermore, interruptins A and B at 10 μg/ml showed a powerful capacity for scavenging intracellular ROS with a 74.5 and 95.4% inhibition, respectively, compared with the control vehicle treatment (Figure 2B). As shown in Figure 2C-D, they combated intracellular ROS leading to a significant reduction in the amount of the DCF fluorescence. The efficient suppression of intracellular ROS production by interruptins A and B indicated that these compounds penetrated the cells and acted as ROS scavengers in the polar intracellular environment with interruptin B having the stronger radical scavenging potency. Therefore, interruptins A and B serve as ROS scavengers that may protect stem cell differentiation induced by ROS. However, interruptin C possessed no ROS scavenging property even though its structure carries a hydroxyl group in the propionyl chain at the 8-substitution. This might indicate that the higher polarity of interruptin C may disturb its entrance through the cell membrane.

ROS are a family of chemicals that include extremely reactive free radicals such as superoxide anion [$O_2^{\bullet-}$] and the hydroxyl radical [OH^-] and non-radical oxidants such hydrogen peroxide [H_2O_2]. Superoxide is rapidly converted to H_2O_2 in the cells, therefore H_2O_2 is one of the major intracellular ROS. We therefore concentrated on H_2O_2 as the principle ROS member. Figure 3A demonstrates that oxidative stress induced by extracellular H_2O_2 damaged the cells as shown by the accumulation of the sub-G1 cells, whereas an anti-apoptotic effect of interruptin B against H_2O_2 was observed, since the population of sub-G1 cells was dramatically diminished after interruptin B treatment (from 23.8% to 3.1%, Figure 3B). A similar effect by interruptin A was also obtained (data not shown). Therefore the ability of interruptin derivatives (A and B) from *C. terminans* to scavenge intracellular and extracellular ROS could be considered to have the potential to protect against cancer or oxidative stress.

In vitro culture experiments from the present study indicated that interruptin A isolated from the fern *C. terminans* was effective as an antibacterial agent against MSSA, MRSA, *S. epidermidis*, and *B. subtilis*. In addition, both interruptins A and B were efficient in suppressing MCF-7

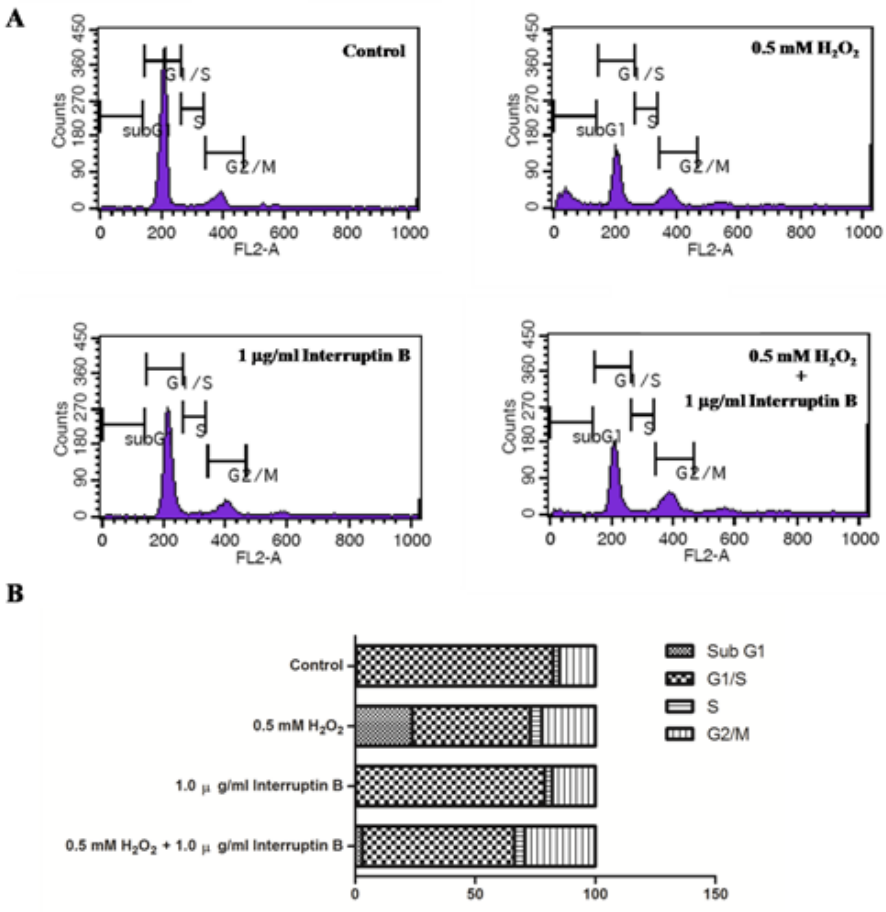

Figure 3. Protective effects of the interruptin B on H_2O_2-exposed ASCs. The cell cycle phases of ASCs incubated with 0.5 mM H_2O_2 and 1 μg/ml interruptin B were determined by flow cytometry (A and B).

human breast and HT-29 human colon cancer cell growth, and were not toxic to normal ASC cells. Furthermore, they potentiated the proliferation of stem cells and scavenged for ROS. Comparing their abilities, interruptin B seems to be the most effective at reducing the growth of human cancer cells and for ROS scavenging. The bacteriostatic, anticancer and free radical scavenging activities of these compounds in addition to their being produced by lower plant makes them attractive for additional investigation on their mechanism of action and for further studies on animal models. These results certainly indicate that these coumarins have a promising potential as natural compounds from *C. terminans* to be used to treat bacterial infection particularly MRSA, to prevent cancer and to attenuate aging caused by free radicals and oxidative damage.

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