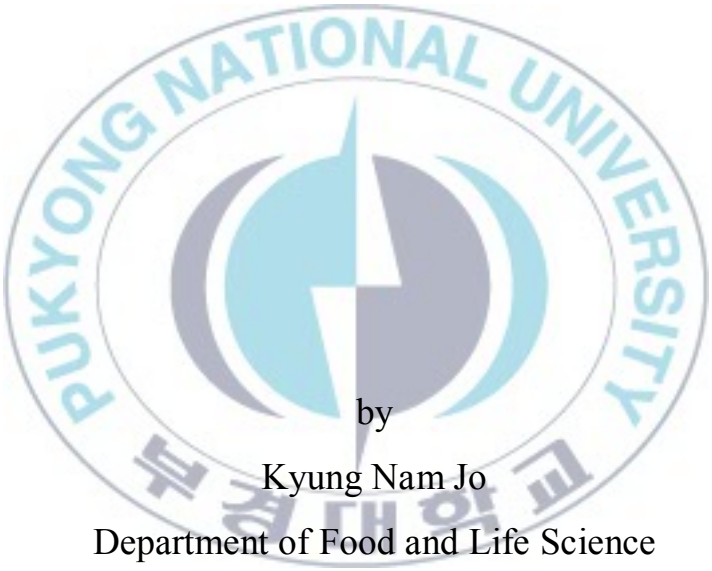
Thesis for the Degree of Science

Hepatoprotective effect of dieckol on the tacrine-induced HepG2 cells



by

Kyung Nam Jo

Department of Food and Life Science

The Graduate School

Pukyong National University

August 2009

Hepatoprotective effect of dieckol on the tacrine-induced HepG2 cells

Tacrine으로 처리된 HepG2 세포에 대한 dieckol의 간보호 효과

Advisor: Prof. Hyeung Rak Kim

By
Kyung Nam Jo

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

Master of Science

In Department of Food and Life Science Graduate School
Pukyong National University

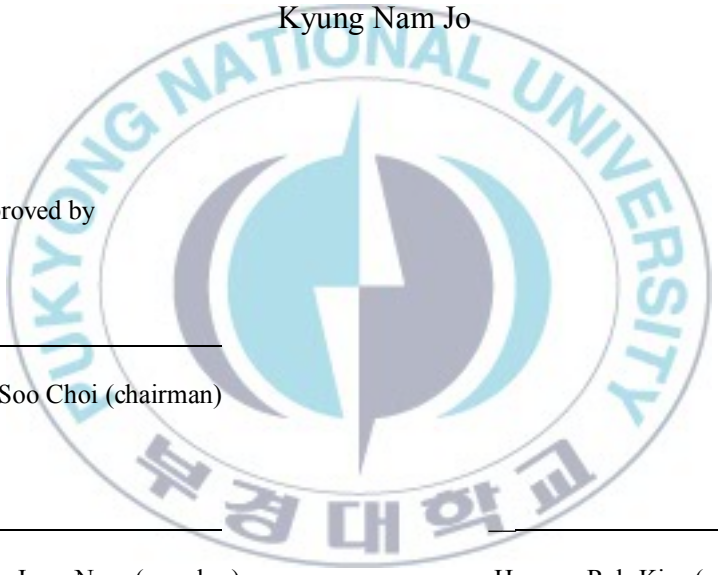
August 2009

Hepatoprotective effect of dieckol on the tacrine-induced HepG2 cells

A dissertation

by

Kyung Nam Jo



Approved by

_____

Jae-Soo Choi (chairman)

_____ _____

Taek-Jung Nam (member) Hyeung-Rak Kim (member)

August 2009

Contents

Abstract

1. Introduction ……. 1

2. Materials and Methods ……... 7

2-1. Plant materials ……..…….... 7

2-2. Materials …… 7

2-3. Extraction and isolation …...……..... 8

2-4. Spectrometry ...…… 18

2-5. DPPH radical scavenging assay ……..... 18

2-6.Measurement of NO and PGE_2 …….. 19

2-7. Meshroom tyrosinase assay ……... 20

2-8. Cell culture and treatment ……. 20

2-9. Cytotoxicity and Hepatoprotection assay ……..…… 21

2-10. Measurement of intracellular ROS ……..…… 22

2-11. Western bolt assay ……..…… 22

2-12. Statistical analysis ……. 23

3. Results and Discussions ……..… 24

3-1.Structural elucidation of dieckol …… 24

3-2. Biological activity of dieckol ……..…… 26

3-3. Cytotoxicity of tacrine on HepG2 cells ……..…… 28

3-4. Protective effect of dieckol
on tacrine-induced HepG2 cells ……...……. 30

3-5. Determination of intracellular ROS
on HepG2 cells 32

3-6. Dieckol protects from tacrine-induced hepatic cell death
by inhibiting Fas-mediated apoptosis ……..…… 34

3.7. The role of JNK in dieckol treated HepG2 cells ..… 36

4. Discussion ……..…… 38

5. References …….. 43

Tacrine으로 처리된 HepG2 세포에 대한 dieckol의 간보호 효과

조경남

부경대학교 대학원 식품생명과학과

요약

해조류는 최근 서구화된 식습관에 의해 증가하고 있는 비만, 당뇨, 고혈압 등의 혈행 장해성 질환과 같은 생활 습관병에 대해 예방 효과를 가지는 높은 함량의 비타민, 무기질은 물론 섬유질이 풍부한 식품으로 알려져 전통적인 웰빙 식품으로 추천 되어 왔다. 또한, 해조류에는 2차 대사 산물로서 phenyl과 phenoxyl기를 가지는 polyphenol류인 phlorotannin 성분이 다량 함유되어 있으며, 그 생리 활성 기능에 대한 관심이 더해지고 있다. 특히 *Ecklonia* 종의 대표적인 해조류 중 하나인 곰피는 (*Ecklonia stolonifera* OKAMURA) 다년생 다시마과 (Laminaricese)에 속하는 갈조류로 아시아에서 미역, 다시마, 톳 등과 같이 식용으로 많이 이용되어 왔다. 최근의 연구를 통하면 곰피의 2차대산물인 phlorotannin류들이 항 산화 활성, 항 돌연 변이 활성 등을 비롯한 여러 가지 생리 활성 기능이 있는 것으로 알려지고 있다. 본 연구에서는 곰피의 phlorotannin류 중 항 산화능이 높은 것으로 확인된 dieckol을 분리한 후, Alzheimer병의 치료제 중 하나인 tacrine을 처리하여 간암 세포의 손상을 유도한 후, dieckol의 간보호 효과를 조사하였다. Tacrine은 acetylcholinesterase의 기능을 억제시켜 Alzheimer병에 대해 치료 효과를 보였으나, 간 독성을 비롯한 여러 가지 부작용으로 인해 현재는 사용이 중단 되어져 있다. 간은 물질 대사에 관여하여 외부로부터 유입된 물질 뿐 아니라 대사 생성물에 의한 독성으로부터 보호와 해독 기능에 깊이 관여함으로, 여러 질병에 대해 치료 목적으로 사용되는 화학 제제들에 의한 손상들도 적지 않게 보고되고 있다. 이에 항 산화능이 높은 것으로 알려진 dieckol을 천연물인 곰피로부터 분리한 후 tacrine과 함께 처리하여 간세포 독성에 대한 보호 효과를 확인하고자 하였다. 곰피의 MeOH 추출물 중, EtOAc 분획물이 높은 DPPH radical 소거능을 가진 것을 확인하고, silica gel chromatography와 HPLC를 반복적으로 수행하여 항 산화 활성을 가진 dieckol을 분리하였다. 본 실험에서 Tacrine 처리(EC_{50}: 0.3 mM)는 HepG2 세포의 손상을 유발시켰고, dieckol의 첨가는 손상된 세포의 보호

효과를 가지는 것이 확인 되었다 (EC_{50}: 43.6 μM). Dieckol은 또한 tacrine에 의하여 유발되는 세포 내 ROS의 생성을 강하게 억제시키는 것으로 나타났으며, Western blot으로 확인한 결과, tacrine은 Fas와 관련된 apoptosis 신호 전달 체계를 활성화시켰다. 반면 dieckol은 Fas 단백질들의 발현을 억제시킴으로써 연차적으로 capsase 8, bid, caspase 3, PARP 등의 단백질의 활성을 저해하여 세포 사멸 기전을 통한 세포 사멸을 막는 것으로 나타났다. 최종적으로, Dieckol은 ROS의 생성을 억제하는 항산화기전과 함께 이와 연관된 Fas 관련 단백질들에 의한 apoptosis를 억제하여 tacrine으로 유도되는 세포 사멸로부터 간세포를 보호하는 것으로 확인되었다.

1. Introduction

Marine brown alga has been widely used for food ingredients as well as industrial purposes in many Asian and some European countries. In the past, brown alga was thought to have only a variety of minerals and vitamins. After that, polysaccharides of brown algae, including alingate, laminarin, fucodian etc, have been noted to be its major bioactive components and considered to inhibit the absorption of over-ingested lipids, absolutely excessive diet components, cholesterols, and chemical additives, etc. (Ara *et al.,* 2002; Amino *et al.,* 2005; He *et al., 2009*) This is beneficial, because the excessive supply of those lipid components can cause metabolic syndrome, including obesity and diabetes. (Elena *et al*., 2008) Metabolic syndrome means a combination of the risk factors of heart attack and diabetes with the conditions of high blood pressure, high serum level of triglycerides and low serum level of high-density lipoprotein cholesterol (HDL) etc. And those conditions can worsen over time within a diet of high lipids and low dietary fiber.

According to the latest studies, the other components of brown alga, polyphenols are suggested to have potential benefits of antioxidation activity (Kang *et al.,* 2003; Nakai *et al.,* 2006), anti-plasmin inhibiting activity (Fukuyama *et al.,* 1989), anti-mutagenic activity (Han *et al.,*

2000), HIV-1 reverse transcriptase and protease inhibiting activity (Ahn *et al.,* 2004), and hepatoprotective activity as well. (Kim *et al.,* 2005).

Phlorotannins, the marine algal polyphenols, are identified to have oligomeric polyphenol of phloroglucinol unit, which is responsible for those biological activities of *Ecklonia sp.* (Kang *et al.,* 2003) Among Phlorotannins from *Ecklonia* species, eckol, 6,6'-bieckol, dieckol, and phlorofucofuroeckol are clarified to have comparatively broad biological activities.(Joe *et al*., 2006) Before this experiment, dieckol was successfully isolated from *Ecklonia* species for the first time, the structure was confirmed to be a hexamer of phloroglucinol. (Yoon *et al*, 2008) It has been reported to have protective function against linoleic acid peroxidation, total intracellular ROS generation (Kang *et al.,* 2003a; Jung *et al.,* 2006), HIV-1 reverse transcripatase and hyperlipemia (Kang *et al.*, 2004a; Ahn *et al*., 2004; Yoon *et al*., 2008). It is also known to have potentially inhibitory effect of acetylcholinestrase (Myung *et al*., 2005). In addition, dieckol has proven much stronger than a well-known antioxidant, L-ascorbic acid, in scavenging radicals and in inhibiting tyrosinase activity (Kang *et al*., 2004b; Kim *et al*., 2004). However, it has not been identified to have protective effect against cytotoxicity in liver cells.

Liver is called the best elaborate chemical factory in a living body.

Its main function is to control metabolic digestion processes, such as glycogen storage, decomposition of erythrocytes, plasmin protein synthesis, and to exclude and detoxify the exoteric toxic materials, playing a primary role in protective and immune system (Taub, 2003).. As the primary role of liver is defense and protection against damage from xenobiotics and viral infections, the liver disorders seems to be more vulnerable to chemicals contained in drugs and food components. [Kaplowitz, 2004]

Some drugs that target many kinds of diseases have caused liver problems in dose dependent manner, and they have often led to acute side effects. (Melzer, 1998) For example, with approximately one-half of Alzheimer's disease cases, serious liver failure and mimics of all forms of chronic liver disease have been reported. It has been suggested that about 1000 drugs may possibly lead tohepatic disorders. (Kaplowitz, 2004)

One of the main factors of Alzheimer's disease is the excess accumulation of choline by activity of acetylcholinesterase (AChE), an enzyme that degrades the neurotransmitter acetylcholine and produces choline and an acetate groups. AChE is mainly found at neuromuscular junctions and cholinergic synapses in the central nervous system(CNS) and serves to terminate synaptic transmission (Mesulam *et al.,* 2002;

Darvesh *et al*., 2003). AChE is the target of many neuropsychiatric problems, including Alzheimer's disease. AChE inhibitors block the activity of acetylcholinesterase, and thus suppress degradation of acetylcholine in the synaptic cleft. (Greig *et al.*, 2001)

Tacrine or 9-amino-1,2,3,4-tetrahydroaminoacridine (THA, also known as Cognex) is a cholinesterase inhibitor, developed for the therapeutic purpose of Alzheimer's disease. However, it had been known to cause a variety of negative symptoms, including nausea, salivation, sweating, bradycardia, hypotention, callapse, and convulsions etc. Particularly, it was reported to induce hepatotoxicity as well, so that now its use is forbidden. (Forsyth *et al.*, 1989; Watkins *et al.,* 1994). Recent reports suggest that hepatic cells are damaged by the reactions between tacrine and cytochrome P-450(CYP), a promoter of xenobiotic metabolism in liver. The electrophilic metabolites produced by the reactions are combined with intracellular glutathione ions and then inactivated. Through these processes, intracellular superoxide anions and other free radicals are produced, and glutathione ion concentration is reduced to eliminate them. The lipid peroxidation is increased and finally mitochondrial dysfunction follows. (Watkins *et al*., 1994; Osseni *et al*., 1999, Meng *et al*., 2007) These toxic metabolites induce reactive oxygen species (ROS), which cause damage DNA and carry on apoptosis

or necrosis to result in cell death. (Um et al., 1996)

Reactive oxygen species (ROS) include free radicals, hydrogen peroxide, and singlet oxygen. Free radicals are atoms, molecules, or ions with unpaired electrons, which are highly unstable and therefore likely to take part in chemical reactions by obtaining an atom from other molecules to be stable, playing an important role in a number of biological processes. The two most important oxygen-centered free radicals are superoxide and hydroxyl radical. Even though some radical reactions are necessary for life, for instance, the intracellular killing bacteria by neutrophil granulocytes, they also have been implicated in unwanted side reactions of certain cell signaling processes to damage cells through apoptosis or necrosis induced by a variety of stimuli. (Zhuang *et al*., 1999).

The increase of intracellular ROS generation activates Fas (also known as CD95) protein in mammalian cells. The increase of expression of TNF(tumor necrosis factor) or growth factors deprivation is accompanied with it. (Groossens *et al.,* 1995; Um *et al*., 1996) Fas receptor clusters at the plasmin membrane induce the activation of caspase-8, and then the released caspase-8 subsequently cleaves downstream signal proteins and the process culminates in apoptosis. By the reason of this, the liver seems to be very sensitive to Fas-mediated

apoptosis and Fas antigen is constitutively shown on hepatocytes. (Haga *et al*., 2003)

In the process of cell proliferation, differentiation, inflammation, cell cycle arrest, and apoptosis, the mitogen-activated protein kinases (MAPKS), c-Jun NH2-terminal kinase (JNK) is activated by various intra- or extra-cellular stresses, and is also known as the stress- activated protein kinase (SAPK). (Dai *et al*., 2000) The Fas also activates caspase 8, bid, caspase 3, and Poly ADP-Ribose Polymerase (PARP), proteins which are related in apoptosis.

Therefore, this study was designed to investigate the protective effect of dieckol isolated from *E. stolonifera* OKAMURA on the tacrine-induced HepG2 cells.

2. Materials and Methods

2.1. Plant material

E. stolonifera was collected along the coast of Busan, Korea, in August 2007. These samples were rinsed using tap water to remove salt on them. Samples were air-dried under shade for 2 weeks and ground with a hammer grinder, and the dried powder was stored at room temperature until used.

2.2. Materials

MEM (Minimum essential medium), penicillin-streptomycin mixture, 0.25% tripsin-EDTA, fetal bovine serum (FBS) were purchased from HyClone Laboratory Inc.,(Logan, UT) sodium pyruvate and non-essential amino acid were purchased from Gibco BRL (Aukland, NZ). CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS) was purchased from Promega (Madison, WI). Tacrine, DMSO (dimethyl sulfoxide), DCFH-DA (dichlorofluorescin diacetate), and HBSS (Hanks' balanced salt buffer) were purchased from Sigma Chemical (St. Louis, MO). WESTSAVE Up (Western bloting substrate) was purchased from AbFrontier (Seoul, Korea). Polyclonal antibodies against PARP, caspase-

3, caspase -8, Bid, Fas, actin, and secondary antibodies were purchased from Santa Cruz Biotechnology (Geumcheon, Seoul, Korea). Cleaved caspase-3 was purchased from Cell Signaling Technology Inc (Danvers, MA).

2.3. Extraction and isolation of dieckol

Extraction and fractionation procedure of methanolic extracts of *E. stolonifera* are shown in Fig 1. The dried powder (4 kg) of *E. stolonifera* was refluxed with MeOH (3 × 9 L) for 3 hr. The filterated liquid extracted from *E. stolonifera* was concentrated by rotary vacuum evaporator. The extract (1.1 kg) was suspended in water (1 L) and partitioned with dichloromethane, ethyl acetate (EtOAc), *n*-butanol, and solvents in sequence, yielding the dichloromethane (114.8 g), EtOAc (314.7 g), *n*-butanol (141.5 g), and water (528.2 g) fractions. (Fig 1)

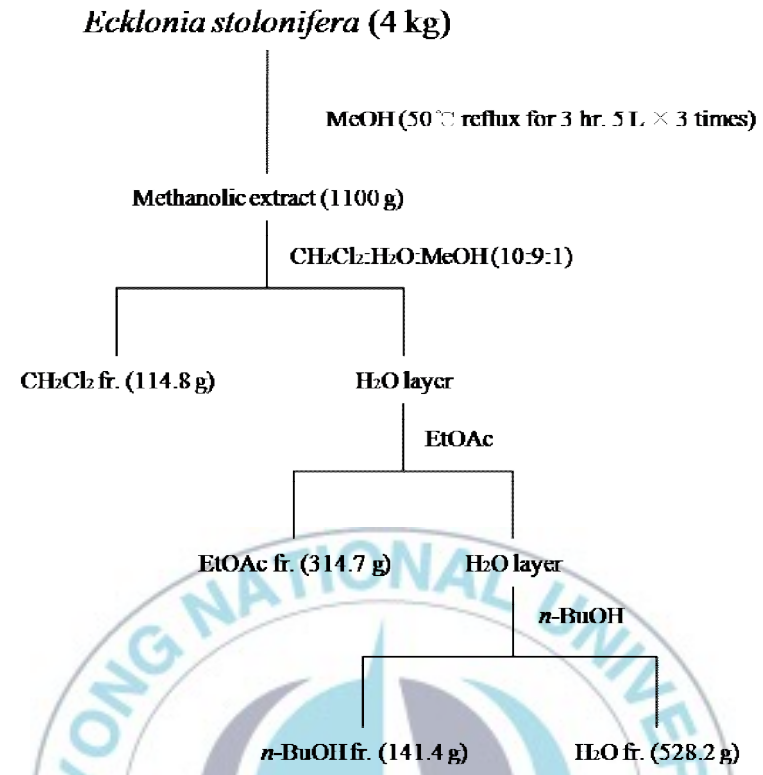


Fig 1. Extraction and fractionation procedure of *E. stolonifera*

The silica gel chromatography and HPLC were conducted to isolate the EtOAc fraction, which exhibited the most potent antioxidant activity on DPPH radical scavenging activity. (Fig 2.) The EtOAc fraction was dissolved in dichloromethane and applied to a silica gel (70-230 mesh, Merck) column (100 cm × 10 cm) and eluted with a stepwise mixture of dichloromethane and methanol (6:1, 5:2, 4:3, 3:4, 2:5, 1:6, v/v, each 1.2 L). The eluates were pooled into 17 fractions based on silica gel thin-layer chromatography (TLC) (250 μm, silica gel GF Uniplate, Analtech, Inc., Newark, DE).

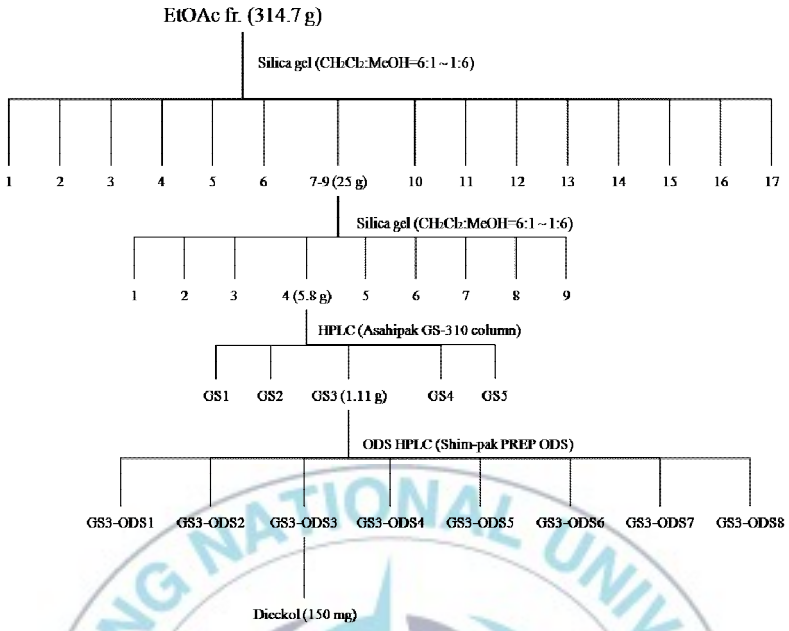


Fig 2. Isolation of dieckol from EtOAc fraction of *E. stolonifera*

The TLC plates were developed in a dichloromethane/methanol/water (65:35:10, v/v/v) solvent system. Fractions 7-9, showed high antioxidant activity on DPPH radical scavenging activities, were pooled, and then dried (25 g). The dried samples were dissolved in dichloromethane and applied on the second silica gel column (100 cm × 5 cm) to enhance the antioxidant activity. The column was eluted with a mixture of dichloromethane and methanol (6:1, 5:2, 4:3, 3:4, 2:5, 1:6, v/v, each 0.5 L) and separated into nine subfractions (Fr.1-Fr.9). Fraction 4 (5.80 g), which showed the highest antioxidant activity, was subjected to preparative size exclusion column of Asahipak GS-310 (500 mm _ 20 mm, Showa Denko, Tokyo, Japan). An exclusion HPLC apparatus consisted of a pump (Shimadzu LC-6AD), a photodiode array detector (Shimadzu SPDM20A), an online degasser (Shimadzu DUG-20A3), an auto sampler (SIL-20A), a fraction collector (Shimadzu FRC-10A), a system controller (CBM-20A), and a Shimadzu LC solution (ver. 1.22sp).

Fraction 4 was chromatographed on an Asahipak GS-310 column eluting with methanol at a flow rate of 5.0 mL/min and monitored at 245 nm. The fraction was separated into five fractions (GS1-GS5, Fig. 3).

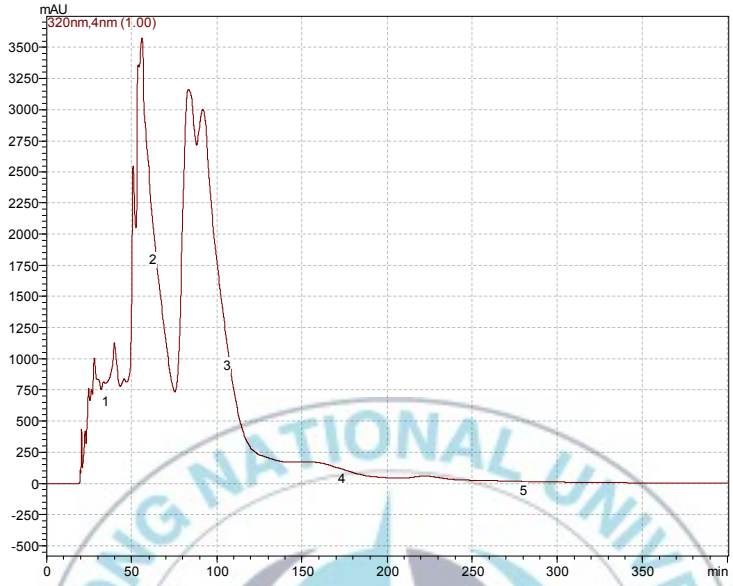


Fig 3. Fraction chromatography of GS column

The GS3 fraction (1.110 g) showing high antioxidant activity was chromatographed over Shim-pack PREP-ODS (5 μm, 100 Å, 250 mm × 20 mm, Shimadzu Co., Tokyo, Japan). A preparative ODS HPLC system was similar to the exclusion HPLC system except for a binary pump (Shimadzu LC-6AD) and a column oven (35℃, Shimadzu CTO-20A). The separation of GS3 fraction was conducted using a mobile phase of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The elution profile consisted of a linear gradient from 20 to 100% B solvent for 40 min and re-equilibration of the column with 20% B solvent for 10 min. The flow rate was 7.0 mL/min, and detection was performed at 245 nm. The fraction gave eight subfractions (GS3-ODS1 - GS3-ODS8, Fig. 4).

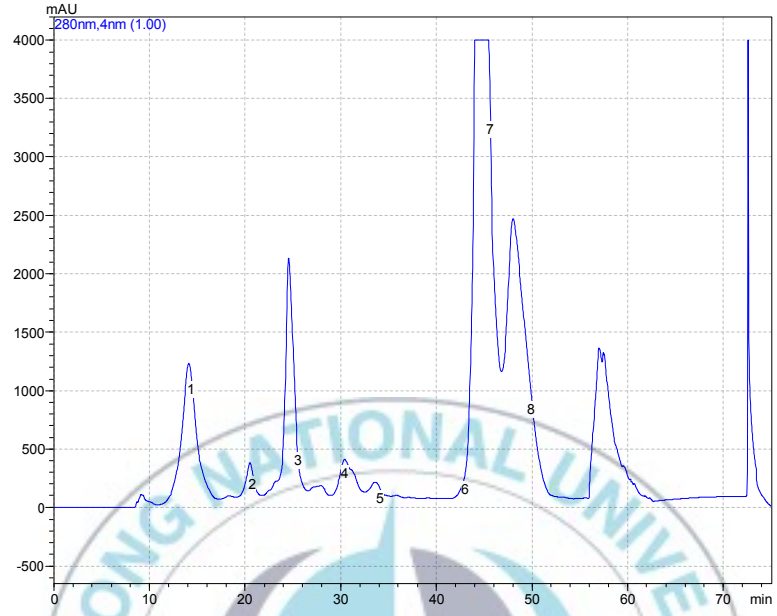


Fig 4. Fraction chromatogram of GS 3 fraction

GS3-ODS-3 (150 mg) was purified by the same HPLC system with a Luna RP-18 column (Luna C18(2), 5 μm, 250 mm × 10 mm, Phenomenex) and with the same mobile phase systems at a flow rate of 3.0 mL/min. The isolated fraction, GS-ODS3 (dieckol, Fig. 4), was used in this study. Isolation of dieckol from *E. stolonifera* was showing in Fig. 5.

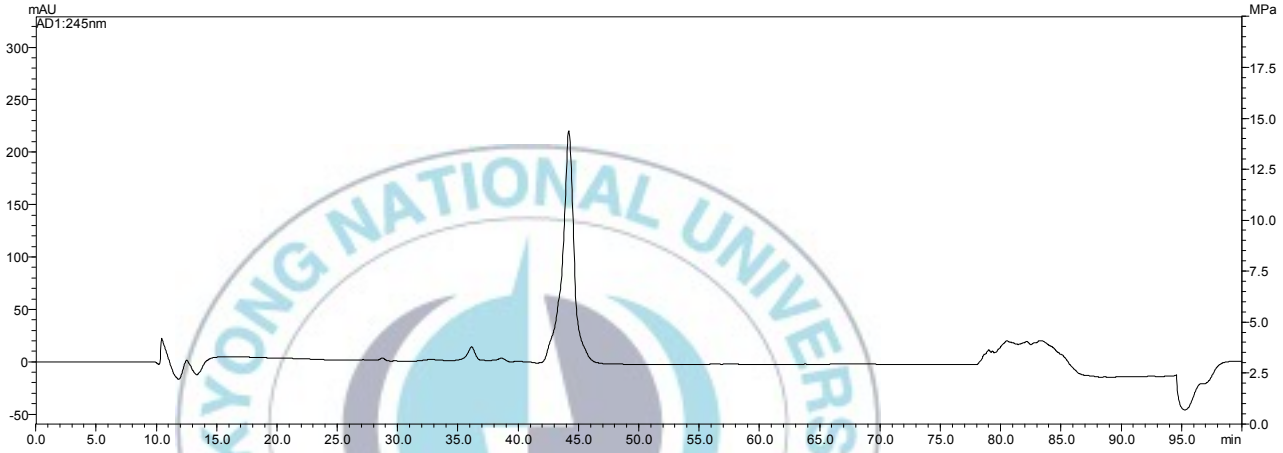


Fig 5. Chromatogram of GS3-ODS3 fraction.

2.4. Spectrometry

^{1}H and ^{1}3C NMR spectra were determined on a JNM ECP-400 spectrometer (JEOL, Japan), using EMSO-d_6 with tetramethylsilane (TMS) as an internal standard. Heteronuclear multiple quantum correlation (HMQC) and heteronuclear multiple bond correlation (HMBC) spectra were recorded using pulsed field gradients

2.5. DPPH radical scavenging assay

DPPH radical scavenging activities of fractions from *E. stolonifera* and isolated compounds were tested according to the modified method of Nanjo *et al.* (1996). Twenty microliters of sample solution (or DMSO as control) was mixed with 40 μL of DPPH (100 μL) in a 96 well microtiter plate and incubated at room temperature for 30 min. The absorbance at 520 nm was measured with a microplate reader (Ultraspec 2100 Pro, Amersham Biosciences, Piscataway, NJ). EC_{50} values were determined as the mean effective concentration to exert half of the antioxidant and were calculated by three different concentrations of sample.

2.6. Measurement of NO and PGE_2

RAW 264.7 cells (5 X 10^5) were plated and incubated with 0-50 μM of dieckol in the absence or presence of LPS (1 μg/ml) for 24 hr. After tratement of LPS and dieckol, RAW 264.7 cell culture medium was saved for the measurement of nitrite and PGE_2. The nitrite concentration in the cultured medium was measured as an indicator of NO production, according to the Griess reaction (Nanjo et al., 1996). One hundred microliters of culture supernatant was mixed with the same volume of Gress reagent (0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% H_3PO_4). The absorbance of the mixture was measured with a microplate reader (Ultraspec 2100 pro) at 540 nm. The concentration of nitrite was calculated with sodium nitrite as a standard.

PGE_2 was measured using the PGE_2 enzyme-linked immunosorbent assay kit (Amersham Biosciences). The concentration of PGE_2 was photometrically determined using a micoplate reader at 405 nm.

2.7. Meshroom tyrosiase activity assay

Tyrosinase activity using L-tyrosine as a substrate was spectrophotometrically determined by the method (Tsuboi et al., 1998) described previously with a little modification. 10 μl of dieckol solution with different concentrations and 20 μl of mushroom tyrosinase (1000 U/ml) in a 50 mM phosphate buffer (pH 6.5) were added to 170 μl of an assay mixture containing with the ratio 10:10:9 of 1 mM L-tyrosine solution, 50 mM potassium phosphate buffer (pH 6.5) and distilled water in a 96-well microplate. The dieckol dissolved in DMSO was diluted to 30 times with distilled water before experiment. After incubation of the reaction mixture at 25°C for 30 min, the absorbance of the mixture was determined at 405 nm in a microplate reader.

2.8. Cell culture and treatment

HepG2 cells (ATCC, MD, US) were maintained in MEM containing 2.0 mM L-glutamine, Earle's BSS, 0,1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 10% heat-inactivated FBS at 37°C in 5% CO_2. Each medium was changed at every 48 hrs. For experiments, cells starved of FBS for 24 hr were cultured in the absence (DMSO alone) or

presence of tacrine (dissolved in DMSO) without or with dieckol (dissolved in DMSO) at indicated concentrations and of time. The final concentration of DMSO did not exceed 0.5%.

2.9. Cytotoxicity and Hepatoprotection assay

Cell viability was determined by MTS assay using CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay Kit (Promega, WI, USA) according to the manufacturer′s manual. Preconfluent HepG2 cells were cultured in 96-well plates at a density of 2.5 x 10^4 cell/well in MEM with 10% FBS. All cells were incubated in serum-free MEM for 24 hr at 37°C under 5% CO_2 before sample treatment. Tacrine (0-0.5 mM) was added on the serum-starved cells with or without dieckol (0-100 μM) in dose-dependent manner and then cells were cultured for 24 hr. The culture medium was removed and replaced by 95 μl of fresh culture medium and 5 μl of MTS solution. After 1 hr, the absorbance was measured using microplate reader (Ultraspec® 2100 *pro*, Amersham Biosciences, Piscataway, NJ) at 490 nm.

2.10. Measurement of intracellular ROS

The intracellular ROS scavenging activity of the sample was measured using the oxidant-sensitive fluorescent probe DCFH-DA. DCFH converted from DCFH-DA by deacetylase within the cells is oxidized by a variety of intracellular ROS to DCF, a highly fluorescent compound. The cells were incubated with tacrine with or without dieckol for 30 min. Cells were harvested by trypsin-EDTA solution (0.05% trypsin and 0.02% EDTA in PBS) and washed with PBS twice. The cells were treated with 20 μM DCFH-DA for 30 min at 37°C. The fluorescence intensity was measured at excitation wavelength of 485 nm and emission wavelength of 528 nm using fluorescence microplate reader. (Dual Scanning SPECTRAmax, Molecular Devices Corporation, CA, USA)

2.11. Western blot analysis

HepG2 cells cultured in 6-well plates were washed twice with ice-cold PBS contained protease and phosphatase inhibitors. Lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% SDS, 1% Nonidet P-40, 1 mM EDTA, 1 mM DTT, 200 nM aprotinin, 20 μM leupetin, 50 μM phenanthroline, 280 μM benzamidine-HCl) was added

on each well and stood on ice for 30 min for lysis. After centrifuge at 12,000 rpm for 20 min at 4°C, protein content of supernatant was measured, and aliquots (20 μg) of protein were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim-milk in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 hr and incubated for 16 hr with primary antibody in TBST. The blots were treated with secondary antibody in TBST for 2 hr, and immune complex was detected using Western blotting substrate.

2.12. Statistical analysis

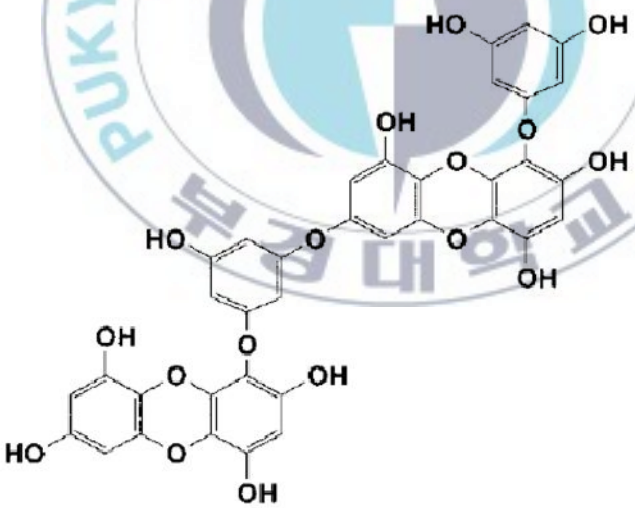
Data were expressed as means ±SD from triplicates of at least three independent experiments. All analysis was done with SPSS (Statistical analysis software package, USA). Statistical significance was established at a P value <0.05, and P value <0.01.

3. Results.

3.1. Structural elucidation of dieckol

The EtOAc-soluble fraction of the E. *stolonifera* led to the isolation of single compound (Fig 5). The structure of isolated compound was identified by comparison with published spectral data of dieckol (Blombitza and Vogels, 1985). The analysis of dieckol with NMR before isolation from *E. stolonifera* is as follows. The structrure is a hexamer of phloroglucinol, which has been reported to protect against linoleic acid peroxidation, total intracellular ROS generation, HIV-1 reverse transcripatase and hyperlipemia, and it has potentially inhibitory effect of acetylcholinesterase.

Dieckol: $C_{36}H_{22}O_{18}$ (MW=742). ^{1}H-NMR (400 MHz, CD_3OD) δ: 6.15 (1H, s, H-3″), 6.13 (1H, s, H-3), 6.09 (2H, s, H-2″, 6″), 6.06 (1H, d, *J*=2.9 Hz, H-8), 6.05 (1H, d, *J*=2.9 Hz, H-6″), 5.98 (1H, d, *J*=2.8 Hz, H-6), 5.95 (1H, d, *J*=2.8 Hz, H-6), 5.92 (3H, s, H-2′, 4′, 6′). ^{13}C-NMR (100 MHz, CD_3OD) δ: 162.7 (C-1′), 161.0 (C-3′, 5′), 158.6 (C-1‴), 156.8 (C-7), 155.3 (C-7″), 153.2 (C-3‴, 5‴), 148.1 (C-2″), 148.01 (C-2), 147.9 (C-9″), 147.7 (C-9), 145.1 (C-5a″), 145.0 (C-5a), 144.2 (C-4″), 144.1 (C-4‴), 139.4 (C-10a), 139.3 (C-10a″), 127.3 (C-4‴), 127.0 (C-9a), 126.5 (C-1), 126.4 (C-1″), 125.7 (C-9a″), 125.5 (C-4a″), 125.4 (C-4a), 100.7 (C-8″), 100.6 (C-8), 100.3 (C-3), 100.2 (C-3″), 98.5 (C-4′), 97.0 (C-2‴, 6‴), 96.7 (C-6″), 96.6 (C-6′), 96.2 (C-2′, 6′).



Structure of dieckol

3.2. Biological activity of dieckol

For clarification of the biological activity of dieckol, we conducted several tests including DPPH radical scavenging activity, mushroom tyrosinase inhibiting activity, NO and PGE2 generation inhibiting activity, and cancer cell proliferation inhibiting activity (Table 1).

Dieckol isolated from *E. stolonifera* was tested for its antioxidant activity by measuring their ability of scavenging free radicals with DPPH. The antioxidant capacity of dieckol was compared with those shown by a known antioxidant, L-ascorbic acid, treated in the same assay. As shown in Table 1, the EC_{50} value (The value of the 50% effective concentration) for the dieckol was 6.2 ± 0.4 μM, which is lower than that of L-ascorbic acid as a positive control.

And dieckol showed to inhibit the mushroom tyrosinase activity. To discover the tyrosinase inhibitory activity of dieckol, each concentration of dieckol (1-10 μM) was treated to investigate their inhibitory effect on mushroom tyrosinase activity using L-tyrosine as a substrate. The EC_{50} value was estimated 2.5 ± 0.5 μM of dieckol. It was compared with kojic acid, as a positive control, with EC50 values of 5.92 ± 0.4 μM.

We also conducted tests about anti-inflammatory and anti-cancer, but

dieckol did not show to affect them by displaying no changes on generation of NO and PGE2 and proliferation of cancer cells.

Table 1. Biological activity of dieckol isolated from *E. stolonifera*

Biological activity	EC_{50} (μM)
Antioxidation	6.2 ± 0.4
Anti-inflammatiory	≥ 50
Anti-cancer	≥ 100
Tyrosinase inhibitory	2.5 ± 0.5

3.3. Cytotoxicity of tacrine on HepG2 cells

To examine cytotoxicity of tacrine on HepG2 cells, serum-starved cells were firstly treated with different concentrations of tacrine (0-0.5 μM) for 24 hr. Cell viability was determined with MTS assay. The result was confirmed that tacrine induced cytotoxicity on HepG2 cells with dose-dependent pattern. EC_{50} of tacrine was estimated to be 0.3 mM of tacrine (Fig 6).

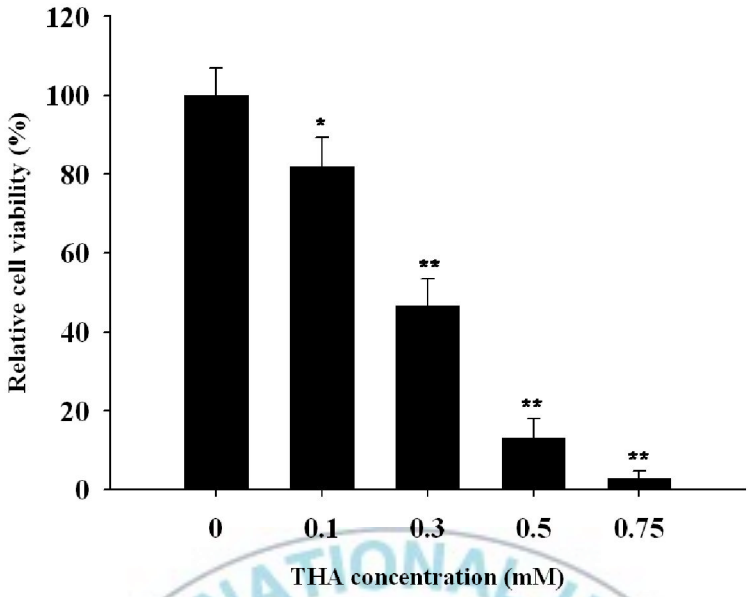


Fig 6. Cytotoxicity of tacrine on HepG2. HepG2 cells preincubated without FBS for 24 hr at 37℃ were cultured with different concentration of tacrine for 24 hr at 37℃. Cell viability was determined by the MTS assay. Data was expressed as means ± SD from triplicates of at least three independent experiments. *P < 0.05 and *P < 0.01 versus tacrine-untreated hepatocytes.

3.4.Protective effect of dieckol on tacrine-induced HepG2 cells

To examine hepatoprotective effect of dieckol on tacrine-induced HepG2 cells, hepatocytes starved with FBS for 24 hr were co-treated by 0.3 mM of tacrine with various concentrations of dieckol (0-100 μM). After incubation for 24 hr, cell viability was measured by MTS assay. (Fig 7) Dieckol showed hepatoprotective effect on tacrine-induced HepG2 cells with dose-dependent pattern. The EC_{50} value of dieckol was determined to be 43.6 μM.

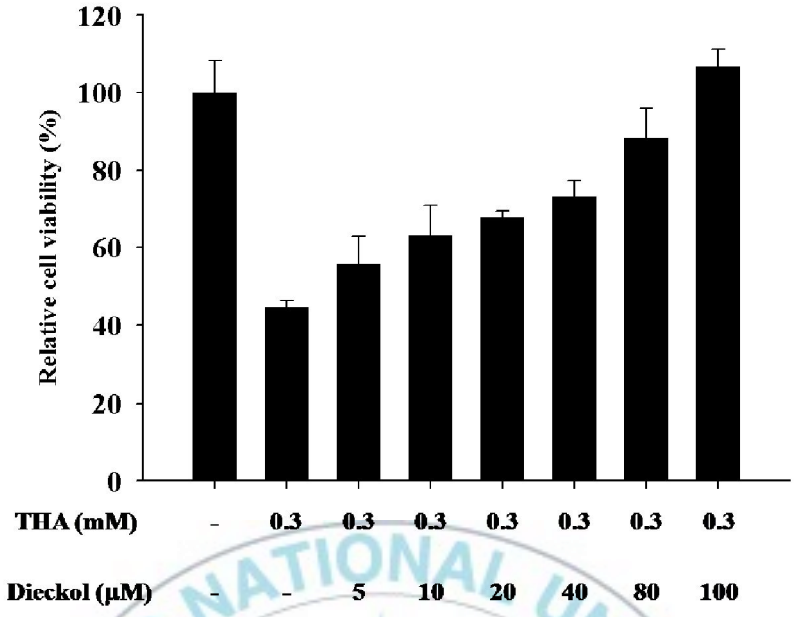


Fig 7. Protective effect of dieckol on tacrine-induced HepG2. HepG2 cells preincubated without FBS for 24 hr were cultured with tacrine (0.3 mM) and dieckol (0-100 μM) for 24 hr at 37℃. After incubation with tacrine for 24 hr, cell viability was determined by MTS kit assay. Data was expressed as means ± SD from triplicates of at least three independent experiments. *P < 0.05 versus untreated HepG2 cells.

3.5. Determination of intracellular ROS on HepG2 cells

To investigate the production of intracellular ROS in tacrine-induced hepatocytes and the inhibitory effects of dieckol on the production of ntracellular ROS in tacrine-induced HepG2 cells, cells starved with FBS for 24 hr were treated with 0.3 mM of tacrine and various concentrations of dieckol (0-50 μM) for 30 min. As showed in Fig 8, dieckol inhibits the production of intracellular ROS on tacrine-treated HepG2 cells. Dieckol treatment with 10, 25, and 50 μM completely blocked the ROS production on tacrine-induced HepG2 cells.

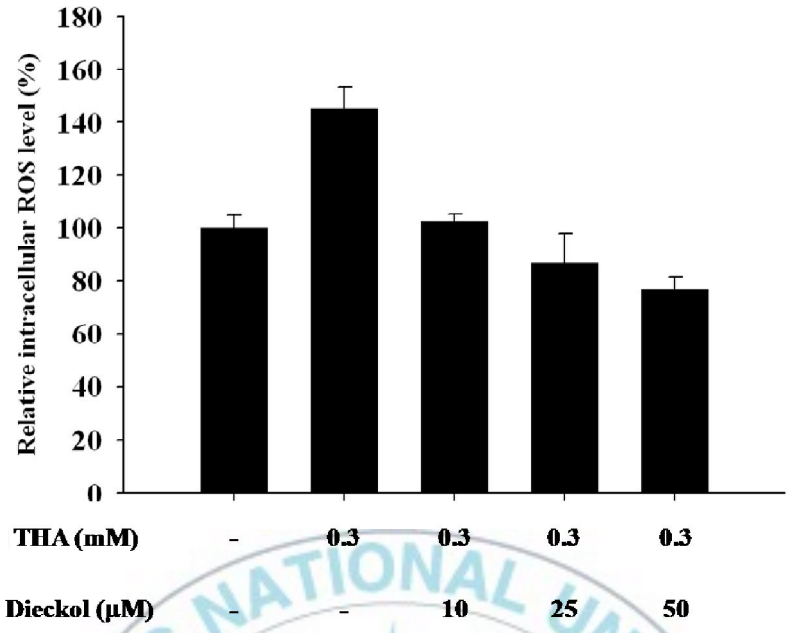


Fig 8. Effect of Dieckol on tacrine-induced intracellular ROS level. HepG2 cells starved with FBS for 24 hr were treated with 0.3 mM of tacrine absence or presence of 10, 25, and 50 μM of dieckol. After incubation with samples for 30 min, intracellular ROS levels were measured by DCFH-DA with fluoroscencemetry. Data was expressed as means ± SD from triplicates of at least three independent experiments. $^{\triangle\triangle}P < 0.01$ versus PFF-A-untreated hepatocyte cultures.

.

3.6.Dieckol protects from tacrine-induced hepatic cell death by inhibiting Fas-mediated apoptosis

Dieckol showed hepatoprotective effect on tacrine-induced cell, as shown in Fig 7. To evaluate the effect of dieckol treatment on cell death signaling proteins, proteins in Fas/Fas L death signaling system were analyzed with Western blot. Fas protein which indicates a potential inducer of apoptosis, expressed at tacrine-treated HepG2 cells. (Fig 9) On the other hand, the Fas, triggered by treatment of tacrine, was dramatically decreased in 10, 25 and 50 μM of dieckol treatment. As the Fas protein reduction with dieckol treatment in dose dependent manner, down-stream proteins of Fas/FasL death signaling system, such as caspase-8 and Bid were significantly changed in protein levels. The expression of caspase-8 induced by tacrine via Fas was decreased with dieckol treatment, and Bid regulated by caspase-8 was increased by dieckol treatment. These changes eventually affected cleaved PARP, which played a pivotal role for apoptosis in mammalian cells, to prevent from tacrine-induced hepatic cell death by decrease of cleavage level of PARP at 24 hr.

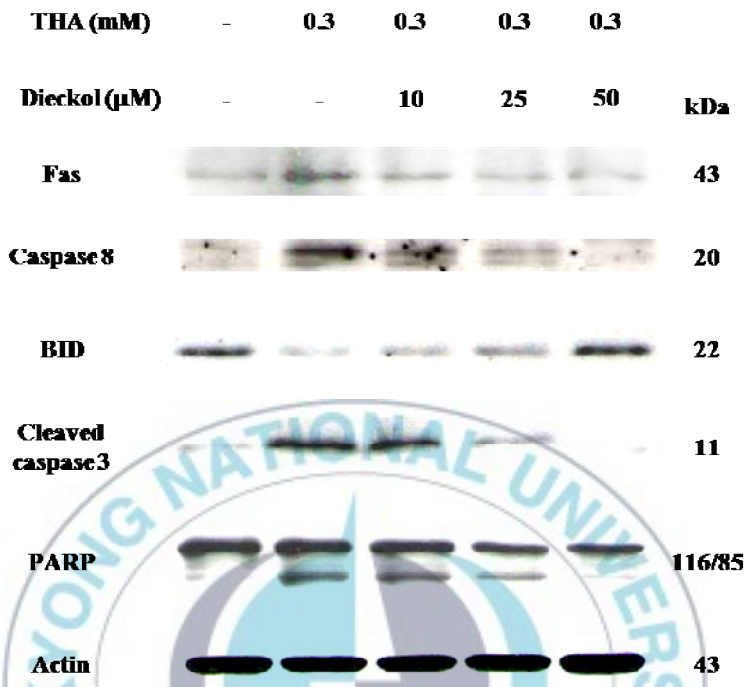


Fig 9. Dose-dependent inhibitory effect of dieckol on tacrine-induced signal trasnduction. 0.3 mM of tacrine was added to serum starved HepG2 cells with 0-50 μM of diekcol for 24 hr to identify the effect of dieckol on tacrine-induced HepG2 cells. Dieckol has a protection effect with dose-dependent manner on tacrine-induced HepG2 cells.

3.7. The role of JNK in dieckol treated HepG2 cells

Among the MAPKs, the activation of pJNK is one of the pathways for apoptosis. To investigate the role of pJNK on tacrine-indcued HepG2 cells, SP600125, inhibitor of JNK phosphorylation, was pretreated on tacrine-treated HepG2 cells. Cells preincubated with 20 μM SP600125 for 1 hr were treated 0.3 mM of tacrine for 24 hr and were co-treated 0.3 mM of tacrine and 50 μM of dieckol without preincubation of SP600125 for 24 hr. Both pre-treatment of SP600125 and co-treatment with dieckol completely inhibited phosphorylation of JNK so that partially inhibited the expression of Fas and fragmentation of PARP on tacrine-induced HepG2 cells (Fig 10).

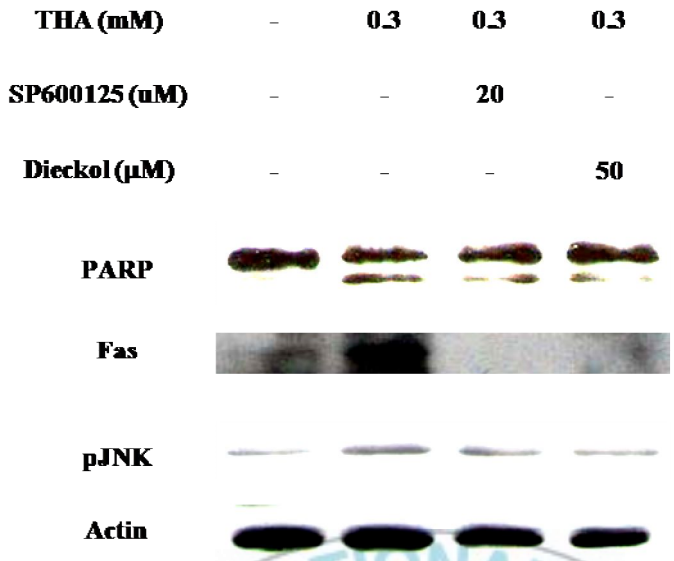


Fig 10. The inhibitory effect of dieckol on phosphorylated JNK by comparing with SP600125, inhibitor of JNK phosphorylation

SP600125, inhibitor of JNK phosphorylation, was pretreated on tacrine-treated HepG2 cells. Cells preincubated with 20 μM SP600125 for 1 hr were treated 0.3 mM of tacrine for 24 hr and cells were co-treated 0.3 mM of tacrine and 50 μM of dieckol without preincubation of SP600125 for 24 hr. Both pre-treatment of SP600125 and co-treatment of dieckol completely inhibited phosphorylation of JNK so that partially inhibited the expression of Fas and fragmentation of PARP on tacrine-induced HepG2 cells..

4. Discussion

Our life has been threatened by so many kinds of antibiotics, drugs, and chemical additives etc. They were originally developed to cure and improve the health conditions in human life, but also have had negative effects which could cause damages in living organs. Especially liver is the representatively protective organ in metabolic digestion and detoxification of ingested agents such as foods, xenobiotics and drugs etc. Thus liver or hepatic disorders are recognized to be severe problem.

Tacrine was developed as a drug to treat Alzheimer's disease, as acting an acetylcholinesterase inhibitor. Tacrine was suggested to increase Central Nerve System (CNS) acetylcholine levels of Alzheimer's disease patients (Mesulam et al., 2002; Darvesh and Hopkins, 2003; Johnson et al., 2004). And tacrine has been reported to have function of antioxidant in vitro and it protects from hydrogen peroxide-induced rat pheochromocytoma line PC12 cells by regulating expression of apoptosis-related genes. Its neuroprotective effects of cholinesterase inhibitor by antioxidation might partly contribute to the clinical efficacy in AD treatment (Xiao et al., 2000; Zhang and Tang. 2000; Wang et al., 2002). However, tacrine had been also reported to cause hepatic injury in 30-50% patients, as indicated by the reversible increasing level of

transaminase (Watkins et al., 1994). In recent reports, tacrine is thought to alter intracellular glutathione concentrations in hepatocytes (Lagadic-Gossmann et al., 1998). Moreover tacrine affects on intracellular ROS generation (Osseni et al., 1999), lipid peroxidation, membrane fluidity (Galisteo et al., 2000) and mtDNA synthesis (Mansouri et al., 2003), indicating that those effects of tacrine might affect on liver cells proliferation through increasing necrosis and/or apoptosis in liver cells. Figure 6 shows that tacrine(0-0.5μM) induces cell death on HepG2 cells as cell viability was reduced in dose dependent manner.

When dieckol, a kind of phlorotannins which had been isolated from *E. stolonifera,* was co-treated with tacrine on HepG2 cells, the cell viability was recovered (Fig 7) and the intracellular ROS (reactive oxygen species) generation was completely blocked compared with only tacrine treated groups (Fig 8). From this result, we confirm that Tacrine increases ROS level, standing for significant damages on hepatic cells(Osseni RA *et al* ., 1999), and dieckol recovers them by eliminating it.

We have also found the expression of Fas/FasL system proteins was induced by tacrine, and was decreased by treatment with dieckol for 24 hr. (Fig 9) Fas, activated as a stimulus of tacrine in HepG2 cells, was decreased by co-treatment with dieckol in dose-dependent manner.

Activation of Fas on the cell membrane by Fas ligand or agonist antibody results to the activation of caspase-8, the capital caspase in this pathway, and then caspase-8 activates caspase-3 through direct or indirect pathway. (Boldin *et al.,*1996; Muzio *et al.,* 1996) Bid, one of the BH3-only Bcl-2 family members, is also activated post-translationally via cleavage by caspase-8/FLICE in response to Fas or TNF receptor activation. (Li *et al.,*1998) The truncated Bid (t-Bid) cleavage product, p15, acts in mitochondria to release cytochrome C to cytosol. (Luo *et al*.,1998) The released cytochrome c to cytosol activates procaspase-3, which usually presents as inactive proenzymes form in cytosol, to active form and the active caspase-3 hydrolyzes PARP. (Zhuang *et al*.,1999; Reed, 1997; Cohen, 1997; Enari *et al*., 1995) The specific cleavage of PARP at the sequence Asp-Glu-Val-Asp (DEVD) has been reported to be a sensitive marker of caspase-3 activation in apoptosis. (Janicke *et* al., 1998; Kaufmann *et al*., 1993; Um *et* al.,1996) In our results, the expression of Fas-related apoptotic signals including caspase-8, Bid, and PARP fragmentation, was ascertained to be down-regulated by dieckol with dose-dependent manner.

JUN NH2-terminal kinases(JNKs) are known to be involved in apoptosis, neurodegradation, cell differentiation and proliferation, inflammatory conditions etc,(Liu *et al*., 2005) to confirm whether

dieckol can block the activation of JNK, SP600125, an antibody of JNK, was treated with tacrine to compare with dieckol. 50μM of dieckol suppressed the phosphorylation and activation of JNK, as much as SP600125, as an inhibitor of JNK. (Fig 10) It is important because the phosphorylated JNK modifies the activity of numerous proteins in mitochondria and nucleus, and regulates several important cellular functions by changing the levels of intracellular ROS. (Xia *et al.,* 1995)

Some compounds extracted from brown alga have been reported to reduce intercellular ROS levels effectively. Choi et al. (1997) reported that methanolic extractions of *E. stolonifera*, its subsequent fractions and its components, phloroglucinol and phlorotannin A, are useful as nitrite scavangers. Brown alga polyphenols have strong antioxidant effects, and phlorotannins, extracted from *E. cava,* were performed as scavengers of DPPH radical in dose-dependent manner. (Kim *et al*., 2004; Kang *et al* ., 2003) (Shibata *et al*., 2002) Our evidence certified that the intracellular events started from generation of ROS by tacrine lead to hepatic cell death sequentially. In the initial step, there is generation of ROS and activation of Fas-mediated protein signaling transduction. At this stage, dieckol suppresses the production of ROS and prevents cell death by blocking these cell signals in apoptosis.

Based on our observation with other latest studies, compounds having antioxidant activities are considered to protect hepatocytes from tacrine-induced cytotoxicity, as its antioxidant function results to reduce formation of ROS or to scavenge free radicals. Therefore, we suppose that tacrine stimulates hepatic cells death through Fas/FasL signal transduction activated by ROS, and dieckol protects the cells from tacrine by inhibiting Fas down-stream proteins with reducing ROS level. It seems that the reductive effect of dieckol is caused both directly and indirectly by redox-reaction, and it triggers the expression of scavenging enzymes such as catalase and superoxide dismutase (SOD) in signaling transduction. Therefore, dieckol should be studied more with the heaptic damages caused by other factors such as acetaminophen or alchol, as they also have the similar side effect of tacrine. (Mitchell *et al*., 1973) Although, in our report, the effect of dieckol on expression of intracellular scavenging enzymes has not covered yet, these findings will provide a biochemical basis for the influence of dieckol on the hepatoprotective effect.

5. References.

Ahn MJ, Yoon KD, Min SY, Lee JS, Kim JH, Kim TG, Kim SH, Kim NG, Huh H, Kim J. Inhibition of HIV-1 reverse transcriptase and protease by phlorotannins from the brown alga *Ecklonia cava*. Biol Pharm Bull. 2004 Apr;27(4):544-7.

Amino H, Kakinuma M, Coury D.A, Ohno H, and Hara T. Effect of a seaweed mixture on serum lipid level abd platelet aggregation in rats. Fish.Sci 2005;71:1160-1166.

Ara J, Sultana V, Qasim R. and Kim Y.C. Hypolipidemic activity of seaweed from Karachi Coast. Phytother Res 2002;16:479-483.

Blombitza KW, Vogels HP. Antibiotics from algae. XXXV. Phlorotannins from *Ecklonia maxima*. Planta Med. 1985;51:308-312.

Boldin MP, Goncharov TM, Goltsev YV, Wallach D. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/Apo1- and TNF receptor induced cell death. Cell 1996;85:803-815.

Cohen GM. Caspase: The executioners of apoptosis. Biochem. J 1997;326:1-16.
Dai R, Frejtag W, He B, Zhang Y, Mivechi NF. c-Jun NH2-terminal kinase targeting and phosphorylation of heat shock factor-1 suppress its transcriptional activity. J Biol Chem. 2000;275(24):18210-8

Darvesh S, Hopkins DA. Differential distribution of butyrylcholinesterase and acetylcholinesterase in the human thalamus. J Comp Neurol 2003;463(1):25-43.

Enari M, Hug H, Nagata S. Involvement of an ICE-like protease in Fas-mediated apoptosis. Nature. 1995;375(6526):78-81.

Forsyth DR, Sormon DJ, Morgan RA, Wilcock GK. Clinical experience with and side effects of tacrine hydrochloride in Alzheimer's disease: A pilot study. Age Ageing 1989;18: 223-229.

Fukuyama Y, Kodama M, Miura I, Kinzyo Z, Kido M, Mori H, Nakayama Y, Takahashi M. Structure of an anti-plasmin inhibitor, eckol, isolated from the brown alga *Ecklonia kurome* Okamura and inhibitory activities of its derivatives on plasma plasmin inhibitors. Chem Pharm Bull (Tokyo). 1989 Feb;37(2):349-53.

Galisteo M, Rissel M, Sergent O, Chevanne M, Cillard J, Guillouzo A, Lagadic-gossmann D. Hepatotoxicity of Tacrine: Occurrence of Membrane Fluidity

Alterations without Involvement of Lipid Peroxidation. JPET 2000;294(1):160-167

Giacobini E. Cholinesterase inhibitors stabilize Alxheimer's diseas. Neurochem Res 2000;36:1185-1190.

Greig NH, Utsuki T, Yu Q, Zhu X, Holloway HW, Perry T, Lee B, Ingram DK, Lahiri DK. A new therapeutic target in Alzheimer's disease treatment: attention to butyrylcholinesterase. Curr Med Res Opin. 2001;17(3):159-65.

Groossens V, Grooteh J, De Vos K, Fiers W. Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. Proc Natl Acad Sci 1995;92:8115-8119.

Haga S, Terui K, Zhang HQ, Enosawa S, Ogawa W, Inoue H, Okuyama T, Takeda K, Akira S, Ogino T, Irani K, Ozaki M. Stat3 protects against Fas-induced liver injury by redox-dependent and –independent mechanisms. J Clin Invest 2003;112:989-998.

Han ES, Kim JW, Eom MO, Kang IH, Jang HJ, Choi JS, Ha KW, Oh HY. Inhibitory effects of *Ecklonia stolonifera* on gene mutation on mouse lymphoma tk+/- locus in L5178Y-3.7.2C cell and bone marrow micronuclei formation in ddY mice. Environ Mutagen Carcinogen 2000;20: 104-111.

Janicke RU, Sprengart ML, Wati MR, Poter AG. Caspase-3 is required for DNA ragmentation and morphological changes associated with apoptosis. J Biol Chem 1998;273:9357-9360.

Johnson CN, Roland A, Upton N. New symptomatic strategies in Alzheimer's disease. Drug Discovery Today 2004;1:13-19.

Kang HS, Chung HY, Jung JH, Son BW, Choi JS. A new phlorotannin from the brown alga *Ecklonia stolonifera*. Chem Pharm Bull 2003;51(8): 1012-1014.

Kang HS, Chung HY, Kim JY, Son BW, Jung HA, Choi JS. Inhibitory phlorotannins from the edible brown alga *Ecklonia stolonifera* on total reactive oxygen species (ROS) generation. Arch Pharm Res. 2004a Feb;27(2):194-8.

Kang HS, Kim HR, Byun DS, Son BW, Nam TJ, Choi JS. Tyrosinase inhibitors isolated from the edible brown alga *Ecklonia stolonifera*. Arch Pharm Res 2004b;27: 1226-1232.

Kang K, Park Y, Hwang HJ, Kim SH, Lee JG, Shin HC. Antioxidative properties of brown algae polyphenolics and their perspectives as chemopreventive agents against vascular risk factors. Arch Pharm Res

2003;26(4):286-293.
Kaplowitz N. Drug-induced liver injury. Clin Infect Dis. 2004;38 Suppl 2:S44-48.

Kaufmann SH, Deasnoyers S, Ottaviano Y, Davidson NE, Poirier GG. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: An early marker of chemotherapy-induced apoptosis. Cancer Res 1993;53:3976-5985.

Kim JA, Lee JM, Shin DB, Lee NH. The antioxidant activity and tyrosinase inhibitory activity of phlorotannins in Ecklonia cava. Food Sci Biotechnol 2004;13(4):476-480.

Kim YC, An RB, Yoon NY, Nam TJ, Choi JS. Hepatoprotective constituents of the edible brown alga *Ecklonia stolonifera* on tacrine-induced cytotoxicity in Hep G2 cells. Arch Pharm Res. 2005;28(12):1376-1380.

Lagadic-Gossmann D, Rissel M, Le Bot MA, Guillouzo A. Toxic effects of tacrine on primary hepatocytes and liver epithelial cells in culture. Cell Biol Toxicol 1998;14:361-373.

Lee JH, Park JC, Choi JS. The antioxidant activity of Ecklonia stolonifera. Arch Pharm Res. 1996;9(3):223-227.

Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 1998;94(4):491-501.

Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell 1998;94(4):481-490.

Mansouri A, Haouzi D, Descatoire V, Demeilliers C, Sutton A, Vadrot N, Fromenty B, Feldmann G, Pessayre D, Berson A. Tacrine inhibits topoisomerases and DNA synthesis to cause mitochondrial DNA depletion and apoptosis in mouse liver. Hepatology. 2003 Sep;38(3):715-25.

Meng Q, Ru J, Zhang G, Shen C, Schmitmeier S, Bader A. Re-evaluation of tacrine hepatotoxicity using gel entrapped hepatocytes. Toxicol Lett. 2007 Jan 30;168(2):140-7.

Mesulam MM, Guillozet A, Shaw P, Levey A, Duysen EG, Lockridge O. Acetylcholinesterase knockouts establish central cholinergic pathways and can use butyrylcholinesterase to hydrolyze acetylcholine. Neuroscience 2002;110:627–639.

Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Scaffidi C, Bretz JD, Zhang M, Ni J, Gentz R. FLICE, a novel FADD-homologous ICE/CED-3-like prote-ase, is recruited to the CD95 (Fas/Apo-1) death-inducing signalingcomplex. Cell 1996;85:817-827.

Myung CS, Shin HC, Bao HY, Yeo SJ, Lee BH, Kang JS. Improvement of memory by dieckol and phlorofucofuroeckol in ethanol-treated mice: possible involvement of the inhibition of acetylcholinesterase. Arch Pharm Res. 2005 Jun;28(6):691-8.

Nakai M, Kageyama N, Nakahara K, Miki W. Phlorotannins as radical scavengers from the extract of *Sargassum ringgoldianum*. Mar biotechnol (NY), 2006;8(4): 409-14.

Nanjo F, Goto K, Seto R, Suzuki M, Sakai M, Hara Y. Scavenging effects of tea catechins and their derivatives on 1,1-diphenyl-2-picrylhydrazyl radical. Free Radical Biol. Med. 1996;21:895-902.

Osseni RA, Debbasch C, Christen MO, Rat P, Warnet JM. Tacrin-induced reactive oxygen species in a human liver cell line: ther role of anethole dithiolethione as a scavenger. Toxicol In Vitro 1999;13:683-688.

Reed JC. Cytochrome c: Can't live with it– can't live without it. Cell 1997;91:559-562.

Taub R. Hepatoprotection via the IL-6/Stat3 pathway. J Clin Invest 2003;112(7):978-980.

Tsuboi T, Kondoh H, Hiratsuka J, Mishima Y. Enhanced melanogenesis induced by tyrosinase gene-transfer increases boron-uptake and killing effect of boron neutron capture therapy for amelanotic melanoma. Pigment Cell Res. 1998 Oct;11(5):275-82.

Um HD, Orenstein DM, Wahl SM. Fas mediates apoptosis in human monocytes by a reactive oxygen intermediate dependent pathway. J Immunol 1996;156:3469–3477.

Wang R, Zhou J, Tang XC. Tacrine attenuates hydrogen peroxide-induced apoptosis by regulating expression of apoptosis-related genes in rat PC12 cells. Molecular Brain Research 2002;107:1-8.

Watkins PB, Zimmerman HJ, Knapp MJ, Gracon SI, Lewis KW. Hepatotoxic effects of tacrine administration in patients with Alzheimer's disease. J Am Med Assoc 1994;271:992–998.

Xiao XQ, Wang R, Tang XC. Huperzine A and tacrine attenuate beta-amyloid peptide-induced oxidative injury. J Neurosci Res. 2000 Sep 1;61(5):564-9.

Yoon NY, Kim HR, Chung HY, Choi JS. Anti-hyperlipidemic effect of an edible brown algae, *Ecklonia stolonifera*, and its constituents on poloxamer 407-induced hyperlipidemic and cholesterol-fed rats. Arch Pharm Res. 2008 Dec;31(12):1564-71. Epub 2008 Dec 20.

Zhang HY, Tang XC. Huperzine B, a novel acetylcholinesterase inhibitor, attenuates hydrogen peroxide induced injury in PC12 cells. Neurosci Lett. 2000 Sep 29;292(1):41-4.

Zhuang S, Lynch MC, Kochevar IE. Caspase-8 mediaes caspase-3 activation and cytochrome c release during singlet oxygen-induced apoptosis of HL-60 cells. Experimental Cee Research 1999;250:203-212.