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A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Bioactive Natural Products from Cultured Marine Microalgae and Sri Lankan Marine Algae

KALPA W. SAMARAKOON

Department of Marine Life Sciences

GRADUATE SCHOOL

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FEBRUARY, 2014

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Bioactive Natural Products from Cultured Marine Microalgae and Sri Lankan Marine Algae

KALPA W. SAMARAKOON

(Supervised by Professor You-Jin Jeon)

A thesis submitted in partial fulfillment of the requirement for the degree of

DOCTOR OF PHYLOSOPHY

FEBRUARY, 2014

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CONTENTS

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

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JEJU NATIONAL UNIVERSITY LIBRARY

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SUMMARY

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 bio-resources 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.

The marine ecosystem represents a vast and dynamic array of bio-resources attributed with its huge diversity and considered as potential untapped reservoirs for the development of functional foods for future health markets. New processing and extraction strategies of marine bio-resources integrated with biotechnology have been cherished recently. Most of the bio-molecular components, such as proteins and lipids can be extracted in large scale, using the modern and advanced biotechnological approaches, are in one hand suitable drug candidates for the pharmaceutical industry, on the other hand functional food materials for the food industry. Moreover, the furtherance of high throughput molecular biological techniques has already been incorporated with identification, mining and extraction of molecular components from marine bio-resource, rendering the promising effects. In this regard, cultured marine microalgae may be considered as a source of alternative material for new functional foods and dietary supplements.

Enzyme-assisted extraction was performed on the cultured marine blue-green algae and targeted to obtain bioactive components in this study. Based on the high contents of crude proteins in the

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marine microalgae, commercially available protease enzymes used to isolate angiotensin I converting enzyme (ACE) inhibitory proteins and peptides from *Nannochloris oculata* and *Chlorella ovalis*, respectively. The isolated novel peptides such as Gly-Met-Asn-Asn-Leu-Thr-Pro (728.3 Da) and Leu-Glu-Gln (369.2 Da) from *N. oculata* showed the profound ACE inhibitory activity with IC_{50} values of 123 µM and 173 µM, respectively. Furthermore, purified proteins and peptides showed the increasing effect of nitric oxide (NO) level in the human umbilical vein endothelial cells (HUVECs) *in vitro*. In addition, molecular docking studies and *in vitro* digestive stability effects also were confirmed that the isolated proteins and peptides from cultured microalgae are having nutraceutical and pharmacological potency. Therefore, cultured marine microalgae proteolytic enzyme extractions can be developed for future therapeutic applications.

In vitro anticancer and anti-inflammatory potentials of cultured marine microalgae including *Chlorella ovalis* and *Nannchloropsis oculata*, *Phaeoductylum tricornutum* and *Amphidinium carterae* were evaluated. Five different compounds were isolated from cultured marine diatom, *P. tricornutum* using the subsequence chromatographic and spectrophotometric analysis. The isolated novel fatty alcohol ester; nonyl 8-acetoxy-6-methyloctanoate (NAMO) was identified among the five isolated compounds as a strong anticancer agent. 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. The protein expression was revealed on the apoptotic

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pathway by activation of Bax and suppression of Bcl-xL as well as by up-regulation of other inducers of apoptosis, caspase-3 and p53, significantly.

Anti-inflammatory effect of the isolated four out of five compounds such as 24-methylcholesta-5(6), 22-diene-3β-ol (MCDO), Cholestra-5(6), 22-dien-3, 24β-diol (CDDO), Cholestra-5(6), 22-dien-3, 24β-diol, methyl ether (CDDME) and Icosa-5, 8, 11, 14-tetraenyl acetate (ITEA) from *P. tricornutum* against LPS-induced RAW macrophages were evaluated. The profound nitric oxide (NO) inhibitory activities were determined by MCDO. CDDO, CDDME and ITEA against LPS-induced RAW macrophages and no cytotoxicity was observed in both LDH and MTT assays. The release of pro-inflammatory cytokines such as interleukin-1β (1L-1β), interleukin-6 (1L-6) and prostaglandin-E_2 (PGE_2) showed to be suppressed by MCDO and CDDO than the other isolated compounds, in a dose-dependent manner. However, tumor necrosis factor-alpha (TNF-α) production was not suppressed significantly by both compounds. In addition, suppression of the nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expressions were determined against LPS-stimulated RAW 264.7 cells significantly. Collectively, the isolated compounds from *P. tricornutum* exhibited the strong anti-inflammatory and anticancer activity and can be employed to develop as new lead compounds for drug discovery.

For the investigation of bioactive components from Sri Lankan seaweeds, three species of red algae (*Chondrophycus ceylanicus*, *Gelidiella acerosa*, *Gelidiopsis cortcata*), two species of green algae (*Chaetomorpha crassa, Caulerpa racemosa*) and four species of brown algae (*Sargassum cassifolium*, *Sargassum sp.* A, *Sargassum sp.* B and *Padina commersonii*) were

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evaluated. Among the selected brown algae, *Sargassum sp*. A, *Sargassum sp.* B and *Padina commersonii* were partitioned by different polar solvents and rest seaweeds were extracted only by methanol. For the bioactivity evaluations, total phenol content (TPC) and free radical scavenging activity using electron spin resonance (ESR) spectroscopy were carried out for all the fractions. In addition, sample cytotoxicity (vero cells), anti-infalmmatory (RAW macrophages) and anticancer effect against different cancer cell lines (human promyelocytic leukemia; HL-60, a human lung carcinoma; A549 and a mouse melanoma; B16F10) were assessed *in vitro*. In fact, most of the fractions showed the significant high activity for at least one or more bioassays, including antioxidant, anti-inflammatory and anticancer were determined. Taken together, some of the Sri Lankan seaweeds were showed the potency for the isolation of bioactive secondary metabolites using further studies.

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LIST OF FIGURES

제주대학교 중앙도서관
JEJU NATIONAL UNIVERSITY LIBRARY

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chloroform fraction; SBE: ethyl acetate fraction and SBW: aqueous fraction with cells in response to LPS (1 μg ml^{-1}) for 24 h, the nitric oxide (NO) levels in the medium was measured. Values are expressed as means ± SD in triplicate experiments.

Fig. 63 Inhibitory effect of *Padina commersonii* solvent extracts by solvent-solvent partition chromatography on (A): LPS-induced NO production (%) and (B) cell viability (%) in RAW 264.7 macrophages. The Incubation of extracts, PCH: hexane fraction; PCC: chloroform fraction; PCE: ethyl acetate fraction and PCW: aqueous fraction with cells in response to LPS (1 μg ml^{-1}) for 24 h, the nitric oxide (NO) levels in the medium was measured. Values are expressed as means ± SD in triplicate experiments.

Fig. 64 The effect of cell viability (%) of HL-60 (A), B16F10 (B) and A549 (C) cancer cells against the methanol extracts of collected seaweeds from Sri Lanka for 24 h. ☐ denoted as the control (no sample treated cancer cells). Values are expressed as means ± SD in triplicate experiments.

Fig. 65 Induction of apoptotic body formation in HL-60 cells was observed under a fluorescent microscope after Hoechest 33342 staining. (A) control: no treated; (B) *Caulerpa racemosa* methanol extract (CRM) treated with 12.5 μg mL^{-1}; (C) CRM treated with 25 μg mL^{-1}; (D) CRM treated with 50μg mL^{-1}; (E) CRM treated with 100 μg mL^{-1}. *Arrows* denoted a typical apoptotic body formation in HL-60 cells.

Fig. 66 Apoptotic Sub-G1content was detected by flow cytometry after stained with PI. (A) control: no treated; (B) *Caulerpa racemosa* methanol extract (CRM) treated with 12.5 μg mL^{-1}; (C) CRM treated with 25 μg mL^{-1}; (D) CRM treated with 50μg mL^{-1}; (E) CRM 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 CRM different concentrations.

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LIST OF TABLES

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BACKGROUND

Nature is an attractive prolific source of a tremendous chemical diversity established with the living organisms on the earth. More than the 70% of the earth surface is covered by oceans and conceived that lives have originated from the sea. Despite the successes of plant and animal diversity in the terrestrial life, marine environment have increased the demand for exploration natural products. Interestingly, marine bio-resources speculate a great deal of chemical diversity and bearing rich sources of secondary metabolites than the terrestrial organisms (Mayer et al., 2010). Among the marine bio-resources, marine algae including macro and microalgae are promising source of organisms that can be rendered the remarkable biological activities which associated with the chemical entities and are useful for finding new drugs with greater efficacy for the treatment of human diseases (Proksch et al., 2002). This phenomenon reflects with evolution in the natural selection and progression through their life cycle. In addition, natural products or secondary metabolites are quite species specific and have demanded for the use of defense against predation, infection, parasitism or interspecies communication and maintenance of homeosatasis in the marine organisms (Jimeno et al., 2004). In fact, it is believed that showing an exceptional reservoir of bioactive secondary metabolites for new therapeutic candidates with the revealing of novel marine metabolites in each year that reported in the literature (Haefner, 2003).

The immersing diversity of marine algae has revealed that its untapped resources are having broad probe to meet the curiosity for the future scientific community. The impressive rates of exploration of the bio-chemicals have been carried out by many research pioneers from different countries in last few decades. Especially, United States, Europe, Korea, Japan and China

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scientists have contributed to a great deal of effort to bring these amazing marine bio-functional ingredients to pharmacological applications. Most of the marine algae secondary metabolites play a physiological role in marine ecological systems and known to involve in specific binding mechanism with desired target receptors. These physiological functions may have conceived to comply with human's biochemical and physiological phenomena. Furthermore, these bio-molecules have not found in the terrestrial analogs and furnished the focus of formulating new classes of drugs (Sode et al., 1996; Mayer et al., 2010). Thus, isolated many of these compounds can be rendered a substantial role for the designing and developments of pharmaceutical products for the future prospects.

Over the few years, protein rich sources have been targeted to figure out their compositions and bioactive constituents due to the health prompting effects (Korhonen & Pilanto, 2006). Recently, marine algae have been used to screen their physiologically active metabolites by focusing on proteins. In this regard, proteolytic extractions have considered significantly (Heo et al., 2005). To explore the potentiality of bioactive resources and the nature of the chemical constituents, method of separation, isolation and characterization techniques have been performed. The isolation of biological active components from the enzymatic extracts was given a high yield and much purity compared to the organic solvent extractions.

Proteases are used to digest the proteins into smaller polypeptides. Most of the research studies have shown that bioactive peptides encrypted in algae proteins (Harnedy & FitzGerald, 2011). Therefore, the physiologically active peptides derived from marine algae are inactive in the parent proteins and only become active when released by proteolytic hydrolisations (Samarakoon

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& Jeon, 2012). There are three kinds of proteases use in enzymatic hydrolysations such as (i) proteolytic digestive enzymes including Pepsin, α-Chymotrypsin and Trypsin by animals, (ii) proteolytic enzymes derived from plants or microorganisms, and (iii) hydrolysis by bacterial or microbial proteases and peptidases during the fermentations (Korhonen & Pihlanto, 2006). Furthermore, the adjustment of physico-chemical environment can be managed to release numerous bioactive peptides *in vitro* from the desired protein sources. However, the encrypted bioactive peptides range in the size from 2 ~ 20 amino acid constituents and can be harvested either by each of proteolytic hydrolysation or serially combination of them together (Fig. 1).

Nowadays, there is a huge demand on the isolation of bioactive peptides from marine organisms. In fact, endogenous marine peptides have opened a new scenario for the development and commercialization of therapeutic agents (Dominic & Danquah, 2011). According to the evidences, the sources of proteins from marine algae would be the treasure to figure out for new therapeutic agents. The findings of consumer interests and the role of dietary proteins will be controlled the influences of health, might be helped to commercialize the bioactive peptides from marine algae. Therefore, the use of algal bioactive peptides in drug development may reduce the cost and time; in addition, peptides have showed a number of advantages such as, toxicologically safe, high bioactivity and bio-specificity to targets during the research and development of clinical trials (Fig. 2). Furthermore, researches have shown that therapeutic activity of peptides with low level toxicity and least or no accumulation in the body tissues are compromising due to the structural diversity.

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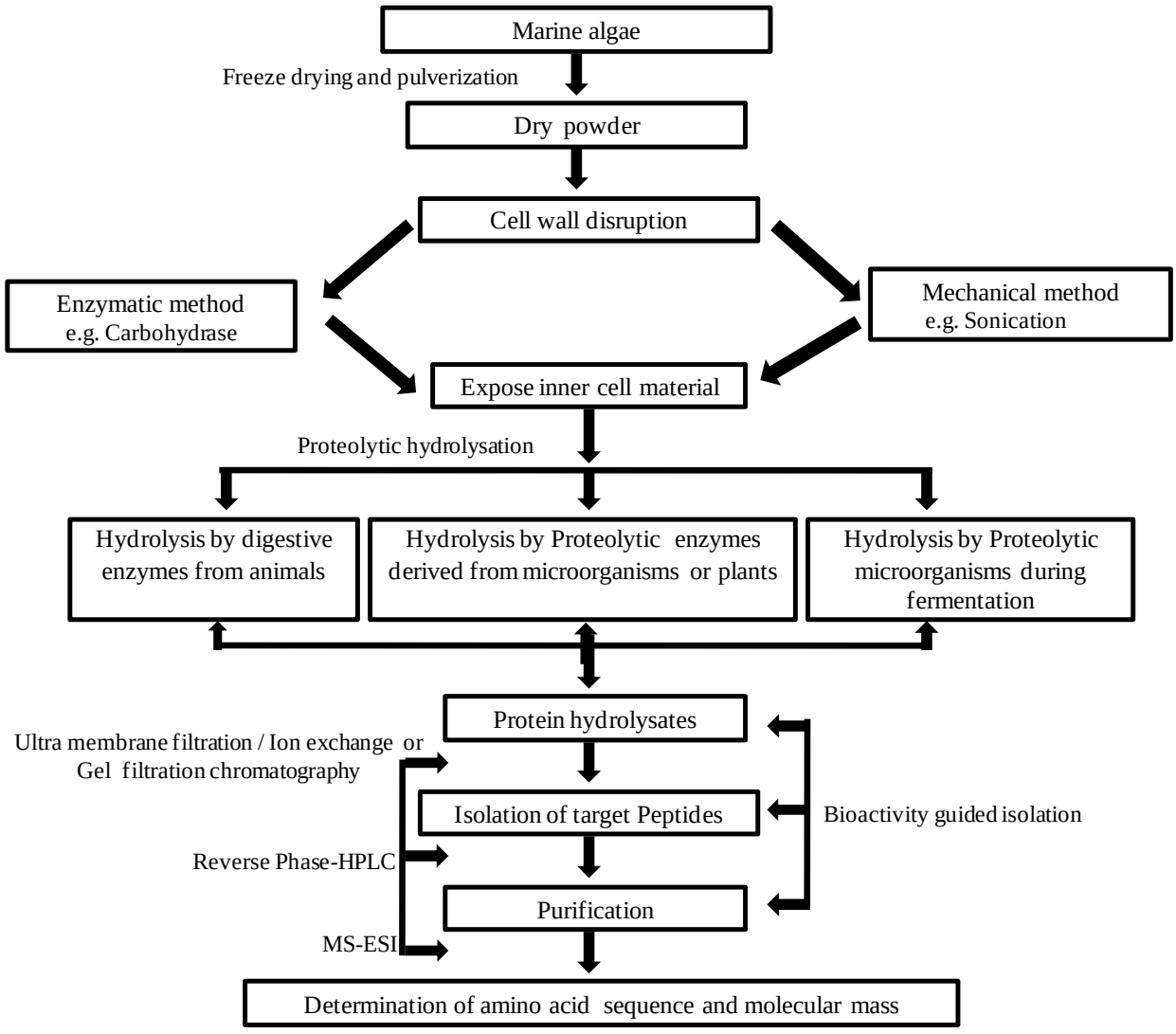

Fig. 1 Schematic diagram for the recovery of bioactive peptides from proteolytic enzyme-assisted extractions of marine algae

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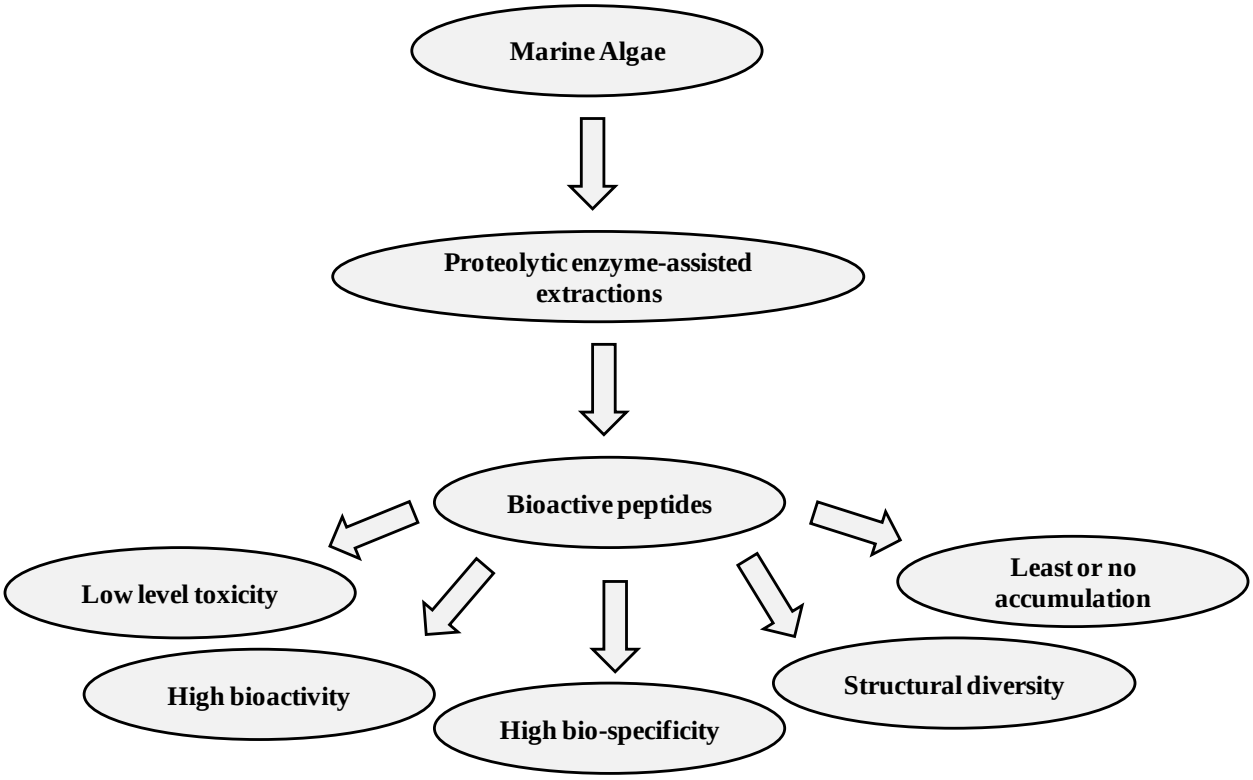

Fig. 2 Physiologically importance of the bioactive peptides derived from marine algae

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Part-I

Enzyme-assisted extractions of cultured marine microalgae and screening bioactivities

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ABSTRACT

In this study, screening of bioactivities including angiotensin I converting enzyme (ACE) inhibitory, antioxidant and anti-inflammatory activity using enzyme-assisted extraction on cultured marine microalgae was evaluated. *Nannochloris oculata*, *Chlorella ovalis* (blue-green algae), *Phyodactylum tricornutum* (diatom) and *Amphidinium carterae* (dinoflagellate) were subjected to culture in laboratory scale and proximate chemical composition was determined. Among the cultured microalgae, two species of blue-green algae *N. oculata* and *C.ovalis* showed high protein content and used for proteolytic hydrolysation against five different proteases. The determined ACE inhibitory activity showed the highest activity against pepsin hydrolysate compared to the other hysrolysates as 0.520 and 2.808 mg/mL on *C. ovalis* and *N. oculata*, respectively. The determined *in vitro* antioxidant and anti-inflammatory effects on the enzymatic hydrolysates indicated that pepsin hydrolysates of *C. ovalis* and *N. oculata* were showed the highest activity compared to other hydrolysates. Taken together, among the screened bioactivities confirmed that pepsin hydrolysates of enzyme-assisted extractions from cultured marine microalgae *C. ovalis* and *N. oculata* can be used to purify the ACE inhibitory proteins and peptides.

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1. INTRODUCTION

Microalgae, as an evolutionary form of organism, are showing an extraordinary adaptation in the ocean. The oldest fossil evidence has shown that appearance of oxygen-producing cyanobacteria and the rise in atmospheric oxygen about 2.45-2.32 billion years ago (Rasmssen et al. 2008). Blue-green algae (cyanobateria), diatoms (bacillariophyta) and dinoflagellates (dinophyceae) have evolved from their origins in a primitive age to contemporary time without much change in structure. This fascinating group of organisms is microscopic, and has been occupied in food chains and long being served as a primary producer in aquatic sources either in fresh or marine, that contributing to almost 70% of global oxygen demand. In fact, abiotic factors such as, light intensity, temperature, nutrients and salinity levels are also influence for their biological functions. Furthermore, extreme fluctuations in climate changes according to the seasons have given a major outbreak through the variations and required for survival in the competitive environmental conditions (Plaza et al., 2008).

The exploration of marine bio-resources is an indefinite challenge for bio-mining researchers. Microalgae have been recognized as rich sources of proteins, lipids, vitamins, pigments and other nutrients (Spolaore et al., 2006). Moreover, studies of the nutritional compositions of many marine microalgae have been showed a high content of proteins than that of the other essential nutrients such as lipids and carbohydrates on the dry weight basis (Becker, 2007). Therefore, isolation of novel bioactive peptides has been demand from the marine microalgae. However, a few studies have been reported in order to investigate the antihypertensive peptides from marine microalgae (Suetsuna and Chen, 2001; Sheih et al., 2009; Ko et al., 2012b).

Reactive oxygen species (ROS) involve in many diseases and these reactive molecules are formed during the physiological process (Heo and Jeon, 2008). Basically, ROS are unstable free

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radicals such as hydroxyl ($^{\bullet}OH$), nitric oxide ($NO^{\bullet}$), peroxyl ($ROO^{\bullet}$) and superoxide ($O_2^{\bullet-}$) and commonly generated with the excessive metabolic oxygen. Under the normal physiological conditions, ROS are effectively eliminated by natural endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Kim et al., 2006) (Fig. 3 A). Non-enzymatic antioxidants such as vitamin C, α-tocopherol and selenium are involved to protect internal organs and tissues from ROS. However, under the certain conditions, imbalances between the antioxidant system and ROS led to damage the biological tissues and macro bio-molecules such as proteins, lipids and nucleic acids (Qian et al., 2008). Moreover, this phenomenon is known as oxidative stress and can be caused to associate with many degenerative diseases such as aging, cancers, coronary heart diseases and atherosclerosis (Finkel and Holbrook, 2000) (Fig. 3 B).

Cardiovascular diseases (CVDs) are the leading causes of death in the world. According to the WHO reports in 2011, CVDs are getting increased frequently and challenge to the health of world-population. As estimated, 17.3 million people were died in 2008 due to this burden disorders. In fact, it is predicted that over 23.6 million people will be died from the cardiovascular health issues in 2030. Hypertension is the main risk factor that associated with CVDs for the high mortality. The renin-angiotensin system (RAS) has two key roles in clinical hypertension (Weber, 1999). Importantly, the inhibition of angiotensin-I generation from angiotensinogen may directly inhibited by rennin, and blocking the conversion of angiotensin-II from angiotensin-I by angiotensin-I-converting enzyme (ACE) (Verdecchia et al., 2008) (Fig. 4). Therefore, ACE inhibitors are a commonly available and widely prescribed medicine for the hypertension (Riordan, 2003). In the RAS, ACE inhibitors work by decreasing the production of angiotensin II, while up-regulating the bioavailability of bradykinin (Fitzgerald et al., 2011).

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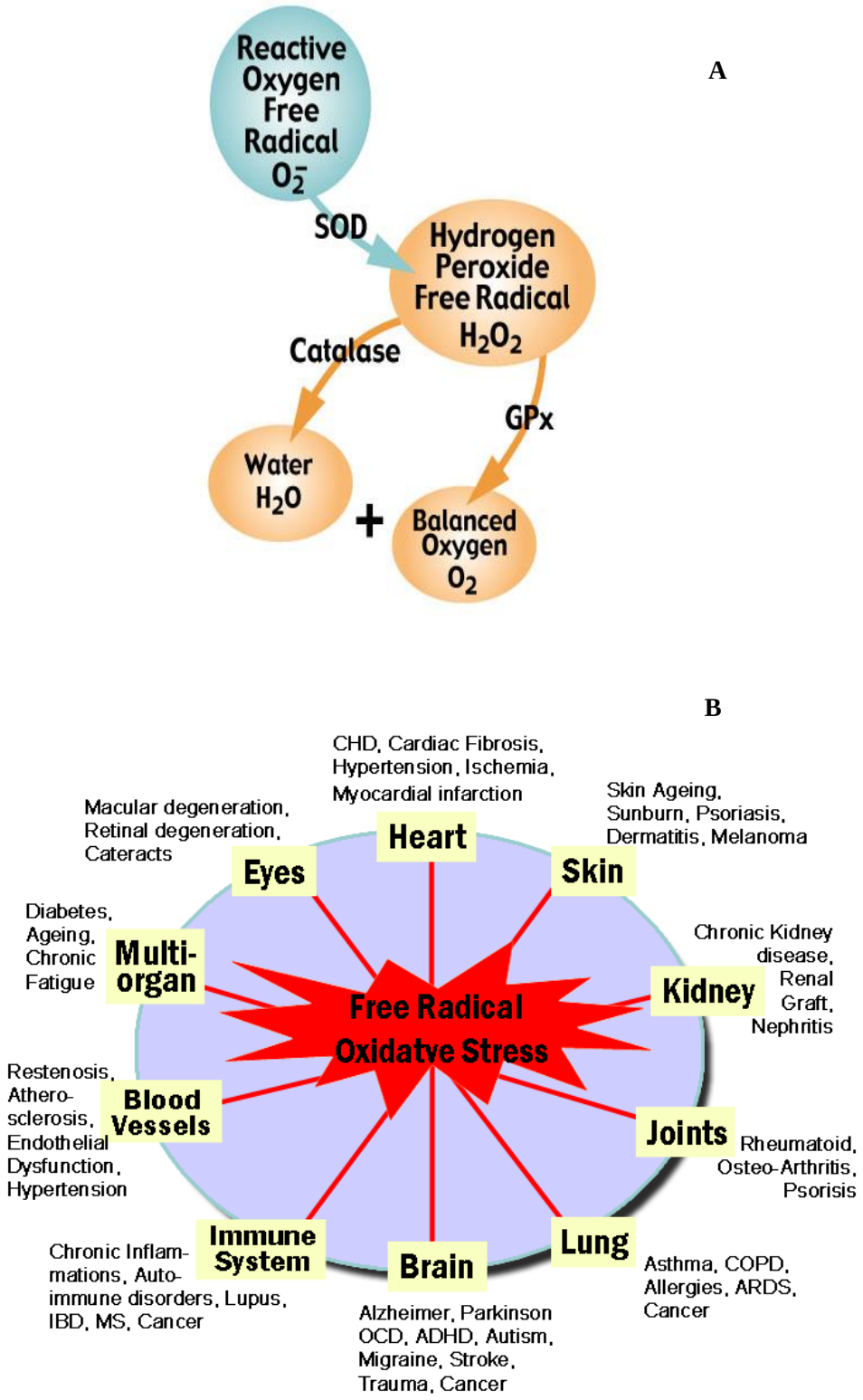

Fig. 3 The effect of natural antioxidants (A) and the causing degenerative effects from oxidative stress

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Bradykinin serves as a potent mediator of endothelium-dependent vasodilator, which releases nitric oxide (NO) by lowering the blood pressure homeostasis. Thus, the inhibition of ACE is considered as the first line of therapy for the treatment against hypertension (Coppey et al., 2006). However, synthesized ACE inhibitors lead to cause undesirable side effects such as lost of taste, chronic dry cough, renal impairment and angioneurotic oedema. Therefore, marine algae derived ACE inhibitory peptides are particularly safe and reduce the side effects by having bio-specificity to the enzymatic reaction.

Inflammation is one of the physiological processes and initiated due to the pathogenic invasion or cell and tissue injury (Wadleigh et al., 2000). This can be influenced by the activation of various immune cells such as macrophages, neutrophils and lymphocytes. Moreover, overproduction of pro-inflammatory cytokines including interleukin (IL) and tumor necrosis factor (TNF-α) can be generated in macrophages as per the inflammatory disease. Wih the effect of LPS, machrophages show a drastic down-regulation of inflammatory cytokines such as TNF-α , IL-6 and IL-1β. In addition, inflammatory mediators such as nitric oxide (NO) and prostaglandin (PGE_2) also activated due to inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Lee et al., 2006). Therefore, finding the better insight of anti-inflammatory agents are gaining an interest view point to regulate the immune related disease, inflammatory disorders and infections (Jung et al., 2009)(Fig 5).

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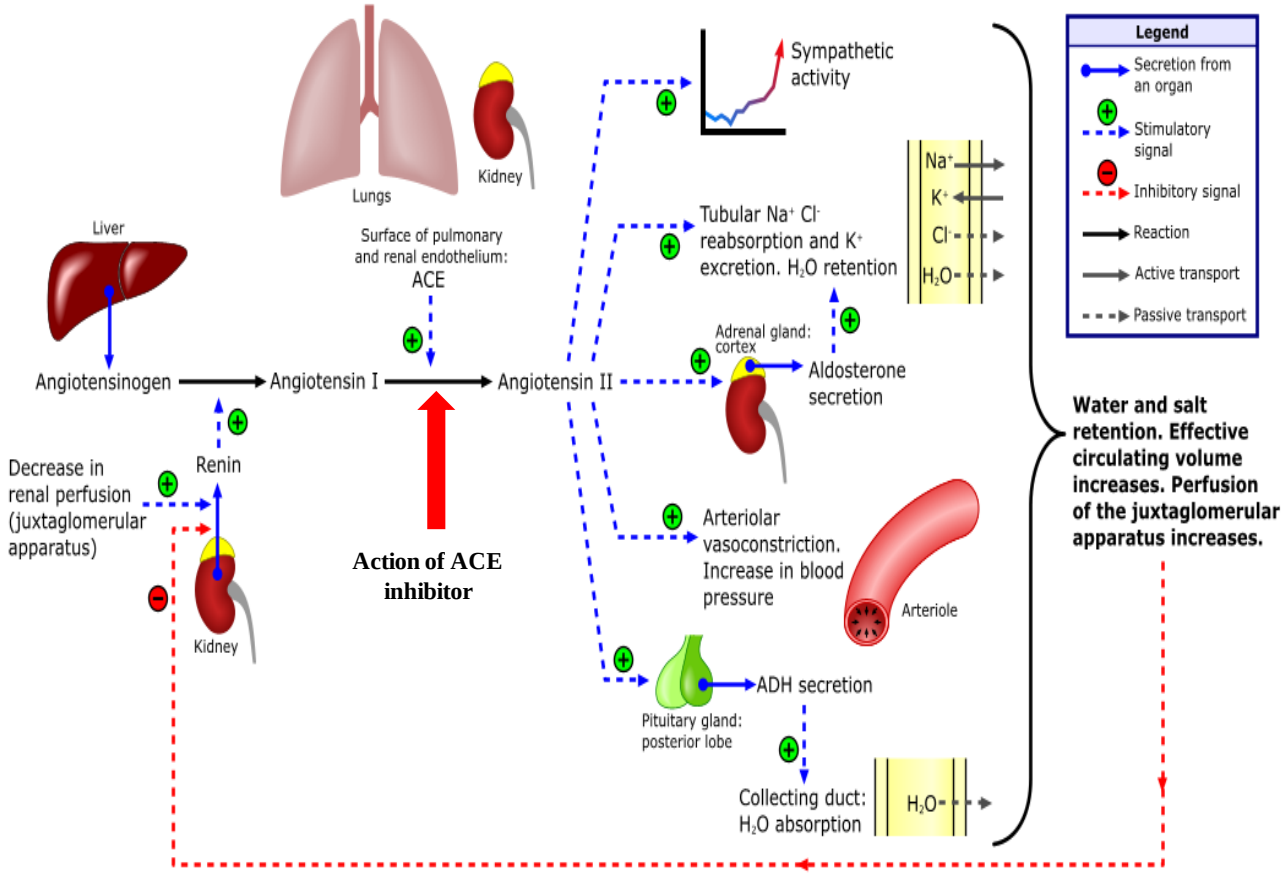

Fig. 4 The renin-angiotensin system (RAS) and the function of ACE inhibitors.

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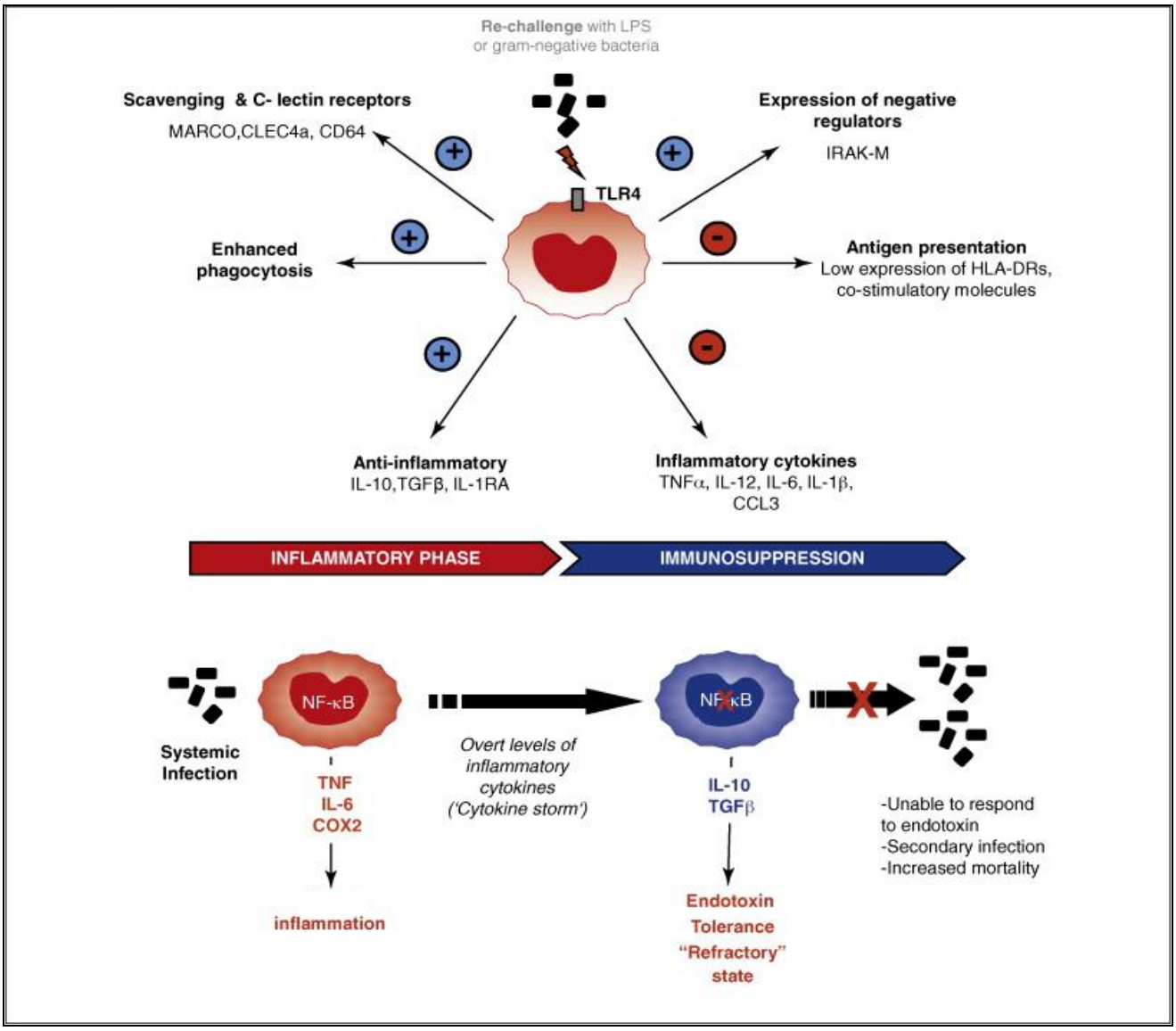

Fig. 5 Schematic diagram of the LPS stimulated inflammation and the regulatory effects of inflammation and immunosupression

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The high content of proteins in the microalgae has been interested target to explore bioactive peptides in the functional food industry. Among the unicellular microalgae, blue green algae, diatoms and dinoflagellates are the most popular organisms that can be cultured and able to biomass production. In this study, *Nannochloris oculata* Droop, *Chlorella ovalis* Butcher (blue-green algae), *Phyodactylum tricornutum* Bohlin (diatom) and *Amphidinium carterae* Hulburt (dinoflagellate) were used to culture in the specific culture medium and subjected to isolate the bioactive proteins and peptides only from blue-green algae. Hence, the principle objective of this part is to screen the bioactivities such as ACE inhibitory, antioxidant and anti-inflammatory activities from the enzyme-assisted extractions of cultured microalgae including *N. oculata* and *C. ovalis* by chemical and *in vitro* assays.

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2. MATERIALS AND METHODS

2.1 Material and reagents

Digestive proteases including Pepsin, Trypsin, α-Chymotrypsin and Papain, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), angiotensin-I-converting enzyme (from rabbit lungs), N-Hippuryl-His-Leu tetrahydrate (HHL) and standard ACE inhibitor, captoprill were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Other proteases including Alcalase 2.4 LFG, Nutrase 0.8 L, Protamex, Kojizyme 500 MG, Flvozymes 500 MG, and carbohydrases including Viscozyme L, Cellulast 1.5 L FG, AMG 300 L, Termamyl 120 L, Ultraflo L were purchased from Novozymes (Bagsvaerd, Denmark). 5,5-Dimethyl-1-pyrrolin N-oxide (DMPO), 2,2-azobis (2-amidinopropane) hydrochloride (AAPH), α-(4-pyridyl-1-oxide)-N-t-butylnitrone (POBN), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, USA). Dulbecco's Modified Eagles Medium (DMEM), Fetal Bovine Serum (FBS), penicillin, streptomycin and other materials required to cell culture were purchased from the manufacturer (Gibco/BRL, Burlington, Ont, Canada). All the other chemicals and reagents were used in this study were highest grade that available commercially.

2.2 Culture conditions for blue-green algae and diaotms

The marine microalgae (*N. oculata*, *C. ovalis* and *P. tricornutum*) were kindly provided by the Korea Marine Microalgae Culture Center (KMMCC-16). 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 2,500 l x (Light: Darkness=24: 0). Microalgae were cultured in Conway medium

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(Walne, 1966) prepared from filter-sterilized seawater and the culture was 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 2,000 rpm using a basket centrifuge (Hanseong Co., Ansan, Korea). The harvested algae biomass was frozen at −25 °C and lyophilized. Then the lyophilized microalgae sample was homogenized (20g) and preserved at -20℃ until use.

2.3 Culture conditions for dinoflagellates

Dinoflagellate strain, *Amphidinium carterae* was collected from sand and the macroalgal samples from the coasts of Jeju Island, Korea during 2011. The species were identified and cell size was measured using the compound microscope (Zeiss Axioplan II, Germany). Single cells were isolated by capillary pipette washing method under inverted microscope (Olympus 1X71, Olympus, Tokyo, Japan) and transferred to multiwell plate containing 2 mL in IMK culture medium (Nihon Pharmaceutical Co., Ltd., Japan) in each well. IMK medium contained the following components: 2.35 mM $NaNO_3$, 50.1 µM NH_4Cl, 9.86 µM Na_2HPO_4, 28.7 µM K_2HPO_4, 12.3 µM Fe–ethylenediaminetetraacetic acid (Fe-EDTA), 0.871 µM Mn-EDTA, 111 µM Na_2EDTA, 0.01 µM $CuSO_4 \cdot 5H_2O$, 0.03 µM $Na_2MoO_4 \cdot 2H_2O$, 0.08 µM $ZnSO_4 \cdot 7H2O$, 49.8 nM $CoSO_4 \cdot 7H_2O$, 0.91 µM $MnCl_2 \cdot 4H_2O$, 0.0132 µM H_2SeO_3, 0.001 µM vitamin B_{12}, 0.006 µM biotin, and 0.593 µM thiamine–HCl. After two weeks, the isolated dinoflagellates were confirmed for growing inside the multi-well plate and then sub-cultured to screw capped test tubes (15 mL) with a round bottom containing 10 mL ¼ IMK medium as stock culture. Natural seawater (35 psu salinity) collected from Jeju coast was filtered through GF/F (47 mm, Whatman) filter, diluted with distilled water to adjust salinity of 30 psu and used for preparation

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of culture media after autoclaving at 121°C for 40 min and filtering (0.21 μm, Millipore). The stock cultures were incubated in duplicate at 20°C under approximately 180 μmol photons m^{-2} s^{-1} of cool-white fluorescent illumination on a 12:12 h L:D cycle. Stock cultures of the dinoflagellate, *A. carterae* was cultured gradually and scaled up to 30 mL, 300 mL, 1 L, and 3 L flasks under the same laboratory conditions. For the 20 L carboys, the culture conditions were changed a little as culture water treated with sodium hypo chloride solution containing 9% active chlorine (at the rate of 1.1 ml L^{-1} seawater) for 30 minutes for chemical sterilization and after that sodium thiosulphate (at the rate of 0.12g L^{-1} Seawater) was added to neutralize chlorine in the water and also medium level of aeration was provided to this cultures. At all scale up stages, the inoculums of exponentially growing phase were used to start next culture. Culture grown in glass flasks (1 and 3 L) were initiated by inoculation of 10 to 20 % by volume of culture stock of 300 mL (maintained by monthly transfer) and culture grown in carboys (20 L) were initiated by inoculation of 10 to 20 % by volume of 3 L cultures.

2.4 Proximate chemical composition

Proximate chemical compositions of the culture marine microalgae were determined according to AOAC method (AOAC 1990). Total nitrogen content was analyzed by the Kjeldahl method (Kejltec 8400, FOSS, USA). Crude protein was determined by calculating a conversion factor of 6.25. Crude lipid was performed by soxhelt method extraction with diethyl ether solvent (Soxtec 2050, FOSS, USA) and crude ash was determined by incineration of samples at 550℃ in the muffle furnace (B180, Nabertherm GmbH, Germany). Moisture of *N. oculata* was determined by oven-drying method at 105 ℃ in the moisture analyzer (mb45, OHAUS, Switzerland). Crude fiber content was measured by fibertec system (Fibertec 2010 Analyzer, FOSS, USA).

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2.5 Preparation of enzymatic hydrolysates

Lyophilized blue-green microalgae samples were ground into a fine powder and 15g of each was hydrolyzed by the commercial enzymes. Enzyme-assisted extractions of cultured microalgae were performed according to the method described by Jung et al., (2006) and Heo et al., (2005) Table 1. In brief, cultured marine microalgae samples were subjected to digest with the commercial digestive enzymes as enzyme/substrate ratio of 1/100 (w/w) with the respective optimum conditions for 24 h. (Fig. 6). All the lyophilized enzymatic hydrolystaes were stored under -80℃ until use.

2.6 Measurement of ACE inhibitory activity

The ACE inhibitory activity assay was performed by measuring the concentration of hippuric acid liberated from HHL by the method described as Cushman and Cheung, (1970) with slight modifications. The reaction mixture containing a 50 μL of the sample solution with 50 μL of ACE solution (25 mU mL^{-1}) was pre-incubated at 37℃ for 10 min. Then incubated with 100 μL of substrate (25 mM HHL in 50 mM Sodium borate buffer containing 500 mM NaCl at pH 8.3) at 37℃ for 60 min. Further reaction was stopped by the addition of 250 μL of 1N HCl. Hippuric acid was extracted with 500 μL of ethyl acetate. After the centrifugation (4000 rpm, for 10 min), 200 μL aliquot of the extract was removed and transferred into a glass tube and evaporated in a dry-oven at 80℃ for 1 h. The residue was dissolved in 1 mL of distilled water and absorbance was measured at 228 nm by UV/VIS Spectrophotometer (Model OPTIZEN POP, Mecasys Co. Ltd, Korea). The IC_{50} value is defined as the concentration of the inhibitor required for 50%

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inhibition of the ACE activity. A standard ACE inhibitor (Captoprill) was used as a positive control.

2.7 DPPH radical scavenging assay

2,2-diphenyl-1-picrylhydrazyl (DPPH) is a free radical donor which can be detected via electron spin resonance **(**ESR) spectrometry (JES-FA machine, JOEL, Tokyo, Japan) (Fig. 7) by the technique described by Nanjo et al., (1996). A 40 μL of each sample was added into 40 μL of DPPH (60 μmol L^{-1}) in ethanol. After 10 sec of vigorous mixing, the solutions were transferred into 100 μL Teflon capillary tubes and fitted into the cavity of the ESR spectrometer. The spin adduct was determined on JES-FA ESR spectrometer exactly 2 min later. The measurement conditions were as follows: Power: 1 mW, Modulation frequency: 5 × 100 kHz, Modulation width: 0.8 mT, Sweep width: 10 mT, Sweep time: 30 sec, Temperature 298 K.

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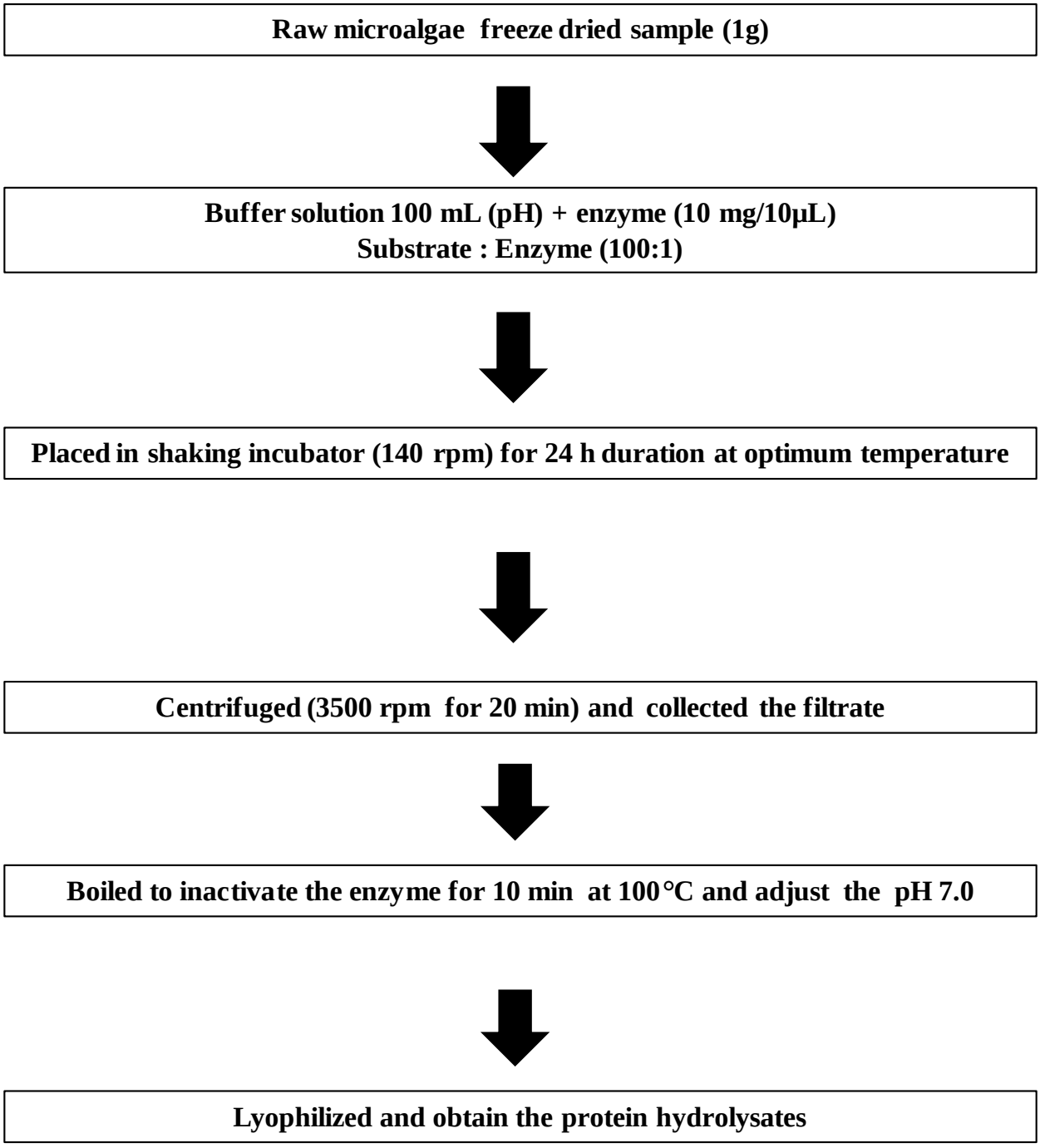

Fig. 6 A flow diagram for enzyme-assisted extraction from marine microalgae

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Table. 1 Used enzymes and their optimum conditions

Enzyme	Buffer	pH	Temperature (°C)
Proteases			
Pepsin	0.1 M Glycine-HCl	2	37
Neutrase	0.1 M Na_2HPO_4- NaH_2PO4	8	50
Alcalase	0.1 M Na_2HPO_4- NaH_2PO4	6	50
Papain	0.1 M Na_2HPO_4- NaH_2PO4	6	37
Trypsin	0.1 M Na_2HPO_4- NaH_2PO4	8	37
α- Chymotrypsin	0.1 M Na_2HPO_4- NaH_2PO4	8	37
Protamex	0.1 M Na_2HPO_4- NaH_2PO4	6	40
Flavourzyme	0.1 M Na_2HPO_4- NaH_2PO4	7	50
Kojizyme	0.1 M Na_2HPO_4- NaH_2PO4	6	40
Carbohydrases			
Viscozyme	0.05 M K_2HPO_4- KH_2PO4	4.5	50
Cellulast	0.05 M K_2HPO_4- KH_2PO4	4.5	50
AMG	0.05 M K_2HPO_4- KH_2PO4	4.5	60
Termamyl	0.1 M Na_2HPO_4- NaH_2PO4	6	60
Ultraflo	0.1 M Na_2HPO_4- NaH_2PO4	7	60

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2.8 Hydroxyl radical scavenging assay

Hydroxyl radicals were generated via the Fenton reaction, and reacted rapidly with nitrone spin trap DMPO; the resultant DMPO-OH adducts was detectable with an ESR spectrometer (Rosen and Rauckman, 1984). The reaction mixtures containing 0.02 mL of 0.3 M DMPO, 0.02 mL of 10 mM $FeSO_4$ and 0.02 mL of 10 mM H_2O_2 were mixed with the tested samples 0.02 mL. The solution was then transferred into 100 μL Teflon capillary tube. The spin adduct was measured on an JES-FA ESR spectrometer (JOEL, Tokyo, Japan) exactly 2.5 min later. The measurement conditions were as follows: Power: 1 mW, Modulation frequency: 1 × 100 kHz, Modulation width: 0.2 mT, Sweep width: 10 mT, Sweep time: 30 sec, Temperature 298 K.

2.9 Alkyl radical scavenging assay

Alkyl radicals were generated via AAPH. The reaction mixtures containing 0.02 mL of deionizsed H_2O, 0.02 mL of 40 mM AAPH and 0.02 mL of 40 mM POBN was mixed with the tested samples 0.02 mL. The solution was then incubated for 30 min at 37°C in a water bath (Hiramoto et al., 1993), and then transferred into 100 μL Teflon capillary tubes. The spin adduct was recorded on JES-FA ESR spectrometer (JOEL, Tokyo, Japan). The measurement conditions were as follows: Power: 7 ~ 8 mW, Modulation frequency: 5 × 100 kHz, Modulation width: 0.2 mT, Sweep width: 10 mT, Sweep time: 30 sec, Temperature 298 K.

2.10 Cell culture

RAW 264.7 (a murine macrophage 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% fetal bovine serum (FBS). The cells were incubated and

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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.

2.11 Determination of nitric oxide (NO) production

RAW 264.7 cells (1×10^5 cell mL^{-1}) were placed in a 24-well plate and after 24 h the cells were pre-incubated with various concentrations of the sample at 37 °C for 1 h. Then further incubation was done for another 24 h with lipo-polysaccharide (LPS) (1 μg mL^{-1}) at the same temperature. After the incubation, quantity of nitrite accumulated in the culture medium was measured as an indicator of NO production (Lee et al., 2007). Briefly, 100 μL of cell culture medium was mixed with 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid), the mixture was incubated at room temperature for 10 min, and the optical density at 540 nm was measured using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Sunrise, Tecan Co. Ltd., Australia). The fresh culture medium was used as a blank in every experiment.

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Fig. 7 Electron Spin Resonance **(**ESR) spectrophotometer (JES-FA machine, JOEL, Tokyo, Japan)

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2.12 Assessment of cell viability (MTT assay)

Cell viability was estimated by a 3-(4, 5-dimehyl-thiazol-2-yl) 2, 5-diphenyltetrazoium bromide (MTT) assay, which is a test of metabolic competence predicted upon the assessment of mitochondrial performance. The conversion of yellow tetrazolium bromide to its purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells can determined as a colorimetric assay (Mosmann, 1983). The cells were seeded in 96-well plate at a concentration of 1×10^5 cells mL^{-1}. After 16h, the cells were treated with the samples at different concentrations and incubated at 37 °C for 24 h. Then the MTT stock solutions (50 µL: 2 mg mL^{-1}) was applied to the wells, to a total reaction volume of 200 µL. After 4 h incubation period the plates were centrifuged for 5 min at 800 × g, and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 µL of dimethylsulfoxide (DMSO), and the absorbance was measured by ELISA at wave length of 540 nm. Relative cell viability was evaluated in accordance with the quantity of MTT converted to the insoluble formazan salt. The optical density of the formazan generated in the control cells were considered to 100% viability. The data are expressed as mean percentages of the viable calls versus the respective control.

2.13 Statistical analysis

For the statistical analysis, all the data were expressed as mean ± standard deviation of three independent determinations. The significance of differences between two samples was analyzed using the student t-test and *P*-value of <0.05 was considered as the level of statistical significance.

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3. RESULTS AND DISCUSSION

3.1 Proximate chemical composition

The proximate chemical composition (%) of the cultured marine microalgae samples were determined by crude dry weight basis according to the AOAC (1990) method. In this study, two cyanobacteria species including *C. ovalis* and *N. oculata*, one diatom species as *P. tricornutum* and one dinoflagellate species as *A. carterae* were cultured and the lyophilized samples were used for the chemical composition analysis. Thus, all samples were showed the high content of ash (%) compared to the other nutrients. *A. carterae* possessed the highest ash content as 41.15 ± 0.2 (%) significantly (Table 2). However, the crude protein content of the cultured marine microalgae was seemed to be high as 1/3 of the total composition after the ash composition. Only the cultured *A. carterae* showed the significantly lower content of protein as 21.5 ± 0.05 (%) compared to the other species. Moreover, *A. carterae* was indicated the higher amount of crude lipids (6.31 ± 0.01 %) than the other cultured microalgae. The higher content of carbohydrates (27.44± 0.06 %) was reported from *C. ovalis* significantly. These chemical compositions render a positive indication of possible extractable components from each of the cultured microalgae samples.

3.2 Angiotensin-I-converting enzyme (ACE) inhibitory activity

Protease enzyme extracts of both blue-green microalgae including *C. ovalis* and *N. oculata* were used to determine the ACE inhibitory activity compared with the commercial ACE inhibitor, captoprill. Among the enzyme extracts, *C. ovalis* showed the highest ACE inhibitory activity compared to the *N. oculata* extracts (Fig. 8). The determined IC_{50} values of ACE inhibitory activity indicated that all the enzymatic extracts of *C. ovalis* and *N. oculata* were in the range of

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0.520 ~ 1.24 mg/mL and 2.808 ~ 3.37 mg/mL, respectively. The highest ACE inhibitory activity was reported by the pepsin enzyme hydrolysate of *C. ovalis* with the IC_{50} 0.52±0.09 mg/mL, whereas the standard ACE inhibitor showed the IC_{50} was 0.665 ± 0.09 μg/mL. Same the enzyme hydrolysate of *N. oculata* was indicted the highest ACE inhibitory activity (IC_{50} was 2.808±0.12 mg/mL) among the *N. oculata* enzymatic extracts. However, trypsin, and α-chymotrypsin hydrolysates were also showed similar IC_{50} values against the ACE inhibitory activity in this study (Table 3).

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Table 2. Proximate chemical composition of microalgae crude dry weight basis

Microalgae	Proximate Chemical Composition (%) dry weight basis*					
	Protein	Lipid	Carbohydrate	Fiber	Ash	Moisture
Chlorella ovalis	32.1 ± 0.13	0.91 ± 0.02	27.4 4± 0.06	2.94 ± 0.05	34.03 ± 0.15	2.58 ± 0.06
Nannchloropsis oculata	30.97 ± 0.14	1.3 ± 0.07	17.78 ± 0.01	4.38 ± 0.08	32.87 ± 0.15	12.7 ± 0.1
Amphidinium carterae	21.5 ± 0.05	6.31 ± 0.01	25.03 ± 0.04	2.45 ± 0.06	41.15 ± 0.2	3.57 ± 0.07
Phaeoductylum tricornutum	34.75 ± 0.25	2.47± 0.05	15.75 ± 0.03	3.65 ± 0.08	35.7 ± 0.03	7.68 ± 0.04

* Analysis was according to the AOAC methods. Values are mean ± SD of three determinations.

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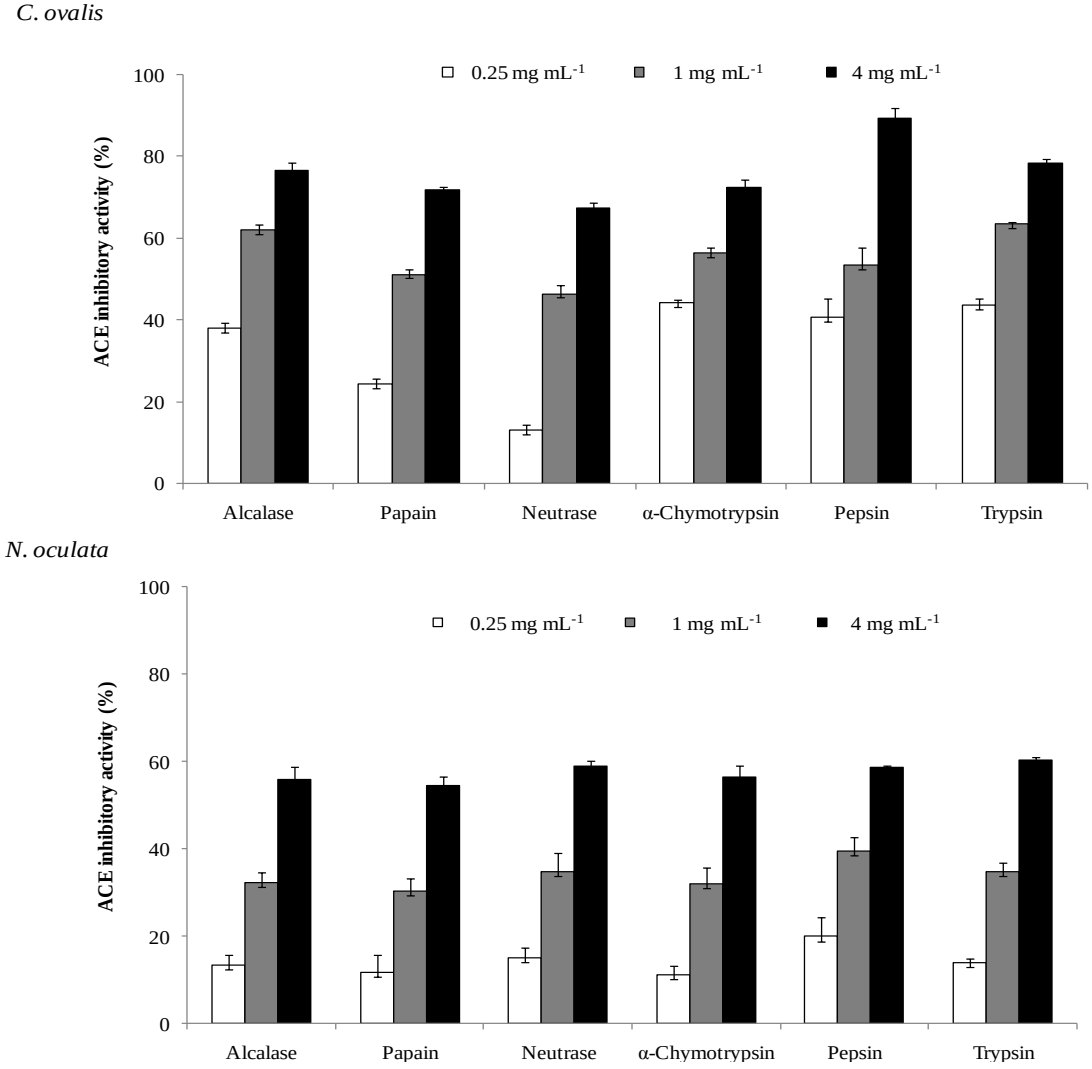

Fig. 8 Inhibitory effect of angiotensin I converting enzyme (ACE) activity from the protyeolytic hydrolyzed cultured marine microalgae *Chlorella ovalis* and *Nannochloris oculata*

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Table 3. ACE inhibitory activity of proteolytic enzyme hydrolysates from microalgae

Protease enzyme	IC_{50} Value (mg/mL)[a]	
	C. ovalis	***N. oculata***
Alcalase	0.942 ± 0.38	3.135 ± 0.31
Papain	0.806 ± 0.23	3.374 ± 0.07
Neutrase	1.24 ± 0.38	2.945 ± 0.72
α-Chymotrypsin	0.862 ± 0.20	2.939 ± 0.23
Pepsin	0.520 ± 0.09	2.808 ± 0.12
Trypsin	0.593 ± 0.14	2.975 ± 0.76
Captoprill [b]	0.665 ± 0.09	

[a] The concentration of an inhibitor required to inhibit 50% of the ACE activity. The values of IC_{50} were determined by at triplicate individual experiments. Values are mean ± SD of three determinations.

[b] Standard ACE inhibitor (concentration used as μg/ mL)

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3.3 Antioxidant activity

In this study, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl radicals were performed to evaluate the scavenging activity using electron spin resonance (ESR) spectrophotometer from various enzymatic extracts of *C. ovalis* and *N. oculata*. Total 10 enzymes including four proteases and six carbohydrases were employed to extract *N. oculata*, and only five proteases were used to hydrolysis *C. ovalis* due to the available amount of microalgae samples. According to the presented results (Fig. 9 A), the highest radical scavenging activity was identified by pepsin hydrolysate of *N. oculata* against DPPH and hydroxyl compared to other hydrolysates of the same sample. In fact, the identified yields of *N. oculata* hydrolysates were indicated that the pepsin hydrolysate was responsible for the highest yield (36.47 ± 1.25 %) compared to other hydrolysates. On the other hand, pepsin hydrolysate of *C. ovalis* was showed the highest radical scavenging activity against both DPPH and hydroxyl significantly compared to other protease extract of *C. ovalis* (Fig. 9 B). The determined yield among the *C. ovalis* protease extracts were indicated that 37.5 ± 2.12% yield was observed from pepsin hydrolystae as the highest value.

In addition, the determined IC_{50} values of radical scavenging activity from microalgae were presented in the Table 4. According to the results, hydroxyl radical scavenging activity of *C. ovalis* pepsin hydrolysate was smallest as 1.89 ± 0.51 mg/mL. Hence, pepsin extracts of *C. ovalis* showed the marked antioxidant activity compared to *N. oculata* pepsin hydrolysate.

3.4 Anti-inflammatory activity

Inflammatory mediators such as nitric oxide (NO) play an important role as the signaling molecule are induced in the macrophages. LPS act as endotoxins for mammals and stimulation of the RAW cells in terms of enhancing the NO concentration in the medium.

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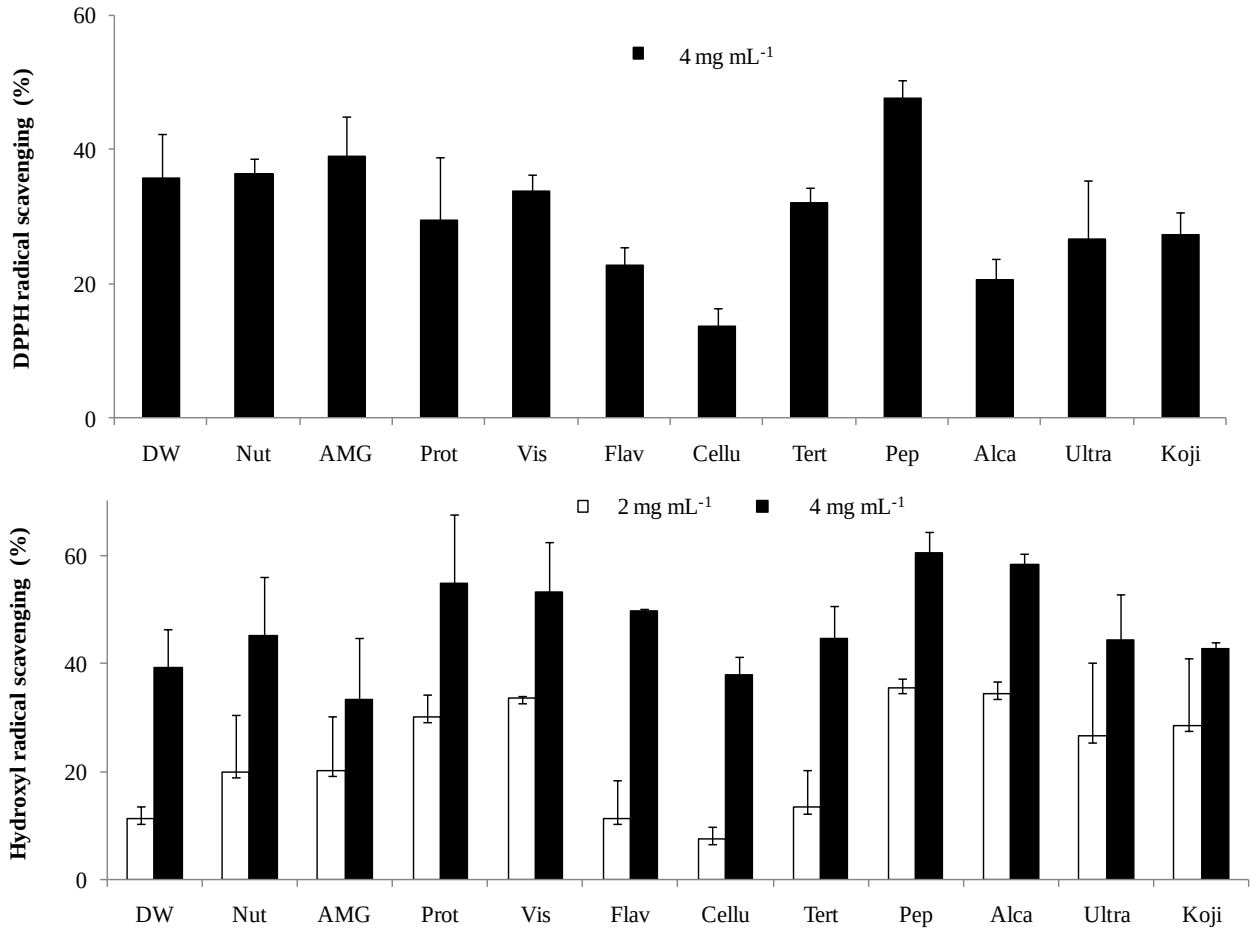

Yield (%)	**31.5±4.95**	**26.0±3.4**	**34.95±4.95**	**16.0±4.95**	**21.5±2.12**	**7.5±2.12**	**13.0±2.83**	**9.5±0.00**	**36.47±1.25**	**13.5±0.71**	**10.0±2.83**	**18.0±0.71**

Fig. 9 (A) The effects of antioxidant activity by DPPH and hydroxyl radical scavenging activity from enzyme-assisted extractions of cultured marine microalga, *Nannochloris oculata* using electron spin resonance (ESR) spectrophotometry.

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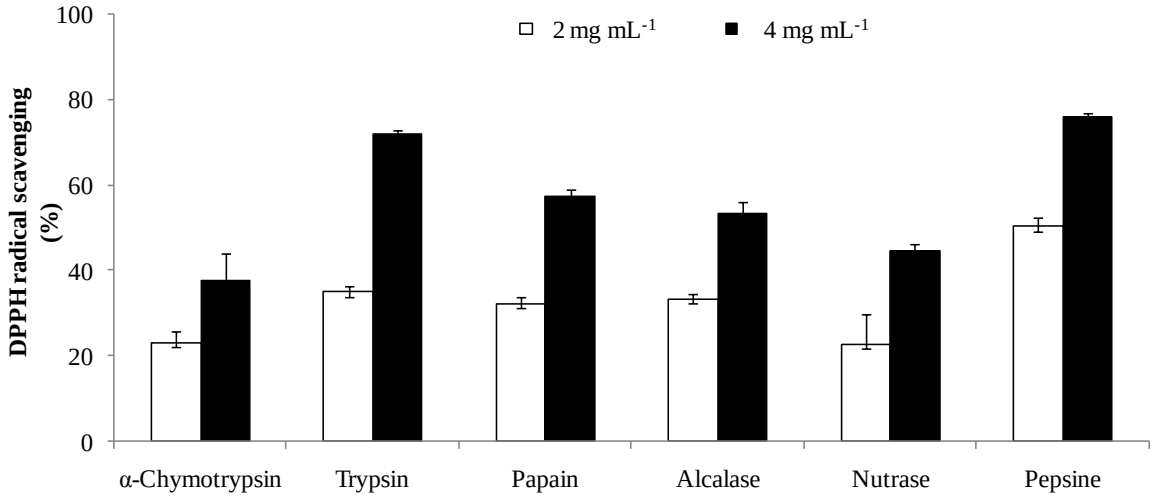

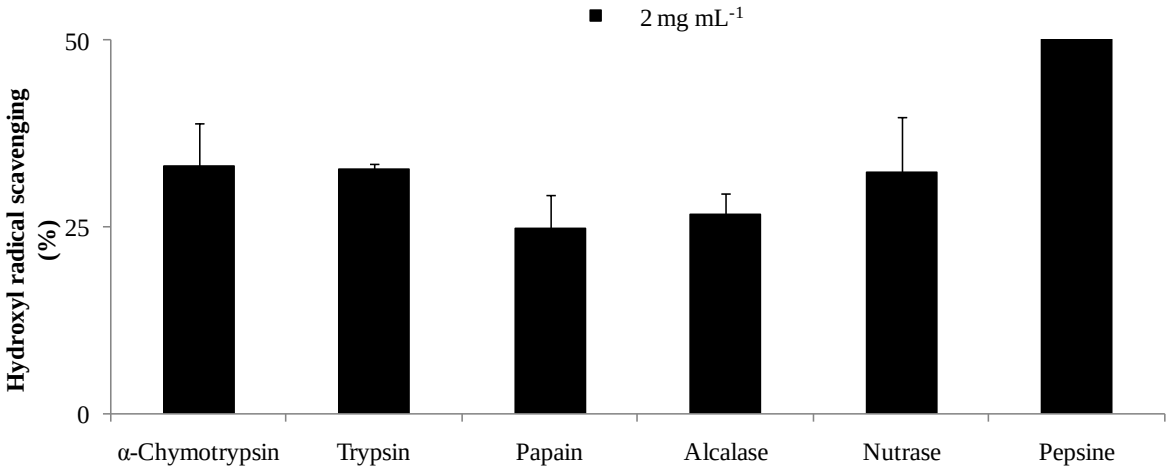

Yield (%)	**34.98±1.75**	**31.24±2.1**	**30.65±3.4**	**28.34.0±2.65**	**31.5±4.16**	**37.5±2.12**

Fig. 9 (B) The effects of antioxidant activity by DPPH and hydroxyl radical scavenging activity from enzyme-assisted extractions of cultured marine microalga, *Chlorella ovalis* using electron spin resonance (ESR) spectrophotometry.

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Table 4. Radical scavenging activity of pepsin enzyme hydrolysates from microalgae

	IC_{50} values (mg/mL) [a]	
Microalgae	**DPPH radicals**	**Hydroxyl radicals**
C. ovalis	1.97 ± 0.24	1.89 ± 0.51
N. oculata	4.305 ± 36	3.16 ± 0.15

[a] The concentration of sample required to scavenge 50 % of the radical scavenging activity. The values of IC_{50} were determined by at triplicate individual experiments. Values are mean ± SD of three determinations.

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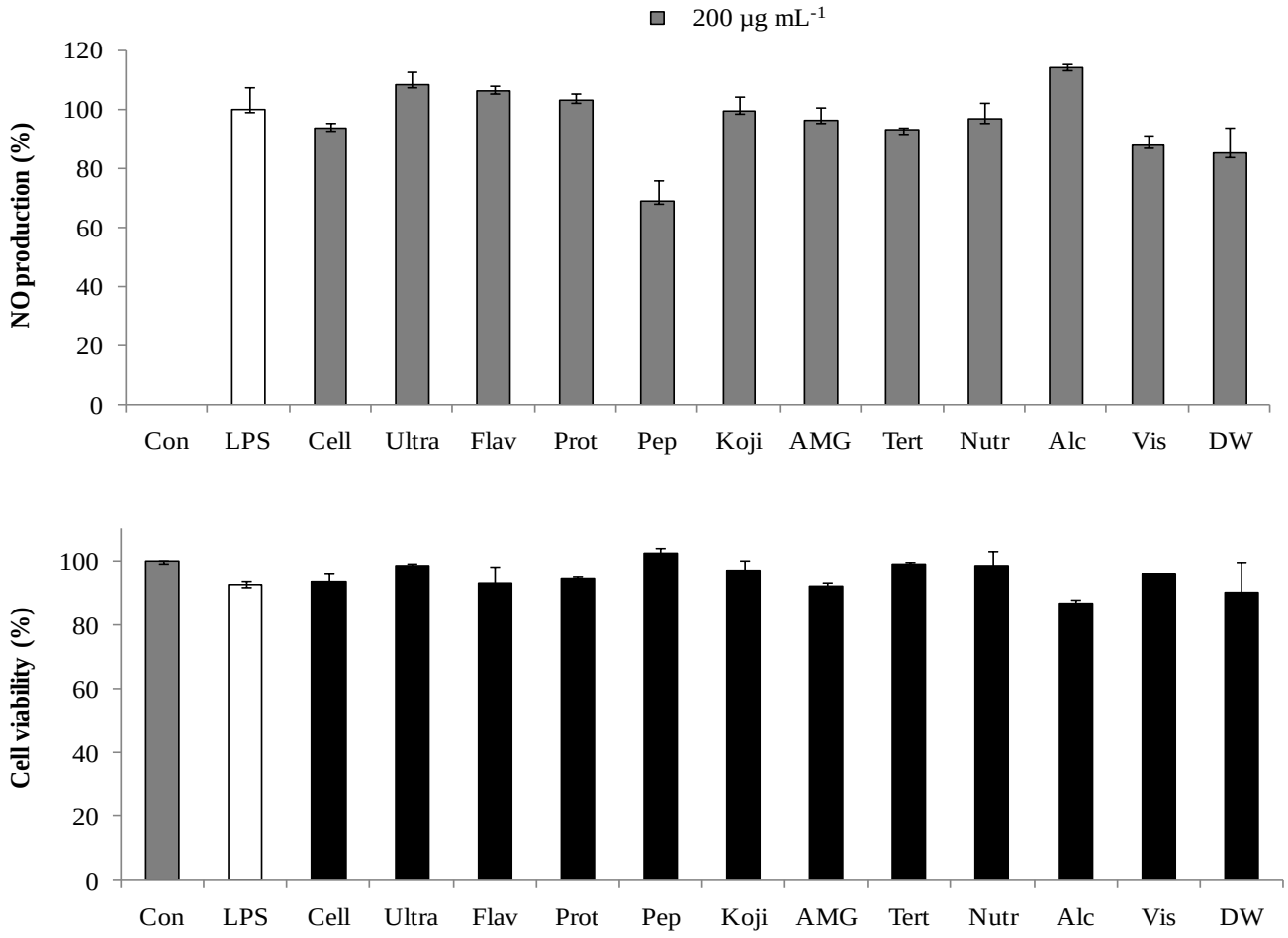

Fig. 10 (A) Inhibitory effect of enzyme-assisted extractions of cultured marine microalgae *Nannochloris oculata* on LPS-induced NO production (%) and cell viability (%) in RAW 264.7 macrophages.

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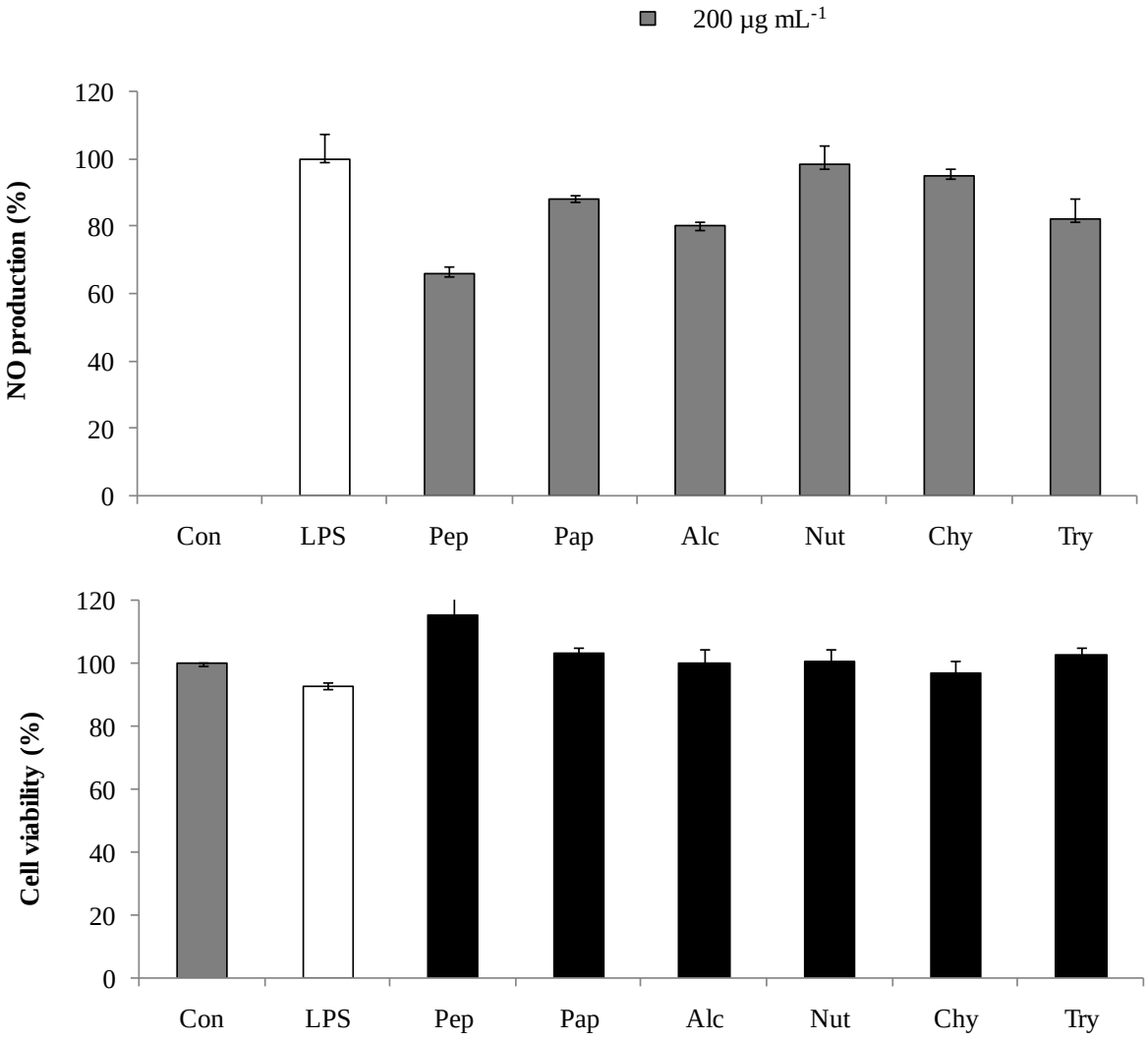

Fig. 10 (B) Inhibitory effect of enzyme-assisted extractions of cultured marine microalgae *Chlorella ovalis* on LPS-induced NO production (%) and cell viability (%) in RAW 264.7 macrophages.

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Hence, the inhibition of NO production (%) level was indicated that the positive evidences of anti-inflammatory activity on LPS-induced RAW macrophages. Fig. 10 (A) is presenting the inhibitory effect of NO production (%) of enzymatic extracts of *N. oculata* against LPS-induced RAW macrophages and cell viability (%) at the incubated concentration (200 μg/mL). Among the treated different enzymatic hydrolysates of *N. oculata*, only the pepsin hydrolysate showed the significant inhibitory activity of NO production (%) on LPS-induced RAW cells. The cell viability was observed more than 100 % and confirmed that no cytotoxicity against RAW macrophages. In fact, protease extracts of *C. ovalis* treated concentration at 200 μg/mL on RAW cells indicated the same results (Fig. 10 B) as pepsin hydrolysate performed the significant NO production inhibitory activity. Moreover, *C. ovalis* pepsin hydrolysate also did not show the cell cytotoxicity.

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4. CONCLUSION

In this part, cultured two species of blue-green algae including *C. ovalis* and *N. oculata* were used to screen the bioactivities after hydrolyzed by different proteases and carbohydrases. Bioassays were conducted through the antioxidant activity using ESR method and anti ACE, anti-inflammatory activities using *in vitro* assays. Among the enzymatic hydrolysates of both the cultured microalgae were indicated the positive results for bioactivity assays. In particular, pepsin hydrolysate of both *C. ovalis* and *N. oculata* microalgae showed the profound effect against all the assays tested. Taken together, this study suggests that the pepsin hydrolysate of both microalgae species can be subjected for further purification and characterization of bioactive proteins and peptides.

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Part-II

Isolation and characterization of angiotensin I converting enzyme (ACE) inhibitory proteins and peptides from cultured microalgae

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ABSTRACT

In this study, the isolation of ACE inhibitory active proteins and peptides from pepsin hydrolysates of blue-green microalgae, *N. oculata* and *C. ovalis* were performed. Consecutive chromatographic steps were used for the purification of ACE inhibitory peptides from *N. oculata* pepsin hydrolysate and identified two novel peptides such as Gly-Met-Asn-Asn-Leu-Thr-Pro (728.3 Da) and Leu-Glu-Gln (369.2 Da) and showd the profound ACE inhibitory activity with IC_{50} values of 123 μM and 173 μM, respectively. Moreover, Reverse-phase high performance liquid chromatography (RP-HPLC-F2-2) fraction was obtained with ACE inhibitory activity as IC_{50} 0.074 ± 0.005 mg/mL from *C. ovalis* pepsin hydrolysate. Furthermore, assessment of nitric oxide (NO) level in the human umbilical vein endothelial cells (HUVECs) was determined as the increasing effect by the purified peptides without cytotoxicity from *N. oculata.* In addition, sephadex gel filtration chromatography fraction 2 (SGFC-F2) from *C. ovalis* showed the increasing effect of NO level significantly at 100 μg/mL concentration without a significant cytotoxicity for HUVECs *in vitro*. Mixed inhibition pattern of ACE inhibitory activity was observed from *C. ovalis* purified protein fraction, SGFC-F2 according to the Lineweaver-Burk plot. Taken together, this study suggests that the ACE inhibitory proteins and peptides from the marine microalgae strains, *N. oculata* and *C. ovalis* pepsin hydrolysates could be a potential candidate to develop neutraceuticals or dietary supplements against hypertension and other cardiovascular diseases.

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1. INTRODUCTION

Nannochloris oculata and *Chlorella ovalis* are the interested unicellular microalga, found in the marine environment. These microalgae are microscopic and do not express any distinct morphological features. However, having the valuable functional ingredients (Banskota et al., 2012) and the ability to biomass production, *Nannochloropsis* strains have been targeted to extensive research areas recently (Raso et al., 2012). Hence, *Nannochloropsis spp* have been investigated for the production of biofuels due to the high rate of lipid accumulation (Chiu et al., 2009; Converti et al., 2009; Huang et al., 2012). On the other hand, the nutritional values of *Nannochloropsis* strains have gained a high demand as a food sources for humans and aquaculture animals in the commercial applications (Spolaore et al., 2006). Although, *C. ovalis* has not been studied and having untapped functional components for further research. Moreover, the high content of proteins in *N. oculata* and *C. ovalis* make an interesting target to search bioactive peptides for functional food industry. Therefore, this is the first study to figure out the functional components including bioactive proteins and peptides from *N. oculata* and *C. ovalis* using protease-assisted extraction.

Antihypertensive peptides from natural sources are getting much attention as an alternative for synthetic drugs in hypertension therapy. There have reported many of food-derived antihypertensive peptides recently, including milk (Quirós et al., 2009), egg (Miguel et al., 2007), soybean (Rho et al., 2009), peanut (Jimsheena and Gowda, 2010), rice (Kuba et al., 2009), cowpea (Campos et al., 2011) and chicken (Saiga et al., 2006). Nowadays, the great deal of interest has been paid on marine organisms. Moreover, many of antihypertensive peptides have been isolated and reported from various marine organisms including, sardine (Matsui et al.,1993), pipefish (Wijesekara et al., 2011), tuna (Je et al., 2009), oyster (Je et al., 2005b), sea

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cucumber (Zhao et al., 2007), blue mussel (Je et al., 2005a), shrimp (Gildberg et al., 2011), squirt (Ko et al., 2012a) and rotifer (Lee et al., 2009).

The vascular endothelium controls either vasodilator or vasoconstrictor action. This is an important effect for regulation of vascular tone and disease etiology. In particular, endothelium-dependent vasodilation is mediated by nitric oxide (NO), prostacyclin (PGI_2) and the non-NO/PGI_2 mechanism (Grayson et al., 2013) In most of the ACE inhibitors regulate the activity of endothelial nitric oxide synthase (eNOS) and subsequently produce NO, an important mediator of vasorelaxation effect. Hence, in this part we demonstrate the isolation and characterization of ACE inhibitory proteins and peptides from cultured marine microalgae including *N. oculata* and *C. ovalis* followed by protease enzyme hydrolysation. Moreover, the identified proteins and peptides subjected to determine the inhibitory pattern and vasodilation effect on Human umbilical vein endothelial cells (HUVECs) *in vitro*.

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2. MATERIALS AND METHODS

2.1 Material and reagents

Angiotensin-I-converting enzyme (from rabbit lungs), N-Hippuryl-His-Leu tetrahydrate (HHL) and standard ACE inhibitor, captoprill were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Other proteases such as alcalase and nutrase were purchased from Novozymes (Bagsvaerd, Denmark). Human umbilical vein endothelial cells (HUVEC) were obtained from the Inno Pharma Screen Inc. (Hoseo University, Asan, Chungnam, South Korea). Cell culture media (endothelium cell growth medium-2; EGM-2) and other materials required to cell culture were purchased from the manufacturer (Clonetics Inc. San Diego, USA). All the other chemicals and reagents were used in this study were highest grade that available commercially.

2.2 Cell culture

Human umbilical vein endothelial cells (HUVECs) were cultured and maintained in EGM-2, supplemented with 20% heat-inactive fetal bovine serum, streptomycin (100 mg mL^{-1}), and penicillin (100 unit/mL). Cell cultures were maintained at 37℃in 5% CO_2 incubator.

2.3 Measurement of nitric oxide (NO) production

After pre-incubation of human umbilical vein endothelial cells (HUVECs, 1×10^5 cells mL^{-1}) with the pepsin hydrolysate (<5 kDa), SGFC fraction F1 and purified peptides of *N. oculata* and SGFC fraction F2 of *C. ovalis* for 24 h, the quantity of nitrite accumulated in the culture medium was measured as an indicator of NO production. Briefly, 100 μL of cell culture medium was mixed with 100 μL Griess reagent (1% sulfanilamide and 0.1% napthylenthylenediamine dihydrochloride in 2.5% phosphoric acid) and the mixture was incubated at room temperature for

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10 min. Then the absorbance was measured at 540 nm with respect to a blank as fresh culture medium in each experiment in a microplate reader (TECAN US Inc, Durham, NC, USA).

2.4 Assessment of cell viability

The procedure has been followed as mentioned in the part I (2.12)

2.5 Purification of ACE inhibitory proteins and peptides

The ACE inhibitory peptides were attempted to purify from the pepsin hydrolysate of *N. oculata* and *C. ovalis.* Then pepsin hydrolystae was fractionated using an ultra-filtration (UF) with 5 kDa and 10 kDa molecular weight cut-off membranes (Millipore Coperartion, Bedford, MA, USA). Two fractions were separated as molecular weights of <5 kDa (5 kDa or smaller) and molecular weights of 5-10 kDa (between 5 and 10 kDa). These two fractions were lyophilized and the highest ACE inhibitory fraction was applied for Sephadex G-25 gel filtration column (2.5 cm × 75 cm), equilibrated with de-ionized, distilled water. The fractions were eluted with a linear gradient of de-ionized distilled water at a flow rate of 2 mL min^{-1} by automated fraction collector (Model Foxy Jr, Dionex softron GmbH, Germering, Germany). Each fraction was monitored at 280 nm and pooled the fractions according to the peaks of the absorbance spectra. Each pooled fractions were analyzed for ACE inhibitory activity and the strongest ACE active fraction lyophilized and used for further purification. The active fraction with the strongest ACE activity was applied to reverse-phase high performance liquid chromatography (RP-HPLC, Waters, Milford, MA, USA.) on a Waters C_{18} Atlantis® (T3, 3µm, 3.0 ×150 mm, Dartford, UK) column with a linear gradient of acetonitrile (0-30% in 40 min) containing 0.1% formic acid (FA) at a flow rate 0.2 mL min^{-1}. The elution peaks were detected at 280 nm and collected the

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active peaks. Then collected peaks were concentrated using a rotary vacuum evaporator (EYELA USA, N-1000, Tokyo Rikakikai Co. Ltd. NY, USA). Finally, the purified fractions with the ACE inhibitory activity were performed by the identification of amino acid sequences.

2.6 Determination of amino acid sequence and molecular mass of purified peptides

Accurate molecular weights of ACE inhibitory peptides were determined with Hybrid Quadrupole-TOF LC/MS/MS Spectrometer (AB Sciex Instruments, CA 94404, USA) coupled with positive mode of electrospray ionization (ESI) source. The quadrupole was operated in deconvolution method of Bayesian Peptide Reconstruct with the scan range m/z 50-2000 and sequenced using the de-novo sequencing program of peptide sequence (AB Sciex Instruments, CA 94404 USA).

2.7 Determination of ACE inhibition pattern

To determine the ACE inhibitory mechanism of the protein hydrolysates and purified peptides, different concentration of protein hydrolysates and purified peptides were added to each reaction mixture. The enzyme activities were measured with different concentrations of substrate (HHL). ACE inhibitory pattern in the presence of inhibitor was obtained with Lineweaver-Burk plot.

2.8 Statistical analysis

For the statistical analysis, all the data were expressed as mean ± standard deviation of three independent determinations. The significance of differences between two samples was analyzed using the student t-test and *P*-value of <0.05 was considered as the level of statistical significance.

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3. RESULTS AND DISCUSSION

3.1 Purification of ACE inhibitory proteins and peptides

In ACE inhibitory activity assay, pepsin hydrolysates of blue - green algae, *N. oculata* and *C. ovalis* showed the highest activity compared to the other hydrolysates. The determined IC_{50} values of pepsin hydrolysate in *N. oculata* and *C. ovalis* were 2.808 ± 0.12 and 0.520 ± 0.09 mg/mL, respectively. Previous reports have shown that pepsin is capable of producing ACE inhibitory peptides when it hydrolysed the protein sources (Sheih et al., 2009). For the purification of pepsin hydrolysates were fractionated by ultra-filtration membrane. Fractionated molecular weights such as <5 kDa, and 5 ~ 10 kDa were determined the ACE inhibitory activity and IC_{50} values are presented in the Table 5. Among the fractions, both microalgae pepsin hydrolysates <5 kDa was indicated the highest ACE inhibitory activity as the IC_{50} value 0.445 ± 0.09 and 2.721 ± 0.13 mg/mL of against *C. ovalis* and *N. oculata,* respectively.

For further purification, the <5 kDa fraction of both microalgae samples were loaded on to a Sephadex G-25 gel filtration column (SGFC) and separated into five fractions respect to *C. ovalis* and three fractions respect to *N. oculata*. These fractions were comprised with different molecular sizes, eluted according to pore size of the gel material (Fig. 11 A and C). Each fractions were pooled, lyophilized and measured the ACE inhibitory activity. The second fraction (F2) of *C. ovalis* and first fraction (F1) of *N. oculata* were found to possess the highest ACE inhibitory effect with the IC_{50} value of 0.245 ± 0.03 and 0.780 ± 0.04 mg mL^{-1}, respectively (Fig. 11 B and D). Moreover, it was indicated that a 2.12 and 3.6-fold higher ACE inhibition than that of the un-fractionated pepsin hydrolyaste of both *C. ovalis* and *N. oculata,* respectively (Table 6).

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Table 5. ACE inhibitory activity of molecular weight fractions of pepsin hydrolysate from microalage

Fraction	IC_{50} value (mg/mL) [a]	
	C. ovalis	***N. oculata***
Un-fractionated	0.520 ± 0.09	2.808 ± 0.12
5-10kDa [b]	0.545 ± 0.04	3.182 ± 0.46
<5kDa	0.445 ± 0.09	2.721 ± 0.13

[a] The concentration of an inhibitor required to inhibit 50% of the ACE activity. The values of IC_{50} were determined by at triplicate individual experiments.

[b] Fractionation was done by an ultra-filtration (UF) with 5 kDa and 10 kDa molecular weight cut-off membranes

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Table 6 Purification of ACE inhibitory peptide of Pepsin hydrolysate from *Nannochloris oculata*

Purification Step	IC_{50} value (mg mL^{-1})[a]	Purification fold [b]
Nannochloris oculata		
Pepsin hydrolysate	2.81 ± 0.12	1
Sephadex Gel filtration (F1)	0.781 ± 0.04	3.6
RP-HPLC (F1-1)	0.064 ± 0.006	43.9
RP-HPLC (F1-2)	0.090 ± 0.002	30.8
Chlorella ovalis		
Pepsin hydrolysate	0.520±0.09	1
Ultrafiltration (<5kDa)	0.445±0.09	1.16
Sephadex Gel filtration (F2)	0.245±0.03	2.12
RP-HPLC (F2-2)	0.074±0.005	7.03

[a] The concentration of an inhibitor required to inhibit 50% of the ACE activity. The values of IC_{50} were determined by at triplicate individual experiments.

[b] Relative value of reciprocal of ACE IC_{50}

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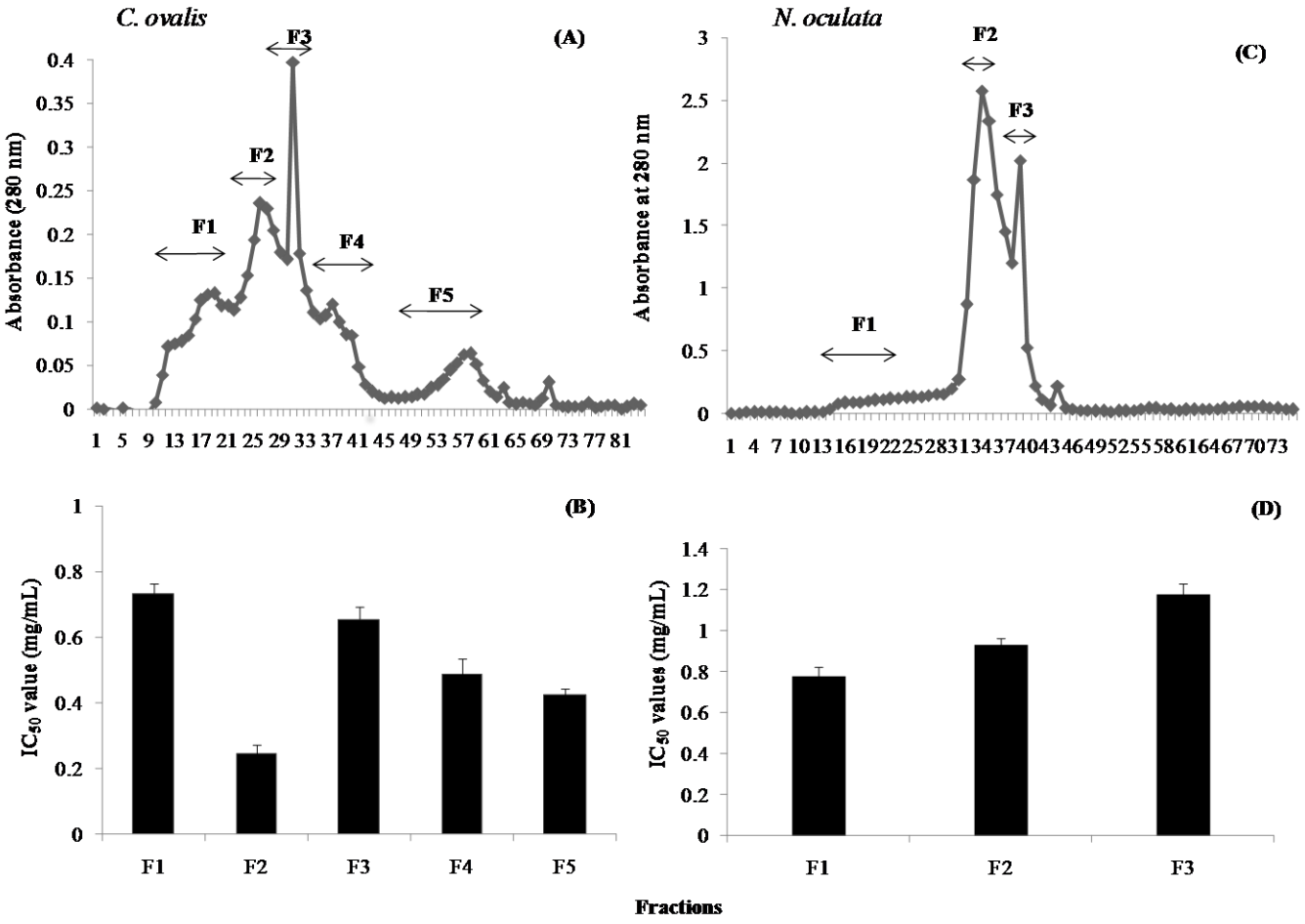

Fig. 11 Sephadex G-25 gel filtration chromatogram of *Chlorella ovalis* (A) and *Nannochloris oculata* (C) Pepsin hydrolysate fraction (< 5 kDa). Separation was performed with 2 mL min^{-1} and collected at a fraction volume (6 mL). ACE inhibitory activity of each separated fractions of *C.ovalis* (B) and *N. oculata* (D)

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Fractionated the highest ACE inhibitory activity shown pepsin hydrolysate SGFC fractions such as F2 from C. *ovalis* and F1 from *N. oculata* were subjected to purify further using RP-HPLC on Waters C_{18} Atlantis® (T3, 3µm, 3.0 ×150 mm) column using a linear gradient of acetonitrile (0-30%) containing 0.1% formic acid. Hence, two fractions were separated from the F1 fraction of *N. oculata* (Fig. 12 A) as F1-1 and F1-2. The fraction F1-1 was shown the most potent ACE inhibitory activity with the IC_{50} value 0.064 ± 0.006 mg mL^{-1} than the F1-2 fraction (IC_{50} value 0.090 ± 0.002 mg mL^{-1}) (Fig. 12 B). Furthermore, the highest ACE-I inhibitory peptide (F1-1) was purified at 43.9-fold from the pepsin hydrolysate and 30.8-fold of purification was reported against the F1-2 using after three-step purification process (Table 6).

The obtained SGFC fraction (F2) of *C. ovalis* was purified using RP-HPLC into four sub-fractions such as F2-1, F2-2, F2-3 and F2-4 (Fig. 13 A). Among the sub-fractions F2-2 was found to show the highest ACE inhibitory activity as IC_{50} value 0.074 ± 0.005 mg mL^{-1}, where as other fractions including F2-1, F2-3 and F2-4 showed 0.142 ± 0.004, 0.096 ± 0.007 and 0.118 ± 0.01 mg mL^{-1}, respectively (Fig 13 B). The highest ACE inhibitory peptide in (F2-2) was purified at 7.03-fold from the pepsin hydrolysate (Table 6).

The isolated peptides from pepsin hydrolysate of *N. oculata* were identified by the hybrid quadrupole-TOF LC/MS/MS spectrometer coupled with positive mode of electrospay ionization (ESI) source. The active fraction was subjected to chromatography by RP-HPLC (ZORBAX 300SB-C18 (1 ×150 mm, 3.5µm, Agilent, USA**)** column using a linear gradient of acetonitrile (0-50%) containing 0.2% formic acid and flow rate at 35µl mL^{-1} for 50 min. Molecular weights and amino acid sequences were identified using MS/MS spectrometer from the fractions F1-1 (388.21 Da) as y-ions mass determination and F1-2 (727.38 Da) as b-ions mass determination. In fact, purified peptides were found to be novel and reported as tripeptide, Leu-Glu-Gln (369.21

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Da) and hepta peptide, Gly-Met-Asn-Asn-Leu-Thr-Pro (728.38 Da), respectively (Fig. 14). In addition, the ACE-I inhibitory activity of purified peptide (Gly-Met-Asn-Asn-Leu-Thr-Pro) was shown the IC_{50} value 123 µM, which was the higher than the IC_{50} value of 173 µM reported on (Leu-Glu-Gln) peptide (Table 7). The identified F2-2 fraction of RP-HPLC of *C. ovalis* was measured the anti ACE effect. In fact, ACE inhibitory activity was determined as IC_{50} 0.074 ± 0.005 mg/mL from RP-HPLC (F2-2) fraction of pepsin hydrolaysate of *C. ovalis.*

The amino acid compositions of the ACE inhibitory peptide are critical factors in possessing the ACE inhibitory activity. Especially, Proline, glycine, leucine, glutamic acid and methionine have been reported in many ACE inhibitory peptides, such as pearl oyster at IC_{50} value 14.2 µM (Leu-Val- Glu) (Wang et al., 2008), skate skin hydrolysate purified two peptides (Pro-Gly-Pro-Leu-Gly-Leu-Thr-Gly-Pro) and (Gln-Leu-Gly-Phe-Leu-Gly-Pro-Arg) at IC_{50} values 95 µM and 148 µM, respectively (Lee et al., 2011). In addition, there have been reported a few of studies on ACE inhibitory peptides from marine microalgae protein hydrolysates. Sheih et al., (2009) has reported that the purified peptide from *Chlorella vulgaris* protein waste at IC_{50} value 29.6 µM (Val-Glu-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Gln-Phe) and another study with the same microalgae, isolated peptides from the pepsin hydrolysate, IC_{50} values of Ile-Val-Val-Glu, Ala-Phe-Leu, Phe-Ala-Leu, Ala-Glu-Leu and Val-Val-Pro-Pro-Ala were 315.3, 63.8, 26.3, 57.1 and 79.5 µM, respectively (Suetsuna and Chen, 2001).

However, the published many of peptides from the marine algae and other food sources showed similar or high ACE inhibitory activities compared to the ACE inhibitory activities of purified peptides from the pepsin hydrolysate of *N. oculata*. Importantly, the Proline (Pro) amino acid at the C-terminus may subject to high ACE inhibitory activity (Lee et al., 2011). Moreover, proline is believed that to be preventing digestion of enzymes and passing across the capillary into the

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blood circulation in the short sequence of peptides (Korhonen and Pihlanto, 2006). In this study, we revealed that the Pro at the C-terminus of the purified peptide (Gly-Met-Asn-Asn-Leu-Thr-Pro) having strong ACE inhibitory effect.

3.2 Inhibition pattern of the purified proteins (SGFC-F2) of *C. ovalis*

The ACE inhibition pattern of the purified protein of sephadex gel filtration chromatography fraction 2 (SGFC-F2) was investigated using Lineweaver-Burk plot. According to the determination (Fig 16) showed that a mixed inhibition pattern, where the ACE inhibitor can bind to the enzyme at the same time as the enzyme's substrate. However, it can be the different site of the enzyme.

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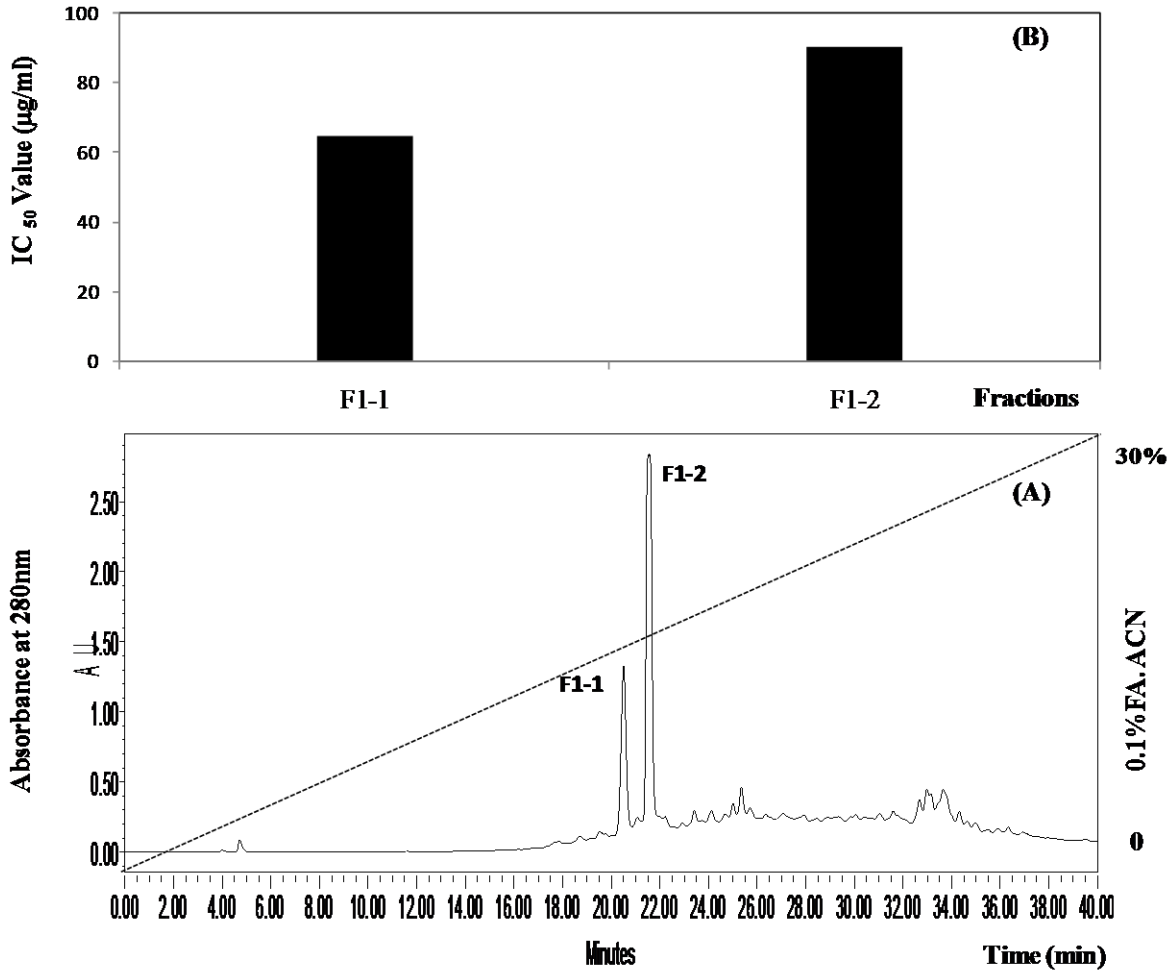

Fig. 12 Reverse-phase gradient HPLC chromatogram of the potent ACE inhibitory fraction (F1) isolated from Sephadex G-25. (A) Separation into sub-fractions (F1-1 and F1-2) were eluted with a linear gradient of 0–30% acetonitrile (ACN) in 0.1% formic acid (FA) for 40 min at flow rate of 0.2 mL min^{-1} and monitored at 280 nm on Atlantis T3 ODS column (3μm, 1.0mm × 150mm). (B) ACE inhibitory activity of each fraction

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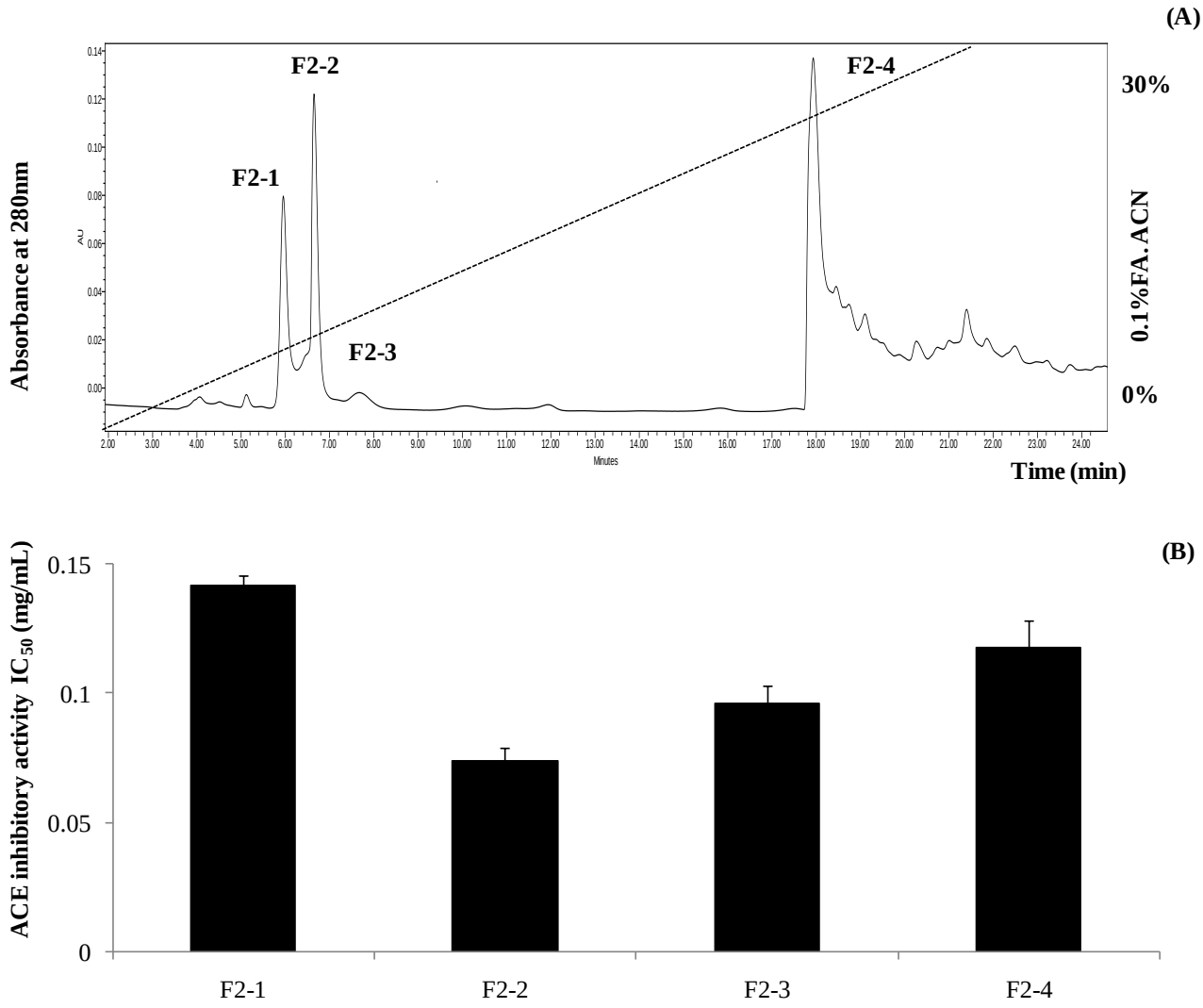

Fig. 13 Reverse-phase gradient HPLC chromatogram of the potent ACE inhibitory fraction (F2) isolated from Sephadex G-25. (A) Separation into sub-fractions (F2-1, F2-2, F2-3 and F2-4) were eluted with a linear gradient of 0–30% acetonitrile (ACN) in 0.1% formic acid (FA) for 35 min at flow rate of 0.2 mL min^{-1} and monitored at 280 nm on Atlantis T3 ODS column (3μm, 1.0mm × 150mm). (B) ACE inhibitory activity of each fraction

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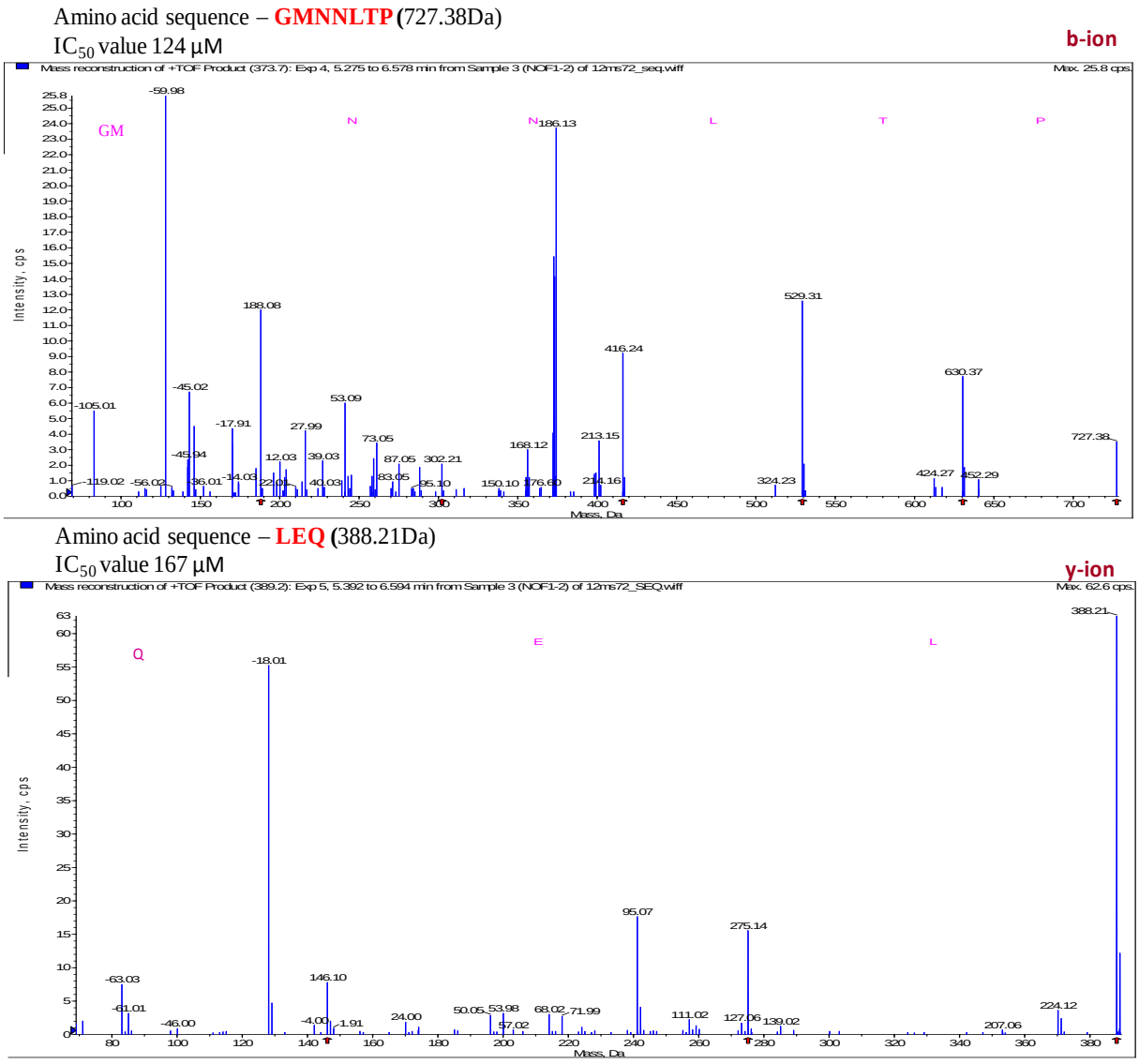

Fig. 14 Identification of molecular mass and amino acid sequence of the purified peptides, GMNNLTP and LEQ from *Nannochloris oculata* Pepsin hydrolysate by RP-HPLC. Calculating of molecular mass of the purified peptide was determined by the charged product ion masses b-ions and y-ions, respectively

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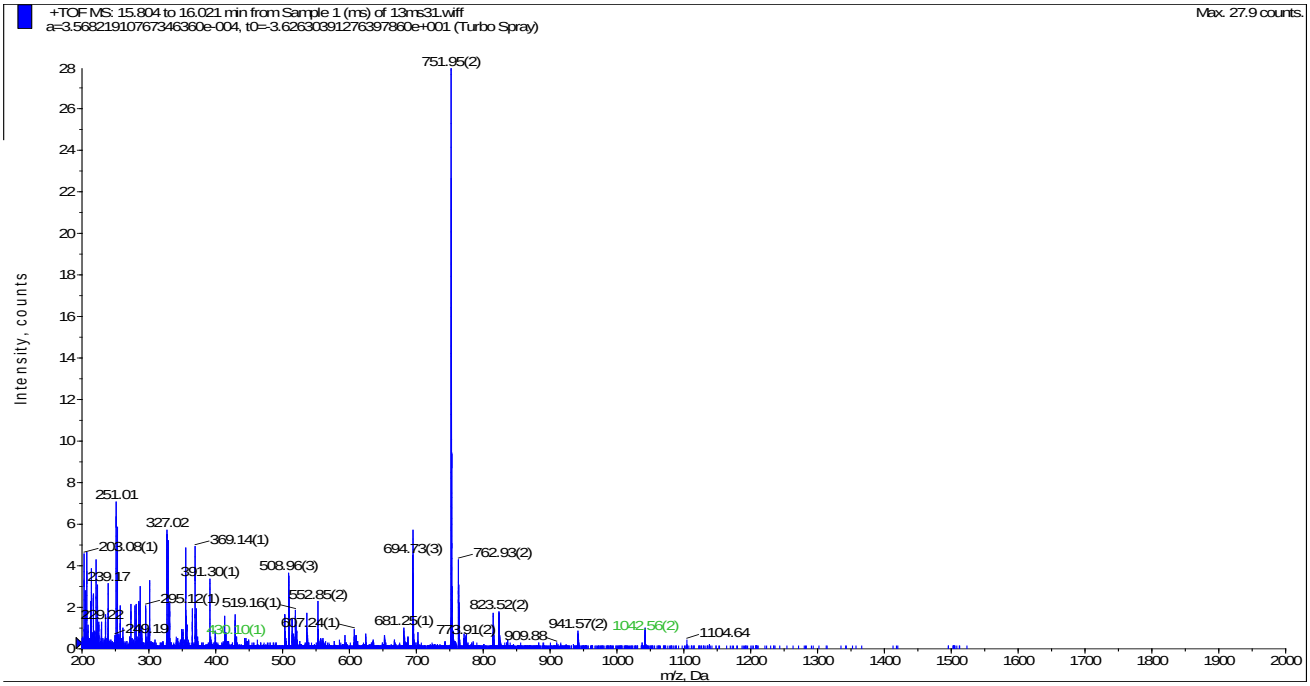

Fig. 15 Identification of molecular mass of the purified protein from *Chlorella ovalis* Pepsin hydrolysate by RP-HPLC.

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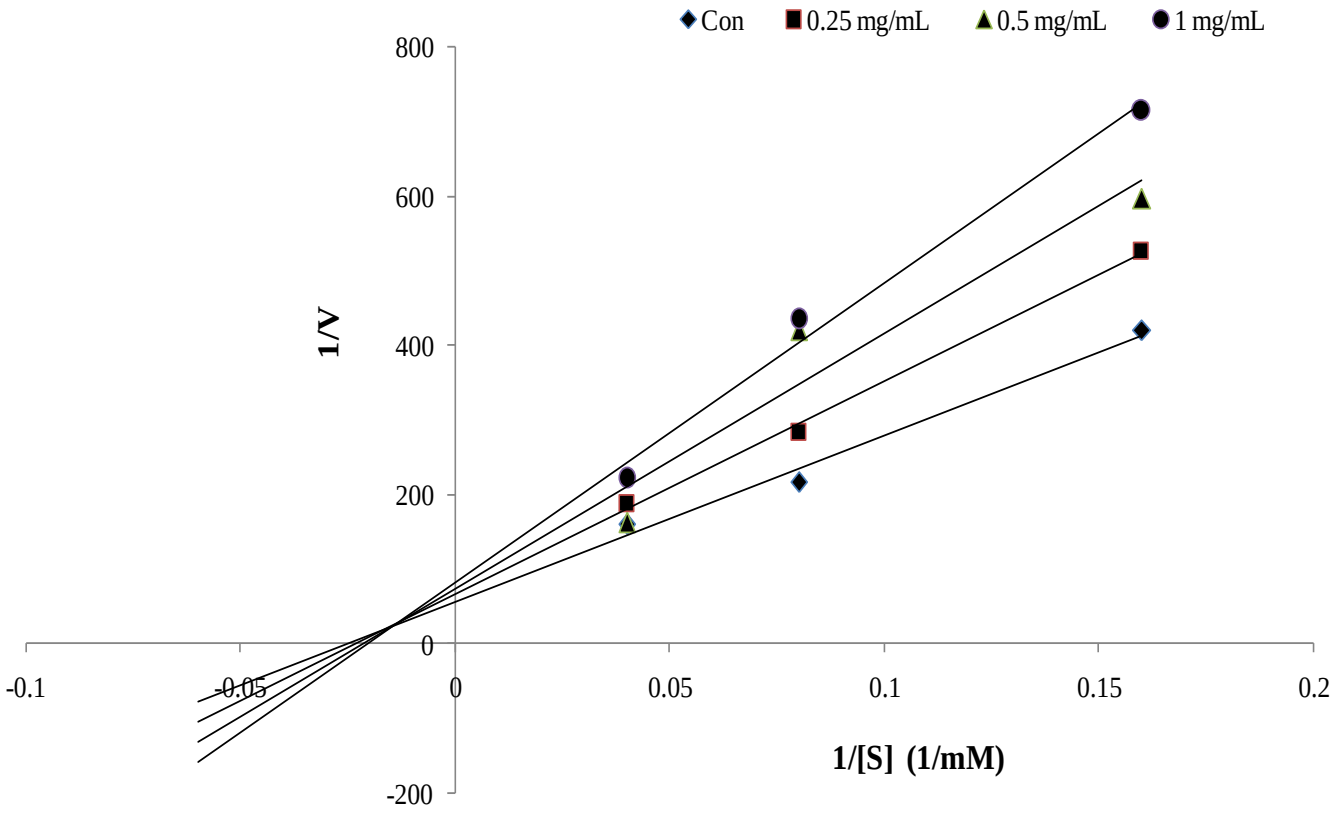

Fig. 16ACE inhibition pattern of the purified protein of sephadex gel filtration chromatography fraction 2 (SGFC-F2) from *Chlorella ovalis* was investigated using Lineweaver-Burk plot and showed the mixed inhibition pattern

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3.3 Assessment of nitric oxide (NO) level

N. oculata pepsin hydrolysate (<5 kDa) fraction, SGFC fraction (F1) and purified peptides were evaluated with the effect of nitric oxide (NO) production level (%) on human umbilical vein endothelial cells (HUVECs) (Fig.17 A). The highest NO production (%) was reported on the purified peptide (Gly-Met-Asn-Asn-Leu-Thr-Pro) with the significant different, (p<0.05) compared to the control (no sample treated cells) at 100 µg mL^{-1} treated concentration. However, purified peptide (LEQ), SGFC fraction F1 and (<5 kDa) fraction were not claimed a significantly different level of NO production (%) dose dependently compared to the control. According to the cell viability assay (Fig. 17 B) HUVECs showed a high or similar survival rate compared to the control (100%) with treated all samples including purified peptides and other fractions at all the concentrations. Therefore, the MTT assay confirmed that no cytotoxcity against HUVECs of purified peptides including LEQ and GMNNLTP, and pepsin hydrolysate (<5 kDa) fraction and SGFC fractions (F1) of *N. oculata* as well.

Furthermore, different concentrations (25, 50 and 100 µg/mL) of SGFC-F2 fraction from the pepsin hydrolysate of *C. ovalis* was determined the effect of nitric oxide (NO) production level (%) on human umbilical vein endothelial cells (HUVECs) (Fig. 18 A). In particular, the increasing effect of NO level was indicated dose dependently and showed a significant difference of NO level at 100 µg/mL concentration compared to the control with the treated SGFC-F2 samples. In the cell viability assay, all the concentrations of SGFC-F2 showed higher cell viability effect (app. 100%) against the HUVECs and confirmed that no cytotoxicity in vitro assay (Fig. 18 B).

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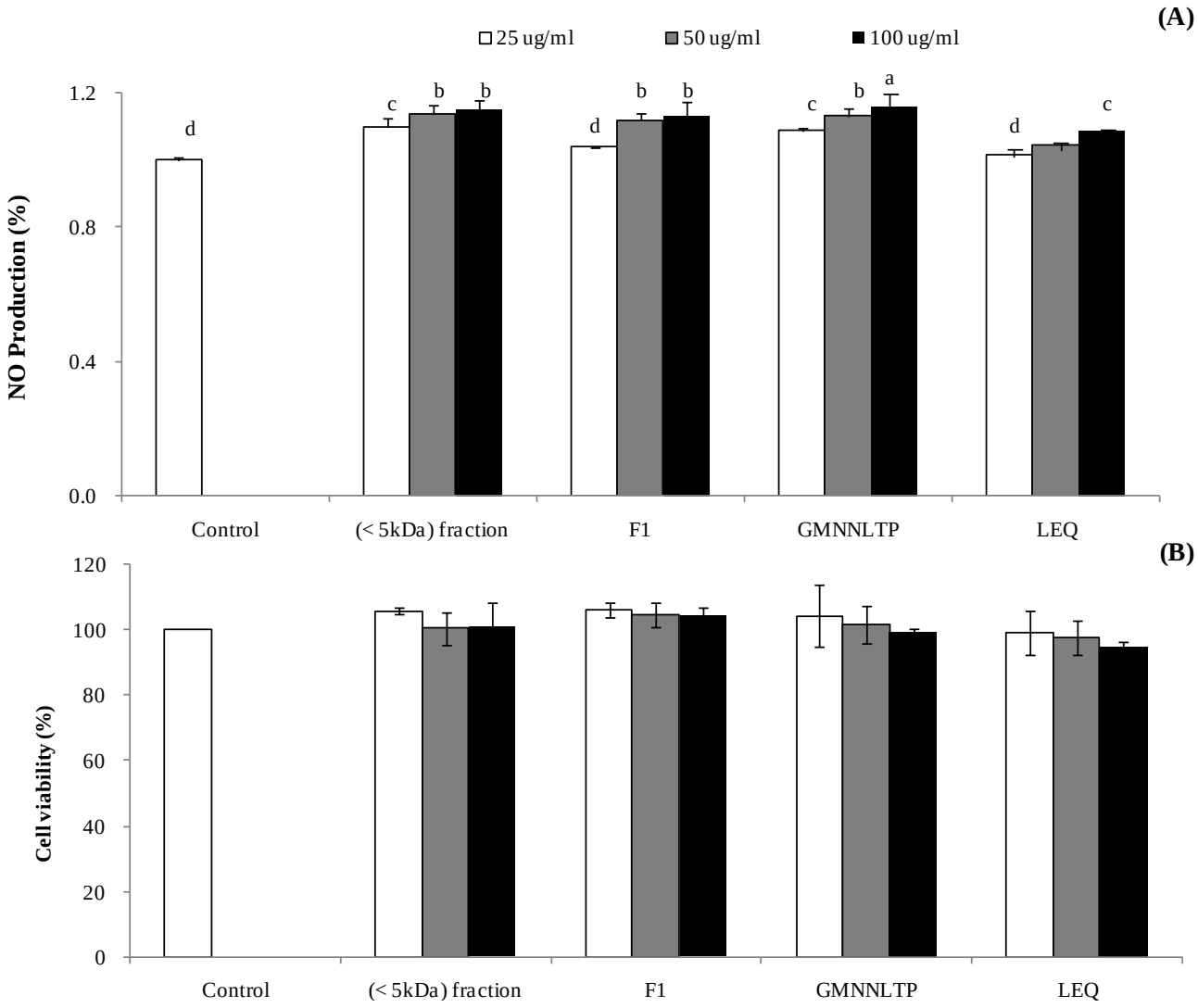

Fig. 17 Effect of the NO production level of pepsin hydrolysate (< 5 kDa) fraction, Sephadex G-25 gel filtration column (SGFC) fraction (F1) and purified peptides (GMNNLTP and LEQ) of *Nannochloris oculata* on human umbilical vein endothelial cells (HUVECs). Values with different alphabets are significantly different at $P < 0.05$

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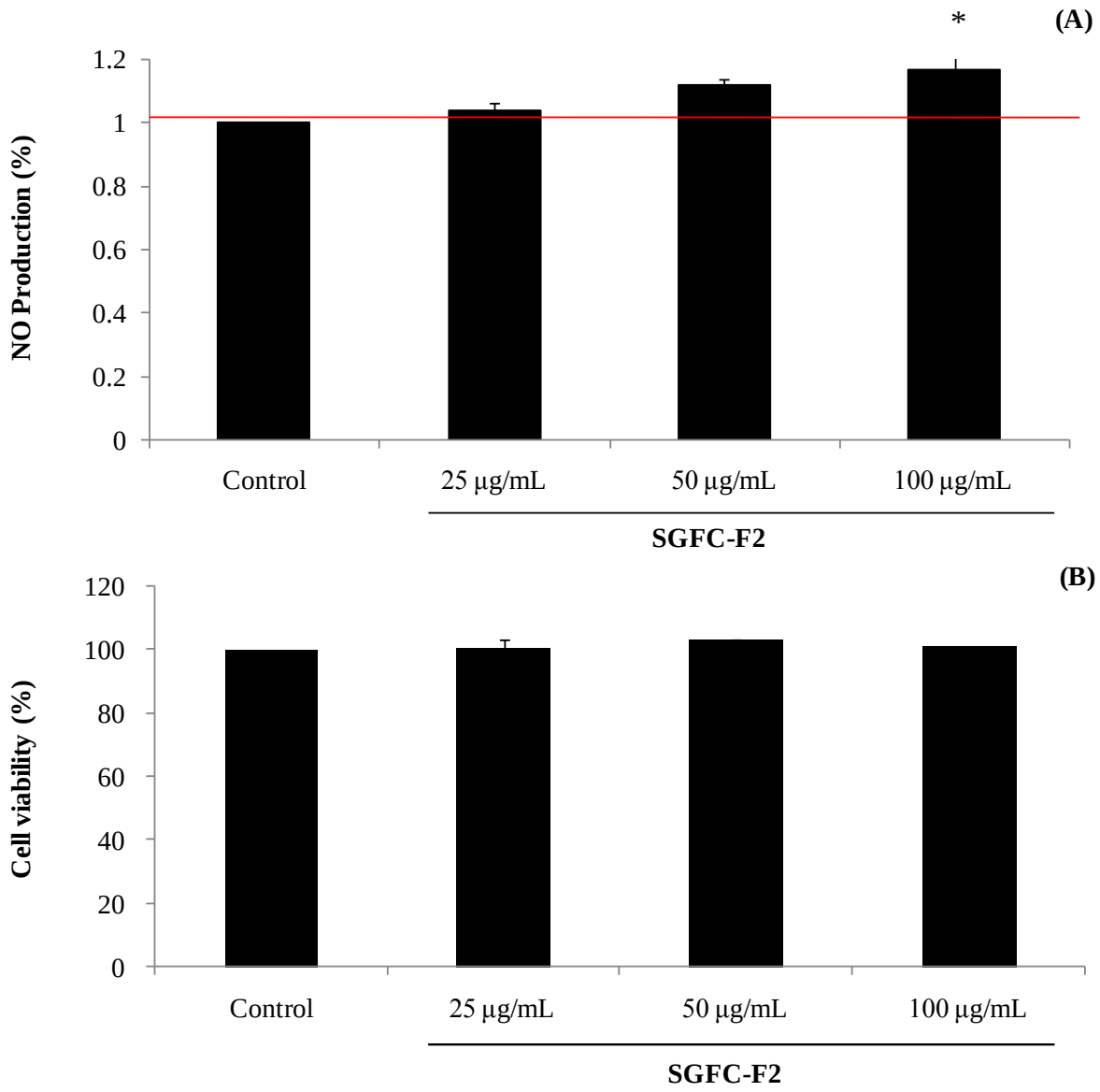

Fig. 18 Effect of the NO production level of pepsin hydrolysate Sephadex G-25 gel filtration column (SGFC) fraction (SGFC-F2) of *Chlorella ovalis* on human umbilical vein endothelial cells (HUVECs). Dose-dependently increased the NO production in HUVECs, significantly.

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Table 7 Identification of molecular weights and amino acid sequences with the ACE inhibitory activity of the purified peptides from Pepsin hydrolysate of *Nannochloris oculata*

Fraction	Peptide	Amino acid sequence	Experimental molecular weight (Da)	Theoretical molecular weight (Da)	IC_{50} value (μM)[a]
F1-1	Tri-peptide	Leu-Glu-Gln	388.21 [b]	369.21	173
F1-2	Hepta-peptide	Gly-Met-Asn-Asn-Leu-Thr-Pro	727.48 [c]	728.38	123

[a] The concentration of an inhibitor required to inhibit 50% of the ACE activity

[b] Mass analysis of product ions : y-ions

[c] Mass analysis of product ions : b-ions

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Nitric oxide (NO) is an important messenger molecule and plays a vital role that producing naturally on the human body. NO molecules are involving in many physiological processes especially in the cardiovascular system. This signaling molecules cause to relax the smooth muscles of the inner lining of the blood vessels, thus resulting to vasodilatation and increased the blood flow (Hou et al., 1999). Recent findings suggested that nitrates may stimulate the enzyme guanylate cyclase, which results in increases in cyclic guanosine monophosphate and vasodilation. Therefore, these molecules may play an important role in the therapy of acute unstable myocardial ischemia, including unstable angina and myocardial infarction (Abrams, 1996). The isolated peptides from *N. oculata* and gel filtration chromatography fraction of *C. ovalis* have evidenced that producing the nitrates in the treated HUVECs. Hence these evidences can be described that the purified peptides of *N. oculata* and oligopeptides of *C. ovalis* may show a greater potential with the promising effect of anti ACE activity *in vitro*.

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4. CONCLUSION

Purification and characterization of ACE inhibitory active proteins and peptides from cultured marine microalgae, *N. oculata* and *C. ovalis* pepsin hydrolysates were performed. Two novel peptides such as Gly-Met-Asn-Asn-Leu-Thr-Pro (728.3 Da) and Leu-Glu-Gln (369.2 Da) isolated from the pepsin hydrolysate of *N. oculata* and showed the profound ACE inhibitory activity with IC_{50} values of 123 μM and 173 μM, respectively. ACE inhibitory active (IC_{50} 0.074 ± 0.005 mg/mL) protein was purified from RP-HPLC (F2-2) fraction of pepsin hydrolaysate of *C. ovalis.* Isolated ACE inhibitory proteins and peptides increased the effect of NO production in HUVECs *in vitro* and indicated no cytotoxcity against HUVECs as well. . These results suggest that the isolated peptides and proteins from cultured marine microalga, *N. oculata* and *C. ovalis* protein sources may have potentiality to use commercially as antihypertensive agents in functional food industry.

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Part-III

Molecular docking, *in vitro* effect of digestive stability in gastrointestinal enzymes and pharmacological effect of the isolated peptides from cultured marine microalga, *Nannochloris oculata*

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ABSTRACT

Isolated novel ACE inhibitory active peptides from the cultured marine microalga, *Nannochloris oculata* for molecular docking studies, *in vitro* digestive stability and pharmacological effects were assessed. The calculated binding energy among the identified peptide, GMNNLTP-isomer 1 showed the lowest (-461.55 kcal/mol) and the highest CDOCK interaction energy (128.24 kcal/mol) compared to the commercial ACE inhibitor, Captopril where as binding energy and CDOCK interaction energy -53.21 and 30.90 kcal/mol, respectively. In fact, LEQ-isomer 1 was indicated the lowest binding energy (-216 kcal/mol) and CDOCK interaction energy (56.83 kcal/mol) compared to the LEQ-isomer 2. However, the determined ACE inhibitory activity of the synthetic peptides either LEQ or GMNNLTP on ACE kit showed the significantly lower activity compared to the captopril. In addition, *in vitro* digestibility assay indicated that LEQ was stabilized in all the gastrointestinal enzymes used including trypsin, pepsin and α-chymotrypsin. However, GMNNLTP stabilized in the trypsin enzymes only. Moreover, the determined ACE inhibitory activity followed by gastrointestinal digestion indicated the strong activity (148.9 μg/mL) reported against α-chymotrypsin enzyme and released the NLT peptide fragment while digestion. Moreover, among the synthetic peptides, LEQ was significantly rendered the *in vitro* anti-inflammatory and anticancer effects against RAW macrophages and HL-60 cancer cells, respectively.

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1. INTRODUCTION

Bio-functionalities or bioactivities of peptides have been described as mimic hormones or showing drug like activities. They could alter the physiological functions or give raise a positive impact through binding to specific receptors and interact on target cells or inhibition by enzyme actions (Kitts & Weiler, 2003; FitzGerald & Murray, 2007). Some algae species have induced biological activities, which are associated with proteins, protein hydrolysates or peptides, that effects standing beyond their nutritional values as antioxidant (Karavita et al., 2007; Kim et al., 2006), antihypertensive (FitzGerald & Murray, 2007), immune-modulatory (Morris et al., 2007), anticancer (Sheih et al., 2010), hepeto-protective (Hwang et al., 2008; Kang et al., 2012) and anticoagulant (Athukorala et al., 2007). Those bioactivities would be added advantage to gain access for multifunctional applications, including functional foods or nutraceuticals (Guil-Guerrero et al., 2004; Mohamed et al., 2012), pharmaceuticals (Dominic & Danquah, 2011), and cosmeceuticals (Stolz & Obermayer, 2005; Sekar & Chandramohan, 2008).

Over the many years, the ACE inhibitory activities of the isolated peptides from different food sources have been executed in many studies. In fact, a few of suggestions have come across with the preferred orientation in the structure-activity relationship studies. Hence, branched amino acid residues at N-terminal positions and aromatic amino acid residues at C- terminal positions in the substrates or competitive inhibitors could be preferred for anti-ACE activity (He et al., 2007). In particular, among the different peptide inhibitors of ACE may influence strongly when binding to substrates with C-terminal tripeptide sequences. However, the intensities of ACE activity were also affected by the adjacent amino acid of the C-terminal proline residue in a particular peptide (Li et al., 2004). Even for the potent activities, this should be a change with the

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hydrophobic amino acids. Hence, this might be the possible mechanism for evaluating the ACE inhibitory activities and antihypertensive effects.

Molecular docking is a method and application which predicts the orientation of one molecule to a second when binding to form a stable complex. In particular, it can provide knowledge of a protein (enzyme) interacts with smaller molecules (ligands) of the preferred orientation and predicts the strength of association or binding affinity using scoring function or QSAR. For the ACE inhibitory activity of the preferred molecule such as purified peptide can be thought as a "key" for the "lock" of ACE proteins active site. Molecular docking describes the best-fit orientation and computationally determine the free energy that to be minimized.

In this study, the isolated two peptides from the pepsin hydrolysate of *Nannochloris oculata* were evaluated for the potentiality of using in the molecular docking system and determined the structure-activity correlation. Moreover, the effects of peptides against digestive enzymes and the efficacy of ACE inhibitory activity of the synthesized peptides were performed *in vitro*. In addition, the effects of anti-inflammatory and anticancer activity of synthesized peptides were also determined.

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2. MATERIALS AND METHODS

2.1 Inhibitory activity of the purified peptides against angiotensin I converting enzyme (ACE) using ACE kit

The purified peptides were performed ACE inhibitory activity using ACE assay kit according to the manufacture's protocol (ACE kit-WST, Dojindo Laboratories). In briefly, enzyme working solution was prepared by adding 1.5 mL of enzyme B to enzyme A. Indicator working solution was prepared by adding 2.8 mL of enzyme C with Coenzyme. Peptide samples were diluted 1/5 ratio using deionized water to obtain the different concentrations and 20 µL of sample was mixed with deinonized water, substrate buffer and of enzyme working solution in each of 20 µL into 96-well plate using multi-channel pipette. For the control, only deionized water was used instead of sample (ACE inhibitor) and followed to add other chemicals. For the blank, enzyme working solution and sample were not added. A 200 µL of indicator solution was mixed to each well pre-incubated the plate at 37 °C for 60 min. and kept again in incubator at room temperature for 10 min. Then absorbance of samples was measured using ELISA micro plate reader (TECAN) at 450 nm.

ACE inhibitory activity (Inhibition rate %) = $[(A_{control}-A_{sample}) / (A_{control}-A_{blank})] \times 100$

2.2 Stability of the peptide during *in vitro* digestion by gastrointestinal enzymes

The 1% (w/w) pepsin solution was prepared in a 20 mM glycine-HCl buffer adjust to pH 2.0, while the 1% (w/w) trypsin and α-Chymotrypsin solution in 50 mM sodium phosphate buffer was adjusted to pH 8.0. The peptide was dissolved at 0.5 mg/mL in the pepsin, trypsin and α-Chymotrypsin solution and reacted at 37°C for 4 h. Reactions were terminated by boiling at 100°C for 15 min. Then, these solutions were centrifuged at 10,000*g* for 25 min. The ACE

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inhibitory activity (IC_{50} values) was then measured. The stability of supernatant was analyzed using by LC-MS.

2.3 Molecular docking and computational studies

The crystal structure of angiotensin I converting enzyme (ACE) (PDB: 1086) was obtained from the Protein Data Bank (PDB, http:/pdb.org) (Fig. 19). We used the docking studies using CDOCKER in Accelrys Discovery Studio 3.1 (Accelrys Inc. USA). We describe the ligand structure of the ACE inhibitory candidate and prepare for the docking procedure using 3D structure as well as calculating charges and determine the binding and CDOCK interaction energy (kcal/mol).

2.4 Determination of nitric oxide (NO) production and cell viability on RAW macrophages

The procedure has been followed as mentioned in the part I (2.11 and 2.12)

2.5 Anticancer activity

The MTT assay was conducted to determine the cell viability against cancer cells as mentioned in the part I (2.12).

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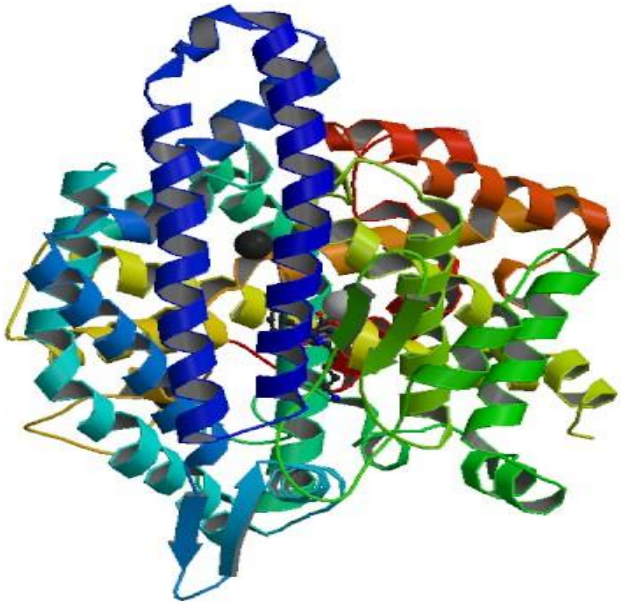
CRYSTAL STRUCTURE OF HUMAN ANGIOTENSIN CONVERTING ENZYME IN COMPLEX WITH LISINOPRIL



Crystal structure of the human angiotensin-converting enzyme-lisinopril complex.
Natesh, R., Schwager, S.L.U., Sturrock, E.D., Acharya, K.R.
Journal: (2003) Nature **421:** 551
PubMed: 12540854
DOI: 10.1038/nature01370

Fig. 19

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3. RESULTS AND DISCUSSION

3.1 ACE inhibitory activity of the isolated peptides using ACE kit

The results obtained from the ACE screening kit were presented in Fig. 21. The isolated two peptides (LEQ and GMNNLTP) from cultured microalga, *N. oculata* different concentrations (50, 100 and 200 µg/mL) were tested the ACE inhibitory activity compared to commercial ACE inhibitor as captopril. Both peptides were showed the lower ACE inhibitory activity compared to the control. In fact, 44.2 and 46.9 % of ACE inhibitory activity were indicated at 200 µg/mL concentration by both peptides, LEQ and GMNNLTP, respectively. However, the captopril showed about 98 % of ACE inhibitory activity at very low concentration (3.34 µg/mL) significantly. Structure-activity relationship of the low molecular weight tripeptides have been reported that possessing the most potent ACE inhibitory activity. In particular, hydrophobic amino acid at the amino-terminus and aromatic amino acid at the carboxy-terminus with middle positive amino acid can be described as the preferred orientation of tripeptide for the strong ACE inhibitory effect (Jimsheena and Govada, 2010). The isolated and purified tripeptide (Leu-Glu-Gln) from *N. oculata* in this study, was evidenced that having a hydrophobic amino acid (Leu) at the N-terminus of the peptide.

3.2 *In silico* molecular docking studies

The identified two peptides were composed with three and seven amino acid residues in the peptide sequences (LEQ and GMNNLTP), respectively. These peptides were drawn by ChemDraw Ultra 10.0 (PerkinElmer Inc.) and determined the molecular formula as well as molecular structure showed with the best orientation in Fig. 20. The 3D structure of native-human ACE was imported from the Protein Data Bank (PDB: 1086). It was allowed to analyze

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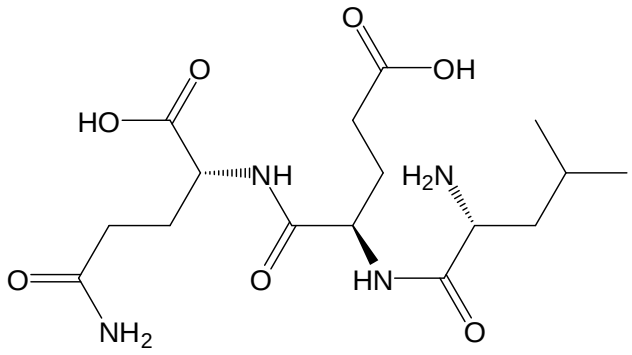
LEQ - $C_{16}H_{28}N_4O_7$



GMNNLTP - $C_{30}H_{51}N_9O_{11}S_1$

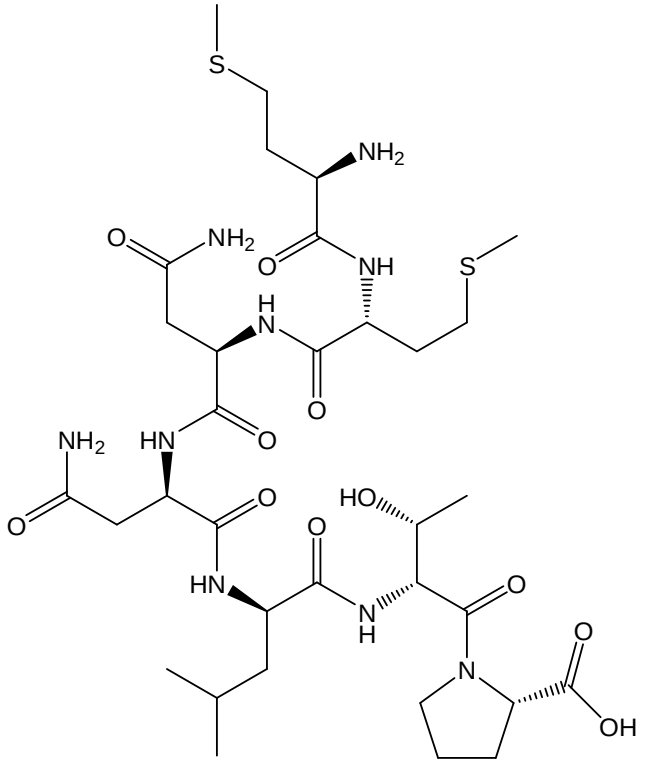


Fig. 20

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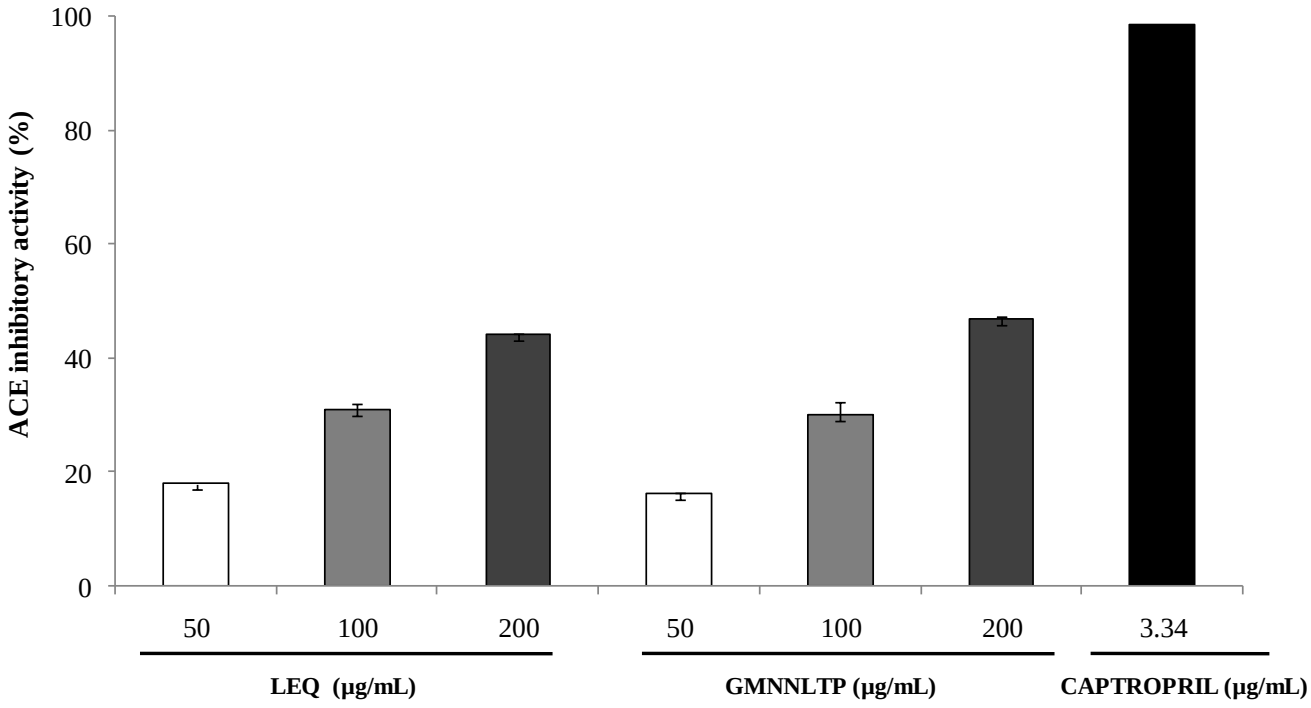

Fig. 21 ACE inhibitory activity of the isolated peptides using ACE kit compared to the commercial ACE inhibitor as captopril.

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the position, type and energy of interactions of the two peptides accomplished in automated molecular docking at the ACE catalytic site in the presence of amino acid residues. The molecular structure of peptides (ACE inhibitors) as ligands was generated in Acclrys Discovery Studio 3.1 software and energy minimizing effect was calculated using CHARM program steepest descent (SD) and conjugate gradient (CG) techniques. SD chooses the gradient of the function as the direction of steepest ascent. CG is to search the direction are conjugate to each other. It takes into account the curvature of the problem and generates improved search directions and coverage faster than steepest descent. Before the docking procedure, water molecules were removed from the protein-crystal structure. Based on the ACE's 3D structure, the possible ACE active sites were obtained from the binding site procedure and identified the hydrogen bonds (H- bonds), hydrophilic, hydrophobic and electrostatic interactions between the residues at the ACE active site and ACE inhibitors (Peptides) poses.

According to the Fig. 22, the commercial ACE inhibitor, Captopril was showed the best pose at the ACE catalytic site ribbon model (ACE-ligand complex) and performed with residual interactions. In the ACE active site, 13 amino acid residues were contained such as Pro^{508}, Lys^{511}, Phe^{460}, Ala^{272}, Asp^{507}, Trp^{182}, Met^{278}, Ala^{510}, Trp^{279}, Gly^{190}, Glu^{183}, Arg^{501} and Trp^{185}. In particular, Captopril binds with Asp507 by H-bond and binding energy values are presented in Table 8.

The molecular docking system of the ACE-ligand complexes was subjected to assess the isolated peptides which are posed in the best orientation in the pocket of ACE active site. In fact, a tripeptide, LEQ showed the two isomeric forms and LEQ-isomer 1 was found to have the best-fit with the lowest binding energy (-216 kcal/mol) than the LEQ-isomer 2 (-204.71 kcal/mol).

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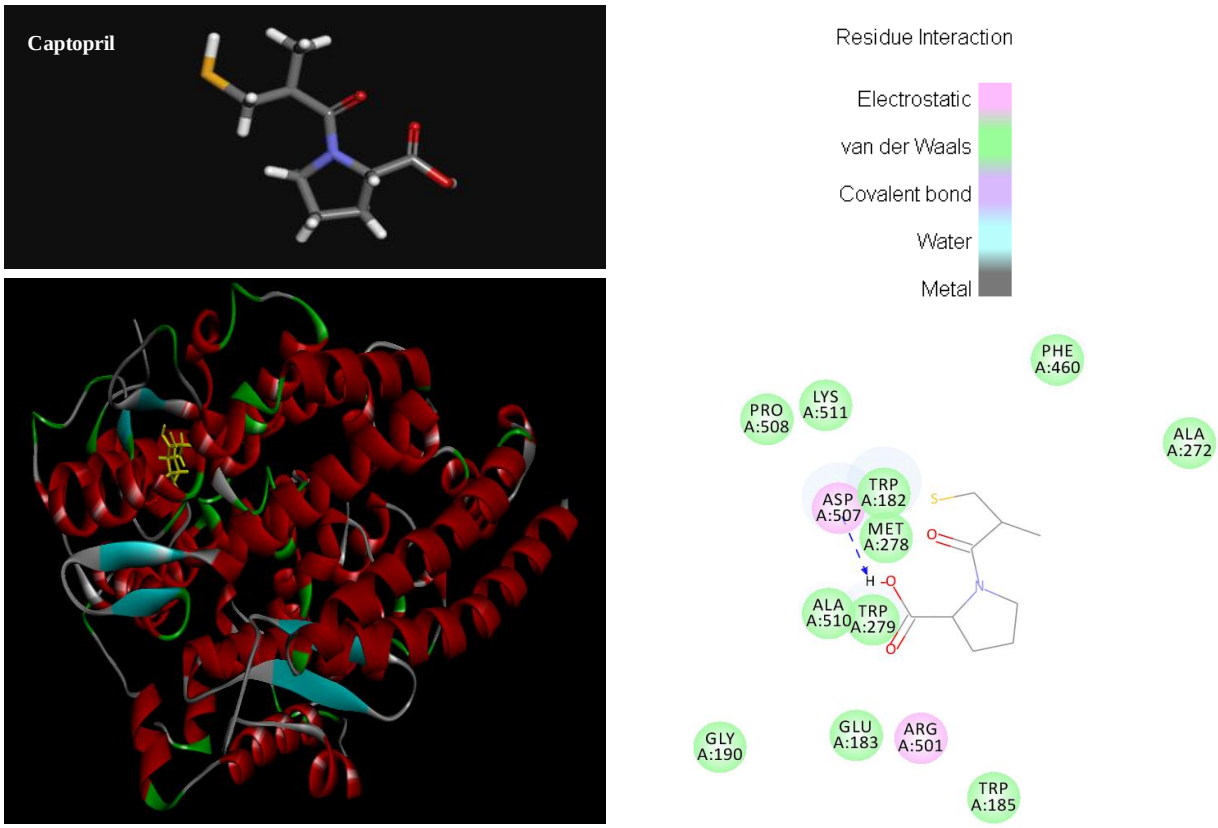

Fig. 22 Computational prediction of the structure of ACE and docking stimulation with commercial ACE inhibitor captopril. Predicted 3D structure of ACE1086 and 2D program (ACE-Ligand Complex)

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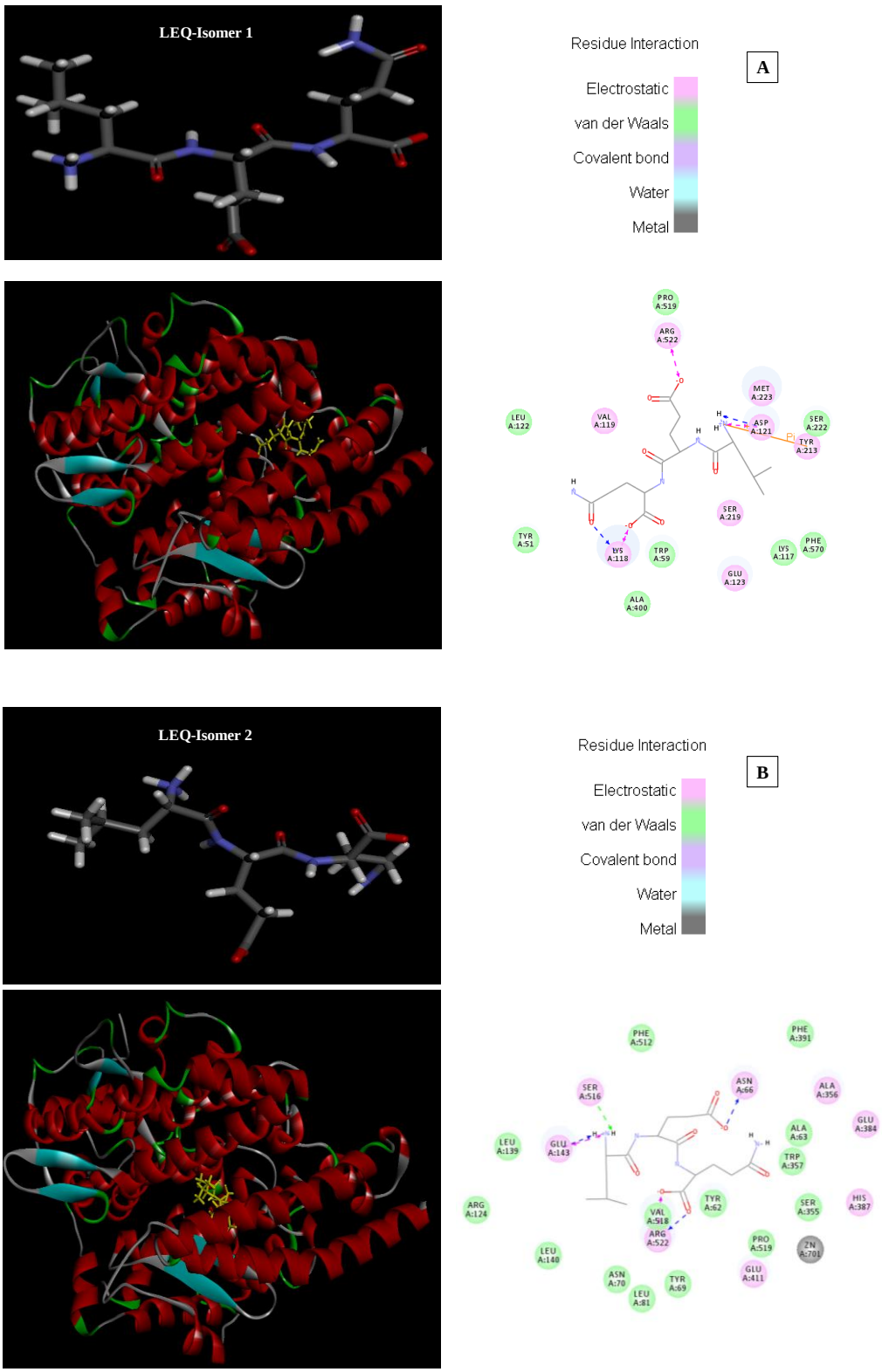

Fig. 23 (A &B) Computational prediction of the structure of ACE and docking stimulation with the isolated peptide LEQ

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In addition, energy of CDOCK interaction showed 58.63 and 73.97 kcal/mol for LEQ isomer 1 and 2, respectively (Table 8). Furthermore, H-bond interactions with residuals in the ACE active site such as, Arg^{522}, Lys^{18}, Met^{223}, Asp^{121}, and Tyr^{213} were observed respect to the LEQ-isomer 1 (Fig. 23 A). In fact, residuals including Glu^{143}, Ser^{516}, Asn^{66}, Val^{518} and Arg^{522} were interacted with the LEQ-isomer 2 also monitored (Fig.23 B).

The isolated hepta-peptide, GMNNLTP was preformed the docking against ACE-ligand active site and the identified isomer configurations as GMNNLTP-isomer 1 and GMNNLTP-isomer 2 were showed in Figure 24 A & B, respectively. In particular, metal ion interaction (Zn701) and H-bond interactions including Arg^{522}, Ala^{356}, Tyr^{523} and Glu^{143} were associated with the GMNNLTP-isomer 1 (Fig. 24 A). Hence, the calculated binding energy of GMNNLTP-isomer 1 was found to be the lowest (-461.55 kcal/mol) and CDOCK interaction energy (128.24 kcal/mol) showed the highest compared to GMNNLTP-isomer 2. In addition, GMNNLTP-isomer 2 interacted with Arg^{124}, Asn^{66}, Ala^{354} and Glu^{384} using H-bond interactions were observed. However, metal ion interaction was not determined with this isomeric form. As a consequence, among the determined molecular docking interactions of ACE inhibitors, GMNNLTP-isomer 1showed the best-fit orientation with the direct co-ordination of the catalytic Zn (II) as observed the lowest binding energy and the highest CDOCK interaction energy compared to the captopril (Table 8). Therefore, GMNNLTP-isomer 1 resembles the substrate may bind selectively with the ACE active site amino acid residuals using hydrogen bond, hydrophilic and metal ion interactions.

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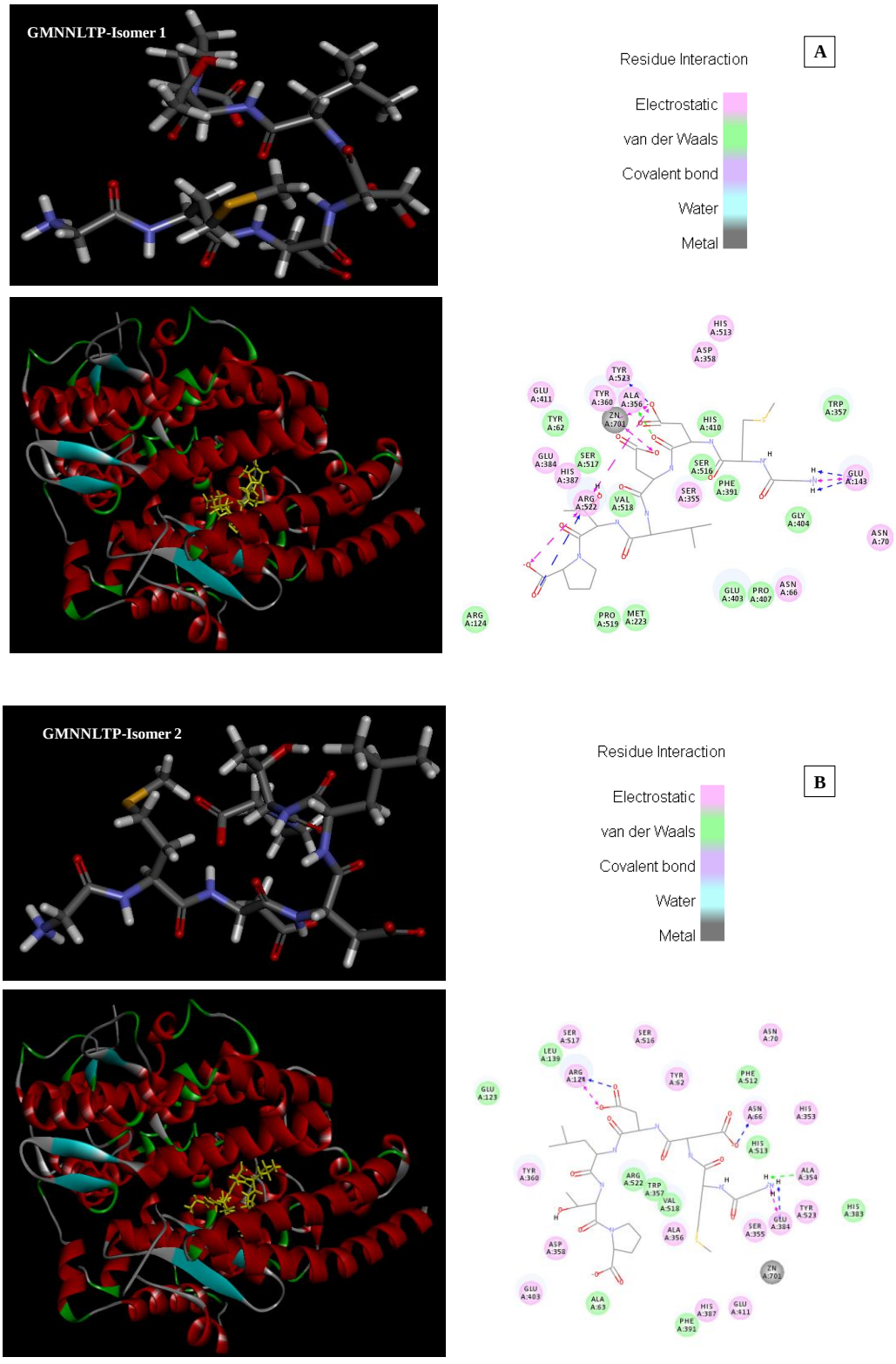

Fig. 24 (A & B) Computational prediction of the structure of ACE and docking stimulation with the isolated peptide GMNNLTP

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Table 8. Results in docking experiments of isolated peptides from *Nannochloris oculata* with the angiotensin I converting enzyme (PDB ID: 1086)

Ligands	**Binding energy (kcal/mol)**	**CDOCK interaction energy (kcal/mol)**
Captopril	-53.21	30.90
LEQ-Isomer 1	-216.00	56.83
LEQ-Isomer 2	-204.71	73.97
GMNNLTP-Isomer 1	-461.55	128.24
GMNNLTP-Isomer 2	-132.82	84.51

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3.3 Stability of ACE inhibitory peptides to simulated gastrointestinal digestion

In this study, ACE inhibitory peptides which isolated from the cultured marine microalga, *N. oculata* were synthesized and subjected to evaluate the stability of peptides against gastrointestinal digestive enzymes. Hence, three digestive enzymes including pepsin, trypsin and α-chymotryspin were intended to stimulate *in vitro* conditions for prevailing during human digestion. Moreover, the digests were subjected to determine the ACE inhibitory activity and further analyzed the HPLC-MS/MS to identify the fragments were released during the digestion.

Therefore, among the synthesized peptides including LEQ and GMNNLTP were showed the ACE inhibitory activity before digestion *in vitro* as IC_{50} values 229.1 and 211.6 μg/mL, respectively. Interestingly, the tri-peptide, LEQ was not showed the significant difference of ACE activity after digestion against the gastrointestinal enzymes compared to the un-digested peptide activity (Table 9). In fact, HPLC-MS/MS profile was suggested that the determined fragments after the *in vitro* digestion were identical with the un-digested peptide (Fig. 25 A & B). Hence, it is considered that the LEQ peptide could not alter the ACE inhibitory effect and stabilized during the human gastrointestinal digestion.

On the other hand, the synthesized hepta-peptide (GMNNLTP) was undertaken for the *in vitro* digestion against gastrointestinal enzymes and found to be stabilized only against the trypsin enzyme. According to the HPLC-MS/MS profile (Fig. 26 A) the determined molecular mass of GMNNLTP (728.4 Da) did not change in either treated or un-treated trypsin enzyme. In addition, ACE inhibitory activity of the digested GMNNLTP by trypsin (IC_{50} value 207.5 μg/mL) did not show a significant difference compared the original activity (IC_{50} value 211.6 μg/mL). Despite that, corresponding digestion against pepsin and α-chymotrypsin enzymes were determined the

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Table 9. Impact of simulated gastrointestinal digestion upon the *in vitro* ACE inhibitory activity measured by IC_{50} [a] and determined peptide fragments after digestion

Peptide	ACE inhibitory effect before digestion (IC_{50} value : μg/mL)	ACE inhibitory effect after digestion (IC_{50} value : μg/mL)		Determined molecular weight (Da)	Theoretical Molecular weight (Da)	Fragment released by digestion
LEQ	229.1	Pepsin	225.4	389.30	369.21	LEQ
		Trypsin	228.1	389.26	369.3	LEQ
		α-Chymotrypsin	234.6	389.23	369.2	LEQ
GMNNLTP	211.6	Pepsin	202.7*	329.22	311.4	LTP
		Trypsin	207.5	746.37	728.4	GMNNLTP
		α-Chymotrypsin	148.9 *	353.44	328.4	NLT

[a] The concentration of an inhibitor required to inhibit 50% of the ACE activity and the values of IC_{50} were determined by at triplicate individual experiments.

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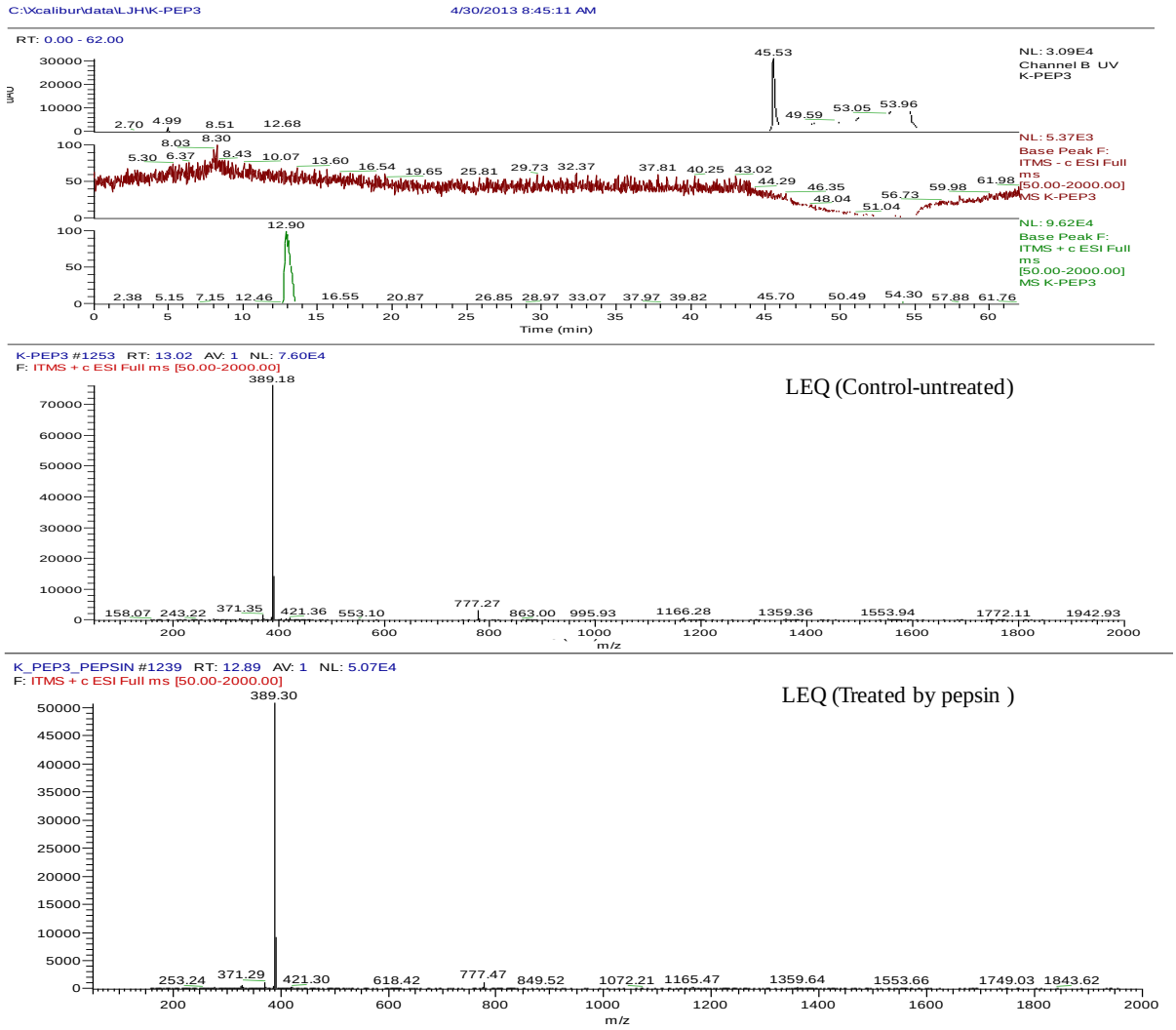

Fig. 25 (A) HPLC-MS chromatogram of synthetic peptides LEQ after and before *in vitro* gastrointestinal digestion against pepsin

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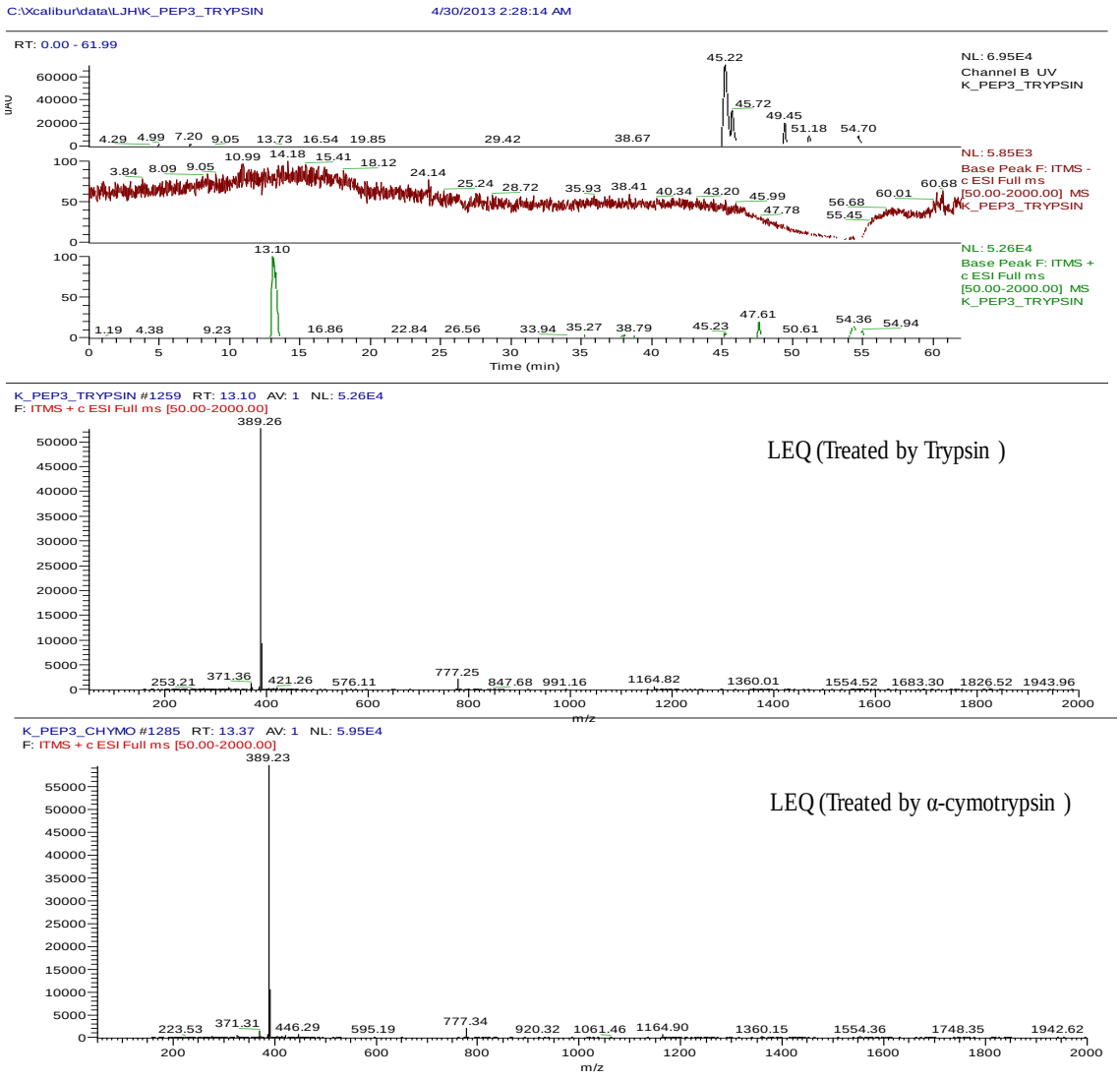

Fig. 25 (B) HPLC-MS chromatogram of synthetic peptides LEQ after and before *in vitro* gastrointestinal digestion against trypsin and α-chymotrypsin enzyme

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ACE inhibitory activities of the GMNNLTP showed 202.7 and 148.9 μg/mL, respectively (Fig. 26 B & C).

It is indicated that the significant difference of the ACE inhibitory activity was monitored after digestion compared to the original ACE inhibitory activity tested. Furthermore, HPLC-MS/MS profile shows the fragmented molecular mass of peptides at 329.22 and 353.44 Da against the pepsin and α-chymotrypsin digestive enzymes, respectively. Further insight on the fragments were evidenced that tri-peptides were released including LTP (311.4 Da) and NLT (328.4 Da) during the pepsin and α-chymotrypsin gastrointestinal digestion respectively (Table 9). Taken together, *in vitro* digestion led to release tri-peptides in both pepsin and α-chymotrypsin gastrointestinal enzymes and they were increased the ACE inhibitory activity even higher than the original hepta-peptide activity.

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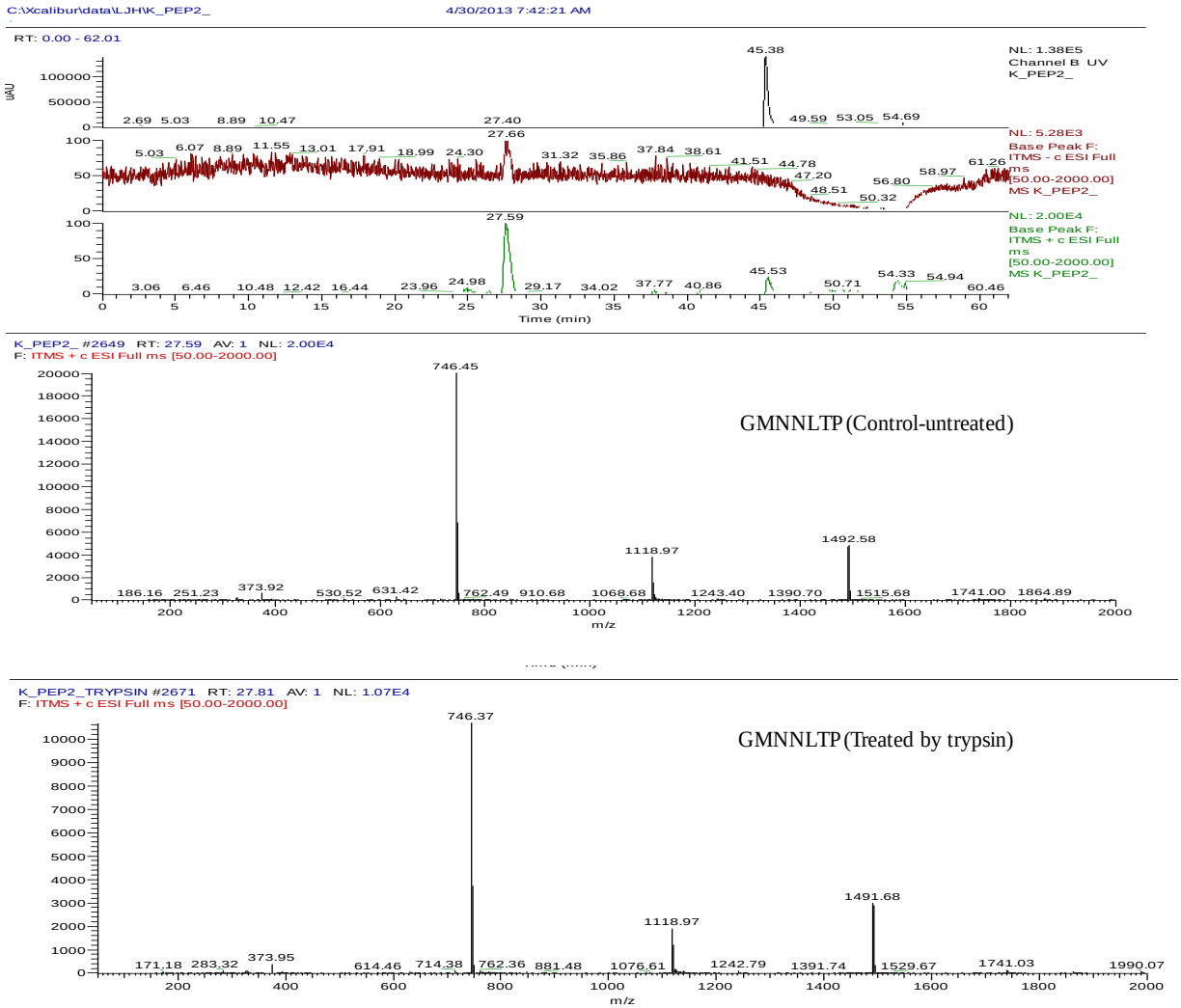

Fig. 26 (A) HPLC-MS chromatogram of synthetic peptides GMNNLTP after and before *in vitro* gastrointestinal digestion against trypsin

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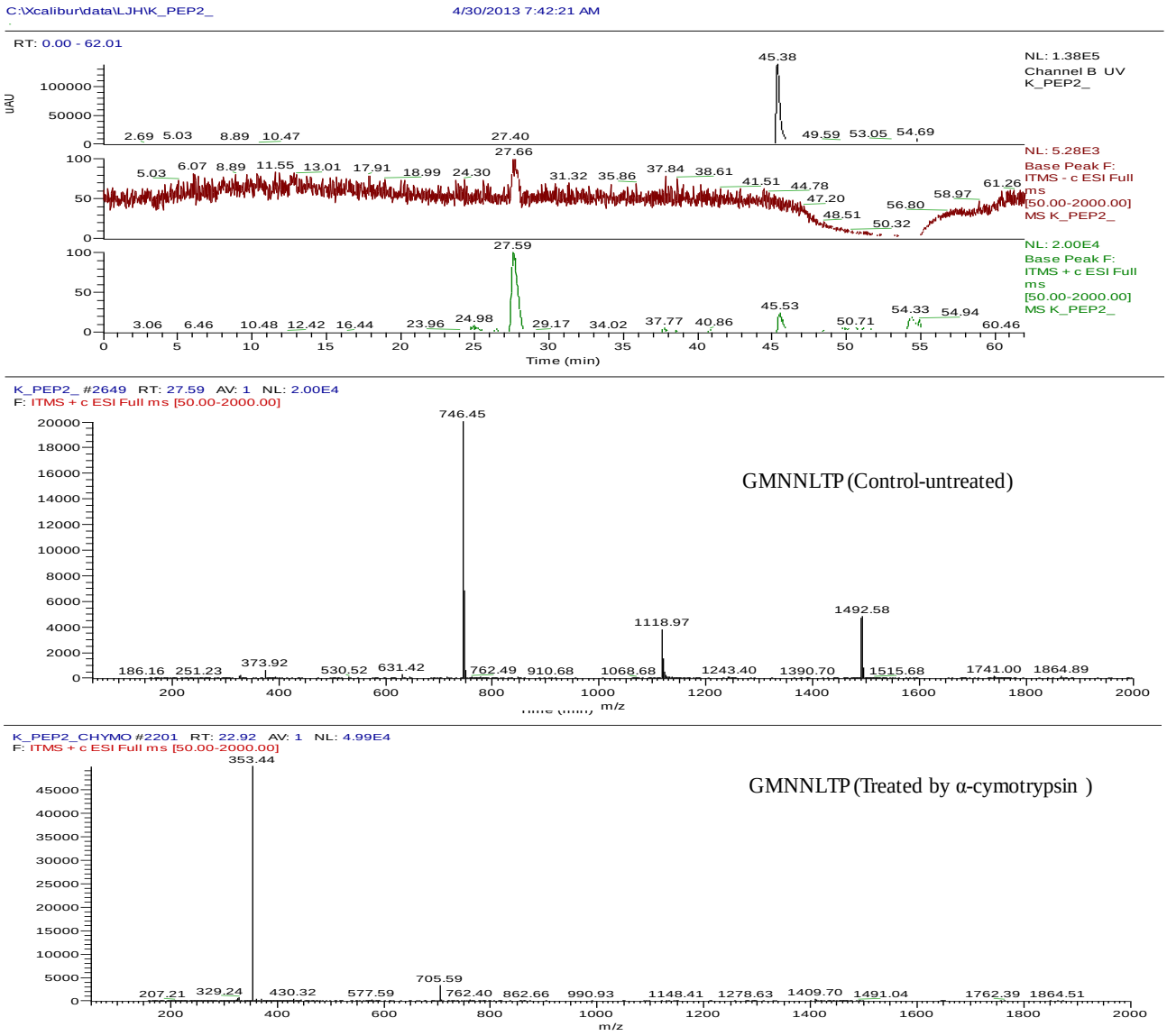

Fig. 26 (B) HPLC-MS chromatogram of synthetic peptides GMNNLTP after and before *in vitro* gastrointestinal digestion against α-chymotrypsin

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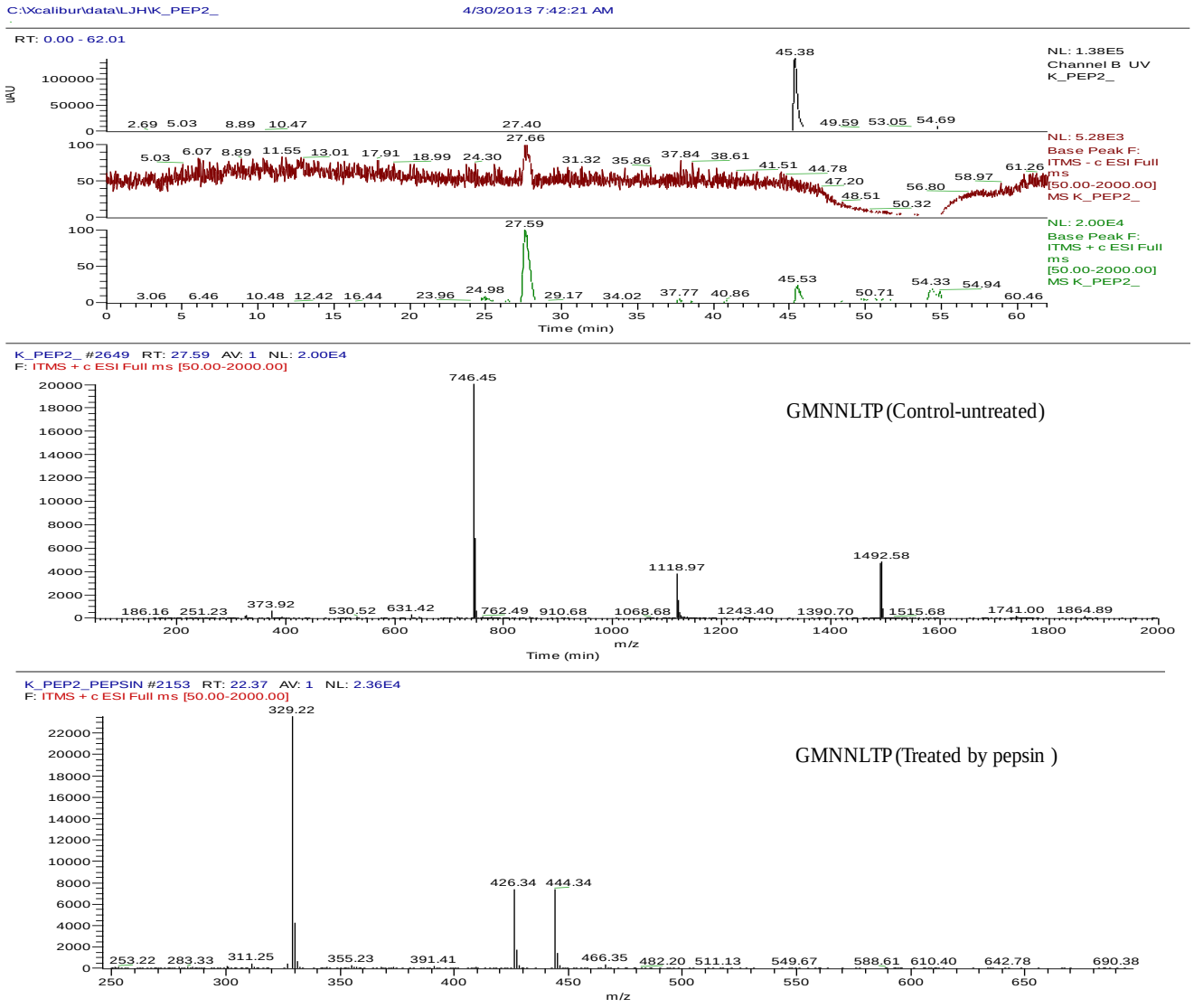

Fig. 26 (C) HPLC-MS chromatogram of synthetic peptides GMNNLTP after and before *in vitro* gastrointestinal digestion against pepsin

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3.4 Assessment of NO production on LPS-induced RAW macrophages

Among the synthesized peptides according to the isolated novel peptides from microalgae, *N. oculata* were determined for anti-inflammatory activity against LPS-induced RAW macrophages. The significant NO production inhibitory effect of the LEQ peptide was showed dose-dependently against LPS-induced RAW cells compared to the other peptide (GMNNLTP) (Fig. 27). Cell viability assay was confirmed that no cytotoxicity for RAW cells at all the treated concentration of LEQ peptide significantly.

3.5 Anticancer activity

The determination of anticancer activity of the synthesized peptides showed to be moderate and only LEQ peptide was performed significant cancer cell growth inhibitory effect against HL-60 cells at 24 and 48h of incubation (Fig. 28). However, the anticancer activity was not significant by the treated LEQ and GMNNLTP peptides against A549 and HL-60 cancer cells, comparatively.

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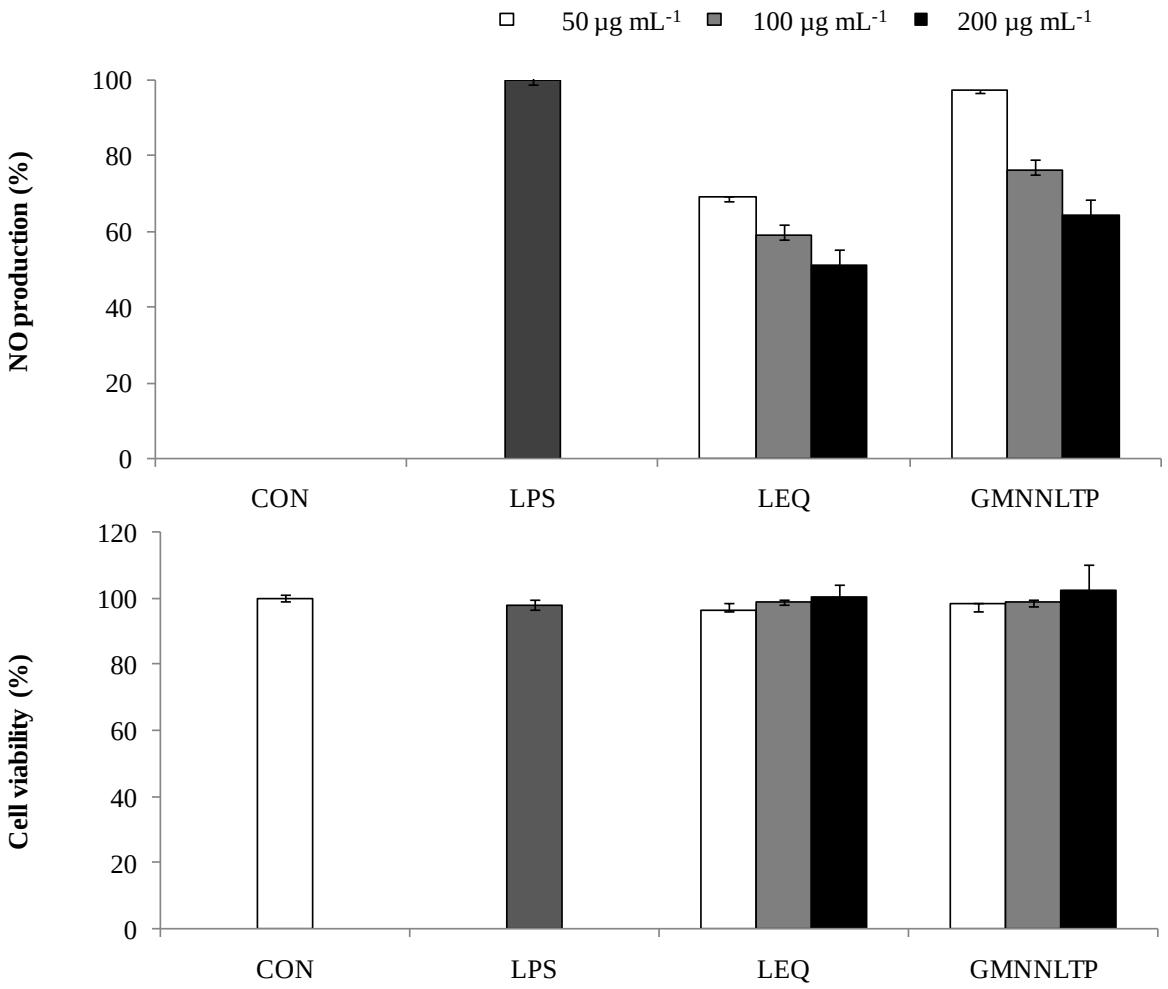

Fig. 27 The effect of anti-inflammatory effect of the synthetic peptides (LEQ and GMNNLTP) against LPS-induced RAW macrophages

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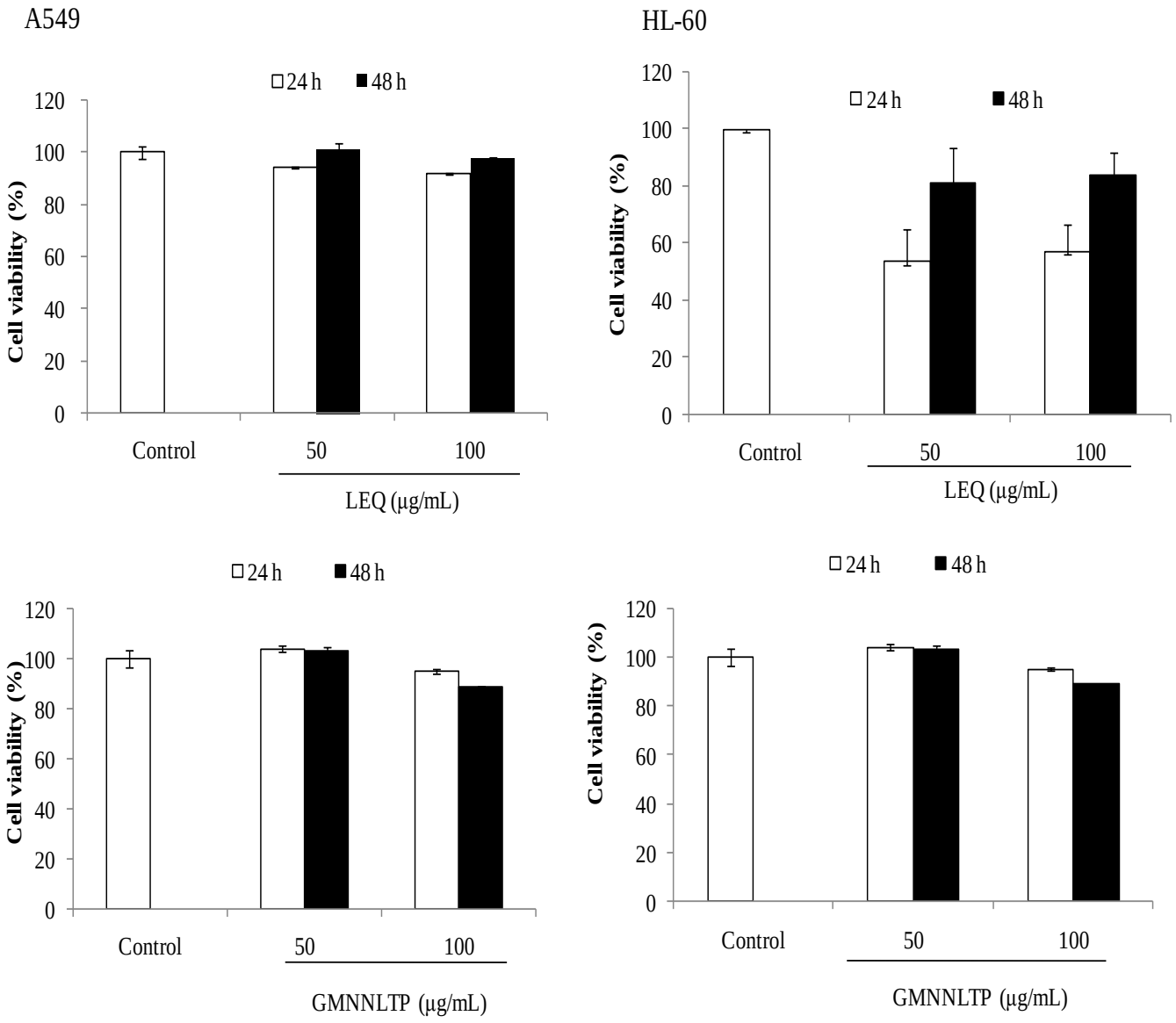

Fig. 28 The effect of anticancer activity of the synthetic peptides (LEQ and GMNNLTP) against A549 and HL-60 cell lines

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4. CONCLUSION

Among the synthesized peptides, GMNNLTP was the most potent ACE inhibitor. Molecular docking simulations of this peptides isomer (GMNNLTP-isomer 1) demonstrated that binds to the active site of ACE and interacted with the best pose compared to the commercial ACE inhibitor, captopril. In addition, LEQ peptide stabilized at the digestion against all the gastrointestinal enzyme tested in this study. However, GMNNLP was stabilized only in trypsin enzyme *in vitro*. During the digestion of synthetic peptide GMNNLTP against pepsin and α-chymotrypsin led to release LTP and NLT as new ACE active peptides respectively. Among the synthetic peptides, LEQ showed the potentiality to use as anticancer and anti-inflammatory agent and can be described as bio-functional effect on the isolated peptides from *N. oculata*.

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Part-IV

Isolation of apoptotic anticancer active compound from cultured *Phaeodactylum tricornutum* through screening bioactivities of cultured marine microalgae

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ABSTRACT

Marine microalgae are a promising source of organisms that can be cultured and targeted to isolate the broad spectrum of functional metabolites. In this study, two species of cyanobacteria, *Chlorella ovalis* and Nannchloropsis *oculata*, one species of bacillariophyta, *Phaeoductylum tricornutum*, and one species of Dinophyceae, *Amphidinium carterae* were cultured and biomasses used to evaluate possible bioactivities. Among the partitioned extracts, *N. oculata* hexane and chloroform fractions showed significantly the strongest anti-inflammatory activity at 6.25 μg mL^{-1} concentration. The anticancer effects of extracts were examined through screening of three different cancer cell lines including human promyelocytic leukemia (HL-60), a human lung carcinoma (A549) and a mouse melanoma (B16F10). *C. ovalis* ethyl acetate and *A. carterae* chloroform fractions suppressed the growth of HL-60 cells significantly at 25 and 50 μg mL^{-1} concentrations. In addition, a novel fatty alcohol ester; nonyl 8-acetoxy-6-methyloctanoate (NAMO) was identified among the five isolated compounds from cultured marine diatom, *P. tricornutum*. 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.

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1. INDRODUCTION

Marine microalgae are gaining a vast diversity due to biochemically and ecologically differences in the ecosystem and associated a broad spectrum of secondary metabolites (Borowitzca, 1995). In particular, utilizing of new bioactive natural products from marine bio-resources for the pharmacological applications have described as a promising aspects. The interest of isolation pharmacologically active metabolites from marine microalgae has been increased throughout the world. Moreover, screening of marine microalgae has received much attention as the rich sources of pharmacologically active functional ingredients in last decades. Microalgae bear an unusual quality of nutrients including rich source of protein, poly unsaturated fatty acids (PUFA), carbohydrates, minerals, vitamins, pigments and secondary metabolites (Becker ,2007). Thus, many of the prominent microalgae natural sources have targeted for the research and revealed that the health effects, for well being of humans (Liang et al., 2004) as well as domestic animal applications (Becker, 2007). However, there are some potential benefits over the nutritional value of microalgae, including antioxidant (Sheih et al., 2009), antitumor (Sheih et al., 2010), immunostimulant (Morris et al., 2007), antibacterial (Desbois et al., 2009), hypocholesterolemic effect (Dvir et al., 2009), and ACE-I inhibition activity (Samarakoon et al., 2013).

Moreover, marine microalgae cultivation has been practiced long time ago. Particularly, there can be described a couple of systems of algae culturing such as open culture and closed (photobioreactors) or artificial culture. Each one of the culturing systems has either desirable or undesirable effect, since the photosynthetic organisms and their mass production may directly correlate with the source of energy. Nevertheless, only selected few microalgae strains are used in controlled conditions for the cultivation in particular environments (Hong et al., 2012). The

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controlled optimum condition with the operational inputs such as salt, dissolved CO_2, water, nutrients, pH and O_2 providing a great opportunity for the steady environment without being contaminated in photo-bioreactors (Plaza et al., 2009). Hence, there has been a great interest among many researches about the cultured marine microalgae because their unrevealed biochemical constituents of the crude extracts and would be more helpful to identify their pharmacological effect (Kim and Wijesekara, 2010).

Marine diatoms are identified as the most important photosynthetic eukaryotes in the marine ecosystems (Wichard et al., 2006). There are estimated around more than ~10^6 species of diatoms and considered as 4-fold more diversity than angiosperms (Lenoci et al., 2008). Most of them are composing with silica derived cell wall and have been evolving from last 40 million years as single-celled algae with various morphological differences (Mann, 1989). Thus, *Phaeodactylim tricornutum*, a diatom of bacillariopyhceae has been known to contain high amount of functional ingredients including pigments (Carreto and catoggio, 1976), lipids (Fajardo et al., 2007), sulphated polysaccharides (Raposo et al., 2013), crude polysaccharide (Guzman et al., 2003) and peptides (Morelli et al., 2002).

In this part, we examine whether the cultured marine microalgae are producing bioactive secondary metabolites and attempted to isolate novel compounds using organic solvent-assisted extractions. Therefore we used the cultured marine microalgae including cyanobateria (*Chlorella ovalis, Nannchloropsis oculata*), bacillariophyta (*Phaeoductylum tricornutum*) and dinophyceae (*Amphidinium carterae*). Moreover, we determined the future potentiality of the isolated bioactive compounds from cultured marine microalgae for pharmacological and nutraceutical applications.

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2. MATERIALS AND MEHTODS

2.1 Chemicals and reagents

The murine macrophage cell line (RAW 264.7), 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), fetal 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) and Dimethyl Sulfoxide (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 microalgae

Marine microalgae culture conditions and harvesting techniques have been described in the Part 1 (2.2 and 2.3).

2.3 Solvent extractions and preparation of samples from cultured marine microalgae

Followed after freeze dried, the cultured marine microalgae were grounded into fine powder and each of the materials was homogenized separately. Then the homogenized marine microalgae samples were sonicated (ultra sound-assisted extraction) at 25 ˚C at 90 min each for three times using (80%) methanol. Crude methanol extracts were concentrated by evaporating the solvent under reduced pressure using rotary evaporator and further subjected to solvent-solvent partition chromatography. Four different fractions such as *n*-hexane, chloroform, ethyl acetate and aqueous extracts were fractionated with the varying of polarity as presented in (Fig. 29). Each of

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the separated fractions from different cultured marine microalgae evaporated the solvents further using rotary evaporator. Prior to the *in vitro* assays, the solvent (*n*-hexane, chloroform, ethyl acetate and aqueous) extractions from the four different samples as *P. tricornutum, C. ovalis, N. oculata* and *A. carterae* were prepared 100 $mgmL^{-1}$concentration each using dimethyl sulfoxide (DMSO). Further dilution was carried out using Dulbecco's Phosphate-Buffered Saline (DPBS) solvent to gain the desired concentration for the *in vitro* assay as appropriately.

2.4 Isolation of secondary metabolites from *Phaeoductylum tricornutum*

The lyophilized and homogenized *P. tricornutum* diatom (38g) 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 solvent under reduced pressure using 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 (3cm × 22 cm) using hexane and ethyl acetate as an increasing hydrophilic solvent system. 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 90:10 (v/v) solvent system and visualized the eluted fractions. Among the eluted 14 fractions from *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 and 90:10 (v/v), respectively. Furthermore, both F1 and F7 fractions were labeled tentatively as (PTH-1; Com 1) and (PTH-2;

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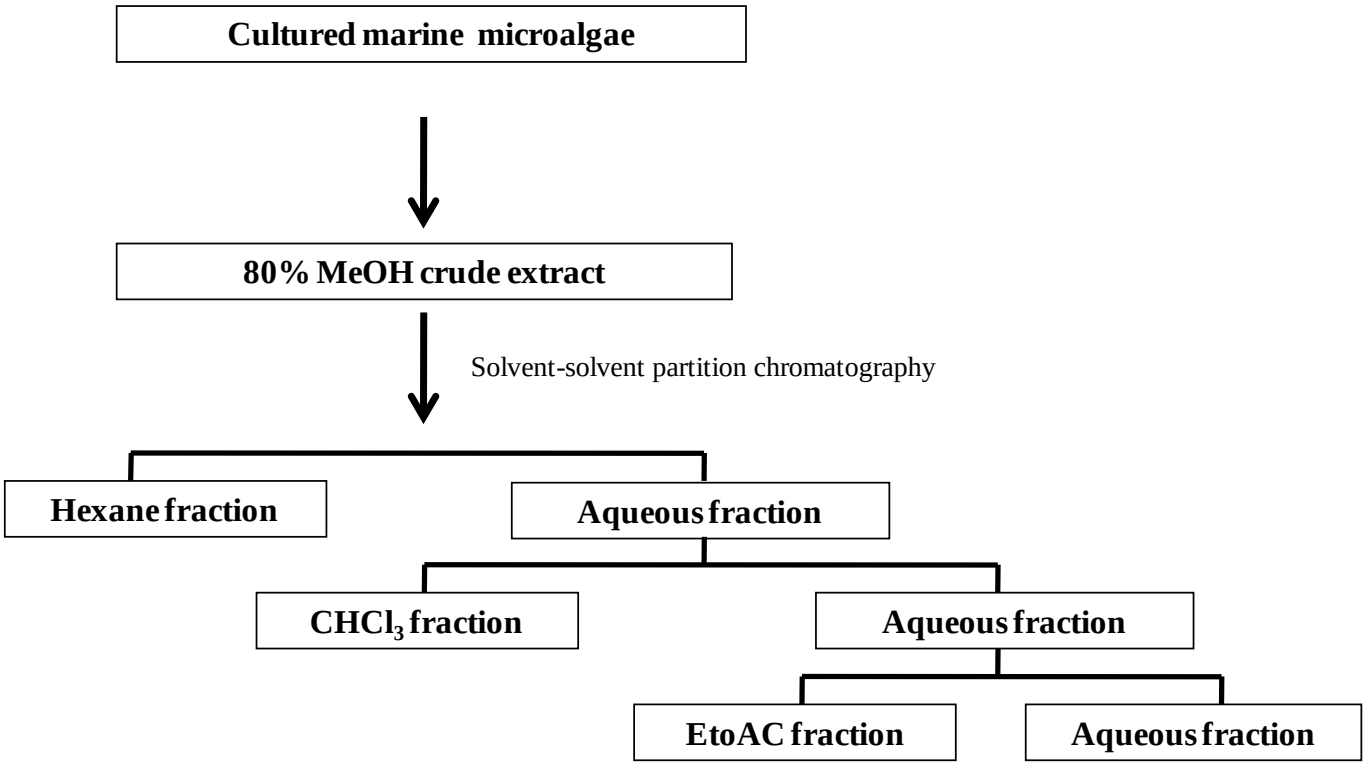

Fig. 29 Extraction approaches of cultured marine microalgae samples using solvent-solvent partition chromatography

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Com 2) until determine the molecular structure. In addition, F8 fraction (90 mg) was eluted at hexane-ethyl acetate 75:25 (v/v) solvent conditions 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.

2.5 Characterization of the active compounds

The molecular mass was measured using high performance liquid chromatography-diode array detector coupled with electro spray ionizing mass spectrometer (HPLC-DAD-ESI/MS) (Hewlett-Packard, Waldbronn, Germany). For the structure elucidation, NMR studies were done using 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 in room temperature conditions.

2.6 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) and RAW 264.7 (a murine macrophage cell line) were 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% fetal 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.

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2.7 Determination of anti-inflammatory activity

Assessment of nitric oxide (NO) and cell viability assays were concisely described in the Part I (2.11 and 2.12).

2.8 Cell growth inhibitory assay for anticancer activity

The cell growth inhibitory activity of cultured marine microalgae crude solvent extracts and isolated compounds against the cancer cells (HL-60, B16F10, and A549) were determined by the colorimetric MTT assay. Suspension cells (HL-60 cells) were seeded (2×10^4 cells mL^{-1}) together with the samples and incubated for 48 h before MTT treatment. Attached cells (B16F10 and A549) were seeded in a 96-well plate at a concentration of 2×10^4 cells mL^{-1}. At 24 h after seeding, the cells were treated with the samples. 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 2000 rpm 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.

2.9 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 to 490nm. 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

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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 color digital camera in order to determine the degree of nuclear condensation.

2.10 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 et al. (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 (Becton–Dickinson, SanJose, 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 et al., 1999).

2.11 Western blot analysis

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HL-60 Cells (2×10^5 cells mL^{-1}) were treated with the isolated compound (NAMO) by different 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 $mmolL^{-1}$Tris–HCl (pH 7.4), 150 $mmolL^{-1}$NaCl, 1% Triton X-100, 0.1% SDS and 1 $mmolL^{-1}$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 30µg of protein were subjected to electrophoresis using sodium dodecyl sulfate–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 $mmolL^{-1}$Tris–HCl, 137 $mmolL^{-1}$NaCl, 0.1% Tween 20, pH 7.4) containing 0.5% 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 an ECL Western blot detection kit.

2.12 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.

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3. RESULTS AND DISCUSSION

3.1 Anti-inflammatory effect of the extracts and fractions from cultured marine microalgae

In this study, the cultured marine microalgae four different strains including *P. tricornutum, C. ovalis, N. oculata* and *A. carterae* were subjected to screen the anti-inflammatory activity after being extracted by different organic solvents (Fig. 29). In fact, each of the microalgae crude (80%) methanol extract was separated into *n*-hexane, chloroform, ethyl acetate and aqueous solvents and determined the nitric oxide (NO) production inhibitory effect against LPS-induced RAW 264.7 cells. Hence, figure 30 A, 31 A, 32 A and 33 A are showing the inhibitory effect of NO production level of cultured marine microalgae including *N. oculata, P. tricornutum, C. ovalis* and *A. carterae* different extracts against LPS-induced RAW 264.7 cells in a dose dependent manner. In particular, *n*-hexane and chloroform fractions (NOH and NOC) from *N. oculata* extract significantly inhibited the NO productions at all the concentrations against LPS-induced RAW 264.7 cells *in vitro* (Fig. 30 A) compared to the positive control. Especially, at the lowest concentration as 6.25 μg mL^{-1} of extracts, *N. oculata* hexane (NOH) and *N. oculata* chloroform (NOC) exhibited less than 40% of NO production against LPS-induced RAW 264.7 cells. Moreover, *C. ovalis* ethyl acetate (COE), *A. carterae* chloroform (ACC), *P. tricornutum* hexane (PTH), and *P. tricornutum* chloroform (PTC) fractions showed a significant inhibition of NO production compared to the other fractions extracted from the cultured marine microalgae (Figs 31 A, 32 A & 33 A), respectively.

In addition, the cytotoxic effects on RAW 264.7 cells with the treated samples were performed by MTT assay.

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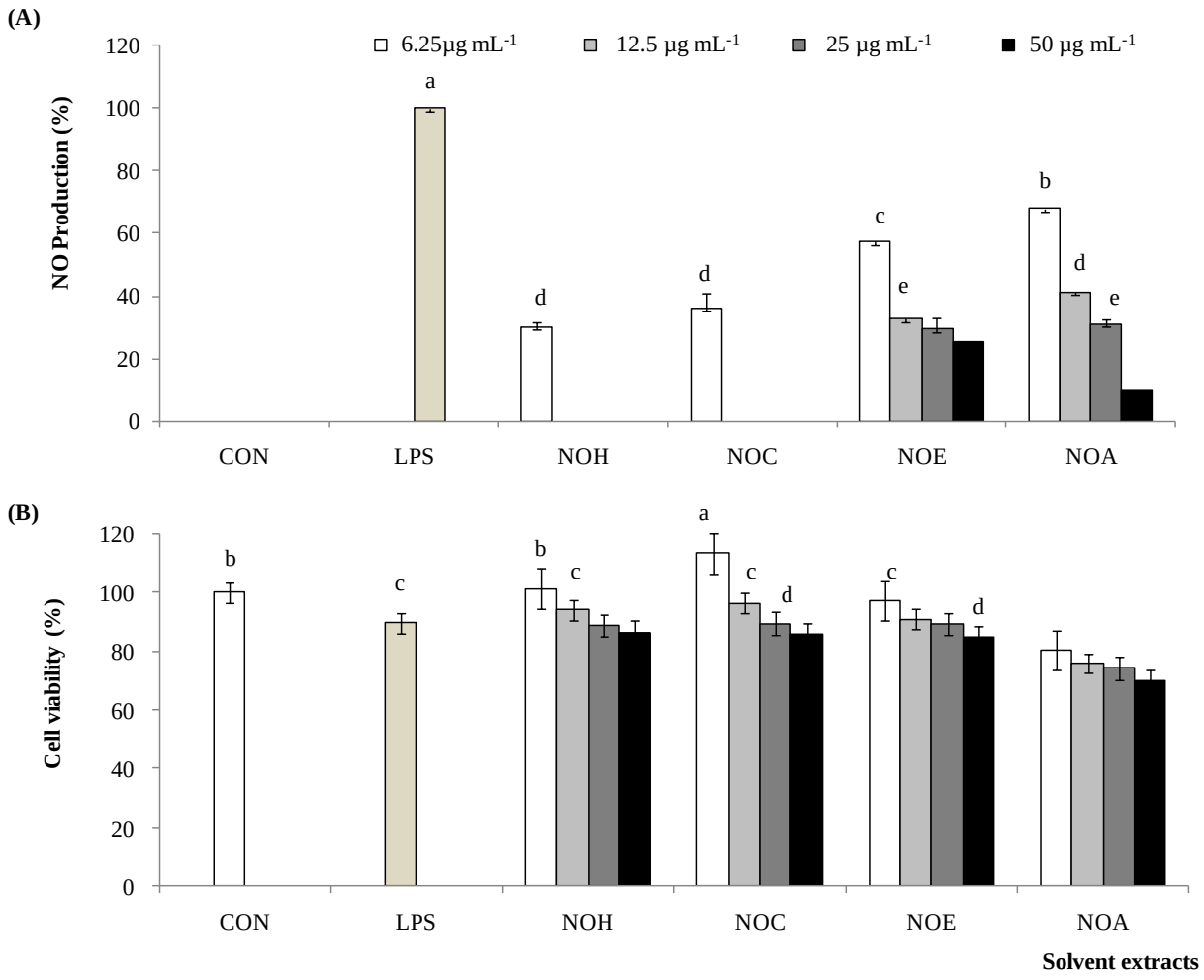

Fig. 30 Inhibitory effect of cultured marine microalga *Nannchloropsis oculata* solvent extracts by solvent-solvent partition chromatography on (A): LPS-induced NO production (%) and (B) cell viability (%) in RAW 264.7 macrophages. The Incubation of extracts, NOH: *N. oculata* hexane fraction; NOC: *N. oculata* chloroform fraction; NOE: *N. oculata* ethyl acetate fraction and NOA: *N. oculata* aqueous fraction with cells in response to LPS (1 μg ml^{-1}) for 24 h, the nitric oxide (NO) levels in the medium was measured. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

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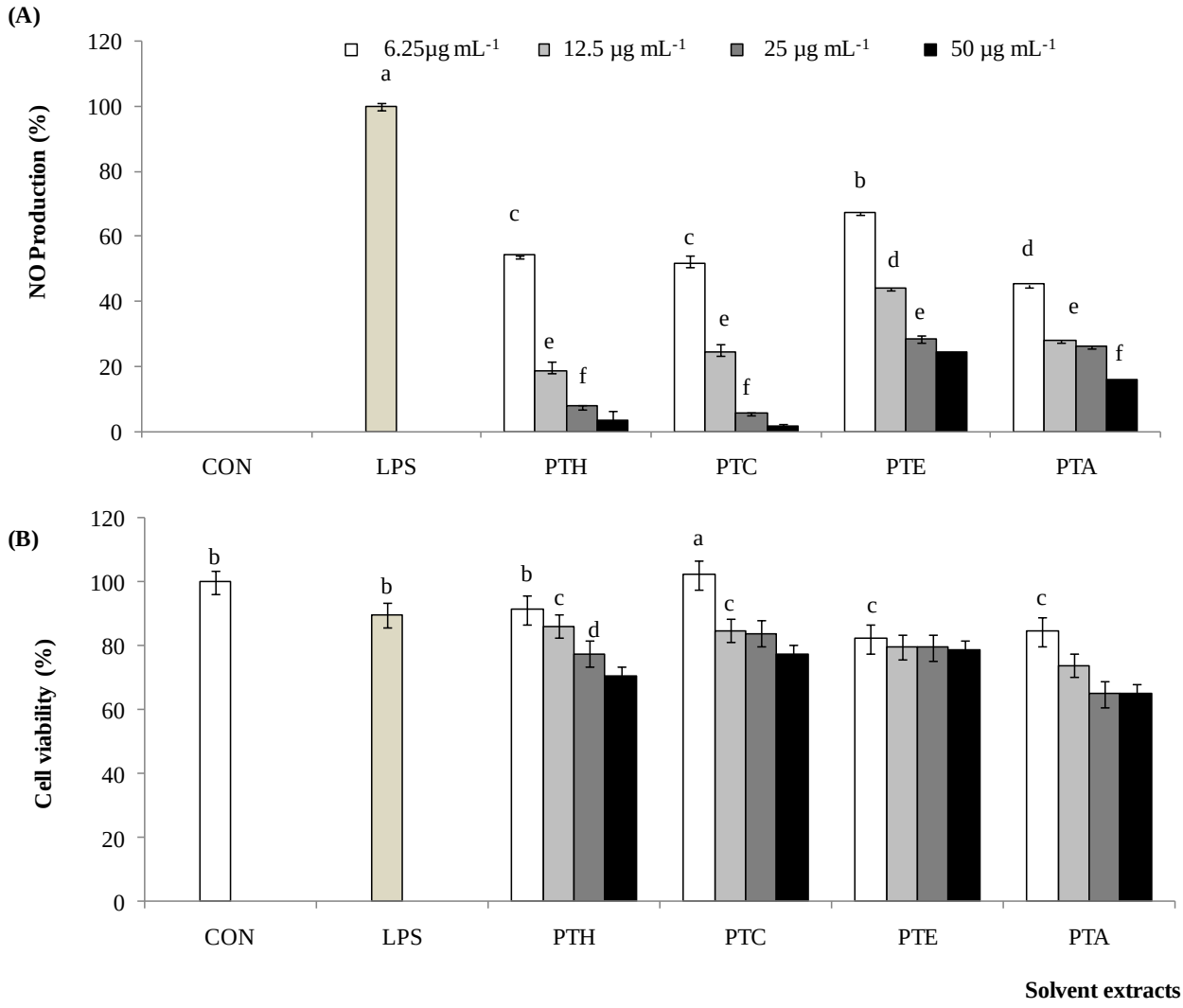

Fig. 31 Inhibitory effect of cultured marine microalga *Phaeoductylum tricornutum* solvent extracts by solvent-solvent partition chromatography on (A): LPS-induced NO production (%) and (B) cell viability (%) in RAW 264.7 macrophages. The Incubation of extracts, PTH: *P. tricornutum* hexane fraction; PTC: *P. tricornutum* chloroform fraction; PTE: *P. tricornutum* ethyl acetate fraction and PTA: *P. tricornutum* aqueous fraction with cells in response to LPS (1 μg ml^{-1}) for 24 h, the nitric oxide (NO) levels in the medium was measured. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

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The cell viability (%) of the extracted samples from the cultured marine microalgae was shown in the Figs 30 B, 31 B, 32 B & 33 B. According to the results, NOH and NOC fractions were reported to have the high cell viability against RAW 264.7 cells compared to the other fractions followed by LPS induced toxicity. More importantly, the concentration of NOC at 6.25 μg mL^{-1} was shown the highest cell viability as nearly 120% compared to the negative control. In addition, same the concentration of PTC and NOH are also reported more than 100 (%) of cell viability with comparatively significant differences. The fractions of *C. ovalis,* (COC) and COE were shown to be nearly 100% of cell viability at 25 μg mL^{-1} and 50 μg mL^{-1} concentrations, respectively. These results are evidenced that the increased cell viability of the samples against LPS treated toxicity followed by significant suppression of NO production as well. Recently reported many publications have indicated that containing of anti-inflammatory components in *P. tricornutum* and *Chlorella stigmatophora* organic solvent extracts (Guzmán et al. 2003). Therefore, these results were suggested that the strong anti-inflammatory activities were observed in the extracts and the fractions derived from the cultured marine microalgae *in vitro* assay.

3.2 Anticancer effect against HL-60, B16F10, and A549 cell lines

The cell growth inhibitory effect was examined against different cancer cell lines followed by pre-treated cultured marine microalgae solvent extracts and fractions for screening anticancer activity of the samples. Figs 34 and 35 represent the cell viability against treated samples from cultured marine microalgae including *C. ovalis* and *A. carterae* extracts on the different cell lines such as a human promyelocytic

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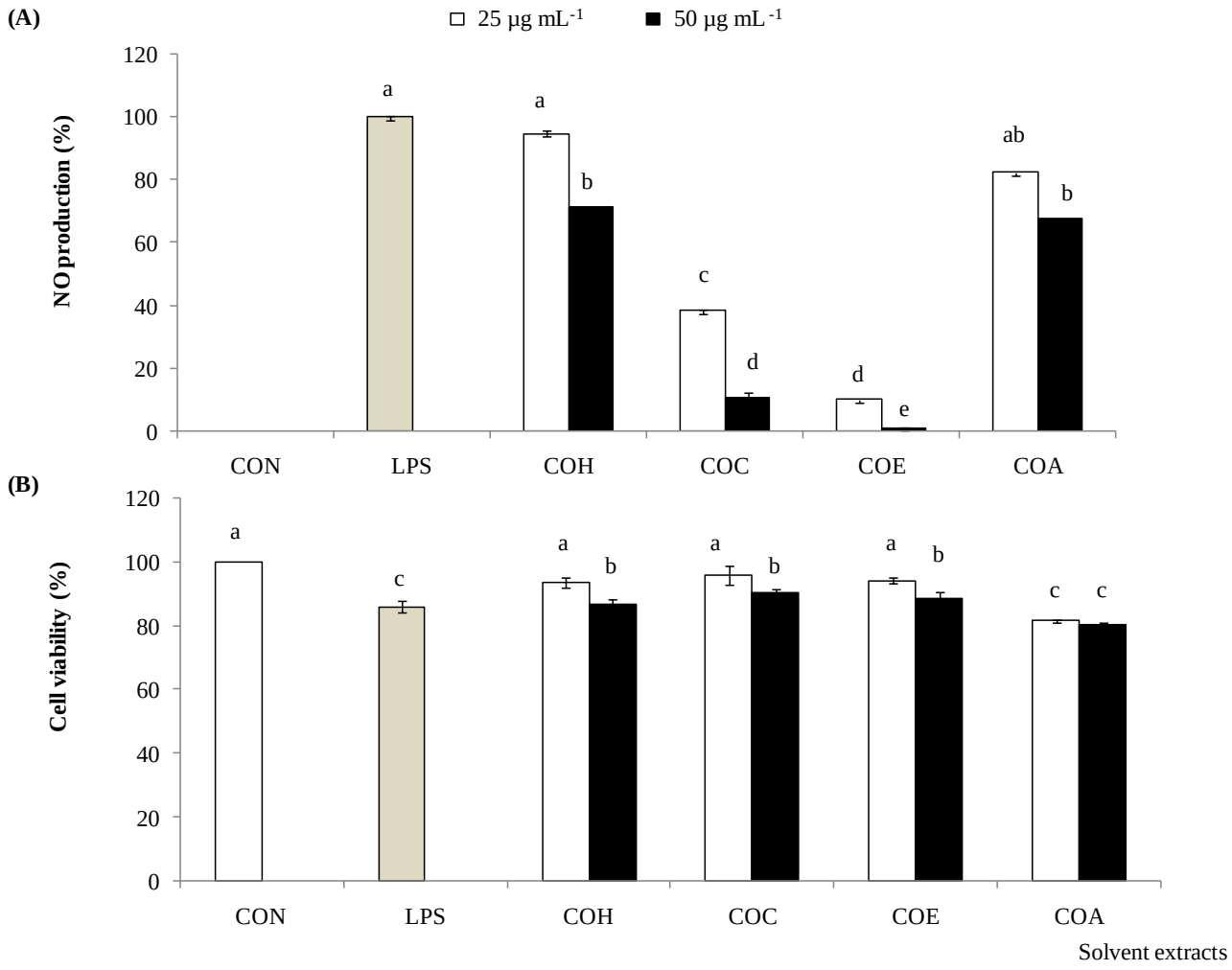

Fig. 32 Inhibitory effect of cultured marine microalga *Chlorella ovalis* solvent extracts by solvent-solvent partition chromatography on (A): LPS-induced NO production (%) and (B) cell viability (%) in RAW 264.7 macrophages. The Incubation of extracts, COH: *C. ovalis* hexane fraction; COC: *C. ovalis* chloroform fraction; COE: *C. ovalis* ethyl acetate fraction and COA: *C. ovalis* aqueous fraction with cells in response to LPS (1 μg ml^{-1}) for 24 h, the nitric oxide (NO) levels in the medium was measured. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

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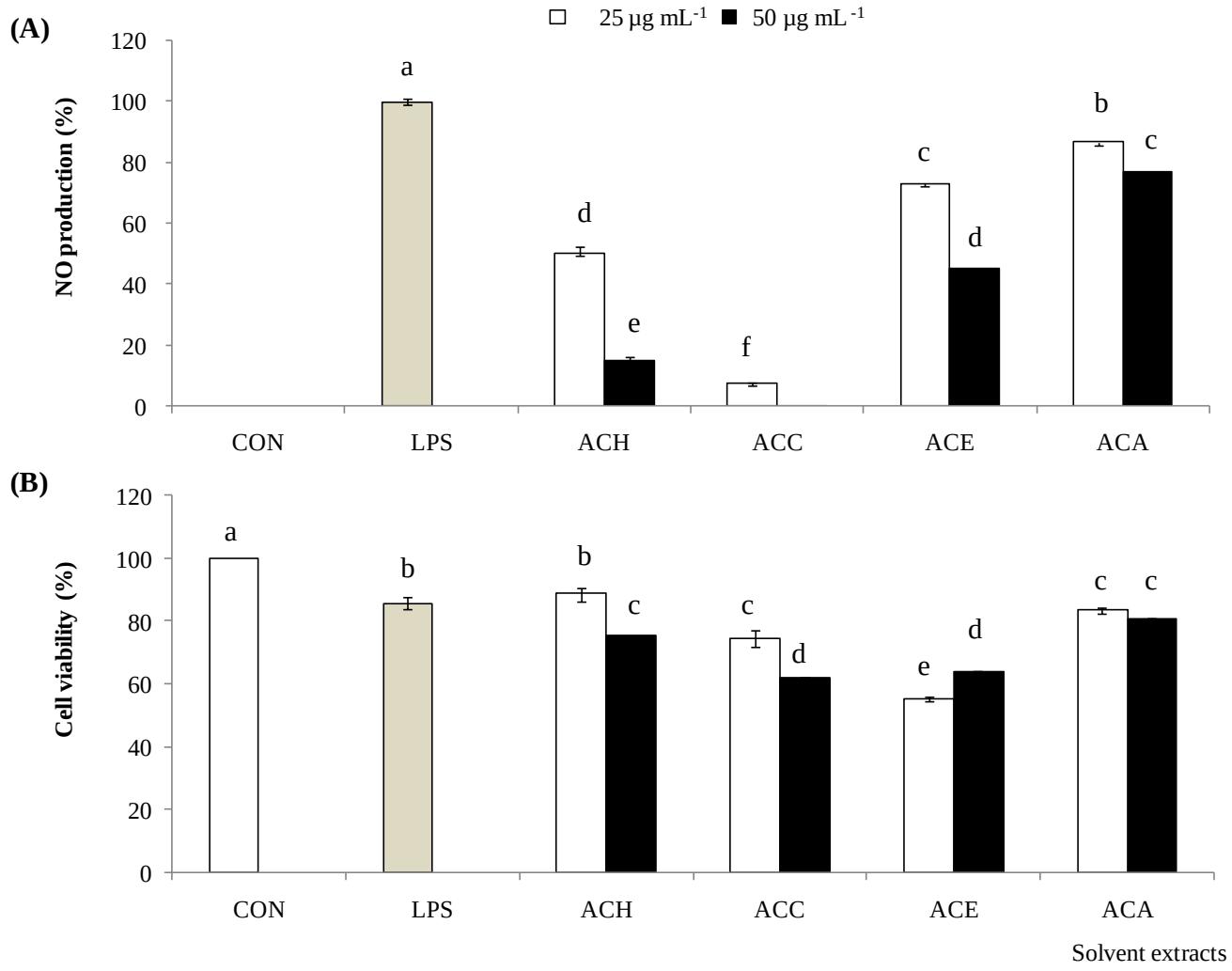

Fig. 33 Inhibitory effect of cultured marine microalga *Amphidinium carterae* solvent extracts by solvent-solvent partition chromatography on (A): LPS-induced NO production (%) and (B) cell viability (%) in RAW 264.7 macrophages. The Incubation of extracts, ACH: *A. carterae* hexane fraction; ACC: *A. carterae* chloroform fraction; ACE: *A. carterae* ethyl acetate fraction and ACA: *A. carterae* aqueous fraction with cells in response to LPS (1 μg ml^{-1}) for 24 h, the nitric oxide (NO) levels in the medium was measured. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

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leukemia cell line (HL-60) (Figs 34 A & 35 A), a mouse melanoma cell line (B16F10) (Figs 34 B & 35 B) and a human lung carcinoma cell line (A549) (Figs 34 C & 35 C), respectively.

In this study, significant growth inhibitory effects were observed from COE and ACC against HL-60 cell lines and the anticancer activities were dose dependent at 25 μg mL^{-1} and 50 μg mL^{-1} concentrations. The cultured dinoflagellate, *A. carterae* extracts such as hexane (ACH), chloroform (ACC), and ethyl acetate (ACE) fractions also effectively suppressed the growth of HL-60, B16F10, and A549 cell lines *in vitro* compared to the control. To our knowledge, few of reports have been published with the extracts of bioactive metabolites from the cultured marine microalgae. Recently, two monogalactosyl diacylglycerols were isolated from the cultured marine *P. tricornutum* and reported as inducing apoptosis in two genetically-matched immortal mouse epithelial cell lines (Andrianasolo et al. 2008). Moreover, these results indicate the potential of cultured marine microalgae species for anticancer activities and should lead to study further on secondary metabolites.

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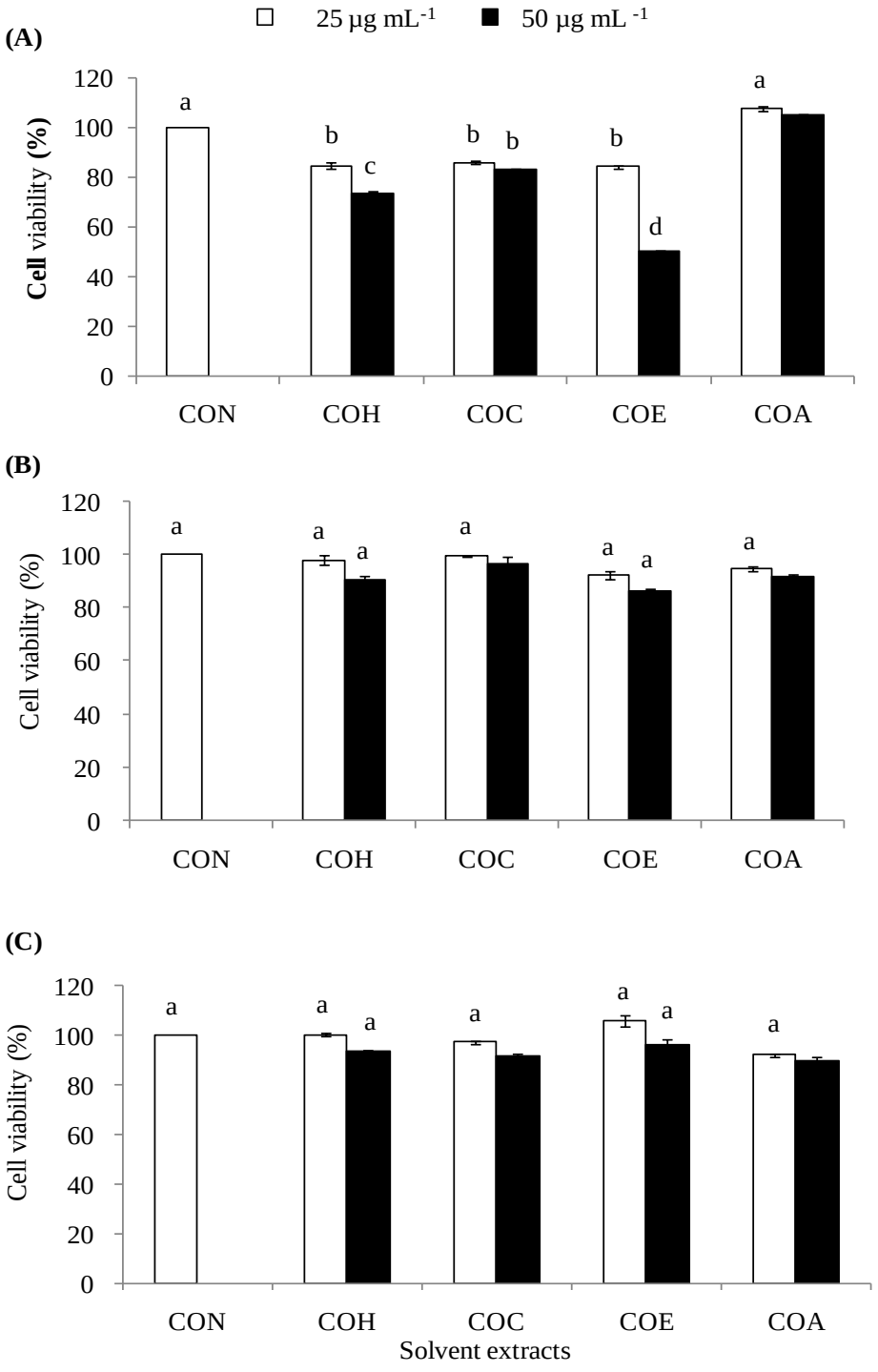

Fig. 34 Inhibitory effect of the growth of cancer cells against cultured marine microalga *Chlorella ovalis* solvent extracts by solvent-solvent partition chromatography on **(**A): HL-60, (B): B16F10, (C): A549 cell lines.

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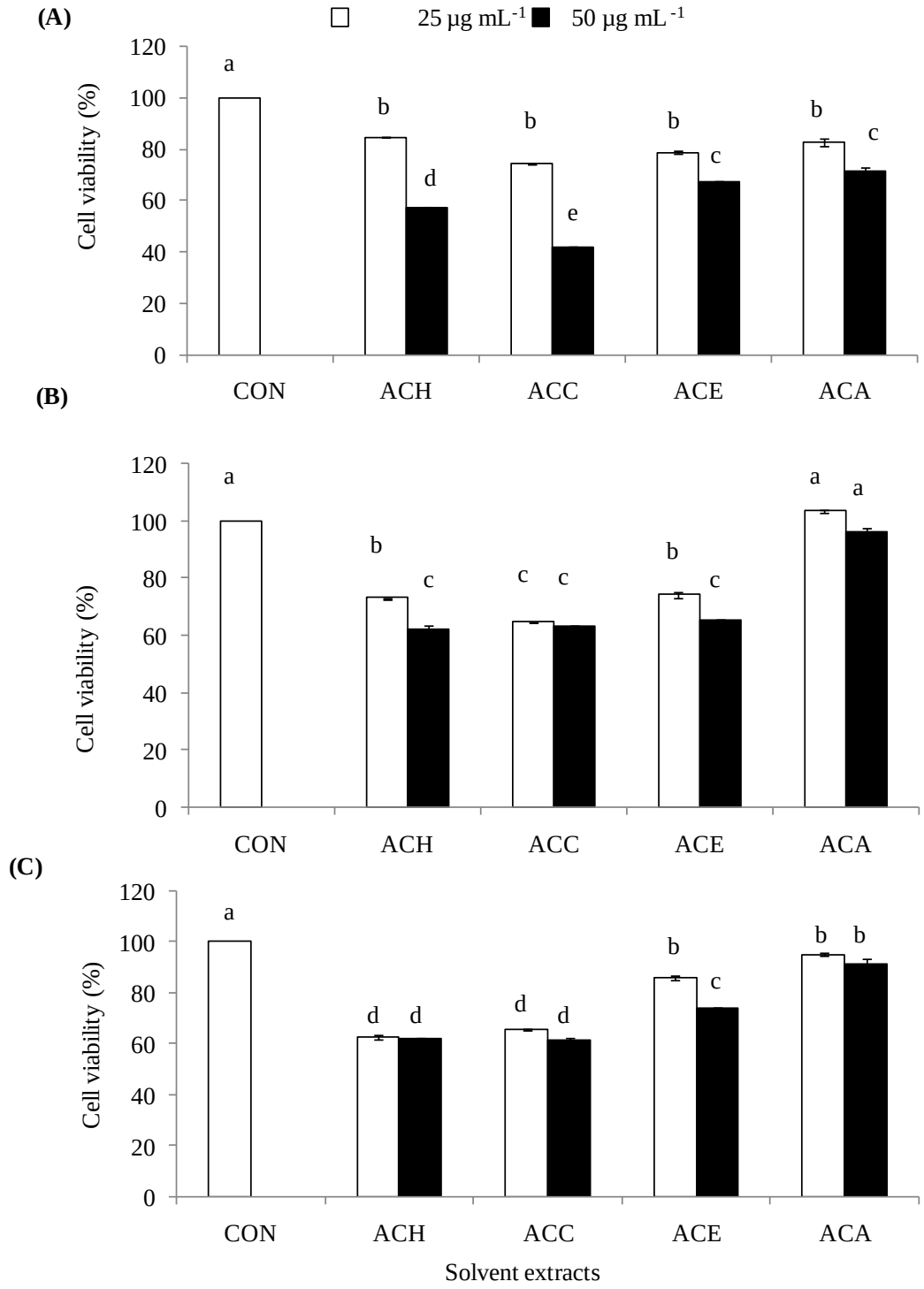

Fig. 35 Inhibitory effect of the growth of cancer cells against cultured marine microalga *Amphidinium carterae* solvent extracts by solvent-solvent partition chromatography on **(**A): HL-60, (B): B16F10, (C): A549 cell lines.

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3.3 Growth inhibitory activity of the isolated compounds from *P. tricornutum* against HL-60, B16F10, and A549 cancer cell lines

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 three cancer cell lines such as HL-60, B16F10 and A549 *in vitro* (Fig. 36). According to the MTT assay results, among the isolated compounds from *P. tricornutum* hexane fraction, Com 2 was only showed the significant lower cell viability 32.8 % and 66.4 % at 50 μg mL^{-1} incubated concentrations for 48 h against HL-60 and A549 cancer cells, respectively. However, the determined cell viability (%) of other compounds against all the cancer cells tested was higher than the Com 2 at 50 μg mL^{-1} concentration significantly. Therefore, the isolated Com 2 was selected for further studies due to inhibit the growth of HL-60 cancer cell growth markedly.

3.4 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 (Fig 37). 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

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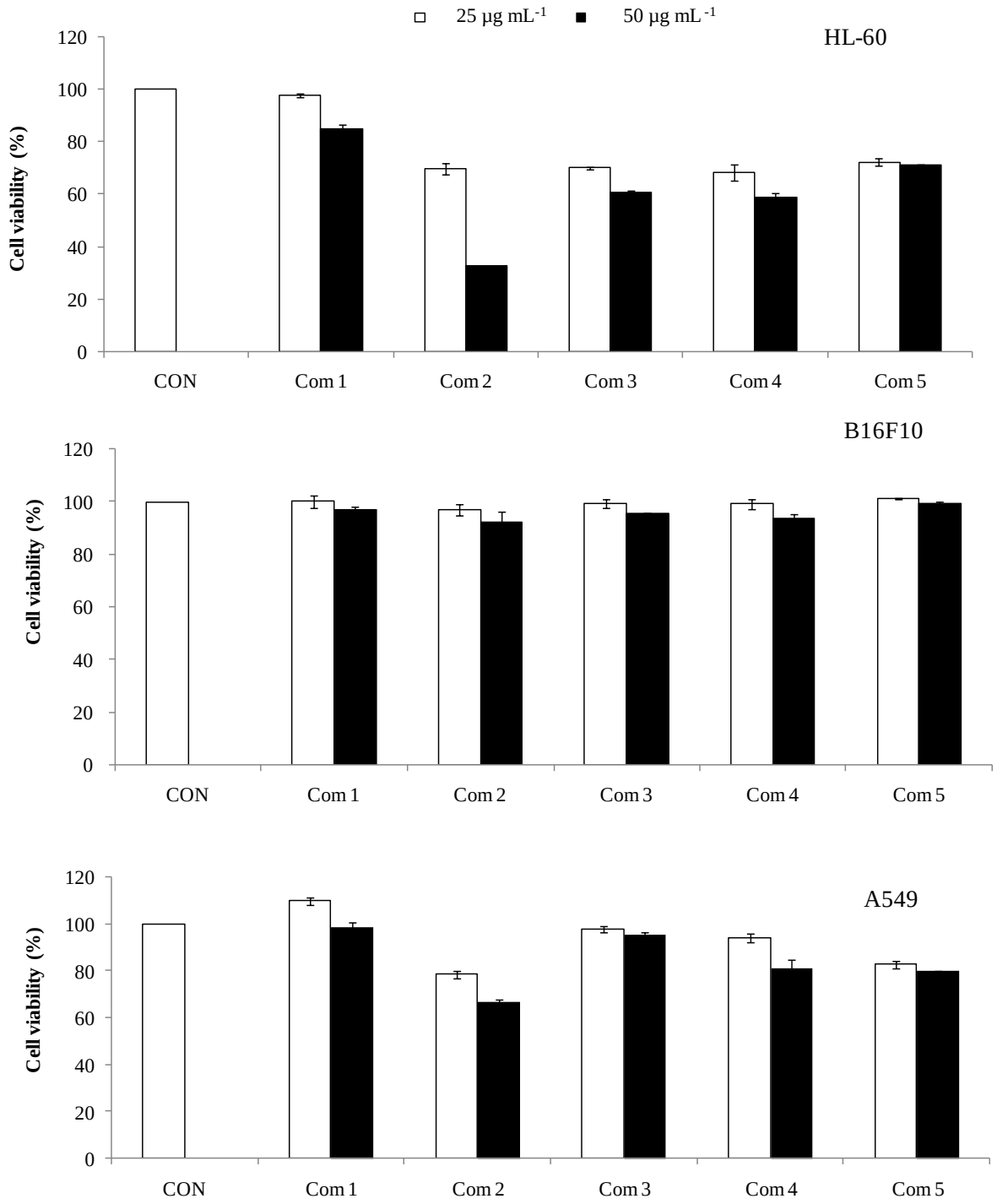

Fig. 36 The effect of cell viability (%) of HL-60, B16F10 and A549 cancer cells against isolated five compounds from the cultured marine diatom, *Phaeodactylum tricornutum* for 48 h.

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and 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, examine the heteronuclear correlation (HSQC) spectrums showed that presence of substitute 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. 37). Furthermore, assigned multiplicity of the isolated compound was represented in Table 10. NMR experiment data are suggested that the molecule has an aliphatic ester character and literature survey at this stage found that the isolated compound is showing a similar skeleton to nonyloctanoate. 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.*

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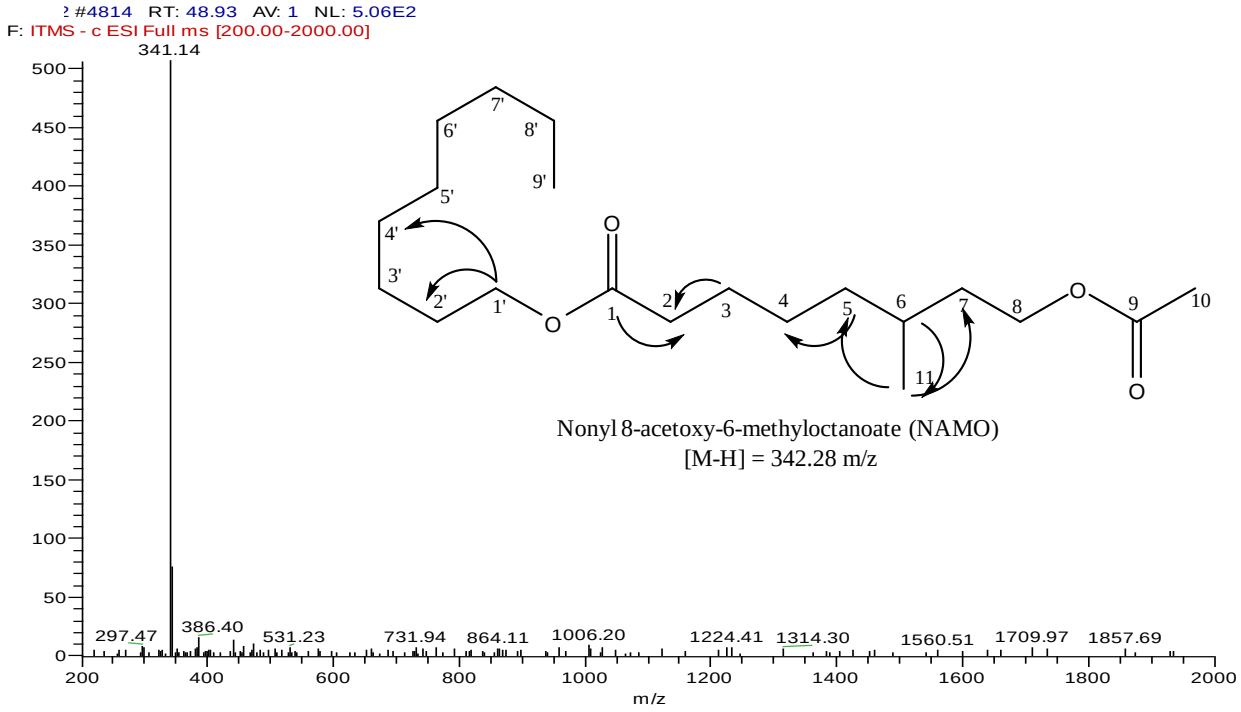

Fig. 37 Mass spectra and chemical structure of the novel fatty alcohol ester: nonyl 8-acetoxy-6-methyloctanoate (NAMO) isolated from the hexane fraction of cultured marine diatom, *Phaeodactylum tricornutum.* The spectrum was determined in negative ionization mode and the HMBC correlations ↷ in the NAMO compound appropriately

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Table 10. 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

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

Figure 38 is presented the cell viability (%) of the cancer cells including HL-60, B16F10 and A549 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 dose dependently compared to the control. In particular, the highest growth inhibitory activity approximately about 70% on HL-60 cells were observed at 50 μg mL^{-1} treated concentration of NAMO compared to the control. However, A549 cell growth suppression was moderate and examined as about 35% at 50 μg mL^{-1} incubated NAMO concentration significantly ($P > 0.05$). The isolated active compound was not significantly reduced the growth of B16F10 cells compared to the control. Therefore, cellular regulatory effect was assessed further to examine whether the apoptosis inducing on HL-60 cancer cells respect to NAMO different concentrations.

3.6 NAMO induced apoptosis in HL-60 cells

The isolated lipid ester compound (NAMO) various concentrations at (12.5~100 μg mL^{-1}) were incubated with the HL-60 cells for 48 h and determined the 50 % of inhibitory concentration (IC_{50}) value as 22.3 μg mL^{-1} (data not shown). 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. 39). In this experiment, the control (no treated sample; A) showed the intact cell nuclei without DNA damage. However, along the treatment NAMO different concentrations, clear DNA damages were observed and led to increase the apoptotic body formations dramatically (Fig. 39 B, C, D and E).

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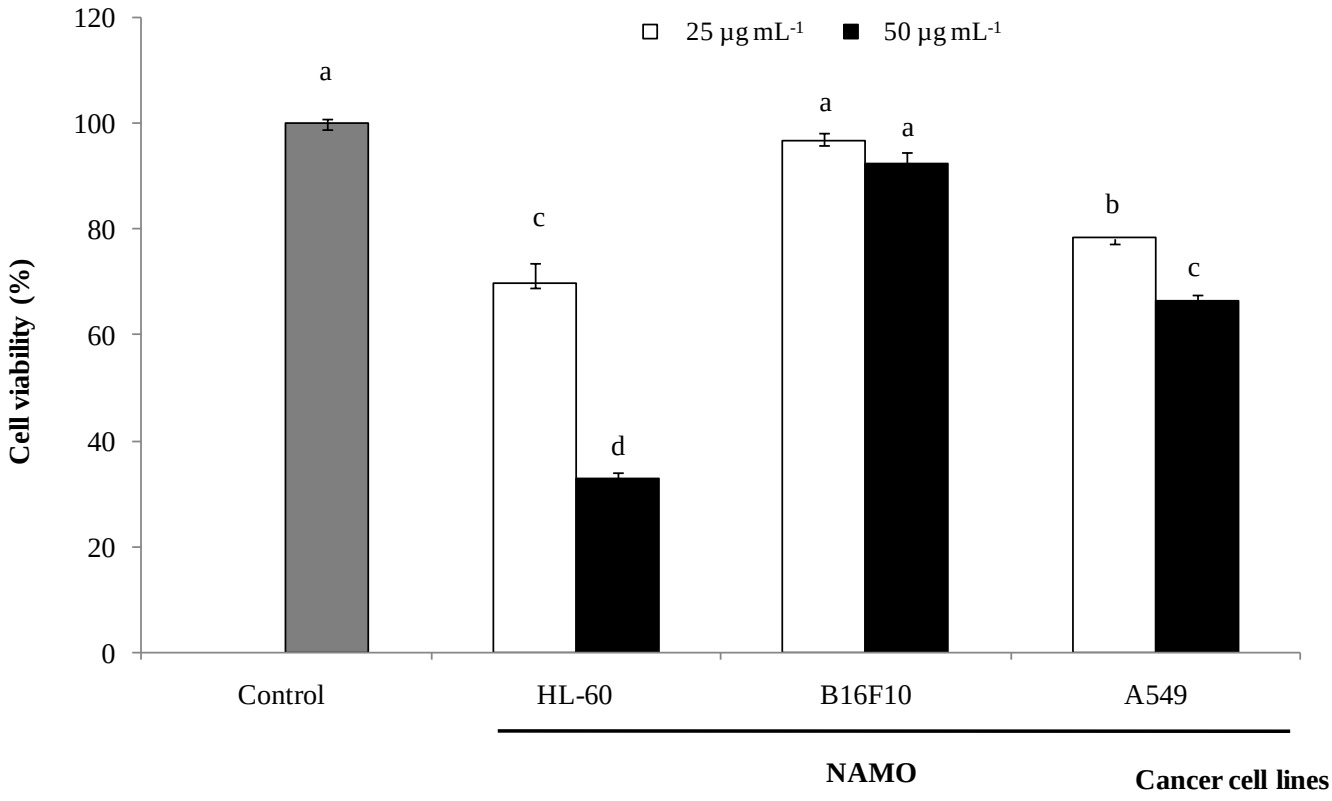

Fig. 38 The effect of cell viability (%) of cancer cell lines including HL-60, B16F10, and A549 against isolated compound, (Com 2) NAMO from the cultured marine diatom, *Phaeodactylum tricornutum* for 48 h. Values are expressed as means ± SD in triplicate experiments. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

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3.7 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 (%) respect to the incubated NAMO different concentrations. According to the results, it is observed that increasing the effect of the cell cycle arrest and the accumulation of cells in the sub-G_1 phase in concentration dependent manner (Fig. 40 B, C, D and E). Moreover, the apoptotic body formations were reflected to the accumulation of sub-G_1 content and further 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. 40 F). These evidences suggest that NAMO induced cell death was caused due to the apoptotic body formations significantly at concentration dependent manner.

3.8 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 regulates the NAMO–induced apoptosis on HL-60 cells (Fig. 41). 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 at after 50 μg mL^{-1} NAMO concentration and p53 protein expression also increased compared to the control. The apoptotic protein expressions were followed to understand the mechanism of regulatory effect of HL-60 cells against anticancer agent.

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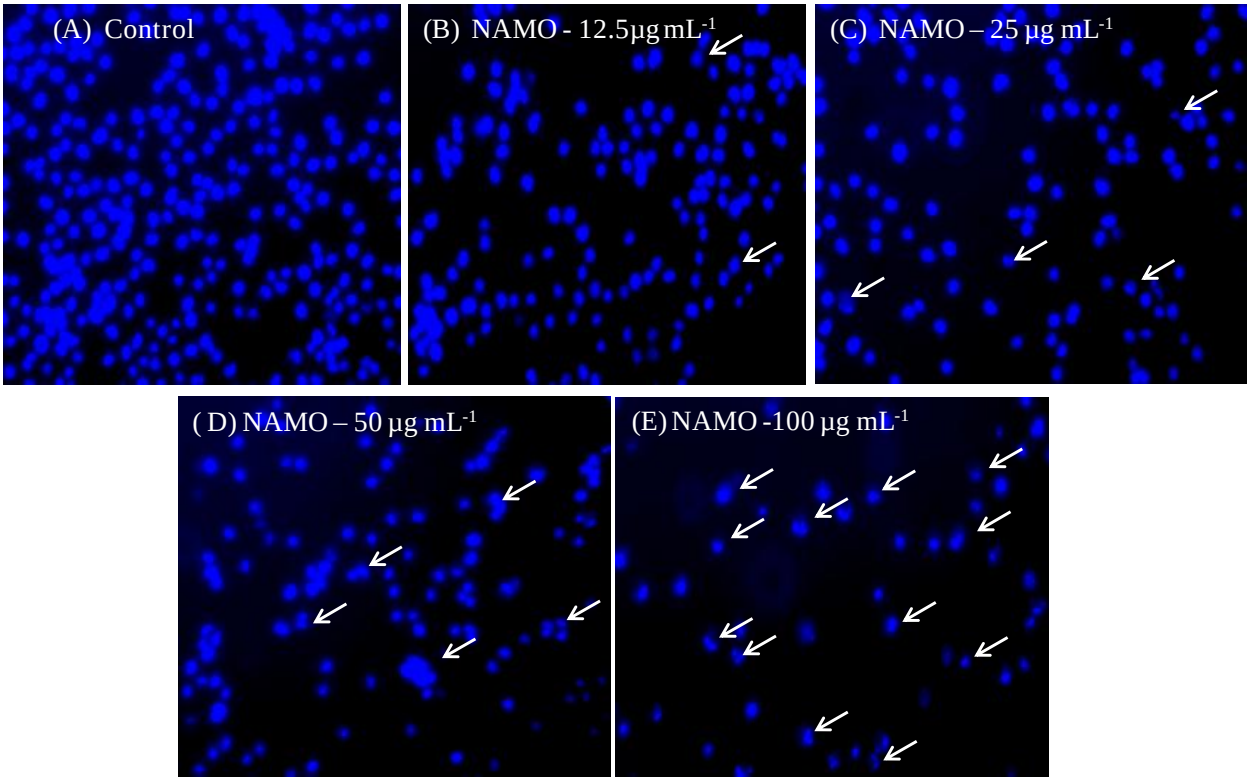

Fig. 39 Induction of apoptotic body formation in HL-60 cells was observed under a fluorescent microscope after Hoechest 33342 staining. (A) control: no treated; (B) NAMO treated with 12.5 µg mL^{-1}; (C) NAMO treated with 25 µg mL^{-1}; (D) NAMO treated with 50µg mL^{-1}; (E) NAMO treated with 100 µg mL^{-1}. *Arrows* denoted a typical apoptotic body formation in HL-60 cells.

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In that case, major apoptotic proteins such as Bax, Bcl-xL, caspase-3 and p53 were studied. In conversely, Bax is pro-apoptotic member of protein

that resides the cytosol, which translocations upon the apoptosis induction to the mitochondria (Cory and Adams, 2002). On the other hand, the Bcl-xL as anti-apoptotic proteins is overproduction due to the proliferations and often found to be promoting the cell survivability (Kang and Reynolds, 2009). Therefore, the ratio between the Bax/Bcl-xL can be suggested to regulate the apoptosis process. In the western blot analysis, we found that the up-regulation of Bax and down–regulation of Bcl-xL proteins significantly as NAMO dose-dependent manner. Therefore, the isolated compound NAMO was increased the Bax/Bcl-xL ratio and led to increase the apoptotic pathway against HL-60 cells growth. The protein expression of the p53 was up-regulated and led to decrease the viability of the HL-60 cells which suppressed the proliferation as well. Thus, p53 mediated apoptosis process was controlled the cell differentiation and change the rate of kinetics in cancer cells (Ronen et al., 1996). Moreover, caspase-3 protein expression was increased markedly at 50 and 100 μg mL^{-1} NAMO incubated concentrations. These evidences are suggesting that rapid cause of induced caspase-3 proteins may lose of mitochondrial transmembrane potential and release the 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 the mitochondrial apoptotic pathway (Budihardjo et al., 1999). The recent reports have been supported that mechanism involving for the suppression of cancer cell growth (Wang et al., 1999).These data suggested that the caspase-3 is the one major executioner of apoptosis that mediated through the p53 apoptotic pathway to regulate the growth of HL-60 cells followed by the NAMO treated concentration at dose-dependently.

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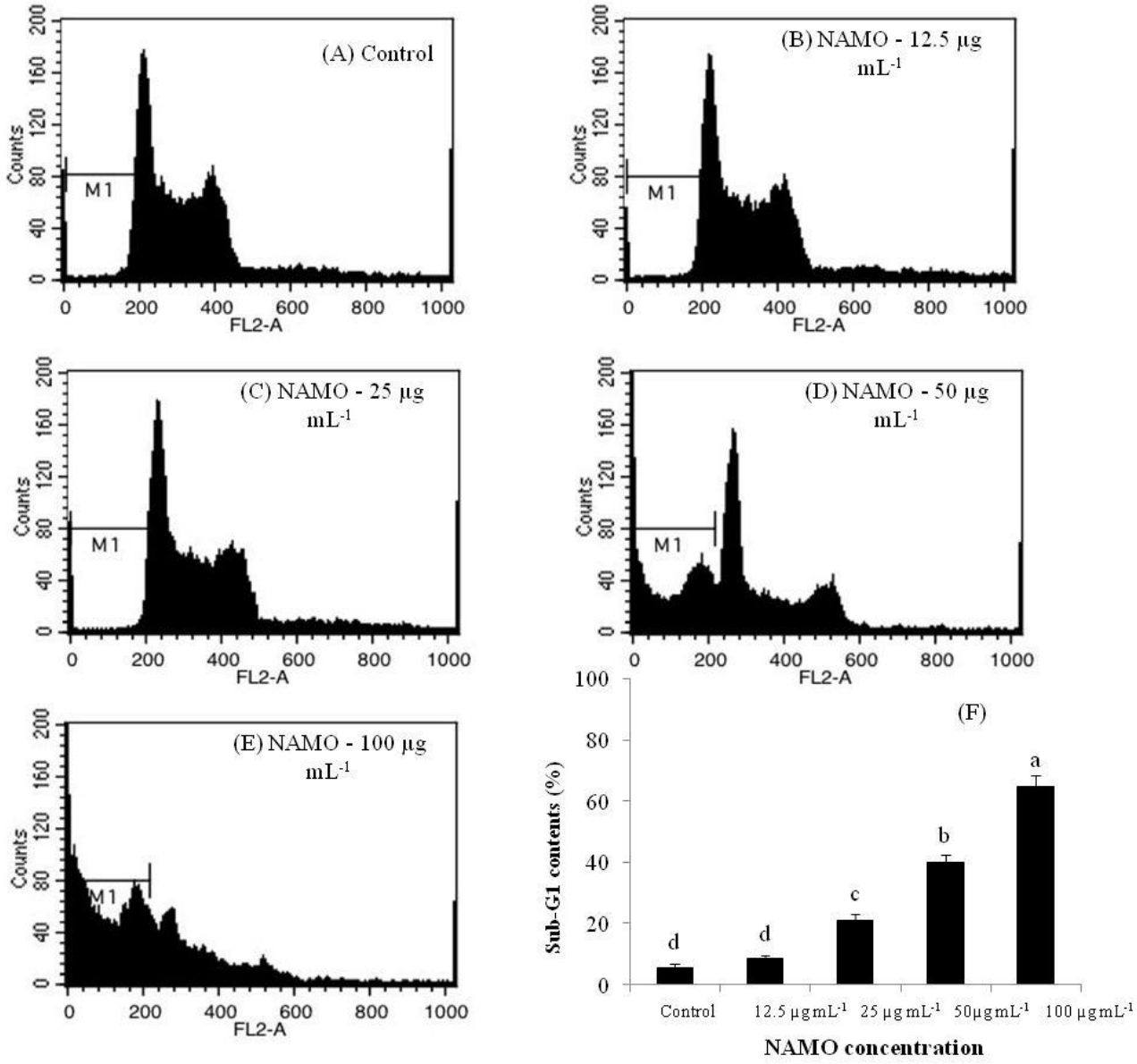

Fig. 40 Apoptotic Sub-G1content was detected by flow cytometry after stained with PI. (A) control: no treated; (B) NAMO treated with 12.5 µg mL^{-1}; (C) NAMO treated with 25 µg mL^{-1}; (D) NAMO treated with 50µg mL^{-1}; (E) NAMO treated with 100 µg mL^{-1}, (F) Accumulation of DNA content (%) in the Sub-G_1 phase of the cell cycle of HL-60 incubated with NAMO different concentrations were determined by flow cytometry.

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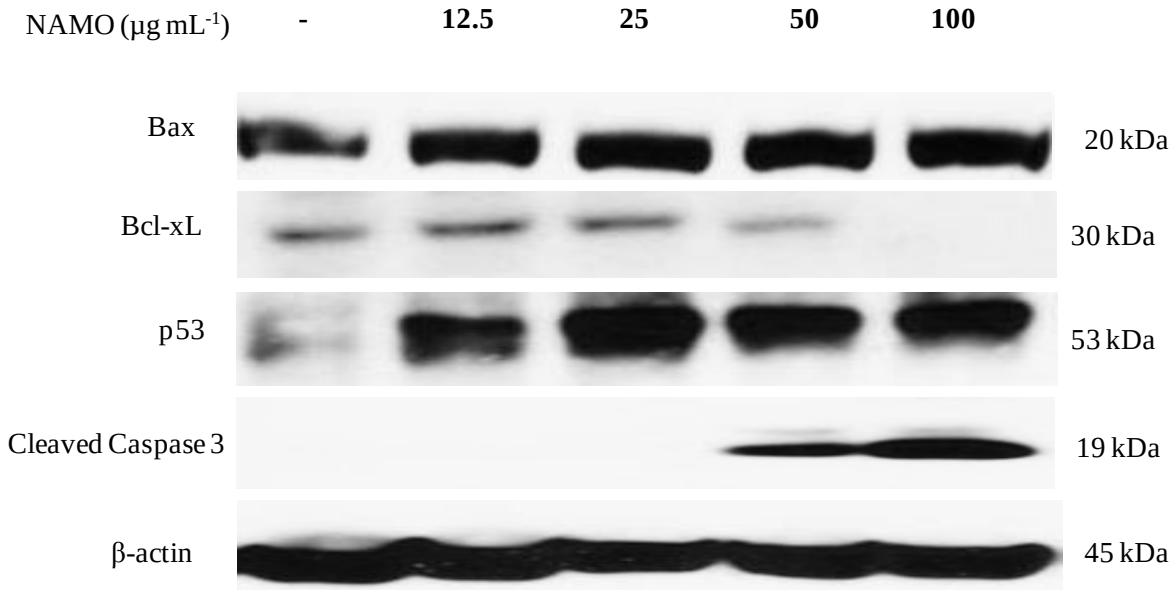

Fig. 41 Effect of NAMO on apoptosis-related proteins in HL-60 cells. The cells were incubated with NAMO different concentrations for 12 h and cell lysates were subjected for the western blot analysis. Antibodies were used as Bax, Bcl-xL, p53 and Cleaved caspase-3. β-actin used as internal control.

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4. CONCLUSION

In this study, four different cultured marine microalgae crude solvent extracts were screened for anti-inflammatory and anticancer effects *in vitro*. In particular, 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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Part-V

Isolation of lipids from cultured *Phaeodactylum tricornutum* with anti-inflammatory effects against lipopolysaccharide (LPS)-induced RAW macrophages

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ABSTRACT

Anti-inflammatory effect of the isolated five compounds such as nonyl 8-acetoxy-6-methyloctanoate (NAMO), 24-methylcholesta-5(6), 22-diene-3β-ol (MCDO), Cholestra-5(6), 22-dien-3, 24β-diol (CDDO), Cholestra-5(6), 22-dien-3, 24β-diol, methyl ether (CDDME) and Icosa-5, 8, 11, 14-tetraenyl acetate (ITEA) from cultured marine diatom, *Phaeodactylum tricornutum* against LPS-induced RAW macrophages were evaluated. All the compounds showed the profound nitric oxide (NO) inhibitory activity dose-dependently and no cytotoxicity was observed by MCDO. CDDO, CDDME and ITEA in both LDH and MTT assays. Among the isolated compounds, MCDO and CDDO were performed to assess the release of pro-inflammatory cytokines, interleukin-1β (1L-1β) and interleukin-6 (1L-6) and showed to be suppressed in a dose-dependent manner. Interestingly, prostaglandin-E_2 (PGE_2) production was significantly inhibited by both the compounds. However, tumor necrosis factor-alpha (TNF-α) production was not suppressed significantly by both compounds. Western blot assay confirmed that the MCDO and CDDO dose-dependently suppressed the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expressions in LPS-stimulated RAW 264.7 cells significantly. Collectively, the isolated lipid compounds from *P. tricornutum* exhibited the strong anti-inflammatory activity and can be employed in numerous functional food applications.

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1. INTRODUCTION

Culture of marine microalgae has been widely used as a food sources for aquaculture. In particular, diatoms are abundant components of the phytoplankton, constitute a major nutritional sources. *Phaeodactylum tricornutum*, a marine diatom and has been characterized that is containing different form of lipids including free sterols, esterified and glycosylated conjugates (Veron et al., 1996). In fact, a few reports have been investigated about the quantification of sterols that available in *P. tricornutum.* Moreover, growth under different conditions has been influenced as temperature, and light spectral quality for the distribution of sterols. The possibility of changing the sterol concentration of microalgae is a very important characteristic that can be used to determine the physiological state of a microalgae population, as a population biomarker (Fabregas et al., 1997). Lipids are a group of molecules occurring naturally that including sterols, fat-soluble vitamins, glyserides, phospholipids and others. Sterols belong to steroid alcohols are described as an important class of organic molecules. The most familiar molecule of sterol is cholesterol. However, many of bioactivities encountered with the different structural variations of sterols can be found extensively in natural sources. Among them, phytosterols are more commonly found as plant sterols. The identification of sterol compounds or compositions can be determined using RP-HPLC separation while GC-MS is the sensitive techniques (Giner et al., 2008). Hence marine microalgae contribute to isolate several forms of phytosterols and the determination of their bio-functionality would be demanded for future drug discovery.

In this study, cultured marine microalgae diatom, *P. tricornutum* hexane fraction was assessed for the anti-inflammatory activity. After, possible isolation was carried out to separate lipids including sterols and poly unsaturated fatty acid. Anti-inflammatory effect was evaluated from the isolated lipids against LPS-stimulated RAW macrophages.

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2. MATERIALS AND METHODS

2.1 Culture conditions of *Phaeodactylum tricornutum*

Marine microalgae culture conditions and harvesting techniques have been described in the Part I (2.2 and 2.3).

2.2 Isolation and characterization of the active compounds from *Phaeoductylum tricornutum*

Cultured marine diatom hexane fraction used to separate possible secondary metabolites and followed procedures were reported in the materials and method section in Part IV (2.4).

2.3 Determination of nitric oxide (NO) production and cell viability on RAW macrophages

The procedure has been followed as mentioned in the part I (2.11 and 2.12)

.

2.4 Lactate dehydrogenase (LDH) cytotoxicity assay

RAW 264.7 cells (1×10^5 cell mL^{-1}) were placed in a 24-well plate and after 24 h the cells were pre-incubated with various concentrations of the sample at 37 °C for 1 h. Then further incubation was done for another 24 h with lipo-polysaccharide (LPS) (1 μg mL^{-1}) at the same temperature. After the incubation, LDH level in the culture medium was determined using an LDH cytotoxicity detection kit (Promega, Madisom, WI, USA) according to the manufacture's instruction. Briefly, 50 μL of reaction mixture was added to each well, and the reaction was incubated for 30 min at room temperature in the dark. Then, 50 μL of stop solution was added to each well and absorbance was measured at 490 nm using a microplate reader (Sunrise, Tecan Co. Ltd., Australia).

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2.5 Determination of prostaglandin-E_2 (PGE_2) production

RAW 264.7 cells (1×10^5 cell mL^{-1}) were pretreated with the sample for 2 h and then treated with LPS (1 μg mL^{-1}) to allow cytokine production for 24 h. The PGE_2 levels in the culture medium were quantified using a competitive enzyme immunoassay kit (R & D systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The release of PGE_2 was measured relative to that of the control value.

2.6 Determination of pro-inflammatory cytokines interleukin-1β (1L-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) production

The inhibitory effect of the sample on the production pro-inflammatory cytokines from LPS-stimulated RAW macrophages was determined according to the previously described method (Cho et al., 2000). Briefly, RAW 264.7 cells (1×10^5 cell mL^{-1}) were pretreated with the sample for 2 h and then treated with LPS (1 μg mL^{-1}) to allow production of pre-inflammatory cytokines fro 24 h. Supernatants were used for the assay using an ELISA kit (R & D systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.7 Western blot analysis

RAW 264.7 cells (1×10^5 cell mL^{-1}) were pretreated for 16 h and then treated with LPS (1 μg mL^{-1}) in the presence or absence of the sample. After incubation for 24 h, the cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS), and the cells lysates were prepared with lysis buffer (50 $mmolL^{-1}$Tris–HCl (pH 7.4), 150 $mmolL^{-1}$NaCl, 1% Triton X-100, 0.1% SDS and 1 $mmolL^{-1}$ EDTA) for 20 min on ice. Cell lysates were centrifuged at 14, 000 × g for 20 min at 4 °C. The protein concentrations were measured using a BCA™ protein assay kit. The

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lysates containing 30μg of protein were subjected to electrophoresis using sodium dodecyl sulfate–polyacrylamide gels (SDS-PAGE) on 12%, and the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were incubated with blocking solution (5 % skim milk in Tris buffered saline containing Tween-20) for 90 min at room temperature. Then the membrane incubated with anti-rabbit Inos (1:1000; Cabiochem, La Jolla, CA, USA) and anti-rabbit COX-2 (1:1000; BD Bioscience Pharmingen, San Jose, CA, USA) for overnight at room temperature. After washing, the blots were incubated with horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody (1:5000; Amersham Pharmacia Biotech, Little Chalfont, UK) for 90 min at room temperature. The bands were visualized on X-ray film using ECL detection reagent (Amersham Biosciences, Piscataway, NJ, USA).

2.8 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.

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3. RESULTS AND DISCUSSION

3.1 Determination of nitric oxide (NO) production inhibitory activity of the isolated compounds from *P. tricornutum* against LPS-induced RAW macrophages

The isolated five compounds (Com 1, Com 2, Com 3, Com 4 and Com 5) from the hexane fraction of cultured marine microalga, *P. tricornutum*, were studied to assess the anti-inflammatory activity using LPS-induced RAW macrophages and determined the NO production inhibitory and cytotoxicity effects (Fig. 42). Each of the compounds with five different concentrations (1, 5, 12.5, 25 and 50 µg mL^{-1}) were pre-incubated with LPS for 24 h and observed the significant inhibitory effect of NO production level dose-dependently. In particular, all the compounds showed the lower then 15 % of NO production level at 50 µg mL^{-1} treated concentration significantly. Cell viability assay was indicated that the isolated compounds including Com 1, Com 3, Com 4 and Com 5 were not cytotoxic at all the treated concentration on RAW macrophages. However, the Com 2 was showed a little cytotoxic effect treated at higher concentrations (12.5, 25 and 50 µg mL^{-1}) significantly. It is noted that Com 2 was having both anticancer and anti-inflammatory activity.

3.2 Identification and structure elucidation of the sterol compounds from *P. tricornutum*

Isolated compounds from *P. tricornutum* were listed in the table 11. Com 2 was identified as a novel fatty alcohol ester, nonyl 8-acetoxy-6-methyloctanoate with potent anticancer activity. In fact, Com 1 was determined as a poly unsaturated fatty acid derivative and other compounds including Com 3, Com 4 and Com 5 were identified as sterols. Each of the isolated sterols characterization was reported in details.

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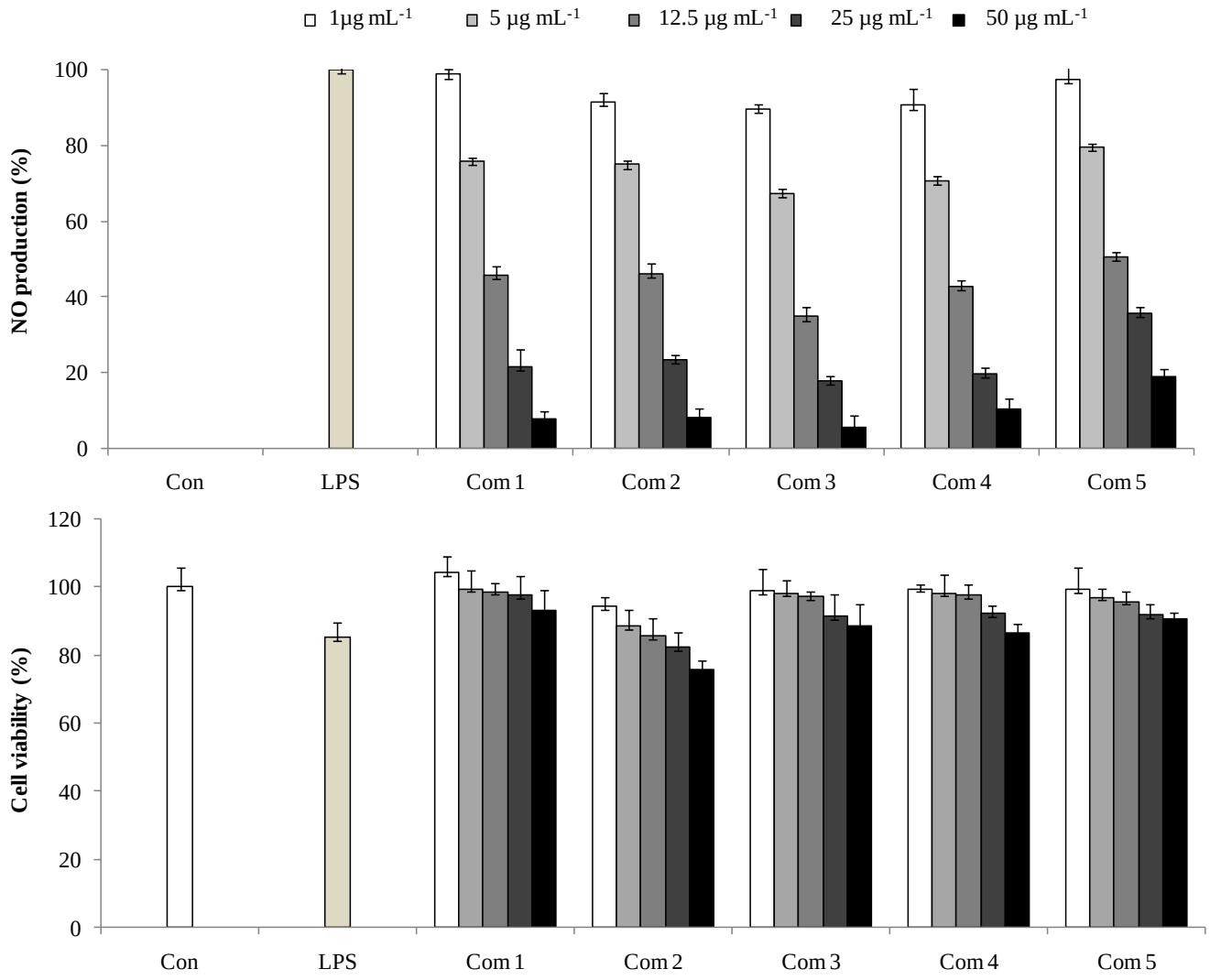

Fig. 42 Inhibitory effect of the isolated five compounds from cultured marine microalga *Phaeodactylum tricornutum* (A): LPS-induced NO production (%) and (B) cell viability (%) in RAW 264.7 macrophages. After 24 h to treat the compounds cell viability was assessed by MTT assay. Values are expressed as means ± SD in triplicate experiments.

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Table 11. Molecular formula and IUPAC names of the isolated compounds from *Phaeodactylim tricornutum*

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

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3.3 Structure elucidation of 24-methylcholesta-5(6), 22-diene-3β-ol

Com 5 was isolated from the eluted fraction 8 (F8) from hexane extract as eluted with hexane: ethyl acetate; 75:25 (v/v) solvent conditions. As determined the one fraction from preparative thin layer chromatography (PTLC) and tentatively named, PTH8-3 (Com 5) with corresponding (6.3 mg) weight. The molecular formula was determined as $C_{28}H_{46}O$ in the ESI (negative mode) as [M-H] peak at 397.35 m/z. The calculated molecular mass (398.35 m/z) was examined with the six degrees of unsaturation (Fig. 43). It is indicating that a C_{28} sterol with the two double bonds, one of which was in the C_5 in the second (B) ring of sterol skeleton and other was in the side chain at C_{22}. ^{13}C-NMR signals at δ 71.9 ppm was indicated that OH group attached at the C_3 in the A ring. In addition, ^{13}C-NMR spectrum showed 28 signals corresponding to 28 carbon atoms in the molecule. The DEPT-90 results indicated the presence of six methyl at δ 12.13, 18.78, 19.49, 19.7, 21.07 and 20.24 ppm signals were corresponded at the molecule C_{18}, C_{28}, C_{19}, C_{27}, C_{21} and C_{26}, respectively. Moreover, eight methylene and ten methane groups while the remaining four carbons were quaternary forms (Table 12). In the ^{1}H-NMR spectrum signals showed the olifinic protons at δ 5.15(1H, H-22) and 5.12 (1H, H-23) were due to presence of double bond as C_{22}.

The signals at δ 5.32 (1H, br, d, H-6) and multiplet at δ 3.52 (1H, m, H-3) were characteristic of Δ^5 – 3-β-hydroxyl sterols. Moreover, examine the heteronuclear correlation (HSQC) spectrums showed that presences of long range coupling are indicated in the Figure 35. Furthermore, the nomenclature was identified as 24-methylcholesta-5(6), 22-diene-3β-ol and it has been previously published from the same diatom, *P. tricornutum* (Fabregas et al., 1997). Among the identified sterols from *P. tricornutum*, 24-methylcholesta-5(6), 22-diene-3β-ol (MCDO) was

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Table 12 NMR data for 24-methylcholesta-5(6), 22-diene-3β-ol in Chloroform-D

Position (C#)	δ_C[a] (ppm)	(mult)	δ_H[a] (mult, J_{HH} Hz)
1	37.32	(CH_2)	1.85/1.02 m
2	31.73	(CH_2)	1.50/1.42 m
3	71.9	(CH)	3.5 m
4	42.38	(CH_2)	2.27/2.19 m
5	140.73	(C)	
6	121.71	(CH)	5.32 (1 H br d, J=4.8 Hz)
7	31.98	(CH_2)	1.98/1.85 m
8	43.15	(CH)	2.24 m
9	51.38	(CH)	0.89 m
10	36.5	(C)	
11	21.15	(CH_2)	1.48/1.42 m
12	39.75	(CH_2)	1.95/2.05 m
13	42.47	(C)	
14	56.85	(CH)	1.16 m
15	24.41	(CH_2)	1.50/1.54 m
16	28.91	(CH_2)	1.24/1.64 m
17	55.93	(CH)	1.14 m
18	12.13	(CH_3)	0.68 s
19	19.49	(CH_3)	0.99 s
20	40.37	(CH)	1.98 m
21	21.07	(CH_3)	0.88 (3H d, J=6.9 Hz)
22	136.63	(CH)	5.15 (1 H, dd, J=8.4, J=15.3 Hz)
23	131.63	(CH)	5.12 (1 H, dd, J=8.4, J=15.3 Hz)
24	50.13	(CH)	0.89 m
25	33.3	(CH)	1.48 m
26	20.24	(CH_3)	0.89 (3 H, d, J=6.3 Hz)
27	19.7	(CH_3)	0.81 (3 H, d, J=6.9 Hz)
28	18.78	(CH_3)	0.99 (3 H, d, J=7.0 Hz)

[a] Multiplicity determined from HSQC-DEPT experiments

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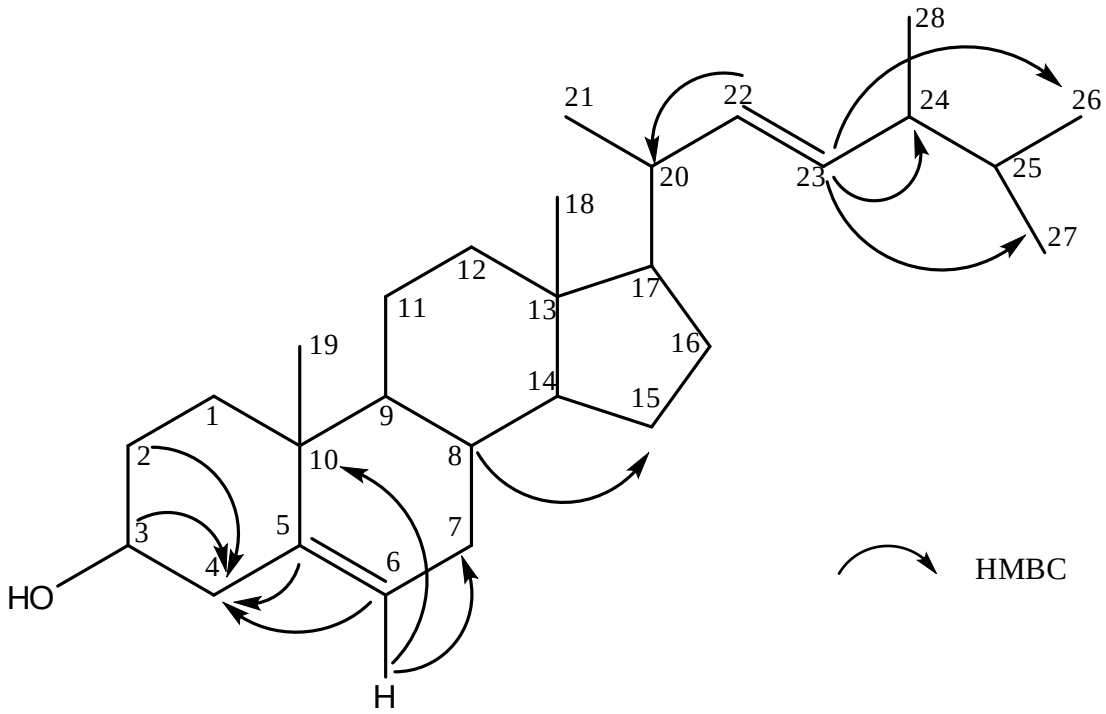

Fig. 43 Structure elucidation of 24-methylcholesta-5(6), 22-diene-3β-ol (MCDO)isolated from *Phaeodactylum tricornutum*

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reported as the (80 to 99%) major component (Veron et al., 1996). However, bioactivity assay has not been determined yet.

3.4 Structure elucidation of Cholestra-5(6), 22-dien-3, 24β-diol and Cholestra-5(6), 22-dien-3, 24β-diol, methyl ether

Molecular formulas of Com 3 and Com 4 were identified as $C_{27}H_{44}O_2$ and $C_{28}H_{46}O_2$ with 400.64 and 414.35 m/z, respectively (Table 8). In fact, IUPAC names were identified as Cholestra-5(6), 22-dien-3, 24β-diol (CDDO) and Cholestra-5(6), 22-dien-3, 24β-diol, methyl ether (CDDME) respectively. In contrast to the molecular structure of Com 5 (MCDO), an OH group is substituted at C_{24} in Com 3 (CDDO). Moreover, in the Com 4 (CDDME), ^{13}C-NMR signals showed an extra methyl group at δ 3.42 ppm (singlet) is attached to 'O' at C_{28} indicated a ether bond. According to the structure elucidation of CDDO and CDDME, molecular structures are presented in Fig 44.

3.5 Structure elucidation of Icosa-5, 8, 11, 14-tetraenyl acetate

Isolated Com 1 determined with molecular mass (332.57 m/z) and identified as $C_{22}H_{36}O_2$ (Table 8). ^{13}C-NMR signals showed 22 carbons and assessed with 5 degree of un-saturation. ^{13}C-NMR signal at δ 171.4 showed a carbonyl group with methyl end. Four olifineic bonds (*cis*-double bonds) were associated at 5, 8 11 and 14 carbon positions and found to be a polyunsaturated omega-6 aliphatic chain 20:4 (ω-6). Moreover, the molecule was identified as the fatty acid derivative, Icosa-5, 8, 11, 14-tetraenyl acetate (ITEA) (Fig. 45).

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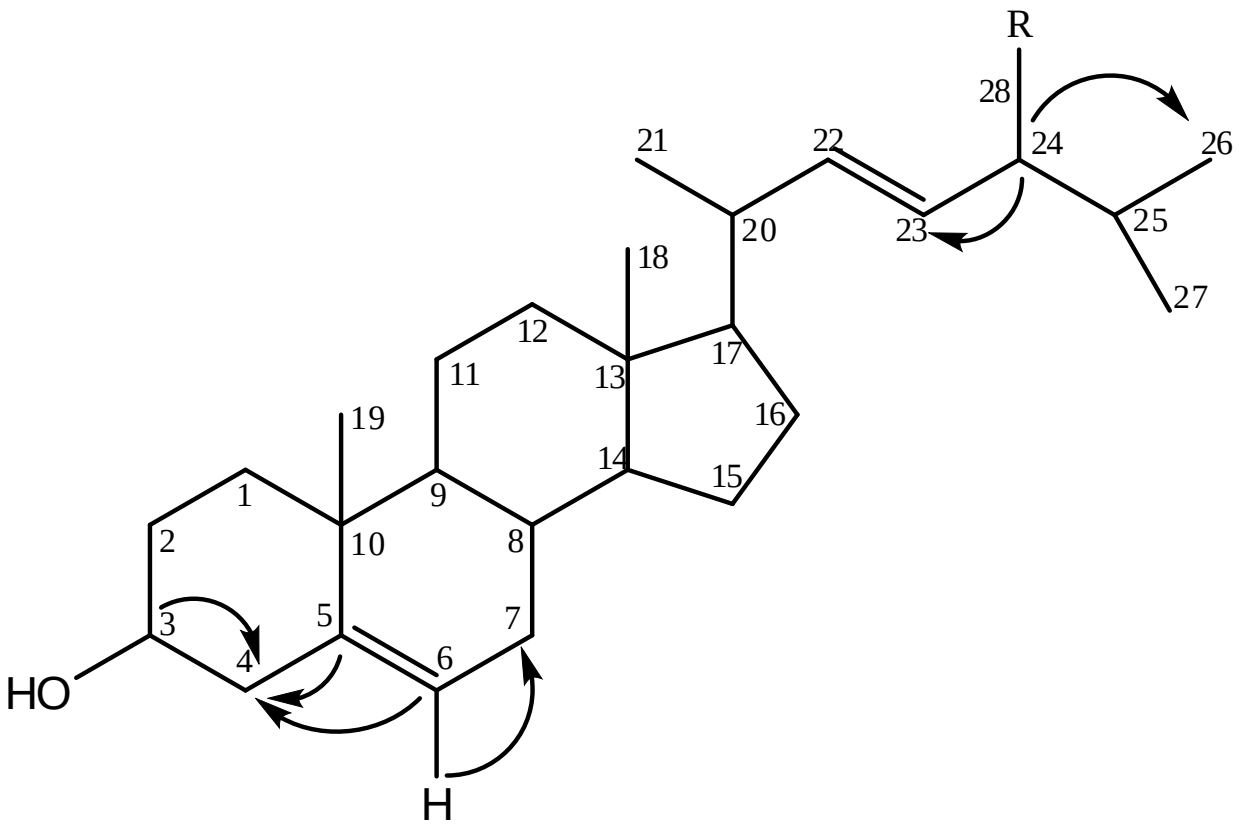

(CDDO) R=OH
(CDDME) R= OCH_3

Fig. 44 Structure elucidation of Cholestra-5(6), 22-dien-3, 24β-diol (CDDO) and Cholestra-5(6), 22-dien-3, 24β-diol, methyl ether (CDDME) isolated from *Phaeodactylum tricornutum*

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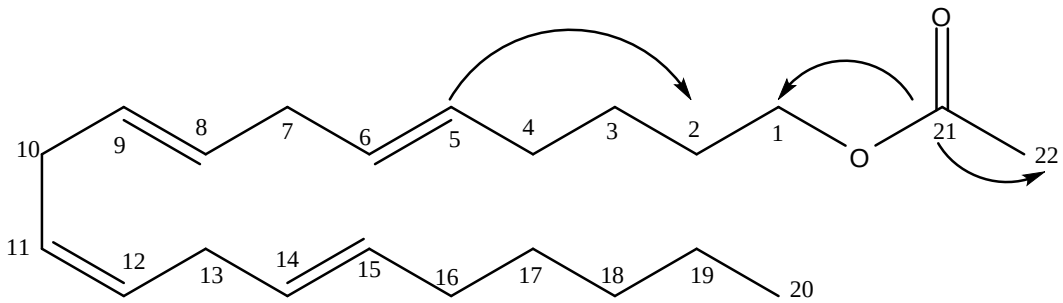

Fig. 45 Structure elucidation of Icosa-5, 8, 11, 14-tetraenyl acetate (ITEA) isolated from *Phaeodactylum tricornutum*

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3.6 Inhibitory effect of nitric oxide (NO) production and cytotoxicity by isolated sterols on LPS-induced RAW macrophages

Isolated sterol molecules such as MCDO, CDDO and CDDME and fatty acid derivative, ITEA from the cultured marine diatom, *P. tricornutum* anti-inflammatory effects against LPS-stimulated RAW cells were evaluated. Stimulation of the RAW cells with LPS leads to enhance of NO concentration in the medium. Since NO production by iNOS is one of the inflammatory mediators. However, pretreatment of isolated lipids such as MCDO, CDDO, CDDME and ITEA the effect of NO production was decreased at all the concentration (Fig. 46, 47, 48 and 49) respectively. LDH and MTT cytotoxicity assays showed that no significant cytotoxic effect on RAW macrophages at all the treated sterols at all the concentrations. Therefore, isolated lipid compounds such as MCDO, CDDO, CDDME and ITEA can be considered as a potential agent for suppressing NO production without a significant cytotoxic effect.

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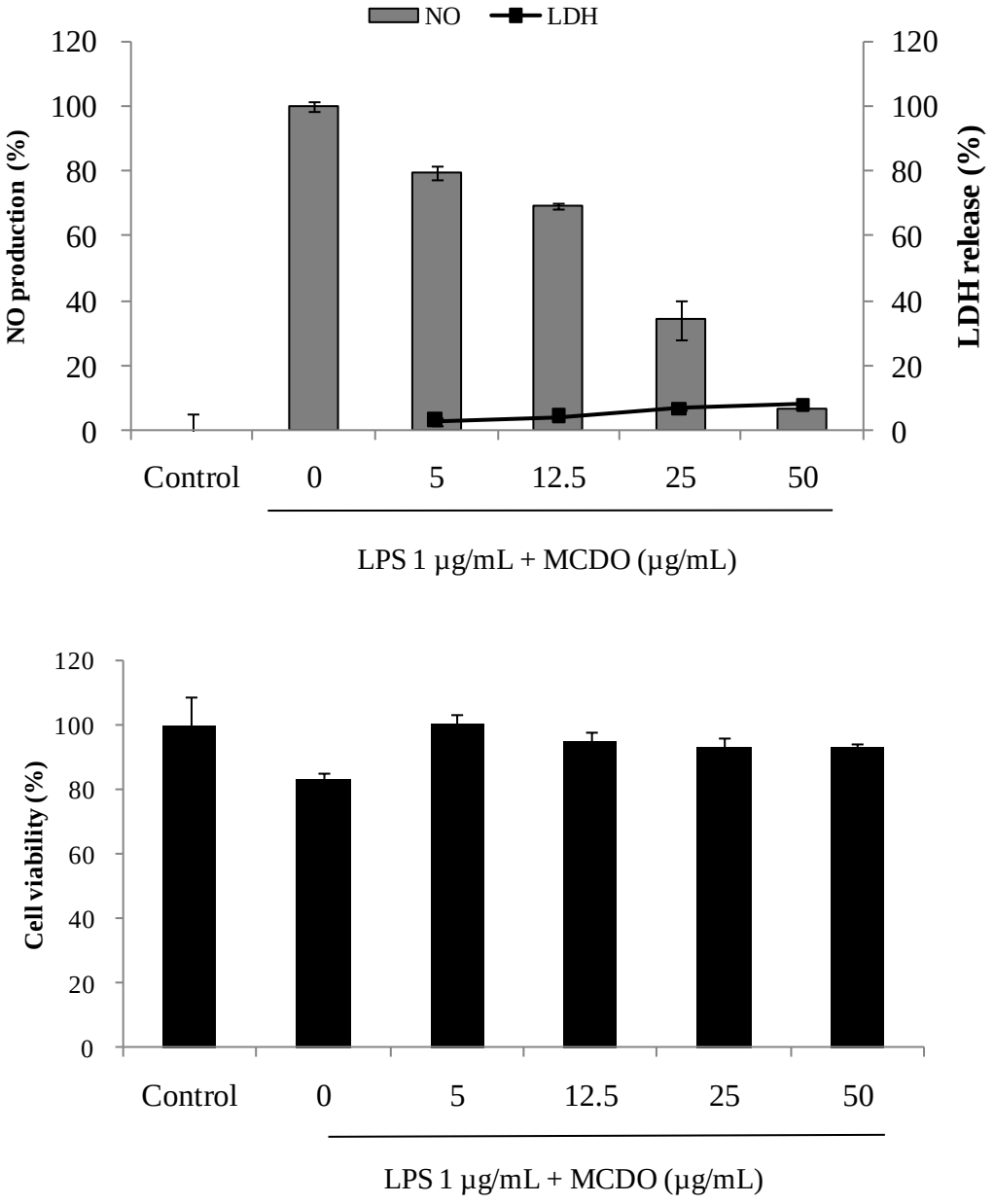

Fig. 46 Inhibitory effect of the isolated MCDO compound from cultured marine microalga *Phaeodactylum tricornutum* against LPS-induced NO production (%) and cell viability (%) by LDH and MTT assay in RAW 264.7 macrophages.

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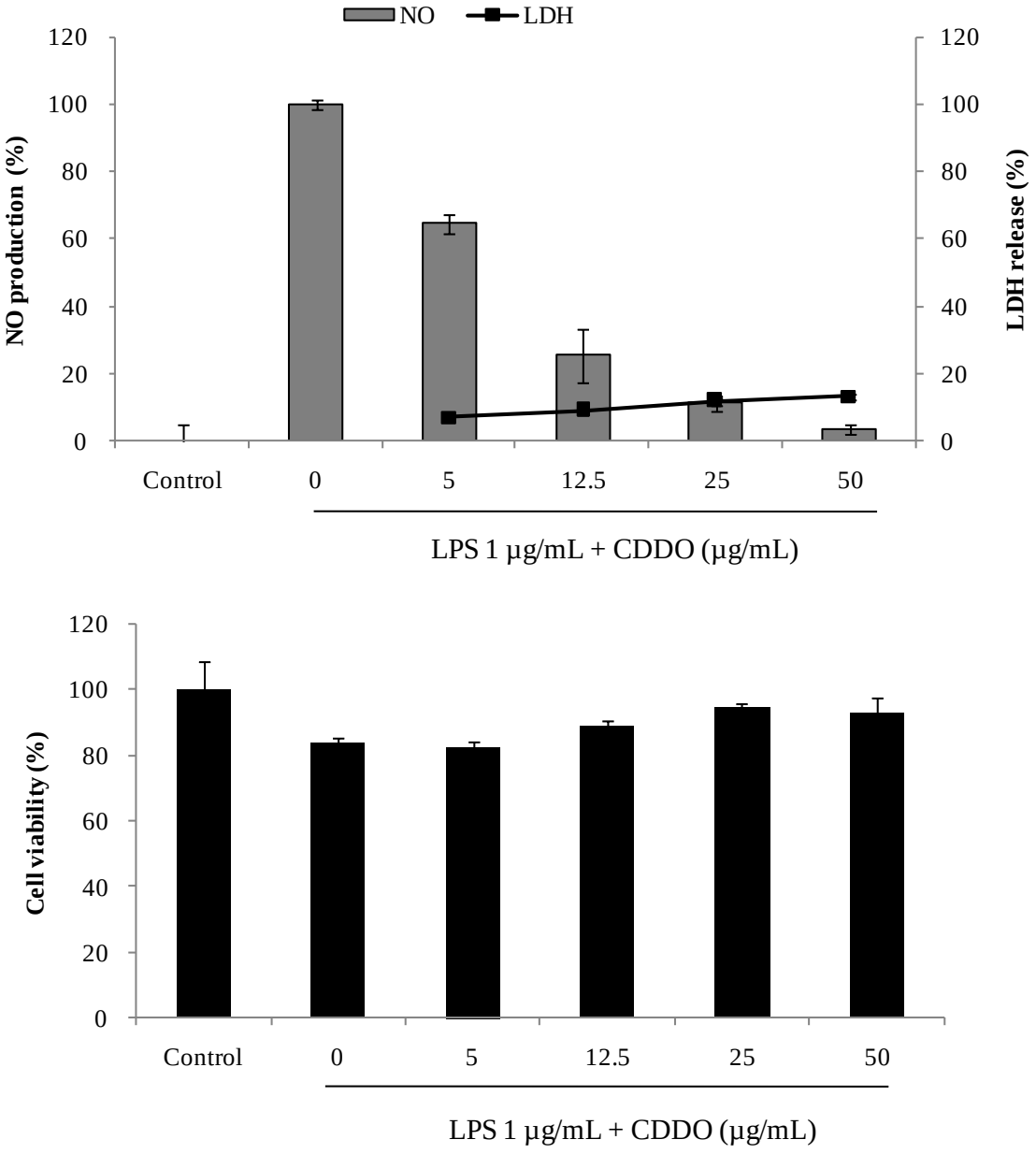

Fig. 47 Inhibitory effect of the isolated CDDO compound from cultured marine microalga *Phaeodactylum tricornutum* against LPS-induced NO production (%) and cell viability (%) by LDH and MTT assay in RAW 264.7 macrophages.

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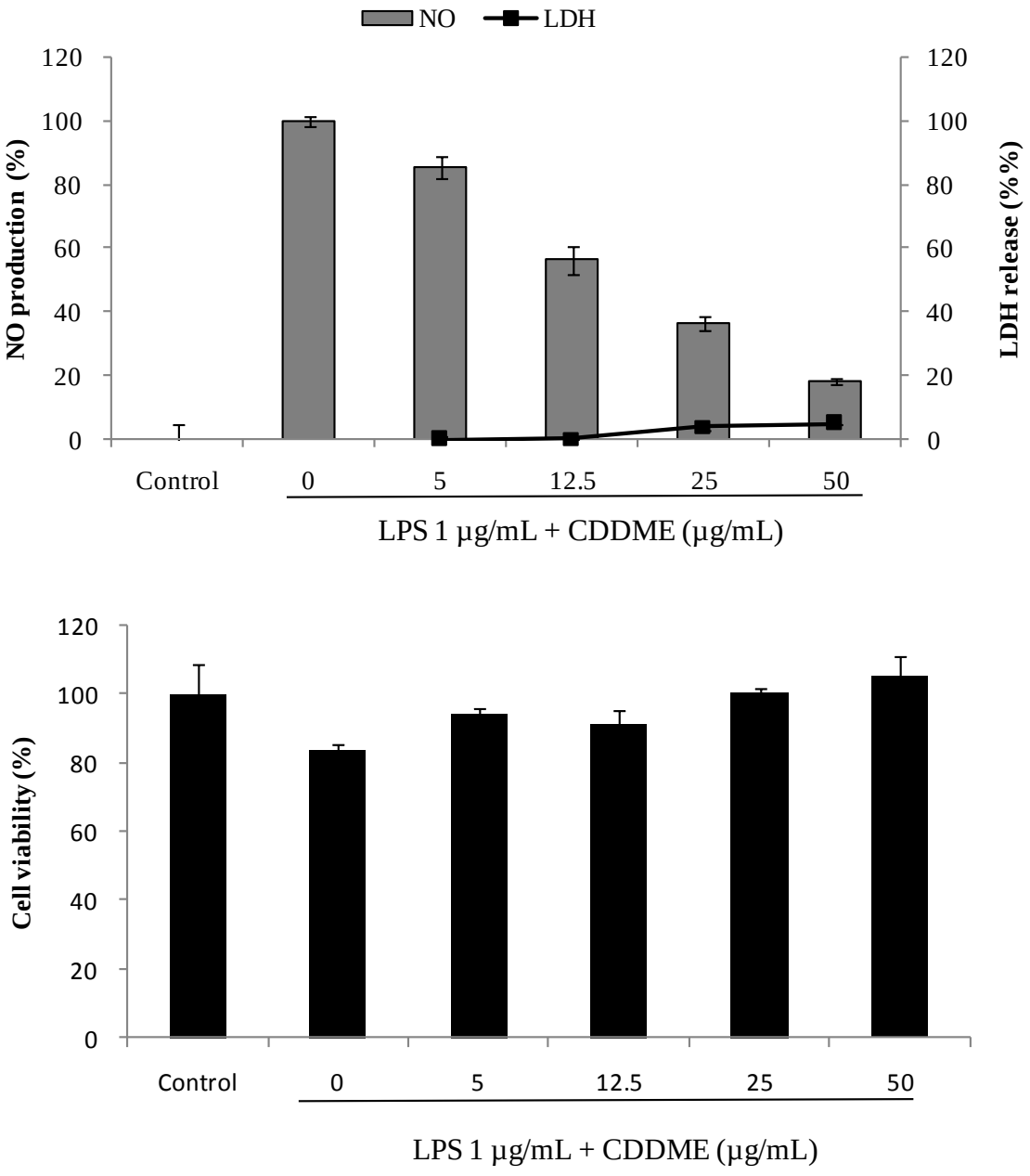

Fig. 48 Inhibitory effect of the isolated CDDME compound from cultured marine microalga *Phaeodactylum tricornutum* against LPS-induced NO production (%) and cell viability (%) by LDH and MTT assay in RAW 264.7 macrophages.

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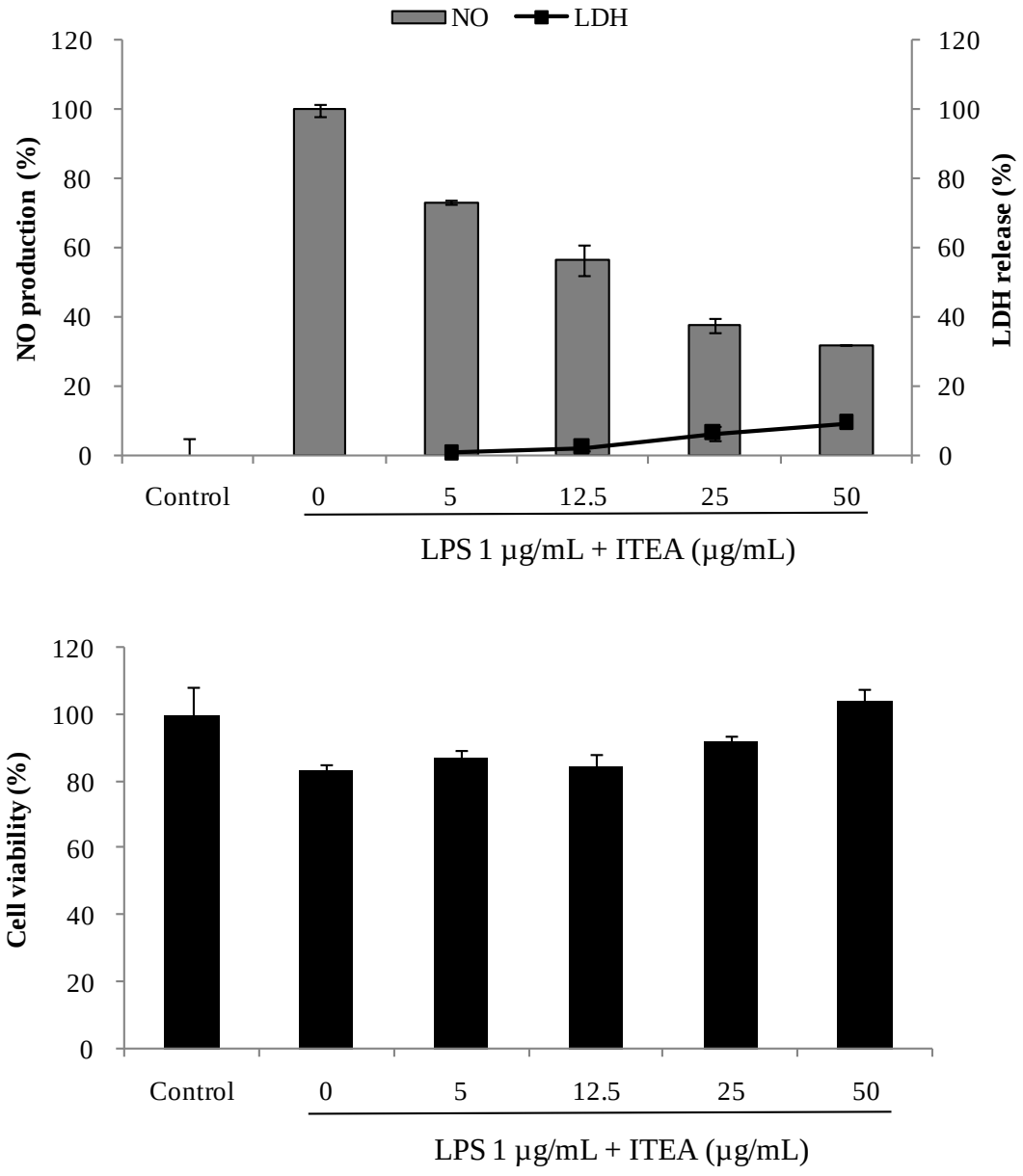

Fig. 49 Inhibitory effect of the isolated ITEA compound from cultured marine microalga *Phaeodactylum tricornutum* against LPS-induced NO production (%) and cell viability (%) by LDH and MTT assay in RAW 264.7 macrophages.

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3.7 Inhibitory effect of isolated sterols on LPS-induced PGE_2 production

Among the isolated sterols, MCDO and CDDO compounds were determined the inhibitory effect of PGE_2 production on LPS-stimulated RAW cells (Fig. 50 A and 51 A). According to the results, the compound CDDO was strongly suppressed the PGE_2 production by 60 % at the 25 μg mL^{-1} (Fig. 50 A). However, the compound MCDO showed the lower inhibitory activity app. 30% of the PGE_2 production suppression at the same concentration (Fig. 51 A) Thus, both the compounds were indicated the PGE_2 production inhibitory effect in a dose-dependent manner against LPS-stimulated RAW cells.

3.8 Inhibitory effect of isolated sterols on LPS-induced pro-inflammatory cytokines (TNF-α, 1L-1β and 1L-6) production

Inflammatory stimulators such as LPS induce cytokines in the process of macrophage activations. In particular, pro-inflammatory cytokines such as TNF-α, 1L-1β and 1L-6 mediate the tissue responses in different phases of inflammation. Hence, the inhibition of cytokine production leads to control the key mechanism of inflammation. In this study, isolated sterol compounds including MCDO and CDDO were determined the inhibitory effects of pro-inflammatory cytokines including TNF-α, 1L-1β and 1L-6 in LPS stimulated RAW macrophages (Fig. 50 B, C, D and 51 B, C, D). According to the results, inhibitory effect of TNF-α production was not showed significantly by both compounds (Fig. 50 B and 51 B). However, a little inhibition of TNF-α production was reported by CDDO at 25 μg mL^{-1}. Even though the production of 1L-1β and 1L-6 in LPS stimulated RAW macrophages were reported to suppress significantly in both compounds at all the treated concentrations. In particular, the strong suppression of 1L-1β production was indicated by CDDO at dose-dependently (Fig 51 C).

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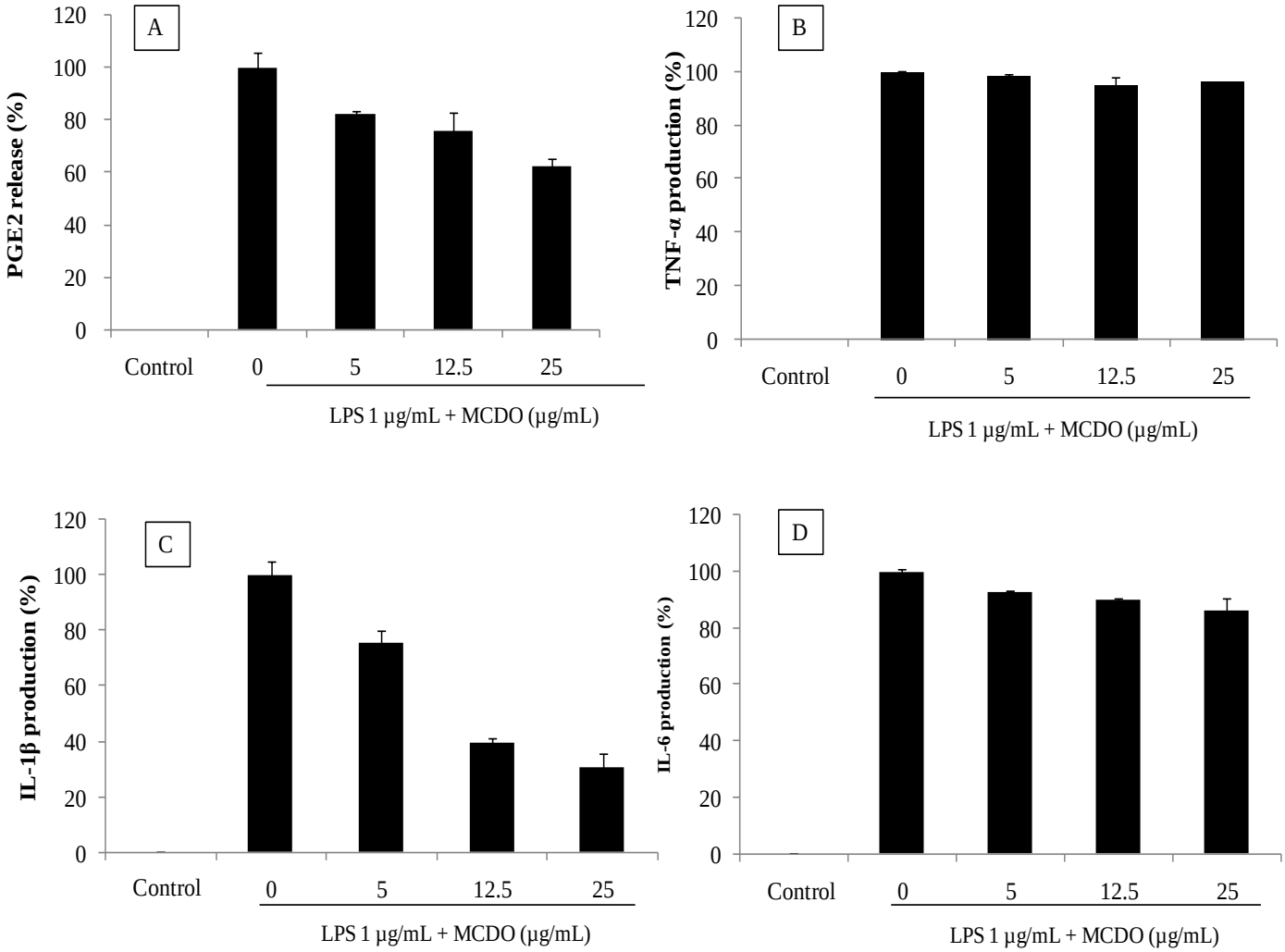

Fig. 50 Inhibitory effect of the isolated MCDO compound from cultured marine microalga *Phaeodactylum tricornutum* on LPS-induced PGE_2 (A), TNF-α (B), 1L-1β (C) and 1L-6 (D) production in RAW 264.7 macrophages. After the incubation of cells with LPS for 24 h in the presence or absence of the MCDO.

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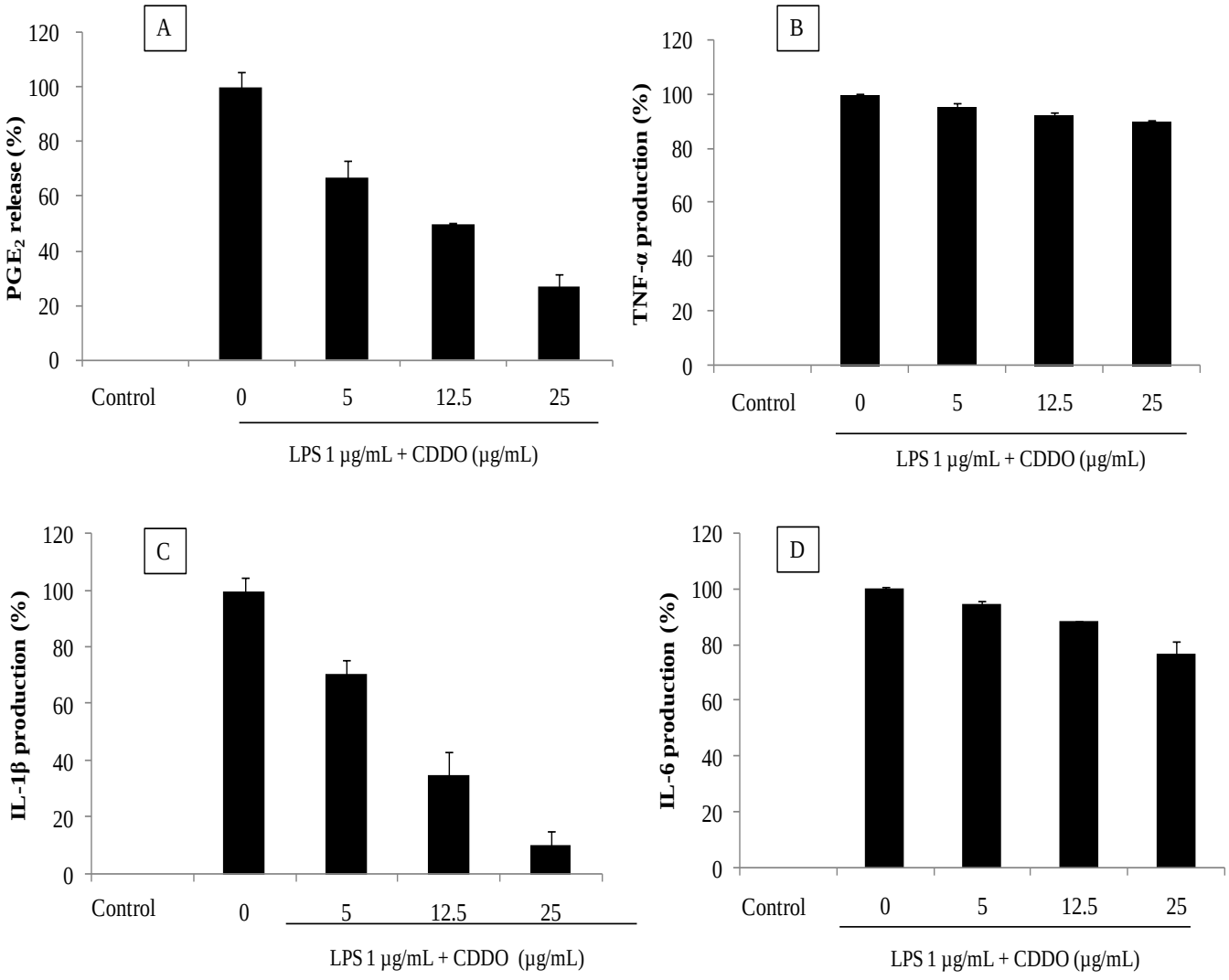

Fig. 51 Inhibitory effect of the isolated CDDO compound from cultured marine microalga *Phaeodactylum tricornutum* on LPS-induced PGE_2 (A), TNF-α (B), 1L-1β (C) and 1L-6 (D) production in RAW 264.7 macrophages. After the incubation of cells with LPS for 24 h in the presence or absence of the CDDO

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Moreover, it was noted the inhibition of 1L-6 in LPS stimulated RAW macrophages by both sterols at lower effect at all the treated concentration (Fig. 50 D and 51 D). In this results demonstrated that the attenuation effect of pro-inflammatory cytokines including TNF-α, 1L-1β and 1L-6 in LPS-stimulated RAW cells by both the isolated sterol compounds markedly.

3.9 Inhibitory effect of isolated sterols on LPS-induced iNOS and COX-2 protein expression

Inhibition of NO production and PGE_2 production is mediated by iNOS and COX-2 enzymes respectively. In order to evaluate the attenuation effects of protein expressions of iNOS and COX-2 western blot assay was performed. Based on the inflammatory stimulation from LPS, NO and PGE_2 productions are increasing with the potential inflammation. These fundamental biological functions may lead to occur in anywhere of the human body and can be a chronic situation. In this study, isolated two sterols such as MCDO and CDDO compounds were used to determine the regulation pattern of protein expressions that involve in the inflammation followed by LPS stimulated RAW macrophages. Figure 52 and 53 showed the western blot analysis of inhibitory effect of MCDO and CDDO on iNOS and COX-2 protein expressions in LPS-stimulated RAW macrophages, respectively. In fact, iNOS and COX-2 protein expressions were down-regulated markedly at the treated 12.5 and 25 μg mL^{-1} concentrations of MCDO compared to the references GAPDH (Fig. 52). In addition, a similar pattern of protein expressions (iNOS and COX-2) were identified with the pre-treated CDDO concentrations dose-dependently compared to β-actin (Fig. 53). Furthermore, it is noted that complete suppression of iNOS protein expression at 25 μg mL^{-1} by CDDO sterol compound significantly.

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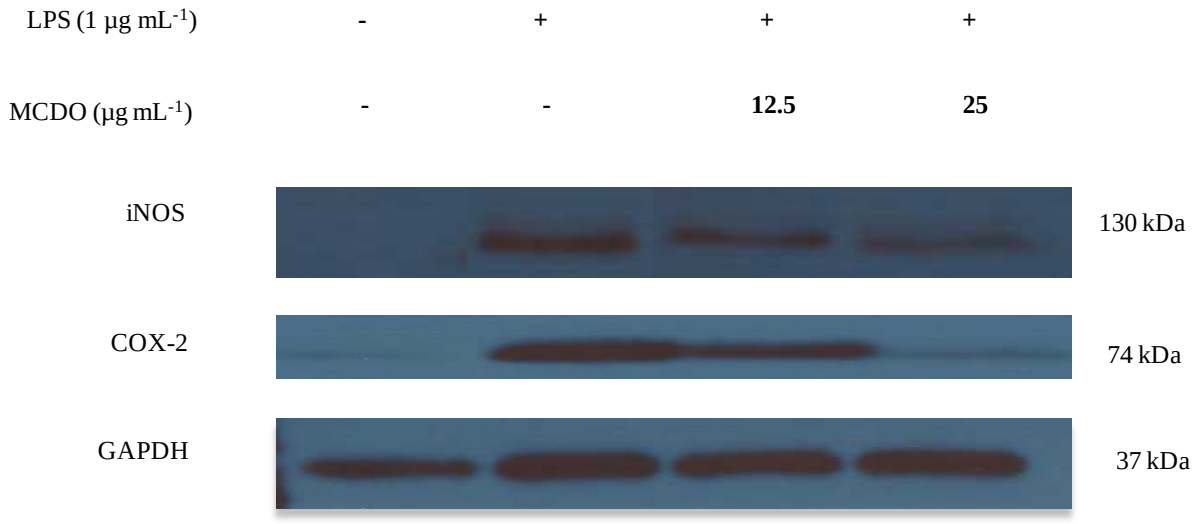

Fig. 52 Inhibitory effect of the isolated MCDO compound from cultured marine microalga *Phaeodactylum tricornutum* on LPS-induced iNOS and COX-2 protein expression in RAW 264.7 macrophages. The cells were incubated with LPS for 24 h in the presence or absence of the MCDO. Then, cell lysates were electrophoreses and the expression levels of iNOS and COX-2 were detected with specific antibodies

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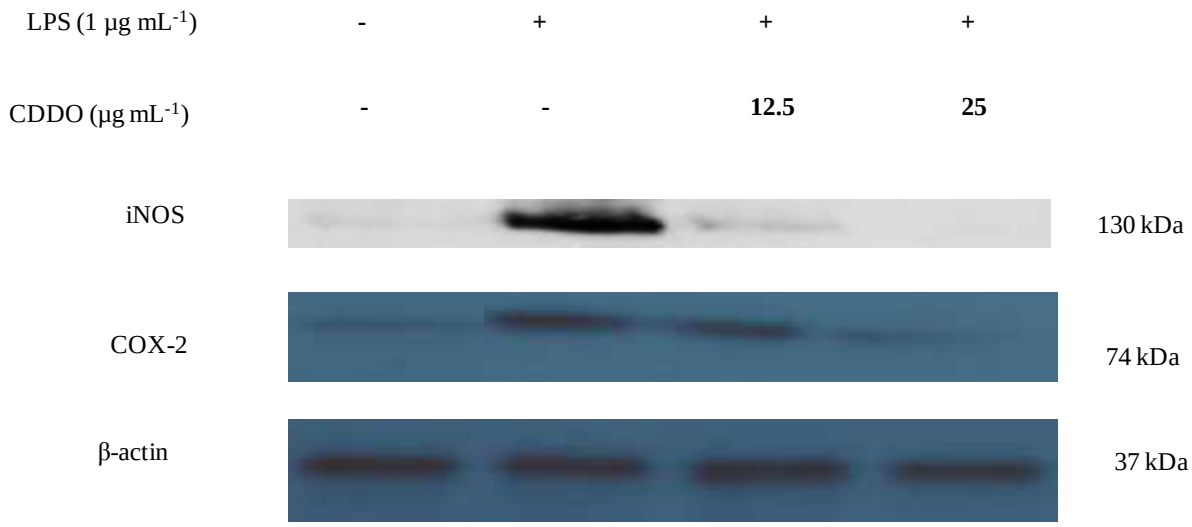

Fig. 53 Inhibitory effect of the isolated CDDO compound from cultured marine microalga *Phaeodactylum tricornutum* on LPS-induced iNOS and COX-2 protein expression in RAW 264.7 macrophages. The cells were incubated with LPS for 24 h in the presence or absence of the CDDO. Then, cell lysates were electrophoreses and the expression levels of iNOS and COX-2 were detected with specific antibodies

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With the profound inhibitory effect of NO and PGE_2 production showed by both sterol compounds such as MCDO and CDDO were correlated with the down-regulated iNOS and COX-2 protein expressions in this study. Some of the previous reports have reported that the induction of COX-2 activity and subsequent production of PGE_2 are closely related to the NO production (Chang et al., 2006). On the other hand, inhibition of iNOS, the enzyme mediates RAW macrophages and NO production has been suppressed as well as blocking the prostaglandin release from macrophages (Ahmed et al., 2002). Hence, the isolated sterols from the cultured marine microalgae, *P. tricornutum* demonstrated that having potentiality to use as anti-inflammatory agent for the therapeutic applications.

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4. CONCLUSION

The cultured marine diatom, *P. tricornutum* led to isolate five different compounds. Among the compounds, NAMO was identified as anticancer active compound and showed the promising effect on apoptosis inducible activity against HL-60 cancer cells markedly. In fact, three sterol molecules and a poly unsaturated aliphatic acetate compound were showed the anti-inflammatory potency on LPS-induced RAW machrophages. Among them, two sterol compounds such as MCDO and CDDO demonstrated the inhibitory effect of NO and PGE_2 and1L-1β production significantly in LPS-induced RAW cells. Moreover, the protein expression of iNOS and COX-2 confirmed that down-regulatory effect in a dose-dependent manner against RAW macrophages. Hence, the isolated lipids can be the novel alternatives with anti-inflammatory efficacy for the future lead compounds in pharmacological application.

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Part-VI

Exploration of bioactive components and pharmacological effects from Sri Lankan marine algae

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ABSTRACT

For the investigation of bioactive components from Sri Lankan seaweeds, three species of red algae (*Chondrophycus ceylanicus*, *Gelidiella acerosa*, *Gelidiopsis cortcata*), two species of green algae (*Chaetomorpha crassa*, *Caulerpa racemosa*) and four species of brown algae (*Sargassum cassifolium*, *Sargassum sp.* A, *Sargassum sp.* B and *Padina commersonii*) were evaluated. Among the selected brown algae, *Sargassum* sp. A, *Sargassum* sp. B and *Padina commersonii* were partitioned by different polar solvents and rest seaweeds were extracted only by methanol. For the bioactivity evaluations, total phenol content (TPC) and free radical scavenging activity using electron spin resonance (ESR) spectroscopy were carried out for all the fractions. In addition, sample cytotoxicity (vero cells), anti-infalmmatory (RAW macrophages) and anticancer effect against different cancer cell lines (human promyelocytic leukemia; HL-60, a human lung carcinoma; A549 and a mouse melanoma; B16F10) were assessed *in vitro*. In fact, most of the fractions showed the significant high activity for at least one or more bioassays, including antioxidant, anti-inflammatory and anticancer were determined. In particular, a significant cancer cell growth inhibitory effect was showed by *C. racemosa* methanol extract against HL-60 cells and it was observed the highest anticancer effects compared to the other extracts. Hence, apoptotic body formation in HL-60 cells and accumulation of DNA in sub-G_1 phase was determined by *C. racemosa* extract in a dose-dependent manner. Taken together, in this study, we demonstrated the first report of Sri Lankan seaweeds with the potentiality for pharmacological effects including antioxidant, anti-inflammatory and anticancer activity and further studied can be employed to isolate bioactive secondary metabolites.

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1. INTRODUCTION

Sri Lanka is an island located in the Bay of Bengal, in the northern Indian Ocean. The drop like island is 435 km long and 225 km wide, covered the total area 65610 km^2 with coastline of 1585 km. Despite the small island, Sri Lanka enriches with marine flora along the coast and identified the most frequent seaweeds by taxonomist. In fact, earliest marine algae collections have been reported in early 19^{th} century by Barton (1903). However, Baldwin (1991) has reported about 440 taxa of marine algae, belonging to 148 genera recorded in the coastline. More recently, a compressive study of marine seaweeds of Sri Lanka has been carried out by a group of scientist (Coppejans et al., 2009).

Marine seaweeds contain rich sources of secondary metabolites and considered as a key ingredient for pharmaceutical applications. Over the many years, marine natural products have been used for the lead compounds and drug discovery in all over the world. However, Sri Lanka seaweeds have not been studied extensively and only a few reports have found in the literature survey. Among them, the study of post-coital contraceptive mechanism of the crude extract of Sri Lankan marine red algae, *Gelidiella acerosa* using rat model was determined by Premekumara et al. (1995). Furthermore, the same group of researchers has isolated the sphingosine derivative as a non-steroidal anti progesterone contragestative agent from *G. acerosa* hexane fraction (Premakumara et al., 1996). In addition, human sperm mortality stimulation activity was reported by the isolated a sulfono glycolipid from *G. acerosa* (Premakumara et al., 2001). In a very recent publication reported that the antioxidant and metal chelating potentials from *G. acerosa* (Suganthy et al., 2013).

These published reports have indicated that only a limited scientific works have been carried out using Sri Lankan marine algae. In this study, the main objective was to evaluate the bioactivity

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of some selected marine algae from the Sri Lankan coastal line. Hence, we attempted to isolate bioactive natural products from the potential seaweeds.

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2. MATERIALS AND METHODS

2.1 Sample collection and preparations

Marine seaweeds were collected freshly from the sample site of Beruwela and Hikkaduwa coral reef in the southern province of Sri Lanka (Fig. 54). All the samples were washed well and removed salt, epiphytes and sand. Seaweed samples were identified based on the algaebase (Table 13) and each of the species were stored at -70°C followed by air drying at room temperature for 24 h. The frozen sample was lyophilized and homogenized using laboratory mechanical blender before extractions. Each of the sample was mixed with 80% methanol and subjected to extract by sonication (Ultra-sound assisted extraction) at 25 °C for 90 min period for three times. The extracts were filtered in a vacuum using Whatman No.1 (Whatman Ltd, Maidenstone, England) filter paper and solvent was evaporated using rotary evaporator (Fisher Scientific, Loughborough, UK**)**. The collected crude methanolic extracts were used for *in vitro* bioassays.

2.2 Determination of total phenolic content

Total phenolic content was determined according to Chandler and Dodds, (1983). One milliliter of sample was mixed in a test tube containing 1 mL of 95% ethanol, 5 mL of distilled water, and 0.5 mL of 50% Folin–Ciocalteu reagent. The mixture was allowed to react for 5 min, and then 1 mL of 5% Na_2CO_3 was added, and it was mixed thoroughly and placed in the dark for 1 h. Absorbance was measured at 725 nm using a UV–VIS spectrometer. A gallic acid standard curve was obtained for the calibration of phenolic content.

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Table 13. Collected Sri Lankan seaweeds with morphology and sample sites.

Seaweed	Morphology *	Type	Sampling site
Chondrophycus ceylanicus	Thalli gregarious, densely arranged robust, rigid, 3-5cm high, dark red	Rhodophyta	Beruwela
Chaetomorpha crassa	Thick, tough, curly, un-branched, mostly dark green filaments	Cholorophyta	Beruwela
Sargassum cassifolium	Erect, tough, leathery, presence of basal stolonoidal branches, air bladders	Phaeophyceae	Beruwela
Gelidiella acerosa	Tufts or clumps of tough and wiry, flexible axes, brownish –black	Rhodophyta	Beruwela
Caulerpa racemosa	Thallus forming, rhizoidal holdfasts, erect parts densely grapes like	Cholorophyta	Beruwela
Gelidiopsis cortcata	Erect, cartilaginous but flexible, dark bright red and creamy, branch axils	Rhodophyta	Beruwela
Sargassum sp. A	Grown in dense populations, straight, plane and flat plant.Elongated leaf and asymmetrical basis	Phaeophyceae	Hikkaduwa
Sargassum sp. B	Leaves thick, oblong with cuneate, broadly rounded apex	Phaeophyceae	Hikkaduwa
Padina commersonii	Plants erect and funnel shaped. Fan shaped bladrs with broad lobes, smooth surface	Phaeophyceae	Hikkaduwa

* www.algaebase.org

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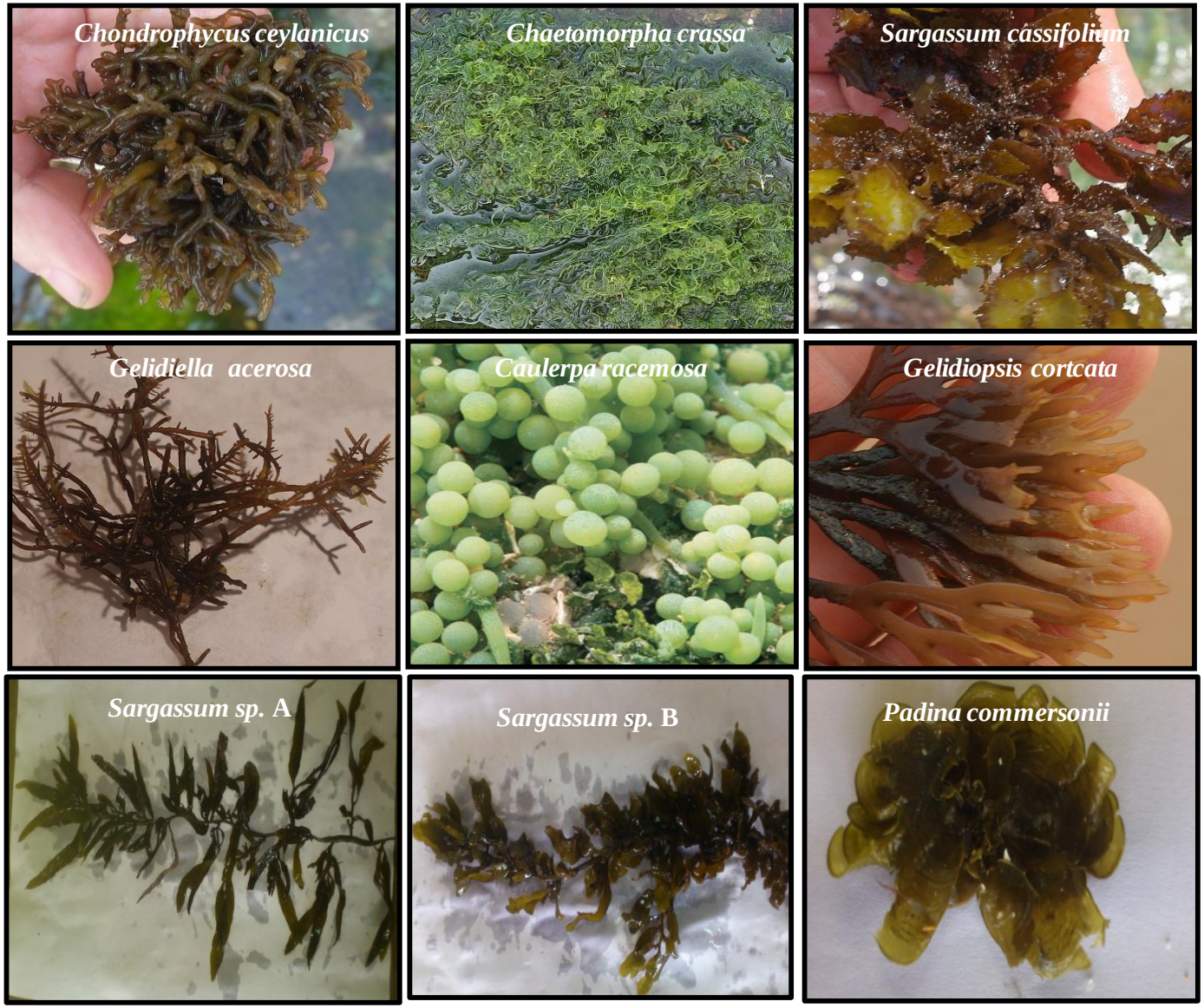

Fig. 54 Collected seaweeds from Beruwela and Hikkaduwa sample site in Sri Lanka

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2.3 Chemicals and reagents

The murine macrophage cell line (RAW 264.7), vero cells (monkey kidney cell line), 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), fetal 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) and Dimethyl Sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals and reagents used in these investigations were of analytical grade.

2.4 Radical scavenging activity using ESR method

The determination of DPPH, Hydroxyl and Alkyl radical scavenging activity using ESR was described in 2.7, 2.8 and 2.9 sub sections, respectively.

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 vero cells (monkey kidney cell line), B16F10 (a mouse melanoma cell line) and RAW 264.7 (a murine macrophage cell line) were 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% fetal 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.

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2.6 Determination of cell viability (MTT assay)

Vero cells were seeded in a 96-well plate at a concentration of 1×10^5 cells mL^{-1}. Sixteen hours after seeding, the cells were treated with samples at 50, 100 and 200 µg mL^{-1}concentrations. The cells were then incubated for an additional 24 h at 37 °C. MTT stock solution (50 µL; 2 mg mL^{-1} in PBS) was then added to each well to a total reaction volume of 250 µL. After 3 h of incubation, the plates were centrifuged (800g, 5 min), and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 µL of dimethylsulfoxide (DMSO) and the absorbance was measured at 540 nm using ELISA plate reader.

2.7 Determination of anti-inflammatory activity

Assessment of nitric oxide (NO) and cell viability assays were concisely described in the procedure as mentioned in the part I (2.11 and 2.12)

2.8 Cell growth inhibitory assay for anticancer activity

The cell growth inhibitory activity of cultured marine microalgae crude solvent extracts against the cancer cells (HL-60, B16F10, and A549) were determined by the colorimetric MTT assay. Suspension cells (HL-60 cells) were seeded (2×10^4 cells mL^{-1}) together with the samples and incubated for 48 h before MTT treatment. Attached cells (B16F10 and A549) were seeded in a 96-well plate at a concentration of 2×10^4 cells mL^{-1}. At 24 h after seeding, the cells were treated with the samples. MTT stock solution (50 µL; 2mg 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 2000 rpm and the supernatants were aspirated. The formazan crystals in each well

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were dissolved in DMSO. The amount of purple formazan was assessed by measuring the absorbance at 540 nm.

2.9 Nuclear staining with Hoechst 33342

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 (50, 100 and 200 μg mL^{-1}) of methanol extracts of *Caulerpa racemosa* (CRM) 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 color digital camera in order to determine the degree of nuclear condensation.

2.10 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 et al. (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 (50, 100 and 200 μg mL^{-1}) of CRM. 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 (Becton–Dickinson, SanJose, CA, USA). The effect on the cell cycle was examined by changes in the percentage of cell distribution at each cell cycle phase,

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and assessed by histograms generated by the Quest and Mod-Fit computer programs as described method (Wang et al., 1999).

2.11 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.

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3. RESULTS AND DISCUSSION

3.1 Total phenolic contents (TPC) and free radical scavenging activity (%)

In this experiment, some selected Sri Lankan marine algae methanol extracts were determined for the total phenolic content (TPC) by Folin–Ciocalteu method and free radical scavenging activity (%) using electron spin resonance (ESR) spectroscopy (Table 14). Among the results, the highest TPC (%) was observed as 5.13 (%) from *G. cortcata*. However, the reported TPC % was not significantly difference to each other and the lowest TPC was recorded from *G. acerosa* as 3.64 %. For the free radical scavenging activity determination, three kinds of radicals, including DPPH, hydroxyl and alkyl were generated in the assay and the scavenging effect was determined using the most senestive, accurate and direct method as ESR spectroscopic measurements. Among the treated at 1 mg/mL concentration of methanol extracts, *C. racemosa* showed the best average radical scavenging activity agasint DPPH (34.34 %), alkyl (85.17 %) and hydroxyl (81.16 %), respectively. In fact, the best alkyl radical scavenging activity was recorded as 91.36% from *C. eylanicus*. In general, alkyl and hydroxyl radical scavenging activity (%) of the methanol extracts were showed markedly compare to DPPH radical scavenging activity from the Sri Lankan marine algae (Table 14). In addition, different solvent extracts from the solvent-solvent partition chromatography of the three selected Sri Lankan brown algae, including *Sargassum sp.* A; *Sargassum sp.* B and *Padina commersonii* were evaluated for TPC and free radical scavenging activity (Table 15). Among the extracted fractions of marine alga, *P. commersonii* including, hexane (PCH: 5.48%), chloroform (PCC: 6.97%), ethylacetate (PCE: 7.44%) and aqueous (PCW: 6.83%) showed the highest TPC (%) compared to the other fractions significantly. For the antioxidant activity assessment of the extracted fractions of marine algae, free radical scavenging activity (IC_{50} mg/mL) was determined by ESR method. Hence, among

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the extracts, the strongest radical scavenging activity was indicated by PCE and PCW fractions against alkyl radical, IC_{50} values as 0.017 and 0.02 mg/mL, respectively. In addition, *Sargassum sp.* A ethyl acetate fraction (SAE) showed the highest hydroxyl radical scavenging activity (IC_{50} 0.07 mg/mL) compared to the other fractions. The highest DPPH radical scavenging activity was identified from the PCE fraction (IC_{50} value of 0.71 mg/mL) significantly. As a consequence, among the partitioned fractions from brown algae, *P. commersonii* showed the profound antioxidant effects at all the treated concentrations agasint DPPH (Fig. 55 C), hydroxyl (Fig. 56 C) and alkyl (Fig. 57 C) radical scavenging activities, respectively.

3.2 Sample cytotoxicity assessment against vero cells

For the determination of sample cytotoxicity effects agasint vero cells, the MTT assay was conducted. The methanol extracted Sri Lankan marine algae samples were incubated at 50, 100 and 200 μg/mL concentrations for 24 h with vero cells and determined the cell viability (%) of the cells *in vitro* (Fig. 58). In fact, all the samples at 50 and 100 μg/mL concentrations were showed the highest cell vability (%) compared to the control cell. In addition, among the samples, *C. ceylanicus*, *C. crassa*, *S. cassifolium* and *G. acerosa* were indicated the least cytotoxicity at even treated 200 μg/mL concentration on vero cells. However, *C. racemosa* and *G. cortcata* methanol extracts showd to be cytotoxic at the treated 200 μg/mL concentration significantly (Fig. 58). On the other hand, the fractionated marine seaeweed samples, including (A) *Sargassum sp.* A; (B) *Sargassum sp.* B and (C) *Padina commersonii* were indicated the sample cytotoxicity effect against vero cells in Figure 59. Among the fractions, the determined cell viability (%) was varied at the different concentrations significantly.

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Table 14. Determined free radical scavenging activity (%) and total phenolic content of some selected Sri Lankan seaweeds [a]

Scientific name	Total phenolic content (mg GAE/g)	Free radical scavenging activity (%)[b]		
		DPPH radical	Alkyl radical	Hydroxyl radical
Chondrophycus ceylanicus	4.05 ± 0.13	6.39 ± 0.68	91.36 ± 2.26*	80.09 ± 1.36
Chaetomorpha crassa	4.69 ± 0.14	20.12 ± 0.08	36.40 ± 4.00	77.08 ± 0.92
Sargassum cassifolium	3.8 ± 0.123	11.16 ± 1.67	60.56 ± 2.86	76.59 ± 1.56
Gelidiella acerosa	3.64 ± 0.16	6.19 ± 1.53	54.25 ± 0.44	74.95 ± 4.854
Caulerpa racemosa	3.78 ± 0.1	34.34 ± 0.03	85.17 ± 0.55*	81.16 ± 0.75
Gelidiopsis cortcata	5.13 ± 0.13*	18.17± 0.90	78.67 ± 0.67	76.28 ± 2.22

[a] The values were determined by at triplicate individual experiments. Values are mean ± SD of three determinations.

[b] Concentration used at 1 mg/mL

* (P> 0.05)

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Table 15. Determined free radical scavenging activity and total phenolic content of the fractions of some selected seaweeds

Scientific name	Fractions	Total phenolic content (mg GAE/g) [a]	Free radical scavenging activity (IC_{50} mg/mL) [a]		
			DPPH radical	Alkyl radical	Hydroxyl radical
Sargassum sp. A	SAH	4.20 ± 0.21	3.18 ± 0.32	0.85 ± 0.06	0.28 ± 0.01
	SAC	5.55 ± 0.56	1.85 ± 0.41	0.55 ± 0.02	0.34 ± 0.05
	SAE	4.61 ± 0.17	3.32 ± 0.18	0.29 ± 0.04	0.07 ± 0.001
	SAW	4.41 ± 0.76	6.55 ± 0.65	2.17 ± 0.2	0.29 ± 0.02
Sargassum sp. B	SBH	4.54 ± 0.07	2.29 ± 0.45	0.56 ± 0.02	0.31 ± 0.06
	SBC	4.74 ± 0.10	5.89 ± 0.56	0.92 ± 0.04	0.32 ± 0.01
	SBE	4.78 ± 0.35	6.29 ± 0.71	0.63 ± 0.06	0.28 ± 0.02
	SBW	4.61 ± 0.17	1.37 ± 0.11	0.22 ± 0.04	0.27 ± 0.09
Padina commersonii	PCH	5.48 ± 0.65	1.12 ± 0.40	0.12 ± 0.03	0.36 ± 0.05
	PCC	6.97 ± 0.15*	0.76 ± 0.01	0.18 ± 0.02	0.31 ± 0.03
	PCE	7.44 ± 0.29*	0.71 ± 0.03	0.017 ± 0.001	0.25 ± 0.01
	PCW	6.83 ± 0.38*	0.78 ± 0.11	0.02 ± 0.003	0.28 ± 0.06

[a] The concentration of sample required to scavenge 50 % of the radical scavenging activity. The values were determined by at triplicate individual experiments. Values are mean ± SD of three determinations. * (P> 0.05)

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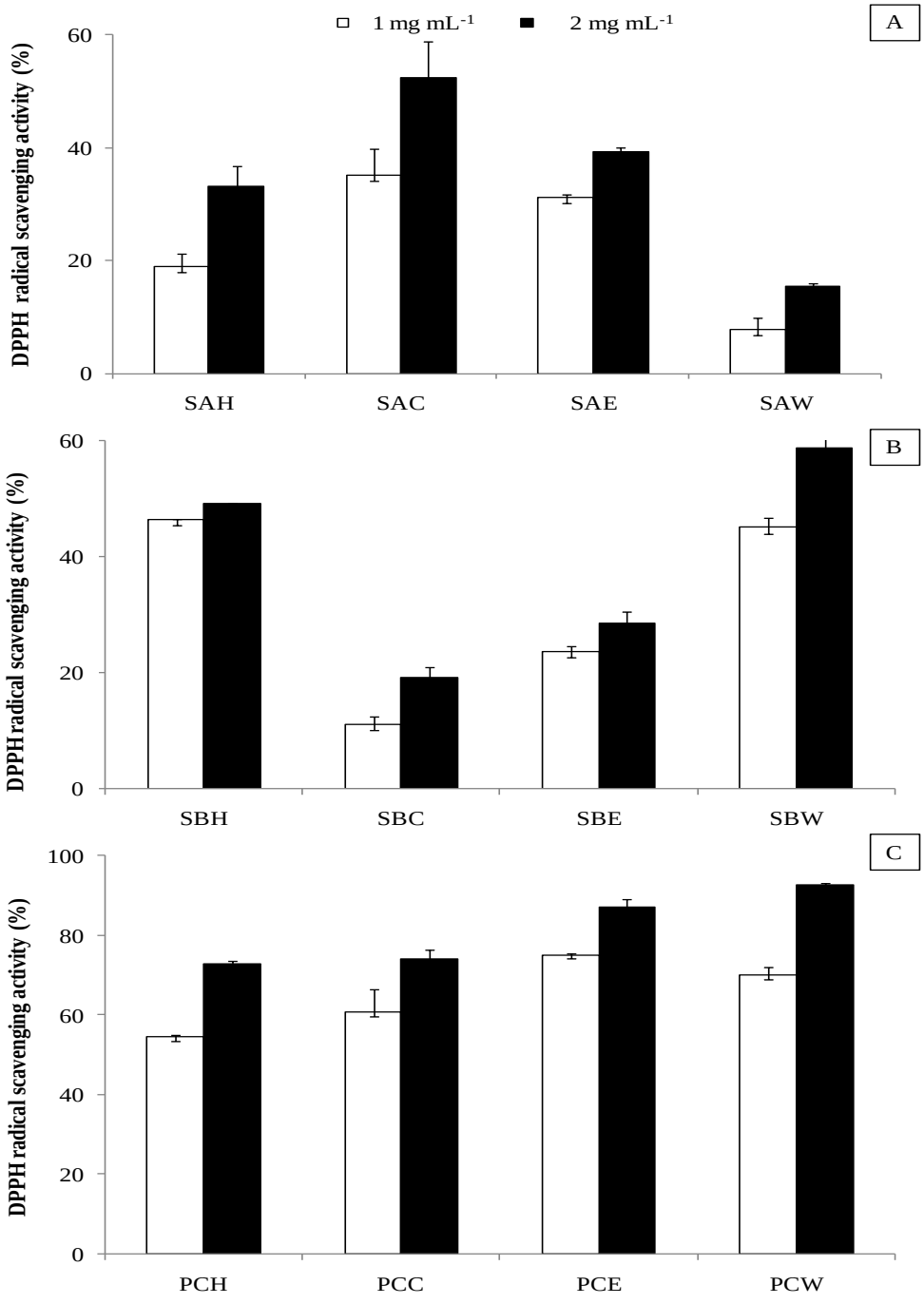

Fig. 55 The effects of antioxidant activity by DPPH radical scavenging activity (%) from the fractions of some selected Sri Lankan seaweeds, including (A) *Sargassum sp.* A; (B) *Sargassum sp.* B and (C) *Padina commersonii* using electron spin resonance (ESR) spectrophotometry.

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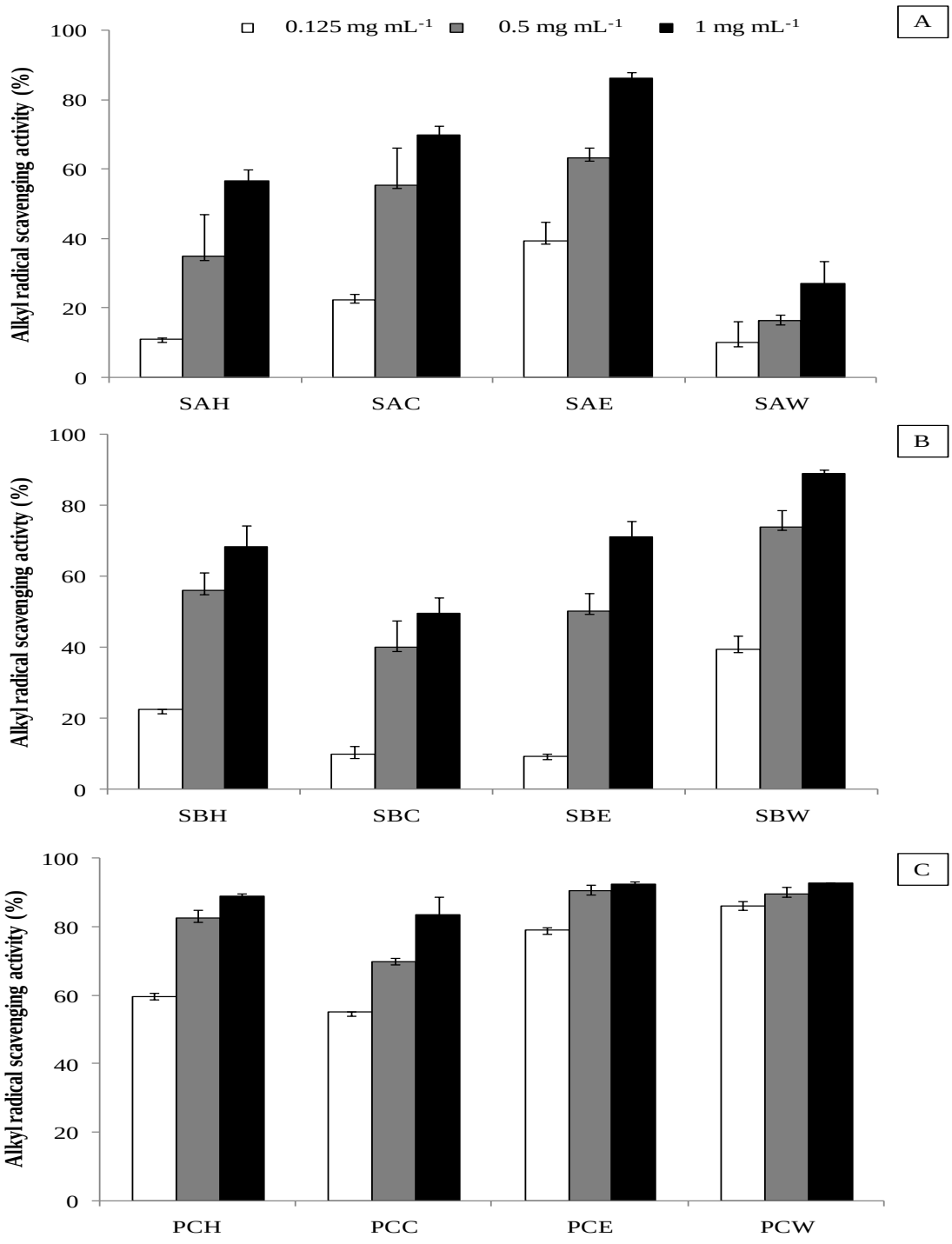

Fig. 56 The effects of antioxidant activity by Alkyl radical scavenging activity (%) from the fractions of some selected Sri Lankan seaweeds, including (A) *Sargassum sp.* A; (B) *Sargassum sp.* B and (C) *Padina commersonii* using electron spin resonance (ESR) spectrophotometry.

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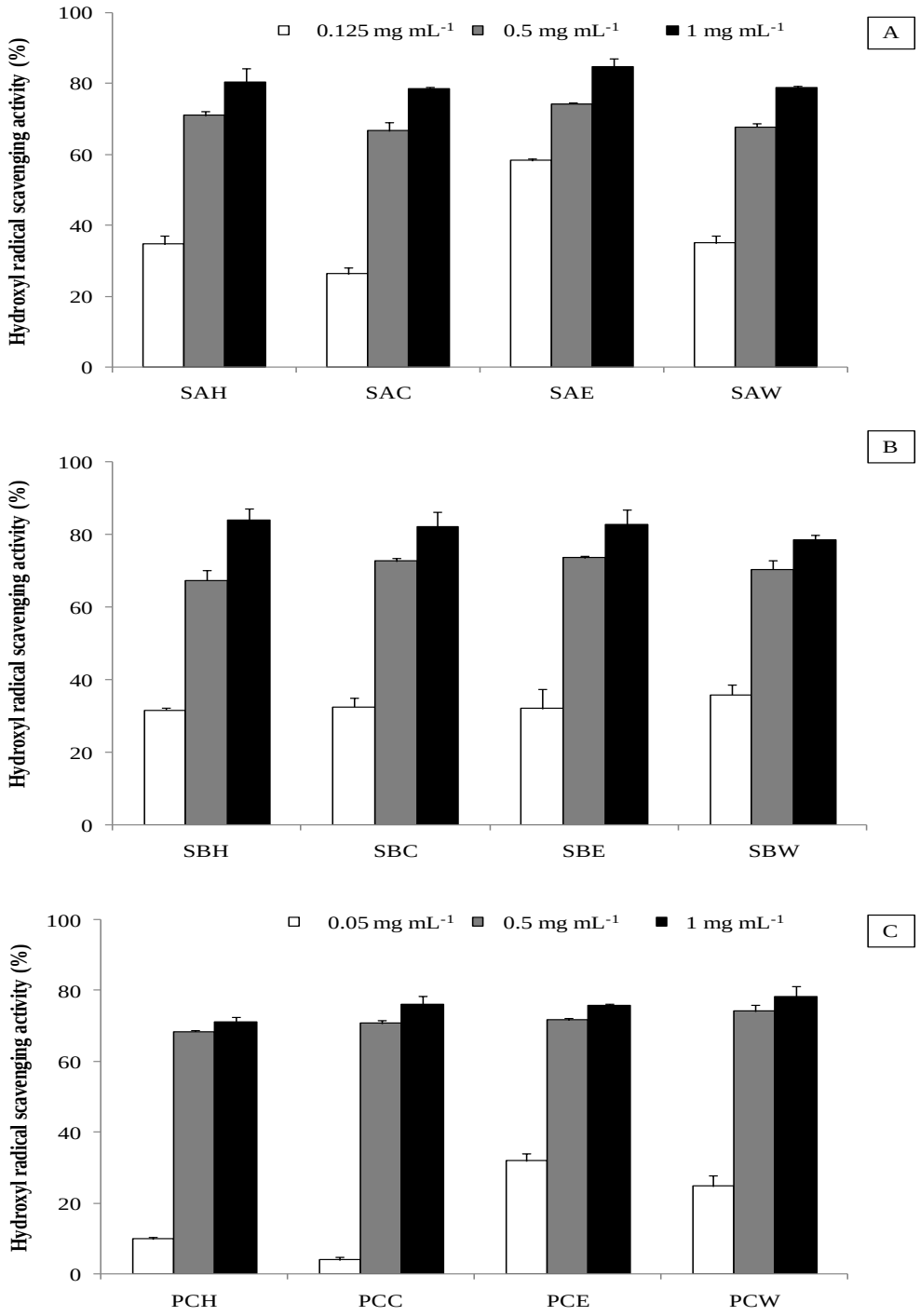

Fig. 57 The effects of antioxidant activity by Alkyl radical scavenging activity (%) from the fractions of some selected Sri Lankan seaweeds, including (A) *Sargassum sp.* A; (B) *Sargassum sp.* B and (C) *Padina commersonii* using electron spin resonance (ESR) spectrophotometry.

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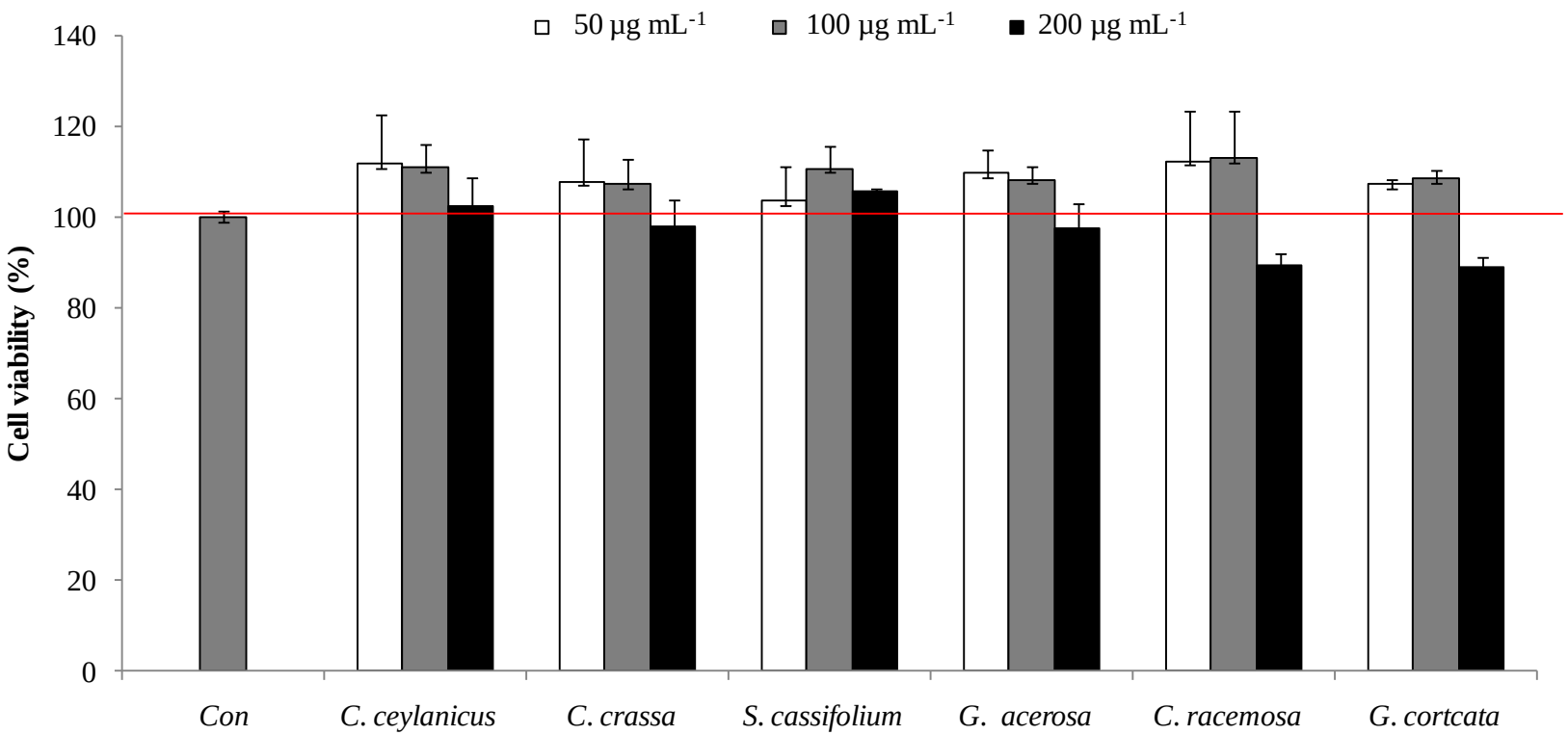

Fig. 58 The effect of cell viability (%) from methanol extracts of some selected Sri Lankan seaweeds agaisnt vero cells, incubated at 24 h by MTT assay.

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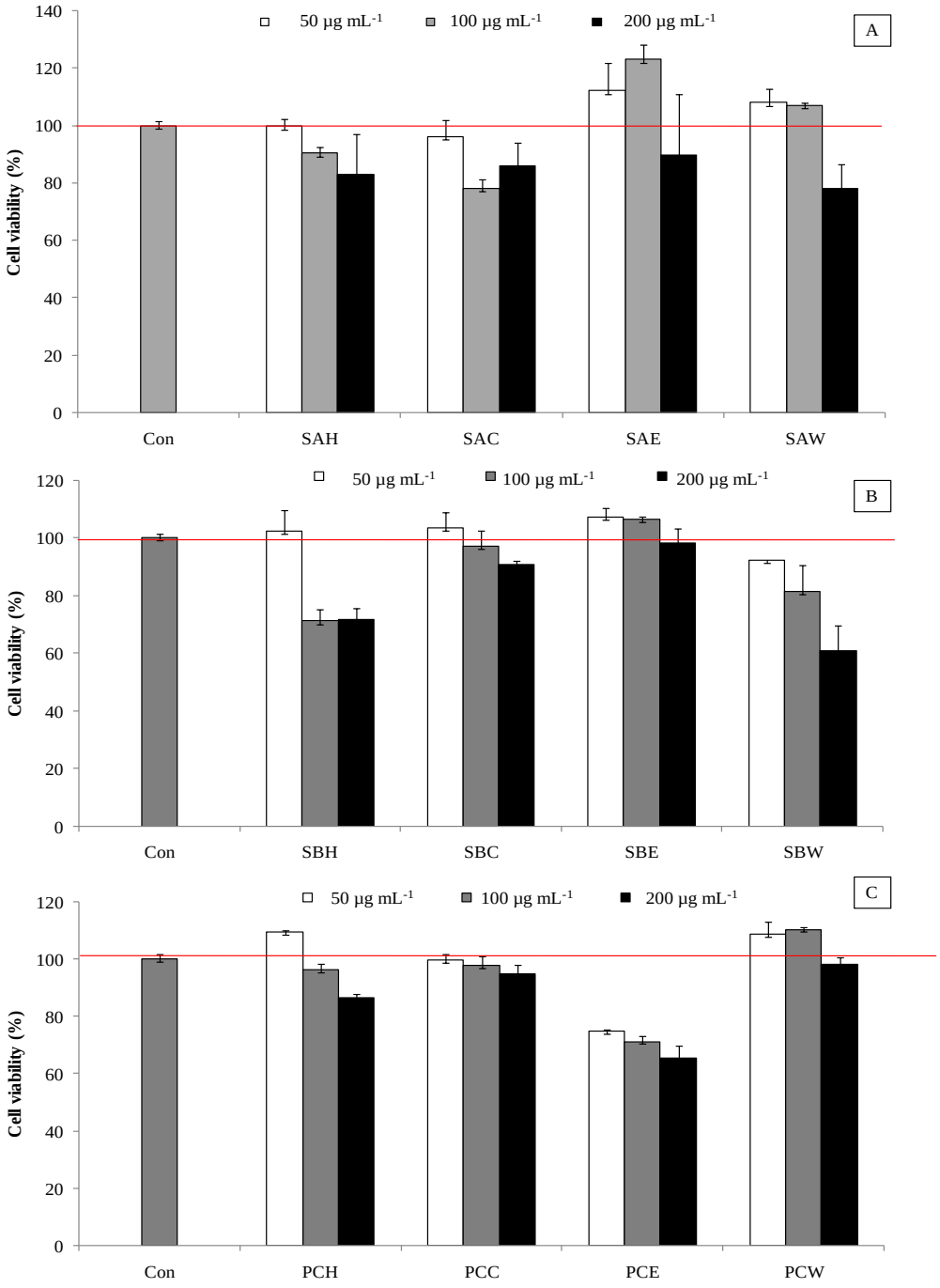

Fig. 59 The effect of cell viability (%) from the different solvent fractions of some selected Sri Lankan seaweeds, including (A) *Sargassum sp.* A; (B) *Sargassum sp.* B and (C) *Padina commersonii* agasint vero cells, incubated at 24 h by MTT assay. (H: hexane; C: chloroform; E: ethyl acetate and W: aqueous fraction)

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In perticualr**,** ethyl acetate fraction of *Sargassum sp.* A (SAE) showed the highest significant cell viability at 50 and 100 μg/mL concentrations. Although the PCE fraction showed the highest cytotoxicity, SBE, PCC, PCW fractions considered as the least cytotoxic effect agasint vero cells at all the concentrations. Thus, cytotoxicity effect of the different fractions can be attributed for screening of *in vitro* antioxidant assays in order to determine the reactive oxygen species (ROS) scavenging effects comparatively.

3.3 Determination of Inhibitory effect of nitric oxide (NO) production and cytotoxicity on LPS-induced RAW macrophages

Inhibitory effects of NO production on LPS-induced RAW macrophages against methanol extracts from Sri Lankan marine seaweeds were evaluated *in vitro*. Among the extracts, *C. ceylanicus*, *C. crassa* and *G. racemosa* showed the profound suppression of NO production on LPS-induced RAW cells compared to the other extracts significantly. Cell viability assay was evidenced for the extracts of *C. ceylanicus* and *C. crassa* are having the lower cytotoxicity at all the treated concentrations. However, *G. racemosa* showed the cytotoxic effect against LPS-induced RAW cells dose-dependently (Fig. 60). The determined IC_{50} values of NO inhibitory activity are shown in the Table 17. Among the extracts, *C.ceylanicus* and *C. crassa* samples showed the NO production inhibitory activity with the IC_{50} values 34.25 and 30.92 μg mL^{-1}, respectively. Hence these extracts can be considered for further studies on anti-inflammatory effects. In addition, the different solvent extractions of Sri Lankan brown algae samples were also considered for evaluation anti-inflammatory activity agasint LPS-induced RAW Cells (Fig. 61, 62 and 63). Among the fractionated samples from *Sargassum sp.* A, ethyl acetate fraction (SAE) showed the profounds NO production inhibitory effect at dose-dependantly (Fig. 61 A).

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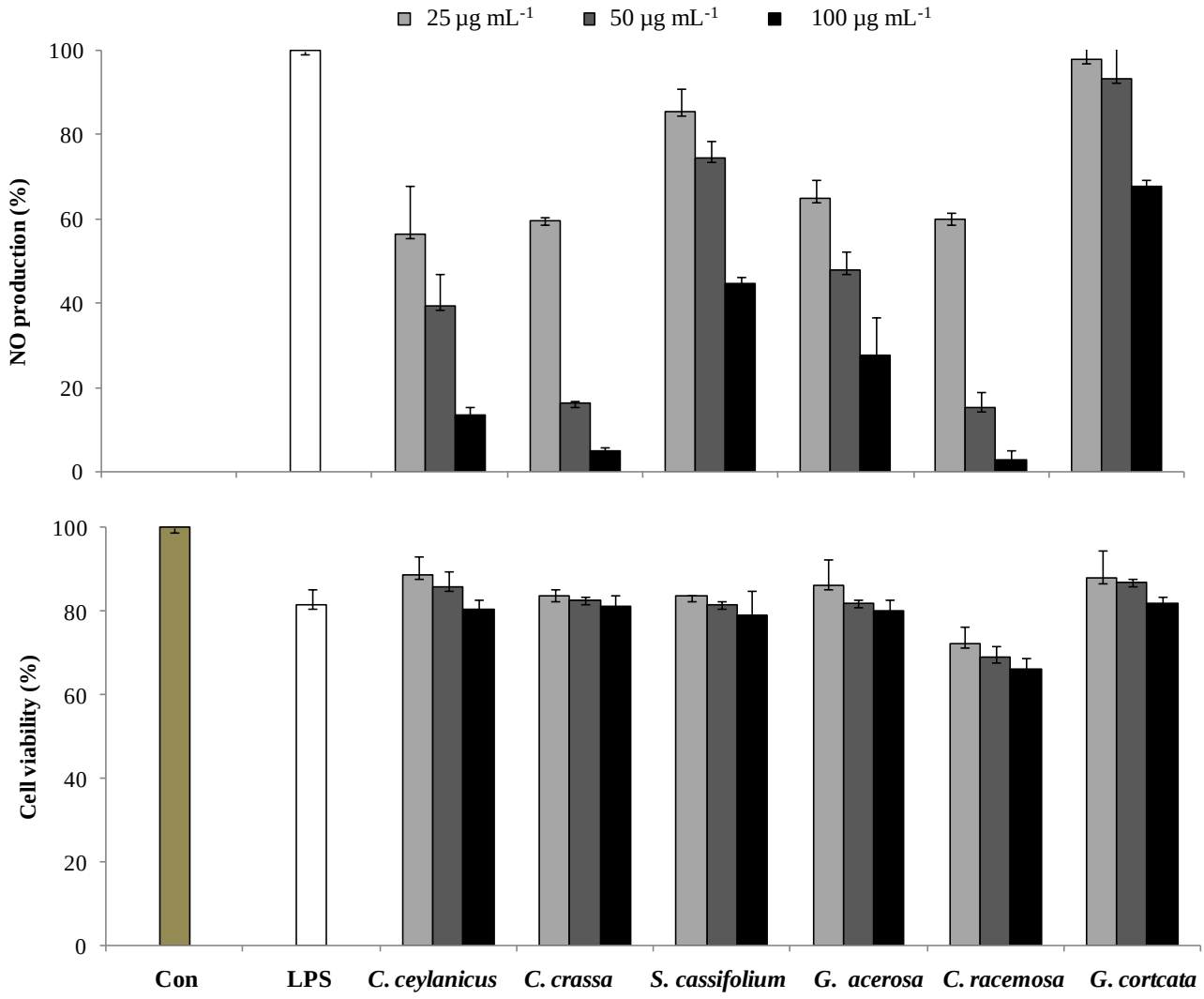

Fig. 60 Inhibitory effect of the methanol extracts of collected seaweeds from Sri Lanka, LPS-induced NO production (%) and cell viability (%) in RAW 264.7 macrophages. After 24 h to treat the compounds cell viability was assessed by MTT assay. Values are expressed as means ± SD in triplicate experiments.

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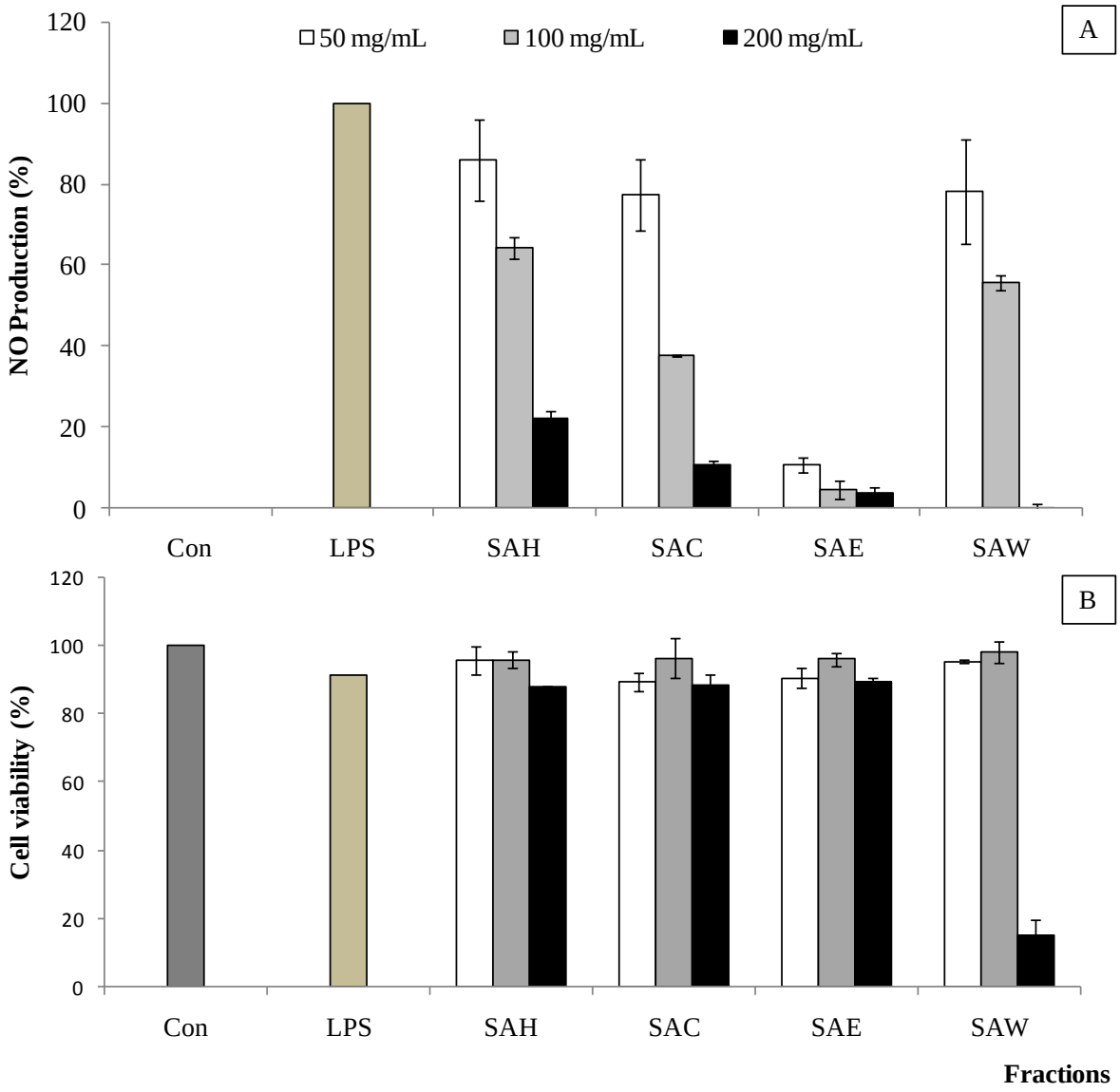

Fig. 61 Inhibitory effect of *Sargassum sp.* A solvent extracts by solvent-solvent partition chromatography on (A): LPS-induced NO production (%) and (B) cell viability (%) in RAW 264.7 macrophages. The Incubation of extracts, SAH: hexane fraction; SAC: chloroform fraction; SAE: ethyl acetate fraction and SAW: aqueous fraction with cells in response to LPS (1 μg ml^{-1}) for 24 h, the nitric oxide (NO) levels in the medium was measured. Values are expressed as means ± SD in triplicate experiments.

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Cell viability assay was confirmed that SAE fraction has the lowest cytotoxicity against LPS-induced RAW macrophages (Fig. 61 B). In addition, Figure 62 A shows the NO production inhibitory activity (%) of the fractions obtained from *Sargassum sp.* B sample. According to the results, SBH and SBE indicated the NO production suppression activity although the cell viability assay showed the SBE fraction has significantly high cytotoxicity effect against RAW cells compared to the LPS treated cells (Fig. 62 B). Hence, SBH fraction was considerd as the highest anti-infalmmatory active fracation among the *Sargassum sp.* B sample significantly. Moreover, the determination of NO production suppression activity on LPS-induced RAW cells by *P. commersonii* samples showed that PCE and PCC fractions are having the significantly high activity (Fig. 63 A). Furthermore, Cell vaibilty assay was confirmed that these fractions are having the least cell toxicity effects compared to the other fractions on LPS-induced RAW cells (Fig. 63 B). Taken together, among the fractions, the determined NO production inhibitory activity (IC_{50} μg/mL) showed in the Table 16. Hence, SAE fraction was exhibited the highest NO production suppression effect as the IC_{50} value 15.07 μg/mL (having the least cell cytotoxicity) agasint LPS-stimulated RAW macrophages and identified as the best fraction among the tested other fractions for promsing anti-inflammatory effect from the Sri Lankan brown algae species.

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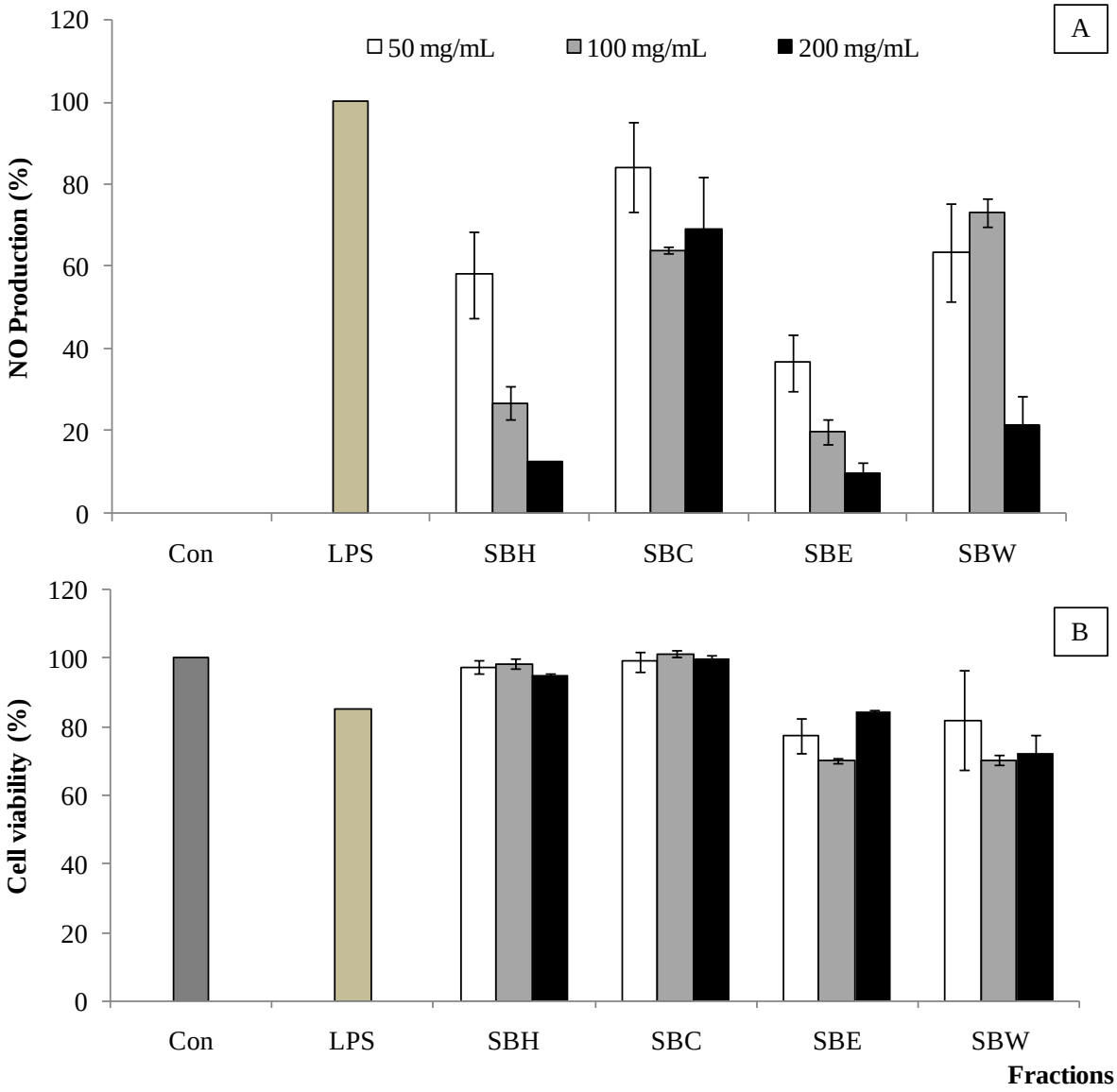

Fig. 62 Inhibitory effect of *Sargassum sp.* B solvent extracts by solvent-solvent partition chromatography on (A): LPS-induced NO production (%) and (B) cell viability (%) in RAW 264.7 macrophages. The Incubation of extracts, SBH: hexane fraction; SBC: chloroform fraction; SBE: ethyl acetate fraction and SBW: aqueous fraction with cells in response to LPS (1 μg ml^{-1}) for 24 h, the nitric oxide (NO) levels in the medium was measured. Values are expressed as means ± SD in triplicate experiments.

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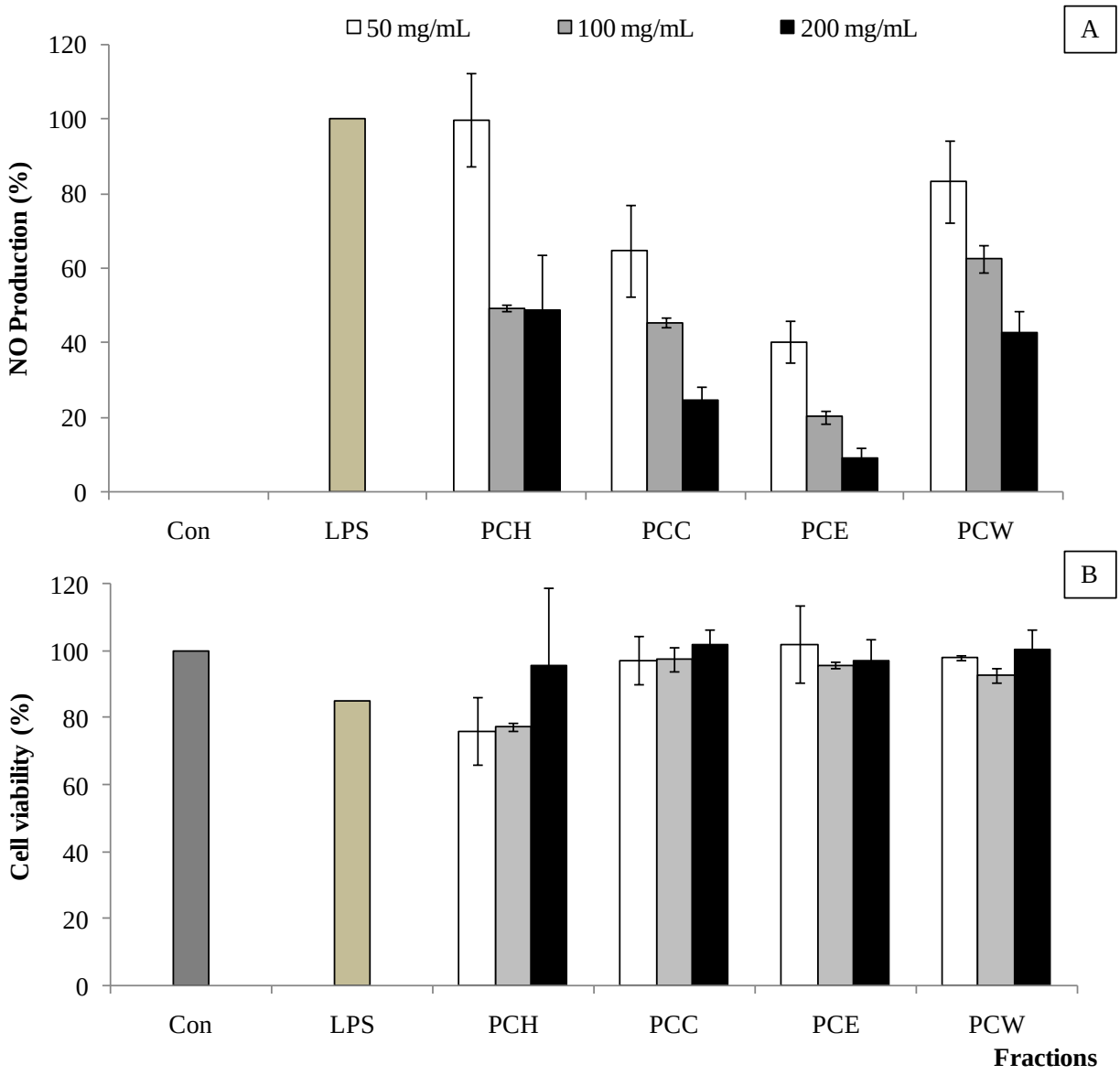

Fig. 63 Inhibitory effect of *Padina commersonii* solvent extracts by solvent-solvent partition chromatography on (A): LPS-induced NO production (%) and (B) cell viability (%) in RAW 264.7 macrophages. The Incubation of extracts, PCH: hexane fraction; PCC: chloroform fraction; PCE: ethyl acetate fraction and PCW: aqueous fraction with cells in response to LPS (1 μg ml^{-1}) for 24 h, the nitric oxide (NO) levels in the medium was measured. Values are expressed as means ± SD in triplicate experiments.

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Table 16. Determined NO production inhibitory activity (IC_{50} values) of solevent extracts from some selected Sri Lankan seaweeds agasint LPS-induced RAW cells

Seaweed	Fractions	NO production Inhibitory activity (IC_{50} µg/mL) [a] on RAW macrophages
Sargassum sp. A	SAH	134.27 ± 1.5
	SAC	97.68 ± 0.41
	SAE	15.07 ± 0.56
	SAW	106.32 ± 3.4
Sargassum sp. B	SBH	62.82 ± 2.3
	SBC	>200
	SBE	80.63 ± 1.9
	SBW	128.69 ± 1.1
Padina commersonii	PCH	99.42 ± 3.1
	PCC	97.15 ± 2.9
	PCE	95.06 ± 1.6
	PCW	165.75 ± 1.8

[a] The values were determined by at triplicate individual experiments. Values are mean ± SD of three determinations.

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3.4 Growth inhibitory activity of Sri Lankan seaweed methanol extracts against HL-60, B16F10, and A549 cancer cell lines

Figure 64 is presented the cell viability (%) of the cancer cells including HL-60, B16F10 and A549 incubated with concentrations (50, 100 and 200 μg mL^{-1}) of the methanol extracts from Sri Lankan seaweeds for 24 h in the MTT assay. Among the cancer cells, the HL-60 cells were suppressed significantly ($P > 0.05$) with the treated *C. racemosa* and *C. ceylanicus* extracts dose dependently compared to the control. In particular, the highest growth inhibitory activity, approximately about 95% on HL-60 cells were observed at 200 μg mL^{-1} treated concentration of *C. racemosa* compared to the control (Fig. 64 A). The same concentration of *C. racemosa* showed the cancer cell growth inhibitory effect markedly against B16F10 and A459 cancer cells, respectively (Fig 64 B & C). The calculated cancer cell growth inhibitory activity, IC_{50} values are listed in the Table 17. According to the results, the lowest cancer cell growth inhibitory activity (IC_{50} value 30.17 μg mL^{-1}) was reported by *C. racemosa* compared to other extracts. Therefore, methanol extract of *C. racemosa* was used in order to determine the mechanism of anticancer activity against HL-60 cells. Hence, the apoptotic body formation and accumulation of DNA content % in the sub-G_1 phase of the cell cycle of HL-60 was also evaluated.

3.5 Apoptosis induced anticancer activity

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 to 490nm. This dye binds preferentially to adenine-thymine (A-T) regions of

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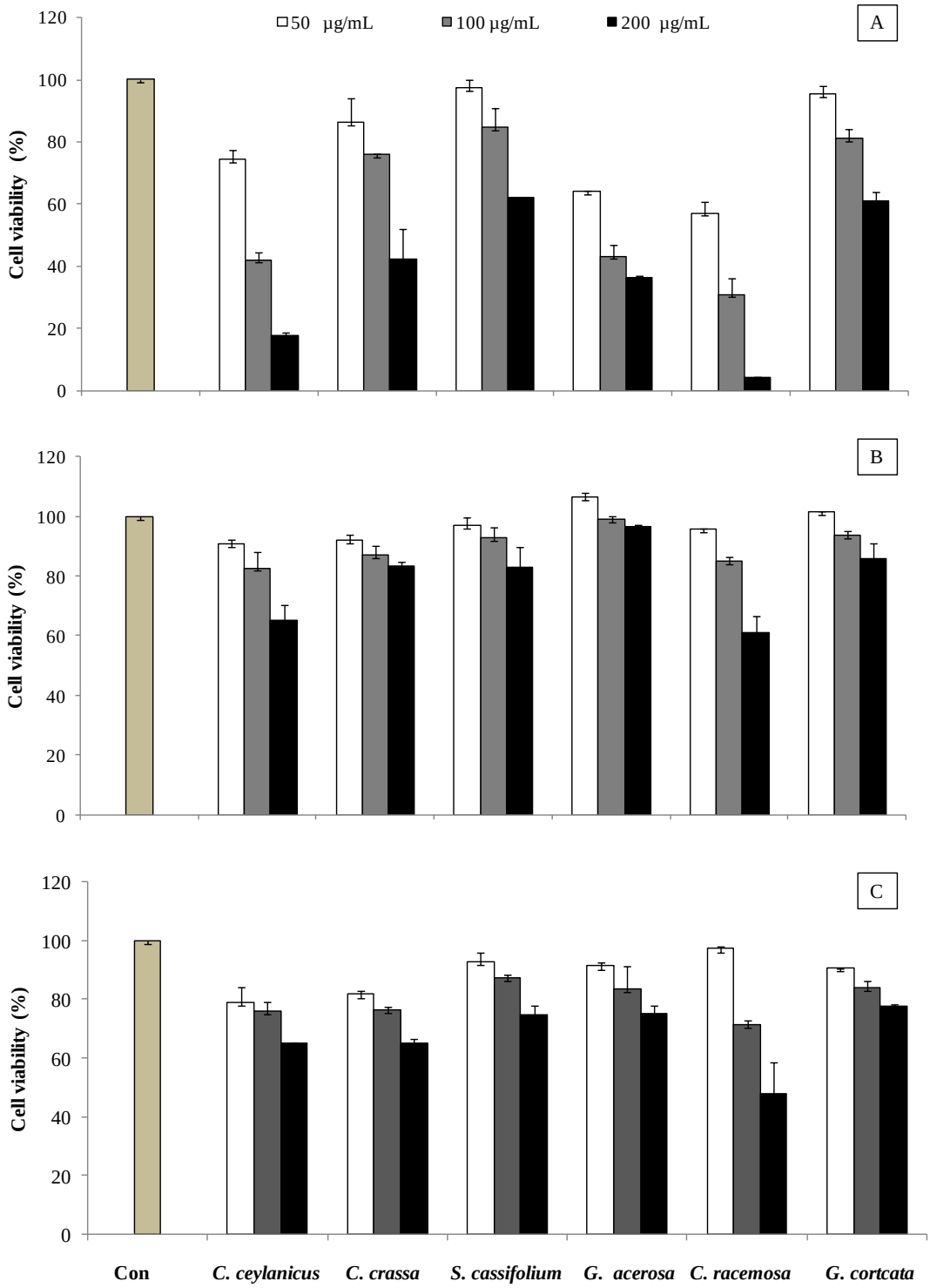

Fig. 64 The effect of cell viability (%) of HL-60 (A), B16F10 (B) and A549 (C) cancer cells against the methanol extracts of collected seaweeds from Sri Lanka for 24 h.

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Table 17. The determined IC_{50} values (μg mL^{-1}) of ant-inflammatory and anticancer activities of some selected Sri Lankan seaweed methanol extracts

***In vitro* assays**	***C. ceylanicus***	***C. crassa***	***S. cassifolium***	***G. acerosa***	***C. racemosa***	***G. cortcata***
Inhibitory effect of NO production on LPS-induced RAW 264.7 cells	34.25	30.92	91.28	51.75	29.32	167.35
Cancer cell (HL-60) growth suppression	102.17	179.88	>200	104.43	30.17	>200

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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). Methanol extract of *C. racemosa* (CRM) various concentrations at (50 ~ 200 μg mL^{-1}) were incubated with the HL-60 cells for 24 h and determined the apoptosis induced nuclear morphology of HL-60 cells stained with Hoechst 33342 and visualized by fluorescent microscopy (Fig. 65). In this experiment, the control (no treated sample; A) showed the intact cell nuclei without DNA damage. However, along the treatment of CRM different concentrations, clear DNA damages were observed and led to increase the apoptotic body formations dramatically (Fig. 65 B, C and D). These results were markedly correlated with the HL-60 cells growth inhibition via the proportion of apoptotic body formation with the treated CRM concentration dose-dependently.

3.6 Induction effect of Sub-G_1 contents in HL-60 cells

The inhibitory effects of the proliferation of HL-60 cells were evaluated by determining the sub-G_1 content population (%) respect to the incubated CRM different concentrations. According to the results, it is observed that increasing the effect of the cell cycle arrest and the accumulation of cells in the sub-G_1 phase in concentration dependent manner (Fig. 66 B, C, and D). Moreover, the apoptotic body formations were reflected to the accumulation of sub-G_1 content and further determined as 3.59, 7.18 and 20.13 % of sub-G_1 population against the CRM concentrations (50, 100 and 200 μg mL^{-1}), compared to the control, respectively (Fig. 66 E). These evidences suggest that CRM induced cell death was caused due to the apoptotic body formations significantly at concentration dependent manner.

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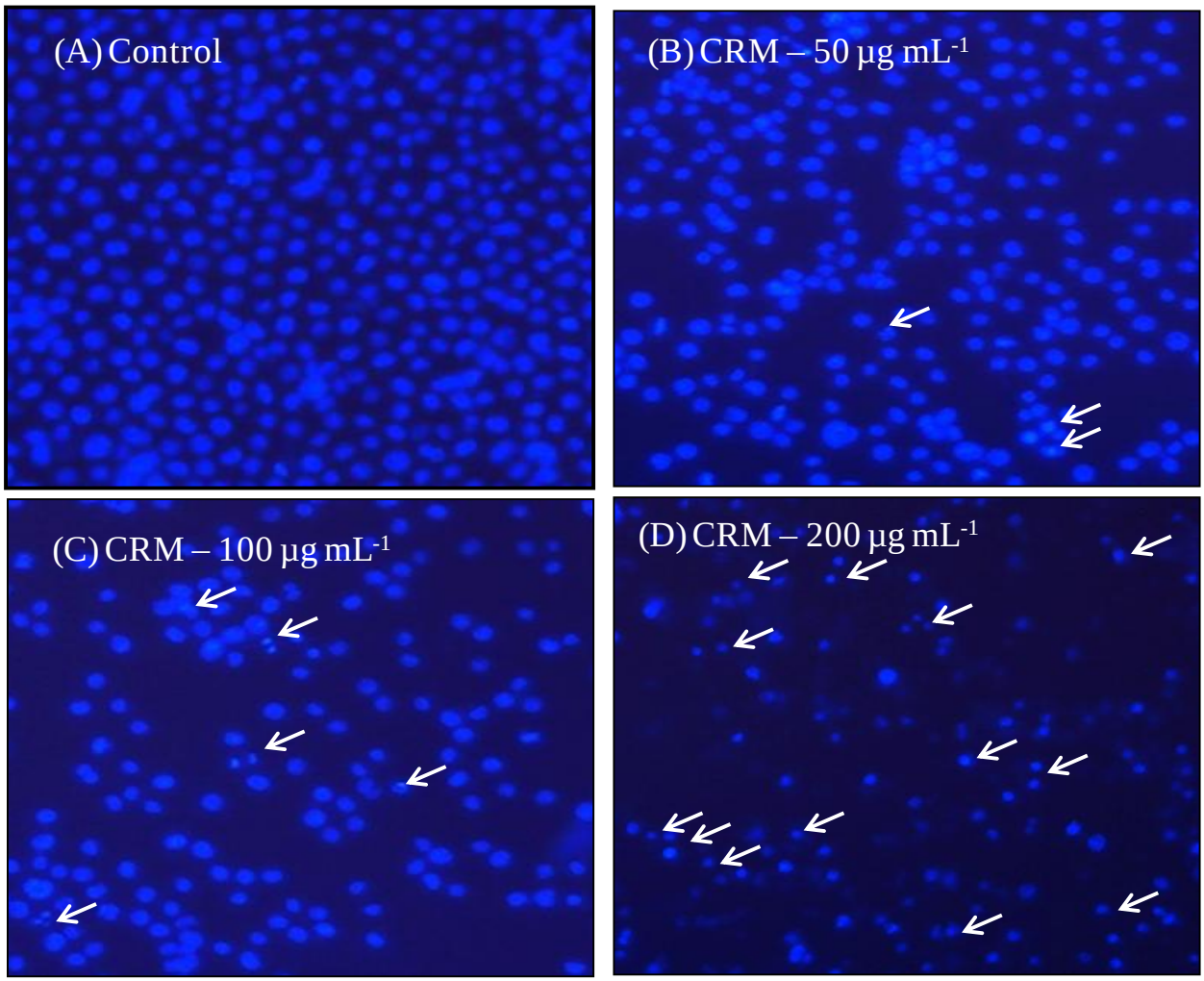

Fig. 65 Induction of apoptotic body formation in HL-60 cells was observed under a fluorescent microscope after Hoechest 33342 staining. (A) control: no treated; (B) *Caulerpa racemosa* methanol extract (CRM) treated with 12.5 µg mL^{-1}; (C) CRM treated with 25 µg mL^{-1}; (D) CRM treated with 50µg mL^{-1}; (E) CRM treated with 100 µg mL^{-1}. *Arrows* denoted a typical apoptotic body formation in HL-60 cells.

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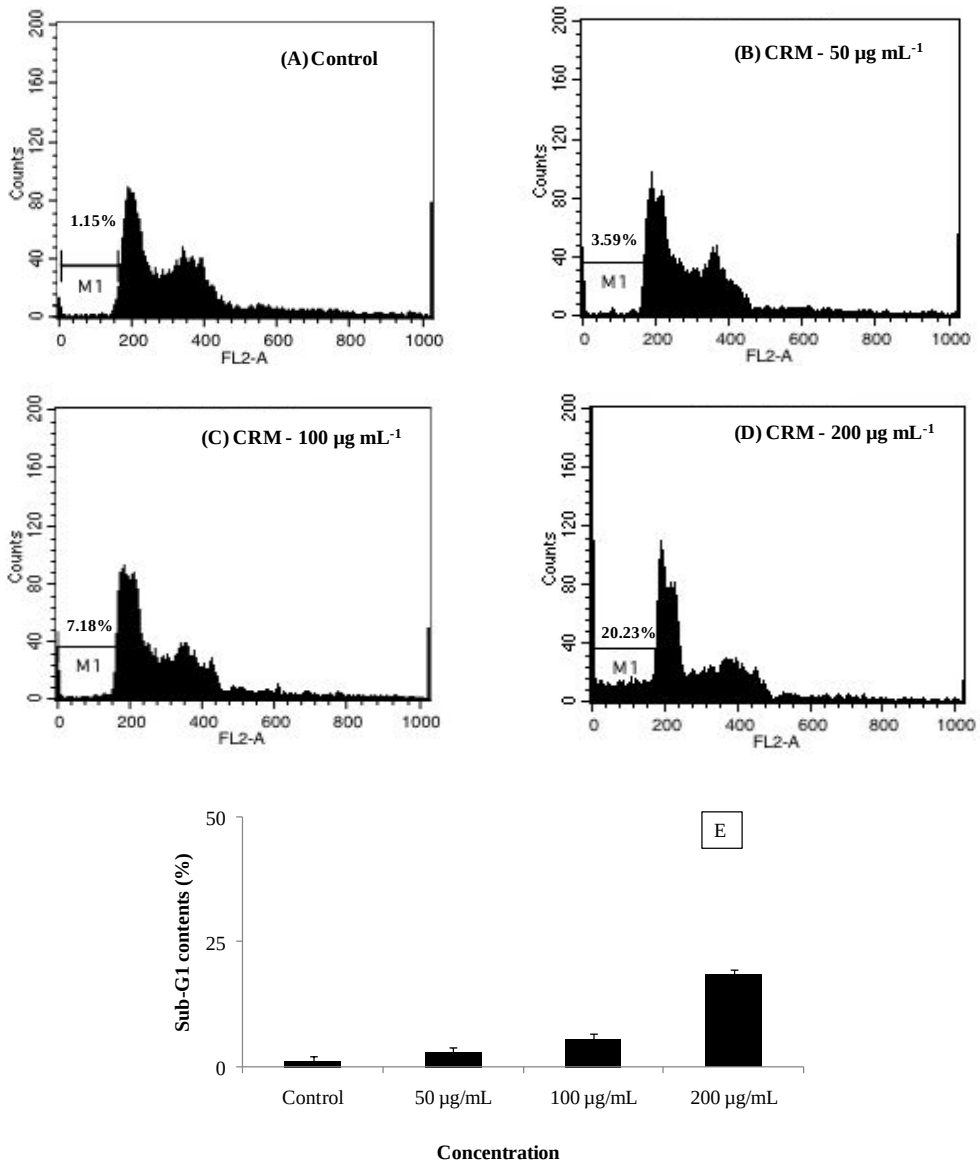

Fig. 66 Apoptotic Sub-G1content was detected by flow cytometry after stained with PI. (A) control: no treated; (B) *Caulerpa racemosa* methanol extract (CRM) treated with 12.5 µg mL^{-1}; (C) CRM treated with 25 µg mL^{-1}; (D) CRM treated with 50µg mL^{-1}; (E) CRM treated with 100 µg mL^{-1}, (F) Accumulation of DNA content (%) in the Sub-G_1 phase of the cell cycle of HL-60 incubated with CRM different concentrations were determined by flow cytometry.

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4. CONCLUSION

Among the collected seaweeds from Sri Lankan water were evaluated for the pharmacological activity. Among the selected marine algae, *C. racemosa* showed the highest anticancer activitity (IC_{50} value 30.17 μg/mL) agaiant HL-60 cells compared to the other methanol extracts. The mechanism of the anticacancer activity with inducing apoptotic body formation and accumulation of DNA in sub-G_1 phase were evaluated *in vitro*. Moreover, among the seaweed fractions, some showed the significant anti-infalmmatory activity against LPS-induced RAW cells. In addition, free radical scavenging activity determined in this study indicated that having the strongest antioxidant activity on Sri Lankan seaweeds. Taken together, the assessed bioactivities of the extracts from Sri Lankan seaweeds are confirmed that having the profound pharmacological properties in order to isolate some of bioactive secondary metabolites using sequencial chromatographic steps.

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