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Antiproliferative and apoptosis-inducing potential of 3β-hydroxy-Δ5-steroidal congeners purified from the soft coral *Dendronephthya putteri**

Thilina U. JAYAWARDENA[1], Won Woo LEE[1], I. P. Shanura FERNANDO[1], K. K. Asanka SANJEEWA[1], Lei WANG[1], Tee Gee LEE[2], Young Jin PARK[3], Chang-ik KO[4], You-Jin JEON[1, **]

[1] *Department of Marine Life Sciences*, *Jeju National University*, *Jeju 690-756*, *Republic of Korea*

[2] *Department of Hotel Cooking Nutrition*, *Jeonnam Provincial College*, *Jeollanam-do*, *Republic of Korea*

[3] *Department of Family Medicine*, *College of Medicine*, *Dong-A University*, *Busan 602-715*, *Republic of Korea*

[4] *Choung Ryong Fisheries Co. Ltd*., *7825*, *Iljudong-ro*, *Namwon-eup*, *Seogwipo-si*, *Jeju-do*, *Republic of Korea*

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Abstract The exploration and identification of antiproliferative phytochemicals have received increased attention in medicinal chemistry. In particular, research focused on the toxicology of marine natural products has increased in recent years. Terpenoids, among many secondary metabolites, have been demonstrated to act as effective anticancer agents. Soft corals, a group of marine invertebrates, produce a variety of terpenoids with biofunctional properties. The current study presents the extraction, purification, and identification of sterol congeners from the soft coral *Dendronephthya putteri*. The method involves 50% chloroform-methanol extraction, polar column fractionation, and analysis through GC-MS^n. Dose-dependent antiproliferative activity was observed within the sterol-rich fraction (DPCMH 2-4), which consisted of 3β-hydroxy-Δ5-steroidal congeners. This fraction inhibited the growth of HL-60 and MCF-7 cells with IC_{50} values of 25.27±1.43 and 22.81±0.15 μg/mL, respectively. Apoptotic body formation, DNA damage, cell cycle arrest, and apoptotic cell signaling pathway activation were also observed, reinforcing the dose-dependent antiproliferative and apoptosis-inducing activity of 3β-hydroxy-Δ5-steroidal congeners. To our knowledge, this is the first report of anticancer agent identification from the soft coral *D. putteri*. Based on the observations, these steroidal congeners are promising candidates for the development of anticancer drugs.

Keyword: *Dendronephthya putteri*; soft coral; antiproliferative agent; HL-60; MCF-7; apoptosis; steroidal congeners

Abbreviation: DP: *Dendronephthya putteri*; DMEM: Dulbecco's modified Eagle's medium; RPMI: Roswell Park Memorial Institute medium; DPCM: 50% mixture of methanol chloroform extract of *D. putteri*; DPCMH: hexane solvent fraction of DPCM; DPCMC: chloroform solvent fraction of DPCM; DPCMEA: ethyl acetate solvent fraction of DPCM; DPCMH 2: column 1 eluate of DPCMH consist of 80% hexane and 20% ethyl acetate; DPCMH 2-4: column 2 eluate of DPCMH consist of 85% hexane and 15% ethyl acetate; MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; GC–MS/MS: gas chromatography tandem mass spectrometry; FBS: fetal bovine serum

1 INTRODUCTION

Bioactive natural products have received enormous attention as sources of therapeutic agents. It has long

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** Corresponding author: youjin2014@gmail.com

T. U. JAYAWARDENA and W. W. LEE contributed equally to this work.

been acknowledged that structures of natural products have a greater chemical diversity and biochemical specificity than those of synthetic compounds do, making them an ideal source for lead compounds in drug discovery (Siddiqui et al., 2014). Secondary metabolites of soft corals represent a substantial portion of the literature on natural products owing to their vast diversity and unusual chemical structures. The majority of these metabolites are terpenes, a class of natural products that comprises compounds containing isoprene building blocks. Terpenes represent one of the most structurally diverse natural product classes. As terpenes are modified chemically either by oxidation or re-arrangement, the resulting compounds are referred as terpenoids. Sesquiterpenoid and diterpenoid metabolites of soft corals are mainly involved in defensive responses. Biologically active terpenoids are used in treating cancer, malaria, inflammation, and a variety of infectious diseases (Mbaveng et al., 2014). Life is believed to have originated in the sea, with more than 70% of earth's surface being covered by oceans. In most marine ecosystems, the biodiversity is greater than that observed in tropical forests. The potency of toxic metabolites in certain marine organisms is likely to be higher, as water acts as a natural diluent for the toxins released. Hence, marine natural products are recognized as a rapidly growing field for the discovery of novel metabolites with a broad range of bioactive properties. Recently, many studies have focused on the anticancer potential of soft corals. Fernando et al. (2017b), reported the antiproliferative potential of steroidal congeners from the soft coral *Dendronephthya gigantea*, harvested from the Jeju Sea of South Korea. The soft coral *Sinularia flexibilis* has also been identified as a source of cytotoxic metabolites with potential anticancer activity. The metabolites responsible for the antiproliferative effects have been identified as cembranoid diterpenes (Bowden et al., 1992).

Cancer is the second leading cause of death worldwide, responsible for nearly one in four deaths. Currently used chemotherapeutic agents cause adverse side effects. Hence, efforts devoted to the discovery of novel anticancer agents with fewer adverse effects have received much attention. In this regard, anticancer agents from natural sources have received significant attention, owing to their biocompatible properties relative to synthetic agents.

Recently, different classes of anticancer drugs isolated from marine organisms have shown cytotoxic activity against multiple tumor types, and in vitro effects include inhibition of protein synthesis, induction of Sub-G_1 cell-cycle arrest, and inhibition of cell-cycle progression via blockade at G_1. The current study is an extension of our previous effort (Sanjeewa et al., 2016) to discover and characterize antiproliferative agents, derived from eight soft coral species, harvested from Jeju Island. The current study aimed to obtain 3β-hydroxy-Δ5-steroidal congeners from the soft coral *D. putteri*. Obtained steroidal congeners were studied in detail for its antiproliferative and apoptosis-inducing properties.

2 MATERIAL AND METHOD

2.1 Material

The cell lines were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). HL-60 (human leukemia) and MCF-7 (human breast cancer) were used as cancer cell lines while "Vero" (monkey kidney epithelial) cell lines were used to evaluate the cytotoxic effects. Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute medium (RPMI), fetal bovine serum (FBS) and penicillin/streptomycin mixtures were purchased from GIBCO INC. 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Hoechst 33342, ethidium bromide and acridine orange were purchased from Sigma (USA). Solvents including methanol, chloroform, hexane, and ethyl acetate were purchased from Fisher Scientific Ltd. (MA, USA). Organic solvents used in the extraction and fractionation process were of analytical grade (Fernando et al., 2017b).

2.2 Sample extraction, fractionation, and purification

Dendronephthya putteri (DP) samples were collected during April 2016 from the Jeju sea at a depth of 10–15 m. The sample identification was assisted by Jeju Biodiversity Research Institute. Samples were reserved in Laboratory of Marine Bioresource Technology at Jeju National University. The samples were washed, lyophilized and ground into a powder (2 kg). Samples were then extracted using 50% solvent mixture chloroform-methanol under 37°C (three times), and the filtrate was evaporated using a rotary evaporator under vacuum. The retrieved crude (DPCM) was dissolved in water and was consequently fractioned between hexane

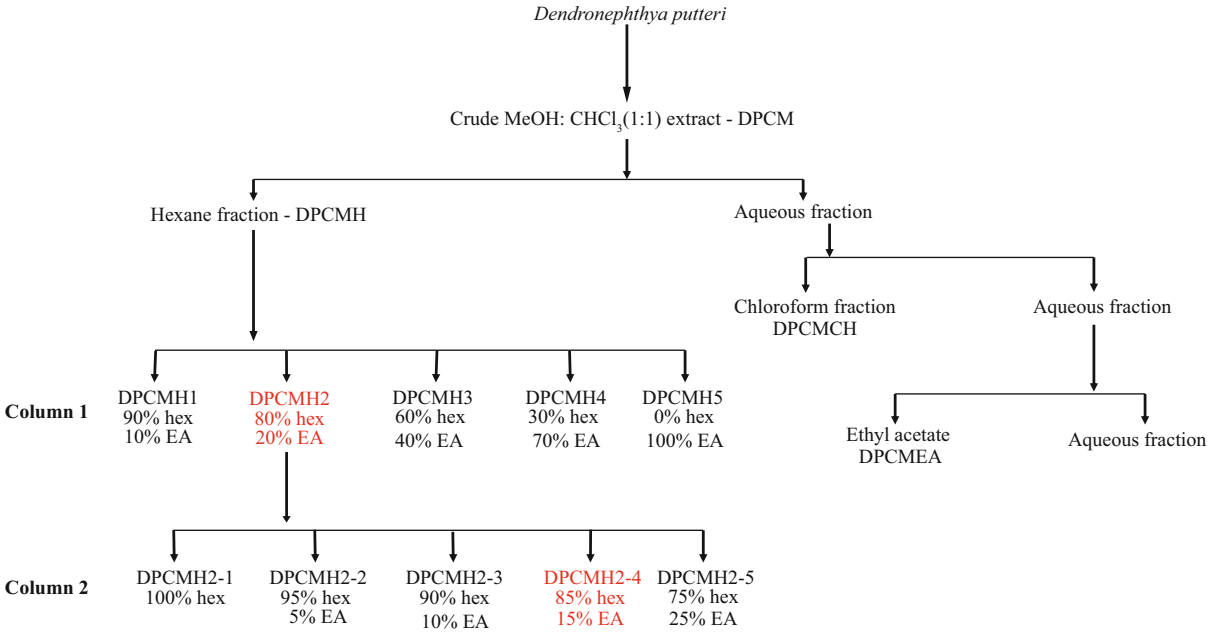

Fig.1 Solvent/ solvent extraction, bioassay-guided fractionation and purification process of *D. putteri* methanol: chloroform (1:1)

Initial purification was carried out through solvent/solvent fractionation, and hexane fraction was further purified using two successive silica open columns. Hexane (H) and Ethyl acetate (EA) were used as the solvent system, varying its percentages. TLC was used to evaluate the eluting fractions detected by UV and 10% sulfuric acid in ethanol staining.

(DPCMH), chloroform (DPCMC), and ethyl acetate (DPCMEA). Each fraction was evaporated using a rotary evaporator under vacuum. Consequentially, bioassay-guided fractionation and purification were implemented. Silica open column was used for the further purification of fraction DPCMH. The elution solvent was prepared using hexane (H) and ethyl acetate (EA) and was performed in the order of increasing polarity. Depending on the bioassay results, 80% H+20% EA (DPCMH 2) was selected and was further purified by a silica open column. Column eluate, 85% H+15% EA designated as DPCMH 2-4 was chosen for the further process. The extraction and purification are illustrated in the flow diagram (Fig.1).

2.3 GC-MS/MS analysis

GC-MS/MS analysis of DPCMH2-4 was performed with Shimadzu GCMS-TQ8040 tandem GC/MS system (Japan). Rtx-5MS fused-silica capillary column, film thickness 0.25 μm coated with non-polar 5% phenylmethyl polysiloxane and length 30 m was used. Helium was used as the carrier gas at a constant flow of 0.73 mL/min. A method programmed with splitless mode injection. Injection temperature at 200°C, oven program 260.0°C start point and was held for 3 min and increased to 320.0°C, ramp 6.0°C/min, again increase to 330.0 at 5.0°C/min incrimination and was held for 5 min. Mass range of analysis was from 50 to 500 m/z (amu) in scan mode (Fernando et al., 2017c).

2.4 Cell culture

Two different media were used depending on the cell type, RPMI, and DMEM. Both media were supplemented with 10% FBS and 1% penicillin, streptomycin mixture. MCF-7 cells were cultured in DMEM media, Vero and HL-60 were cultured in RPMI media. The cells were maintained at 37°C with 5% CO_2. Subcultures were performed periodically until the cells reach the exponential phase and were used for experiments. Cells were seeded in a 1×10^5 cells/mL concentration in 96 well plates followed by sample treatment after a 24-h incubation. The cell viability was measured by MTT assay after 24 h (Mosmann, 1983; Kang et al., 2017). Readings were taken using the ELISA plate reader at 540 nm wavelength.

2.5 Apoptotic body formation

Hoechst 33342 staining method was used to evaluate the apoptotic nuclear morphology of the cells. Cells were cultured in 24 well plates with a cell density of 1×10^5 cells/mL. Sixteen hours after the

seeding, the cells were treated with various concentrations of the DPCMH 2-4 sample and further incubated for 24 h at 37°C in a humidified atmosphere. Then, Hoechst 33342 dye (10 μg/mL) 5 μL was added and incubated at 37°C for 10 min. A fluorescence microscope, Lionheart™ FX Automated Microscope system BioTek Instruments, Inc. (Winooski, Vermont, USA) was used to observe the cells to examine nuclear condensation and fragmentation (Fernando et al., 2017b).

2.6 Comet assay

The alkaline comet assay was carried out accordingly with the method described by Fernando et al. (2017b) with minor modifications. Briefly, the cells were seeded and incubated with different DPCMH 2-4 concentrations for 24 h. The harvested cell suspension was mixed with 1% low melting agarose at 40°C. The cell suspension in low melting agarose was gently pipetted on to the surface of agarose pre-coated microscopic slides and lysed using a lysis buffer containing TritonX-100. The electrophoresis was carried out at 30 V/300 mA for 30 min. After rinsing with the neutralization buffer, the slides were stained with ethidium bromide, and the images were taken using a fluorescent microscope (Lionheart™ FX Automated Microscope BioTek, USA) using the 469/525 (GFP) filter. OpenComet plugin in ImageJ software was used to analyze the images. The analysis parameter of DNA damage was the average of tail DNA percentage.

2.7 Cell cycle analysis

The fraction of apoptotic hypodiploid cells in Sub-G1 was analyzed as a measure of the proportion of apoptotic cells. The cancer cell lines, HL-60 and MCF7, were seeded on 5-well plates with a cell density of 2×10^5 cells/mL. Different sample concentrations were treated, and the cells were harvested after 24 h and fixed in 70% ethanol. Before the analysis, the cells were washed with EDTA in PBS by centrifugation and resuspension, and the samples were then prepared for flow-cytometric analysis by suspending the cells in a solution containing propidium iodide and RNase and was incubated for 30 min at room temperature. The analysis was performed with a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). The cell distribution at each cell cycle phase was used to examine the effect of DPCMH 2-4 on cell cycle and was assessed by histograms generated (Nicoletti et al., 1991).

2.8 Western blot analysis

Both cell lines were cultured in separate culture dishes (2×10^5 cells/mL). Sample treatment was done after 24 h of incubation time. Given 24 h period, cells were harvested and homogenized in lysis buffer (Samarakoon et al., 2014). Centrifugation was used to obtain the cell lysates (12 000×*g* for 20 min). The protein contents in the supernatants were analyzed by BCA protein assay kit. Gels containing 12% polyacrylamide (SDS-PAGE) were used to load the extracted proteins. Protein bands were resolved using electrophoresis and were blotted on to nitrocellulose membranes. The membranes were incubated overnight with specific primary antibodies as β-actin, Bax, Bcl-xL, Caspase-3, p53, and PARP (Santa Cruz Biotechnology) in 5% skim milk. After the incubation, the HRP-conjugated secondary antibodies (anti-mouse IgG, Santa Cruz Biotechnology) were added into the membrane. Finally exposed against the chemiluminescent substrate (Cyanagen S.r.l, Bologna, Italy) using Fusion Solo Vilber Lourmat system (Paris, France) for images. Protein expression analysis was done using Image J program (Fernando et al., 2017a, b).

2.9 Statistical analysis

All the data are expressed as the mean±standard deviation of minimum three determinations. Statistical comparison of significant differences was performed via IBM SPSS statics using one-way ANOVA. *P* values of less than 0.05 ($P<0.05$) were considered as significant.

3 RESULT

3.1 Extraction and bioassay-guided purification

Dendronephthya putteri sample powder was extracted in a mixture of 1:1 chloroform and methanol (DPCM) and the extract was then suspended in deionized water and fractionated with hexane, chloroform, and ethyl acetate. Designated hexane fraction (DPCMH) expressed better anticancer properties against HL-60 and MCF-7 cells than the other fractions. The hexane fraction designated DPCMH was continued in the process. DPCMH was subjected to open column purification, resulting in five fractions. Improved antiproliferative effects were observed in the DPCMH 2 fraction (results are given in supplementary 1). DPCMH 2 was further resolved using a second open column, resulting in five column

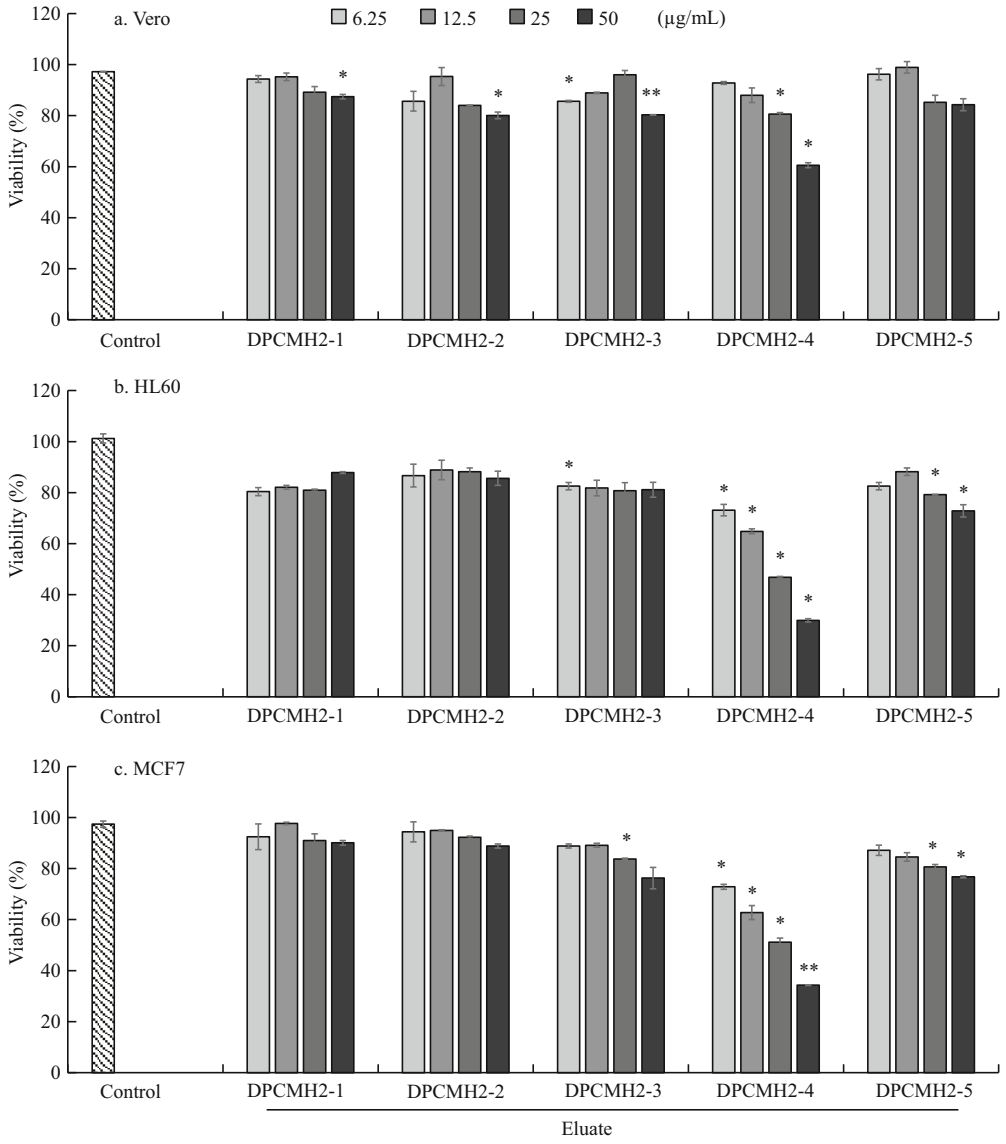

Fig.2 Cell viability as a measure of cell proliferation against treatment with DPCMH 2-4

a. Vero cells; b. HL-60; c. MCF-7 cells. The experiments were performed in triplicates, and SD represented as±with respect to the mean. Compared to control, significance was considered as, *P<0.05, **P<0.001.

fractions. Among them, DPCMH 2-4 exhibited significantly higher antiproliferative effects. Figure 1 briefly illustrates the extraction and purification procedure.

3.2 Anti-proliferation and cytotoxic behavior of DPCMH 2 and DPCMH 2-4 on selected cell lines

Following the initial observation of antiproliferative effects within fraction DPCMH 2 on HL-60 and MCF-7 cancer cell lines, and relatively higher viability on Vero cells, DPCMH 2 was separated using a second open column. From the second column eluates, fraction 4 (DPCMH 2-4) displayed significantly higher activity against cancer cell lines, relative to other fractions (Fig.2). The IC_{50} values for DPCMH 2-4 treatments were 25.27±1.43 and 22.81±0.15 μg/mL respectively, for the HL-60 and MCF-7 cell lines. For the particular IC_{50} values, Vero cells expressed viability as following, 81.24% for HL-60 and 85.94% for MCF-7 cells. Hence, DPCMH 2-4 was selected for further studies.

3.3 Analysis of compound composition in DPCMH 2-4 fractions

Terpenoids have been isolated from a variety of marine organisms. Soft corals are an abundant source of sesquiterpenes and diterpenes, which possess profound anticancer and anti-inflammatory properties. Soft corals benefit from toxic terpenes as defensive molecules and paralyzing agents that help to capture

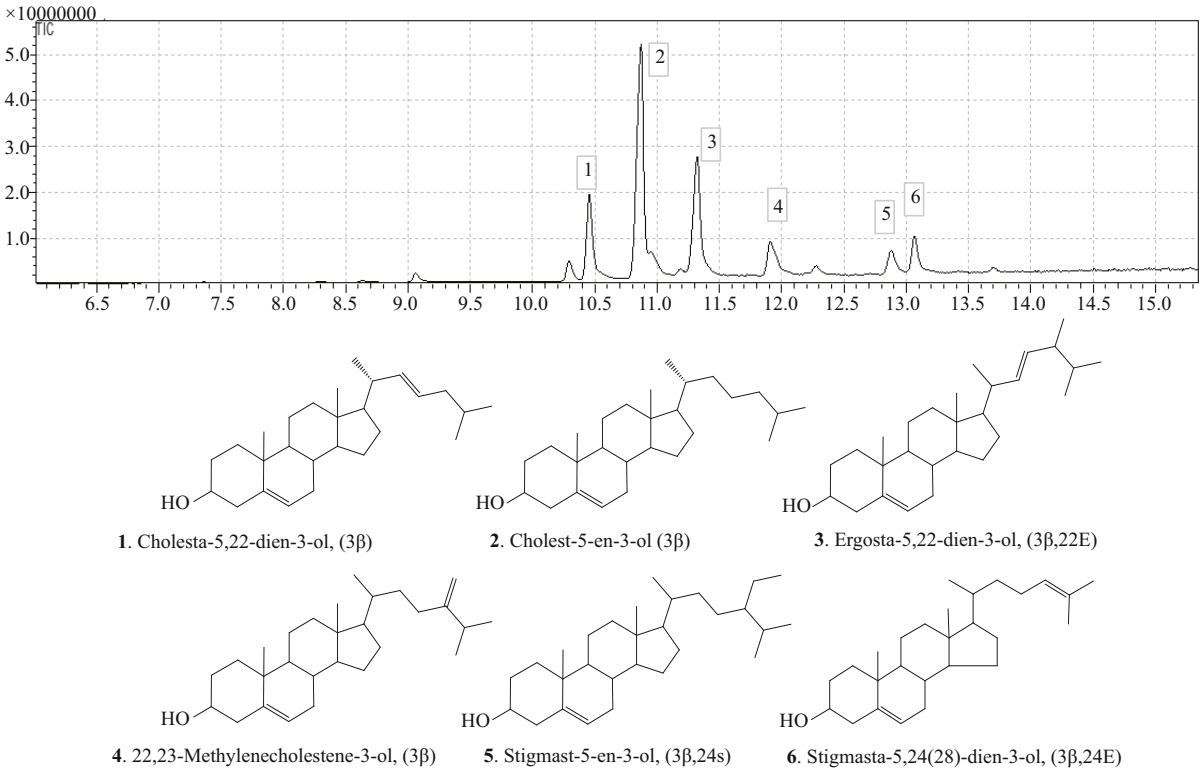

Fig.3 GC-MS/MS data of DPCMH 2-4

Mass spectroscopic data analysis was supported by the NIST 17 and Wiley 11 libraries. **(1)** Cholesta-5,22-dien-3-ol, (3β); **(2)** Cholest-5-en-3-ol (3β); **(3)** Ergosta-5,22-dien-3-ol, (3β,22E); **(4)** 22,23-Methylenecholestene-3-ol, (3β); **(5)** Stigmast-5-en-3-ol, (3β,24s); **(6)** Stigmasta-5,24(28)-dien-3-ol, (3β,24E).

their prey. Terpenes exist as complex and inseparable mixtures that are typically analyzed by GC-MS^n. The current study uses GC-MS/MS for the analysis of these potential antiproliferative fractions. As shown in Fig.3, the isolated fraction DPCMH 2-4, consisted of Cholesta-5,22-dien-3-ol, (3β), Cholest-5-en-3-ol (3β), Ergosta-5,22-dien-3-ol, (3β,22E), 22, 23-Methylenecholestene-3-ol, (3β), Stigmast-5-en-3-ol, (3β,24s), and Stigmasta-5,24(28)-dien-3-ol, (3β,24E).

3.4 DPCMH 2-4 induced apoptosis in both HL-60 and MCF-7 cell lines

MCF-7 and HL-60 cell lines were treated with various concentrations of DPCMH 2-4 fraction. Cells were stained with Hoechst 33342 and visualized by fluorescent microscopy post-treatment. Homogeneously stained nuclei were interpreted as viable cells, whereas the presence of chromatin condensation and/or fragmentation indicated an apoptotic cell. Viable cells are indicated as homogeneously stained nuclei, whereas nuclear condensation and fragmentation mark the presence of apoptotic cells. Intact nuclei without DNA damage were observed in control cells (Fig.4). Significant DNA damage was observed in cells treated with different concentrations of DPCMH 2-4. The proportion of apoptotic cells increased with increasing DPCMH 2-4 concentrations.

3.5 DPCMH 2-4 induced DNA damage

The single cell gel electrophoresis assay is a rapid and graphically subtle technique for analyzing DNA breakage in cells. The single strand breakage is made more obvious by using an alkaline medium. A bright fluorescent core possessing a less sharp edge, facing the anode, can be observed in the undamaged cells. In possible breaks, a fluorescent tail is expressed, which extends from the core (comet tail), and is indicative of DNA damage. As indicated in the Fig.5a, b, the length of the comet tail improved with increasing DPCMH 2-4 concentrations in both cell lines. The degree of DNA damage is indicated as proportional to the length of the tail and its intensity. The tail DNA percentage for HL-60 and MCF-7 cells was 56.73% and 55.85%, respectively, at 50 μg/mL DPCMH 2-4, which was the highest concentration tested. Results of the comet assay are consistent with the results obtained through Hoechst staining. Apoptotic body formation was also increased in DPCMH 2-4-treated cells. Hence, the

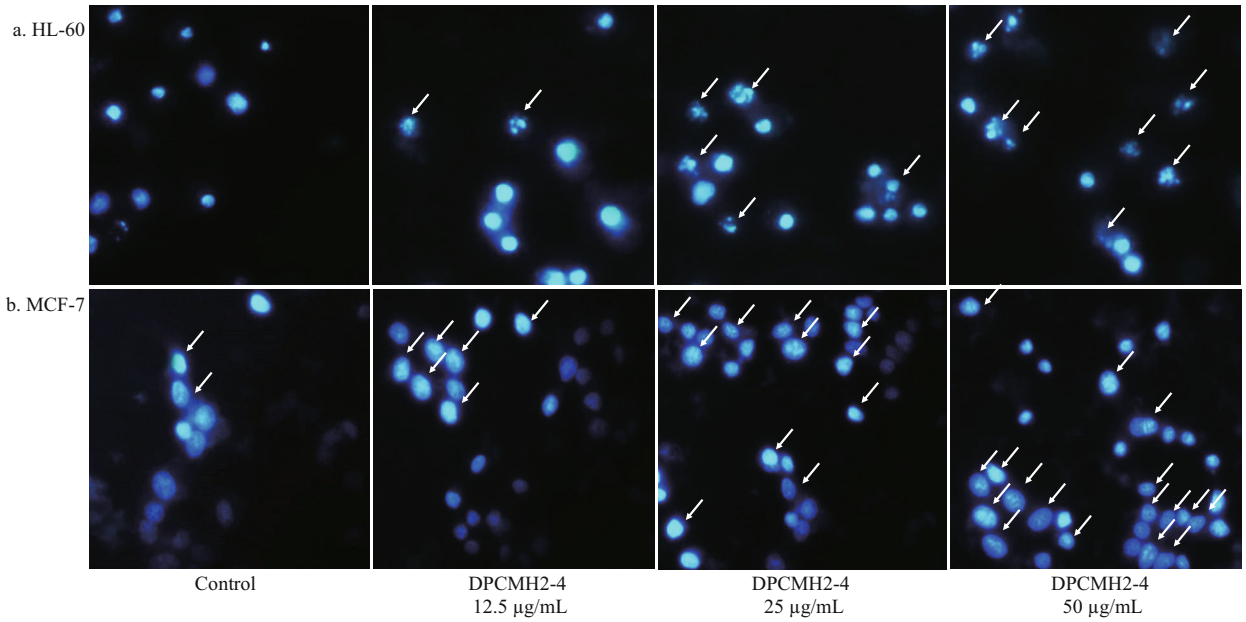

Fig.4 Apoptotic body formation indicated via chromatin condensation and nuclear fragmentation

a. HL-60; b. MCF-7 cells. The nuclear morphology was observed by Hoechst 33342 staining, via a fluorescent microscope. DPCMH 2-4 treatment was carried out as: control (no treatment), 12.5 μg/mL, 25 μg/mL, and 50 μg/mL.

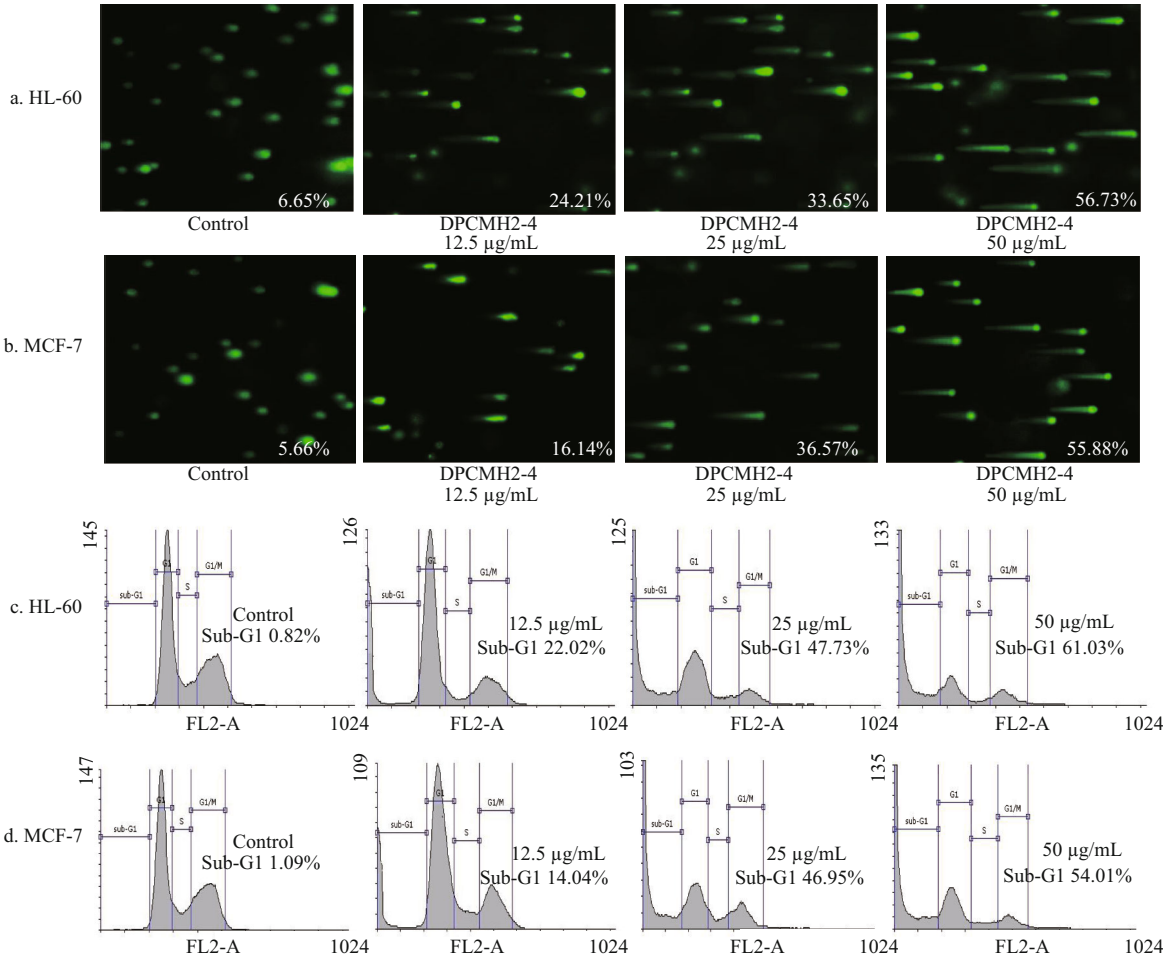

Fig.5 Comet assay and cell cycle progression against DPCMH 2-4 treatment

Comet tails of HL-60 (a) and MCF-7 (b) cells incline dose-dependently. Accumulation of apoptotic cells in sub-G1 phase HL-60 (c) and MCF-7 (d). Results are determined after successive triplicate determinations.

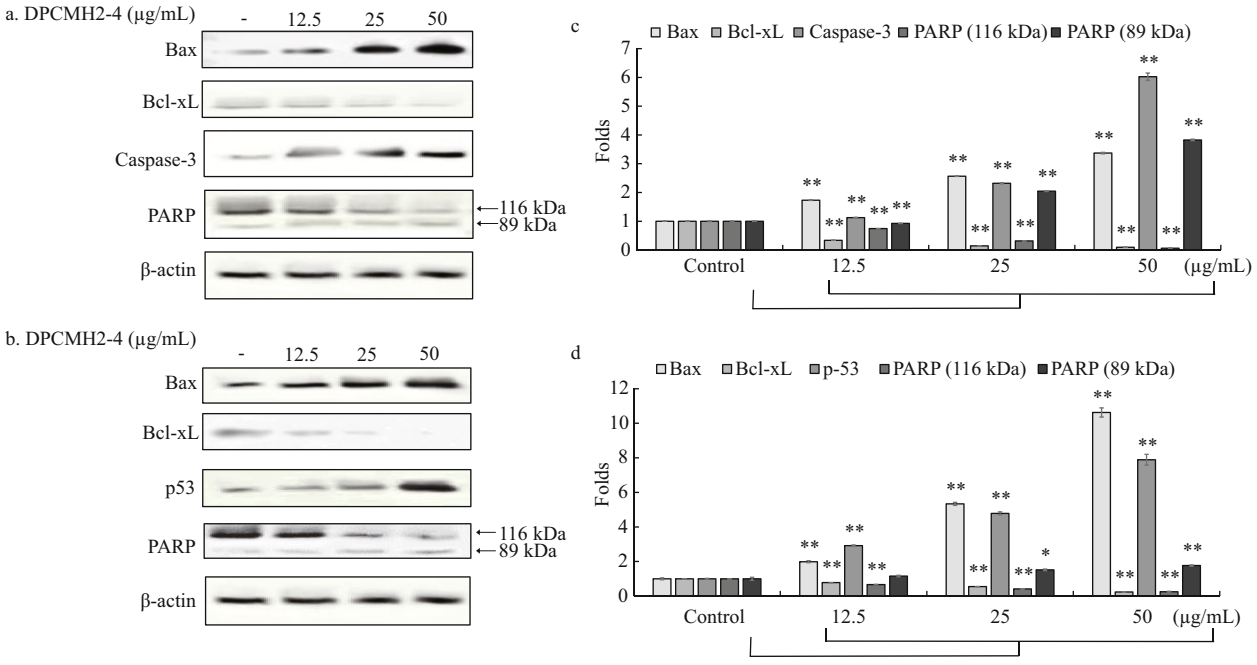

Fig.6 Effect of DPCMH 2-4 on the mediation of apoptosis-related protein levels

a, c. HL-60; b, d. MCF-7 cells. Results are determined after three independent determinations. Cells were treated with DPCMH 2-4 different concentrations and cell lysates were subjected to western blot analysis. Expression of Bax, Bcl-xL, p53, caspase-3 and PARP. β-actin was used as the internal control. The experiments were performed in triplicates, and SD represented as±with respect to the mean. Compared to control, significance was considered as, **P*<0.05, ***P*<0.001.

antiproliferative effects of DPCMH 2-4 are due, in part, to the induction of apoptosis and cell cycle arrest.

3.6 Cell cycle analysis and increased sub-G1 apoptotic cell population

To determine the anti-proliferative effect of DPCMH 2-4, the sub-G1 cell population percentage was evaluated in each cell line using a range of DPCMH 2-4 concentrations. The results reveal that sub-G1 population percentage increased in a dose-dependent manner in both HL-60 and MCF-7 cell lines. As Fig.5c, d indicates, the sub-G1 accumulation in HL-60 cells was 0.82%, 22.02%, 47.73%, and 61.03% for the control, 12.5, 25, and 50 μg/mL concentrations, whereas that in MCF-7 cells was 1.09%, 14.04%, 46.95%, and 54.01%.

3.7 Apoptotic protein expression by DPCMH 2-4

Western blot analysis was conducted to investigate the levels of several key apoptosis regulatory molecular mediators, including Bax, Bcl-xL, caspase-3, p53, and PARP. As indicated in Fig.6, a shift toward conditions favoring apoptosis was observed, such that Bax, caspase-3, p53, and cleaved PARP were dose-dependently upregulated, while Bcl-xL was downregulated, in both cancer cell lines. HL-60 lacks p53 (Wolf and Rotter, 1985), whereas MCF7 is short of caspase-3 (Jänicke, 2009). The results suggest that both intrinsic and extrinsic apoptotic pathways are activated in response to DPCMH 2-4 treatment. PARP was present in cleaved and non-cleaved forms following DPCMH 2-4 treatment.

4 DISCUSSION

Cancer has now become a global adversity. The pathophysiology of cancer is dependent on the tissue from which cancer originates. A central hallmark of cancer is uncontrolled cellular proliferation and avoidance of programmed cell death. Chemotherapy and radiotherapy cause various side effects, such as mental stress, diarrhea, mouth sores, and fatigue. As a result, the discovery and development of naturally occurring therapeutics that could enhance immunocompetence and reduce the level of side effects are sought.

Soft corals are known as rich sources of terpenoids (sesqui-, di-, and tri-terpenoids), which act as part of the defensive mechanism of the organism. The present study identified a cluster of six terpenoids. *Dendronephthya* sp. has been exploited in several studies and has been reported to contain terpenoids (mainly sterols) with anticancer properties. According to Duh et al. (2004), sesquiterpenoids extracted from

D. gigantea possess potential antiproliferative effects against P338 and HT-29 cancer cells. Byju et al. (2014), reported the potential anticancer effects of 26, 26-dimethyl-5,24(28)-ergostadien-3β-ol and β-sitosterol purified from the soft coral *Subergorgia reticulata*, based on cytotoxicity effect on Dalton's lymphoma ascite cells.

The current study identified six sterols, Cholesta-5,22-dien-3-ol, (3β), Cholest-5-en-3-ol (3β), Ergosta-5,22-dien-3-ol, (3β,22E),22,23-Methylenecholestene-3-ol, (3β), Stigmast-5-en-3-ol, (3β,24s), and Stigmasta-5,24(28)-dien-3-ol, (3β,24E). Yang et al. (2006) have reported sterols isolated from the South China Sea coral *S. reticulata*. Soft corals are reported to contain more than 200 different types of sterol derivatives with many possessing potential antiproliferative properties. Sarma et al. (2009) provided an illustrative report on the sterols that are present in soft corals, giving a detailed description of the types of steroidal congeners present. The current study focused on the potential antiproliferative and apoptotic activities of DPCMH 2-4 fraction, rich in steroidal metabolites, against HL-60 and MCF-7 carcinoma cells, and their cytotoxic dose boundaries in Vero cells. To our knowledge, this represents the first description of anticancer compounds found in *D. putteri* extracts. Investigation of the apoptotic pathway was a key aspect of the current study. DPCMH 2-4 extracts inhibited the proliferation of HL-60 and MCF-7 cells by multiple mechanisms, including the induction of apoptosis and cell cycle arrest. This effect was further corroborated by observing aberrant nuclear morphology via Hoechst staining following DPCMH 2-4 treatment. During apoptosis, cells exhibit a unique morphology, wherein the cell starts forming blebs, starts to shrink, forms DNA fragments, and eventually becomes an apoptotic body to facilitate the removal by macrophages (Hassan et al., 2014). DPCMH 2-4 dose-dependently increased DNA damage and fragmentation. This effect was confirmed by observing a marked increase of comet tail DNA percentage following DPCMH 2-4 treatment. Another indicator of apoptosis is cellular DNA fragmentation and chromatin condensation. At 50 μg/mL DPCMH 2-4, the tail DNA percentages for HL-60 and MCF-7 cells were 56.73% and 55.85%, respectively. These results provide mechanistic insight into the antiproliferative and pro-apoptotic effects of DPCMH 2-4 on HL-60 and MCF-7 cells. Flow cytometric analysis is one of the most widely used assays to determine the fractional DNA content. During apoptosis, genomic DNA is cleaved into smaller fragments, thereby providing a specific marker of apoptosis. The procedure involves cell staining with propidium iodide (PI) and de-convolution of the cellular DNA content frequency through histograms. This method identifies three major phases of the cycle (G1, S, and G2/M) within cell populations, making it possible to distinguish apoptotic cells with fractional DNA content. The results of the current study suggest a dose-dependent increase in sub-G1 cell population percentages following DPCMH 2-4 treatment.

Levels of the apoptotic proteins such as Bax, Bcl-xL, caspase-3, and p53 were determined by western blot to provide further mechanistic insight. Bax, a pro-apoptotic protein present in the cytosol, is inserted into the mitochondrial membrane, inducing the release of cytochrome c ultimately resulting in apoptotic cell death. Bax proteins are often triggered by the cleavage of caspases, inhibition of protein kinases, and/or activation of phosphatases (Pawlowski and Kraft, 2000). Another protein family that is a key regulator of apoptosis is Bcl-2. This family includes both anti and pro-apoptotic proteins that function either as inhibitors or promoters of apoptosis (Huang, 2000). Bcl-xL, which is the focus of this study, is an anti-apoptotic protein which forms due to cell proliferation and promotes cell survival. Cytochrome c release is inhibited by Bcl-xL, which resides in the outer mitochondrial wall. This suggests that Bcl-xL has an anti-apoptotic effect. Therefore, the apoptotic process is controlled by the relative levels of Bax and Bcl-xL (Sharifi et al., 2015). The observed upregulation of Bax and downregulation of Bcl-xL upon DPCMH 2-4 treatment suggests a shift towards a pro-apoptotic state. The p53 protein acts as a transcription factor central to the cell's apoptotic mechanisms. To maintain cellular genetic integrity, this particular transcription factor can induce different processes such as growth arrest and apoptosis. Many anticancer drugs are designed to reactivate p53 in cancer cells. However, p53 has the opposite effect in normal cells. Consequently, many of these drugs could specifically act against cancer cells (Bremer and Helfrich, 2011). HL-60 cells lack p53 expression, while MCF-7 cells are deficient in caspase-3. Caspases act as mediators of the apoptotic process. Within the family of caspases, caspase-3 is a death protease often activated during apoptosis. In the current study, the proliferation rate was suppressed by the upregulation of p53 levels in MCF-7 cells. Simultaneous caspase-3

levels were upregulated in HL-60 cells, whereas MCF-7 cells lack the ability to synthesis caspase-3. Poly (ADP-ribose) polymerase (PARP) is a protein involved in programmed cell death. PARP is involved in the action of caspases, and plays an important role in the repair of damaged DNA. However, in situations of extensive DNA damage, PARP is deactivated by cleavage. This helps in saving energy being allocated to DNA repair, which in turn leads to necrosis. Although PARP is cleaved into segments via caspase-3 activity in the apoptotic cell pathway, PARP also plays a major role in chromatin structure regulation (Doetsch et al., 2012). PARP exhibited a similar cleavage pattern in both the cell lines following DPCMH 2-4 treatment. Non-cleaved (116 kDa) and cleaved (89 kDa) PARP fragments were observed during the analysis. Cleaved PARP levels increased in a dose-dependent manner, with a corresponding decrease in non-cleaved PARP. The results suggest that these proteins collectively contribute to a loss of mitochondrial transmembrane potential, releasing mitochondrial cytochrome *c*, subsequently inducing apoptosis.

5 CONCLUSION

The soft coral *D. putteri* collected from the Jeju Sea contains 3β-hydroxy-Δ5-steroidal derivatives with antiproliferative and apoptosis-inducing activities. Taken together, DPCMH 2-4 treatment caused a dose-dependent increase in apoptotic body formation, DNA damage, and sub-G1 apoptotic cell population. The steroidal congeners in DPCMH 2-4 were capable of significantly inhibiting the growth of HL-60 and MCF7 cells through the mitochondria-mediated apoptotic pathway. Therefore, the steroidal congeners of *D. putteri* have the potential to be used as candidates for anticancer therapeutics. In vivo studies through the use of animal models like zebra fish and mouse models are applicable. As this study partially isolates the compound that exhibit synergistic effect, pure compound isolation could lead to in-depth studies.

6 DATA AVAILABILITY STATEMENT

Due to organizational restrictions, the data and materials will not be publicly available.

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