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Apoptotic anticancer activity of a novel fatty alcohol ester isolated from cultured marine diatom, *Phaeodactylum tricornutum*

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ABSTRACT

A novel fatty alcohol ester; nonyl 8-acetoxy-6-methyloctanoate (NAMO) was identified among the five isolated compounds from cultured marine diatom, *Phaeodactylum tricornutum* Bohlin. The anticancer effects of NAMO were examined through screening of three different cancer cell lines including a human promyelocytic leukemia (HL-60), a human lung carcinoma (A549) and a mouse melanoma (B16F10). A strong suppression of cancer cells growth was observed in HL-60 and its IC_{50} value was 65.15 μM compared to the other cancer cells *in vitro*. The apoptotic occurrence in HL-60 cells by NAMO was evidenced as the accumulation of DNA in sub-G_1 phase and nuclear condensations dose-dependent manner. From the protein expression results it was revealed on the apoptotic pathway that NAMO switched on the apoptosis by activation of Bax and suppression of Bcl-xL through apoptotic inducing control of pro- and anti-apoptotic proteins, and by up-regulation of another inducers of apoptosis, caspase-3 and p53.

1. Introduction

Functional foods and food supplements are closely related in the manipulation of functional ingredients for the health and well-being of an individual. The reason for developing new functional foods or dietary supplements from marine bioresources can be a growing and interesting area which can provide health enhancing ingredients in a convenient form. Therefore, it is indeed a distinct importance of the relationship between food supplements and health and well-being. Furthermore, chemically engineered food supplements are gaining interest from the view-point of their purity and containing high quality components for therapeutic uses. In this regard, cultured marine microalgae may be considered as a source of alternative material for new functional foods and dietary supplements.

Cancer is the leading threat for the world population and first-leading cause of death in economically developed coun-

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tries and the second in the developing countries (Ezzati, Lopez, Rodgers, Hoorn, & Murray, 2002). According to the global cancer statistics, about 12.7 million cancer cases were reported with an estimated mortality of 7.6 million in 2008 (Jemal et al., 2011). In addition, breast cancer in females and lung/bronchus cancer in male are the most frequently diagnosed type of cancers reported in the world. Therefore, finding cancer cure therapeutics from marine food materials is highly demanded. Apoptosis is considered as a programmed cell death and characterized cell changes including blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation (Kang et al., 2012; Kroemer, Petit, Zamzami, Vayssiere, & Mignotte, 1995). Apoptosis provides a conceptual framework to understand the cancer genetics and cancer therapy (Lowe & Lin, 2000). Moreover, most cytotoxic anticancer agents are inducing the apoptosis that can be compelled to characteristic cell changes and diminishing cancer cells.

Over the many years, the interest in isolation of pharmacologically active metabolites from marine natural sources has increased throughout the world. In particular, utilization of new bioactive natural products from marine bio-resources for the pharmacological applications has been considered as being promising. Thus, functional ingredients are quite species specific and demanded for the defense against predation, infections, parasitism and homeostasis (Jimeno, Faircloth, Sousa-Fero, Scheuer, & Rinhart, 2004). During the last three decades, more than 35,000 novel marine natural products have been isolated and structurally identified from marine sources (Fenical, 2006). Moreover, microalgae have been widely used as rich sources of pharmacologically active functional ingredients. Along with this trend, the possibility of replacing synthetic compounds with natural ones is receiving much attention (El Baky Abd & El Baroty, 2013).

Marine diatoms are identified as the most important photosynthetic eukaryotes in the marine ecosystems (Wichard & Pohnert, 2006). There are an estimated about $\sim 10^6$ species of diatoms and considered as 4-fold more diversity than angiosperms (Lenoci & Camp, 2008). Most of them are included with silica derived cell wall and have been evolving for the last 40 million years as single-cell algae with various morphological differences (Mann, 1989). Thus, *Phaeodactylum tricornutum*, a diatom of bacillariopyhceae has been known to contain high amounts of functional ingredients, including pigments (Carreto & catoggio, 1976), lipids (Fajardo et al., 2007; Zhukova & Aizdaicher, 1995), sulphated polysaccharides (Raposo, Morais, & Morias 2013), crude polysaccharide (Guzman, Gato, Lamela, Garabal, & Calleja, 2003) and peptides (Kawaguchi & Maita, 1990; Morelli, Cruz, Somovigo, & Scarano, 2002).

The isolation of pharmaceutical materials from *P. tricornutum* has not previously been carried out. However, two monogalactosyldiacylglycerols were isolated from cultured marine *P. tricornutum* and reported as inducing apoptosis in two genetically matched immortal mouse epithelial cell lines (Andrianasolo et al., 2008). Therefore, in this study we examined whether the cultured marine diatom, *P. tricornutum* is capable of producing novel compounds with anticancer activities through *in vitro* assays. Hence, we evaluated the apoptotic anticancer effect of an isolated novel compound from the hexane fraction of the cultured marine diatom, *P. tricornutum* against a human promyelocytic leukemia cell line (HL-60).

2. Materials and methods

2.1. Chemicals and reagents

A human promyelocytic leukemia cell line (HL-60), a mouse melanoma cell line (B16F10) and a human lung carcinoma cell line (A549) were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). Roswell Park Memorial Institute (RPMI-1640) medium, Dulbecco's Modified Eagle's Medium (DMEM), Foetal Bovine Serum (FBS), and penicillin–streptomycin were purchased from Gibco/BRL (Burlington, ON, Canada). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazole trihydrochloride trihydrate (Hoechst 33342) and dimethyl sulphoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals and reagents used in these investigations were of analytical grade.

2.2. Culture of marine diatom: P. tricornutum

The marine diatom *P. tricornutum* Bohlin was kindly provided by the Korea Marine Microalgae Culture Center (KMMCC). The algae were inoculated in 30-L plastic cylinders at 20 °C after pre-cultivated in 5 L glass vessels (medium 4 L), and air was continuously supplied at 5 L min^{-1} by air-lift. Light was provided by 60-W fluorescent lamps at an intensity of 34 μmol photons m^{-2} s^{-1} (light:darkness = 24:0). Microalgae species were cultured in Conway medium (Walne 1966) prepared from filter-sterilized seawater and the culture remained continuously active during the 8–10 days after inoculation. The cells were flocculated with 200 ppm $Al_2(SO_4)_3$ (v/v) (Ilshin biochemical, Magicpool-99) and then recovered with centrifugation at 500×*g* using a basket centrifuge (Hanseong Co., Ansan, Korea). The harvested diatom biomass was frozen at −80 °C and preserved until freeze dry.

2.3. Extraction and isolation

The lyophilized and homogenized *P. tricornutum* diatom (38 g) was extracted three times with (80%) methanol in each 90 min sonication period at 25 °C. Concentrated crude methanol extract was further subjected to solvent-solvent partition chromatography after evaporating the solvent under reduced pressure using a rotary evaporator. Then, four different fractions such as *n*-hexane, chloroform, ethyl acetate and aqueous extracts were separated with the varying of polarity. The *n*-hexane fraction (550 mg) was found to be active and subjected to fractionate using solid-liquid phase chromatography (normal phase-silica) column (3 cm × 22 cm) using hexane and ethyl acetate as solvents with increasing hydrophilic character. In fact, 14 fractions were obtained after pooling the elution followed by thin layer chromatography (TLC) studies and labeled as F1–F14. TLC studies were done by staining ethanol-sulphuric acid (90:10, v/v) solvent system and visualized the eluted fractions. Among the eluted 14 fractions from

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n-hexane extract of cultured *P. tricornutum*, F1 (10.4 mg) and F7 (19 mg) fractions were separated with enough purity at the following solvent conditions, hexane-ethyl acetate (95:5, v/v) and (90:10, v/v) respectively. Furthermore, both F1 and F7 fractions were labeled tentatively as (PTH-1; Com 1) and (PTH-2; Com 2) until their molecular structure was determined. In addition, F8 fraction (90 mg) was eluted using hexane-ethyl acetate (75:25, v/v) solvents and further fractionated using preparative thin layer chromatography (PTLC) into three sub-fractions such as PTH8-1 (Com 3), PTH8-2 (Com 4) and PTH8-3 (Com 5) with corresponding weights (16.8, 10.0 and 6.3 mg), respectively. Molecular formula and IUPAC names of the structure elucidated compounds are listed in Table 1.

2.4. Characterization of the active compound

The molecular mass was measured using high performance liquid chromatography-diode array detector coupled with electrospray ionization mass spectrometer (HPLC-DAD-ESI/MS) (Hewlett-Packard, Waldbronn, Germany). For the structure elucidation, NMR studies were carried out using a JEOL JNM-ECX400 spectrometer (JEOL, Tokyo, Japan), operated at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. The isolated compounds were prepared in deuterated solvents in 5 mm NMR tubes. Chloroform deuterated solvent was used and the chemical shifts were measured relative to the TMS signal. All the experiments were conducted at room temperature. Only the determined NMR data on compound (Com 2) is presented in Table 2.

2.5. Cell culture

HL-60 (a human promyelocytic leukemia cell line), A549 (a human lung carcinoma cell line) cells were grown in RPMI-1640 medium, and B16F10 (a mouse melanoma cell line) was cultured in Dulbecco's modified eagle medium (DMEM). Both culture media were supplemented with 100 U mL^{-1} of penicillin, 100 μg mL^{-1} of streptomycin and 10% foetal bovine serum (FBS). The cells were incubated and maintained in an atmosphere of 5% CO_2 at 37 °C. The cells were sub-cultured every 2 days and exponential phase cells were used throughout the experiments.

2.6. Cell growth inhibitory assay

The cell growth inhibitory activity (cytotoxicity) of the isolated five compounds with different concentrations (25 and 50 μg mL^{-1}) was assessed from the cultured marine diatom: *P. tricornutum* against the cancer cell lines (HL-60, B16F10, and A549) using the colorimetric MTT assay. In fact, suspension cells (HL-60) and attached cells (B16F10 and A549) were seeded in a 96-well plate at a concentration of 2×10^4 cells mL^{-1} together with the samples with various concentrations and incubated for 48 h before MTT treatment. MTT stock solution (50 μL; 2 mg mL^{-1} in PBS) was added to each well to achieve a total reaction volume of 250 μL. After 4 h of incubation, the plates were centrifuged for 10 min at 800×*g* and the supernatants were aspirated. The formazan crystals in each well were dissolved in DMSO. The amount of purple formazan was assessed by measuring the absorbance at 540 nm.

2.7. Nuclear staining with Hoechst 33342

The cell permeable DNA dye, Hoechst 33342 staining was used to examine the nuclear morphology of the cells. Hoechst 33342 is excited by ultraviolet light and emits blue fluorescence at 460–490 nm. This dye binds preferentially to adenine-thymine (A–T) regions of DNA and stained nuclei were considered as viable. In addition, the presence of DNA fragmentations and chromatin condensations were visualized as apoptosis (Lizard et al., 1995). HL-60 cells were placed in 24-well plates at a concentration of 2×10^4 cells mL^{-1}. The cells were then treated with various concentrations (12.5, 25, 50 and 100 μg mL^{-1}) of NAMO and incubated for an additional 24 h. Then, Hoechst 33342, a DNA-specific fluorescent dye was added to the culture media at a final concentration of 10 μg mL^{-1} and the plates were incubated for an additional 10 min at 37 °C. The stained cells were then observed under a fluorescence microscope equipped with a CoolSNAP-Pro colour digital camera in order to determine the degree of nuclear condensation.

2.8. Cell cycle analysis

The cell cycle analysis was performed to examine the proportion of apoptotic sub-G_1 hypodiploid cells according to described by Nicoletti, Migliorati, Pagliacci, Grignani, and Riccardi (1991). The HL-60 cells were seeded on 6-well plates at a concentration of 2×10^5 cells mL^{-1}. The cells were treated with different concentrations (12.5, 25, 50 and 100 μg mL^{-1}) of isolated compound (NAMO) from the hexane fraction of *P. tricornutum*. The cells were harvested after 24 h and fixed in 1 mL of 70% ethanol for 30 min at 4 °C. Then the cells were washed twice with PBS and incubated in darkness in 1 mL of PBS containing 100 μg of propidium iodide (PI) and 100 μg RNase A for 30 min at 37 °C. After that the flow cytometric analysis was performed with a FACS Calibur flow cytometer

Table 1 – Molecular formula and IUPAC names of the isolated compounds from *Phaeodactylum tricornutum*.

Compounds	Molecular formula	IUPAC name
1	$C_{22}H_{36}O_2$	Icosa-5,8,11,14-tetraenyl acetate
2	$C_{20}H_{38}O_4$	Nonyl 8-acetoxy-6-methyloctanoate
3	$C_{27}H_{44}O_2$	Cholestra-5(6),22-diene-3,24β-diol
4	$C_{28}H_{46}O_2$	Cholestra-5(6),22-diene-3,24β-diol, methyl ether
5	$C_{28}H_{46}O$	24-methylcholesta-5(6),22-diene-3β-ol

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Table 2 – NMR data for nonyl 8-acetoxy-6-methyloctanoate (NAMO) compound in chloroform-d.

Position (C#)	δ_C[a] (ppm)	(mult)	δ_H[a] (mult, J_{HH} Hz)
1	179.2	(C)	
2	34.1	(CH_2)	2.32 *t* (7.53)
3	24.9	(CH_2)	1.68 m
4	24.7	(CH_2)	1.25 m
5	37.5	(CH_2)	1.25 m
6	32.9	(CH)	1.65 m
7	29.4	(CH_2)	1.53 m
8	63.3	(CH_2)	4.08 dd (7.40,14.61)
9	171.3	(C)	
10	21.2	(CH_3)	2.21 s
11	22.8	(CH_3)	0.96 s
1′	60.6	(CH_2)	4.13 dd (7.40, 14.61)
2′	29.2	(CH_2)	1.50 m
3′	24.6	(CH_2)	1.43 m
4′	29.6	(CH_2)	1.29 m
5′	29.9	(CH_2)	1.25 m
6′	29.9	(CH_2)	1.25 m
7′	32.1	(CH_2)	1.29 m
8′	22.9	(CH_2)	1.31 m
9′	14.3	(CH_3)	0.87 *t* (6.72)

[a] Multiplicity determined from HSQC-DEPT experiments.

(Becton–Dickinson, San Jose, CA, USA). The effect on the cell cycle was examined by changes in the percentage of cell distribution at each cell cycle phase, and assessed by histograms generated by the Quest and Mod-Fit computer programs as described method (Wang, Shiau, & Lin, 1999a; Wang et al., 1999b).

2.9. Western blot analysis

HL-60 Cells (2×10^5 cells mL^{-1}) were treated with the isolated compound (NAMO) at concentrations of 12.5, 25, 50 and 100 μg mL^{-1} and allowed to harvest after 24 h. The cell lysates were prepared with lysis buffer (50 mmol L^{-1} Tris–HCl (pH 7.4), 150 mmol L^{-1} NaCl, 1% Triton X-100, 0.1% SDS and 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA)). Then the cell lysates were washed via centrifugation, and the protein concentrations were measured using a BCA™ protein assay kit. The lysates containing 30 μg of protein were subjected to electrophoresis using sodium dodecyl sulphate–polyacrylamide gels (SDS–PAGE) on 12%, and the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were incubated with primary antibody against Bax, Bcl-xL, cleaved caspase-3, p53, and β-actin in TTBS (25 mmol L^{-1} Tris–HCl, 137 mmol L^{-1} NaCl, 0.1% Tween 20, pH 7.4) containing 0.5% concentration of nonfat dry milk for 1 h period. Then the membranes were washed with TTBS and incubated with secondary antibodies. Signals were developed by exposing to X-ray films and used to visualize according to the described as a Western Lightning-ECL detection kit (PerkinElmer, MA, USA).

2.10. Statistical analysis

All the data are expressed as mean ± standard deviation of three determinations. Statistical comparison was performed via a one-way analysis of variance followed by Duncan's multiple range test (DMRT). *P* values of less than 0.05 ($P < 0.05$) were considered as significant.

3. Results

3.1. Growth inhibitory activity of the isolated compounds from P. tricornutum against HL-60 cancer cell line

The cultured marine microalga, *P. tricornutum* hexane fraction was led to isolate five different compounds and named tentatively as Com 1, Com 2, Com 3, Com 4 and Com 5. The growth inhibitory effects of the isolated five compounds were performed by MTT assay for screening the anticancer activity using cancer cell line (HL-60) *in vitro* (Fig. 1). According to the MTT assay results, Com 2 was showed the significant lower cell viability (32.8%) at 50 μg mL^{-1} incubated concentration for 48 h. However, the determined cell viability (%) of other compounds was higher than Com 2 at 50 μg mL^{-1} significantly. Therefore, the isolated Com 2 was selected for further studies due to inhibit the growth of HL-60 cancer cell growth markedly.

3.2. Identification and structure elucidation of the isolated anticancer compound from P. tricornutum

The isolated compound (Com 2) from the cultured microalga, *P. tricornutum* was indicated the strongest cytotoxic effect against HL-60 cancer cells and it was subjected to assess the molecular mass and structure. Hence, the molecular formula was determined as $C_{20}H_{38}O_4$ in the ESI (negative mode) as [M−H] peak at 341.14 m/z. The calculated molecular mass (342.28 m/z) was examined with the two degrees of unsaturation. The ^{13}C NMR spectrum showed 20 signals and two signals were appeared at δ179.2 and 171.3 ppm assigned as carbonyl carbons (C=O; ester) at C-1 and C-9 positions, respectively. In addition, the ^{13}C NMR signals at δ 63.3 and

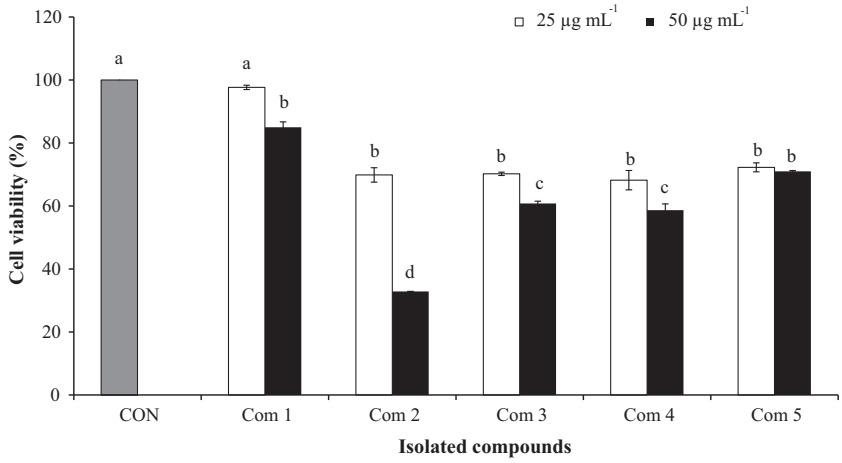

Fig. 1 – The effect of cell viability (%) of HL-60 cancer cells against isolated five compounds from the cultured marine diatom, *Phaeodactylum tricornutum* for 48 h. □ Denoted as the control (no sample treated HL-60 cells). HL-60 cells were treated with the compounds including Com1, Com 2, Com 3, Com 4 and Com 5 at the indicated concentrations denoted as □ 25 μg mL^{-1} and ■ 50 μg mL^{-1} respectively. Values are expressed as means ± SD in triplicate experiments. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

60.6 ppm revealed that two carbons which linked as (C–O) bond for ester linkage at the C-1′ and C-8 positions in the carbon skeleton, respectively. Further insight to the molecular structure was indicated that C-1′ linked to a nine carbon aliphatic chain (nonyl group). In fact, the carbonyl carbon positioned at C-1 was linked with alcohol aliphatic chain started at C-8 position which interconnected between two carbonyl carbons of ester molecules. Moreover, the heteronuclear correlation (HSQC) spectrums showed the presence of substitution at the C-6 position as exo-methyl group and heteronuclear multiple bond correlation (HMBC) confirmed the coherence to C-5 and C-7 positions, respectively (Fig. 2). Furthermore, assigned multiplicity of the isolated compound is represented in Table 1. NMR data suggest that the molecule has an aliphatic ester character and literature survey at this stage found that the isolated compound had a similar skeleton to nonyl octanoate (Shmuel, 2004). However, the analyzed HSQC spectral data complying to report that the isolated compound (Com 2) was a novel fatty alcohol ester, and given name as nonyl 8-acetoxy-6-methyloctanoate (NAMO) from the cultured marine diatom, *P. tricornutum*.

3.3. *Growth inhibitory effect of NAMO against HL-60, B16F10 and A549 cancer cell lines*

In this study, anticancer activity was screened against three different cell lines such as a human promyelocytic leukemia cell line (HL-60), a human lung carcinoma cell line (A549) and a mouse melanoma cell line (B16F10).The active compound, Com 2 (nonyl 8-acetoxy-6-methyloctanoate; NAMO) was assessed the inhibitory effect of the growth of cancer cell lines compared to the control (cancer cells without treated NAMO). Fig. 3 presents the cell viability (%) of the cancer cells which incubated with the NAMO at 25 and 50 μg mL^{-1} concentrations for 48 h *in vitro* assay, respectively. Among the cancer cells, the HL-60 cells were suppressed significantly ($P > 0.05$) with the treated NAMO at all the concentrations when compared to the control. In particular, the highest growth inhibitory activity of about 70% on HL-60 cells was observed at 50 μg mL^{-1} treated concentration of NAMO compared to the control. However, A549 cell growth suppression was moderate at about 35% at 50 μg mL^{-1} incubated NAMO concentration ($P > 0.05$). The isolated active compound did not significantly reduce the growth of B16F10 cells compared to the control. Hence, cellular regulatory effects on HL-60 cancer cells for inducing apoptosis with the different NAMO concentrations were examined.

3.4. *NAMO induced apoptosis in HL-60 cells*

The isolated lipid ester compound (NAMO) at various concentrations (12.5–100 μg mL^{-1}) was incubated with the HL-60 cells for 48 h and the 50% of inhibitory concentration (IC_{50}) value as 22.3 μg mL^{-1} (65.15 μM) was determined. To determine the apoptosis induced nuclear morphology of HL-60 cells respect to the various treated NAMO concentrations were stained with Hoechst 33342 and visualized by fluorescent microscopy (Fig. 4). In this experiment, the control (no treated sample; A) showed the intact cell nuclei without DNA damage. However, during the NAMO treatment at different concentrations, clear DNA damages were observed and led to increase the apoptotic body formations dramatically (Fig. 4(B–E)). These results were markedly correlated with the HL-60 cells growth inhibition via the proportion of apoptotic body formation with the treated NAMO concentration dose-dependently.

3.5. *Induction effect of Sub-G_1 contents in HL-60 cells by NAMO*

The inhibitory effects of the proliferation of HL-60 cells were evaluated by determining the sub-G_1 content population (%) with respect to the incubated NAMO different concentrations. According to the results, it is observed that increased effect of cell cycle arrest and accumulation of cells in the sub-G_1 phase was concentration dependent (Fig. 5(B–E)). Moreover, the apoptotic body formations corresponded with the accumula-

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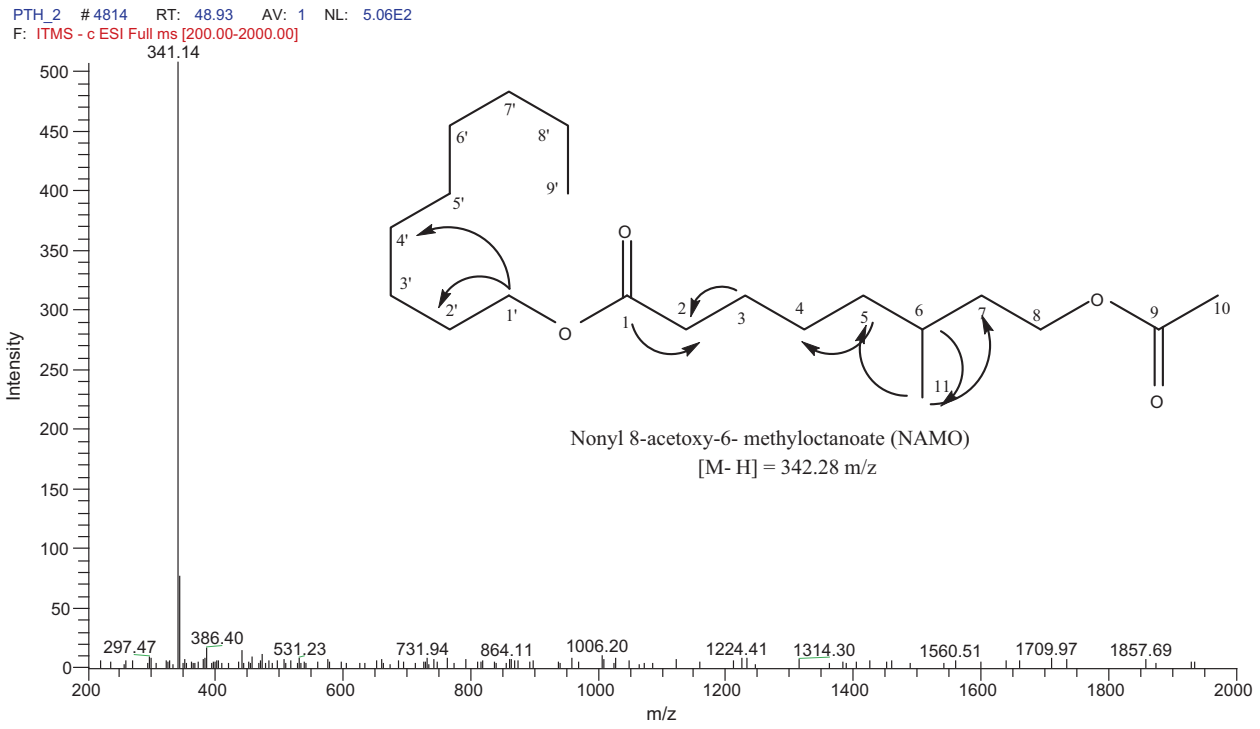

Fig. 2 – Mass spectra and chemical structure of the novel fatty alcohol ester: nonyl 8-acetoxy-6-methyloctanoate (NAMO) isolated from the hexane fraction of cultured marine diatom, *Phaeodactylum tricornutum*. The spectrum was determined in negative ionization mode and the HMBC correlations (↷) denoted in the NAMO compound appropriately.

tion of sub-G_1 content and further were determined as 8.53%, 20.8%, 39.9% and 64.5% of sub-G_1 population against the NAMO concentrations (12.5, 25, 50 and 100 μg mL^{-1}), compared to the control, respectively (Fig. 5(F)). These evidences suggest that NAMO induced cell death due to the apoptotic body formation in a concentration dependent manner.

3.6. *The expression of apoptosis-related proteins by NAMO*

Western blot analysis was carried out to determine the protein expressions of Bax, Bcl-xL, caspase-3 and p53 that regulate the NAMO-induced apoptosis on HL-60 cells (Fig. 6). The pro-apoptotic protein, Bax expression was up-regulated dose dependently at the NAMO treated concentrations. In addition, the expression of Bcl-xL as anti-apoptotic protein was down-regulated dose dependently followed by NAMO treatment. Interestingly, the Bcl-xL expression was diminished completely at the 100 μg mL^{-1} of NAMO concentration. Moreover, cleaved caspase-3 expression increased markedly above 50 μg mL^{-1} NAMO concentration and p53 protein expression also increased compared to the control.

4. Discussion

Apoptosis is an important biological mechanism that effectively plays a role in maintaining cellular integrity and homeostasis (Yan et al., 2008). In fact, this process regulates cell division and intrinsic suicide or programmed cell death (Chang & Yang, 2000). Uncontrolled cell proliferations were characterized for the growing of cancer cells. Therefore, a key to activate apoptotic pathway might be the mechanism for kill cancer cells (Xu et al., 2009). Hence, the possible development of a new lead compound from marine sources for cancer therapy was an important aspect.

P. tricornutum is a well known marine diatom that accumulated fatty acids with varying degrees of unsaturation. In particular, the compositions of the long or short chain aliphatic compounds which associated with *P. tricornutum* biomasses as lipids are having different functional value that are of significant importance for the advancement of marine biotechnology (Radakovits, Eduafo, & Posewitz, 2011). Moreover, fatty acid rich substrate of a high proportion of eicosapentaenoic acid (EPA) was extracted and purified from *P. tricornutum*. The high content of polyunsaturated fatty acids (PUFA) at around 30–45% was extracted that included EPA as is major component up to 20–40% of the total fatty acids in the cultured *P. tricornutum* (Fajardo et al., 2007). Therefore, the cultured marine diatom is supposed to be a good source of bioactive lipids for the neutraceutical industry.

In the present study, we isolated five different compounds from the hexane fraction of cultured marine microalga, *P. tricornutum*. Among the isolated compounds, Com 2 was determined as a novel fatty alcohol ester, nonyl 8-acetoxy-6-methyloctanoate (NAMO) and examined the growth inhibitory effects against human leukemia HL-60 cells *in vitro*. The

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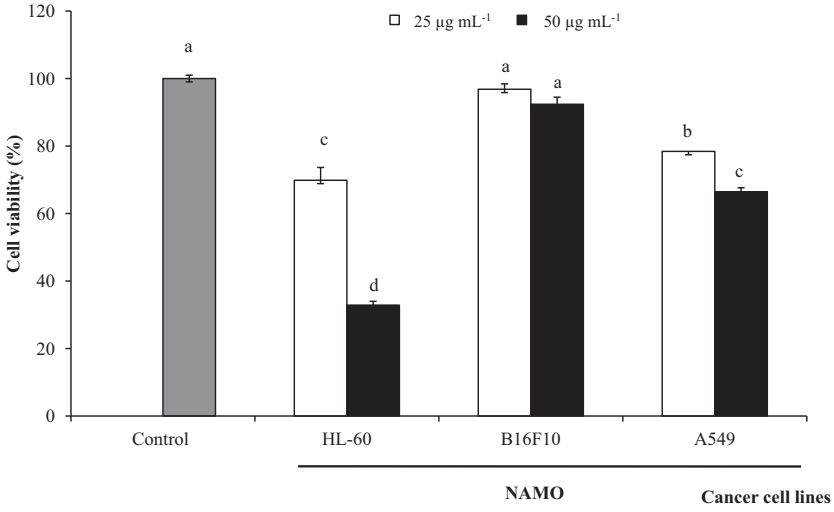

Fig. 3 – The effect of cell viability (%) of cancer cell lines including HL-60, B16F10, and A549 against isolated compound, (Com 2) NAMO from the cultured marine diatom, *Phaeodactylum tricornutum* for 48 h. ▒ Denoted as the control (no sample treated cancer cells). Cancer cells were treated with the NAMO compound at the indicated concentrations denoted as □ 25 μg mL^{-1} and ■ 50 μg mL^{-1} respectively. Values are expressed as means ± SD in triplicate experiments. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

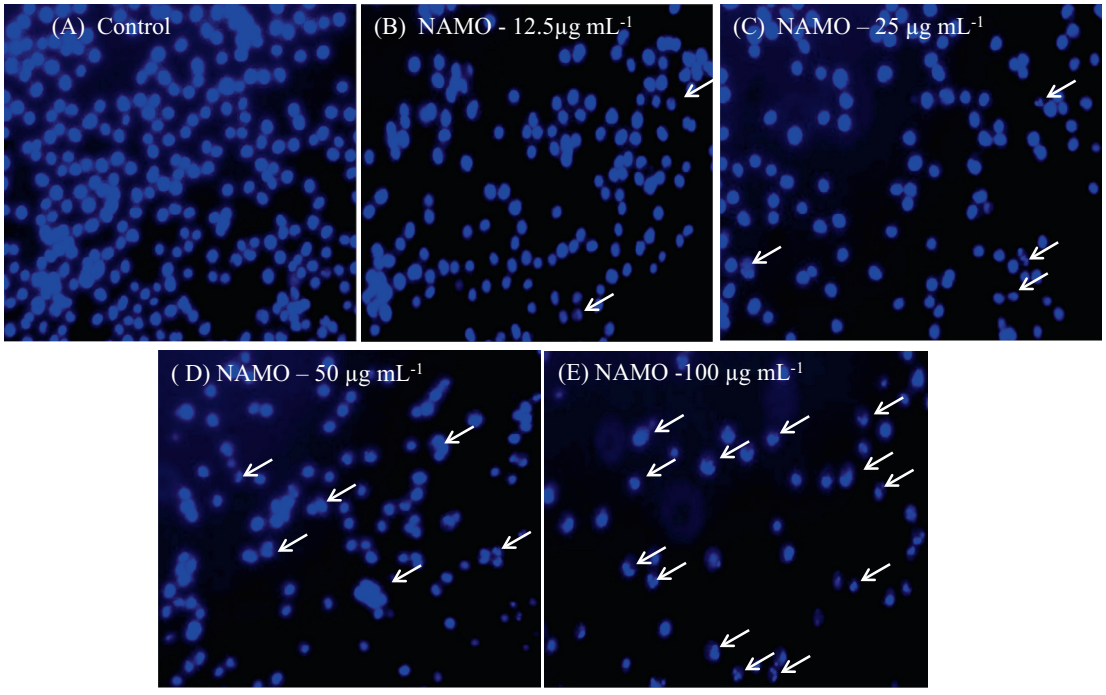

Fig. 4 – Induction of apoptotic body formation in HL-60 cells was observed under a fluorescent microscope after Hoechest 33342 staining. (A) control: no treated; (B) NAMO treated with 12.5 μg mL^{-1}; (C) NAMO treated with 25 μg mL^{-1}; (D) NAMO treated with 50 μg mL^{-1}; (E) NAMO treated with 100 μg mL^{-1}. *Arrows* denoted a typical apoptotic body formation in HL-60 cells.

inhibition of HL-60 cell proliferation was strongly reduced by apoptosis induction or cell cycle arrest or combination of both with the incubated NAMO concentration dependently manner. This was confirmed that the observed typical nuclei morphology characteristics which induced the nuclear condensation or DNA fragmentation and sub-G_1 DNA accumulations with respect to the treated NAMO at different concentrations.

The apoptotic protein expressions were subsequently followed to understand the mechanism of regulatory effect of HL-60 cells against anticancer agents. In that case, major apoptotic proteins such as Bax, Bcl-xL, caspase-3 and p53

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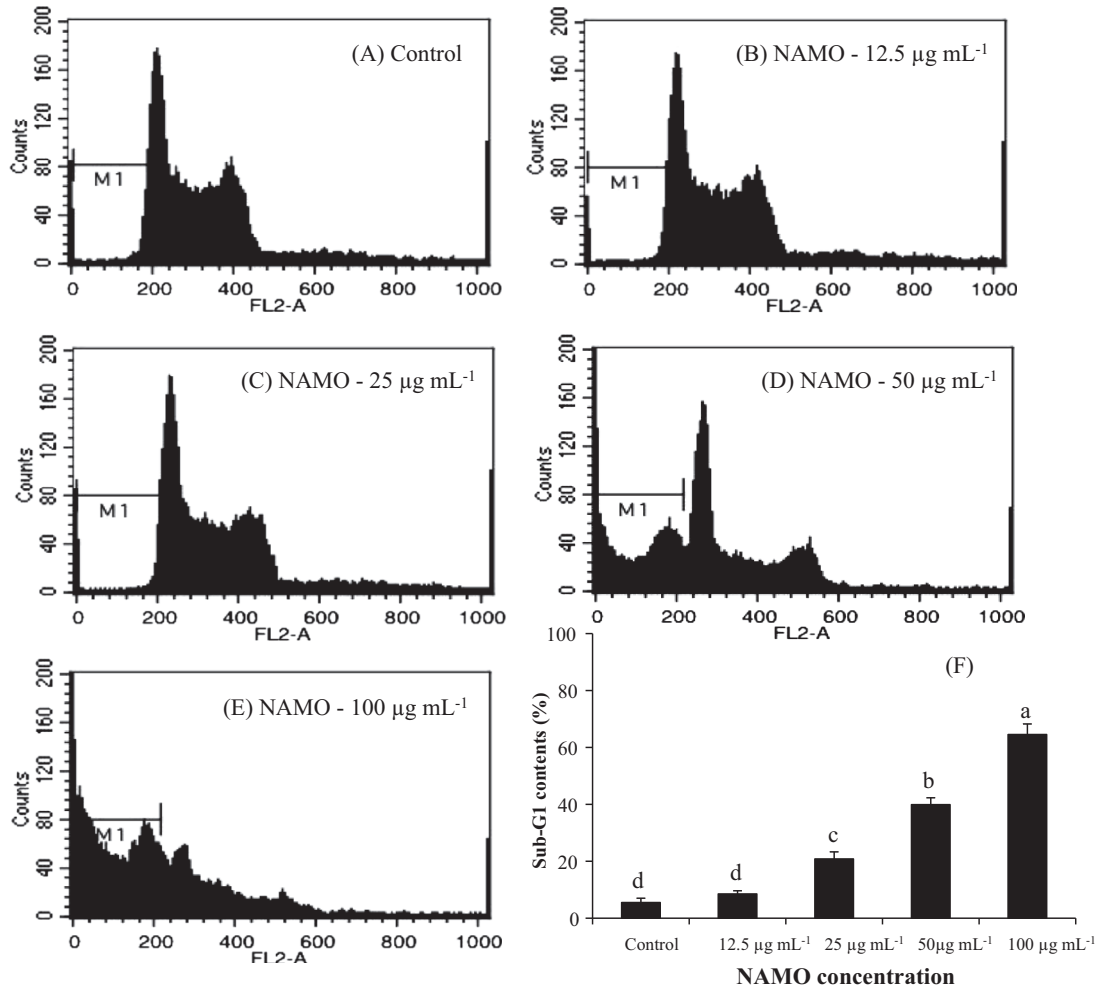

Fig. 5 – Apoptotic Sub-G1 content was detected by flow cytometry after stained with PI. (A) control: no treated; (B) NAMO treated with 12.5 μg mL^{-1}; (C) NAMO treated with 25 μg mL^{-1}; (D) NAMO treated with 50 μg mL^{-1}; (E) NAMO treated with 100 μg mL^{-1}, (F) Accumulation of DNA content (%) in the Sub-G$_1$ phase of the cell cycle of HL-60 incubated with NAMO different concentrations were determined by flow cytometry.

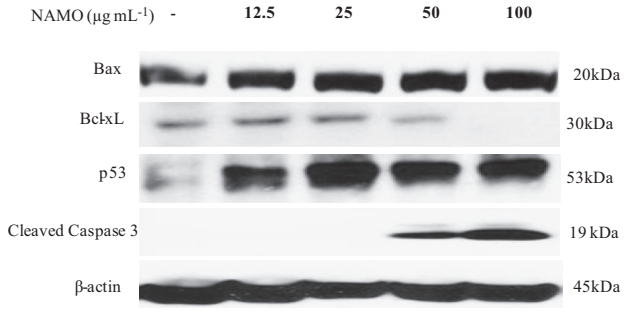

Fig. 6 – Effect of NAMO on apoptosis-related proteins in HL-60 cells. The cells were incubated with NAMO different concentrations for 12 h and cell lysates were subjected for the western blot analysis. Antibodies were used as Bax, Bcl-xL, p53 and Cleaved caspase-3. β-actin used as internal control.

were studied. In particular, Bax is pro-apoptotic member of protein which resides the cytosol. However, it translocates to mitochondria following death signaling as the apoptosis induction and promotes the release of cytochrome *c* (Cory & Adams, 2002). However, the Bcl-xL as anti-apoptotic proteins was overproduced due to proliferation and often found to be promoting the cell survivability (Kang & Reynolds, 2009). Bcl-xL resides in the outer mitochondrial wall and inhibits cytochrom *c* release. Therefore, the ratio between the Bax and Bcl-xL can be suggested to regulate the apoptosis process. In the western blot analysis, we found a significant up-regulation of Bax and down-regulation of Bcl-xL proteins as NAMO in a dose-dependent manner. Therefore, the isolated compound NAMO increased the Bax/Bcl-xL ratio and led to increased apoptotic pathway against HL-60 cells growth. The protein expression of the p53 was up-regulated and led to decreased viability of the HL-60 cells which suppressed the proliferation as well. Thus, p53 mediated apoptosis process controlled cell differentiation and changed the rate of kinetics in cancer cells (Ronen, Schwartz, Teitz, Goldfinger, & Rotter, 1996). Moreover, caspase-3 protein expression was increased markedly at 50 and 100 μg mL^{-1} NAMO incubation concentrations. These evidences suggest that rapid cause of induced

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caspase-3 proteins may lead to loss of mitochondrial transmembrane potential and release of mitochondrial cytochrome *c* into the cytosol. Furthermore, the activation of these pro-apoptotic proteins is also accompanied by the release of cytochrome *c* into the cytosol. Importantly, release of cytochrome *c* may lead to mitochondrial apoptotic pathway (Budihardjo, Oliver, Lutter, Luo, & Wang, 1999) and the suppression of cancer cell growth (Ham et al., 2012; Wang et al., 1999a,b). These data suggest that caspase-3 is one major executioner of apoptosis. Moreover, apoptosis is mediated through p53 apoptotic pathway to regulate the growth of HL-60 cells with the treatment of anticancer compound dose-dependently.

Taken together, the isolated fatty alcohol ester (NAMO) compound from the cultured marine diatom, *P. tricornutum* was capable of inhibiting the growth of human leukemia cells significantly through the p53 and caspace-3 mediated cell apoptotic pathway. Hence, this functional lipid molecule has the potential to be used in pharmacological and netraceutical industries since the cultured marine microalgae could be the good alternative for future bio-resources. Therefore, further studies on *in vivo* and phase trail experiments can be supported for describing the efficacy of the isolated novel compound for future therapeutic uses.

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