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Apoptotic and antiproliferative effects of Stigmast-5-en-3-ol from *Dendronephthya gigantea* on human leukemia HL-60 and human breast cancer MCF-7 cells

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ABSTRACT

The genus *Dendronephthya* encompasses marine soft corals that produce a wide spectrum of biofunctional terpenoids. Anticancer properties of these metabolites are widely exploited as potential chemotherapeutic agents. The present study reports the purification and isolation of a potential antiproliferative constituent, stigmast-5-en-3-ol from the 70% ethanol extract of the soft coral *Dendronephthya gigantea*. Among several other 3β-hydroxy-Δ5-steroidal congeners, stigmast-5-en-3-ol indicated prominent antiproliferative effects on HL-60 (leukemia) and MCF-7 (breast cancer) cell lines with IC_{50} values of 37.82 and 45.17 μg/ml respectively. Stigmast-5-en-3-ol increased apoptotic body formation, accumulation of sub G1 apoptotic cells, and DNA damage in HL-60 and MCF-7 cells. It increased the expression of Bax, caspases, and PARP cleavage while decreasing Bcl-xL levels in both cancer cell lines indicating that the effects are arbitrated via the mitochondria-mediated apoptotic pathway. Steroidal derivatives were identified by GC MS/MS and the identity of stigmast-5-en-3-ol was confirmed by NMR spectra. The present study suggests that stigmast-5-en-3-ol could be a promising candidate for anticancer drug research.

1. Introduction

Cancer is rated the second most devastating disease throughout the globe. In 2012 nearly 14.1 million patients were diagnosed with cancer, with 8.2 million deaths (Ferlay et al., 2016). In 2013, an estimated 0.2 million patients were diagnosed with cancer in South Korea. The number of cancer incidents is increasing with the population growth and modernizing lifestyle (Jung et al., 2017). Cancer research receives a high priority in the medical sector. The major risk factors contributing cancer are the use of tobacco, aging, consumption of food containing artificial flavoring, coloring agents, and preservatives, gaining excessive body weight, physical inactivity, exposure to carcinogenic and radioactive agents and pathogenesis of certain disease conditions (Madigan et al., 1995). Although a variety of anticancer drugs are available, development of efficient and safe anticancer medications is one of the major challenges for researchers. Many chemotherapeutic agents cause severe side effects. Lack of selectivity towards cancer cells, implying metastasis, heterogeneous nature of the disease and multidrug resistance are among the major drawbacks in anticancer drug development (Chen et al., 2011).

Natural products have long served as drugs against a wide range of disease conditions, and many of them are renounced to be biocompatible. Recently marine natural products have gained admiration for their unique structural characteristics and bioactive properties (Blunt et al., 2011). Soft corals are among the fascinating marine organisms rich in bioactive natural products. Especially the terpenoid derivatives of soft corals such as diterpenes, sesquiterpenes, polyhydroxylated steroids, and polyamines have gained popularity for their cytotoxic, anti-inflammatory and antimicrobial properties (Chen et al., 2012). Soft corals of the genus *Dendronephthya* have proven to be a rich source of steroids with a broad structural diversity (Li et al., 2005). Their bioactive potential is yet to be unraveled. The current study is an extension of our previous work on identifying potential antiproliferative activities of a partially purified mixture of 3β-hydroxy-Δ5-steroidal congeners (Fernando et al., 2017c). The present study aimed to further purify and isolate individual sterol metabolites in the pre-described

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mixture to explore their antiproliferative effects in detail.

2. Materials and methods

MCF-7 human breast cancer, HL-60 human leukemia, and "Chang" human liver epithelial cells were purchased from the Korean Cell Line Bank (Seoul, Korea). Roswell Park Memorial Institute medium (RPMI), Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin mixture and fetal bovine serum (FBS) were purchased from GIBCO INC. Hoechst 33342, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), acridine orange, ethidium bromide, and propidium iodide (PI) were purchased from Sigma (USA). High purity grade organic solvents (hexane, ethyl acetate, and ethanol) were purchased from Fisher scientific Inc. (USA). Deuterated chloroform was purchased from Cambridge Isotope Laboratories, Inc. (USA). Primary antibodies were purchased from Cell Signaling Technology, Inc. (USA).

2.1. Purification and isolation of active components

The partial purification method of the active fraction is presented in our previous study (Fernando et al., 2017c). Briefly, the *D. gigantea* dry powder was extracted with 70% ethanol to obtain the crude extract. The extract was suspended in water and fractionated with hexane. Further purification was done by eluting the hexane fraction via two consecutive open columns. The selected fraction that indicated synergistic antiproliferative effects were identified to be composed of eight 3β-hydroxy-Δ5-steroidal congeners. Further purification of the fraction was done by using an open silica column of length 1.00 m to achieve a fine resolution. The elution was carried out using a gradient of 90% hexane +10% ethyl acetate to 70% hexane +30% ethyl acetate. The eluates were collected into test tubes and analyzed by thin layer chromatography (TLC). The tubes were pooled according to the separation and obtained five fractions (DGEH21–1 to DGEH21–5). The fractions were further separated using silica preparative TLC plates obtaining a total of eight fractions (DGEH21–1-1, DGEH21–3-1, DGEH21–3-2, DGEH21–3-3, DGEH21–4-1, DGEH21–4-2, DGEH21–5-1, and DGEH21–5-2). The bands on the PTLC were scraped off and recovered by dissolving in a mixture of hexane and ethyl acetate.

2.2. Structural characterization

Proton and ^{13}C NMR spectra were obtained by a JEOL JNM-ECX-400, 400 MHz spectrometer (Japan) at 33 k. Compounds were dissolved in deuterated chloroform for the NMR analysis. Mass spectra were obtained by a Shimadzu GCMS-TQ8040 system (Japan) as mentioned by (Fernando et al., 2017b).

2.3. Maintenance of the cell lines and evaluation of antiproliferative effects

HL-60 and Chang cell cultures were maintained in RPMI media, and MCF-7 cells were maintained in DMEM media whereas both media were supplemented with 10% FBS and 1% penicillin, streptomycin mixture. Cell cultures were maintained at 37 °C in a humidified atmosphere supplemented with 5% CO_2. Cells were periodically subcultured until they attain the exponential growth. Then the cells were seeded in microwell plates at a 1×10^5 cells/ml concentration. Samples were treated after a 24 h pre-incubation period. The cell viability was determined by MTT assay following a 24 h post-incubation period (Fernando et al., 2017a).

2.4. Analysis of apoptotic body formation

Cells pre-cultured for 24 h in 24 well plates were treated with different sample concentrations and incubated for another 24 h period. The wells were either treated with Hoechst 33342 (10 μg/ml) or the mixture of acridine orange and ethidium bromide (10 μg/ml) (double staining method). After 10 min incubation, cellular morphologies were evaluated using a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera.

2.5. Flow cytometry analysis

The hypodiploid cells population in sub-G1 were analyzed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, USA) as described by (Sanjeewa et al., 2016). Briefly, the cells pre-seeded for 24 h were treated with different sample concentrations and incubated for a 24 h period. The cells were then harvested, washed with PBS, fixed in 70% ethanol for 30 min and again washed with Sodium Ethylenediaminetetraacetate (EDTA) in PBS. The cells were then suspended in a solution of PI and RNase for 30 min and used for the analysis.

2.6. Analysis of DNA damage by agarose gel electrophoresis and alkaline comet assay

The DNA damage was analyzed by both DNA laddering analysis and by Comet assay. DNA laddering analysis was performed according to the method described by (Jayasooriya et al., 2012). Briefly, the cells were seeded at 4×10^5 cells/ml concentration and treated with different sample concentrations. After 24 h the cells were lysed using a solvent containing 10 mM Tris–HCl (pH 7.4), 5 mM EDTA, 150 mM NaCl, and 0.5% Triton X-100. After centrifugation 10,000 *g* for 20 min DNA was collected from the supernatant using a solution of phenol, isoamyl alcohol and chloroform (25:1:24, *v*/v/v). Collected DNA was electrophoretically analyzed using an agarose gel. The comet assay was carried out as described by (Fernando et al., 2017c). Briefly, the cells were collected 24 h after the sample treatment and mounted on pre-coated agarose plates. Then the cells were lysed in a lysis buffer containing 1% TritonX-100, and the electrophoresis was carried out at 30 V/300 mA for 30 min. After the DNA was stained with ethidium bromide and the comets were visualized using a BioTek Lionheart™ FX Automated Microscope (USA) using the 469/525 (GFP) filter. The comet tail DNA percentages were analyzed by ImageJ software using the OpenComet plugin. The DNA damage was analyzed as a measure of average tail DNA percentages (Gyori et al., 2014).

2.7. Western blot analysis

Intracellular levels of apoptosis-related key molecular mediators including Bax, Bcl-xL, caspase-3, caspase-9, p53, and PARP cleavage were investigated by western blot analysis. The analysis was performed as described by (Fernando et al., 2017d). Briefly, the pre-seeded cells were treated with different sample concentrations and incubated for 24 h. The cells were lysed, and the proteins were standardized using a BCA protein assay kit. The proteins (50 μg) were resolved by polyacrylamide gel (12%) electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were consecutively incubated with selective primary and secondary antibodies. The signals were developed by a chemiluminescent substrate (Cyanagen Srl, Bologna, Italy) and the fluorescence images were obtained by a FUSION SOLO Vilber Lourmat system (Paris, France). Densitometry analysis of the proteins was done using Image J program.

2.8. Statistical analysis

The data values are represented as mean ± SD based on at least three independent evaluations ($n = 3$). Statistical analysis was performed using IBM SPSS Statistics 20 software using one-way ANOVA by Duncan's multiple range test. *P*-values < .05 ($P < .05$), * and 0.01 ($P < .01$), ** were considered significant.