A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Endothelium dependent vasorelaxation of Gallic acid isolated from *Spirogyra* sp. and its antihypertension effect

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Endothelium dependent vasorelaxation of Gallic acid isolated from *Spirogyra* sp. and its antihypertension effect

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CONTENTS

국문초록

순환계는 신체의 모든 기관으로 산소와 영양분을 운반하는 역할을 하는 기관계이며, 순환계에서 혈관은 매우 중요한 조직이다. 혈관의 항상성이 유지되지 않을 경우에는 고혈압, 심근경색, 동맥경화 등의 심각한 심혈관계 질환에 노출된다. 이러한 질환에 직·간접적인 영향을 미치는 혈관확장은 혈관의 이완 운동에 따라 일어나는 현상이다. 혈관은 크게 평활근 세포와 내피 세포로 이루어져 있는데, 평활근 세포가 이완 운동을 하고, 내피 세포는 평활근 세포가 운동할 수 있도록 신호를 전달한다. 내피 세포에 의존적인 혈관확장 신호 물질 중에서 가장 중요한 것은 산화질소이다. 내피 세포에 존재하는 endothelial Nitric Oxide Synthase (eNOS)에서 생성된 산화질소는 평활근 세포로 확산되어 cyclic Guanosine Monophosphate (cGMP)를 생성시킴으로써 혈관을 확장시킨다.

본 연구에서는 수생식물인 해캄에서 분리한 에틸아세테이트 층의 혈관확장 효과를 인형 제대정맥 내피세포에서 확인하였다. 해캄 에틸아세테이트 층은 고성능 원심분배 크로마토그래피를 통하여 분리되었으며, 분리된 물질들의 혈관확장 효과는 산화질소 생성, eNOS의 인산화, 내피세포로의 칼슘의 유입과 같은 내피 세포에 의존적인 요인들을 측정함으로써 확인하였다.

해캄 에틸아세테이트 층에서 분리한 물질들 중에서 SPE.Ⅳ 는 유의적으로 내피 세포에 의존적인 혈관확장 효과를 나타내었으며, eNOS의 저해제인 N^{G}-nitro-$_{L}$-arginine methyl

ester (L-NAME)를 전처리 할 경우, SPE.IV에 의한 혈관확장 효과가 저해되는 것을 확인하였다. LC/MS 및 1H NMR data 를 비교·분석한 결과, SPE.IV는 항산화제로 널리 알려진 갈산임을 확인하였다.

평활근 세포에서 갈산의 혈관확장 효과는 분자 모델링 연구를 통하여 cGMP를 분해하는 효소인 Phosphodiesterase 5 (PDE 5)에 대한 결합 작용을 예측·분석하였다. 그 결과, 갈산은 비교적 안정적으로 PDE 5의 활성 부위에 결합함으로써 효소의 cGMP 분해 작용을 억제하는 것으로 예측된다.

혈관확장과 관련된 질환인 고혈압은 동맥경화, 뇌졸중, 심근경색 등의 합병증 발병의 원인이 되는 심각한 만성 질환이다. 본 연구에서는 혈관확장과 더불어 갈산의 항고혈압 활성을 확인하기 위해서 안지오텐신 I 전환 효소의 저해 활성을 평가하였다. 그 결과, 122.9 μg/ml 의 IC_{50} value 을 나타내어 비교적 우수한 활성을 나타내는 것을 확인하였다. 효소 저해 패턴을 예측·분석하기 위해 분자 모델링 연구를 수행한 결과, 효소의 활성 부위의 입구를 막음으로써 안지오텐신 I 전환 효소의 작용을 저해하는 것으로 예측된다.

결론적으로 갈산은 혈관확장 효과와 항고혈압 효과를 동시에 가짐으로써 혈관 질환 개선의 효과를 나타낼 것으로 사료된다.

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ABSTRACT

In the present study, vasorelaxation effect of ethyl acetate fraction of aquatic plant, *Spirogyra* sp. was investigated using human umbilical vein endothelial cells (HUVECs). *Spirogyra* sp. ethyl acetate fraction (SPE) was isolated through high performance centrifugal partition chromatography (HPCPC), and identified its vasorelaxation effect by measuring endothelium dependent factors including nitric oxide (NO) levels, phosphorylation of endothelial Nitric Oxide Synthase (eNOS) and influx of calcium into HUVECs.

Among the SPE fractions, SPE.IV significantly induced endothelium dependent vasorelaxation. Moreover, this vasorelaxation effect induced by SPE.IV was attenuated by pretreatment with N^G-nitro-$_L$-arginine methyl ester (L-NAME), eNOS inhibitor. SPE.IV was identified as gallic acid (GA) by comparing LC/MS and 1H NMR data to the literature report.

Vasorelaxation effect of GA in vascular smooth muscle cell was evaluated by simulating binding mode of GA to Phosphodiesterase 5 (PDE 5), enzyme interrupting vasorelaxation, using the crystal structure of PDE 5 (PDB ID: 1UDT). The molecular modeling study was successful (calculated binding energy value: -90.00 kcal/mol), suggested that GA interacts with Phe820, Gln817 and Ser663.

Hypertension directly related with vasorelaxation is the most common serious chronic disease because it is a high risk factor for arteriosclerosis, stroke, and myocardial infarction (Je et al., 2005).

Antihypertensive effect of GA was estimated as angiotensin-I converting enzyme (ACE) inhibitory activity using ACE kit (DOJINDO Laboratories, Kumamoto Japan). GA exhibited relatively high ACE inhibitory activity with IC_{50} value of 122.9 μg/ml . For further insight, binding mode of GA to ACE using the crystal structure of ACE (PDB ID: 1O86). The molecular modeling study was successful (calculated binding energy value: -128.33 kcal/mol), suggested that GA interacts with Glu384, Tyr523, Ala356 and Arg522.

Therefore, GA could be a profound enhancer on vascular diseases by possessing endothelium-dependent vasorelaxation effect and antihypertensive effect.

1. INTRODUCTION

Blood vessels are vital tissue of the circulatory system which transports oxygen and nutrients to all vertebrate organs, supporting organ development as well as metabolism and homeostasis (Villasenor et al., 2012). Blood vessels consist of endothelial cells, the inner lining of vessels, and smooth muscle cells, within the vessel walls (Golpon et al., 2003). Vasorelaxation is relaxation of smooth muscle cells and is an important physiological phenomenon related in various vascular diseases such as hypertension, myocardial infarction and atherosclerosis even erectile dysfunction. Vasorelaxation improves these vascular diseases by alleviating high blood pressure and peripheral resistance (Shou et al., 2012).

Mechanism of vasorelaxation is various and may be exerted in endothelium-dependent and/or-independent manners (Akhlaghi et al., 2009). In the endothelium-dependent relaxation effect, endothelial cells regulate the function of the underlying vascular smooth muscle cells and the diameter of the vessels (Kim et al., 2011).

The endothelium-dependent relaxation effect is mediated by nitric oxide (Akhlaghi et al., 2009). NO is generated via oxidation of $_{L}$-arginine catalyzed by NO synthase (NOS), which exists in three isoforms: inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS) expressed in a variety of tissues (Alderton et al,.2001, Jin et al., 2011).

Depending on isoforms of NOS, generated NO plays respectively different role. NO generated by iNOS is a critical player in the development of various airway inflammatory diseases and is a part of complex signaling systems associated with airway inflammatory diseases. (Lee et al., 2010). On the other hands, NO generated by eNOS is an endothelium-derived messenger molecule and plays an important role in the control of cardiovascular homeostasis by maintaining coronary vasodilatory tone, inhibiting platelet aggregation and inhibiting adhesions of neutrophils and platelets to vascular endothelium (Akhlaghi et al., 2009). After Ca^{2+}/calmodulin pathways, NO generated in Endothelial cells diffuses to vascular smooth muscle cells and increases intracellular cyclic Guanosine Monophosphate (cGMP) with activation of the soluble guanylyl cyclase (sGC), leading to relaxation of underlying vessels (Rapoport and Murad, 1983, Jin et al., 2011).

Hypertension caused by vasoconstriction has become a worldwide major problem threat to human health. It is the most common serious chronic health problem, because it is a high risk factor for arteriosclerosis, stroke, and myocardial infarction (Je et al., 2005).

Angiotensin I-converting enzyme (ACE) performs a pivotal function in the regulation of blood pressure (Lee et al., 2009). ACE converts an inactive form of decapeptide, angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu), to octapeptide angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), a potent vasoconstrictor, and inactivates bradykinin, which exerts a depressor effect (Je et al., 2005).

ACE inhibition has been used extensively in therapeutic strategies for the prevention and treatment of hypertension. Several synthetic ACE inhibitors have been developed, including alacepril, captopril, benazepril, enalapril, fosinopril, ramipril, and zofenopril, all of which are currently extensively used in the treatment of essential hypertension and heart failure in humans (Ondetti et al., 1997). However, these synthetic ACE inhibitors are believed to exert certain side effects, including cough, taste disturbances, and skin rashes (Kato et al., 1972). Therefore, the development of ACE inhibitors from natural products has become a major area of research.

In this study, vasorelaxation effect of ethyl acetate fractions of aquatic plant, *Spirogyra* sp. was investigated using human umbilical vein endothelial cells (HUVECs) and vascular smooth muscle cell. Moreover, its antihypertension, caused by vasoconstriction, effect was investigated on ACE inhibitory activity.

2. MATERIALS AND METHODS

2.1. Materials

Antibodies including phospho-eNOS (Ser1177), iNOS and phospho-Akt (Ser473) were purchased from Cell signaling Technology (Beverly, MA). Calcium flux assay kit was purchase from BD Biosciences. Diamino fluorophore 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) and N^G-nitro-$_L$-arginine methyl ester (L-NAME) was purchase from Sigma Chemical Co. (St. Louis, MO, USA). The other chemicals and reagents used were of analytical grade.

2.2. Preparation of ethyl acetate fraction of *Spirogyra* sp. (SPE)

Spirogyra sp. was collected from several shallow pond in Kongju, Korea (36˚20'34", 127˚12'28"). Samples were cleaned and cultured in modified Bold's Basal Medium at 20℃ under 50 μmol photoms m-2s-1 (12L/12D) for 3years, and ground and shifted through a 50 mesh standard testing sieve after dried by freeze dryer SFDSMO6, and then dried *Spirogyra* sp. was stored in refrigerator until use.

Dried *Spirogyra* sp. (20 g) was extracted three times for 3hrs using 80% MeOH under

sonication at room temperature. The extract was concentrated in a rotary vacuum evaporator and partitioned with ethyl acetate, and then the dried ethyl acetate fraction was stored in a refrigerator for CPC separation.

2.3. Procedure of CPC

The CPC experiments were performed using a two-phase solvent system composed of ethyl acetate/methanol/water (10:1:9, v/v). The two phases were separated after thoroughly equilibrating the mixture in a separating funnel at room temperature. And, for dual mode operation, the upper organic phase was used as the stationary phase (0~150 min) and mobile phase (150~300 min), whereas the lower aqueous phase was employed as the mobile phase (0~150 min) and stationary phase (150~300 min).

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) and makes change to ascending mode after 150 min. When the mobile phase emerged from the column, indicating that hydrodynamic equilibrium had been reached (back pressure : 2.1 MPa), The concentrated ethyl acetate fraction (500 mg) from the 80% MeOH extracts from *S. varians* was dissolved in 6 mL of a

1:1 (v/v) mixture of the two CPC solvent system phases and was injected through the Rheodyne injection valve. The effluent from the CPC was monitored in the UV at 254 nm and fractions were collected with 6 mL in 8mL tube by a a Advantec CHF 122SC fraction collector (Toyo seisakusho kaisha LTD., Japan).

2.4. Cell culture

Human umbilical vein endothelial cells (HUVECs) and endothelial cell basal medium-2 (EBM-2) with endothelial cell growth medium-2 (EGM-2) bullet kit were purchased from Clonetics Inc. (San Diego, USA). Cells were maintained in culture at 37 ℃ in a humidified atmosphere containing 5% CO_2, in EBM-2 supplemented with ascorbic acid, 2% fetal bovine serum (FBS), hydrocortisone, human fibroblast growth factor (hFGF), vascular endothelial growth factor (VEGF), human epidermal growth factor (hEGF), long R insulin-like growth factor-1 (R3-lGF-1), gentamicin sulfate (CA-1000) and heparin as described by the manufacturer. Cells were employed between passage 3 and 6.

2.5. Cell viability

Cell viability was quantified through a colorimetric MTT assay that measured the mitochondrial

activity in viable cells. Cells ($1*10^5$ cells/mL) in 96-well plates were incubated without or with the sample of various concentrations (25 and 50 ug/ml) for 1.5 h prior to MTT treatment. 50 uL of MTT stock solution (2 mg/mL in PBS) was added to each well. After incubation of 4 h, 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.

2.6. Assay of nitric oxide (NO) levels

Generation of NO was analyzed using a fluorescent probe dye, diaminofluorophore4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) which is converted via a NO-specific mechanism to an intensely fluorescent triazole derivatives (Itoh et al., 2000). Cells ($1*10^5$ cells/mL) in 96-well plates were incubated without or with the sample of indicated concentrations for 1.5 h. Then, DAF-FM DA solution (5 μM) was treated and cells were incubated at 37 ℃ for 1 h in the dark. After incubation, fluorescence was measured using a fluorescence plate reader.

2.7. Immunoblotting

Phospho-eNOS, iNOS and phospho-Akt (Ser473) expressions were determined by western blot analysis (Yamabe et al., 2007). Total protein (10 ug) levels were electrophoresed through 12% sodium dodecyl sulfate–polyacrylamide gel. Separated proteins were transferred electrophoretically to a pure nitrocellulose membrane, blocked with 5% skim milk solution for 2 h, and then incubated with primary antibodies overnight at 4 ℃. After washing of the blots, they were incubated with goat anti-rabbit or goat anti-mouse IgG HRP conjugated secondary antibody for 1 h at room temperature. Signals were developed using an enhanced chemiluminescence (ECL) western blotting detection kit and exposed to X-ray films.

2.8. Measurement of intracellular calcium levels

Intracellular calcium levels were analyzed using the BD™ Calcium Assay Kit. Cells ($1*10^5$ cells/mL) in 96-well plates were incubated without or with the sample of indicated concentrations for 1.5 h. Then, loading solution was treated and the cells were incubated at 37 ℃ for 1 h in the dark as described by the manufacturer. After incubation, fluorescence was measured using a fluorescence plate reader. The images of the cells were observed using a fluorescent microscope, which was equipped with a Moticam color digital camera (Motix, Xiamen, China).

2.9. Procedure of HPLC

The HPLC system in this experiment consisted of two mono Waters 515 HPLC pump, a Waters 2998 photodiode array detector, Waters 2707 autosampler, and Waters pump control module II (Waters, USA). A 10 µL of 5 mg/ml sample solution was directly injected on Atlantis T3 3µm 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 ~ 40 min : 5:95 v/v ~ 50:50 v/v, ~ 50 min : ~ 100: 0 v/v, ~ 60min : ~ 100:0 v/v). The flow rate was 0.2 mL/min with UV absorbance detection at 254 nm.

2.10. In silico docking of proteins and new inhibitor candidate

Molecular docking is an application that molecular modeling techniques are used to predict how a protein (enzyme) interacts with small molecules (ligands) (Perola E. 2006). The ability of a protein to interact with small molecules plays a major role in the dynamics of that protein, which may enhance or inhibit its biological function (Kang et al., 2012)

For docking studies, the crystal structures of protein were allocated from Protein Data Bank (PDB, http://www.pdb.org). The docking studies were performed using CDOCKER in Accelrys Discovery Studio 3.0 (Accelrys, Inc). To prepare for the docking procedure, we performed the following steps:

(1) conversion of the 2D structure into 3D structure; (2) calculation of charges; and (3) addition of hydrogen atoms using the CDOCKER docking program.

2.11. Statistical analysis

All data were expressed as mean ± standard deviation (SD) of three determinations. Statistical comparison was performed via a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRP). P-values of less than 0.01 ($P < 0.01$), 0.05 ($P < 0.05$) and 0.1 ($P < 0.1$) was considered as significant.

3. Results and Discussion

3.1. NO production by influx of calcium into HUVECs.

To experimentally identify whether calcium flow into the cell and then NO was produced, we measured intracellular calcium levels and NO levels after treatment of $CaCl_2$ to HUVECs. As shown in fig. 1, fluorescent intensity of intracellular calcium increase compared with non treatment, although the difference of value is small and it is not concentration-dependent manner. Calcium play an important role in the physiology and biochemistry of organisms and cells such as signal transduction pathways, neurotransmitter release from neurons, contraction of all muscle cell types and fertilization. However, calcium levels in mammals are tightly regulated. Martin et al. (2002) studied that calcium influx to endothelial cells and appeared amount of calcium as nM (Martin et al.,2002). Likewise, fluorescent intensity of intracellular calcium in fluorescent image is slightly increased when $CaCl_2$ was treated at relative high concentration (30~50 μg/ml) (Fig. 2).

Moreover, NO levels produced by treatment of $CaCl_2$ increased compared with non treatment, although it is not concentration-dependent manner as well as intracellular calcium levels (Fig. 3).

As a result, the fact that calcium inflow to endothelial cells and then NO is produced was experimentally verified.

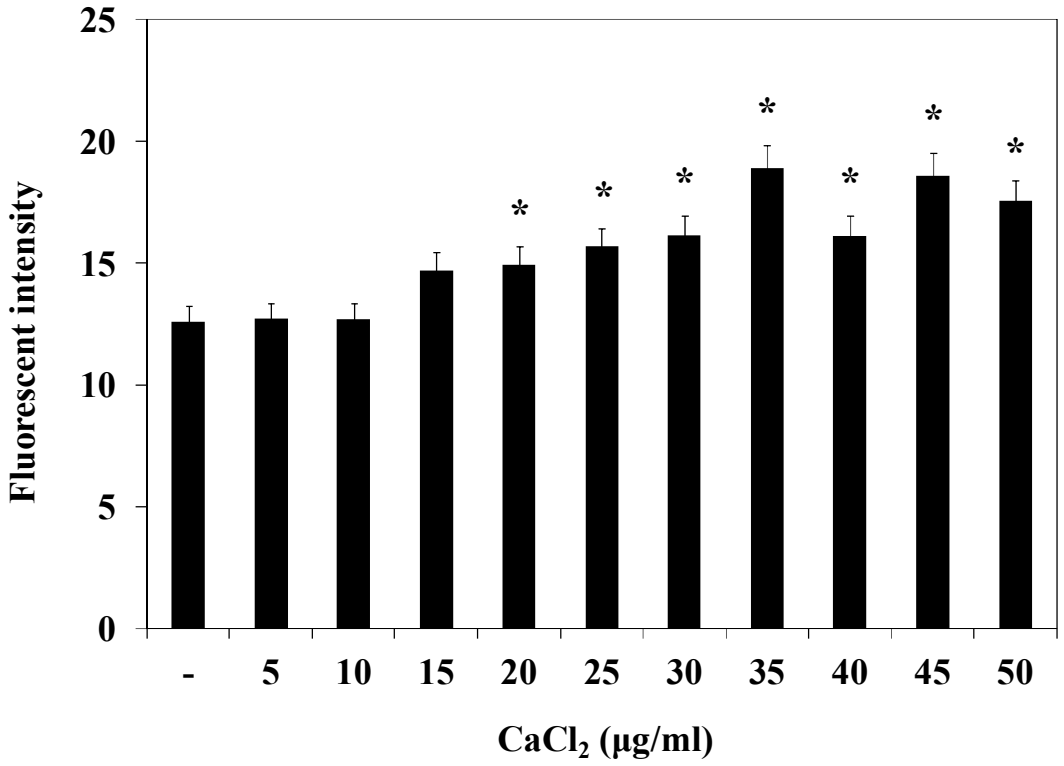


Figure 1. Fluorescent intensity of intracellular calcium levels after treatment of $CaCl_2$ in HUVECs. Values are mean ± SD of three determinations. Values are significantly different at $P < 0.01$ as analyzed by DMRT.

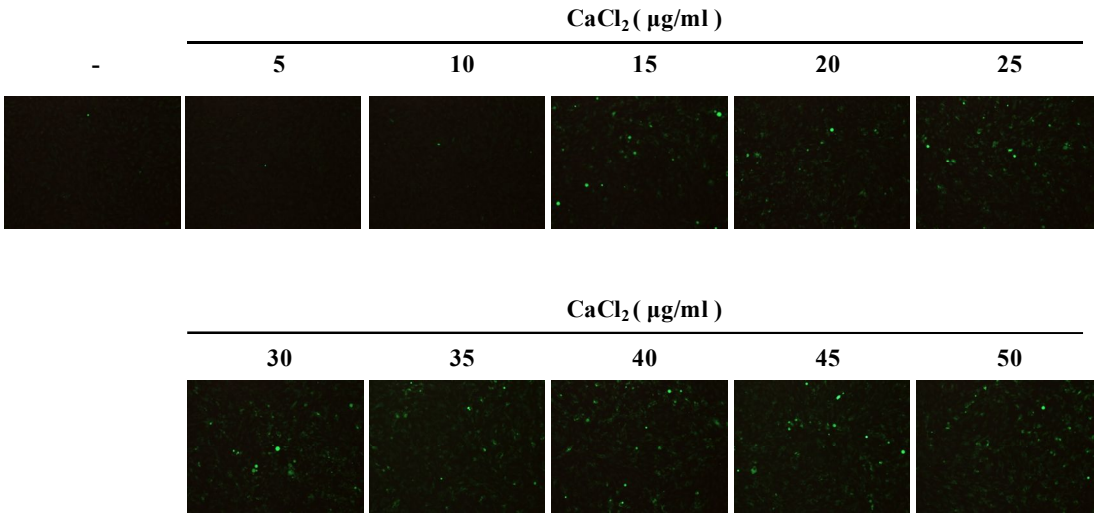


Figure 2. Fluorescent image of intracellular calcium levels after treatment of $CaCl_2$ in HUVECs.

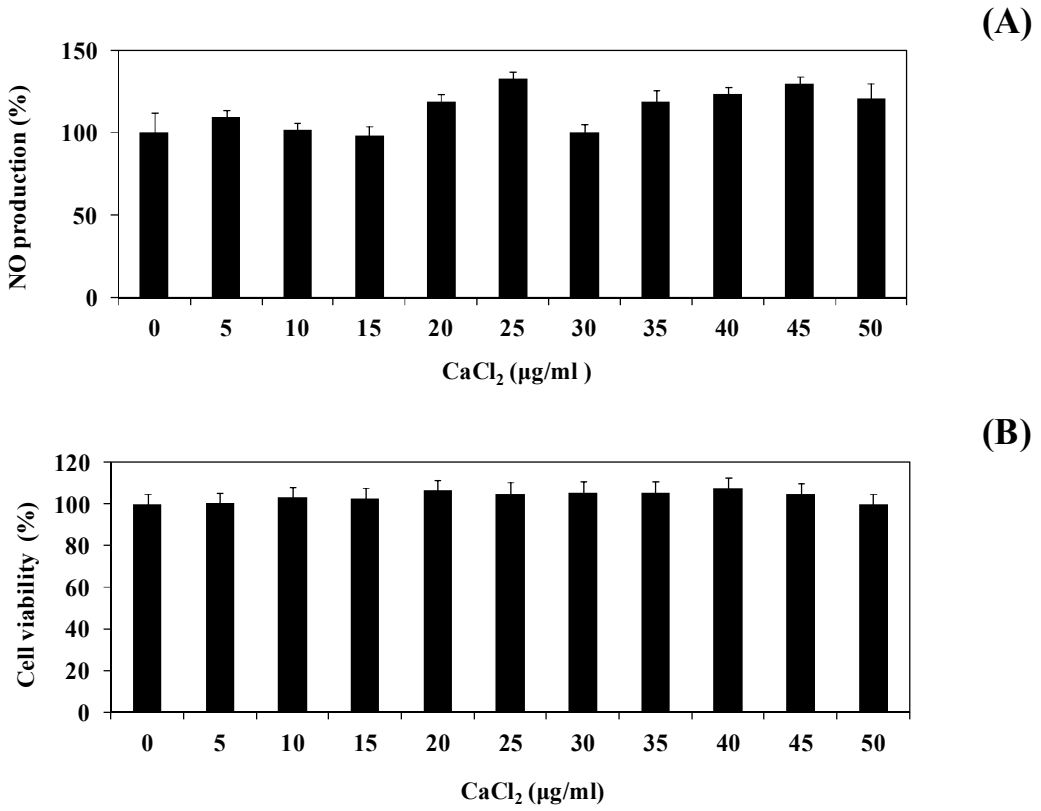


Figure 3. NO levels (A) and cell viability (B) after treatment of $CaCl_2$ in HUVECs. NO levels were measured after staining with DAF-FM DA.

3.2. Isolation of five fractions from ethyl acetate fraction of *Spirogyra* sp. (SPE)

The five fractions (SPE.I~V, respectively) were successfully isolated from SPE by CPC with a two-phase solvent system composed of n-hexane / EtOAc / methanol / water (0:10:1:9, v/v) exhibited good K values for dual mode operation (Fig. 4).

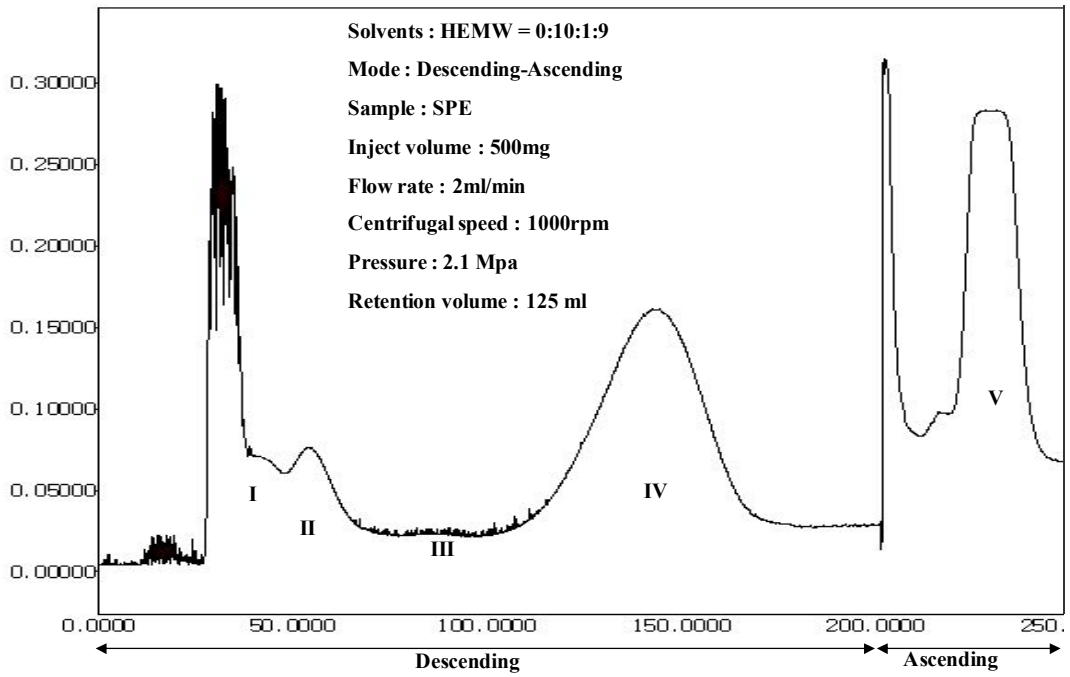


Figure 4. Isolation of ethyl acetate fraction of *Spirogyra* sp. (SPE)

3.3. NO production of SPE.IV in HUVECs.

Among the signaling molecule in relation to vasorelaxation, NO production is most important (Akhlaghi et al., 2009, Woldman et al., 2009). To identify vasorelaxation effect of SPE fractions in HUVECs, first of all, NO production was examined.

As shown in Fig. 5, most of fractions not showed the cytotoxicity at dose of 25, 50 μg/ml excluding the SPE.V (Fig. 5 (B)) meanwhile NO level in SPE.IV-treated HUVECs was significantly increased at 25, 50 μg/ml compared with other fractions (Fig. 5 (A)). Therefore, SPE.IV was taken for the further experiments.

To identify the effect of SPE.IV in HUVECs, we measured that NO production of SPE.IV-treated HUVECs with different concentrations. As shown in Fig. 6, SPE.IV significantly increased NO levels in a concentration-dependent manner at doses of 12.5, 25, 50 and 100 μg/ml.

Moreover, in order to exactly confirm whether NO production of SPE.IV related with endothelial nitric oxide synthase (eNOS), N^{G}-nitro-$_{L}$-arginine methyl ester (L-NAME), eNOS inhibitor, was pretreated to HUVECs. In consequence, NO levels increased by SPE. IV was significantly attenuated by L-NAME at a concentration of 100 μM. Therefore, NO produced by SPE.IV in HUVECs is induced through eNOS.

To investigate whether SPE.IV influences eNOS protein expression levels, HUVECs was incubated

with SPE.IV of 25 μg/ml. As shown in Fig 8, no change on the eNOS protein levels however, p-eNOS (Ser 1177) expression level was increased in SPE.IV-treated HUVECs than in non-treated HUVECs. On the other hands, iNOS expression level was little higher in SPE.IV-treated HUVEC than in non-treated HUVECs but the difference has not influence on NO production. Therefore, SPE.IV induced NO production though phosphorylation of eNOS.

eNOS can be phosphorylated by other kinases such as phosphoinositol 3-kinase (PI3K)/Akt (Mount et al., 2007). To investigate whether SPE.IV influences Akt protein expression levels during eNOS phosphorylation by SPE.IV, HUVEC was incubated with SPE.IV of 25 μg/ml and western blotting analysis was performed. As shown in Fig 8, SPE.IV induced the phosphorylation of Akt (Ser473) at 25 μg/ml.

As a result, the increase of NO production by SPE.IV in HUVECs is mediated through the phosphorylation of eNOS (Ser 1177) via phosphorylation of Akt (Ser473).

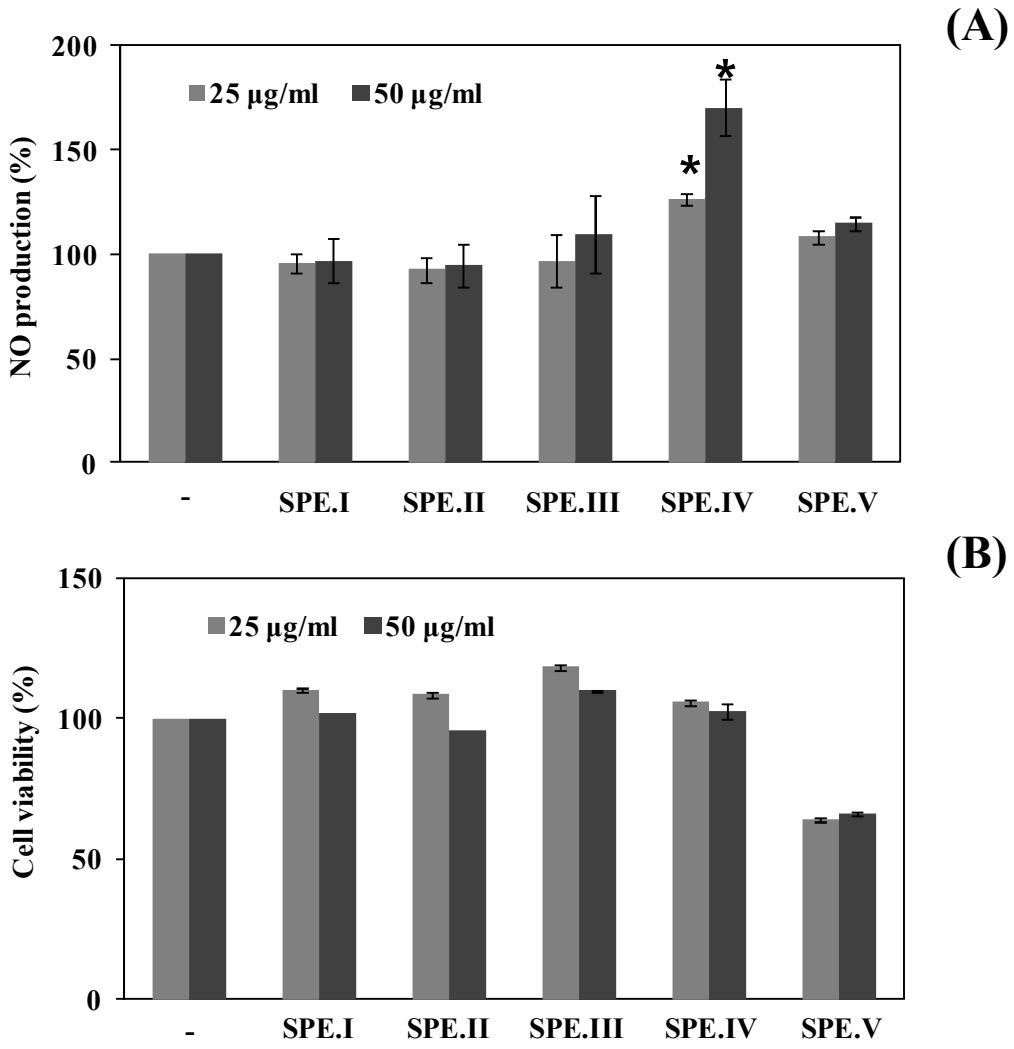


Figure 5. NO production (A) and cell viability (B) of SPE fractions in HUVECs. Values are mean ± SD of three determinations. Values are significantly different at P < 0.01 as analyzed by DMRT.

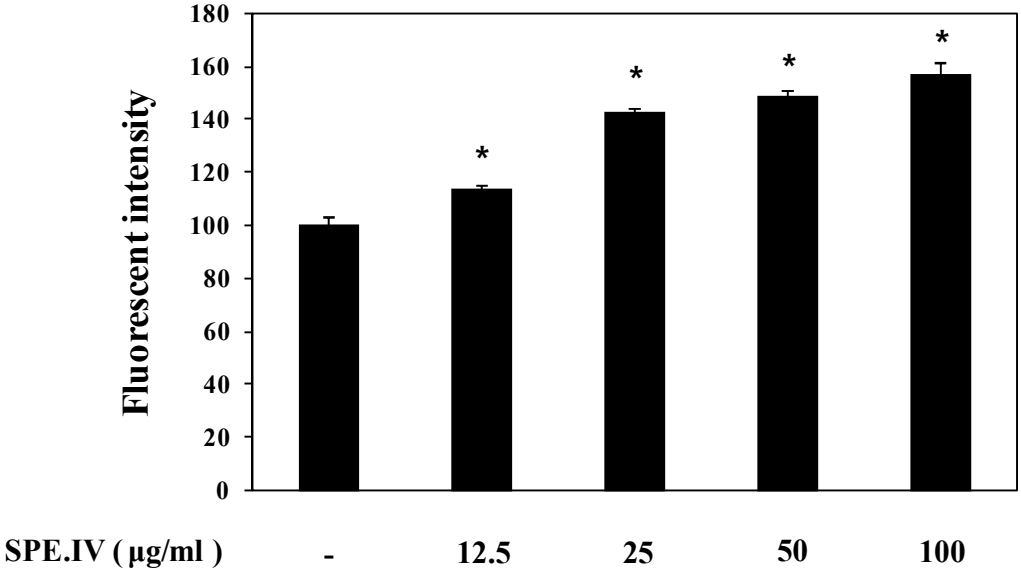


Figure 6. NO production of SPE.IV in HUVECs. NO levels were measured after staining with DAF-FM DA. Values are mean ± SD of three determinations. Values are significantly different at P < 0.01 as analyzed by DMRT.

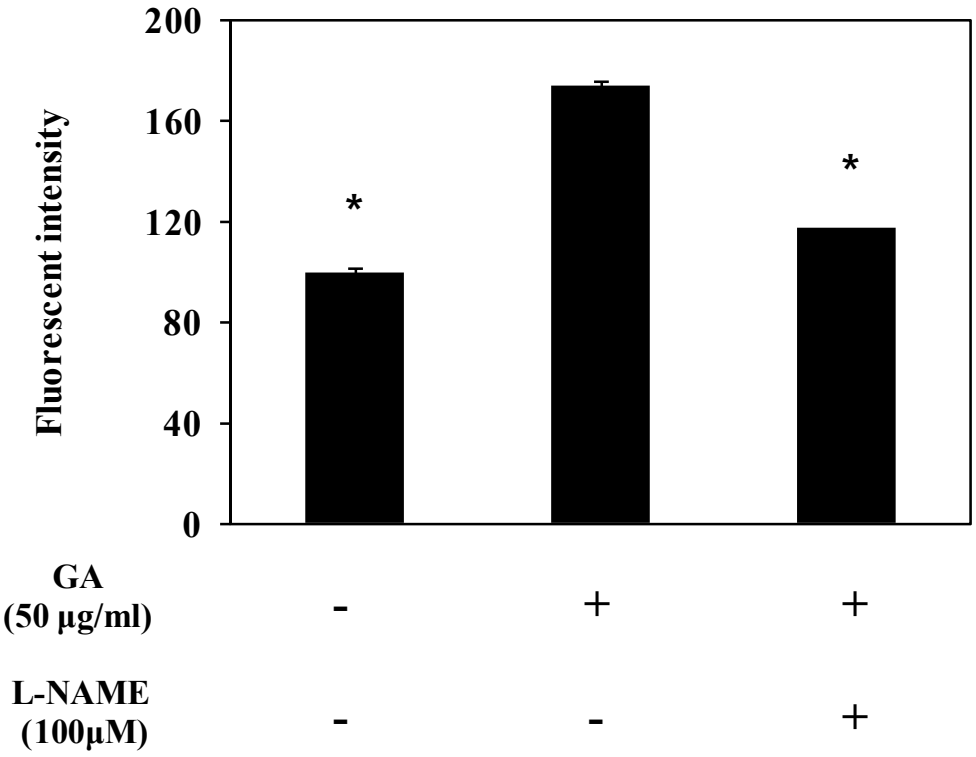


Figure 7. Inhibition of L-NAME on NO production of SPE.IV in HUVECs. NO levels were measured after staining with DAF-FM DA. Values are mean ± SD of three determinations. Values are significantly different at P < 0.01 as analyzed by DMRT.

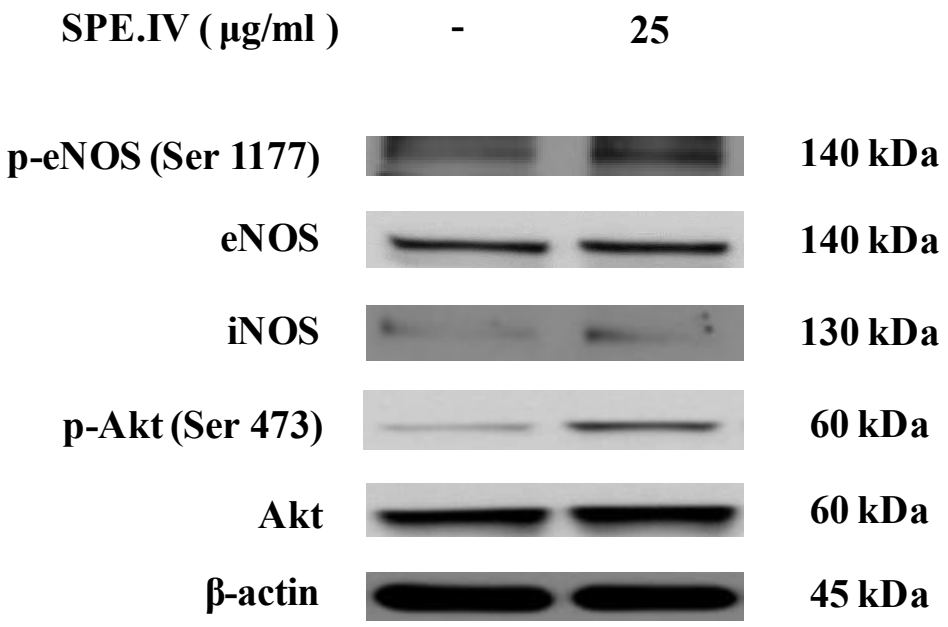


Figure 8. Effect of SPE.IV on eNOS, iNOS and Akt expression in HUVECs. Equal amounts of cell lysates (10 μg) were subjected to electrophoresis and analyzed for diverse protein related in NO production such as phospho-eNOS(Ser 1177), phospho-Akt (Ser 473). β-actin was used as an internal control.

3.4. SPE.IV induces influx of calcium into HUVECs

eNOS is a calcium-dependent enzyme (Akhlaghi et al., 2009). In order to evaluate the direct role of SPE.IV on the regulation of intracellular calcium levels, we incubated HUVECs with SPE.IV of 12.5, 25 and 50 μg/ml and measured intracellular calcium levels using the BD™ Calcium Assay Kit. The intracellular calcium levels were increased in SPE.IV-treated HUVECs, although it is not significant (Fig. 9). Therefore, NO production of SPE.IV-treated HUVECs was induced by influx of calcium.

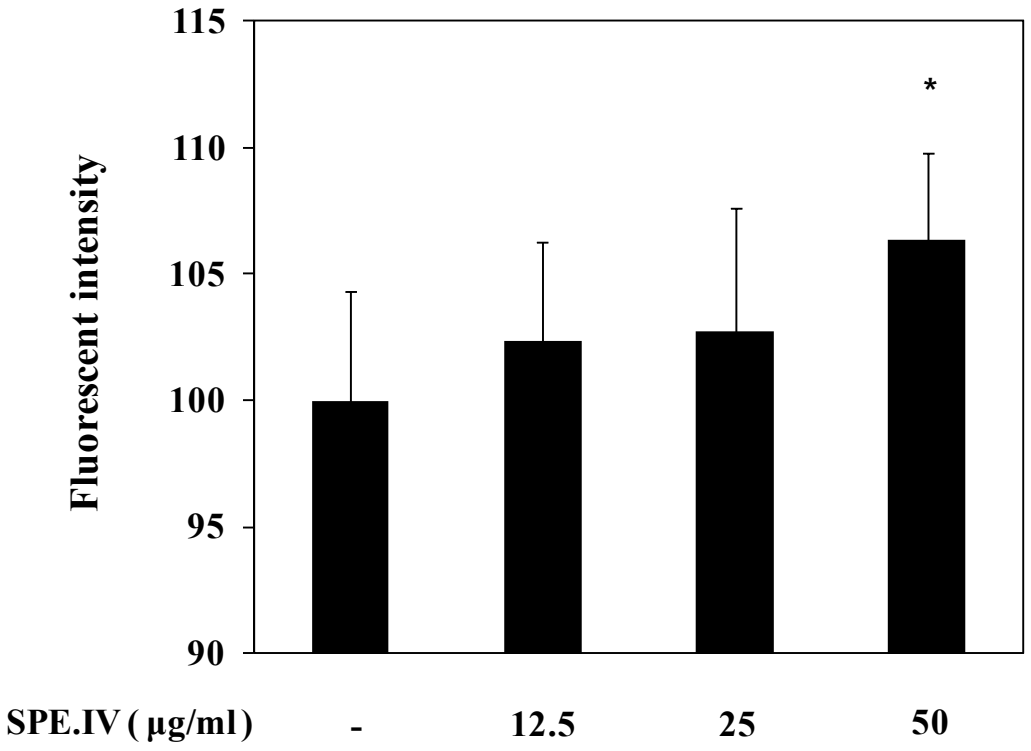
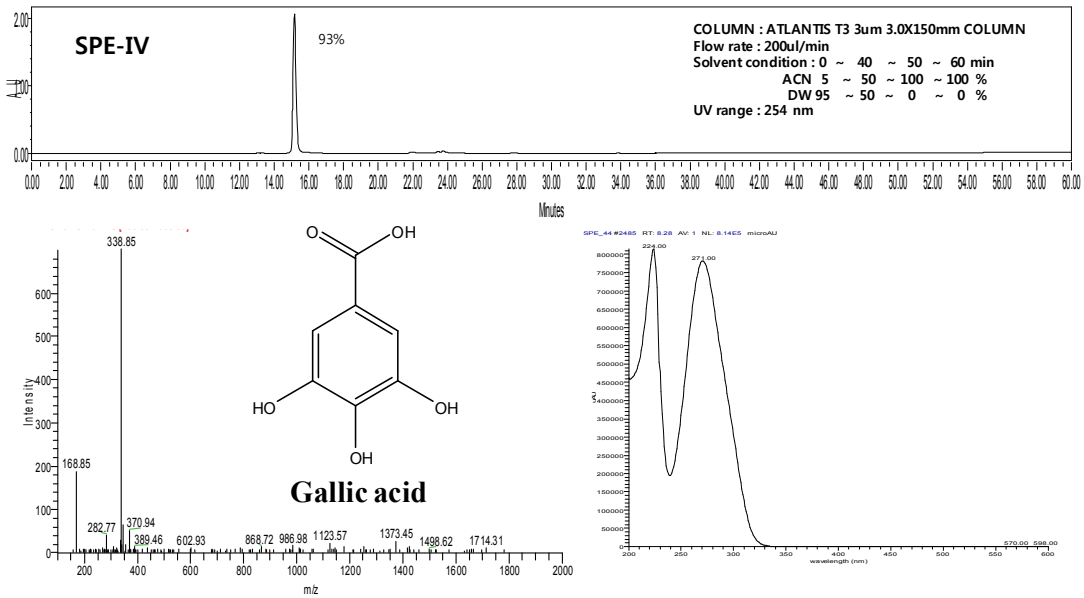


Figure 9. Fluorescent intensity of intracellular calcium levels with treatment of SPE.IV in HUVECs. Values are mean ± SD of three determinations. Values are significantly different at P < 0.1 as analyzed by DMRT.

3.5. Identification of SPE.IV

SPE.IV mass values and UV spectra by LC-DAD-ESI/MS, SPE.IV was identified as gallic acid (m/z 170) (Fig. 10). The structural identification of gallic acid was carried out by 1H NMR and 13C NMR spectra as follows: gallic acid: 1H NMR (400 MHz, methanol-d4) δ 7.05(2H, s, H-2, 6); 13C NMR (400 MHz, methanol-d4): δ 169.9(–COOH), δ 145.5(C-3, 5), δ 138.5(C-4), δ 121.7(C-1), δ 109.4(C-2, 6).



^{1}H NMR (400 MHz, methanol-d4) δ 7.05(2H, s, H-2, 6); ^{13}C NMR (400 MHz, methanol-d4): δ 169.9(–COOH), δ 145.5(C-3, 5), δ 138.5(C-4), δ 121.7(C-1), δ 109.4(C-2, 6).

Figure 10. Identification of Gallic acid (GA)

3.6. In silico docking of Phosphodiesterase 5 (PDE 5)

Phosphodiesterases (PDEs) are a class of enzymes that cleave the phosphodiester bond in cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) which relaxes smooth muscle, resulting in vasorelaxation. (Rahimi et al., 2010, Sung et al., 2003, Schwartz et al., 2012). PDEs inhibitors are the drugs targets for the medical management of diverse physiological dyfunctions including heart failure, depression, asthma, inflammation and erectile dysfunction (Sung et al., 2003).

Of the 12 PDE families, cGMP-specific PDE 5 carries out the principal cGMP-hydrolysing activity in human corpus cavernosum tissue. It is well known as the target of sildenafil citrate (Viagra) and other similar drugs for the treatment of erectile dysfunction (Sung et al., 2003).

The PDE 5 inhibition mode of the GA was predicted by molecular docking analysis. To simulate docking of the protein-ligand complex, Discovery Studio 3.0 (DS) was utilized. For docking studies, the crystal structure of PDE 5 was allocated from Protein Data Bank (PDB ID : 1UDT) and active site of PDE 5 involved the following residues : Phe820, Gly819, Gln 817, Met816, Leu804, Ala783, Ala779 and Tyr664.

The docking of the PDE 5-ligand complexes was well-performed with GA stably posed in the pocket of the PDE 5 by DS 3.0 (Fig. 11). The binding site predicted by the 2D program of DS 3.0 (Fig. 11)

was formed by the following residues : Phe820 (pi interaction bond), Gln817 (hydrogen bond) and Ser663 (hydrogen bond). Moreover, the docking analysis results indicated that the following highest docking binding energy and lowest total binding energy confirmation of the most proposed complex had to be taken into account when using the CDOCKER interaction energy program of DS 3.0: 22.91 kcal/mol and in the calculate program of DS 3.0: -90.00 kcal/mol (Table 1).

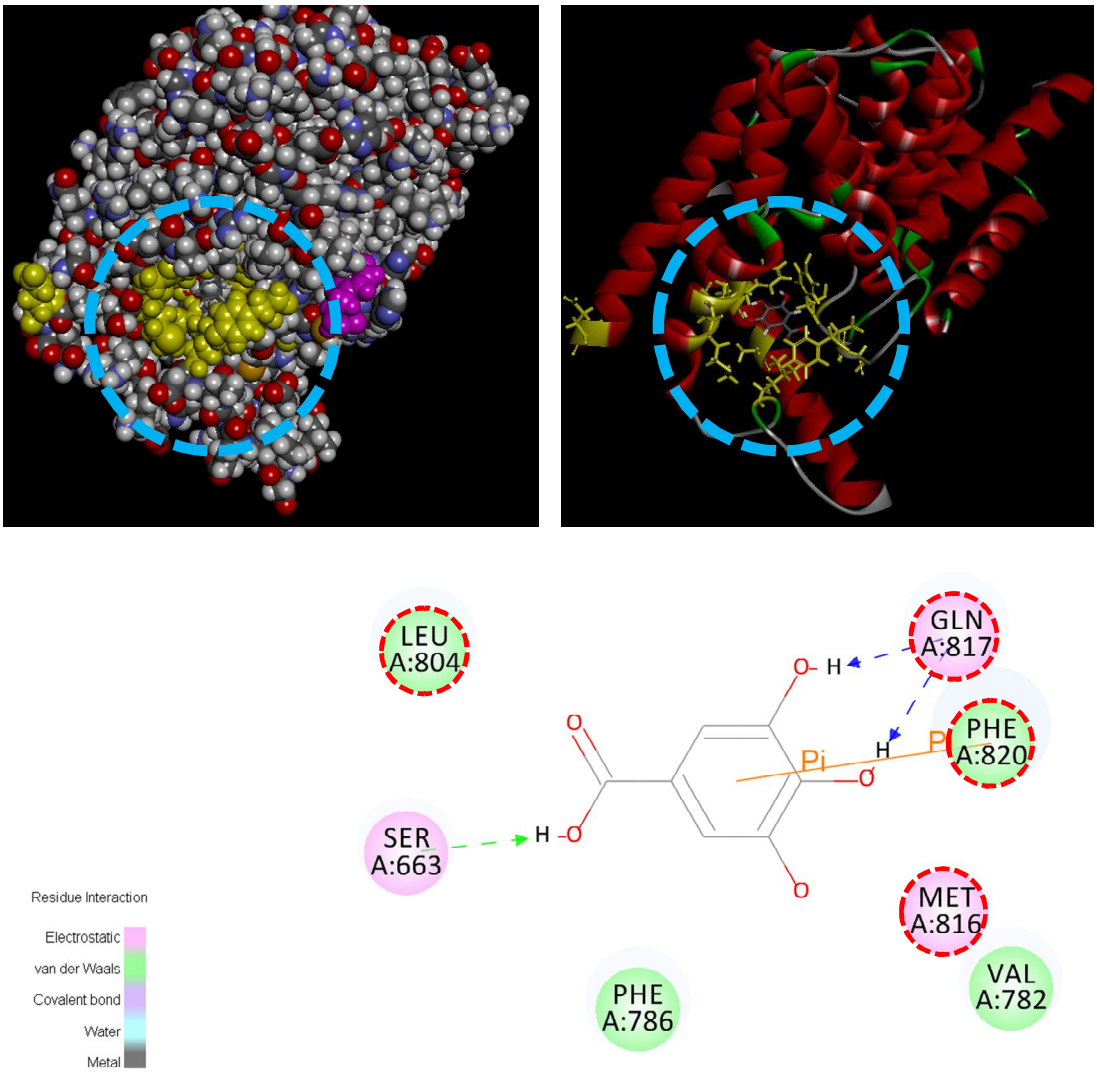


Figure 11. Computational prediction of the structure for PDE 5 and docking simulation with GA. Predicted 3D structure of PDE 5 (PDB ID : 1UDT). 2D diagram (PDE 5-GA complex).

Table 1. Results of docking experiments of GA with the PDE5 (PDB ID: 1UDT)

Ligand	Binding energy (kcal/mol)	CDOCK interaction energy (kcal/mol)
Gallic acid	-90.00	22.91

3.7. Angiotensin-I Converting Enzyme (ACE) inhibitory activity of gallic acid (GA)

Antihypertensive treatment with Angiotensin-I Converting Enzyme (ACE) inhibitors can improve endothelium-dependent vasorelaxation (Clozel, Kuhn and Hefti, 1990). The most potent improvement of endothelium function might be obtained by ACE inhibitors, and reduce blood pressure by inhibiting not only the production of angiotensin II, but also via the degradation of bradykinin, the vasorelaxation activity of which is caused by NO (Asselbergs et al., 2005).

The antihypertensive effect of GA was evaluated by measuring Angiotensin I converting enzyme (ACE) inhibitory activity using ACE kit (Dojindo). As shown in Figure 12, GA showed the relatively high activity with an IC_{50} value of 122.9 ug/mL.

The ACE inhibition mode of the GA was predicted by molecular docking analysis. To simulate docking of the protein-ligand complex, Discovery Studio 3.0 (DS) was utilized. For docking studies, the crystal structure of ACE was allocated from Protein Data Bank (PDB ID : 1O86) and active site of ACE involved the following residues : Tyr520, His513, Tyr523, Lys511, Gln281, Glu411, His353, Glu162, Ala354, His383, Glu384, His387

The docking of the ACE-ligand complexes were well-performed by blocking the active site pocket of the ACE by DS 3.0 (Fig. 13). The binding site predicted by the 2D program of DS 3.0 (Fig. 12) was formed by the following residues : Tyr523 (hydrogen bond), Glu384 (hydrogen bond). Moreover,

the docking analysis results indicated that the following highest docking binding energy and lowest total binding energy confirmation of the most proposed complex had to be taken into account when using the CDOCKER interaction energy program of DS 3.0: 45.62 kcal/mol and in the calculate program of DS 3.0: -128.33 kcal/mol (Table 2).

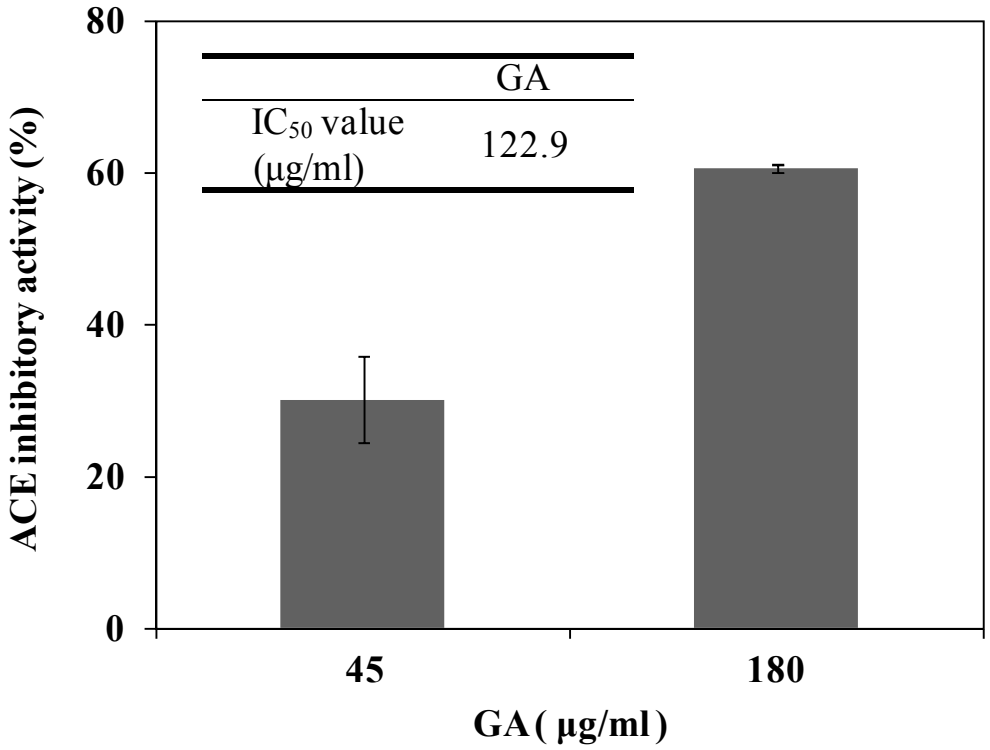
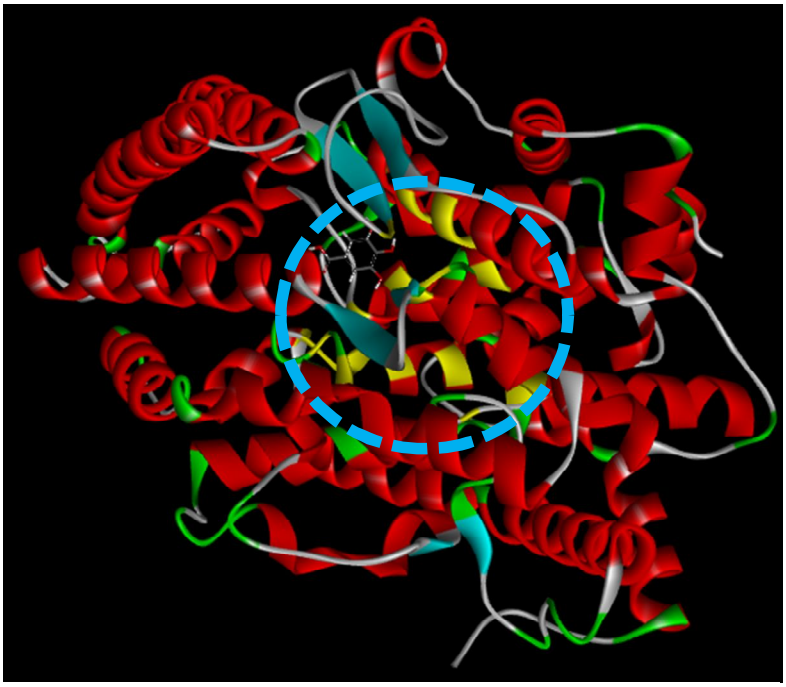


Figure 12. ACE inhibitory activity of GA isolated from *spirogyra* sp. Values are mean ± SD of three determinations. Values are significantly different at P < 0.01 as analyzed by DMRT.



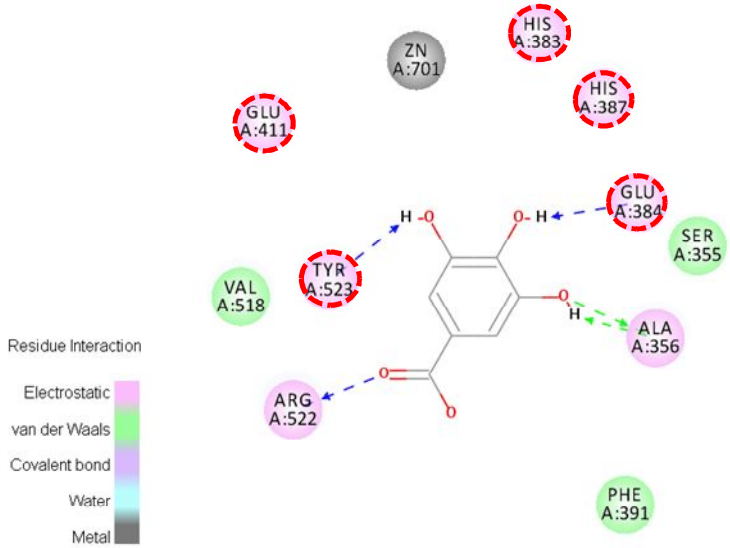


Figure 13. Computational prediction of the structure for ACE and docking simulation with GA.

Predicted 3D structure of ACE (PDB ID : 1O86). 2D diagram (ACE-GA complex).

Table 2. Results of docking experiments of GA with the ACE (PDB ID:1O86)

Ligand	Binding energy (kcal/mol)	CDOCK interaction energy (kcal/mol)
Gallic acid	-128.33	45.62

4. Conclusion

In this study, we investigated vasorelaxation effect of GA isolated from the aquatic plant, *Spirogyra* sp. Our results demonstrate that the profound vasorelaxation effect of GA on NO production in HUVECs. Vasorelaxation effect of the GA is mediated through the phosphorylation of eNOS (Ser 1177) via phosphorylation of Akt (Ser473). Also, PDE 5 inhibition effect of the GA was predicted through molecular docking program (DS 3.0). Also, the GA possesses antihypertensive effect related with vasorelaxation. Therefore, GA is considered profound enhancer on vascular diseases by exhibiting vasorelaxation and antihypertension effect, simultaneously.

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부족한 저에게 관심을 주시고, 연구의 길을 걸을 수 있도록 이끌어주신 전유진 교수님. 실험실에 처음 들어와서부터 지금까지도 변함없이 믿어주시고 기운을 주시는 교수님께 실망시켜 드리지 않으려고, 포기하지 않고 달려왔습니다. 교수님의 믿음과 사랑으로 석사과정을 무사히 마칠 수 있었습니다. 감사합니다. 교수님을 본받아 언제나 변함없이 최선을 다하도록 하겠습니다.

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