A THESIS

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Preparative isolation and identification of functional compounds from *Pyropia yezoensis* and evaluation on their biological effects

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GRADUATE SCHOOL

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Preparative isolation and identification of functional compounds from *Pyropia yezoensis* and evaluation on their biological effects

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CONTENTS

Part II. Identification of chemical structure of anti-inflammatory and anti-diabetes compounds from ethyl acetate fraction of *Pyropia yezoensis*

국문초록

방사무늬김은 예로부터 동양아시아인 한국, 중국, 일본 등 에서 양식되어 오면서, 식품 및 의약소재로 널리 이용되고 있는 식용 해조류이다. 이들은 황산기 다당류인 포피란, 글라이코프로테인 및 파이코에리스린인 기능성 단백질 등 다양한 기능성 성분이 풍부하여, 항산화, 항암, 항염 및 항당뇨 등 다양한 효능이 규명되어 왔다. 하지만 대부분의 연구가 상대적으로 함량이 높은 단백질(30~40%) 및 다당류(40~50%) 에 대한 연구가 활발히 진행되어 왔으나, 그 외 성분에 대한 연구는 매우 미흡한 실정이다. 따라서 본 연구에서는 우리나라에서 양식되고 있는 주요 품종인 방사무늬김(*Pyropia yezoensis*)으로부터 항염증 및 항당뇨 성분에 대한 규명을 수행하였다.

1. 방사무늬김 유래 추출물에서 각 n-hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc), water 분획물로 나누었으며, 기존 보고에서 항염증 및 항당뇨 효능 및 성분에 대한 알려진, n-hexane 분획물을 제외한 $CHCl_3$ (PYC) 및 EtOAc (PYE) 분획물에 대한 lipopolysaccharide (LPS)에 의해 염증반응이 유도된 마우스 대식세포인 Raw 264.7 cell내 염증메게체인 nitro oxide (NO)에 감소효능을 평가한 결과, PYC 및 PYE에서 각각 16.88 μg/ml 과 10.40 μg/ml의 IC50 값을 나타내었다. 그리고 항당뇨 평가에서 alloxan에 의해 췌장이 파괴된 zebrafish에 2% glucose를 처리하였을 때 무처리 군에 비해 혈중 glucose 함량이 크게 증가하였으며, PYC 및

PYE 0.5~2μg/ml에서 농도 구배적으로 혈중 glucose 함량을 낮추었다. 따라서, 먼저 PYC에서 항염증 및 항당뇨 효능을 갖는 성분을 규명하기 위하여, 항염증은 LPS에 의해 대식세포에 생성된 NO 생성 억제능으로 평가하였으며, 항당뇨 효능은 근원섬유세포인 C2C12내 glucose 함량과 zebrafish 혈중 내 glucose 함량을 측정하여 평가하였다. PYC는 silica column을 EtOAc (PYCE) 과 MeOH (PYCM) 분획물로 나누었다. 해조류내 $CHCl_3$ 분획물에는 일반적으로 지방산, 지용성 색소체, 스테롤 및 폴리페놀 등의 기능성 성분이 있다고 알려져 왔으며, 특히 폴리페놀과 스테롤은 항산화, 항염, 항암, 항당뇨 및 항비만 등 매우 다양한 효능이 있다고 보고되어 왔다. 따라서 이 연구에서는 방사무늬김 에탄올 추출물유래 PYC에서 타깃성분에 대한 확인을 위하여 total phenol 및 total sterol 함량을 측정한 결과 PYCE에서 28%의 높은 스테롤 함량을 보였으며, total phenol 함량의 경우에는 모든 분획물에서 5% 이하의 낮은 함량을 보였다. 따라서 PYCE내 주요 기능성 성분으로 sterol을 타깃으로 설정하였으며, 이들의 유리화된 형태의 생성을 위하여 saponification 공정을 수행하여 PYCES를 획득하였다. 상기에서 획득한 모든 분획물에 대한 항염증 및 항당뇨 *in vitro*, *in vivo* 효능을 평가한 결과 PYCES에서 전체적으로 가장 우수한 효능을 보였다. 홍조류내 스테롤 성분에는 desmosterol이 80%이상 차지하고 있으며, 그 외는 cholesterol, stigmasterol, campesterol등이 알려져 있다. 우리는 Silica TLC를 통해 PYCE내 총 5개의 스테롤 성분을 확인

하였으며, 이중 기존에 알려진 desmosterol과 cholesterol로 확인되었으며, 그 외 성분은 기존에 알려진 성분과는 다른 스테롤로 확인되었다. 따라서 그 외 성분은 PYCES1~3으로 설정하였으며, liquid liquid chromatography의 일종인 고속원심분배크로마토 그래피를 이용한 이들의 분리정제를 위하여 최적 분배조건을 수립하였다. 9:1:9:1(n-Hexane:EtOAc:MeOH:Water) ~ 6:4:6:4 용매 조건에서 최적 분배 조건을 확인한 결과, PYCES1, desmosterol 및 cholesterol은 9:1:10:1 조건으로 확립되었으며, PYCES2 및 3는 5:5:7:3 조건으로 확립되었다. 따라서 다음과 같은 조건을 적용한 CPC를 이용하여 5개의 물질을 분리하였으며, 그들은 preparative TLC를 통하여 각각 정제되어 사용되었다. 기존에 알려진 desmosterol과 cholesterol을 제외한 PYCES1~3에 대한 항염증 효능을 평가한 결과 PYCE2, 3이 독성이 없는 상태에서 높은 항염증 효능을 보였으며, 항당뇨 in vitro, in vivo 효능평가에서는 PYCES3이 가장 우수한 항당뇨 효능을 보였다. 따라서 전체적으로 가장 우수한 항염증 및 항당뇨 효능을 보인 PYCE3에 대한 구조 규명을 위해 GC-MS와 MS/MS, NMR 분석을 진행하였다. GC-MS 분석 결과26,27-Dinorergosta-5,23-dien-3-ol과 82%의 유사성을 보였으며, ^{13}C NMR 분석 결과 PYCES3은 27개의 carbon 수를 갖는 것으로 확인되었다. 따라서 기존 연구 보고와 NMR 분석 결과 26,27-Dinorergosta-5,23-dien-3-ol 을 기본골격으로 하는 10,13-dimethyl-17-(6-methylheptan-2-yl)-2,3,8,9,10,11,12,13,14,15,16,17-dodecahydro-1H-

cyclopenta[a]phenanthren-3-ol로 확인되었다. 이들에 대한 염증성 cytokines에 대한 생성억제 효능을 평가한 결과 염증성 매게 채인 COX-2에 의해 생성되는 PGE_2 는 PYCES3에 의해 강력하게 억제 되었으며, 염증 및 암 생성 cytokine인 IL-6는 농도구배적으로 억제되었다. 그 외 염증성 cytokine인 IL-1β는 50μg/ml에서 억제되었다.

2. PYC외 항염증 및 항당뇨 평가에서 우수한 효능을 보인 PYE에서도 기능성 성분을 규명하기 위하여 PYE를 silica column을 사용하여 EtOAc (PYCE) 과 MeOH (PYCM) 분획물로 나누었다. 항염증 효능은 LPS에 의해 대식세포에 생성된 NO 생성 억제 능으로 평가하였으며, 항당뇨 효능은 근원섬유세포인 C2C12내 glucose 함량과 alloxan에 의해 췌장이 파괴된 zebrafish 혈중내 glucose 함량을 측정하여 평가한 결과, PYE, PYEE 및 PYEM 중 에서 PYEE가 가장 우수한 항염증 및 항당뇨을 나타내었다. 따라서, 이 연구에서는 방사무늬김 유래 PYE에서 타깃성분에 대한 확인을 위하여 항염증 및 항당뇨 효능이 가장 좋은 PYEE를 TLC와 HPLC 분석을 한 결과 다량의 색소채가 함유된 것을 확인하였고, 그 외에 지방산과 극성의 화합물이 함유된 것을 확인을 하였다. PYEE로부터 활성물질을 분리 하기 위해 open column chromatography를 사용하였고, 이동상 용매인 MeOH의 비율을 20, 70 100% 비율로 순차적으로 올리며 총 7개의 분획물 (PYEE1~7)을 얻었다. PYEE로부터 얻어진 7개의 분획물에 대한 항염증 및 항당뇨 *in vitro*, *in vivo* 효능을 평가한 결과

PYEE6이 전체적으로 가장 우수한 효능을 보였다. PYEE6을 HPLC로 분석한 결과 다량 극성화합물이 함유된 것을 확인 하였고, TLC 분석을 통하여 최적의 분리 조건을 설정한 결과, 이동상 용매의 비율을 55% MeOH로 설정을 하여 open column chromatography 분리정제를 한 결과, 총 2개의 화합물 PYEE6-1, 6-2를 분리하였으며, 그들은 preparative TLC를 통하여 각각 정제되어 사용되었다. PYEE6-1, 2에 대한 항염증 효능을 평가한 결과 PYCE1, 2 모두 독성이 없는 상태에서 높은 항염증 효능을 보였으며, 및 항당뇨 *in vitro, in vivo* 효능평가에서도 PYEE6-1, 2 모두 우수한 항당뇨 효능을 보였다. 따라서, PYEE6-1, 2 두 개의 화합물에 대한 구조 규명을 위해 GC-MS와 MS/MS, NMR 분석을 통해 진행하였다. 먼저 PYEE6-1의 GC-MS 분석 결과 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester와 97%의 유사성을 보였으며 NMR 분석 결과 PYEE6-1와 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester은 동일한 화합물로 확인되었다. 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester은 *Polygonum chinense* 내에서 존재하는 천연물로, 보고된 효능으로는 항균효과 정도 만이 보고되어있다. 이어서, PYEE6-2의 GC-MS 분석 결과 3,4-dichloro[1,6]naphthyridine $$ pyrido[4,3-B]pyridine, 3,4-dichloro 와 79%의 유사성을 나타냈으며, 이 성분에 대한 효능 보고는 전무 하였다.

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2. INTRODUCTION

Inflammation represents a highly coordinated set of events that allow the tissues to respond against an injury or infection. It involves the participation of various cell-types expressing and reacting to the diverse mediators along a very precise sequence of events (Babu *et al*., 2009). Usually, inflammation is initiated through the production of specific cytokines or chemokines characterized by the recruitment of leukocytes to the damage sites. Initial features are of nonspecific flu-like symptoms, common to almost all acute viral infections and may include malaise, muscle and joint aches, fever, nausea or vomiting, diarrhea, and headache. However, the sustained or excessive inflammation can lead to various diseases including rheumatoid arthritis, psoriasis and inflammatory bowel disease (Simon and Green, 2005). Macrophages play a key role in the inflammatory and immune reactions by releasing a variety of inflammatory mediators such as nitric oxide (NO), tumor necrosis factor-α (TNF- α), interleukin-1 β (IL-1β), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Yoon et al., 2003; Ramana and Srivastava.,2006; Nunez Miguel et al., 2007).

Lipopolysaccharide (LPS), a component of the gram negative cell wall, was used to stimulate peritoneal macrophages and induce the production of anti-inflammatory cytokines in the infection response (Pan et al., 2008). Suppression of these inflammatory mediators may be an effective therapeutic strategy to prevent diseases caused by inflammatory disorders (Kim et al., 2015). It is commonly used to inhibit inflammatory mediators using drugs such as non-steroidal anti-inflammatory analgesics (NSAIDs) and corticosteroids (Benjamini and Sunshine, 1996). However, various side effects of long term use of these drugs have been described (Benjamini and Sunshine, 1996). Recently, development of anti-inflammatory and anti-ulcerogenic drugs has recently focused on discovering the favourable application of herbal plant-derived extracts that are potent and safer to use (Smit AJ., 2004; Dhargalkar and Pereira, 2005). The red algae has been reported to contain active compounds that may help ameliorate inflammation of the alimentary tract (Kang et al., 2008), prevent or treat gastric ulcers and cancers caused by oxidative stress (Gonzalez et al., 1999; Yeh et al., 2012),

inhibit inflammatory activities by suppressing the production of inflammatory mediators (Khan et al., 2007; Khan et al., 2008; Lee et al., 2009).

Pyropia species is cold-water seaweed that grows in cold, shallow seawater. More specifically, it belongs to red algae phylum of laver species, comprising approximately 70 species (Brodie and Irvine, 2003). It grows in the intertidal zone, typically between the upper intertidal zone and the splash zone in cold waters of temperate oceans. In East Asia, it is used to produce the sea vegetable products Nori (in Japan) and Gim (in Korea). There are considered to be 60 to 70 species of *Pyropia* worldwide (Kain, 1991). It grows like moss and is about 14 ~ 25 cm wide and 5 ~ 12 cm long. It is long elliptical shape with wrinkles on the edge and brown on the top and blue on the bottom. It will be cultivated from October to March. As of 2015, about 320,000 tons of laver has been cultivated in Korea and its production amounted to 330 billion won. There are various kinds of processed lavers, and the most common ones are seasoned lavers with oil, salt and baking, and others kinds of salted lavers, lavers cup noodles, lavers snacks. As of 2015, processed food exports have reached $ 300 million and are continuing to increase. Lavers is mainly composed of 40 ~ 50% of carbohydrates and 30 ~ 40% of proteins and has recently been attracting attention as a diet food. The dietary consumption of *Porphyra yezoensis* was weakly associated with a lower incidence of diabetes in Korean men (Lee et al. 2010).

According to the World Health Organization approximately 150 million people have diabetes mellitus worldwide, and this number may double by the year 2025, due to population growth, ageing, unhealthy diets, obesity and sedentary lifestyles (Panagiotakos et al., 2005) Diabetes mellitus (DM), commonly referred to as diabetes, is a group of metabolic diseases in which there are high blood sugar levels over a prolonged period. Symptoms of high blood sugar include frequent urination, increased thirst, and increased hunger. If left untreated, diabetes can cause many complications. Acute complications include diabetic ketoacidosis and nonketotic hyperosmolar coma (Kitabchi et al., 2009). Serious long-term complications include cardiovascular disease, stroke, kidney failure, foot ulcers and damage to the eyes. Diabetes mellitus is classified into four broad categories: type 1, type 2, gestational diabetes, and "other specific types" (David G. Gardner, 2011). Type 1 diabetes mellitus is

characterized by loss of the insulin-producing beta cells of the islets of Langerhans in the pancreas, leading to insulin deficiency. Type 2 diabetes mellitus is characterized by insulin resistance, which may be combined with relatively reduced insulin secretion (David G. Gardner, 2011). Previous study, sulfated polysaccharide from *Pyropia* sp. has been reported an anti-diabetes effect, but there are various continents such as sterol, pigments, and polar compounds in red algae. Therefore we focused on identification of anti-diabetes compounds from *P. yezoensis* and performed the evaluation of *in vivo*, zebrafish model for diabetes.

Pyropia species, the important edible red algae, are well known as food and medicine in East and Southeast Asia including Korea, Japan, Taiwan and China. The species was functional component contains sulfated polysaccharide, mycosporine like amino acids, sterols, carotenoids, protein, essential fatty acids, vitamins, and minerals (Kazlowska et al., 2013; Senevirathne et al., 2010).

Among various functional components, porphyran, sulphated polysaccharide has been known to have a variety of physiological effects such as anti-oxidant, - cancer, -hyperlipidemic, -fatigue, improvement of immunology and hypercholesterolemic activities (She et al., 2005; Ren et al., 1994; Guo et al., 2005; Inoue et al., 2009). As another one, mycosporine-like amino acids (MAAs) are small, colorless and water-soluble compounds composed of a cyclohexenone or cyclohexenimine chromophore conjugated with the nitrogen subsituent of an amino acids or its imino alcohol (Nakamura et at., 1982; Carreto et al., 1990; Shailendra et al., 2008), and have been identified in a wide variety of marine organisoms including, fungi, heterotrophic bacteria, cyanobacteria, eykaryotic algae and fish (Sinha et al., 1999). And among various functional minor compounds such as β-carotene, chlorophyll a, phenolic compounds, sterols, several amino acids and fatty acids are also existed in *Pyropia* sp., and especially, β-carotene from *Pyropia* sp. exhibited protective effect against mutagenesis probably associated with carcinogenesis and reactive oxygen species (ROSs) (Okai et al., 1996; Nakayama et la., 1999). Sterols, including cholesterol, β-sitosterol and campesterol showed potential for protecting an organism from 4T1 cell-vased tumor genesis (Kazlowska et al., 2013). Phytosterols, which encompass plant sterols and stanols, are steroid compounds similar to cholesterol which occur in plants and vary only in carbon side chains and/or presence or absence of a double

bond (Akhisa., 1991). Sterols include sitosterol, campesterol, β-sitosterol, cholesterol and stigmasterol. These plant sterols may possess cholesterol lowering effect, anti-cancer (Awad et al., 2003), anti-atherosclerosis (Moghadasian et al., 1997; Moghadasian et al., 1999), anti-inflammation (Bouic et al., 2001) and anti-oxidation activities (Rensburg et al., 2000).

And Kazlowska et al. (2012) reported phenolic compounds from *Porphyra dentate* suppressed NO production in lipopolysaccharide (LPS)-stimulated macrophages via NF-κB-dependent iNOS gene transcription. Therefore, existences of these functional continents in *Pyropia* sp. are very important factors for revelation of various biological effects.

One of liquid-liquid chromatographic techniques, preparative CPC system is a non-solid support preparative liquid-liquid separation process chromatographic technique which is based on the difference in distribution of components over two immiscible liquid phases and is possible to large isolate and purify large quantities of the compounds with a purity of over 90% by one step process (Michelet al., 1997, Delannayet al., 2006, Bourdat-Deschampset al., 2004). In addition, CPC system also offers the following technological advantages such as versatile products, faster, less expensive product development, and retention of bioactivity integrity, higher throughput, higher yields and reduced operating costs. The solutes are separated according to their partition coefficient (K) expressed as the ratio of their concentration in the stationary phase to their concentration in the mobile phase (Berthodet al., 1988). CPC system has been widely used at separation of bioactive compounds from land plants (Marstonet al., 1988, Bourdat – Deschampsetal., 2004, Kim et al., 2006). But, in case of seaweeds, only a few algae such as *Ascophyllum nodosum* have been subjected to CPC (Chevolotet al.,1998, 2000).Therefore, in this study, we preparation isolation and identification of chemical structure of functional compounds from *P. yezoensis* by using centrifugal partition chromatography (CPC). Additionally, we examined anti-inflammation and anti-diabetes effects and mechanisms of these compounds.

Figure 1. The red alga *Pyropia yezoensis*

Part I.

Identification of chemical structure of anti-inflammatory and anti-diabetes compounds from chloroform fraction of *Pyropia yezoensis*

1. ABSTRACTS

This study was focus on isolation and identification of anti-inflammatory and anti-diabetic compounds from *Pyropia yezoensis*, a marine red alga. The chloroform fraction from *P. yezoenesis* 70% EtOH extract was fractionated using EtOAc (PYCE) and MeOH (PYCM) by a silica column. The saponification of PYCE was performed to efficiently obtain the free sterols and saponified PYCE had highest sterol content. Among them, PYCE saponification fraction (PYCES) showed the strongest inhibitory activity against nitric oxide (NO) generated from lipopolysaccharide (LPS) stimulated RAW 264.7 macrophages without the cytotoxicity, and PYCES enhanced the uptake of glucose into C2C12 cells in a concentration-dependent manner and PYCES showed strong anti-diabetic activity to lower the blood glucose level in alloxan damaged zebrafish. The three fractions (PYCES1~3) were prepared from PYCES by preparative centrifugal partition chromatography (CPC) with solvent condition consisted with n-hexane:EtOAc:MeOH:water (9:1:10:1 and 5:5:7:3, respectively). PYCES3 exhibited higher anti-inflammatory and anti-diabetic effects than others. Therefore, we identified chemical structures of PYEE6-1 using NMR and GC-MS and they were confirmed as 10,13-dimethyl-17-(6-methylheptan-2-yl)-2,3,8,9,10,11,12,13,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthre-n-3-ol.

2. MATERIALS & METHODS

2.1. Materials

Pyropia yezoens is which was cultivated at the Coast of Wando Island in South Korea, was washed three times with freshwater, and then immediately frozen and stored at –20℃ until use. The frozen samples were lyophilized and ground with a grinder. The dried sample powder was stored in refrigerator until use. All solvent used for preparation of crude sample and CPC separation were of analytical grade (Daejung Chemicals & Metals CO., Seoul, Korea). HPLC grade solvents were purchased from Burdick & Jackson (MI, USA). Lipopolysaccharide (LPS) was purchased from sigma chemical Co., Ltd (ST. Louis, MO).Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) penicillin-strptomycinand trypsine-EDTA were obtained from Gibco/BRL (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium, dimethyl sulfoxide (DMSO)were purchased from Sigma (St. Louis, MO, USA).M-MuLV reverse transcriptase were purchased from Promega (Madison, WI, USA). The enzyme-linked immunosorbent assay (ELISA) kit for IL-1β, TNF-α and Prostaglandin E_2 (PGE_2) were purchased from R & D Systems Inc. (Minneapolis, MN, USA).Protein assay kit and ECL detection reagent were bought from Bio-Rad (Richmond, CA, USA) and Amercham Biosciences (Piscataway, NJ, USA), respectively. Other all reagents and solvents were purchased from Sigma (St. Louis, MO, USA).

2.2. Apparatus

LLB-M high performance CPC (Sanki Engineering, Kyoto,Japan) was used in preparative CPC. The total cell volume is 240 mL. A four-way switching valve incorporated in the CPC apparatus allows operating in either the descending or the ascending mode. This CPC system was equipped with a Hitachi 6000 pump (Hitachi, Japan), anL-4000 UV detector (Hitachi), and a Gilson FC203B fraction collector (Gilson, France). The samples were manually injected through a Rheodynevalve (Rheodyne, CA, USA) with a 2 mL sample loop. ^{1}H-NMR spectra were measured with a JEOL JNM-LA300 spectrometer and ^{13}C-NMR spectra with a Bruker AVANCE 400 spectrometer. Mass spectra (FAB-

MS and EIMS) were recorded on a JEOL JMS 700 spectrometer. The HPLC system in this experiment consisted of two mono Waters 515 HPLC pumps, a Waters 2998 photodiode array detector, a Waters2707 autosampler, and the Waters pump control module II (Waters Corporation). Gas chromatography-mass spectrometry (GC-MS) by Shimadzu GCMS-TQ8040 (Shimadzu Corporation, Japan).

2.3. Preparation of crude sample from *P. yezoensis*

The dried *P. yezoenesis* (2 kg) was extracted three times for 3hr with 70% EtOH under shaking at room temperature. The extract, concentrated in a rotary vacuum evaporator, partitioned with ethyl acetate, and then the dried ethyl acetate fraction was stored in a refrigerator for CPC separation. The whole process was illustrated in Fig. **I**-1

2.4. Preparation of two-phase solvent system

The CPC experiments were performed using a two-phase solvent system composed of n-hexane:ethyla cetate:methanol:water (9:1:10:1, v/v) and (5:5:7:3, v/v). The two phases were separated after thoroughly equilibrating the mixture in a separating funnel at room temperature. The upper organic phase was used as the stationary phase, whereas the lower aqueous phase was employed as the mobile phase.

2.5. CPC separation procedure

The CPC column was initially filled with the organic stationary phase and then rotated at 1000 rpm while the mobile phase was pumped into the column in the descending mode at the flow rate used for the separation (2 mL/min). When the mobile phase emerged from the column, indicating that hydrostatic equilibrium had been reached (back pressure : 34 and 37 MPa), The concentrated $CHCl_3$

fraction saponification (PYCES) (500mg) was dissolved in 6 mL of a 1:1 (v/v) mixture of the two CPC solvent system was injected through the Rheodyne injection valve. The effluent from the CPC was monitored in the UV at 290 nm and fractions were collected with 6ml in 10mltube by a Gilson FC 203 B fraction collector.

2.6. HPLC analysis

A 10ul of 5mg/ml sample solution was directly injected on Atlantis T3 3um 3.0 x 150mm column (Waters, USA) using a gradient acetonitrile–water solvent system. The mobile phase was acetonitrile –water in gradient mode as follows: acetonitrile with 0.1% formic acid – water with 0.1% formic acid (0 min ~ 50 min : 5:95 v/v ~ 100:0 v/v, ~ 50 min : ~ 100:0 v/v, ~70min : ~ 100:0 v/v). The flow rate was 1 mL/min with UV absorbance detection at 290 nm.

2.7. Cell culture

The murine macrophage cell line RAW 264.7 cells was grown in Dulbecco's modified Eagle'smedium (DMEM; Sigma) supplemented with 10% (v/v) heat-inactivated FBS, penicillin (100U/ml), and streptomycin (100 μg/ml). Cultures were maintained at 37 ℃ in a 5% CO2 incubator. Mouse myoblast C2C12 cells were maintained in high glucose-DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 μg/ml) and streptomycin (100 μg/ml). Cultures were maintained at 37℃ in 5% CO2incubator. For differentiation 1312 the cells were seeded in appropriate culture plates, and after sub-confluence (about 80%), the medium was changed to DMEM containing 2% horse serum for 7 days, with medium changes every day. All experiments were performed in differentiated C2C12 myotubes after 7 days.

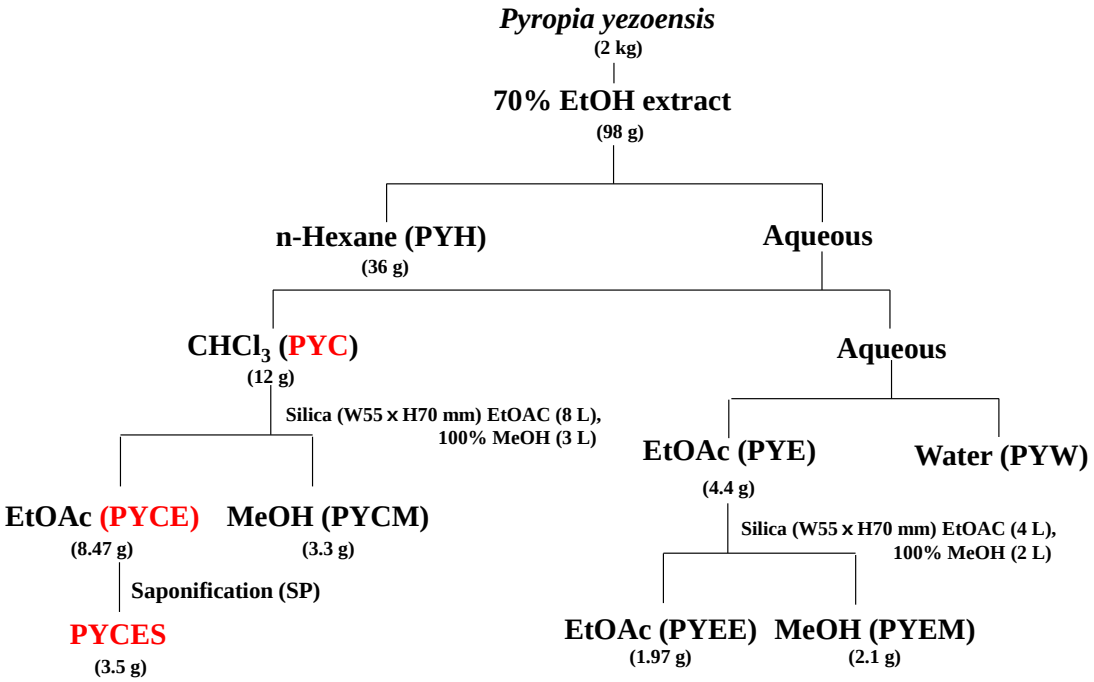


Figure I-1. Fractionation scheme of crude extracts from *Pyropia yezoenesis*

2.8. Determination of nitric oxide (NO) production

After a 24 h pre-incubation of RAW 264.7 cells (1.5×105 cells/ml) with LPS (1 μg/ml), the quantity of nitrite accumulated in the culture medium was measured as an indicator of NO production. In brief, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent (1%sulfanilamide and 0.1% naphthylethylenediamine dihydrochloridein 2.5% phosphoric acid), the mixture was incubated at room temperature for 10 min, and the absorbance at 540 nm was measured in a microplate reader. Fresh culture medium was employed as a blank in every experiment. The quantity of nitrite was determined from a sodium nitrite standard curve.

2.9. Measurement of pro-inflammatory cytokines (IL-1, IL-1β) and PGE_2 production

All samples solubilized with DMSO were diluted with PBS before treatment. The inhibitory effect of samples on the pro-inflammatory cytokines (IL-1β, TNF-α) and PGE_2 production from LPS induced RAW 264.7 cells was determined using a competitive enzyme immunoassay (ELISA) kit according to the manufacturer's instructions.

2.10. Glucose uptake assay

C2C12 cells were seeded in a 24-wells plate. After differentiation, the cells were starved in serum-free low glucose DMEM for 12 h, and then washed with PBS and incubated with fresh serum-free low glucose DMEM. After that, the cells were treated without or with 100 μg/ml of samples for 3 h. Glucose uptake was measured by glucose concentration in the media solution using glucose oxidase assay kit (Asan Pharmaceutical corp., Korea).

2.11. Experimental animals

Adult zebrafish were obtained from a commercial dealer (Seoul Aquarium, Seoul, Korea) and 15 fishes were kept in a 3.5 L acrylic tank under the following conditions; 28.5 ± 1 ^{o}C, and fed twice a day (Tetra GmgH D-49304 Melle Made in Germany) with a 14/10 h light/dark cycle. The zebrafish were exposed of 2 mg/mL alloxan for 1 h and transferred to 1% glucose during 1 h. And then, the solution was changed to water for 1 h. The zebrafish were anesthetized using 2-phenoxy ethanol (1:1000 dilutions). The zebrafish were divided to 4 groups, the normal (alloxan-untreated) as well as alloxan-induced diabetic zebrafish without (control) and with PYH4 or Metformin(Met) (2 μg/g body weight).

2.12. Measurement of blood glucose level

The zebrafish was anesthetized by 2-phenoxy ethanol and removed from the water by Kim wipe. After, blood sample was taken from the heart at 0, 60, 90, 120 min. Approximately 1 μL of blood rapidly transferred to a glucometer strip (Roche Diagnostics Gmbh, Germany).

2.13. GC/MS analysis

Sterol analysis was performed by gas chromatography-mass spectrometry (GC-MS) by Shimadzu GCMS-TQ8040 (Shimadzu Corporation, Japan) on a Rxi-5MS capillary column (30 m × 0.25 mm id, film thickness 0.25 μm, Shimadzu, Japan). The carrier gas was helium at a flow rate of 1.36 mL/min. The GC-MS method was carried out using the following temperature program: initial temperature at 180 ℃ hold for 3 min, followed by 6 ℃/min ramp to 240 ℃ and hold for 3 min, followed by 3 ℃/min ramp to 275 ℃ and hold for 3 min, followed by 5 ℃/min ramp to 300 ℃ and hold for 3 min, followed by 5 ℃/min ramp to a final temperature of 330 ℃ and hold for 5 min. The injection temperature was set at 290 ℃, and the injection volume was 1 μl (splitless mode). Detector parameters used for GC-MS analyses were as follows: interface temperature, 320 ℃; ion source temperature, 300 ℃ and Mass spectrometry was performed using Q3 scan with an m/z 45-500

scanning range. Chromatograms and mass spectra were evaluated using the GC-MS solution software (Shimadzu Corporation, Japan).

2.14. ^{1}H-NMR and ^{13}C -NMR analysis of purified compound

^{1}H-NMR spectra and ^{13}C-NMR spectra were measured with a JEOL JNM-LA300 spectrometer. Mass spectra (FAB-MS and EIMS) were recorded on a JEOL JMS 700 spectrometer.

2.15. Statistical analysis

All the measurements were made in triplicate and all values were represented as means ± standard error. The results were subjected to an analysis of the variance (ANOVA) using the Turkey test to analyze the difference. A value of $p < 0.05$ was considered to indicate statistical significance.

3. Result & discussions

3.1. Total phenolic content of each fraction of PYC

Phenolic or polyphenolic compound are widely distributed secondary metabolite in the plants. Large number of studies were reported that occurrence of polyphenolic compounds among the marine algae species. They synthesized by plants via two main primary synthetic pathways: the sikimate pathway and the acetate pathway (Paixao et al. 2007). Acidic character of the phenolic functional group and nucleophilic character of the benzene ring is responsible for the reactivity of these compounds. Polyphenolic compounds have been reported to demonstrate anti-inflammatory antibacterial, antimutagenic and vasodialatory action and these activities are associated with polyphenol content and antioxidant properties (Kusuma et al. 2011). Therefore, total polyphenol content of each fraction from $CHCl_3$ fraction of *Pyropia yezoenesis* was quantitated. The total polyphenol content of PYC,

PYCE, PYCES, and PYCM fractions are shown in Table 01 (Data of total phenolic contents are expressed as grams of gallic acid (GAE) equivalents per 100 gram dry weight.). They were 4.03±0.15 g GAE/100g, 2.75±0.15 g GAE/100g, 3.60±0.15 g GAE/100g and 4.99±0.60 g GAE/100g in PYC, PYCE, PYCES, and PYCM respectively. According to the results, all four fractions showed a low content of polyphenol content.

3.2. Total sterol content of each fraction of PYC

Phytosterols have chemical structures similar to that of cholesterol; however, they are only made by plants and algae. These include β-sitosterol, stigmasterol, campesterol, sitostanol and campestanol (Moghadasina., 2000). Regardless of their chemical forms or origins, phytosterols interfere with cholesterol absorption, and thereby reduce serum total and low density lipoprotein (LDL) cholesterol levels (Ikeda et al., 1988). In addition to cholesterol-lowering effects, sporadic reports suggest beneficial effects of phytosterols against inflammation, infection, colitis, cancer development, Alzheimer's disease, and other abnormalities (Solati et al., 2015). Therefore, the total sterol content of each fraction (PYC, PYCE, PYCES, PYCM) derived from the $CHCl_3$ fraction was measured and shown in Table 1. They were 15 %, 27 %, 30 % and 12% in PYC, PYCE, PYCES, and PYCM respectively. According to the results PYCES have high amount of sterol content.

Saponification is to free the esterified sterols, tocopherols, and xanthophylls (including lutein) of their side chains, which simplifies analysis by allowing quantification of only the core molecules. Saponification also liberates free fatty acids from triacylglycerol molecules, and may be performed directly upon a homogenized sample or on its extraction (Fernandes et al., 2007). As a result of the saponification of PYCE, it was confirmed that the sterol content was increased. Therefore, fatty acids and pigment can be removed through the saponification process to obtain increased sterols.

3.3. Thin layer chromatographic (TLC) analysis of each fraction of PYC

Chromatography is a separation technique and the separation is depending on the differential distribution of the mixture of compounds or components between mobile phase and stationary phase. Stationary of the TLC is thin layer adhering to the suitable plate of supporting material such as aluminum plate, glass plate or plastic plate. Mobile phase is flowed ascending way by capillary action (Vogle 1989). Normal phase silica TLC was used during this study and mixture of n-hexane and ethyl acetate was used as mobile phase with different ratio. Developed TLCs were observed under UV lamp and stain using mixture of ethanol and H_2SO_4 and heated at 100˚C for some time to visualization. TLC chromatographs of PYCE, PYCES, PYCM and PYCMS are shown in Figure **I**-2. PYCE and PYCM show that after saponification the fatty acid and pigment are removed and only the sterol appears. Therefore, we propose that the saponification process is a way to efficiently separate homogeneity sterols.

3.4. NO inhibitory effects of each fraction of PYC

Nitric oxide plays crucial roles in many cellular functions in the nervous, cardiac, vascular, and immune systems, and also acts as an intracellular and intracellular signal molecule (Wang et al., 2006). Low levels of NO production are important in protecting organs such as liver from ischemic damage. However, the chronic expression of NO is associated with various carcinomas and inflammatory conditions, and NO is also generated by macrophages as a part of the human immune responses (Kassim et al., 2010). Moreover, under pathological conditions, NO production is increased by iNOS (Kim et al., 1999; Suh et al., 2003). Therefore, inhibition of NO production by suppression of iNOS expression may have potential therapeutic value when related to inflammation.

LPS is found in outer membrane of the gram negative bacteria and it is endotoxin for mammalian cells. LPS is able to activate anti-inflammatory mediators and induced to produce nitric oxide. Sample pre-treated RAW 264.7 cells were stimulated with LPS to produce the NO and NO concentration was quantified after 24 h incubation using colorimetric method. The culture supernatants were used for the

evaluation of NO production by Griess reaction. To evaluate the anti-inflammatory activity of each fraction of PYC, their inhibitory activities against NO production (%) were measured in the LPS-stimulated RAW 264.7 cells (Fig. **I**-3A) and the cytotoxicity was shown in Fig. **I**-3B. Among PYC -derived fractions, PYCES showed the strongest inhibitory activity against NO production (%), and its IC50 value was 12.7 ± 0.28 μg/ml. Cytotoxicity did not show at below 25 μg/ml, therefore all following experiments were progressed at concentrations below 25 μg/ml. In the previous studies, the hexane fraction from *Pyropia yezoensis* showed strong NO inhibitory activity (IC_{50} value : < 12.5 μg/ml), and PYCES showed similar activities (Lee et al. 2015).

3.5. Glucose uptake effect of each fraction of PYC in C2C12 myotube

Diabetes mellitus (DM) is a heterogeneous group of metabolic disorders characterized by high levels of blood sugar, termed hyperglycemia (Capiotti et al., 2014). Chronic blood sugar elevation is the major biochemical diagnostic parameter that is seen in the two major forms of diabetes: Type 1 diabetes or insulin-dependent, caused by autoimmune destruction of insulin producing pancreatic β-cells, and Type 2 diabetes or non-insulin dependent characterized by insulin insensitivity, and most diabetes belongs to type 2 (Harris and Zimmet, 1997).

Generally, muscles play a key role in the regulation of energy balance and comprise the primary tissue for glucose uptake and disposal (Lee et al., 2010). Therefore, we used C2C12 skeletal muscle cells to evaluate whether PYC each fraction possesses anti-diabetic properties.

To measure the effects of PYC, PYCE, PYCES and PYCM on glucose uptake, we first evaluated its effects on the uptake of 2-NBDG, a fluorescent glucose analog widely used for monitoring glucose uptake in cells. As shown in Fig. **I**-4, all samples effectively stimulated glucose uptake in a concentration-dependent manner in C2C12 myotube, especially PYCES exhibited a very strong glucose uptake activity than metformin (100 μg/ml) used as a positive control.

3.6. Blood glucose levels inhibitory effects of each fraction of PYC in alloxan stimulated diabetic zebrafish

There are various classes of anti-diabetic drugs, with different mechanisms of action. As a positive control in this experiment, Metformin is a biguanide that reduces glucose production in the liver by promoting glucose transporter activity into cells. The zebrafish model system is becoming one of the most widely used animal models for developmental research because of its fecundity and its genetic and physiological similarities to mammals (Vascotto et al. 1997). Blood sampling was done by cutting the head of the zebrafish and glucose levels determined using a glucosimeter. The change of level of glucose in the blood by alloxane stimulated diabetic zebrafish model, exhibited in Figure I-5. The blood glucose level of control group was slightly decreased as increase of time whereas PYCES similarly decreased the glucose level of blood with positive control, metformin (0.5 μg/g).

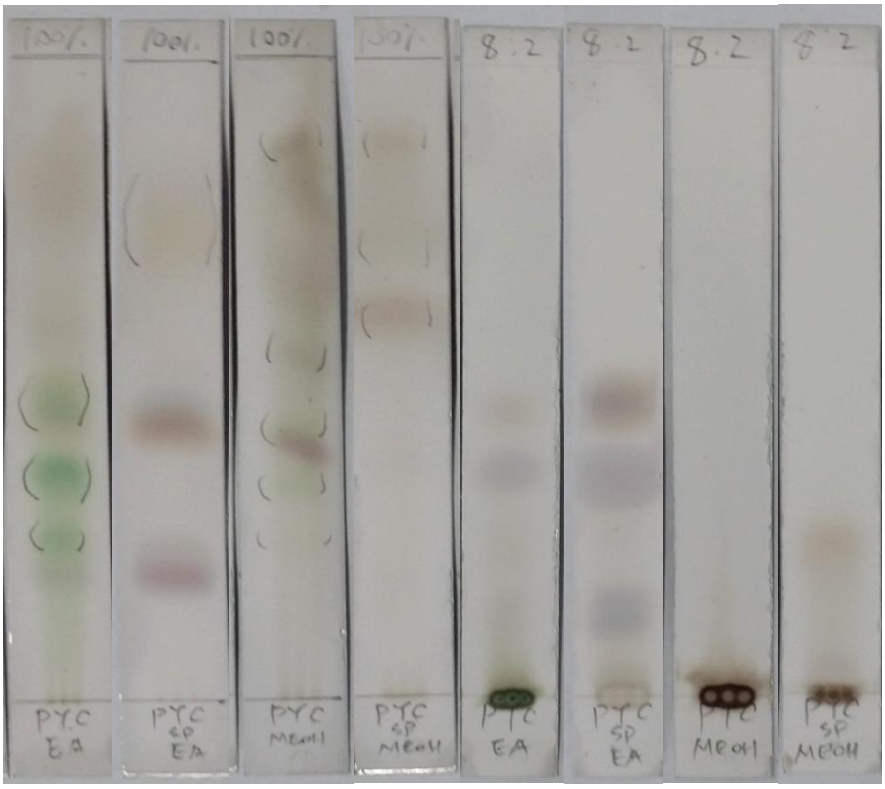
3.7. Optimization of the two-phase solvent system

Partition coefficient (K) for the selection of a suitable two-phase solvent system was the most important for the successful separation of the target samples by the preparative CPC. In order to choose the most efficient separation, several two-phase solvent ratios were applied with the different compositions and volume ratios of the two immiscible solvents such as n-hexane:EtOAc:MeOH:water (v/v). In PYCES, four sterols were selected as targets, and the most efficient isolation condition was then selected as 9:1:10:1 and 5:5:7:3 (n-hexane:EtOAc:MeOH:water, v/v) through compare of target band size produced after reaction with H_2SO_4 (Fig. **I**-6). There is a great the band size gap between both top phase and bottom phase of most target compounds.

Table 1. Total phenolic content and total sterol content of the fractionated *Pyropia yezoenesis*

	Fractions			
	PYC	**PYCE**	**PYCES**	**PYCM**
Total phnolic content (g GAE/100g)	**4.03 ± 0.15**	**2.75 ± 0.15**	**3.60 ± 0.15**	**4.99 ± 0.6**
Total sterol content (%)	**15 ± 0.15**	**27 ± 0.27**	**30 ± 0.15**	**12 ± 0.25**

(A) **(B)**



PYCE PYCES PYCM PYCMS PYCE PYCES PYCM PYCMS

Figure I-2. TLC chromatograph of the PYCE, PYCES, PYCM and PYCMS in different solvent condition (A) RP-TLC was heat with sulfuric and ethanol staining reagent.(B) Silica TLC was heat with sulfuric and ethanol staining reagent.

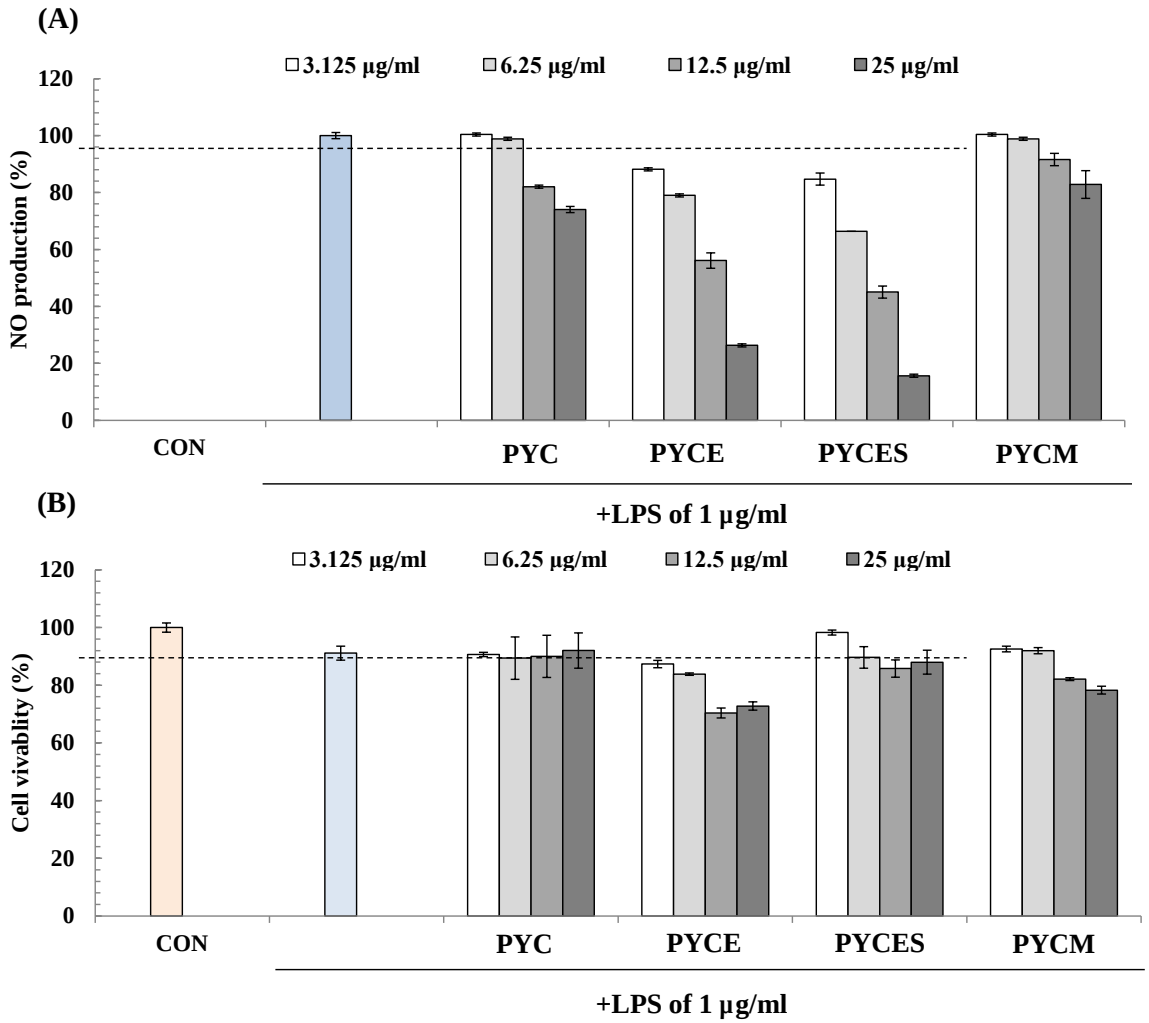


Figure I-3. Inhibitory effect of NO production of fractions of PYC (A) and their cytotoxicity (B) in LPS induced Raw 264.7 macrophages. Cells were incubated with LPS for 24 h presence or absence of desire fraction.

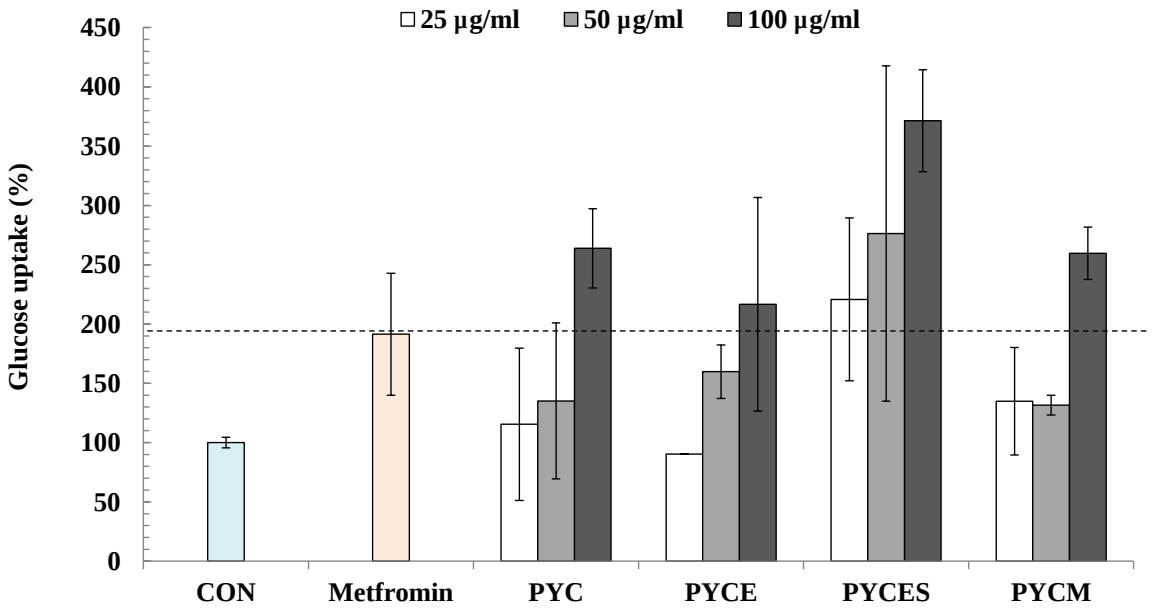


Figure I-4. PYC, PYCE, PYCES and PYCM on glucose uptake in C2C12 myotubes. C2C12 cells were treated with increasing concentrations of sample or metformin (100 µg/ml), and following this treatment.

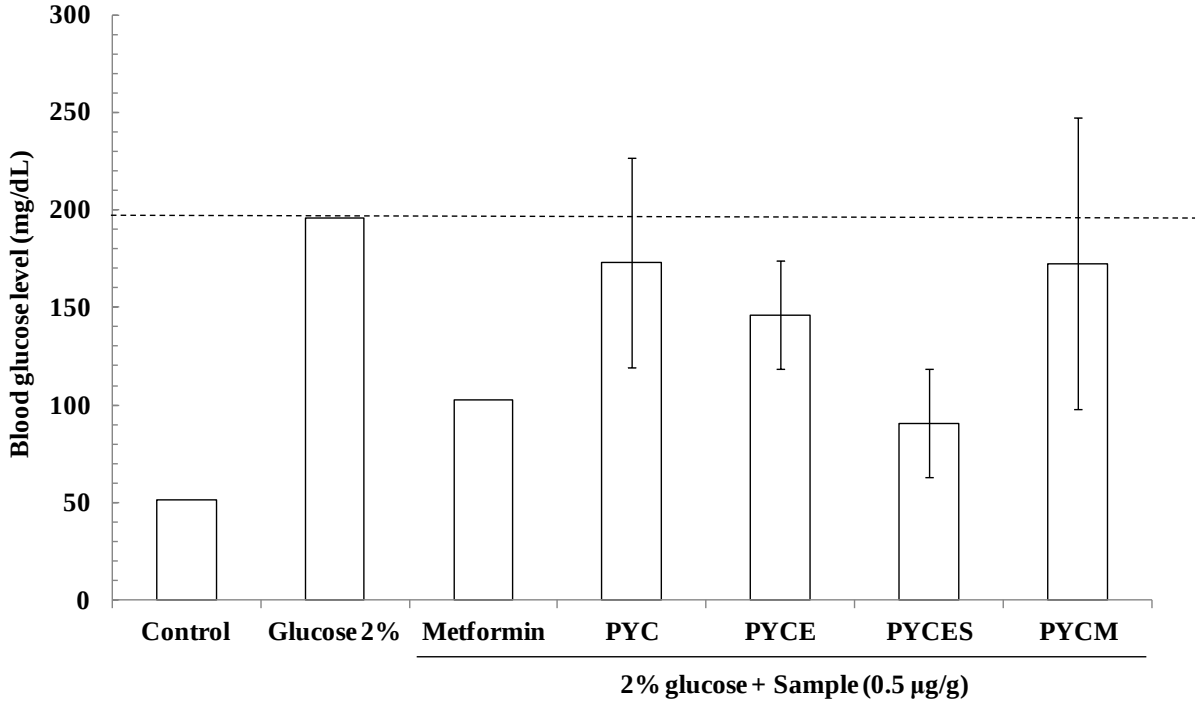


Figure I-5. Blood glucose level inhibitory effect of PYC, PYCE, PYCES and PYCM alloxan stimulated zebrafish.

3.8. Separation of compounds by CPC

The preparative CPC was operated in the ascending mode (lower phase: stationary phase and upper phase: mobile phase, respectively) for efficiently isolation of the non-polar target compounds and, descending mode (lower phase: mobile phase and upper phase: stationary phase, respectively) for efficiently isolation of the polar target compounds. Ascending mode and descending mode of retention of the stationary phase in the coil retained 65%, and the pressure exhibited 3.4 and 3.7 MPa during the operation. The TLC data of vials collected through preparative CPC system was described in Fig. **I**-7 and 8. We confirmed that there were three fraction (PYCES1~3) including with the each same compounds and three compounds were purified by preparative TLC. The yields of fraction 1 ~ 3 isolated from 500 mg of the PYCES by the one-step of CPC system were 1.5 mg, 1.4 mg, and 2.8 mg, respectively.

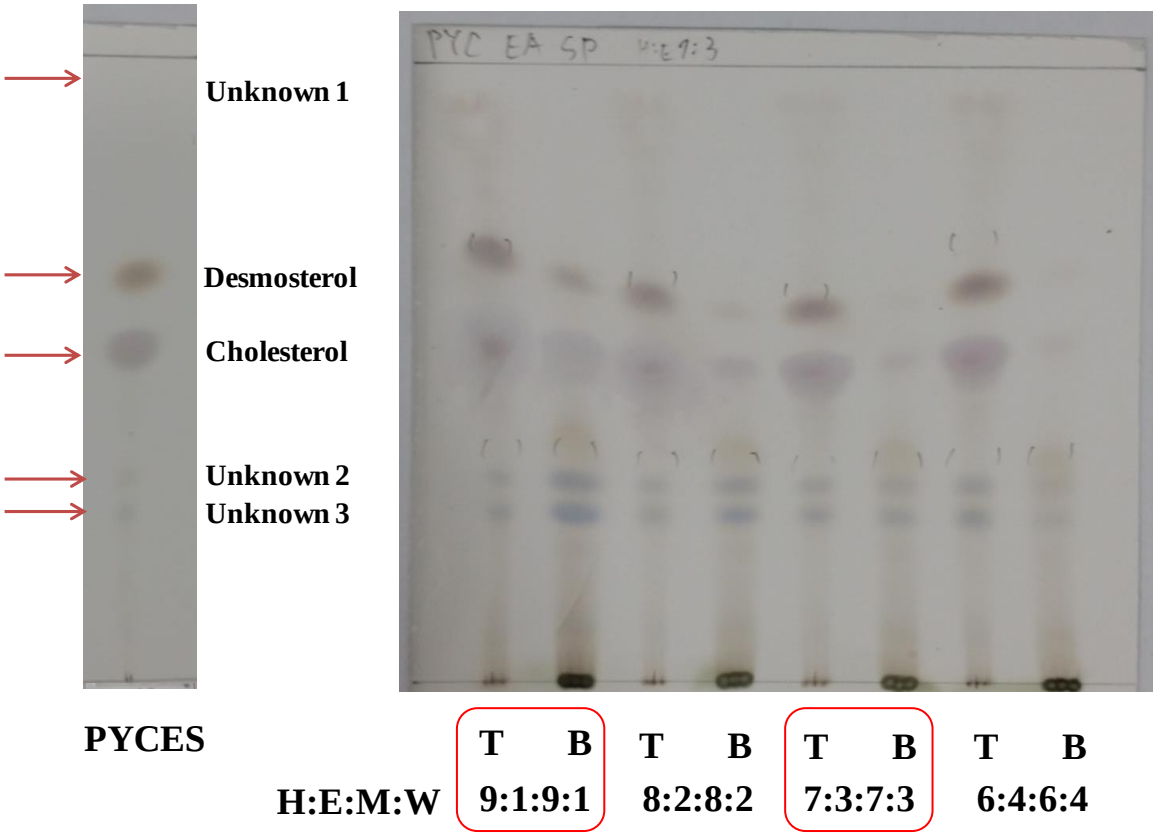


Figure I-6. Silica TLC analysis data of PYECS to efficiently operate the preparative CPC

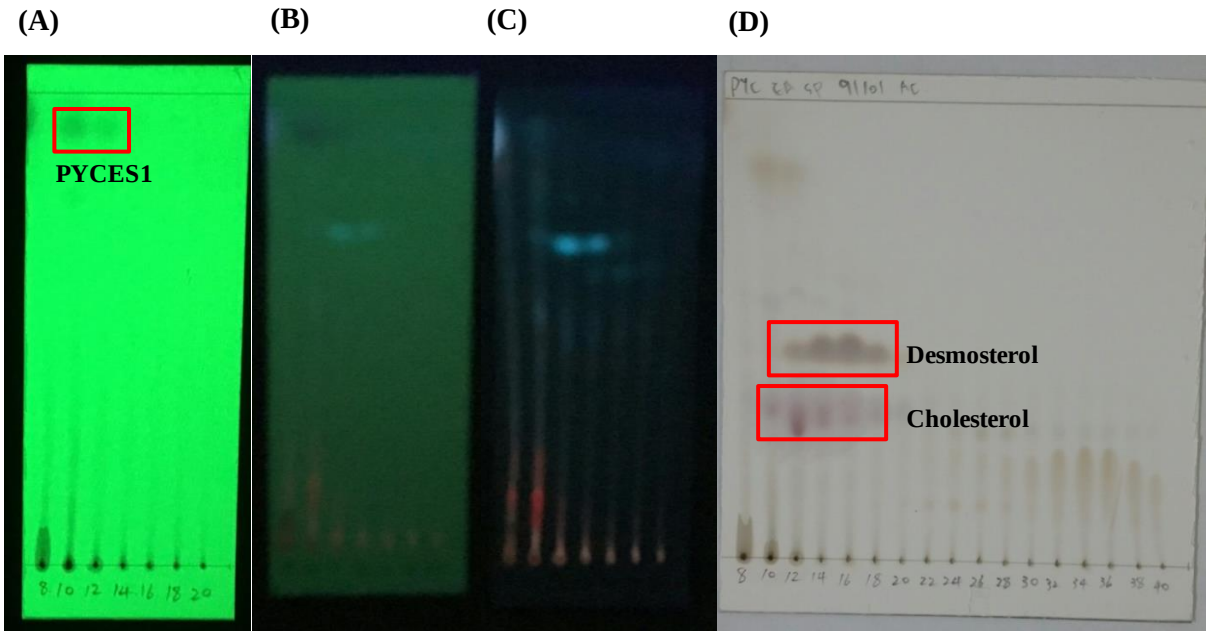


Figure I-7. Silica TLC analysis data of the CPC (n-hexane:EtOAc:MeOH:water - 9:1:10:1, v/v) fractions from PYCES. (A) under 254 nm UV radiation (B) under 312 nm UV radiation (C) under 365 nm UV radiation (D) heat with sulfuric and ethanol staining reagent.

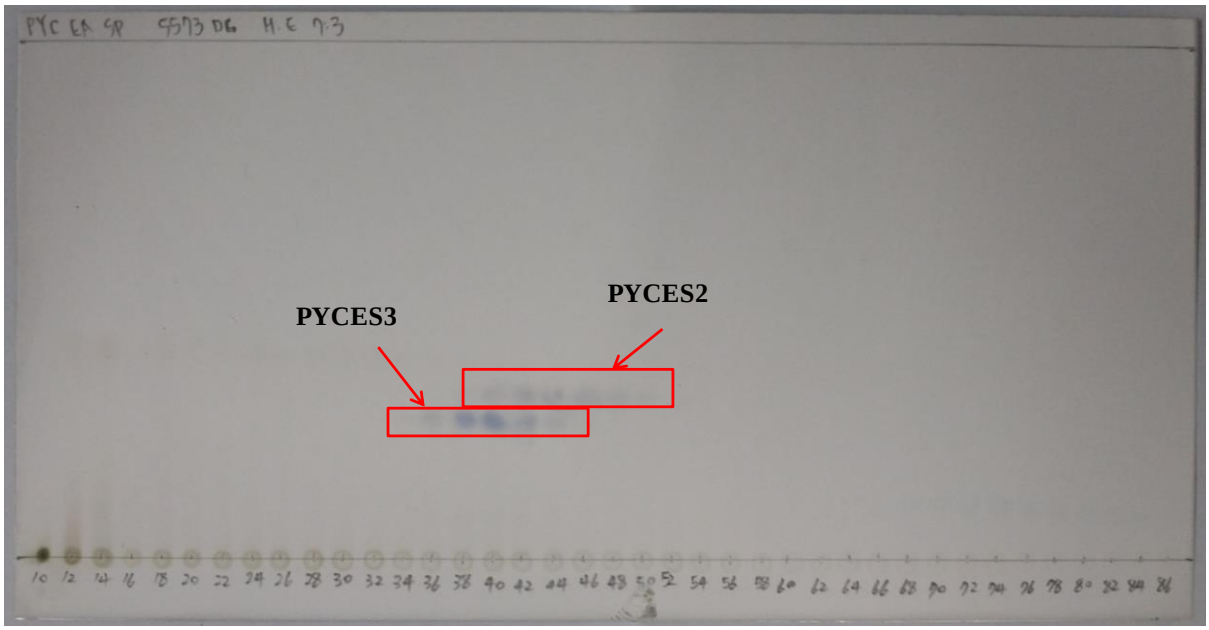


Figure I-8. Silica TLC analysis data of the CPC (n-hexane:EtOAc:MeOH:water - 5:5:7:3, v/v) fractions from PYCES. Heat with sulfuric and ethanol staining reagent.

3.9. NO inhibitory effects of the compounds from PYCES

To evaluate the anti-inflammatory activity of the compounds isolated from the PYCES, the inhibitory activity against the NO production (%) were measured in the LPS induced 267.4 RAW cells (Fig. **I**-9). RAW 264.7 cells treated with the compounds for 2h were then stimulated with 1 μg/ml LPS for the 24 h incubation. The culture supernatants were used for the evaluation of NO production by Griess reaction. PYCES1, PYCES2 and PYCES3 showed the inhibitory activity against NO production (%) and PYCES2 and PYCES3 cytotoxicity did not show at below 25 μg/ml, but PYCES1 cytotoxicity show at below 12.5 μg/ml (Fig. **I**-9B). Therefore, all subsequent experiments were carried out at a concentration of 25 μg / ml or less except for PYCES1.

3.10. Glucose uptake effect of the compounds from PYCES in C2C12 myotube

To measure the PYCES2 and PYCES3 on glucose uptake, we first evaluated its effects on the uptake of 2-NBDG, a fluorescent glucose analog widely used for monitoring glucose uptake in cells. As shown in Fig. **I**-10, all samples effectively stimulated glucose uptake in a concentration-dependent manner in C2C12 myotube, especially PYCES2 and PYCES3 showed strong glucose uptake effect. Under the same condition, metformin (100 μg/ml) was used as a positive control for glucose uptake.

3.11. Blood glucose levels inhibitory effects of the compounds from PYCES in alloxan stimulated diabetic zebrafish

There are various classes of anti-diabetic drugs, with different mechanisms of action. Metformin is a biguanide that reduces hepatic glucose production by facilitating glucose transporter activity into cells. Blood sampling was done by cutting the head of the zebrafish and glucose levels determined using a glucosimeter. The change of level of glucose in the blood by PYCES2 and PYCES3 alloxane stimulated diabetic zebrafish model, exhibited in Figure **I**-11. The blood glucose level of control

group was slightly decreased as increase of time whereas PYCES2 and PYCES3 similarly decreased the glucose level of blood with positive control, metformin.

3.12. Structural identification of anti-inflammatory compounds from PYCES

The identification of PYCES3 was carried out by ^{1}H NMR, ^{13}C NMR and GC-MS/MS. Figure I-12. PYCES3 was confirmed as 26,27-Dinorergosta-5,23-dien-3-ol. This compound has been reported to belong to the giant timber bamboo species *Phyllostachys edulis*, Wheat straw and *Piper longum* linn, and it was first isolated from seaweed except for land plants (Bhuiyan et al, 2008; Zhang et al., 2011; Zhang et al, 2012). In addition, no results have been reported on the biological activity of this compound in previous studies. Thus, this study confirmed that the compound has anti-inflammatory and anti-diabetic effects.

3.13. PYCES3 inhibited release of PGE_2, and decreased pro-inflammatory cytokines released by LPS-stimulation in RAW 264.7 macrophages

The LPS stimulation induces the expression of iNOS which triggers the production of its downstream effector, NO which ultimately results in the tissue damage by NO itself or its more toxic metabolite, peroxynitrite ($ONOO^-$) (Dringen, 2005; Li et al., 2005). In addition, inducible cyclooxygenase, COX-2 drives the onset of inflammation through the production of pro-inflammatory PGE_2 (Tzeng et al., 2005). Pro-inflammatory cytokines such as TNF-α and IL-1β are largely produced in the inflammatory condition and these cytokines act as messengers which stimulate the inflammatory process (Jung et al., 2009). Therefore, the down-regulation of the pro-inflammatory cytokine production is one of the most important strategies in the anti-inflammatory therapy.

The production of PGE_2 was significantly increased upon the LPS treatment when compared with the untreated control group. However, PYCES3 dose-dependently decreased the LPS-stimulated

PGE_2 production (Fig. **I**-13A). The pro-inflammatory cytokines produced from macrophages, are the key components of inflammation. Therefore, the effect of PYCES3 on the production levels of the inflammatory cytokines IL-1ß and IL-6 were investigated by ELISA kit using the conditioned media of LPS-stimulated RAW 264.7 macrophages. The LPS stimulation significantly increased the production of pro-inflammatory cytokines IL-1ß and IL-6 compared to the non-stimulated blank groups. The LPS induced production of IL-1ß and IL-6 showed a significant concentration dependent decrease following the treatment of PYECS3 (Fig. **I**-13).

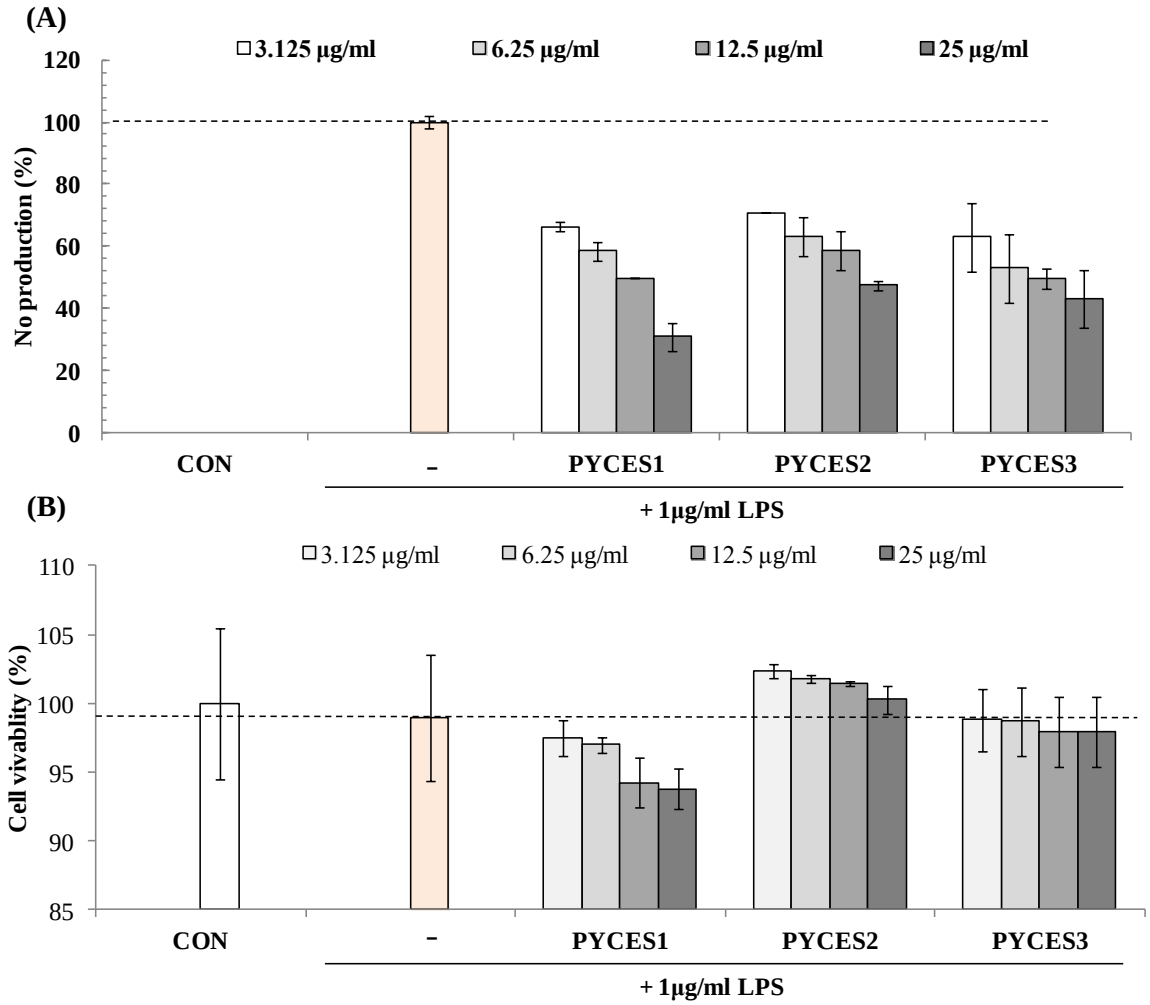


Figure I-9. Inhibitory effect of NO production of compounds from PYCES (A) and their cytotoxicity (B) in LPS induced Raw 264.7 macrophages. Cells were incubated with LPS for 24 h presence or absence of desire fraction.

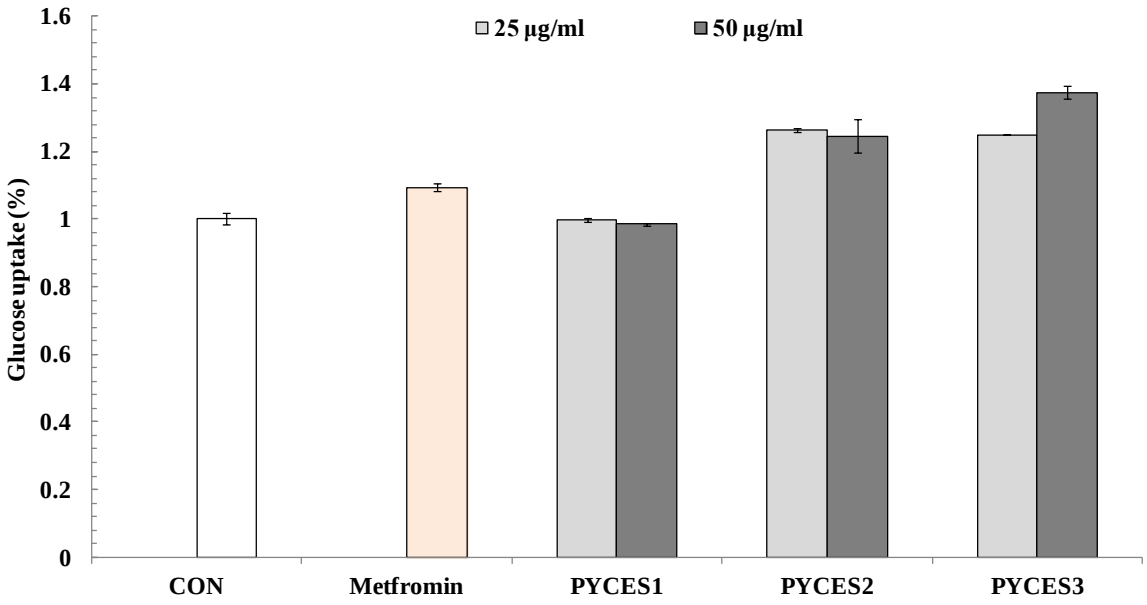


Figure I-10. Compounds from PYCES on glucose uptake in C2C12 myotubes. C2C12 cells were treated with increasing concentrations of sample or metformin (100 μg/ml), and following this treatment.

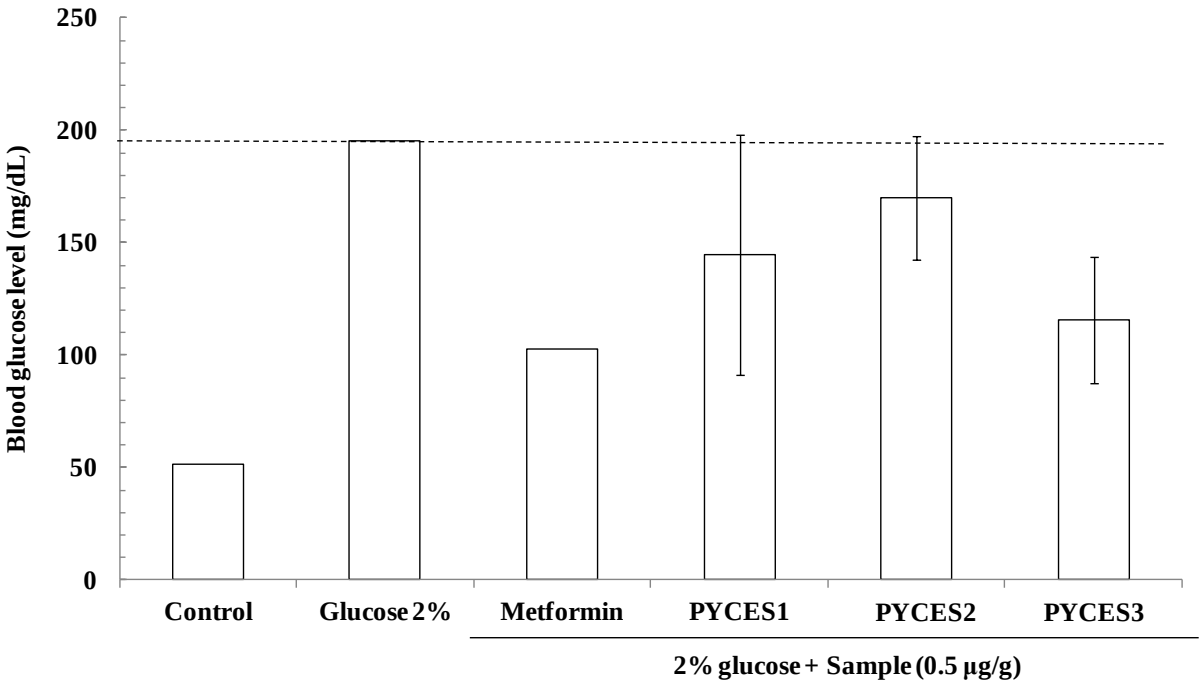


Figure I-11. Blood glucose level inhibitory effect of Compounds from PYCES alloxan stimulated zebrafish.

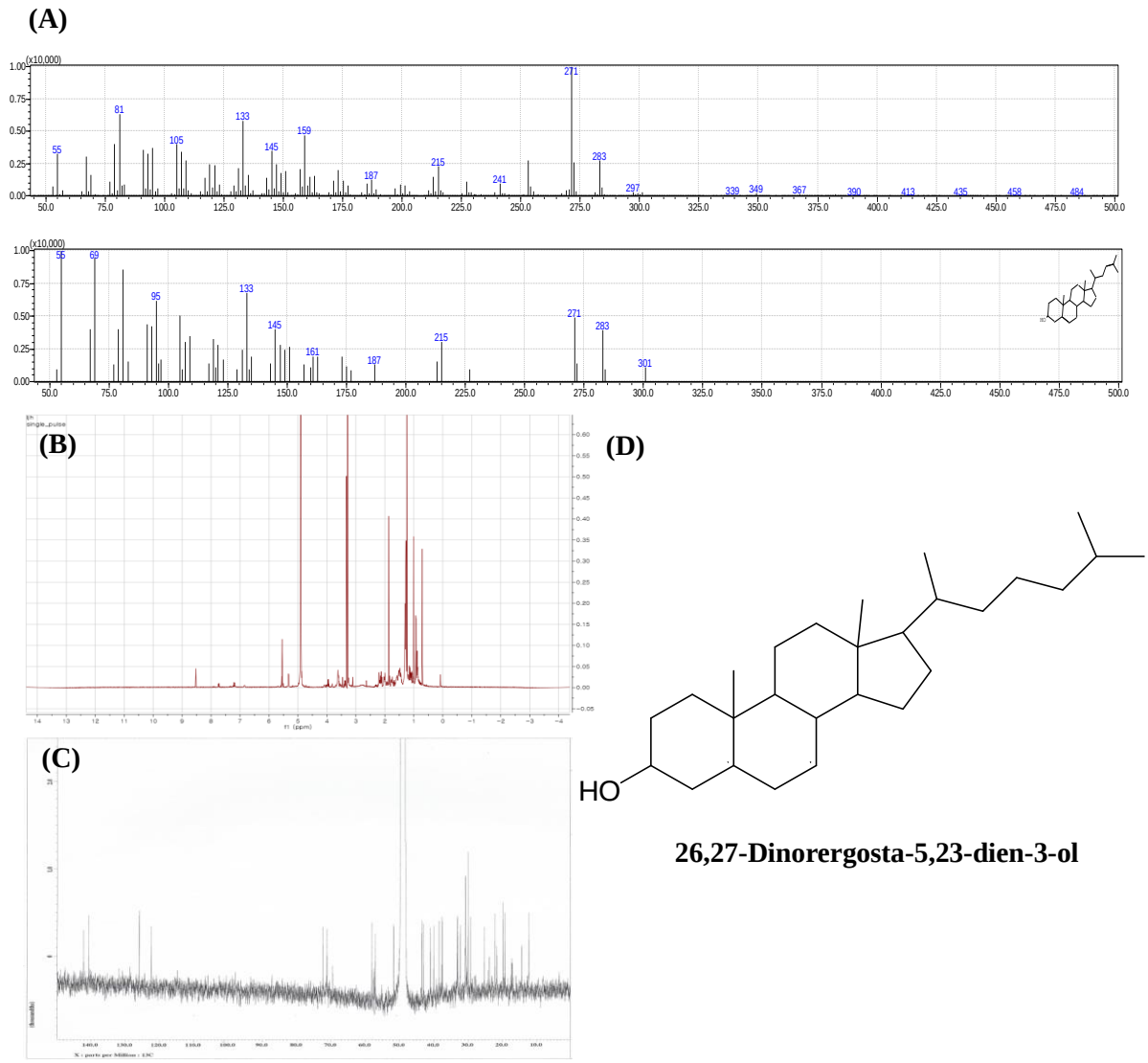


Figure I-12. GC/MS library search results of PYCES3 (A). ^{1}H NMR (B) and ^{13}C NMR data of PYCES3. Chemical structures of PYCES3 (D).

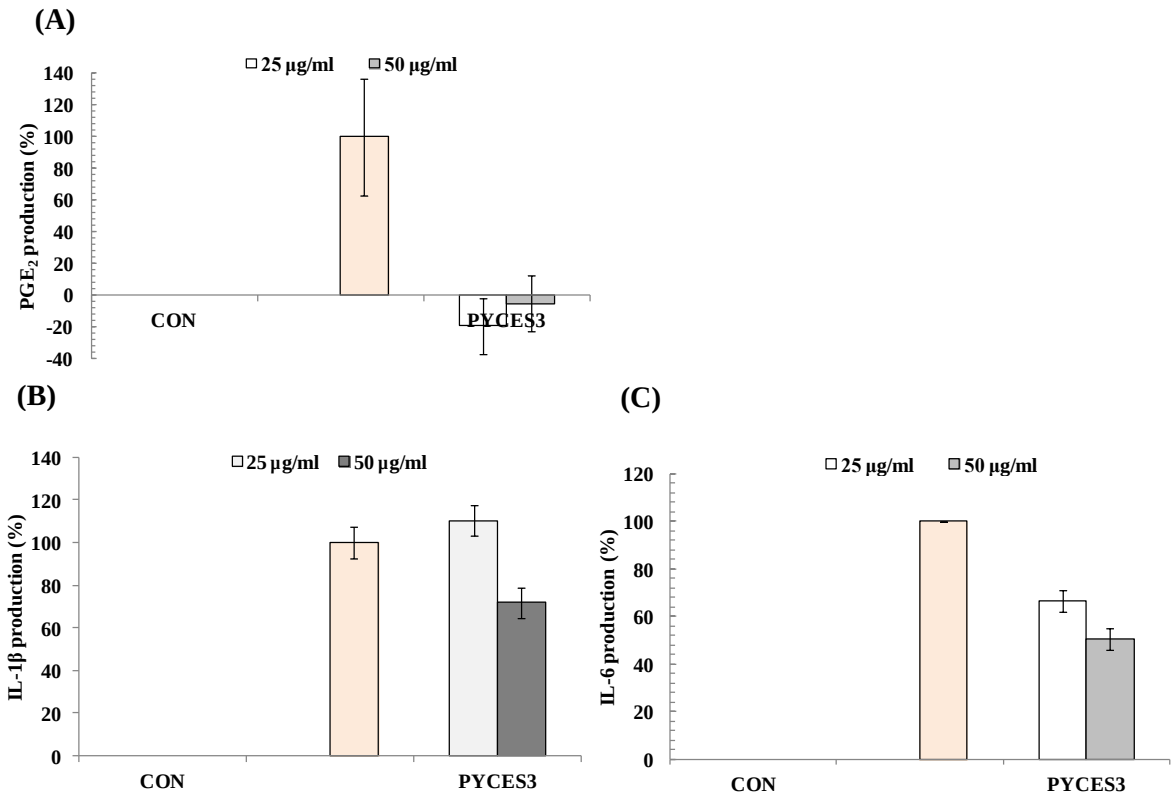


Figure I-13. Pro-inflammatory cytokines inhibitory effect of PYCES3 in LPS-induced RAW 264.7 cells. (A) PGE_2, (B) IL-1β, and (C) IL-6.

4. CONCLUSION

In this study, we isolation and identification of anti-inflammation and anti-diabetic compounds from $CHCl_3$ fraction from *P. yezoensis* by using centrifugal partition chromatography (CPC). These compound showed strong anti-inflammatory and anti-diabetes effects.

Part-II

Identification of chemical structure of anti-inflammatory and anti-diabetes compounds from ethyl acetate fraction of *Pyropia yezoensis*

1. ABSTRACT

This study was focus on isolation and identification of anti-inflammatory and anti-diabetic compounds from *Pyropia yezoensis*, a marine red alga. The ethyl acetate fraction from *P. yezoenesis* 70% EtOH extract was fractionated using EtOAc(PYEE) and MeOH (PYEM) by a silica column. Among them, PYEE showed the strongest inhibitory activity against nitric oxide (NO) generated from lipopolysaccharide (LPS) stimulated RAW 264.7 macrophages without the cytotoxicity and, PYEE enhanced the uptake of glucose into C2C12 cells in a concentration-dependent manner and PYEE showed strong anti-diabetic activity to lower the blood glucose level in alloxan damaged zebrafish. The seven fractions (PYEE1~7) were prepared from PYEE by open column chromatography. PYEE6 exhibited higher anti-inflammatory and anti-diabetic effects than others. The two compounds (PYEE6-1~2) were isolated from PYEE6 by open column chromatography. Therefore, we identified chemical structures of PYEE6-1 using NMR and GC-MS and they were confirmed as 1,2-Benzenedicarboxylic acid, mono ester and 3,4-dichloro[1,6]naphthyridine$$pyrido[4,3-B]pyridine, 3,4-dichloro, respectively.

2. MATERIALS & METHODS

2.1. Materials

Pyropia yezoens is which was cultivated at the Coast of Wando Island in South Korea, was washed three times with freshwater, and then immediately frozen and stored at –20℃ until use. The frozen samples were lyophilized and ground with a grinder. The dried sample powder was stored in refrigerator until use. Lipopolysaccharide (LPS) was purchased from sigma chemical Co., Ltd (ST. Louis, MO).Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) penicillin-strptomycinand trypsine-EDTA were obtained from Gibco/BRL (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium, dimethyl sulfoxide (DMSO)were purchased from Sigma (St. Louis, MO, USA).M-MuLV reverse transcriptase were purchased from Promega (Madison, WI, USA). The enzyme-linked immunosorbent assay (ELISA) kit for IL-1β, TNF-α and Prostaglandin E_2 (PGE_2) were purchased from R & D Systems Inc. (Minneapolis, MN, USA).Protein assay kit and ECL detection reagent were bought from Bio-Rad (Richmond, CA, USA) and Amercham Biosciences (Piscataway, NJ, USA), respectively. Other all reagents and solvents were purchased from Sigma (St. Louis, MO, USA).

2.2. Apparatus

^{1}H-NMR spectra were measured with a JEOL JNM-LA300 spectrometer and ^{13}C-NMR spectra with a Bruker AVANCE 400 spectrometer. Mass spectra (FAB-MS and EIMS) were recorded on a JEOL JMS 700 spectrometer. The HPLC system in this experiment consisted of two mono Waters 515 HPLC pumps, a Waters 2998 photodiode array detector, a Waters2707 autosampler, and the Waters pump control module II (Waters Corporation). Gas chromatography-mass spectrometry (GC-MS) by Shimadzu GCMS-TQ8040 (Shimadzu Corporation, Japan).

2.3. Preparation of crude sample from *P. yezoensis*

The dried *P. yezoenesis* (2 kg) was extracted three times for 3hr with 70% EtOH under shaking at room temperature. The extract, concentrated in a rotary vacuum evaporator, partitioned with ethyl acetate, and then the dried ethyl acetate fraction was stored in a refrigerator for CPC separation. The whole process was illustrated in Fig. **II**-1

2.4. HPLC analysis

A 10 ul of 5 mg/ml sample solution was directly injected on Atlantis T3 3um 3.0 x 150 mm column (Waters, USA) using a gradient acetonitrile–water solvent system. The mobile phase was acetonitrile –water in gradient mode as follows: acetonitrile with 0.1% formic acid – water with 0.1% formic acid (0 min ~ 50 min : 5:95 v/v ~ 100:0 v/v, ~ 50 min : ~ 100:0 v/v, ~70min : ~ 100:0 v/v). The flow rate was 1 mL/min with UV absorbance detection at 290 nm.

2.5. Cell culture

The murine macrophage cell line RAW 264.7 cells was grown in Dulbecco's modified Eagle'smedium (DMEM; Sigma) supplemented with 10% (v/v) heat-inactivated FBS, penicillin (100U/ml), and streptomycin (100 μg/ml). Cultures were maintained at 37 ℃ in a 5% CO2 incubator. Mouse myoblast C2C12 cells were maintained in high glucose-DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were maintained at 37℃ in 5% CO2incubator. For differentiation 1312 the cells were seeded in appropriate culture plates, and after sub-confluence (about 80%), the medium was changed to DMEM containing 2% horse serum for 7 days, with medium changes every day. All experiments were performed in differentiated C2C12 myotubes after 7 days.

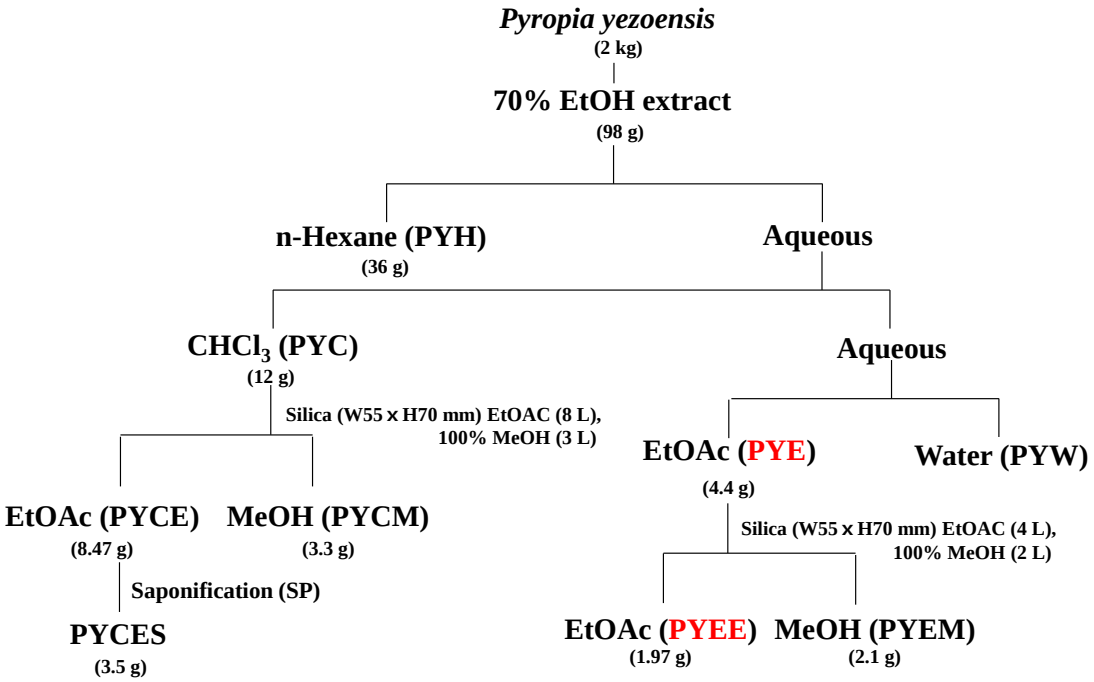


Figure II-1. Fractionation scheme of crude extracts from *Pyropia yezoenesis*

2.6. Determination of nitric oxide (NO) production

After a 24 h pre-incubation of RAW 264.7 cells (1.5×105 cells/ml) with LPS (1 μg/ml), the quantity of nitrite accumulated in the culture medium was measured as an indicator of NO production. In brief, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloridein 2.5% phosphoric acid), the mixture was incubated at room temperature for 10 min, and the absorbance at 540 nm was measured in a microplate reader. Fresh culture medium was employed as a blank in every experiment. The quantity of nitrite was determined from a sodium nitrite standard curve.

2.7. Measurement of pro-inflammatory cytokines (IL-1, IL-1β) and PGE_2 production

All samples solubilized with DMSO were diluted with PBS before treatment. The inhibitory effect of samples on the pro-inflammatory cytokines (IL-1β, TNF-α) and PGE_2 production from LPS induced RAW 264.7 cells was determined using a competitive enzyme immunoassay (ELISA) kit according to the manufacturer's instructions.

2.8. Glucose uptake assay

C2C12 cells were seeded in a 24-wells plate. After differentiation, the cells were starved in serum-free low glucose DMEM for 12 h, and then washed with PBS and incubated with fresh serum-free low glucose DMEM. After that, the cells were treated without or with 100 μg/ml of samples for 3 h. Glucose uptake were measured by glucose concentration in the media solution using glucose oxidase assay kit (Asan Pharmaceutical corp., Korea).

2.9. Experimental animals

Adult zebrafish were obtained from a commercial dealer (Seoul Aquarium, Seoul, Korea) and 15 fishes were kept in a 3.5 L acrylic tank under the following conditions; 28.5 ± 1 ^{o}C, and fed twice a day (Tetra GmgH D-49304 Melle Made in Germany) with a 14/10 h light/dark cycle. The zebrafish were exposed of 2 mg/mL alloxan for 1 h and transferred to 1% glucose during 1 h. And then, the solution was changed to water for 1 h. The zebrafish were anesthetized using 2-phenoxy ethanol (1:1000 dilutions). The zebrafish were divided to 4 groups, the normal (alloxan-untreated) as well as alloxan-induced diabetic zebrafish without (control) and with PYH4 or Metformin (Met) (2 μg/g body weight).

2.10. Measurement of blood glucose level

The zebrafish was anesthetized by 2-phenoxy ethanol and removed from the water by Kim wipe. After, blood sample was taken from the heart at 0, 60, 90, 120 min. Approximately 1 μL of blood rapidly transferred to a glucometer strip (Roche Diagnostics Gmbh, Germany).

2.11. GC/MS analysis

Sterol analysis was performed by gas chromatography-mass spectrometry (GC-MS) by Shimadzu GCMS-TQ8040 (Shimadzu Corporation, Japan) on a Rxi-5MS capillary column (30 m × 0.25 mm id, film thickness 0.25 μm, Shimadzu, Japan). The carrier gas was helium at a flow rate of 1.36 mL/min. The GC-MS method was carried out using the following temperature program: initial temperature at 180 ℃ hold for 3 min, followed by 6 ℃/min ramp to 240 ℃ and hold for 3 min, followed by 3 ℃/min ramp to 275 ℃ and hold for 3 min, followed by 5 ℃/min ramp to 300 ℃ and hold for 3 min, followed by 5 ℃/min ramp to a final temperature of 330 ℃ and hold for 5 min. The injection temperature was set at 290 ℃, and the injection volume was 1 μl (splitless mode). Detector

parameters used for GC-MS analyses were as follows: interface temperature, 320 ℃; ion source temperature, 300 ℃ and Mass spectrometry was performed using Q3 scan with an m/z 45-500 scanning range. Chromatograms and mass spectra were evaluated using the GCMS solution software (Shimadzu Corporation, Japan).

2.12. ^{1}H-NMR and ^{13}C -NMR analysis of purified compound

^{1}H-NMR spectra and ^{13}C-NMR spectra were measured with a JEOL JNM-LA300 spectrometer. Mass spectra (FAB-MS and EIMS) were recorded on a JEOL JMS 700 spectrometer.

2.13. Statistical analysis

All the measurements were made in triplicate and all values were represented as means ± standard error. The results were subjected to an analysis of the variance (ANOVA) using the Turkey test to analyze the difference. A value of $p < 0.05$ was considered to indicate statistical significance.

3. RESULT & DISCUSSTIONS

3.1. Thin layer chromatographic (TLC) analysis of each fraction of PYE

Chromatography is a separation technique and the separation is depending on the differential distribution of the mixture of compounds or components between mobile phase and stationary phase. Stationary of the TLC is thin layer adhering to the suitable plate of supporting material such as aluminum plate, glass plate or plastic plate. Mobile phase is flowed ascending way by capillary action (Vogle 1989). Normal phase silica TLC was used during this study and mixture of n-hexane and ethyl acetate was used as mobile phase with different ratio. Developed TLCs were observed under UV lamp and stain using mixture of ethanol and H_2SO_4 and heated at 100˚C for some time to visualization. TLC

chromatographs of PYEE and PYEM are shown in Figure **II-2**. Components in PYEE and PYEM were separated in RP-TLC with increasing the methanol amount in the mobile phase. According to the observed results, little number of components was separated from the base of the RP-TLC line at 40% methanol and more separated spots were appeared in 100% methanol. It was confirmed that most of them contained pigments and contained trace amounts of polyphenols and fatty acids.

3.2 HPLC analysis of each fraction of PYE

High-performance liquid chromatography (HPLC) is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out the column (Gerber et a.l, 2004). PYE, PYEE and PYEM were analyzed by RP-HPLC. Observed chromatograms are shown in Figure **II-3**. According to the results PYE was consist with mostly of nonpolar compounds and small amounts of polar compounds. PYEE shows not only the non-polar compounds of the PYE but also the polar compounds. PYEM shows mostly consist of non-polar compounds.

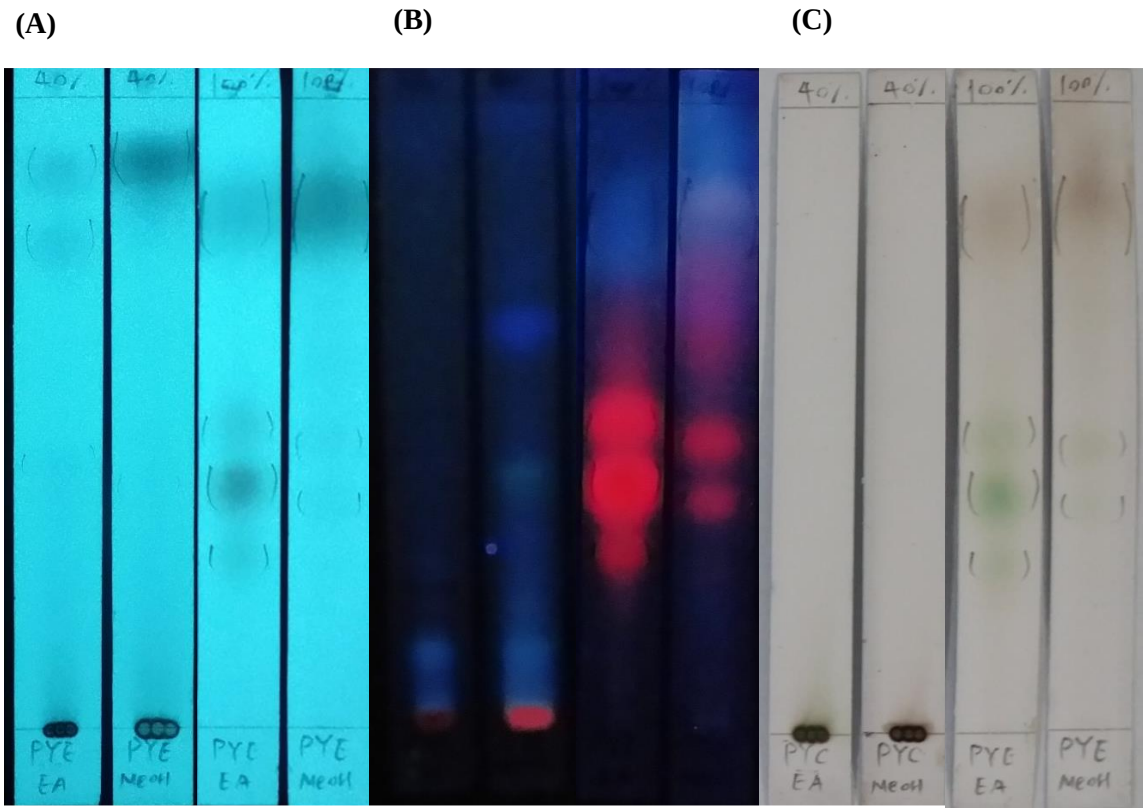


Figure II-2. RP-TLC chromatograph of the PYEE and PYEM in different solvent condition (A) under 254 nm UV radiation (B) under 365 nm UV radiation (C) heat with sulfuric and ethanol staining reagent.

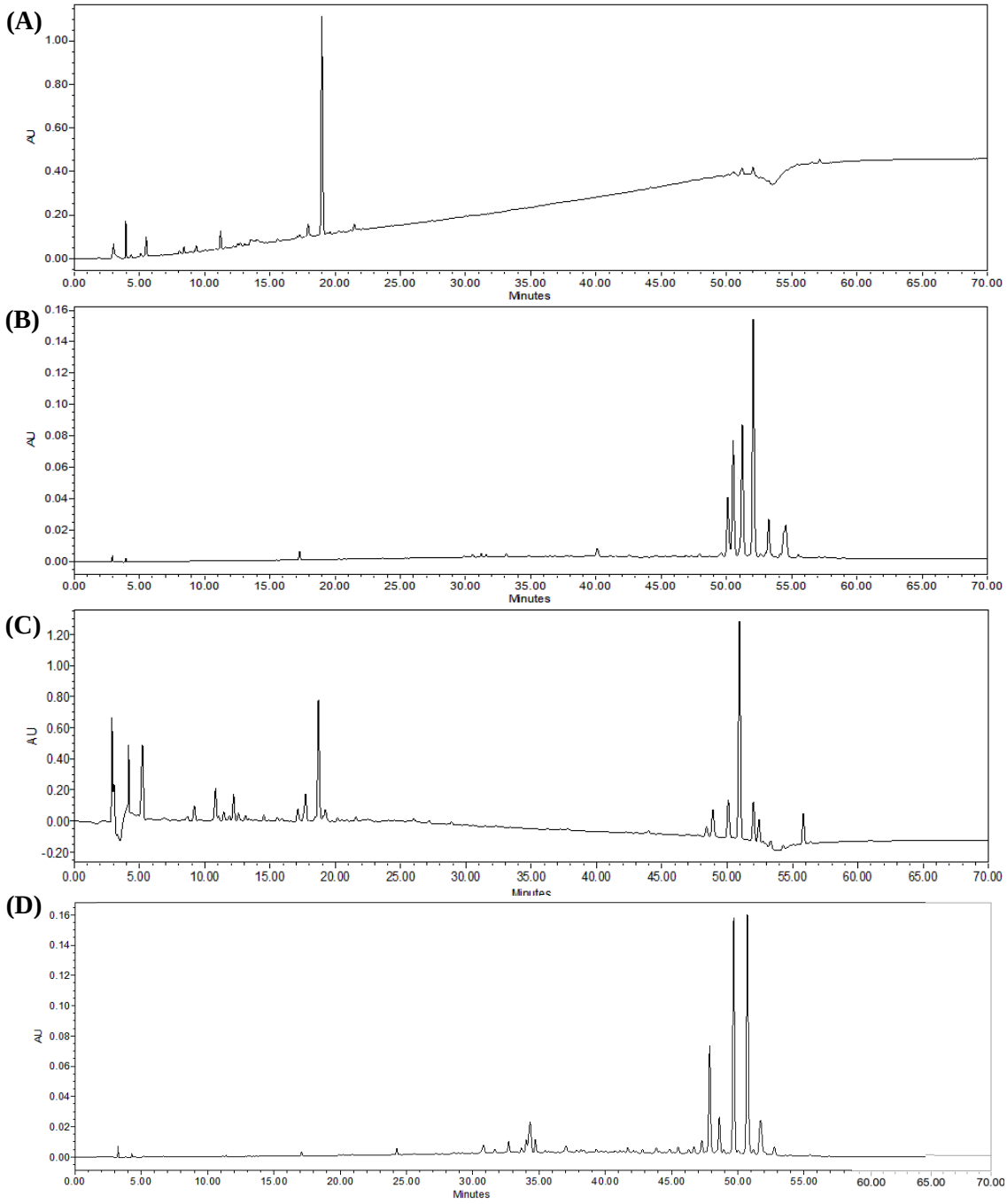


Figure II-3. RP-HPLC chromatogram of (A) PYE (detector wave length 228 nm), (B) PYE (detector wave length 400 nm), (C) PYEE (detector wave length 228 nm) and (D) PYEM (detector wave length 400 nm), Column: Sunfire ODS 5 µm ODS column (250 mm 4.6 mm i.d.); mobile phase: acetonitrile-0.01% acetic acid (5:95 v/v to 100:0 v/v at 0–50 min, 100:0 v/v to 100:0 v/v at 50–70 min); flow rate: 1 mL/min.

3.3. NO inhibitory effects of each fraction of PYE

Nitric oxide plays crucial roles in many cellular functions in the nervous, cardiac, vascular, and immune systems, and also acts as an intracellular and intracellular signal molecule (Wang et al., 2006). Low levels of NO production are important in protecting organs such as liver from ischemic damage. However, the chronic expression of NO is associated with various carcinomas and inflammatory conditions, and NO is also generated by macrophages as a part of the human immune responses (Kassim et al., 2010). Moreover, under pathological conditions, NO production is increased by iNOS (Kim et al., 1999; Suh et al., 2003). Therefore, inhibition of NO production by suppression of iNOS expression may have potential therapeutic value when related to inflammation.

LPS is found in outer membrane of the gram negative bacteria and it is endotoxin for mammalian cells. LPS is able to activate anti-inflammatory mediators and induced to produce nitric oxide. Sample pre-treated RAW 264.7 cells were stimulated with LPS to produce the NO and NO concentration was quantified after 24 h incubation using colorimetric method. The culture supernatants were used for the evaluation of NO production by Griess reaction. To evaluate the anti-inflammatory activity of each fraction of PYE, their inhibitory activities against NO production (%) were measured in the LPS-stimulated RAW 264.7 cells (Fig. II-4A) and the cytotoxicity was shown in Figure II-4B. Among PYE - derived fractions, PYEE showed the strongest inhibitory activity against NO production (%), and its IC50 value was 24.33 ± 0.45 μg/ml. cytotoxicity was shown at 6.25 μg/ml, but the NO inhibitory activity was shown in PYEE rather than PYEM (Fig. II-4B). In previous studies, the hexane fraction of *Pyropia yezoensis* exhibited strong NO inhibitory activity (IC50 value: <12.5 μg/ml) and PYEE showed slightly lower activity (Lee et al., 2015).

3.4. Glucose uptake effect of PYE, PYEE and PYEM in C2C12 myotube

To measure the effects of PYE, PYEE and PYEM on glucose uptake, we first evaluated its effects on the uptake of 2-NBDG, a fluorescent glucose analog widely used for monitoring glucose uptake in

cells. As shown in Figure **II-5**, PYE and PYEE effectively stimulated glucose uptake in a concentration-dependent manner in C2C12 myotube, especially PYEE showed strong glucose uptake effect. Under the same condition, metformin (100 μg/ml) was used as a positive control for glucose uptake.

3.5. Blood glucose levels inhibitory effects of each fraction of PYE, PYEE and PYEM in alloxan stimulated diabetic zebrafish

There are various classes of anti-diabetic drugs, with different mechanisms of action. As a positive control in this experiment, Metformin is a biguanide that reduces glucose production in the liver by promoting glucose transporter activity into cells. The zebrafish model system is becoming one of the most widely used animal models for developmental research because of its fecundity and its genetic and physiological similarities to mammals (Vascotto et al. 1997). Blood sampling was done by cutting the head of the zebrafish and glucose levels determined using a glucosimeter. The change of level of glucose in the blood by alloxane stimulated diabetic zebrafish model, exhibited in Figure II-6. The blood glucose level of control group was slightly decreased as increase of time whereas PYEE similarly decreased the glucose level of blood with positive control, metformin (0.5 μg/g).

3.6. Reverse phase open column fractionation of the PYEE

Reversed phase open column is packing was packed with octadecyl-silica (ODS). ODS is a non-polar hydrophobic stationary phase. Non polar compounds are having more affinity for the stationary phase and run slowly through the open column. Polar solvents are using for the RP-open column such as methanol and water. Polar compounds are eluting very first and non-polar compounds are eluting later. In this study, the first mobile phase was 20% methanol and the five fractions eluted from this mobile phase were PYEE1-5. As well as other fractions were labeled as PYEE6 and PYEE7 which were eluted with 70% and 100% methanol respectively. Each fraction of PYEE was analyzed by RP-

HPLC. The observed chromatogram is shown in Figure **II-7**. The yields of fraction 1 ~ 7 isolated from 1 g of the PYEE by the RP-open column were 28.4 mg, 14.2 mg, 6.2 mg, 6.2 mg, 62.4 mg, 152 mg and 392 mg, respectively. The whole process was illustrated in Figure **II-8**.

3.7. NO inhibitory effects of each fraction of PYEE

To evaluate the anti-inflammatory activity of the fractions (PYEE1~7, Fig. **II-8**) isolated from the PYEE, the inhibitory activity against the NO production (%) were measured in the LPS induced 267.4 RAW cells (Fig. **II-9**A). RAW 264.7 cells treated with the fractions for 2 h were 1then stimulated with 1 μg/ml LPS for the 24 h incubation. The culture supernatants were used for the evaluation of NO production by Griess reaction. Only PYEE6 showed the strongest inhibitory activity against NO production (%), and its IC50 value was 15.19 μg/ml. Cytotoxicity did not show at below 25 μg/ml, therefore all following experiments were progressed at concentrations below 25 μg/ml (Fig. **II-9**B).

3.8. Glucose uptake effect of PYEE in C2C12 myotube

To measure the effects of PYEE1~7 on glucose uptake, we first evaluated its effects on the uptake of 2-NBDG, a fluorescent glucose analog widely used for monitoring glucose uptake in cells. As shown in Figure **II-10**, PYEE1, 2, 3, 6 and 7 effectively stimulated glucose uptake in a concentration-dependent manner in C2C12 myotube. In particular, PYEE6, a fraction with anti-diabetic effect in anti-inflammatory effect fractions, showed strong glucose uptake. Under the same condition, metformin (100 μg/ml) was used as a positive control for glucose uptake.

3.9 HPLC analysis of PYEE6

PYEE6 was analyzed by RP-HPLC. Observed chromatograms are shown in Figure 23. According to the results, PYEE6 consists mainly of several compounds of polarity (Fig. **II-11**A). Therefore, optimal HPLC analysis conditions for PYEE6 were set (Fig. **II-11**B).

3. 10. Thin layer chromatographic (TLC) analysis of PYEE6

TLC chromatographs of PYEE6 are shown in Figure **II-1**2. Components in PYEE6 were separated in RP-TLC with increasing the methanol amount in the mobile phase. In order to establish the optimal separation conditions, the ratio of mobile phase solvent methanol was increased from 15% to 60% by 5%. According to the observed results, 55% methanol ratio was set as the optimal separation condition. Therefore 55% methanol was selected as mobile phase for the open column separation.

3.11. Reverse phase open column fractionation of the PYEE6

The ODS column was used to apply the 55% methanol mobile phase solvent determined by TLC analysis. The mobile phase solvent was isocratic 55% methanol, and the collections were collected in 6 ml volumes in glass test tubes. As a result of collection, two main compounds were isolated and named PYEE6-1 and 2. PYEE6-1 and 2 was analyzed by RP-HPLC and, observed chromatograms are shown in Figure **II-13**. We confirmed that there were two fractions (PYEE6-1 and 2) including with the each same compound and two compounds were purified by preparative TLC. The yields of PYEE6-1 and 2 isolated from 250 mg of the PYEE6 by the RP-open column were 6.3 mg and 6.7 mg, respectively. The whole process was illustrated in Figure **II-13**.

3.12. NO inhibitory effects of the compounds from PYEEF6

To evaluate the anti-inflammatory activity of the fractions (PYEE6-1 and 2, Fig. **II-14**) isolated from the PYEE, the inhibitory activity against the NO production (%) were measured in the LPS induced

267.4 RAW cells (Fig. **II-15**A). RAW 264.7 cells treated with the fractions for 2 h were then stimulated with 1 μg/ml LPS for the 24 h incubation. The culture supernatants were used for the evaluation of NO production by Griess reaction. PYEE6-1 and 2 showed the strongest inhibitory activity against NO production (%), and its IC_{50} value was 15.19 μg/ml. Cytotoxicity did not show at below 25 μg/ml, therefore all following experiments were progressed at concentrations below 25 μg/ml (Fig. **II-15** B).

3.13. Glucose uptake effect of the compounds from PYEEF6 in C2C12 myotube

To measure the effects of PYEE6-1 and 2 on glucose uptake, we first evaluated its effects on the uptake of 2-NBDG, a fluorescent glucose analog widely used for monitoring glucose uptake in cells. As shown in Figure **II**-16, PYEE6-1 and 2 effectively stimulated glucose uptake in a concentration-dependent manner in C2C12 myotube. All samples were showed strong glucose uptake effect. Under the same condition, metformin (100 μg/ml) was used as a positive control for glucose uptake.

3.14. Structural identification of anti-inflammatory compounds from PYEE6

The identification of PYEE6-1 and 2 was carried out by ^{1}H NMR, ^{13}C NMR and GC-MS/MS. Figure **II-17** and **II-18**. PYEE6-1 and 2 was confirmed as 2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester and 3,4-DICHLORO[1,6]NAPHTHYRIDINE $$ PYRIDO[4,3-B]PYRIDINE, 3,4-DICHLORO.

2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester has been studied from leaves of *Andrographis Paniculata*. And it is known as a compound of the plasticizer compound series (Krishnamoorhty et al, 2016).3,4-DICHLORO[1,6]NAPHTHYRIDINE $$ PYRIDO[4,3-B]PYRIDINE, 3,4-DICHLORO has been no studies on the biological activities and characteristic of these compounds.

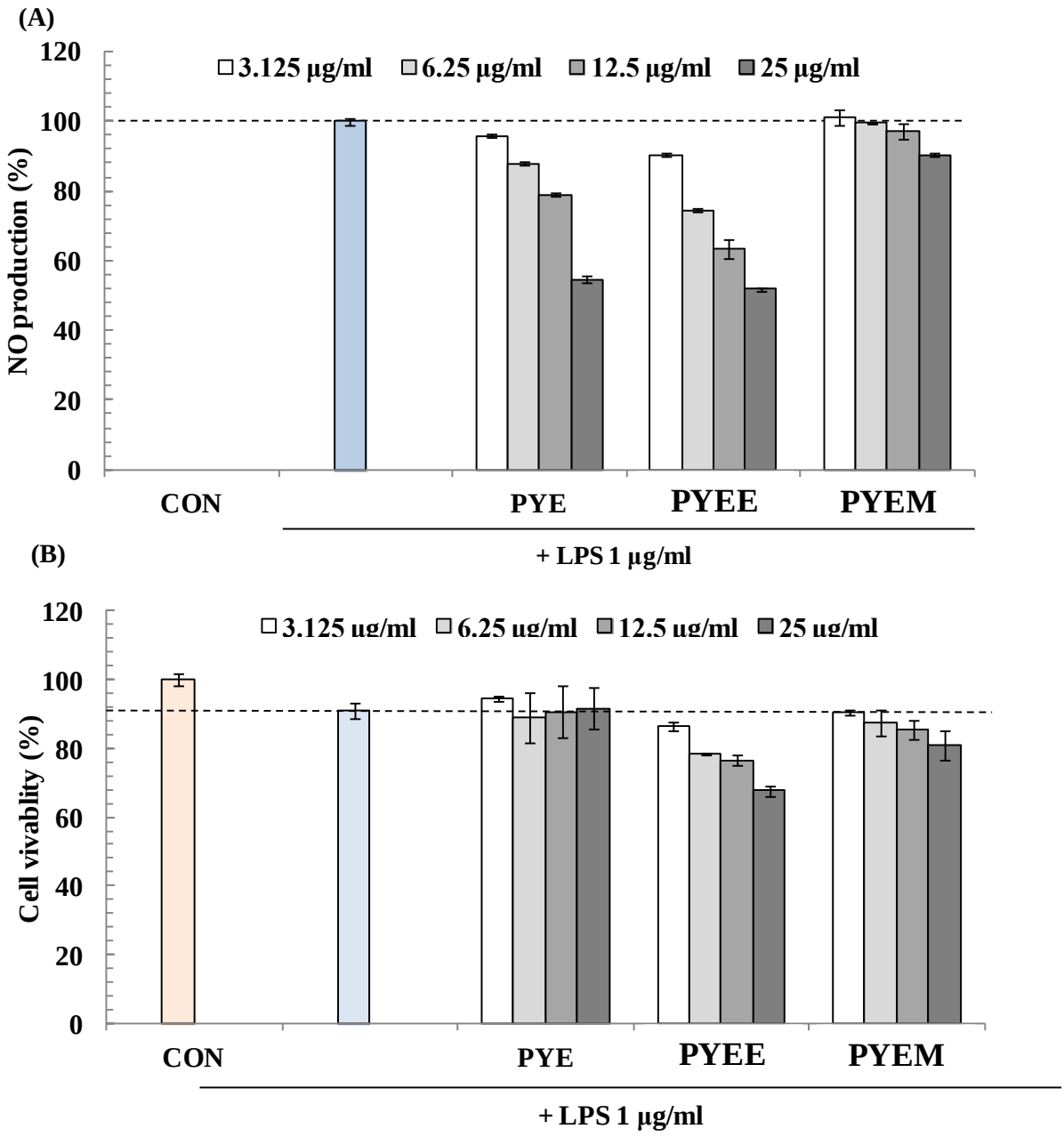


Figure II-4. Inhibitory effect of NO production of fractions of PYC (A) and their cytotoxicity (B) in LPS induced Raw 264.7 macrophages. Cells were incubated with LPS for 24 h presence or absence of desire fraction.

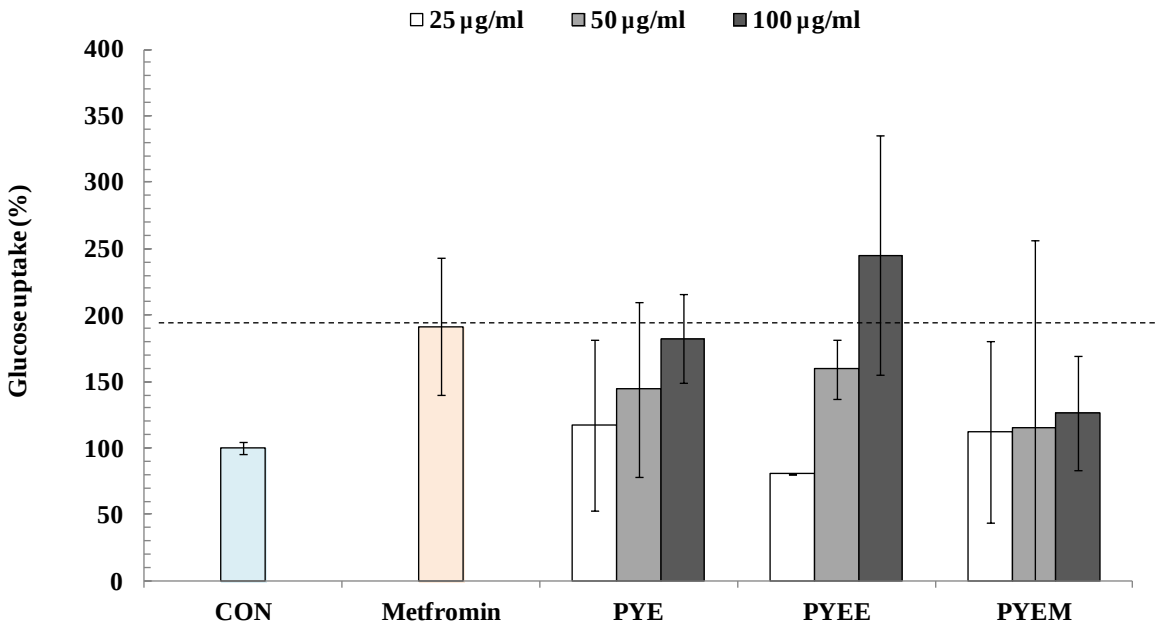


Figure II-5. PYE, PYEE and PYEM on glucose uptake in C2C12 myotubes. C2C12 cells were treated with increasing concentrations of sample or metformin (100 μg/ml), and following this treatment.

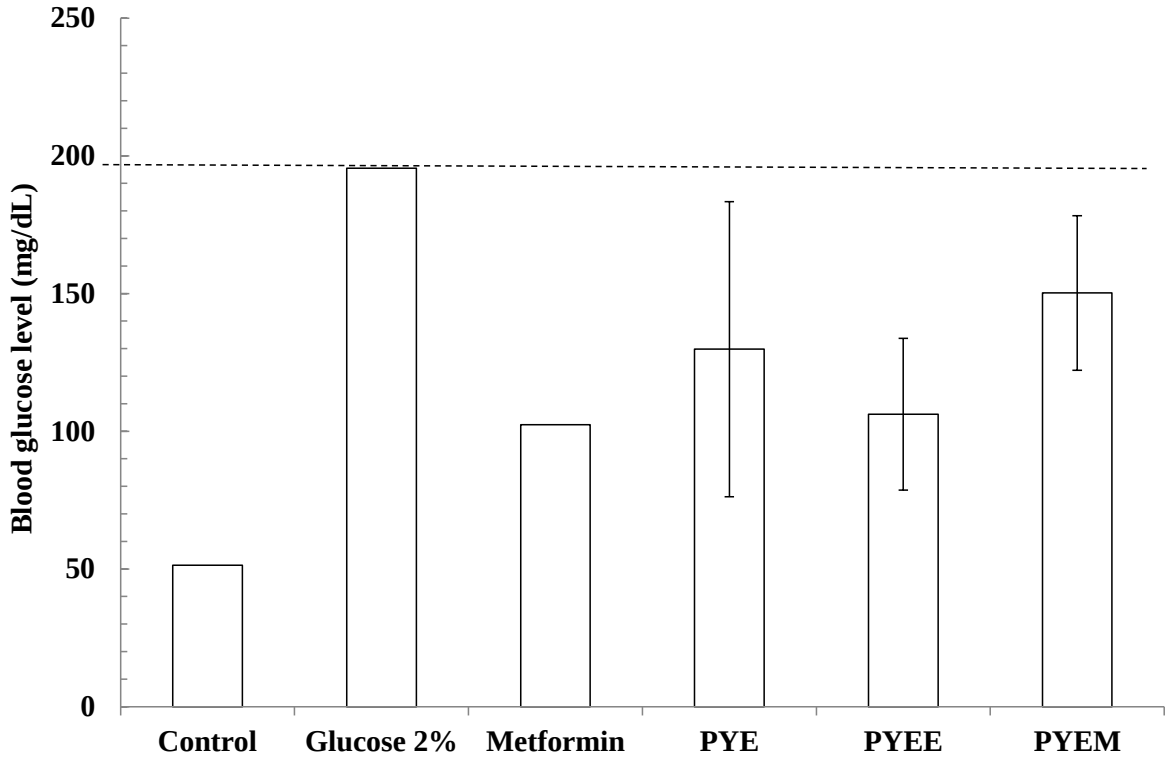


Figure II-6. Blood glucose level inhibitory effect of PYE, PYEE and PYEM alloxan stimulated zebrafish.

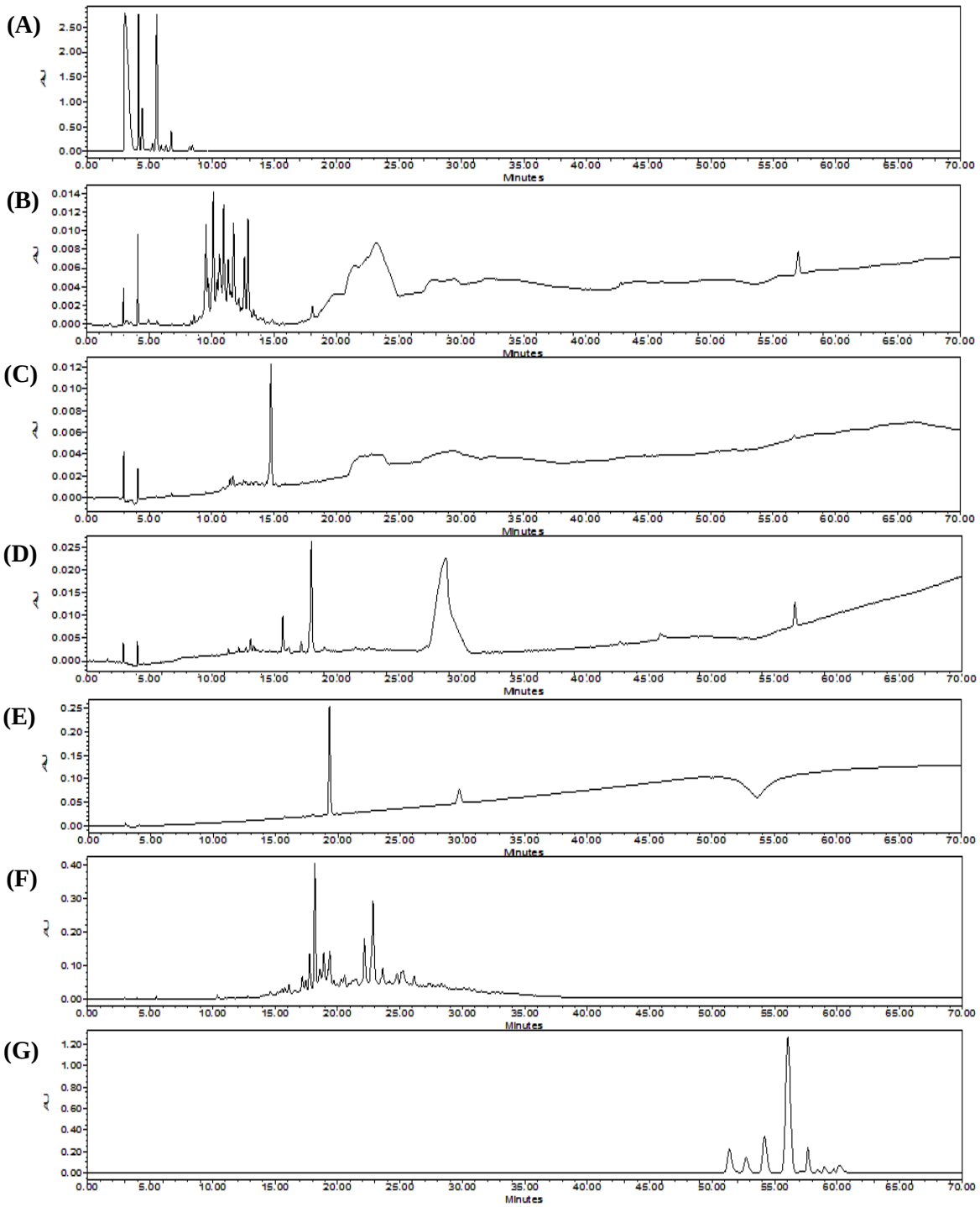


Figure 19. RP-HPLC chromatogram of (A) PYEE1 (detector wave length 254 nm), (B) PYE E2 (235 nm), (C) PYEE3 (284 nm) and (D) PYEE4 (220 nm), (E) PYEE5 (228 nm), (F) PYEE6 (278 nm) and (G) PYEE7 (400 nm), Column: Sunfire ODS 5 μm ODS column (250 mm 4.6 mm i.d.); mobile phase: acetonitrile-0.01% acetic acid (5:95 v/v to 100:0 v/v at 0–50 min, 100:0 v/v to 100:0 v/v at 50–70 min); flow rate: 1 mL/min.

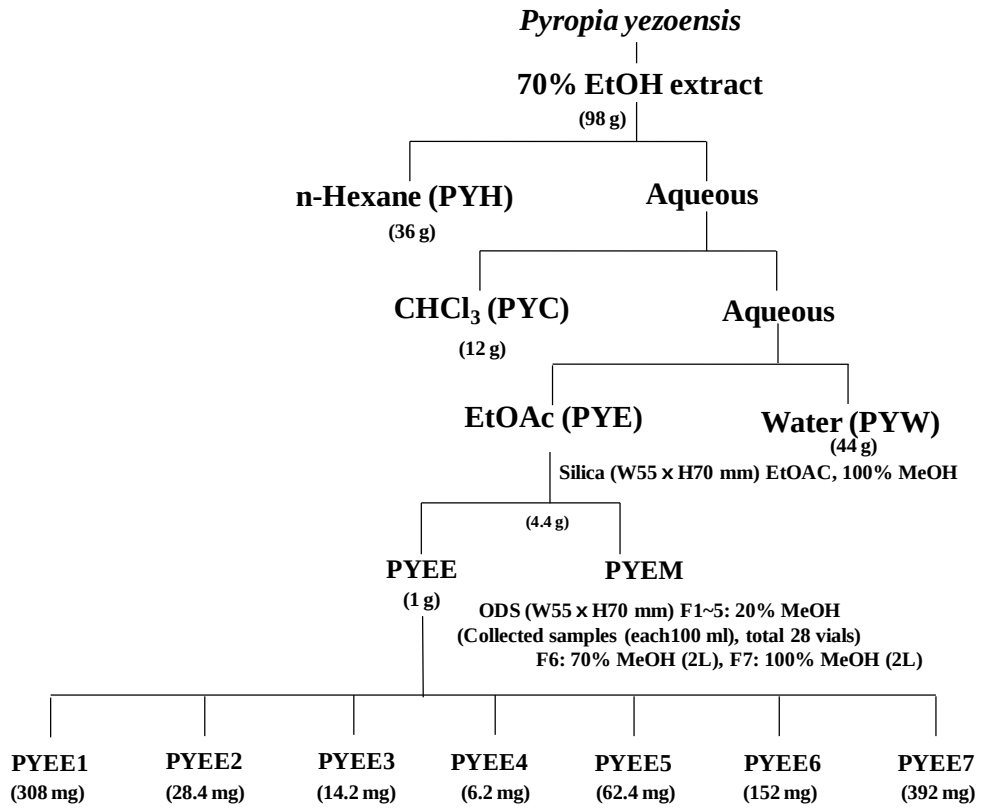


Figure II-8. Fractionation scheme of PYEE

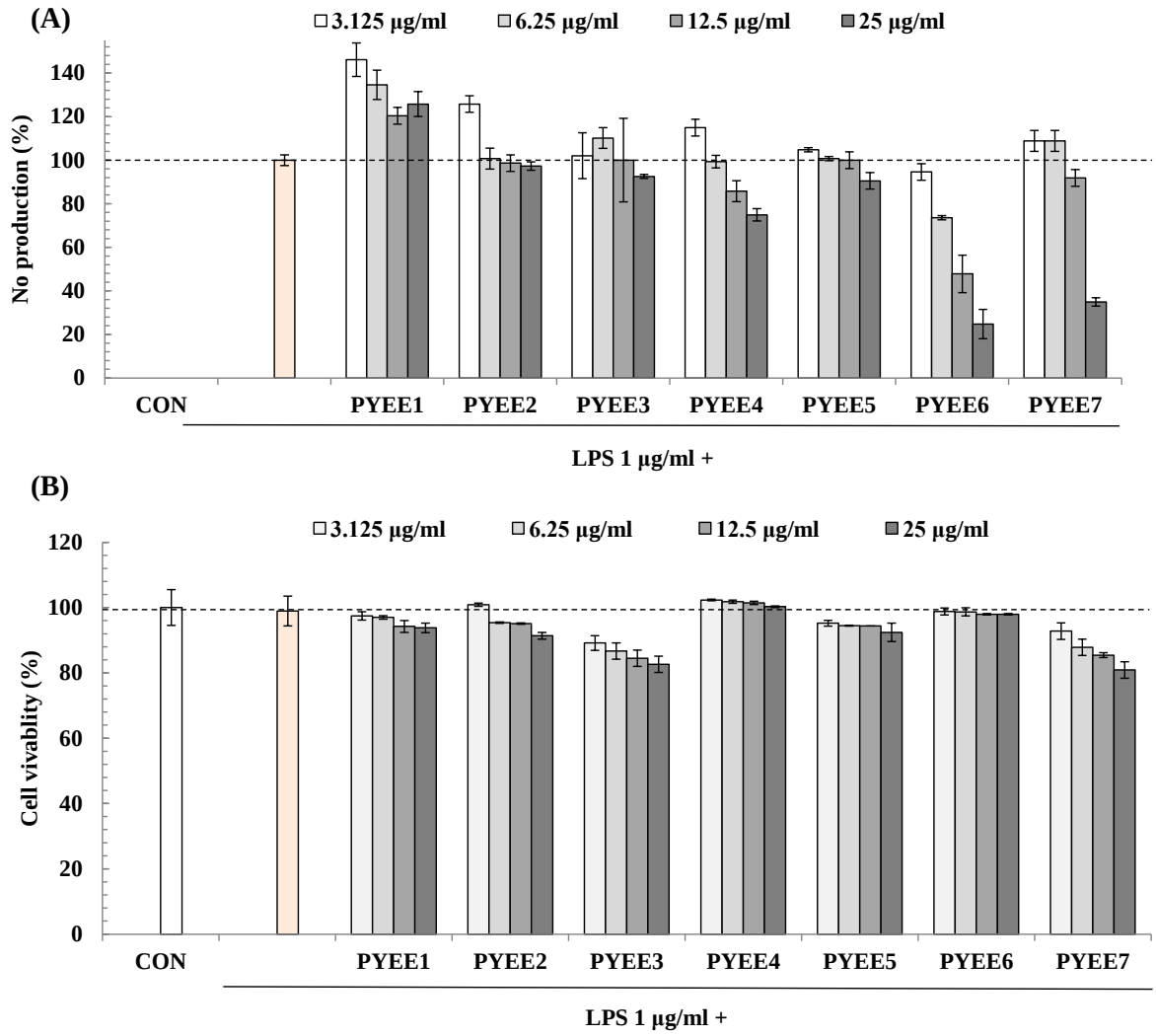


Figure II-9. Inhibitory effect of NO production of fractions of PYEE (A) and their cytotoxicity (B) in LPS induced Raw 264.7 macrophages. Cells were incubated with LPS for 24 h presence or absence of desire fraction.

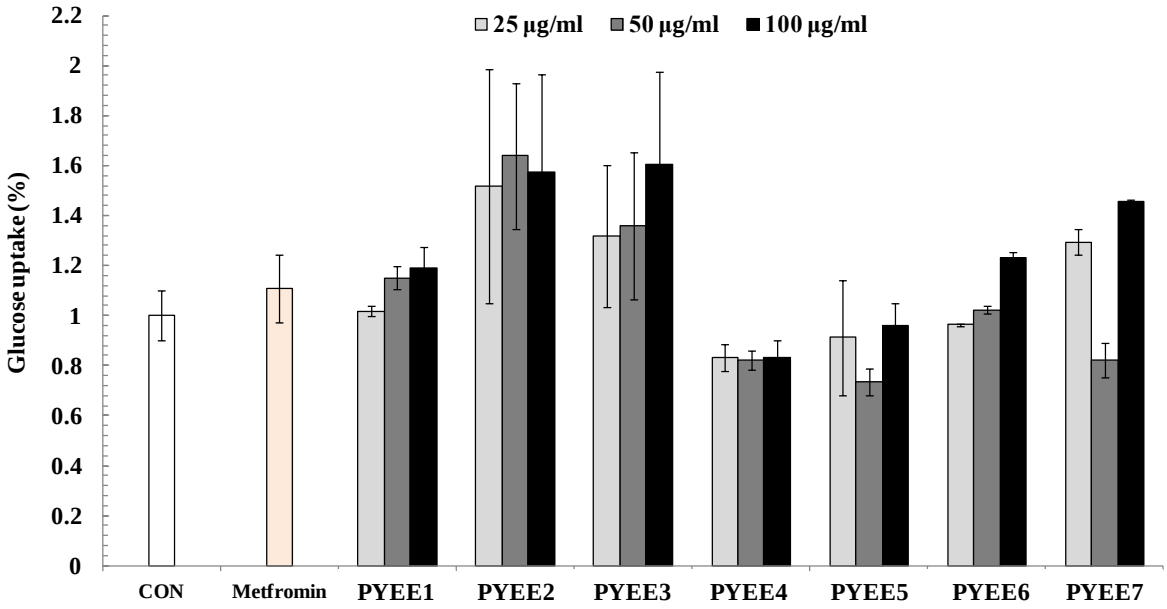


Figure II-10. PYEE1~7 on glucose uptake in C2C12 myotubes. C2C12 cells were treated with increasing concentrations of sample or metformin (100 μg/ml), and following this treatment.

3.15. PYEE6-1 and 2 inhibited release of PGE_2, and decreased pro-inflammatory cytokines released by LPS-stimulation in RAW 264.7 macrophages

The LPS stimulation induces the expression of iNOS which triggers the production of its downstream effector, NO which ultimately results inthe tissue damage by NO itself or its more toxic metabolite, peroxynitrite ($ONOO^-$) (Dringen, 2005; Li et al., 2005). In addition, inducible cyclooxygenase, COX-2 drives the onset of inflammation through the production of pro-inflammatory PGE_2 (Tzeng et al., 2005). Pro-inflammatory cytokines such as TNF-α and IL-1β are largely produced in the inflammatory condition and these cytokines act as messengers which stimulate the inflammatory process (Jung et al., 2009). Therefore, the down-regulation of the pro-inflammatory cytokine production is one of the most important strategies in the anti-inflammatory therapy.

The production of PGE_2 was significantly increased upon the LPS treatment when compared with the untreated control group. However, PYEE6-1 and 2 dose-dependently decreased the LPS-stimulated PGE_2 production (Fig. **II-19**A). The pro-inflammatory cytokines produced from macrophages, are the key components of inflammation. Therefore, the effect of PYEE6-1 and 2 on the production levels of the inflammatory cytokines IL-1ß and IL-6 were investigated by ELISA kit using the conditioned media of LPS-stimulated RAW 264.7 macrophages. The LPS stimulation significantly increased the production of pro-inflammatory cytokines IL-1ß and IL-6 compared to the non-stimulated blank groups. The LPS induced production of IL-1ß and IL-6 showed a significant concentration dependent decrease following the treatment of PYEEE6-1 and 2 (Fig. **II**-19).

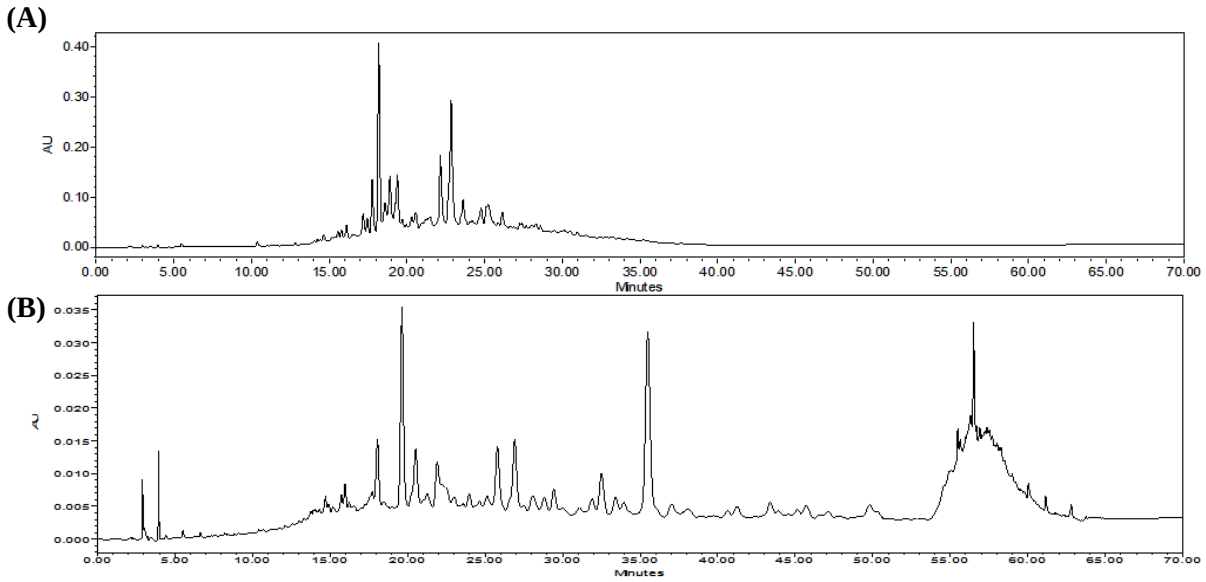


Figure II-11. RP-HPLC chromatogram of PYEE (A), mobile phase: acetonitrile-0.01% acetic acid (5:95 v/v to 100:0 v/v at 0–50 min, 100:0 v/v to 100:0 v/v at 50–70 min); flow rate: 1 mL/min. RP-HPLC chromatogram of PYEE (B), mobile phase: acetonitrile-0.01% acetic acid (5:95 v/v to 25:75 v/v at 0–10 min, 25:75 v/v to 33:67 v/v at 10–50 min, 33:67 v/v to 100:0 v/v at 50–60 min, 100:0 v/v to 100:0 v/v at 60–70 min); flow rate: 1 mL/min. Column: Sunfire ODS 5 μm ODS column (250 mm 4.6 mm i.d.), monitored at 260 nm.

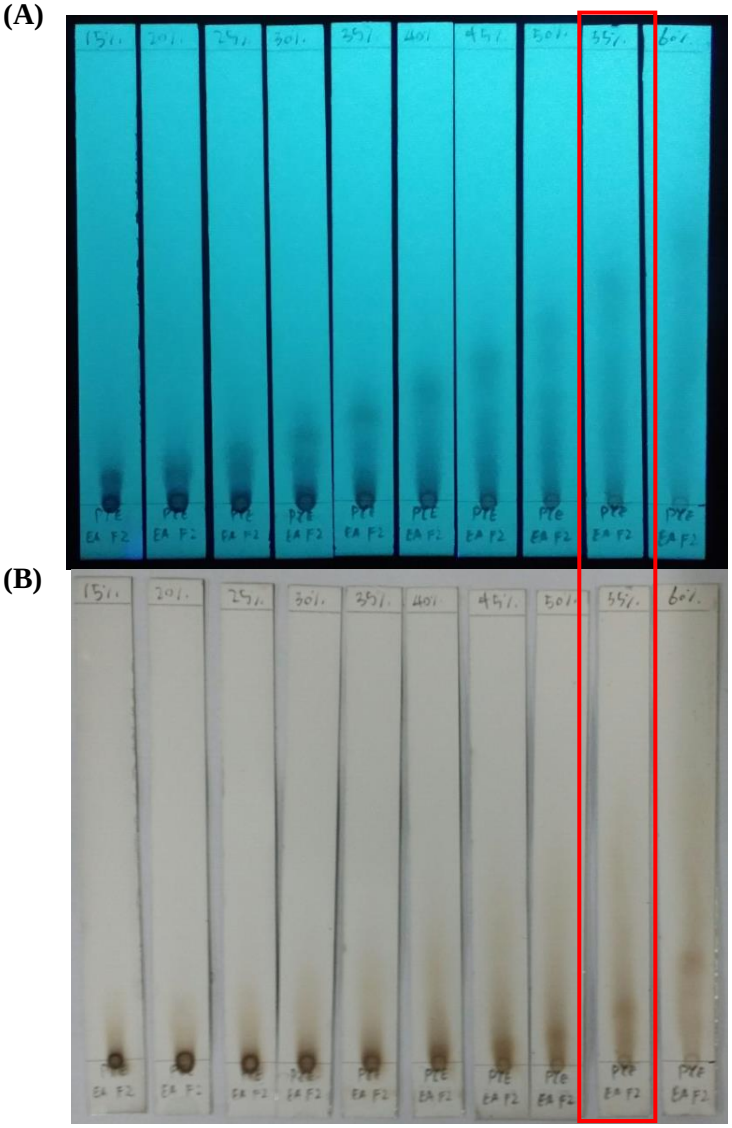


Figure II-12. RP-TLC analysis data of the PYEE6 (A) under 254 nm UV radiation (B) heat with sulfuric and ethanol staining reagent.

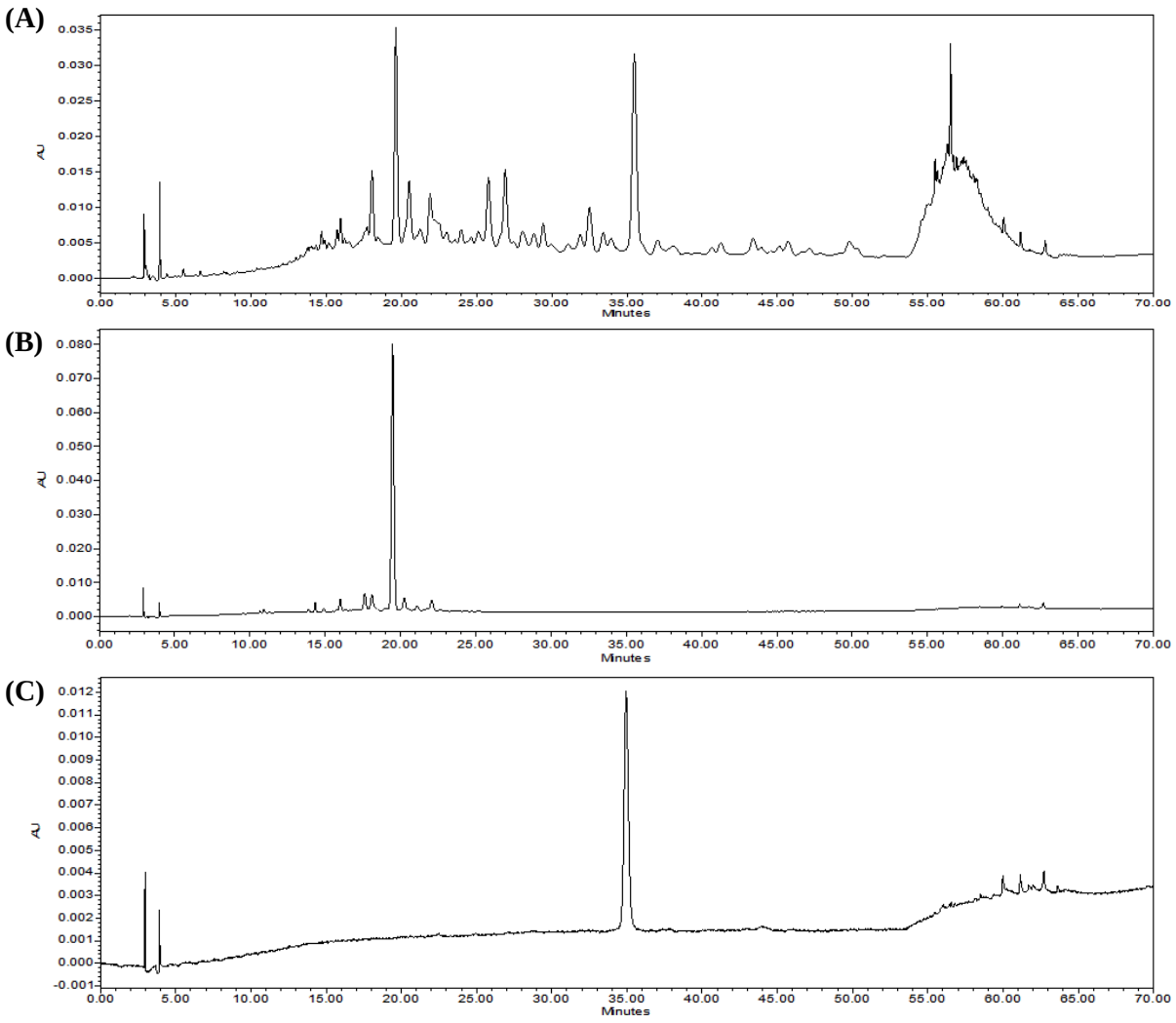


Figure II-13. RP-HPLC chromatogram of PYEE6 (A) PYEE6-1 (B) PYEE6-2 (C), mobile phase: acetonitrile-0.01% acetic acid (5:95 v/v to 25:75 v/v at 0–10 min, 25:75 v/v to 33:67 v/v at 10–50 min, 33:67 v/v to 100:0 v/v at 50–60 min, 100:0 v/v to 100:0 v/v at 60–70 min); flow rate: 1 mL/min. Column: Sunfire ODS 5 μm ODS column (250 mm 4.6 mm i.d.), monitored at 260 nm.

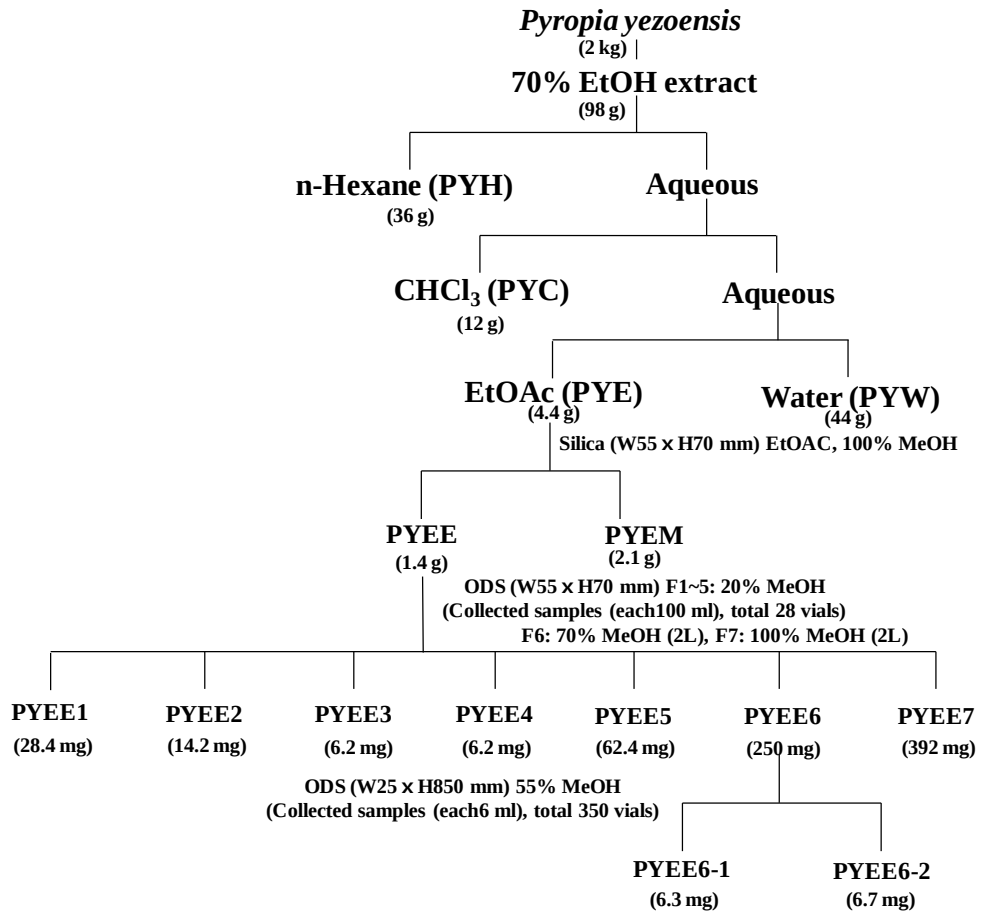


Figure II-14. Fractionation scheme of PYEE6

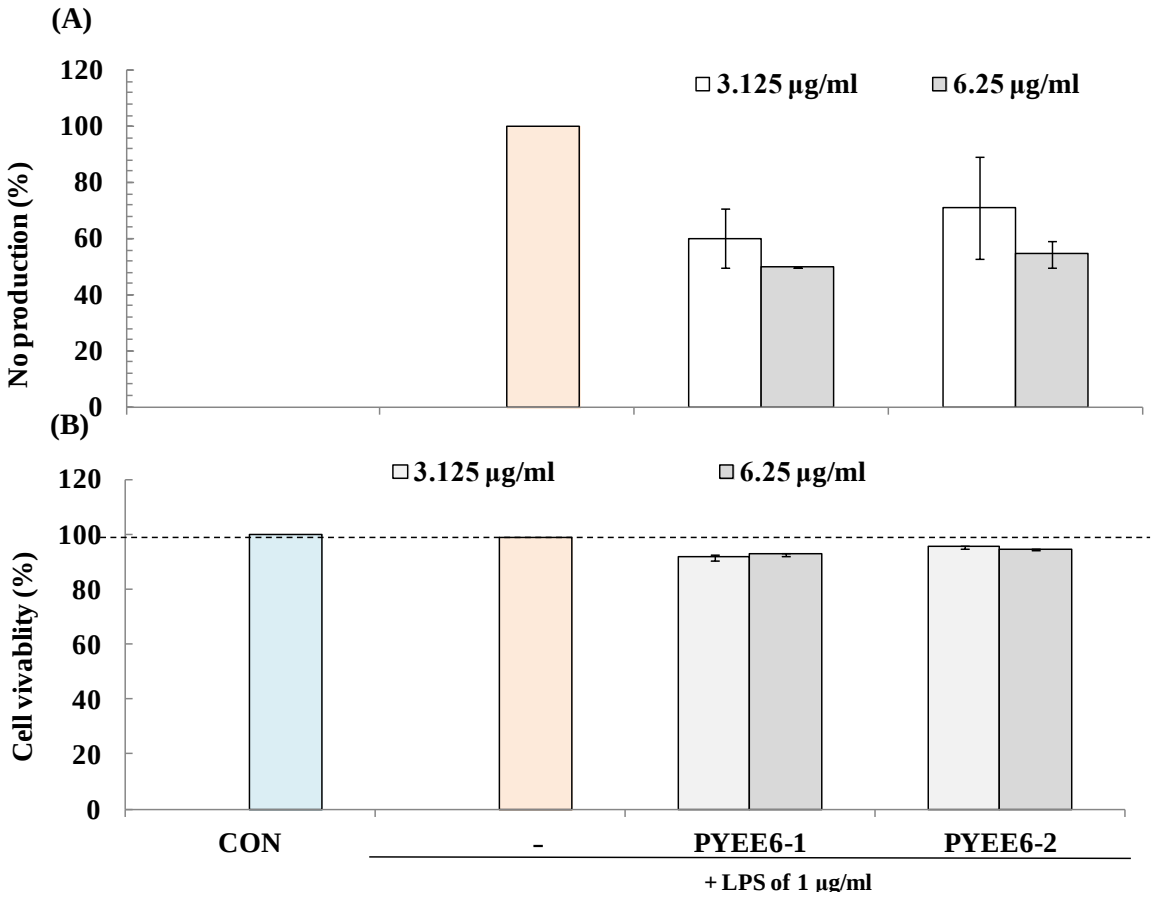


Figure II-15. Inhibitory effect of NO production of PYEE6-1 and 2 (A) and their cytotoxicity (B) in LPS induced Raw 264.7 macrophages. Cells were incubated with LPS for 24 h presence or absence of desire fraction.

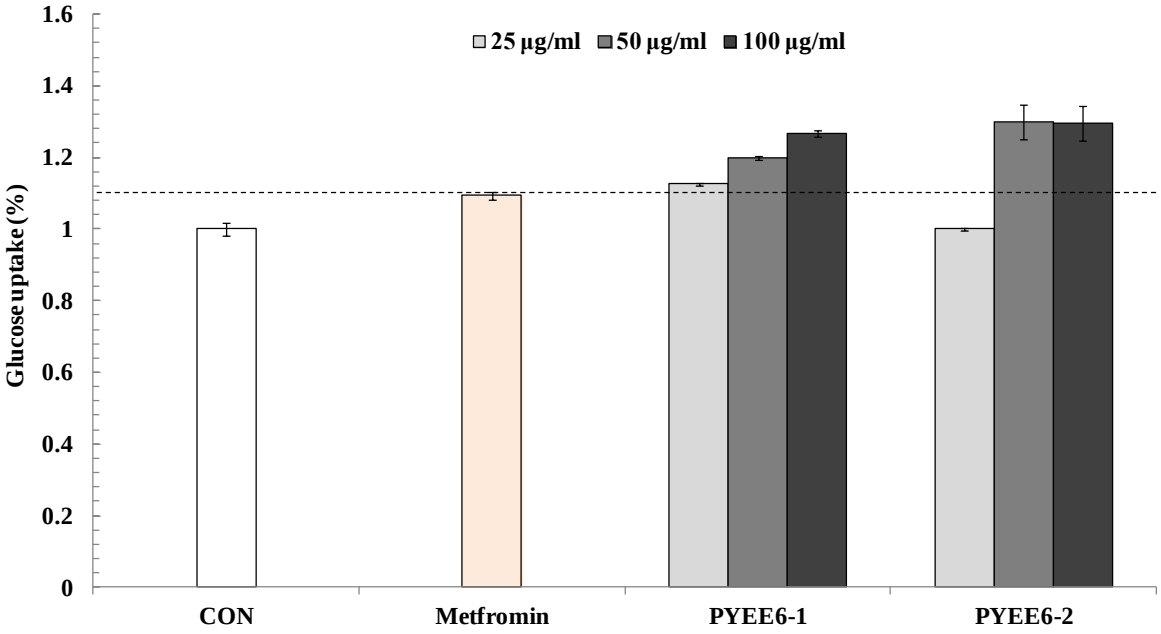


Figure II-16. PYEE6-1 and 2 on glucose uptake in C2C12 myotubes. C2C12 cells were treated with increasing concentrations of sample or metformin (100 μg/ml), and following this treatment.

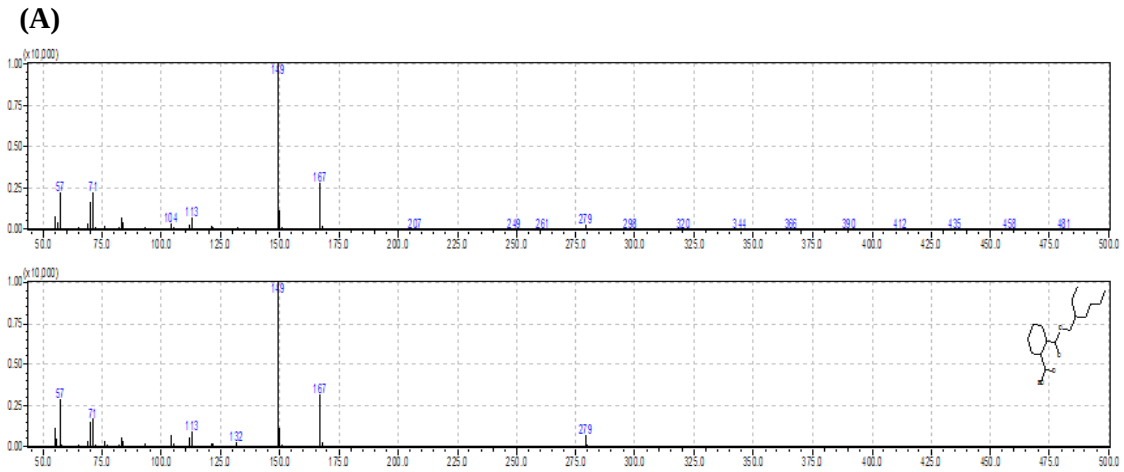
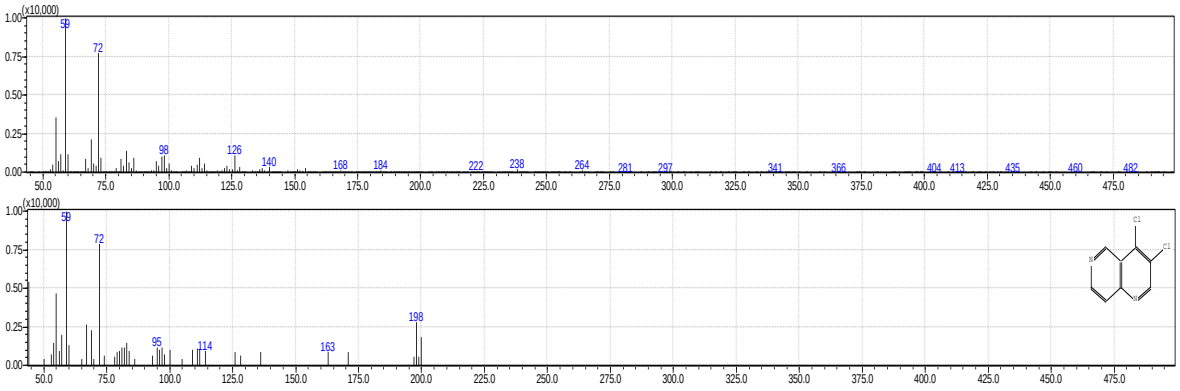
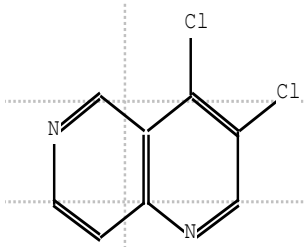


Figure II-17. GC/MS library search results of PYEE6-1 (A). ^{1}H NMR data of PYEE6-1 (B). Chemical structures of PYEE6-1 (C).

(A)



(B)



3,4-DICHLORO[1,6]NAPHTHYRIDINE $$ PYRIDO[4,3-B]PYRIDINE, 3,4-DICHLORO

Figure II-18. GC/MS library search results of PYEE6-2 (A). Chemical structures of PYEE6-2 (B).

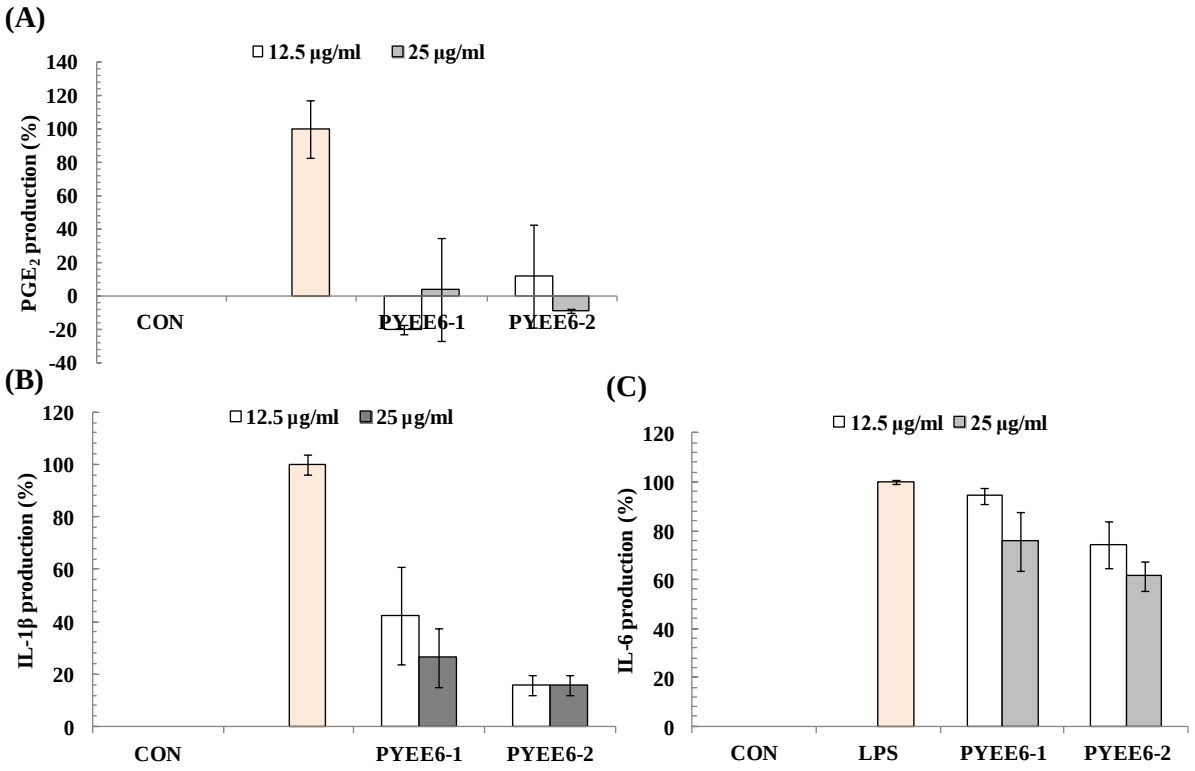


Figure II-19. Pro-inflammatory cytokines inhibitory effect of PYEE6-1 and 2 in LPS-induced RAW 264.7 cells. (A) PGE_2, (B) IL-1β, and (C) IL-6.

4. CONCLUSION

In this study, we isolation and identification of anti-inflammation and anti-diabetic compounds from EtOAc fraction from *P. yezoensis* by using centrifugal partition chromatography (CPC). These compounds showed strong anti-inflammatory and anti-diabetes effects.

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제가 실험실 생활을 잘 해나갈 수 있게 이끌어주시고 좋은 말씀을 해주신 우리 실험실원들에

게 감사를 표합니다. 실험실 생활 뿐만 아니라 사회생활을 하는 법을 가르쳐 주신 이승홍 교수님, 친근하게 웃어주시며 다가와 주시는 김길남 박사님, 누구보다도 많은 도움을 주시고, 부족한 점을 언제나 채워주시고, 큰 버팀목이 되어준 지혁이형, 무심한 듯 항상 잘 챙겨주시는 주영누나, 친근감 있게 많은 이야기를 해주신 원우형, 항상 유쾌한 준성이형, 겉으로 무섭지만 아무 말 없이 도와주는 재영이형, 항상 웃으며 반겨주는 은아누나와 나래누나, 학부때부터 실험실에 들어오기까지 같이 지내온 현수형, 묵묵히 도와주는 윤택이형, 실험실 생활에 많이 배우고 도움 받은 서영누나와 혜원이, 장난많은 외국인 친구들 샤누라와 아산카, 왕뢰, 도움을 많이 준 부끄럼쟁이 바로,

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제가 석사 학위를 마칠 수 있게 도와주신 모든 분들께 다시 한번 감사의 말씀 드리며, 이 분들의 도움이 가치 있는 것이 되게끔 더욱더 노력하고 발전하는 모습을 보여 드리겠습니다.