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In vivo and *In vitro*Effects of Chestnut (*Castanea crenata*) Inner Shell on Antioxidant and Cardiovascular-Related Parameters

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INTRODUCTION

Cardiovascular disease (CVD) is one of the leading causes of death and disability in the world.[1)]An estimated 17.3 million people died from CVE in 2008, representing that 30% of all global deaths and almost 23.6 million people will die from CVD, mainly from heart disease and stroke by 2030.[2)]According to 2010 statistics in Korea, CVD was the second cause of death following cancer, corresponding 23.0% of all death.[3)]Incidence of CVD is associated with multiple risk factors such as raised total cholesterol and LDL-cholesterol, hypertension, increased platelet reactivity, alterations in glucose metabolism, and smoking.[4)]Recent findings have suggested that oxidative stress, vascular inflammation, and endothelial dysfunction may play roles in developing atherosclerosis which is the major incidence of CVD.[5)]

Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are commonly used drugs to treat hyperlipidemia, consequently preventing coronary artery disease and stroke. Statin is known to deplete coenzyme Q10 (CoQ10) by halting production of mevalonate, a CoQ10 precursor in cholesterol biosynthesis. In fact, the use of statins can decrease the synthesis of CoQ10 up to 40%.[6)]CoQ10 acts as electron carrier in mitochondrial electron transport system (ETS), and depletion of CoQ10 directly impacts on active muscles. Myopathic complications such as myalgia and rhabdomyolysis are most serious side effects after statin therapy and constipation, diarrhea, dizziness, headaches, rashes, and upset stomach are also reported as minor symptoms. Besides function in ETS, the reduced form of CoQ10 (CoQH2), ubiquinol plays role in protecting cell membrane from oxidative damage by scavenging free radical generated in physiological system. Dhanasekaran *et*

al.[7]proposed that a lipophilic antioxidant CoQH2 participates regeneration of α–tocopherol and ascorbate in plasma membrane and prevents lipid peroxidation.

Recently, the emphasis in medical care has changed from “treatment” to “prevention” in which functional food and food therapy play an important role. This approach reflects the oriental medicine paradigm of “prevent illness before it began” and “food and medicine have the same root”. With this concepts, there has been growing interest in complementary and alternative medicine (CAM) for treatment of diseases, illness prevention and maintenance of health. Many Americans are now pursuing CAM as an alternative or supplement to conventional medicine, and a 2007 National Health Interview Survey (NHIS) showed that approximately 38% of adults in America use CAM therapy.[8)]

Over the past few years, various epidemiological studies have shown an inverse relation between the consumption of polyphenols or polyphenol-rich foods and the risk of CVD.[9)10)]Much attention has been paid to natural polyphenols of silymarin, curcumin, green tea, and grape seed extracts,[11)] which provide strong antioxidants against free radicals, thereby reducing cell damage and risk of certain diseases such as cancer,[12)] inflammation,[13)]and CVD.[9)10)]

Chestnut is one of the favorite nuts cultivated for a long time and widely used including Korean traditional ceremonies. In the last few years, the consumption of fresh or previously transformed chestnuts has gradually increased due to their nutritional qualities and palatability as foods. In the process of getting edible nut, considerable amounts of the inedible waste part, the pericarp (outer shell) and integument (inner shell) are generated, and much efforts has been made to regenerate the chestnut bark (inner and outer shells) to valuable coproduct with economic view

point in European countries.[14)]

Ellagitannin and gallotannin in chestnut bark are hydrolyzed to strong antioxidant polyphenols, ellagic acid and gallic acid. Despite valuable phenolic compounds of chestnut bark, there are not many studies have been reported on its protective effects against CVD.

Accordingly, the first part of this study attempted to investigate the effects of chestnut inner shell of powder and extract *in vivo* on cardiovascular-related parameters, such as plasma and liver lipids, platelet aggregation, erythrocyte Na efflux, ACE activity, and plasma GOT and GPT levels using animal model of guinea pigs which were fed with statin-added cholesterol diets. In the second part, study focused on *in vitro* or *ex vivo* effects of ethanol extract of chestnut inner shell and the major polyphenol compounds such as ellagic acid, gallic acid, and catechin using lung tissue, platelet, lymphocytes, and RAW 264.7 cells. This study also included the comparison of anti-inflammatory activity in fractions partitionated by polarity (*n*-hexane, ethyl acetate, butanol, and water) of ethanol extract of the chestnut inner shell, providing valuable data to elucidate the association of the chestnut inner shell with CVD development.

LITERATURE REVIEW

1. Risk factors of CVD

1) Atherosclerosis

Atherosclerosis, the primary cause of CVD and stroke, is a progressive disease characterized by the build-up of lipids and fibrous elements in the large arteries. The prevalent view of spontaneous atherosclerosis is the "response-to-injury" hypothesis based on early proposals made by Virchow[15] and subsequently modified by Ross[16]more than a century later.

In this hypothesis,[17]atherosclerosis is initiated as a response to various forms of injury to arterial endothelium. Endothelial cell injury from hyperlipidemia, hypertension, diabetes mellitus, or smoking *etc*, disrupts normal endothelial cell function. Endothelial dysfunction leads to increased permeability so that lipids and circulating cells including monocytes and T-lymphocytes, enter the sub-endothelial space and form the initial characteristic lesion of atherosclerosis, the fatty steak. Accumulation of cells and lipids may increased endothelial disruption leading to thrombogenic surfaces to which platelets adhere. Platelets, macrophages, endothelial cells and probably smooth muscle cells (SMCs) themselves can release growth modulatory factors, leading to proliferation of SMCs and fibroblasts and this process leads to the formation of the fibrous plaque and ultimately to the complex advanced lesion of atherosclerosis (Figure 1). Intimal vascular SMCs synthesize extracellular matrix proteins such as collagen, elastin, and proteoglycans that lead to the development of a fibrous cap.[18][19]Vulnerable plaque are characterized by lipid

accumulation that expands the intima, degradation of the extracellular matrix, a decrease in connective tissue proteins, and a necrotic core. It is thought that lesions with large amounts of such lipids are particularly unstable and liable to rupture, leading to thrombosis and vessel occlusion. Plaque rupture and thrombosis induce acute cardiovascular events that result in myocardial infarction and stroke.[20)]

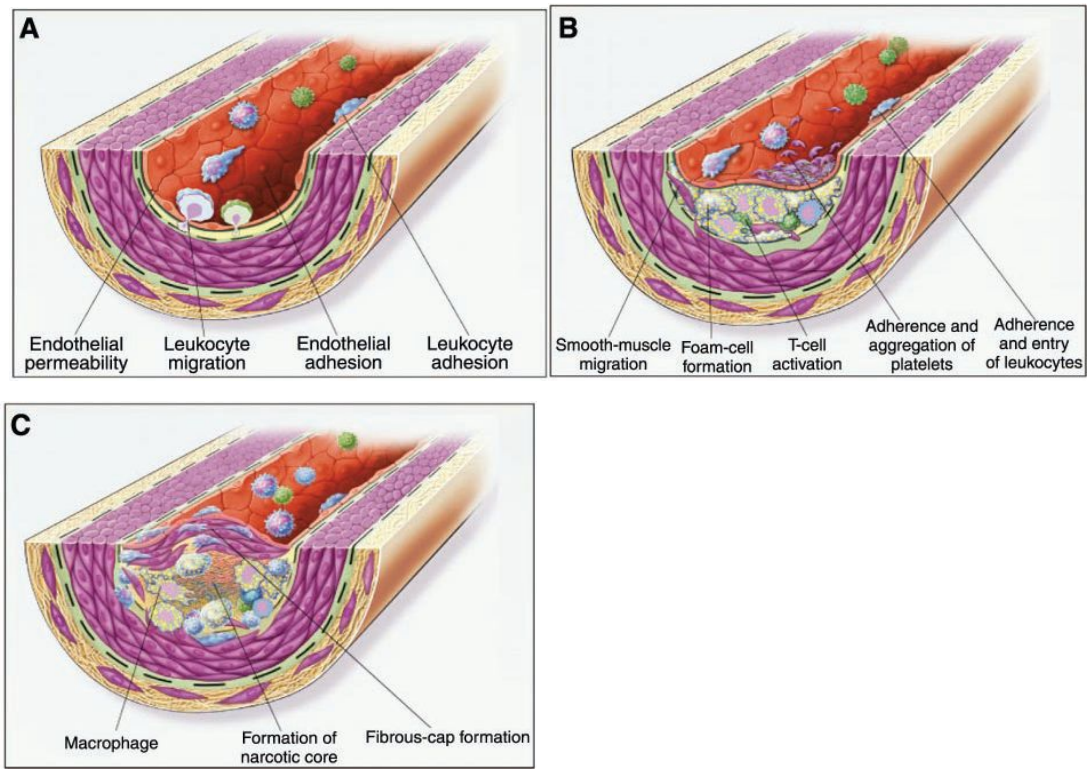


Figure 1. Response-to-injury hypothesis of atherosclerosis

In this hypothesis, atherosclerosis begins with endothelial injury or dysfunction (*A*) that is characterized by enhanced endothelial permeability and low-density lipoprotein (LDL) deposition in the subendothelial space. This is followed by leukocyte adhesion and transmigration across the endothelium. In intermediate stages (*B*), atherosclerosis is characterized by foam cell formation and an inflammatory response including T-cell activation, the adherence and aggregation of platelets, and further entry of leukocytes into the arterial wall along with migration of smooth muscle cells into the intima. Finally, advanced atherosclerosis (*C*) is characterized by continued macrophage accumulation, fibrous cap formation, and necrosis in the core of the lesion. [From Stocker *et al*,[21]copyright 2004 Physiol Rev.]

2) Oxidative stress and inflammation in atherosclerosis

It is now widely accepted that oxidative stress and inflammation play a pivotal role in the initiation and progression of atherosclerosis and CVD.[22]Oxygen is essential for metabolic processes in the body. However, oxygen derived free radicals, namely reactive oxygen species (ROS) are reported to exert detrimental effects, such as membrane lipid peroxidation, alteration of lipid-protein interactions, enzyme inactivations, and DNA breakage.[23]

The production of free oxidative radical is believed to induce endothelial dysfunction through several mechanisms. Firstly, ROS, especially hydroxyl radicals, directly injure cell membranes and nuclei. Lipid peroxidation by ROS causes particularly destructive effects on cell membranes, furthermore severe lipid peroxidation causes myocardial necrosis.[24]H_2O_2 has been demonstrated to induce vascular smooth muscle cell (VSMC) death,[25]which may occur by apoptosis.[26]

Secondly, ROS was proposed to modulate vasomotion and the atherogenic process by interacting with endogenous vasoactive mediators formed in endothelial cells. Endogenous nitric oxide (NO) known as endothelium-derived relaxing factor (EDRF) not only acts as potent endogenous vasodilator, but also inhibits vascular smooth muscle cell (VSMC) migration and proliferation, platelet adhesion and aggregation, LDL oxidation, and vascular inflammation.[27][28]Endogenous NO synthesized from *L*-arginine by endothelial nitric oxide synthase (eNOS) get dampened by ROS. ROS inactivate NO via three different mechanisms: 1) direct inactivation of NO by superoxide (O_2^-), resulting in the formation of peroxynitrite ($ONOO^-$),[29]2) reducing NOS activity with increased levels of asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor,[30]and 3) uncoupling

eNOS with increased oxidation of cofactor tetrahydrobiopterin (BH_4).[31]Decreased NO bioavailability with decreased NO production and/or increased NO inactivation causes endothelial dysfunction by an imbalance of NO and ROS, that cause atherosclerosis.[32]

Thirdly, ROS expedites atherosclerosis by formation of the key mediator, oxidized lipoprotein (LDL) via peroxidizing lipid components. Hydroxyl radicals may initiate the peroxidation of long-chain polyunsaturated fatty acids within LDL molecule, giving rise to conjugated dienes and lipid hydroperoxy radicals ($LOO^{\cdot}$). This process is self-propagation, such that $LOO^{\cdot}$ can attack adjacent fatty acids until completion of chain fragmentation. Unlimited amount of oxidatively modified LDL (oxLDL) is taken up by sub-endothelial macrophages via the "scavenger" receptor pathway to form a "foam cell" in the arterial intima.[33]In addition, oxidized LDL exerts several biological effects; induction of inflammation, inhibition of eNOS and vasoconstriction, stimulation of cytokines such as interleukin-1 (IL-1), and activation of platelets.[34]

Inflammatory processes play an important role in all stages of atherosclerosis.[35]Unlike circulating monocytes in the blood, resident monocytes in healthy arteries patrol healthy tissue for sites of inflammation. In response to oxLDL, local tissue produces directional chemical signals, called chemokines that direct monocytes to areas of inflammation where they differentiate into macrophage. In genetically altered mice, inhibition of chemokine receptors results in almost complete prevention of macrophage accumulation and atherosclerosis.[36]Once differentiated into macrophages, they secrete pro-inflammatory mediators, resulting in upregulation of vascular cell adhesion molecule-1 and intercellular adhesion

molecule-1 on activated endothelial cell surfaces. This leads to further recruitment of T-cells and macrophages to the arterial wall. Smooth muscle cells also participate synthesis of pro-inflammatory cytokines including interleukin-1 (IL-1) and tumor necrosis factor-alpha (THF-α). In atheroma, T-cells undergo activation after interacting with macrophages and dendritic cells, both of which process and present antigen such as oxLDL.[37)]

Inflammation not only promotes development of atherosclerosis but also influence plaque vulnerability. The immediate site of plaque rupture or erosion is marked by an active inflammatory process where activated monocytes, macrophages and T-cells may play roles in destabilizing the fibrous cap and rupturing plaque. Atherosclerotic plaques from unstable symptomatic patients exhibit significant infiltration by leukocytes, which secrete matrix-degrading enzymes and thrombogenic substances, resulting in plaque disruption and local thrombosis.[38)]

Inflammatory processes, which play a key role in the development and progression of atherosclerosis, are characterized by increased circulating levels of pro-inflammatory cytokines [interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), and inducible nitric oxide synthase (iNOS)], soluble adhesion molecules, and cytokine-responsive acute phase protein like C-reactive protein (CRP).[39)40)]

Prostaglandins (PGs) and nitric oxide (NO) exert numerous vascular and inflammatory effects. Production of PGs or NO by the constitutive isoenzymes, cyclooxygenase-1 (COX-1), or eNOS, is implicated in regulation of vascular tone and homeostatic functions. In contrast, COX-2 and iNOS are not generally expressed in resting cells, but are induced following appropriate stimulation with pro-

inflammatory agents such as cytokines and lipopolysaccharide (LPS).[41]Activation of these inducible enzymes results in overproduction of PGs and NO, which play a key role in the pathophysiology of arthritis and other inflammatory conditions.[42]NO is also able to enhance the production of TNF-α and IL-1β, which participate in the macrophage dependent inflammation.[43]

3) Hypertension

Hypertension is a known risk factor for the development of atherosclerosis and in hypertensive patients with elevated plasma rennin-angiotensin activity, a five folds increased incidence of myocardial infarction was demonstrated[44]Rennin-angiotensin system (RAS) plays a part in blood pressure regulation, sodium homeostasis, and blood volume maintenance in the human body.[45]The angiotensin-converting enzyme (ACE) is a highly glycosylated zinc dipeptidyl-carboxypeptidase that plays an important role in the RAS, where the latter regulated the arterial blood pressure and the electrolyte balance in mammals.[46]ACE catalyzes the degradation of angiotensin I to angiotensin II, a potent vasoconstrictor, by removing the carboxyl terminal dipeptide His-Leu.[47]Accordingly, ACE hydrolyzes the angiotensin I analogue, hippuryl-L-histidyl-L-leucine (HHL) forming hippuric acid (His-Leu), levels of which determine ACE activity.Clinical studies have demonstrated that ACE inhibitorssignificantly reduced the morbidity and mortalityin myocardial infarction patients and theincidence of recurrent myocardial infarction and ischemicevents in patients with coronary artery disease,even in the absence of blood pressure lowering.[48)~50)]The mechanism by which ACE inhibitors affectatherosclerosis is not

well understood, but it has beenpostulated that these agents may have multiple effects,including blood pressure lowering, anti-proliferative effecton vascular cells, inhibitory effect on platelet aggregationand inhibition of lipid peroxidation.[51)52)]Currently, several synthetic drugs that act as ACE inhibitors havebeen synthesized and are used to treat arterial hypertension inhumans, such as Captopril and Enalapril. Nevertheless,the identificationof natural sources that act as ACE inhibitors had also beenreported. Epidemiological studies have associated the consumptionof flavonoid-rich foods with reduced risk of cardiovasculardiseases and decreased blood pressure in humans.[53)54)]

Electrolytic Na^+ and K^+ are important in controlling extracellular fluid volume including blood. It has long been recognized that alternation of Na^+ distribution within the cells can be related to the trigger mechanism of hypertension. If the amount of Na^+ is increased within the cells, there can be change in cell volume followed by increased cell membrane Ca^{2+} and electric pressure. These changes cause an increase in blood vessel resistance and blood pressure. There are four Na^+ transport pathways; Na-K ATPase, Na-K cotransport, Na-(Li+) counter transport and Na-passive transport. Na-K ATPase is known to be sensitive and inhibited by a cardiac glycoside, ouabain and Na-K cotransport is known to be sensitive and inhibited by furosemide. Both ouabain and furosemide have natriuretic and diuretic effects, thereby being used for hypotensive drugs. Use of red blood cells for measurement of Na^+ efflux has great advantage, because it can be gotten easily.Na-K ATPase isused for the indicator of the study as the pathological adjunctive factors of diseases such as hypertension, arthritis andcardiac disorder.[55)~57)]Na-K ATPase in blood vessel and heartventricle was more suppressed in the hypertensive patients

than thenormotensive entity.[58)59)]

2. Prevention properties of polyphenols on cardiovascular disease

Epidemiologic studies suggest that consumption of fruits and vegetables has been linked with lower prevalence of coronary heart disease.[60)]Drinking tea has also been linked with reduced mortality arising from cardiovascular disease,[61)]although some epidemiological data are inconclusive.[62)]Fruits, vegetables and teas contain a wide range of antioxidant compounds, including phenolic compounds and vitamins. Phenolic compounds, such as anthocyanins, flavan-3-ols, flavonols, and tannins, are widespread in fruits and vegetables, with especially high quantities being found in berries and teas. Among the most well-known polyphenols are the flavonoids, and they have long been recognized to possess anti-inflammatory, antioxidant, anti-allergic, hepato-protective, anti-thrombotic, anti-viral, and anti-carcinogenic activities.[63)]

Tannins are a unique group of phenolic metabolites that are widely distributed in almost all plant foods and beverages.[64)]They are classified into two main groups, hydrolysable tannins and condensed tannins. The hydrolysable tannins[65)]are readily hydrolyzed by acids or enzymes into a polyalcohol and a phenolic carboxylic acid. Depending on the nature of the phenolic carboxylic acid, the hydrolysable tannins are usually subdivided into gallotannins and ellagitannins. Hydrolysis of gallotannins yields gallic acid while that of ellagitannins, hexahydroxydiphenic acid, which is isolated normally as its stable dilactone, ellagic acid (Figure 2). The condensed tannins or proanthocyanidins[64)]are polyflavonoids in nature, consisting of chains of

flavan-3-ol units. The most common class of proanthocyanidins are the procyanidins which consist of chains of catechin and/or epicatechin linked 4→6 or 4→8 (Figure 3).

Tannins exhibit a variety of bioactivities such as anti-inflammatory and anti-allergic properties,[66] anti-diabetic effect,[67] anticancer activity,[68] and antimicrobial and anti-viral properties.[69][70] Several studies have reported on the anti-oxidant, anti-mutagenic, and anti-inflammatory activities of ellagic acid.[71][72]

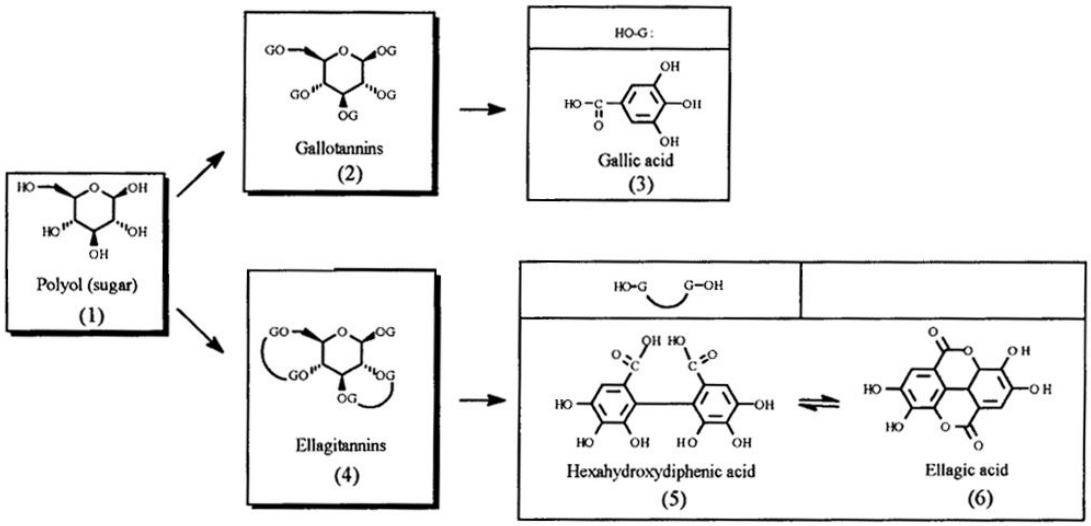


Figure 2. Hydrolysable tannins

The hydrolysable tannins are readily hydrolyzed by acids (or enzymes) into a sugar (1) or a related polyhydric alcohol and a phenolic carboxylic acid. Depending on the nature of the phenolic carboxylic acid, the hydrolysable tannins are usually subdivided into gallotannins (2) and ellagitannins (4). Hydrolysis of gallotannins yields gallic acid (3) while that of ellagitannins, hexahydroxydiphenic acid (5), which is isolated normally as its stable dilactone, ellagic acid (6) [From Garro Galvez *et al*,[73] copyright 1997Holzforschung]

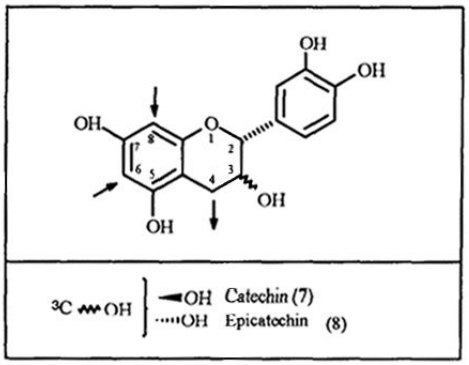


Figure 3. Precursor of condensed tannins

The condendsed tannins or proanthocyanidins are polyflavonoids in nature, consisting of chains of flavan-3-ol units. The most common class of proanthocyanidins are the procyanidins which consist of chains of catechin (7) and/or epicatechin (8) linked 4→6 or 4→8. [From Garro Galvez *et al*,[73] copyright 1997Holzforschung]

Gallic acid is one of the constituents of hydrolysable tannins and possesses anti-carcinogenic, anti-oxidative, anti-allergic, and anti-inflammatory activities.[74)]Tannin with gallate group has various physiological functions, such as anti-bacterial, anti-allergic, scavenging free radical, lowering blood pressure and serum and hepatic cholesterol concentrations and increasing fecal sterol excretion in rats with hypercholesterolemia.[75)]Hydrolyzable gallotannins from *Rhus coriaria* (Sumac) show anti-ischemic activity and endothelium-dependent vasorelaxant effect in isolated rabbit heart and thoracic aorta.[76)]

Condensed tannins are known to have antioxidant properties.[77)]Yokozawa *et al*.[78)]fed rats with condensed tannins and found that lipid peroxidation in plasma and tissues decreased significantly in the presence of the supplemented polymeric tannins, and the antioxidant effect of condensed tannins was as effective as that of vitamin E. The lower incidence of coronary heart disease in France has been linked to the high consumption of proanthocyanidin-rich red wine, since proanthocyanins possess long lasting antihypertensive and vasorelaxing properties linked to endothelium-related factors in normotensive and hypertensive rats, in which nitric oxide is involved.[79)]A diet rich in flavanols and procyanidins can improve oxidant defense and reduce tissue markers for oxidative stress, although these effects can be tissue specific.[80)]As a result, tannins have recently attracted attention for their potential cardiac protective properties.[81)]Chestnut inner shell also contains relatively high amount of polyphenols, with gallic acid, ellagic acid, and catechin predominant among hydrolysable and condensed tannins.[82)]

3. Potential possibility of utilization of chestnut inner shell as bio-resource

The chestnut belongs to the genus *Castanea* in the *Fagaceae* family and 13 *Castanea* species in total are recognized. They are native to warm temperate areas of the Northern Hemisphere, but many countries cultivate different varieties of chestnut mainly for fruit as *Castanea crenata Sieb.et Zucc.*(Japan and Korea), *Castanea mollissima Blume* (China), *Castanea sativa Mill.*(Europe), and *Castanea dentate Borkhausen* (America). All *Castanea* species and their hybrids are edible and some are commercialized as nut products around the world.

Chestnut trees have been cultivated long time ago in Korea and chestnut fruit are used in traditional Korean ceremonies. Chestnuts are still consumed as a favorite food because of their nutritional values. Chestnuts are not only excellent energy source due to its high starch content, but also good food source containing unsaturated fatty acids and polyphenolic compounds. Several studies have investigated the presence of bioactive antioxidant molecules, not only in edible kernels of chestnut,[82)~84)]but also in chestnut waste.[82)86)]There is strong evidence that polyphenols provide protection against harmful effects of free radicals and are known to reduce the risk of several diseases including certain types of cancer, coronary heart disease (CHD), type-2 diabetes, and inflammation. The action of polyphenols on health is also to protect against environmental stresses.[87)]The fruit of chestnut is typically consumed whole (raw, boiled, or roasted, without skin) or used as ingredient in a variety of processed foods, especially in bakery and confectionery products. From the processing of chestnut productions, large amount of inedible byproduct is generated, representing about 8~14% of outer shell and 6~10% of inner shell.

In 2005, Son *et al*.[88)]foundthat the extract of chestnut inner shell contains a

relatively high amount of tannins, which are well-known polyphenolic antioxidants. Barreira *et al.*[82]analyzed the phenolic contents in chestnut leaves, flowers, skins and fruits, and then reported that contents of polyphenols and flavonoids showed in the following order:outer skins > inner skins > flowers > leaves > fruits.The other study on the chestnut shell, also showed high content of total phenolics as well as a good antioxidant activity in comparison with the results from the same analysis performed with eucalyptus bark samples.[89]Despite these reports, the byproducts of chestnut, including inner shell, are usually discarded without recycling. The other hand, extraction yields of chestnut shell with boiling water were higher in inner shell (21.6%) than outer shell (4.98%), therefore the resource recycling with chestnut inner shell is more effectively.

According to trade trends of annual agricultural products in Korea rural economic institute, Korea's chestnut harvests were about 70,000 tons, and 15~25% are exported, the remaining is consumed in the nation. A large amount of inner shell is discarded from chestnut production processing. Hence, if bioactive materials which obtained from chestnut inner shell by extraction or fraction can re-product as medicinal materials or health supplements, the results can enhance public health with the economic benefit.

PART Ⅰ

In vivo study for effects of chestnut (*Castanea crenata*) inner shell on antioxidant and cardiovascular-related parameters

ABSTRACT

The first part of this study focused to compare *in vivo* effects of simvastatin versus chestnut inner shell on cardiovascular-related parameters and CoQ10 versus chestnut inner shell on antioxidant-related parameters using guinea pigs.

Fifty guinea pigs were divided into five groups and fed one of the following diets; 0.25% cholesterol-based control diet, control diet plus 10% chestnut inner shell powder (Pch), control diet plus 0.03% simvastatin (STN), control diet plus 0.03% simvastatin and 0.3% CoQ10 (STN + CoQ10), and control diet plus 0.03% simvastatin and 3% ethanol extract of Pch (STN + Ech) for four weeks.The final weights significantly decreased in all groups fed statin-containing diet, compared to cholesterol-fed control group, which is paralleled with the pattern of significant decreasing in average daily feed intake (ADFI) ($P< 0.01$ for both final body weight and daily feed intake). Plasma total cholesterol were significantly lower in the statin plus CoQ10 group than those of other groups ($P< 0.01$). Liver total cholesterol decreased significantly in groups of statin and statin plus CoQ10 compared with that of the control ($P< 0.05$). Hematocrit was significantly higher in the statin plus CoQ10 group than that of Pch group ($P< 0.05$). GOT increased and GPT decreased significantly in all groups fed statin diets compared with those of the control ($P< 0.01$, $P< 0.05$, respectively). Erythrocyte Na-K ATPase and intracellular Na and K in groups of Pch and statin decreased significantly compared to those of the control (all, $P< 0.01$) with no difference in ACE of lung tissue between groups. Na-K cotransport and Na passive transport decreased significantly in group of Pch compared with the control or statin group ($P< 0.05$,$P< 0.01$, respectively). Na leak in 2,2-azobis-(2-

amidinopropane) dihydrochloride (AAPH) treated erythrocyte decreased significantly in groups of statin plus CoQ10 and statin plus Ech compared with that of statin alone ($P< 0.01$). TBARS productions in liver and platelet rich plasma increased in statin group and decreased in group of statin plus CoQ10, and the difference between these two groups was significant ($P< 0.01$). Present study showed thatstatin or chestnut inner shell which contains high amount of tannins did not affect plasma cholesterol. Statin with CoQ10 decreased cholesterol and triglyceride more efficiently than statin alone, assuming synergic effects of CoQ10 with statin. Ethanol extract of chestnut inner shell and CoQ10 seemed to enhance thepositive effects of statin by reducing oxidative stress and cell damage, thereby protecting liver and cardiovascular systems from CVD such as atherosclerosis.

MATERIALS AND METHODS

1. Materials

1) Animals and diets

Fifty of six weeks old guinea pig (Orient Bio Co Ltd, Gapyung, Korea) were divided into five groups and fed the following diets : 0.25% cholesterol-based control diet; control diet plus 10% chestnut (*Castanea crenata*)inner shell powder; control diet plus 0.03% simvastatin (20 mg/kg BW); statin plus 0.3% CoQ10 (200 mg/kg BW); statin plus 3% ethanol extract of chestnut inner shell powder. Doses in detail for chestnut inner shell (herbal medicine shop in Jeju), CoQ10 (Yunjin Pharm Co, Korea) and simvastatin (Choongwae Pham Co, Korea) are in diet composition (Table 1-1). Diet was pelletized by the assistance of Korea Food Research Institute. Guinea pig had free access to water and were housed individual cages in a room maintained at 20 ~ 25℃ with a 12-hour dark-light cycle.

(1) Preparation of chestnut inner shell extract

Chestnut inner shell powder was purchased from herbal medicine shop in Jeju. Chestnut inner shell powder (4 kg) were extracted with 12ℓ of 80% ethanol (EtOH) three times for 24 hours each at room temperature and filtered. The filtrate was then concentrated *in vacuo* at 40℃. Residual ethanol was removed by freeze dryer. The yields of the chestnut inner shell powder extrat (Ech) was 526.5 g (13.16%). The prepared extracts were stored at -20℃until further examined.

Table 1-1.Diet composition (%)

Components	Control	Pch	STN	STN+CoQ10	STN+Ech
Soy protein	21.5	21.5	21.5	21.5	21.5
Methionine	0.5	0.5	0.5	0.5	0.5
Sucrose	21	21	21	21	21
Corn Starch	20	17	20	19.7	17
Fat mix[1)]	15	15	15	15	15
Cellulose	10	3	10	10	10
Guar gum	2.5	2.5	2.5	2.5	2.5
Mineral Mix[2)]	8.2	8.2	8.2	8.2	8.2
Vitamin Mix[2)]	1.1	1.1	1.1	1.1	1.1
Cholesterol	0.25	0.25	0.25	0.25	0.25
Simvastatin[3)]	-	-	0.03	0.03	0.03
CoQ10[3)]	-	-	-	0.3	-
Pch[4)]	-	10	-	-	-
Ech[5)]	-	-	-	-	3
Total	100.05	100.05	100.08	100.08	100.08

[1] Fat mix contains olive oil, palm oil, and safflower oil (1:2:1.8), high in lauric and myristic acids

[2] Mineral and vitamin mix adjusted to meet NRC requirements for guinea pigs. Detailed composition of the vitamin and mineral mix has been reported elsewhere.[90)]

[3] Simvastatin, Choongwage Pharma Co. Korea; 0.03% statin equivalent to 20mg/kg BW/day.
0.3% CoQ10 (Yungjin Pharm Co) equivalent to 200mg/kg BW/day when calculated from the daily foodconsumption of 40g/day

[4] Pch : Chestnut inner shell powder

[5] Ech : Chestnut inner shell extract (extracted with 80% ethanol)

2. Collection of samples

After 4 weeks ad libitum feeding, guinea pigs were anesthetized with ether after fasted for 12 hours, and blood samples were obtained by cardiac puncture into heparinized vacuum tubes. Whole blood platelet aggregation, hematocrit, and erythrocyte Na efflux were performed with fresh blood.

The Platelet rich plasma (PRP) was obtained by centrifugation at 300 × g for 10 minutes and stored at -70°C for TBARS analysis. The plasma samples were obtained by centrifugation at 1,000 × g for 10 minutes and stored at -70°C for lipid profiles, glutamic oxaloacetic transaminase (GOT), and glutamic pyruvic transaminase (GPT) analysis.

The liver and lung samples were quickly removed and measured weight, then stored at -70°Cfor later assays.

3. Sample analysis

1) Whole blood platelet aggregation

Platelet aggregation was measured using a Chronolog Whole Blood Aggregometor (model 500-Ca, Havertown, Pennsylvania, USA). Fresh whole blood was diluted with isotonic saline (1: 4) to give platelet concentration of approximately 200,000 platelets/μℓ. Adenosine diphosphate (ADP, 2μM; Chronolog, Havertown, Pennsylvania, USA) was added to initiate aggregation, and three readings of impedance changes were averaged for each guinea pig to determine the maximum

aggregation and the initial slope. The instrumental principle is based on the increase in impedance (Ω) across two platinum electrodes as platelet aggregation proceeds.

2) Plasma and liver lipid assay

Plasma total cholesterol, HDL-cholesterol and triglyceride were assayed using enzymatic kits (Asan Pharmaceuticals Co., Ltd, Seoul, Korea). Ten μℓ of plasma was used for the assays of total cholesterol and triglyceride For HDL cholesterol, 200 μℓ plasma was incubated with dextran sulfate to precipitate apo B containing lipoprotein, and 50 μℓof the supernatant was used.

Solvents for liver extract were supplied by Merck (Merck KcaA, Darmstadt, Germany). Liver lipids were extracted by a modified Folch method.[91]One gram of liver tissue was homogenized for 5 min in 6mℓ of Folch solution [chloroform (2): methanol (1)] and 2 mℓ H_2O. After centrifugation for 10 minutes, the lower phase that contains liver lipids was separated. Lower phase of lipid fractions was assayed after treating with tritonX-100 : chloroform (25 μℓ : 475 μℓ) for total cholesterol or with methanol for triglyceride, using enzymatic kits (Asan Pharmaceuticals Co., Ltd, Seoul, Korea).

3) Platelet rich plasma (PRP) and liver TBARS productions

Platelet rich plasma (PRP) were obtained after centrifuging whole blood at 300 × g for 10 minutes. PRP TBARS (Thiobarbaituric acid reactive substance) production was measured with a modified Buege and Aust method.[92]An half mℓ PRP in 1.5

mℓPBS (Phosphate buffered saline) was incubated at 100°Coil bath for 20 minutes after adding 2 mℓTBA solution (15g TBA, 0.139g TCA, 1.81mℓ 12N HCl in 85mℓ D-H_2O). After cooled under tap water, the incubated mixture was centrifuged at 1,000 × g for 10 minutes. Finally the TBARS in supernatant was measured with the absorbance at 532 nm on spectrophotometer using deionized H_2O as blank.

Buege and Aust method[92]was also used for the liver TBARS. One gram of minced liver sample in 2 mℓ PBS was incubated at 100°C oil bath for 20 minutes after adding 2 mℓ TBA or non-TBA solution (0.139 g TCA, 1.81 mℓ 12N HCl in 85mℓ D-H_2O). The incubated mixture was cooled down and centrifuged at 1,000 × g for 10 minutes. The TBARS in supernatant was measured of spectrophotometer at 532 nm using deionized H_2O as blank. All samples were performed in duplicate and the values ofTBARS was the outcome subtracting the concentration of thenon-TBA treated from the TBA treated.

The liver protein was measured with Lowry *et al.*[93]method using bovine serum albumin (BSA) as standard protein. The absorbance for protein concentration was read at 750 nm with spectrophotometer using D-H_2O as blank.

4) Erythrocyte Na efflux channels

(1) Red cell preparation

Chemicals for mediums including ouabain, furosemide and MOPS were purchased from Sigma chemical company (MO, USA). Blood was centrifuged at 1,000 × g for 10 minutes, and the plasma and buffy coat were removed. Red blood cells were washed 5 times with a cold isotonic washing solution [150 mM choline chloride, 10

mM Tris-4 morpholinopropane sulfonic acid (MOPS), pH 7.4 at 4 °C], and centrifuged at 1,000 × g for 5 minutes after each wash. The RBC pellet was resuspended in the choline chloride washing to give 40~50% hematocrit, then hematocrit was measured.The RBC suspension was kept in ice for measurement intracellular Na and K concentration, Na-K ATPase, Na-K cotransport, and Na-passive transport.

(2) Na-K ATPase activity

Ouabain sensitive Na-K ATPase can be blocked by ouabain, and Na^+ efflux via Na-K ATPase is calculated from the difference between the efflux into $MgCl_2$ medium with and without ouabain.

Fourmℓeach of erythrocyte was added to 40 mℓ medium 1 [70mM $MgCl_2$, 10mM KCl, 85mM sucrose, 10mM glucose, 10mM Tris-MOPS, pH 7.4 at 37°C] and medium 2 [medium 1 plus 10mM ouabain] then mixed gently and aliquot 10 tubes. The tubes were transferred in duplicates to an ice bath after incubation at 37°C in a shaking water bath for 0, 2, 4, 6, 8 minutes. The tubes were subsequently centrifuged at 1,000 × g for 10 minutes, the supernatant was removed.

(3) Na-K cotransport

Furosemide sensitive Na-K cotransport can be blocked by furosemide, and Na efflux via Na-K cotransport is calculated from the difference between the efflux into choline chloride medium with and without furosemide.

Twomℓ each of erythrocyte was added to 30 mℓ medium 3 [150mM choline chloride, 1.0mM ouabain, 10mM glucose, 10mM Tris-MOPS, pH 7.4 at 37℃] and

medium 4 [medium 3 plus 0.3308g/ℓ furosemide] then mixed gently and aliquot 10 tubes. The tubes were transferred in duplicates to an ice bath after incubation at 37°Cin a shaking water bath for 0, 10, 20, 30, 40 minutes. The tubes were subsequently centrifuged at 1,000 × g for 10 minutes, the supernatant was removed for determining Na concentration.

(4) Na-passive transport

Na-passive transport is the efflux into choline chloride medium containing ouabain and furosemide under condition of both Na-K ATPase and Na-K cotransport blocked.

For Na-passive transport measurement, erythrocyte was added medium 4 [150mM choline chloride, 1.0mM ouabain, 10mM glucose, 0.3308g/ℓ furosemide, 10mM Tris-MOPS, pH 7.4 at 37°C].

(5) Intracellular Na and K concentration

For intracellular Na^+ concentration measurement, 50μℓ of the RBC suspension was added to 5 mℓ of 0.02% acationox (a metal free detergent, Scientific products, McGraw Park, Illinois, USA) and Na concentration was measured using atomic absorption spectrophotometer (AAS, AA6701F Shimazu Co., Japan). For intracellular Kmeasurement, RBC suspension in acationox was diluted with distilled water in 1 to 10 and K concentration was measured using AAS.

(6) Erythrocyte Na-leak

Sodium leak is defined as sodium efflux through passive sodium channels occurring under inhibition of ouabain-sensitive Na-pumps and furosemide-sensitive

Na-K cotransport. Sodium leak can be increased through damage of erythrocyte membranes after exposure to a free radical generating system such as 2,2-azobis-(2-amidinopropane)dihydrochloride (AAPH).

All mediums and tubes were kept and handled in ice, and Na concentrations from Na efflux channels (Na-K ATPase, Na-K cotransport, Na-passive transport) were measured using AAS (AA6701F Shimazu Co., Japan).

[Calculations]

- Na efflux ;

$$\frac{[\text{Na } \mu g/m\ell]}{[\text{min}]} \times \frac{[60\text{min}]}{[\text{hr}]} \times \frac{[\mu\text{mole}]}{[23\ \mu g]} \times \frac{[44m\ell-(4\ m\ell\times\text{Hct})]}{[4m\ell\times\text{Hct})]} = \text{Na mmole}/\ell\text{RBC}\cdot\text{hr}^{-1}$$

- Intracellular Na ;

$$\frac{[\text{Na } \mu g]}{[m\ell]} \times \frac{[\mu\text{mole}]}{[23\ \mu g]} \times \frac{[101]}{[\text{Hct}]} = \text{Na mmole}/\ell\text{RBC}$$

- Intracellular K ;

$$\frac{[\text{K } \mu g]}{[m\ell]} \times \frac{[\mu\text{mole}]}{[39\ \mu g]} \times \frac{[101]}{[\text{Hct}]} = \text{Na mmole}/\ell\text{RBC}$$

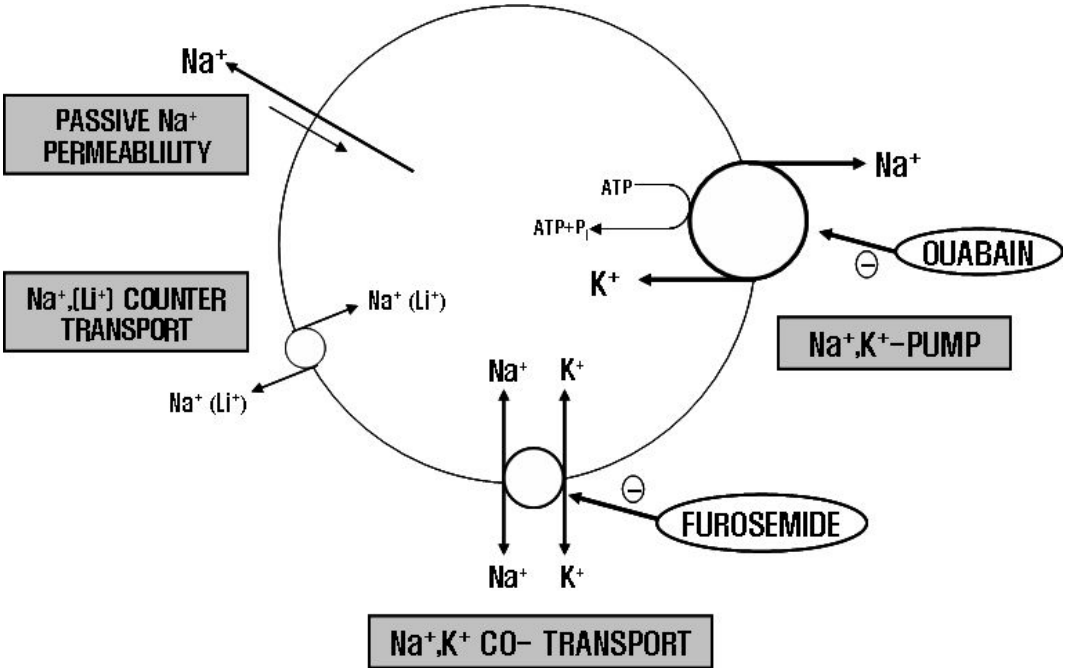


Figure 1-1. Model of erythrocyte Na efflux channels

5) Plasma GOT and GPT levels

The plasma glutamic oxaloacetic transaminase (GOT) and glutamic pyruvictransaminase (GPT) was measured with a spectrophotometric diagnostic kit purchased from the Asan pharmaceutical Company (Seoul, Korea). Briefly, 1 mℓ GOT or GPT substrate solution was incubated at 37°Cshaking water bath for 5 minutes and 0.2 mℓ plasma sample was added. After GOT or GPT incubated at 37°Cfor 60 minutes and 30 minutes respectively, 1 mℓ of color reagent was added to each of samples and incubated for 20 minutes at room temperature. After incubation, 10 mℓ 0.4 N NaOH was added mixed well, incubated for 10 minutes at room

temperature. The absorbance at 505 nm was read, using spectrophotometer and results are expressed as IU/ℓ.

6) Lung angiotensin converting enzyme (ACE) activity

ACE activity was determined modified methods of Cushman and Cheung.[94)]Tissue ACE activity is determined by measuring hippuric acid (HA) released from hippuryl-L-his-tidyl-L-leucine (HHL) in incubation medium. Two hundreds mg of lung tissue was homogenized with 2mℓ 20 mM phosphate buffer (1M K_2HPO_4, plus 1M KH_2PO_4, pH 8.3) containing 0.01% tritonX-100 and centrifuged at 600 × g for 10 minutes. Supernatant containing membrane bound ACE was transferred to a clean tube. Total 250μℓ medium including 10μℓ supernatant, 100 μℓ 5 mM HHL, 190μℓ100 mM K-phosphate buffer with 30 mM NaCl in effendorf tube was incubated at 37°Cfor 30 minutes, then reaction was stopped by 500 μℓ1 N HCl and medium was centrifuged at 1,000 × g for 10 minutes. Supernatant of 500 μℓwas transferred to a glass tube containing 1.5 mℓ ethyl acetate, vortexed for 15 seconds and centrifuged at 1,000 × g for 5 minutes. One mℓ of upper ethyl acetate layer was transferred to a new tubeand dried for 30 minutes on 120°C heating block. Dried HA was dissolved in deionized waterand read the absorbance at 228 nm on UV spectrophotometer using each blank prepared with the same procedure without HHL in incubation medium. Standard HA was prepared in the same procedure as tissue samples and ACE activity is expressed as HA in μmole/mg lung tissue.

4. Statistical analysis

Values were analyzed using the SPSS version 18.0 (SPSS, Inc Chicago, IL, USA). Analysis of variance were conducted in a completely randomized block design. Duncan's multiple test was applied to compared individual means when F-value was significant. A value of $P< 0.05$ was considered significant.

RESULTS

1. Body weight, plasma and liver lipids

Final body weight, average daily feed intake (ADFI), and liver weight/body weight ratio (L.W/B.W) are shown in Table 1-2. Body weight increased in the Pch group and the control group with no weight gain in all groups fed statin containing diet for 4 weeks, the final body weight was significantly different between the control and any of statin groups. Similarly, average daily feed intake (ADFI) increased in Pch group and decreased in the other groups. There was no difference in liver weight / body weight (L.W/B.W) ratio among groups. Considering the proper weight gain in animals, chestnut inner shell is safe as diet material and can be used for complementary medicine.

As listed in Table 1-2, Pch or statin alone rather increased plasma cholesterol compared to the control showing no cholesterol lowering effect. Statin plus CoQ10 effectively decreased plasma total cholesterol and the difference between group with statin plus CoQ10 and any of other groups was statistically significant ($P < 0.01$).No difference was observed in plasma HDL cholesterol among groups, and plasma triglyceride levels somewhat decreased in groups of the Pch and statin plus CoQ10, but the difference was not significant.

Liver cholesterol levels of the statin and statin plus CoQ10 groups were significantly lower than that of control ($P < 0.05$) (Table 1-2). Groups of Pch and statin plus Ech tended to be lower in liver cholesterol levels than the control group. All groups fed diets containing statin decreased in liver triglyceride with the lowest

in statin plus CoQ10 group, but not statistically different. In present study with guinea pigs, statin at dose of 20 mg/kg BW decreased liver cholesterol and triglyceride without affecting plasma total cholesterol and HDL-cholesterol. Statin with CoQ10 was more effective in cholesterol lowering than statin alone.

Table 1-2. Effects of chestnut inner shell powder (Pch) and extract (Ech), statin and coenzyme Q10 on body weight and plasma and liver lipid in rats fed with cholesterol-based diet

	Control	Pch	STN	STN+CoQ10	STN+Ech
Final B.W(*g*)**	433.6±37.1[a]	492.2±47.8[a]	351.7±42.9[b]	329.5±59.3[b]	369.3 ± 44.1[b]
ADFI[1)](*g/d*)**	20.2±2.3[a]	22.1±4.0[a]	15.4±2.1[b]	13.9± 4.1[b]	18.4±2.8[ab]
L.W/B.W[2)](%)	4.55±0.63	4.77±0.61	4.55±0.62	4.77±1.10	4.76±0.80
Plasma (mg/dℓ)					
Total-cholesterol**	104.5±22.4[a]	112.8±20.3[a]	120.9±28.2[a]	79.8±20.5[b]	105.8±17.1[a]
HDL-cholesterol	15.3±1.2	15.4±0.6	15.7±1.4	15.5±1.3	15.4±1.0
Triglyceride	83.7±25.3	73.7±14.6	84.1±14.1	73.7±12.9	80.4±12.2
Liver (mg/g)					
Total-cholesterol*	15.2± 5.8[a]	12.4±4.7[ab]	9.3±1.7[b]	9.3±2.4[b]	11.1±2.7[ab]
Triglyceride	62.4±13.7	61.9±11.6	56.5±7.7	49.3±17.2	61.5±15.6

[1)]ADFI : Average daily feed intake

[2)] L.W/B.W : Liver weight / Body weight ratio

Values are means ± SD of 9 guinea pigs.

* Values in the same row not sharing the same superscript differ ($P< 0.05$).

** Values in the same row not sharing the same superscript differ ($P< 0.01$).

2. Hematocrit and whole blood platelet aggregation

Hematocrit and platelet aggregation are shown in Figure 1-2. Hematocrit was increased in groups with statin and decreased in Pch group, showing statistical difference between Pch and statin plus CoQ10($P< 0.05$).

Maximum of platelet aggregation tended to decrease in groups of Pch and statin plus CoQ10 and tended to increase in group of statin. Initial slope of platelet aggregation decreased in all experimental groups compared with the control, but not statistically different. Both of the maximum and initial slope of platelet aggregation were the lowest in Pch group.

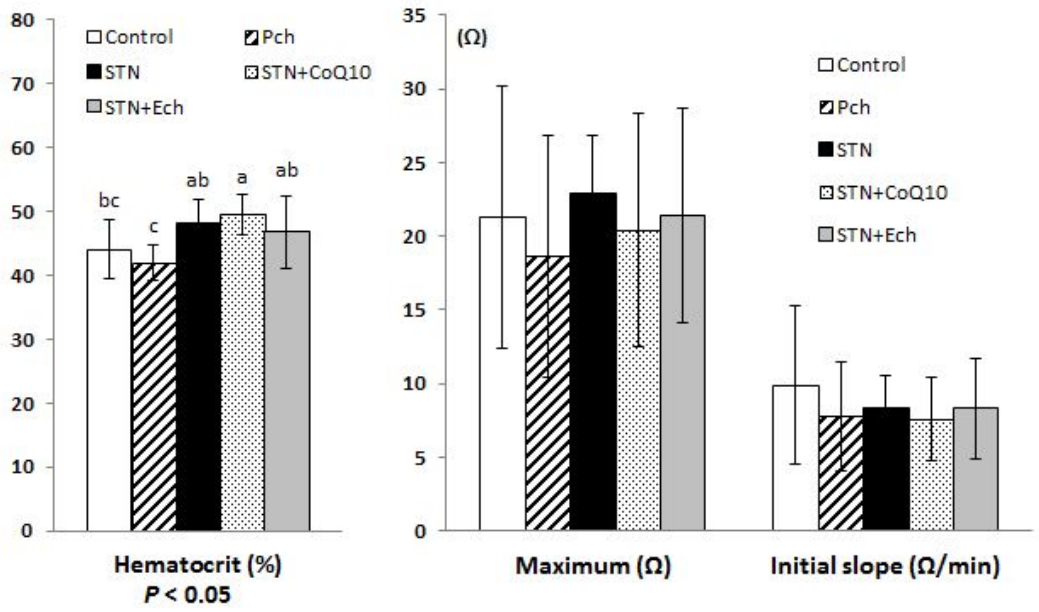


Figure 1-2. Effects of chestnut inner shell powder (Pch) and extract (Ech), statin and coenzyme Q10 on the hematocrit and platelet aggregation in rats fed with cholesterol-based diet

Maximum aggregation in ohm at the point where platelet aggregate is dissociated.
Initial slope is the ohm change for the first one minute of aggregation.
Values are mean ± SD of 9 guinea pigs.

3. Plasma GOT and GPT levels

Plasma glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) are shown in Figure 1-3. Plasma GOT increased in groups of statin, statin plus CoQ10, and statin plus Ech, and the differences between these groups and Pch group were significant ($P< 0.01$). Unlikely, plasma GPT decreased in all groups fed statin containing diets, and the difference between these groups and the control were significant ($P< 0.05$).

GOT is present in most organs including liver, heart, skeletal muscle, kidney, brain and lung, while GPT located predominantly in liver with significant quantity. They are released into the blood when cells are damaged. Even though they changed in range of normal levels, high GOT with low GPT in present study can hardly be explained.

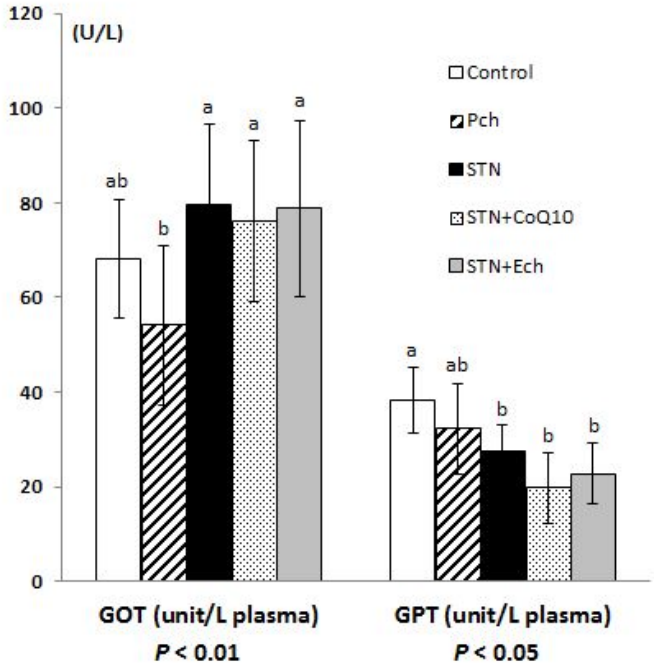


Figure 1-3. Effects of chestnut inner shell powder (Pch) and extract (Ech), statin and coenzyme Q10 on plasma GOT and GPT levels in rats fed with cholesterol-based diet

GOT; plasma glutamic oxaloacetic transaminase
GPT; plasma glutamic pyruvic transaminase
Values are mean ± SD of 9 guinea pigs.

4.Erythrocyte Na efflux and ACE inhibitory effect

Intracellular Na and K concentrations and erythrocyte Na efflux channels are shown in Figure 1-4. Intracellular Na and K showed the similar tendency with decrease in groups of Pch and statin compared to the control. Both of intracellular Na and K levels were significantly decreased in the statin group compared with those in the control ($P< 0.01$). The major efflux channel, Na-K ATPase decreased in groups of Pch and statin compared with the control, which were the same trend with intracellular Na and K concentrations. Group of Pch consistently decreased in both Na-K cotransport and Na passive transport and the differences between Pch and the control were significant ($P< 0.05$, $P< 0.01$ respectively). Total Na efflux in group of Pch was significantly low compared to the control group ($P< 0.01$). Group of statin significantly decreased in Na-K ATPase ($P< 0.01$) and Na passive transport compared to the control, resulting an overall decrease in total Na efflux compared to the control group. Total Na efflux was mostly contributed by Na-K ATPase channel, consequently depending on Na-K ATPase activity. Angiotensin converting enzyme (ACE) activity which is involved in Na transport channels was measured to examine the correlations between ACE and Na channels (Figure 1-5). ACE activity of lung tissue in group of Pch increased compared with the control and group of statin, but not statistically different.

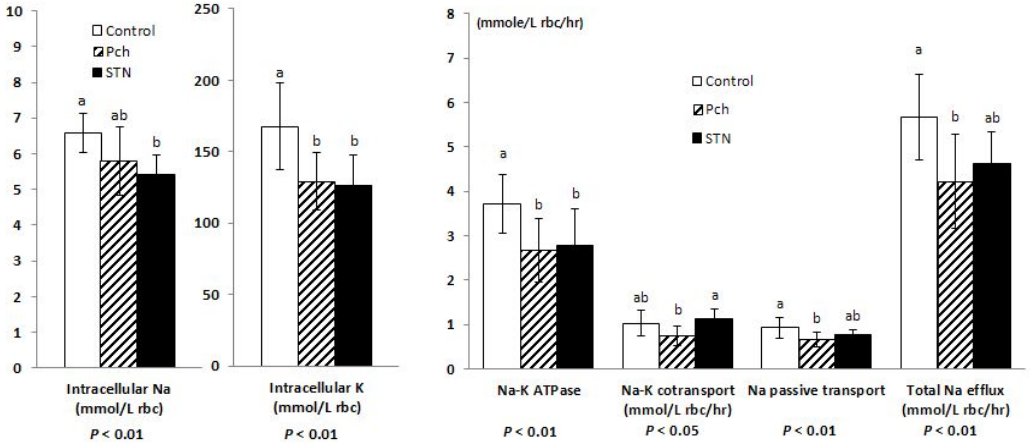


Figure 1-4. Effects of chestnut inner shell powder (Pch) and statin on erythrocyte sodium efflux in rats fed with cholesterol-based diet

Intracelluar Na and K; upper values are for intact red blood cells.
Na-K ATPase is ouabain sensitive Na efflux.
Na-K cotransport is furosemide sensitive Na efflux.
Na passive transport is Na efflux under the blockage of Na-K ATPase and Na-K cotransport.
Values are mean ± SD of 9 guinea pigs.

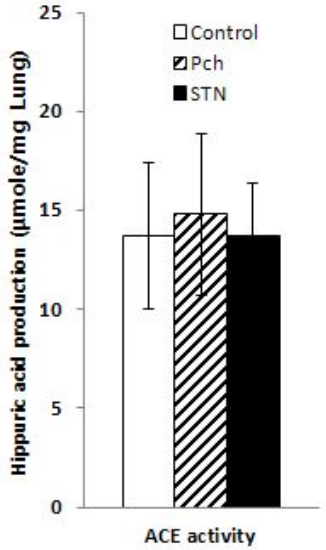


Figure 1-5. Effects of chestnut inner shell powder (Pch) and statin on angiotensin converting enzyme activity in rats fed with cholesterol-based diet

The angiotensin converting enzyme (ACE) activity expressed as hippuric acid (HA) concentrated released from HHL (2mM/mg lung tissue) using 0.2mg lung tissue. Values are mean ± SD of 9 guinea pigs.

5. Erythrocyte Na-leak

Depletion of reduced form ubiquinol by statin therapy leads to a loss of antioxidant capacity and erythrocyte membrane stability. Accordingly, Na passive leak through erythrocyte membrane after exposure to free radical generating system such as AAPH [2,2'-azobis(2-amidinopropane)dihydrochloride] was measured as parameter.

Assessment of Na leak lowering effects by erythrocyte membrane protection was performed among the groups with statin. In present study, Na leak through the erythrocyte are observed effects of statin plus coenzyme Q10 and chestnut inner shell ethanol extract compared with the statin only.

Intracellular Na and K, and Na passive leak in intact cells are shown in Figure 1-6. Intracellular Na and K in intact cells were not different. Na passive leak in intact cells was not also different among the groups.

Na leak in AAPH treated cells was significantly increased in statin control group but decreased in statin plus CoQ10 and statin plus chestnut inner shell extract groups, and there was significant difference ($P< 0.01$).

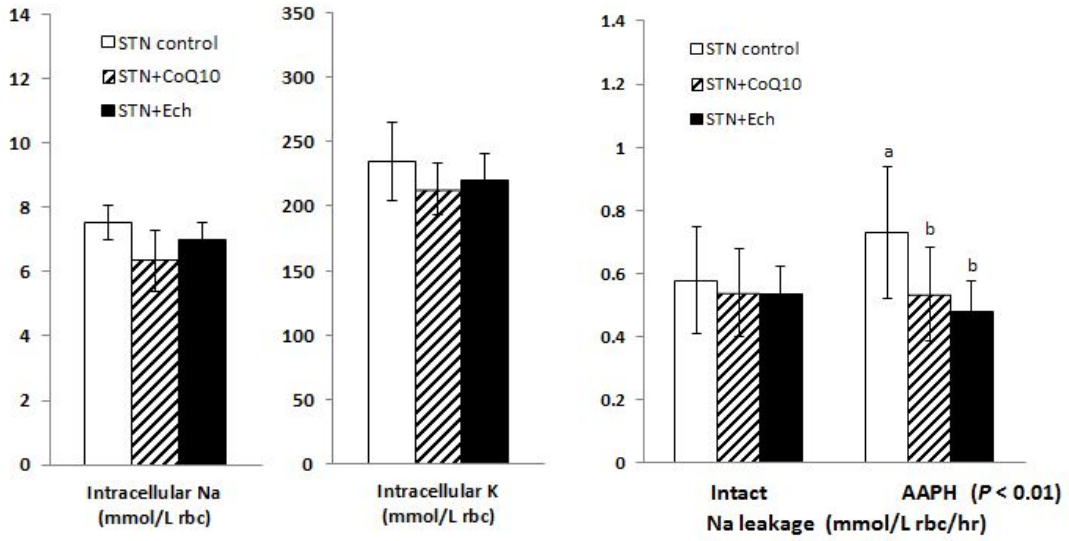


Figure 1-6. Effects of statin, coenzyme Q10, and chestnut inner shell extract (Ech) on intact and AAPH treated erythrocyte Na-leak in rats fed with cholesterol-based diet

Intact Na-leak is erythrocyte Na efflux through passive pathway in intact red blood cells.
AAPH; Na leakage is erythrocyte Na efflux through passive pathway in AAPH [2,2'-azobis(2-amidino-propane) dihydrochloride] treated red blood cell.
Values are mean ± SD of 9 guinea pigs.

6. Platelet rich plasma (PRP) and liver TBARS productions

Thiobarbituric acid reactive substance (TBARS) productions in liver and platelet rich plasma (PRP) are shown in Figure 1-7. TBARS are formed as a byproduct of lipid peroxidation and can be measured as productions of the damage produced by oxidative stress, because reactive oxygen species (ROS) have extremely short half-lives.

Liver TBARS production decreased in all experimental groups compared to the control group, showing statistical difference between the control and statin plus CoQ10 or the control and the statin plus Ech (both $P< 0.01$).

However, PRP TBARS productions tended to increase in groups of statin and statin plus Ech, showing statistical difference between the control and statin or the control and statin plus Ech (both $P < 0.01$). There was no difference between the control and Pch or statin plus CoQ10 in PRP TBAR production.

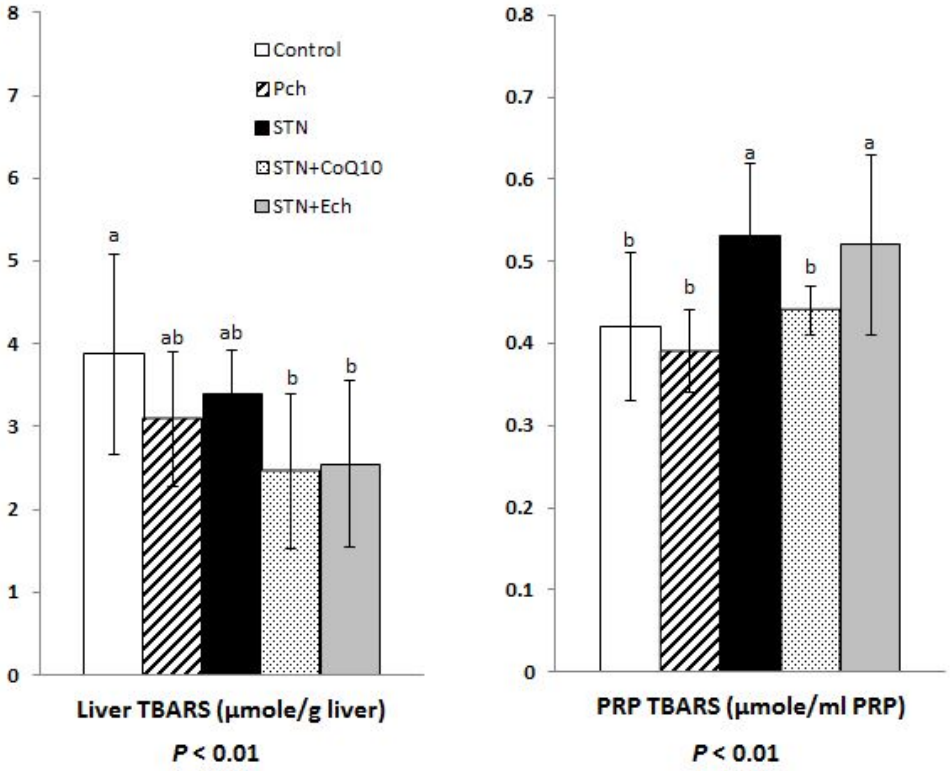


Figure 1-7. Effects of chestnut inner shell powder (Pch) and extract (Ech), statin and coenzyme Q10 on platelet rich plasma (PRP) and liver thiobarbituric acid reactive substance (TBARS) in rats fed with cholesterol-based diet

Platelet rich plasma (PRP) was obtained after centrifuging whole blood at 300 X g for 10 minutes.
Values are mean ± SD of 9 guinea pigs.

DISCUSSION

In present study, guinea pigs fed chestnut inner shell containing diet showed normal growth rate and feed intake, suggesting that chestnut inner shell in its dietary composition is safe and can be a useful source for complementary alternative medicine. Unlike normal growth in rat study,[95]guinea pigs fed 0.03% simvastatin containing diet had low growth rate with low feed intake in present study. Similarly, West *et al*.[96]reported that final weights of guinea pigs fed 0.05% simvastatin diet containing bile absorption inhibitor (SC-435) were significantly lower than those of the other groups. WT (wild type) mice and apo E knockout mice fed 0.5 mg/kg BW rosuvastatin did not have normal weight gain during 18 weeks experimental period.[97]Statin with CoQ10 seemed to further suppress growth rate with decreased feed intake in present study. Although NOAEL dose (No Observed Adverse Effect Level) of CoQ10 is reported to be 600 mg/kg BW in male rats[98]and low dose of CoQ10 (15 mg/kg BW) induced normal growth rate in rats.[95]In present study, high dose of CoQ10 (200 mg/kg BW) seemed to negatively influence on weight gain in guinea pigs when it was fed with statin. Although many studies have reported that the supplementation of CoQ10 is necessary for statin user in antioxidant view point, further studies are needed to confirm the beneficial effects of the combined therapy of statin and CoQ10. High doses of statin and CoQ10 may have metabolic impact affecting appetite, feed intake and growth rate. In present study with guinea pigs, statin of decreased liver cholesterol and triglyceride without affecting plasma total cholesterol and HDL-cholesterol. In rat studies, high simvastatin of 30 mg/kg BW did not decrease liver and serum cholesterol[95]and low simvastatin of 2 mg/kg BW

even increased in serum cholesterol.[99]Grothusen *et al.*[100]reported that atorvastatin of 1 mg/kg BW in ApoE (-/-) mice tended to increase serum total cholesterol and LDL-cholesterol levels. Unlike these results, Aggawal *et al.*[101]reported that high atorvastatin (0.05 % or 30 mg/kg BW) in guinea pigs significantly decreased serum cholesterol and triglyceride. In present study, statin at dose of 20 mg/kg BW did not decrease plasma cholesterol, but decreased liver cholesterol. Statin plus CoQ10 (200 mg/kg BW) decreased plasma and liver cholesterol as well as liver triglyceride more efficiently.

One of the most serious side effects from statin use is the exhaustion of CoQ10 which shares the cholesterol biosynthetic pathway. The alternative plan that is minimized these side effect would be to prescribe statin with CoQ10. In present study showed that supplemented CoQ10 seemed to potentiate hypocholesterolemic and hypolipidemic effects of statin, particularly lowering effect of plasma and liver triglyceride levels. Statin, a HMG-CoA reductase inhibitor and CoQ10 may have synergistic actions in lowering cholesterol. CoQ10, an intermediate product of cholesterol synthetic pathway may have negative feedback inhibition on HMG-CoA reductase. Further systemic researchesare necessary to clarify metabolic action of statin and CoQ10 in cholesterol metabolism. Although Pch did not affect cholesterol, but tended to decrease plasma triglyceride.Insoluble tannins and soluble fibers are known to inhibit the absorption of cholesterol and lipid.[102)103)]It is assumed that the tannins and soluble fibers of chestnut inner shell also seemed to inhibit intestinal absorption of triglyceride. Considering lipid lowering effect of Pch for 4 weeks, Pch can be a good dietary source for complementary alternative medicine for statin therapy. Functions of statin are known to inhibition of HMG-CoA reductase activity

and cholesterol synthesis because of similar structure to HMG-CoA reductase. However, hypocholesterolemic effects of statin were different among mouse, rat, and guinea pigs within rodents, which should be further studied.

In present study, hematocrit increased in all groups with statin, and especially increase above normal in group of statin plus CoQ10 suggested that there might be some compensations for depleting status of oxygen in metabolizing statin and/or CoQ10.

Kim *et al.*[95]observed simvastatin of 30 mg/kg BW did not affect the maximum platelet aggregation in rat, but statin plus CoQ10 (15 mg/kg BW) tended to decrease platelet aggregation. In present study with guinea pig, statin plus high dose CoQ10 of 200 mg/kg BW caused the lowest initial slope. Dajani *et al.*[104]reported pravastatin treatment in primary hypercholesterolemic patients inhibited platelet aggregation and TXB_2synthesis. It is known that hypercholesterolemia itself increased hyper-sensitivity of platelet. Dajani*etal.*[104]explained that inhibition effect of pravastatin on platelet aggregation coincided with cholesterol lowering effect of it. In present study, there was no cholesterol lowering effect in statin group with an increased platelet aggregation, inferring that increased cholesterol correlated with increased platelet aggregation. Considering no cholesterol lowering effects in guinea pigs with high statin (20 mg/kg BW) above applicable dose to human, there might be species difference between animal and human in metabolism and cardiovascular system. Plant antioxidants like herbal extracts decreased platelet aggregation,[105]but 5% green tea powder diet increased somewhat in Kim's study.[95]Pch in present study reduced platelet aggregation of both the initial and the maximum compared with the control, whereas statin plus Ech did not affect. Kawaguchi *et al.*[106] reported that the platelet

activating factor raised activity of angiotensin converting enzyme (ACE). Unlike these results, there was no correlation between platelet aggregation and ACE activity in present study.

All animals supplemented with statin in present study showed overall increased plasma GOT and overall decreased GPT level. Plasma GOT did not correlated with liver weight and liver lipid, but plasma GPT somewhat correlated with liver cholesterol and triglyceride. Cholesterol supplemented diet is one factor causing accumulation of cholesterol and triglyceride in liver, leading fatty liver and liver damage. Cholesterol of 0.25% with 15% fat mix in present study presumably caused fat accumulation in liver and liver damage. We assumed that higher plasma GOT with statin treatment may be associated with liver function on statin detoxification. However, statin with CoQ10 decreased both GOT and GPT compared with statin used alone, suggesting CoQ10 may have liver protective effect. Chestnut inner shell in form of powder may have beneficial effects, lessening detoxification burden of liver following high fat and high cholesterol diets.

Intracellular Na and K concentrations showed similar tendency among groups, and correlated well with Na-K ATPase activity. Although intracellular Na and K ratio normally falls 1 : 10, but the ratio in present study was 1 : 25 giving 2 times higher K concentration, which might affect Na efflux channels. Statin and Pch in present study suppressed Na-K ATPase reducing the total Na efflux. Although consensus is that suppressed Na-K ATPase correlated with high blood pressure, different cell types in different species would differ in Na-K ATPase activity in given physiological milieu. Physiologically, Na-K ATPase plays roles in Na reabsorption in kidney and glucose absorption in intestinal epithelium.

Statin induced nitric oxide synthase (NOS) increasing cellular NO which in turn activates Na-K ATPase.[107] Uyuklu *et al.*[108] also reported that atrovastatin decreased cholesterol contents in erythrocyte membrane decreasing cholesterol/phospholipid ratio, giving membrane stability with increased Na-K ATPase in guinea pig. Despite these reports, simvastatin in present study was not increase Na-K ATPase without affecting plasma total cholesterol. The total Na efflux through Na channels was lower in chestnut inner shell powder group. But chestnut inner shell did not have favorable effects on cholesterol reduction and Na channel activation, despite cholesterol lowering potency of tannin and antioxidant components. There are four Na transport pathways; Na-K ATPase, Na-K cotransport, Na passive transport, and Na-(Li+) counter transport on erythrocyte membrane. It is known that counter transport may not actually affect on Na efflux. Kim *et al.*[99] and Kang *et al.*[109] reported that activity of Na-K ATPase in rats was 1.5 times more than that of Na-K cotransport in rat study. Unlike these results, Na-K ATPase in present study with guinea pigs was decreased regardless of diet, suggesting difference regulation of electrolyte in the same rodent family.

Protective effect of erythrocyte membrane against AAPH-induced damage was assessed quantity of Na leak among the groups with statin, statin plus CoQ10, and statin plus Ech, comparing the antioxidant effects of CoQ10 versus Ech in assumably antioxidant deplete state by statin. In present study, Na leak in intact cells was not different among the groups, but Na leak in AAPH treated cells significantly decreased in the groups of statin plus CoQ and statin plus Ech, which means that CoQ10 and Ech had strong antioxidant activity protecting cell membrane from radical damage. AAPH [2,2-azobis-(2-amidinopropane) dihydrochloride], a water-

soluble radical initiator, like H_2O_2 and $CuSO_4$, induce cell damages. Erythrocyte Na leak is Na efflux through cell membrane in the manner of passive diffusion by concentration gradient. Upon exposure to AAPH radical generating system, Na leak would increase with the extent of membrane damage. Therefore, this result suggested that CoQ10 and Ech have potential protective properties of erythrocyte membrane against oxidative damage.

In the present study, TBARS production in liver did not correlated with TBARS production in platelet rich plasma (PRP). Liver TBARS production decreased overall and PRP TBARS production increased overall with statin treatment. CoQ10 and Ech efficiently reduced TBARS production compared with the control in liver and the statin control in PRP, which agreed with their antioxidant actions in AAPH treated erythrocyte Na leak. Inhibitory effect on TBARS production of chestnut inner shell might be associated with strong antioxidant components such as ellagic acid and gallic acid, and supplemented CoQ10 in present study play roles as antioxidant in form of reduced form ubiquinol (CoQ10) as reported in other studies.[110)111)]

PART Ⅱ

In vitro study for effects of chestnut (*Castanea crenata*) inner shell on antioxidant and cardiovascular-related parameters

ABSTRACT

The second part of this study focusedon the *in vitro* effects of ethanol extract of chestnut inner shell (Ech), solvent fractions of Ech, and the major compounds of chestnut inner shell such as ellagic acid, gallic acid, and (+)-catechin on antioxidant and the cardiovascular related parameters.

Compared scavenging activities against DPPH and hydroxyl radicals on ESR showed that ellagic acid > gallic acid > (+)-catechin > Ech with similar activity of Ech 2.5 μg/ml to (+)-catechin 5μM. The relative DPPH radical scavenging activities of BuOH fraction, EtOAc fraction, and Ech was 5.5:3:1. All tested substance had high protective effect on DNA from H_2O_2-induceddamage. Ellagic acid was theonly effective compound in reducing the angiotensin converting enzyme (ACE) activity of lung tissue. The initial slope of platelet aggregation decreased significantly when platelets exposed to *n*-hexane and BuOH fraction.In LPS-stimulated RAW 264.7 cells, the *n*-hexane and EtOAc fractions had inhibitory effects on nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression. Additionally, the *n*-hexane fraction inhibited prostaglandin E_2 (PGE_2) production and cyclooxygenase-2 (COX-2) expression with suppressing effects on pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in LPS-stimulated cells.

Although there were no detectable effects on platelet aggregation and lung ACE activity, Ech and its *n*-hexane and BuOH fractions showed strong activity in inhibiting iNOS and COX-2 expression and suppressing production of anti-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 which are known to be associated with the development of atherosclerosis and complications. This study

suggested that chestnut inner shell with its strong antioxidant components may play beneficial roles in prevention of cardiovascular diseases and its use as alternative medicine is promising to enhance human health and to produce the industrial application and economic values using the remnants of the chestnut inner shell components.

MATERIALS AND METHODS

1. Materials

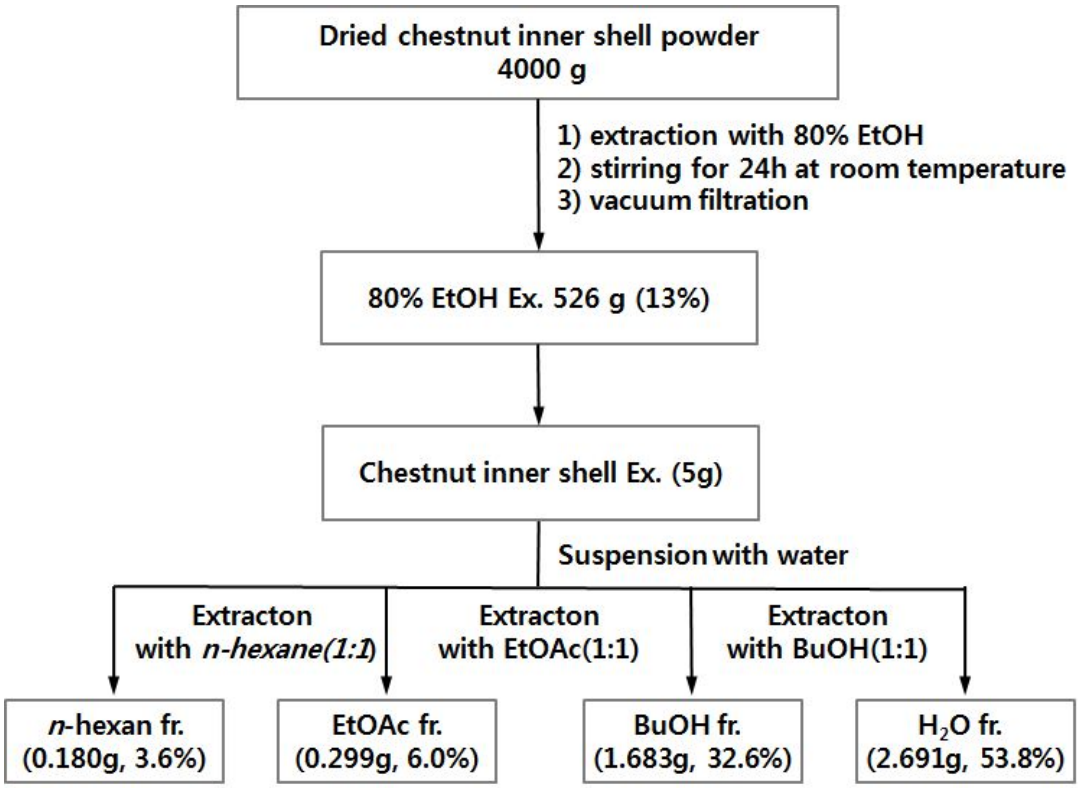
1) Chemical materials

Lipopolysaccharide (LPS) was purchased from sigma Chemical Co (St. Louis, MO) Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) penicillin–streptomycin and trypsine–EDTA were obtained from Gibco/BRL (Grand Island, NY, USA). The lactate dehydrogenase (LDH) cytotoxicity detection kit was purchased from Promega (Madison, WI, USA). The enzyme-linked immunosorbent assay (ELISA) kit for IL-1β, IL-6, 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 Amersham Biosicences (Piscataway, NJ, USA), respectively. Antibodies against iNOS, eNOS, and COX-2 were obtained from Calbiochem (La Jolla, CA, USA) and BD Biosciences Pharmingen (San Jose, CA, USA), respectively. The other chemicals and reagents used were of analytical grade.

2) Solvent fractionation of chestnut inner shell extract

Five gram of the chestnut inner shell ethanol extractwas dissolved in water, and then sequentially partitioned with *n*-hexane (Hx), ethyl acetate (EtOAc), butanol (BuOH). Each solvent was removed under reduced pressure to yield then *n*-hexane-

soluble (0.180g, 3.6%), EtOAc-soluble (0.299g, 6.0%), BuOH-soluble (1.683g, 32.6%), and H_2O-soluble (2.691g, 53.8%). (Scheme 2-1)



Scheme 2-1. Systematic purification using solvent partitioning from chestnut (*Castanea crenata*) inner shell powder

3) Blood and lung samples preparation

The blood and lung samples were obtained from Sprague-Dawley male rat (Orient Bio Co., Ltd, Korea) fed normal pellet diet. The blood samples were collected by

cardiac puncture into heparinized vacuum tube, and platelet aggregation and comet assay were performed with fresh blood. The lung for ACE inhibition activity analysis were quickly removed and used fresh.

4) Lymphocyte isolation

Heparin-treated blood was diluted to 1:1 with phosphate buffered saline (PBS). Six mℓ of the mixture of blood plus PBS layered onto 3mℓ of Histopaque 1077. The prepared blood was centrifugated 1,000 × g for 10 minutes in condition of no break. The lymphocyte layer (buffy coat) was removed carefully and washed twice with PBS.

2. Methods

1) Free radical scavenging activity

(1) Assay of free radical scavenging activity using ESR spectrometer forextractand major polyphenols of chestnut inner shell

① DPPH radical scavenging activity

DPPH (1,1-diphenyl-2-picrylhydrazl) radical scavenging activity was measured using the method described by Nanjo *et al.*[112]Sixtyμℓ of each sample was added to 60 μℓ of DPPH (60 μmole/ℓ) in methanol solvent. The sample mixed roughly. And then after 2 minutes transferred in to a capillary tube and recorded spectrum by ESR

(electron spin resonance) spectrometer (JES-FA machine, JEOL, Tokyo, Japan). The measurement conditions were as followed; magnetic field 336.0 ± 10 mT, power 1 mW, modulation frequency 100 kHz, amplitude 6 × 100, modulation width 0.8 mT, sweep width 10 mT, sweep time 30 sec, time constant 0.03 sec. The extent of scavenging activity was calculated as followed.

Scavenging activity % = [($H_{control}$ - H_{sample}) / $H_{control}$] × 100

$H_{control}$: The relative peak heights of the radical signals without a sample

H_{sample} : The relative peak heights of the radical signals with a sample

All experiments were performed in triplicate

② **Hydroxyl radical scavenging activity**

Hydroxyl radicals were generated by the iron-catalyzed Haber-Weiss reaction (Fenton-driven Haber Weiss reaction; $Fe^{2+} + H_2O_2 \rightarrow {}^1OH + OH^-$) and the hydroxyl radicals rapidly reacted with nitrone spin trap DMPO.[113]The resultant DMPO-OH adducts was detectable with the ESR spectrometer. Sample 20μℓ of various concentrations were mixed with 0.3 M DMPO 20 μℓin phosphate buffer solution (PBS; pH 7.4), 10 mM $FeSO_4$ 20μℓ and 10 mM H_2O_2 20 μℓ. And then after 2.5 minutes transferred into a capillary tube and recorded spectrum by ESR. The experimental conditions were as followed; magnetic field 336.0 ± 10 mT, power 1mW, modulation frequency 100 kHz, amplitude 2 × 100, modulation width 0.1 mT, sweep width 10 mT, sweep time 30 sec, time constant 0.03 sec. All experiments were performed in triplicate.

(2) Assay of DPPH radical scavenging activity for solvent fractions

DPPH radical-scavenging activity was determined according to a slightly modified method described by Blois.[114] Five different concentrations of each fraction in methanol (MeOH; 100μℓ) were added to 100μℓ of 0.4 mM DPPH in MeOH, and the reaction mixture was left to stand for 10 minutes at room temperature in darkness. The scavenging activity was estimated by measuring the absorption of mixture at 517 nm, reflecting the amount of DPPH radical remaining in the solution. Gallic acid was used as positive controls. Scavenging activity is expressed as SC_{50}, the concentration required for scavenging 50 % of DPPH radical in the solution. The scavenging activity (%) was calculated as follows:

DPPH radical scavenging activity (%) = [$(A_{control} - A_{sample})/A_{control}$] × 100

$A_{control}$: The absorbance of the reaction mixture without a sample

A_{sample} : The absorbance of the reaction mixture with a sample

All experiments were performed in triplicate

2) Determination of total polyphenolic content

The total phenolic content was determined according to the method described by Chandler and Dodds.[115] Each 1 mℓ of Ech or solvent fractions of Ech, 1 mℓ 95% EtOH, 5 mℓ of distilled water, and 0.5 mℓ of 50% Folin-Ciocalteu reagent were mixed. The mixtures were allowed to react for 5 minutes, and then 1mℓ of 5% Na_2Co_3 was added, and the mixture was thoroughly mixed and placed in the dark for

1 h. Absorbance was measured at 725 nm and gallic acid standard curve was obtained for the calibration of phenolic content.

3) Comet assay for determination of DNA damage

A comet assay was performed to determine the oxidative DNA damage.[116] The lymphocyte suspension was incubated with 0.5 mM of sample (or 0.5 mg/mℓ) at 37°C for 10 minutes and then oxidatively damaged with 400μM H_2O_2.Immediately after treatment, the lymphocyte suspension was centrifuged at 1,000 × g for 5 minutes and removed the supernatant. The lymphocytes were mixed with 75μℓ of 0.5% low melting agarose (LMA), and added to the slides precoated with 1.0% normal melting agarose (NMA). After solidification of the agarose, the slides were covered with another 75μℓ of 0.5% LMA and allowed to solidify at 4°C for 40 minutes. The slides were then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, and 1% sodium laurylasarcosine; 1% Triton X-100 and 10% DMSO) for 1h at 4 °C. The slides were next placed into an electrophoresis tank containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for DNA unwinding. For electrophoresis of the DNA, an electric current of 25 V/300 mA was applied for 20 minutes at 4 °C, and then treated with ethanol for another 5 minutes before staining with 50 μℓof ethidium bromide (20 μg/mℓ). Measurements were made by image analysis (Kinetic Imaging, Komet 5.0, U.K) using a fluorescence microscope (LEICA DMLB, Germany), determining the percentage of fluorescence in the tail (tail intensity, TI; 50 cells from each of two replicate slides).

4) Angiotensin converting enzyme (ACE) inhibition activity

The ACE inhibition activity assay was performed according to the method of Cushman and Cheung[94] and Dominik *et al.*[117] with modification. The amount of hippuric acid (HA) formed from hippuryl-L-histidyl-L-leucine (HHL) is determined by a spectrophotometric assay. Two hundreds mg of lung tissue was homogenized with 2 mℓ of 20 mM potassium phosphate buffer (1 M K_2HPO_4 plus 1 M KH_2PO_4, pH 8.3) containing membrane bound ACE was transferred to a clean tube. A sample solution 10μℓ with 60μℓ of ACE solution and 80 μℓof 100 mM K-phosphate buffer containing 300 mM Nacl at pH 8.3 was pre-incubated at 37 ℃ for 10 minutes, and then incubated with 100μℓ of substrate (5 mM hippuryl-His-Leu in 100 mM K-phosphate buffer at pH 8.3) at 37 ℃ for 30 minutes. The reaction was stopped by added 500 μℓof 1 N HCl and medium was centrifuged at 1,000 × g for 10 minutes. Supernatant of 500 μℓwas transferred to a glass tube containing 1.5 mℓ ethyl acetate, vortexed for 15 seconds and centrifuged at 1,000 × g for 5 minutes. One mℓ of upper ethyl acetate layer was transferred to a eppendorf tube and dried for 3 days on room temperature.Because the absorbance of HA dried at high temperature (120 ℃) was degradation, we performed dry at room temperature (Figure 2-1).The hippuric acid was dissolved in 1mℓ deionized water, and the absorbance was measured at 228 nm UV spectrophotometer using each blank prepared with the same procedure without HHL in incubation medium. The extent of inhibition was calculated as followed.

ACE inhibition activity (%) = [($A_{control}$ - A_{sample})/$A_{control}$] × 100

$A_{control}$: The absorbance of the solution without a sample

A_{sample} : The absorbance of the solution with a sample

All experiments were performed in triplicate

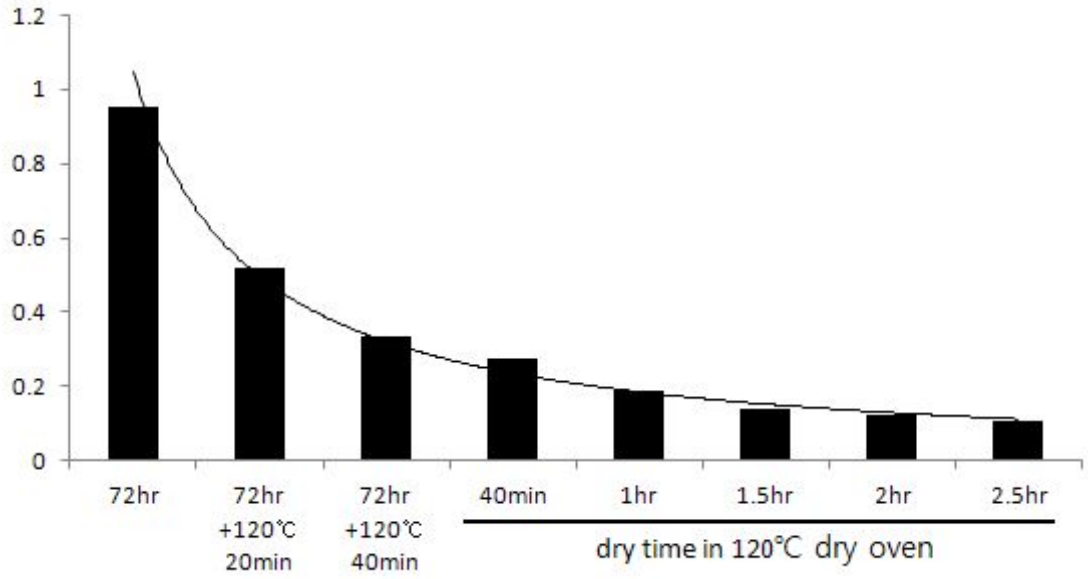


Figure 2-1. Absorbance changes by difference drying conditions.

5) Whole blood platelet aggregation

The impedance method using the fresh whole blood has the advantage of measuring platelet aggregation under almost *in vivo* conditions in the presence of other blood components. Platelet aggregation was measured using a Chronolog Whole Blood aggregometor (model 500-Ca, Havertown, Pennsylvania, USA). The whole blood incubated with 100 μM of sample for 10 minutes, was diluted with isotonic saline solution (1:4) to give a platelet concentration of 200,000/μℓ. Adenosine diphosphate (ADP, 1μM) was added to initiate aggregation, and three

readings of impedance changes were averaged for each sample.Increased platelet aggregation caused an increase in impedance across two platinum electrodes. All experiments were performed in triplicate.

6) Assay of pro-inflammatory mediators

(1) Cell culture

The murine macrophage cell line RAW 264.7 was purchased from the Korean Cell Line Bank (KCLB; Seoul, Korea). RAW 264.7 cells were cultured in DMEM supplemented with 100 U/mℓof penicillin, 100 μg/mℓ of streptomycin and 10% FBS. The cells were incubated in an atmosphere of 5% CO_2 at 37 ℃ and were subcultured every 3 days.

(2) Lactate dehydrogenase (LDH) cytotoxicity assay

RAW 264.7 cells (1.8×10^5 cells/mℓ) plated in 24-well plates were pre-incubated and then treated with LPS (1 μg/mℓ) plus samples at 37 ℃ for 24 h. The medium was carefully removed from each well, and the LDH activity in the medium was determined using an LDH cytotoxicity detection kit (Promega, Madison, WI, USA). In briefly, 100 μℓ of reaction mixture were added to each well, and the reaction was incubated for 30 minutes at room temperature in darkness. The absorbance of each well was measured at 490 nm using a UV spectrophotometer. All experiments were performed in triplicate.

(3) Determination of nitric oxide (NO) production

The NO production in the culture medium was measured by the Griess method.[118)]RAW 264.7 cells (1.8×10^5 cells/mℓ) were treated with LPS (1μg/mℓ) plus samples at 37 ℃ for 24 h. The culture supernatant (100μℓ) was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2.5% phosphoric acid) and incubated at room temperature for 10 minutes. The NO production was determined by absorbance at 540 nm, using $NaNO_2$ as a standard. All experiments were performed in triplicate.

(4) Determination of prostaglandin E_2 (PGE_2) production

RAW 264.7 cells plated at 1.8×10^5 cells/mℓwere stimulated with LPS (1μg/mℓ) and then incubated with samples at 37 ℃ for 24 h. The concentration of PGE_2 in the culture supernatant was determined using an ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instruction. The assay is based on competition between the unlabelled PGE_2 and a fixed quantity of peroxidase-labeled PGE_2 for a limited number of binding sites on a PGE_2-specific antibody. All experiments were performed in triplicate.

(5) Measurement of pro-inflammatory cytokines (TNF-α IL-1β, and IL-6) production

Samples solubilized with DMSO was diluted with DMEM prior to treatment. The inhibitory effect of samples on the production of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) from LPS-treated RAW 264.7 cells was determined as described in the Cho *et al.*[119] protocols. Supernatants were used for the pro-inflammatory cytokines assays using a mouse ELISA kit (R&D Systems, Minneapolis, MN, USA).

(6) Western blot analysis

RAW 264.7 cells were pre-incubated for 18 h, and then stimulated with LPS (1 μg/mℓ) in the presence samples for the indicated times. After incubation, the cells were collected and washed twice with cold-PBS. The cells were lysed in a lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/mℓaprotinin, 25μg/mℓ leupeptin] and kept on ice for 30 minutes. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatant. The protein concentrations were determined by using BCA™ protein assay kit. Aliquots of the lysates (30~50 μg of protein) were separated on a 12% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (BIO-RAD, HC, USA) with a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% MeOH (v/v)]. After blocking the nonspecific site with 1% bovine serum albumin (BSA) the membrane was then incubated overnight with specific primary antibody at 4 °C. The membrane was further incubated for 60 min with a peroxidase-conjugated secondary antibody (1:5000, Vector Laboratories, Burlingame, USA) at room temperature. The

immunoactive proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detection kit.

3. Statistical analysis

All data are presented as mean ± SE. Significant differences among the groups were determined using the Student's t-test.A value of $P< 0.05$ was considered to be statistically significant.

RESULTS

1. Free radical scavenging activities of Ech and the major polyphenolsin Ech

The activity of test substances against DPPH and hydroxyl radicals was assessed by electron spin resonance (ESR). The activity was evaluated by measuring the peak area of the corresponding signal obtained under the instrumental conditions previously described. The higher antioxidant activity was observed as the lower peak area of the radical. ESR spin trapping provides a sensitive, direct, and accurate means of monitoring reactive species.[120)]Therefore, in this study, ESR technique was used to compare the DPPH and hydroxyl radical scavenging abilities of the chestnut inner shell ethanol extract with the main polyphenols of those.

The scavenging activities of Ech (extract of chestnut inner shell) and the major polyphenols in Ech on DPPH free radicals are provided in Figure 2-2. It was observed that ellagic acid, gallic acid, and (+)-catechin scavenged 67.7, 47.8, and 15.5% of DPPH radical at 5 μM, respectively, while Ech showed 67.7% DPPH radical scavenging activity at 50 μg/mℓ. Those radical scavenging levels illustrated in Figure 2-2C, which values were dose- dependent manners. Among the three polyphenolic compounds, ellagic acid and gallic acid possess higher DPPH radical scavenging activity (SC_{50} = 2.99, 5.49 μM, respectively) than (+)-catechin (SC_{50} = 23.87 μM) (Table 2-1). The SC_{50} value of Ecis exhibited 29.01 μg/ml, and was equivalent to 1/4 of (+)-catechin (SC_{50} = 6.93 μg/ml).

Hydroxyl radicals are the major oxygen species, which are generated by Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{-}OH + {}^{\cdot}OH$) and are trapped by DMPO, forming a

spin adduct that can be detected by the ESR system. We therefore also examined whether the test substances can scavenge hydroxyl radicals generated by this reaction. The ability of Ech and the major polyphenols in Ech to scavenge hydroxyl radicals was shown in Figure 2-3. Scavenging activity of ellagic acid, gallic acid, and (+)-catechin against hydroxyl radical recorded 90.4, 59.4, and 36.3% at 2.5 mM, respectively, whereas Ech showed 49.1% at 2.5 mg/mℓ. The decreased amount of DMPO-OH adduct was expressed by ESR signals after addition of test substances and the values were dose-dependent manners (Figure 2-3C). Among the three phenolic compounds, ellagic acid had the highest hydroxyl radical scavenging activity with SC_{50} values 0.24 mM, whereas the SC_{50} values of gallic acid and (+)-catechin were 1.06 and 7.73 mM, respectively (Table 2-1). The SC_{50} value of Ech recorded 2.77 mg/mℓ, and was similar to that of (+)-catechin (SC_{50}= 2.24 mg/mℓ).

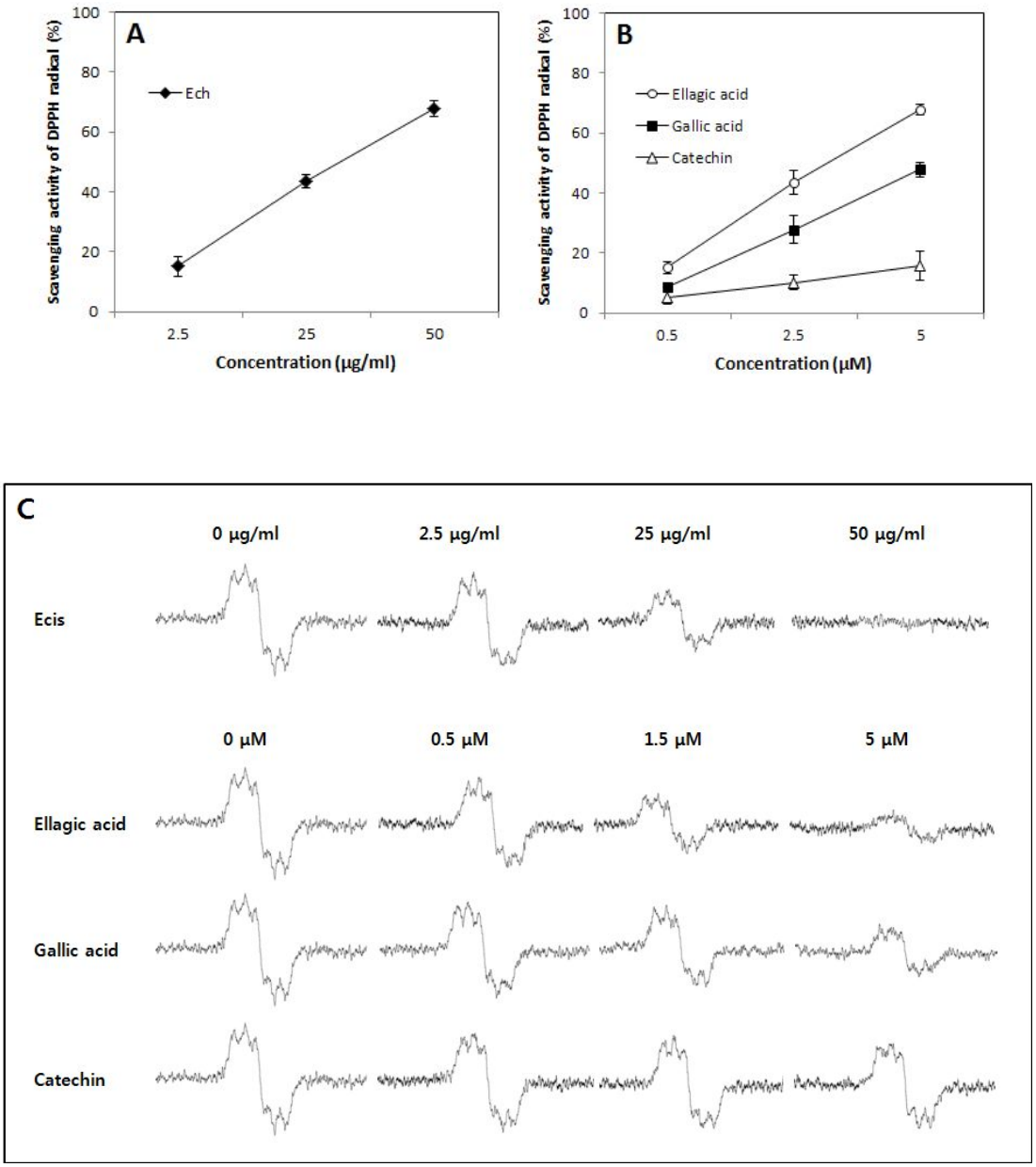


Figure 2-2. DPPH radical scavenging activities of Ech and the major polyphenols in Ech.

◆, 80% ethanol extract of chestnut inner shell (Ech); ○, Ellagic acid; ■, Gallic acid; Δ, (+)-catechin.

Experiments were performed in triplicate and the data are expressed as mean ± SE.

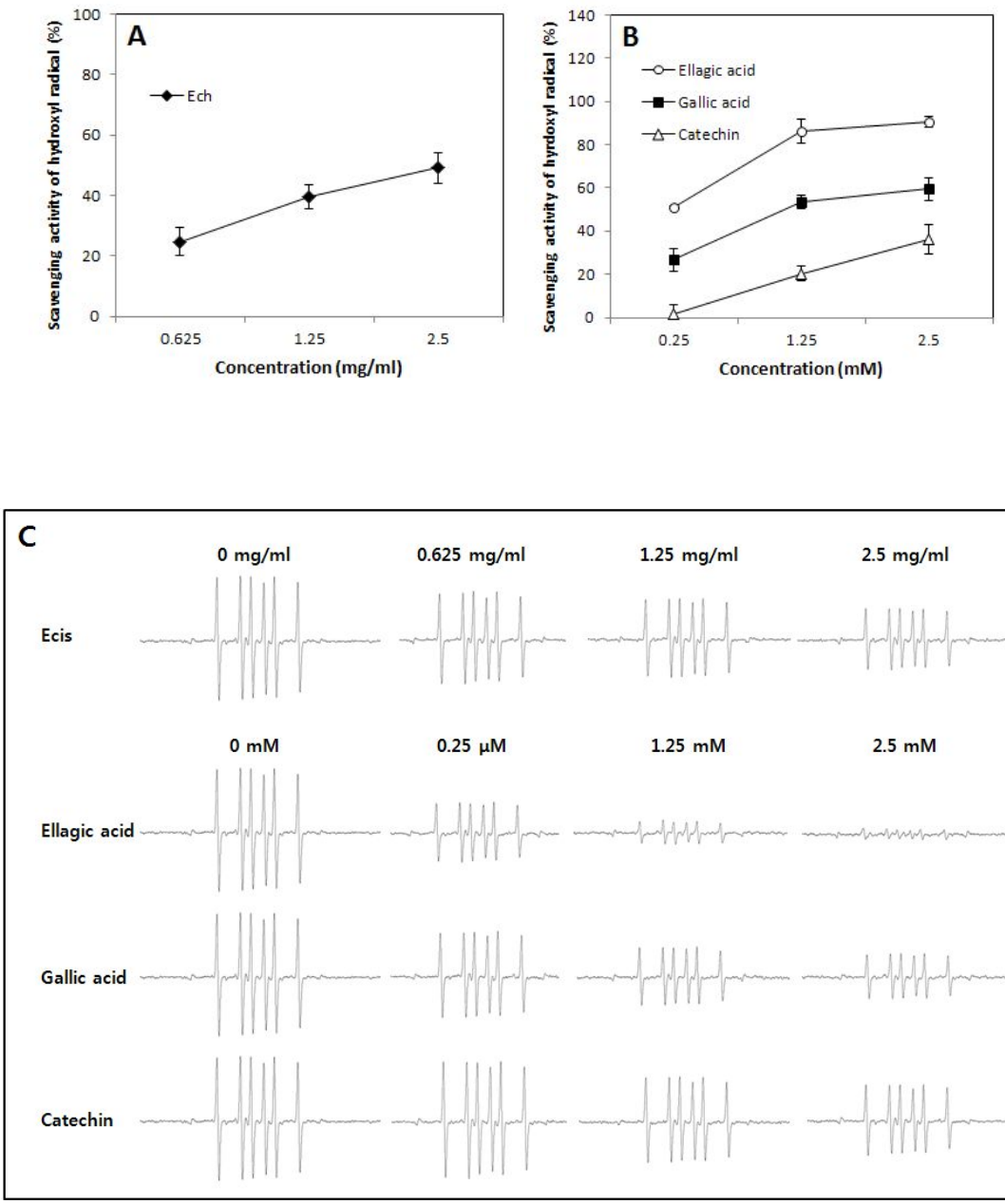


Figure 2-3. Hydroxyl radical scavenging activities of Ech and the major polyphenols in Ech.

◆, 80% ethanol extract of chestnut inner shell (Ech); ○, Ellagic acid; ■, Gallic acid; Δ, (+)-catechin.

Experiments were performed in triplicate and the data are expressed as mean ± SE.

2. DPPH radical scavenging activities and total polyphenolic contents of Ech and its solvent fractions

The DPPH radical was one of the few stable radical sources. Thus the DPPH radical was widely used to investigate the scavenging activity of natural compounds. DPPH radical displays a maximum absorbance at 517 nm in methanol. When DPPH encounters a proton-donating substance, the radical would be scavenged, as visualized by changing its color from purple to yellow, and the absorbance is reduced.[121)]

Antioxidative activities of solvent fractions from Ech were examined by DPPH methods. The SC_{50} values of the organic solvent fractions were shown in Table 2-2. It was observed that the BuOH fraction exhibited the highest scavenging activities. The antioxidative activity of Ech and its solvent fractions showed in the following order: BuOH fr. > EtOAc fr. > Ech > H_2O fr. >*n*-Hx fr.

Ech and the solvent fractions of Ech were subjected to total polyphenolic assay to their polyphenolic contents. The total polyphenolic amount of the test substances are shown in the Table 2-3. The highest polyphenolic content (29.24 g/100g) was recorded in the BuOH fraction of Ech. The polyphenolic contents of Ech and its solvent fraction showed that is paralleled with the pattern of DPPH radical scavenging activity.

Table 2-1. SC_{50} of Ech and the major polyphenols in Ech against DPPH and hydroxyl radicals

	Ech[1)]	Ellagic acid	Gallic acid	(+)-Catechin
	μg/mℓ		*μM*	
SC_{50} for DPPH radical[2)]	29.01	2.99	5.49	23.87
SC_{50} for H_2O_2 radical[2)]	2,770	240	1,060	7,730

[1)] Ech: 80% ethanol extract of chestnut inner shell.

[2)] The concentrations of test reagent : DPPH 30μM, H_2O_2 2.5mM

Table 2-2. SC_{50} of Ech and its solvent fractions against DPPH radical

	Gallic acid	Ech[1)]	*n*-Hx fr.	EtOAc fr.	BuOH fr.	H_2O fr.
	μM			*μg/mℓ*		
SC_{50}[2)]	11.43	68.08	120.99	24.31	12.55	115.76

[1)] Ech: 80% ethanol extract of chestnut inner shell.

[2)] The concentrations of test reagent : DPPH 0.2mM

Table 2-3. Total polyphenolic contentsof Ech and its solvent fractions

	Ech[1)]	*n*-Hx fr.	EtOAc fr.	BuOH fr.	H_2O fr.
	(*ggallic acid/100g*)				

Total polyphenolic content	8.87	5.70	20.70	29.24	5.15

[1)] Ech: 80% ethanol extract of chestnut inner shell.

3. Protective effect against H_2O_2-induced DNA damage

The protective effects of test substances on cell damages were confirmed by a comet assay, which is a rapid and sensitive fluroescence microscopic method for detecting primary DNA damage at the individual cell level, and is extensively used to evaluate the genotoxicity of test substances.[122)]In the present study, tail DNA in the negative control was significantly different from the positive control (H_2O_2-treated control, 400 μM). This increase in DNA damage induced by H_2O_2 was significantly inhibited in all of test substances including Ech (Figure 2-4). The inhibitory activities of Ech, ellagic acid, gallic acid, and (+)-catechin on DNA damage were 53.8%, 64.2%. 64.2%, and 68.7% at concentrations of 0.5 μg/mℓ and 0.5 mM, respectively.

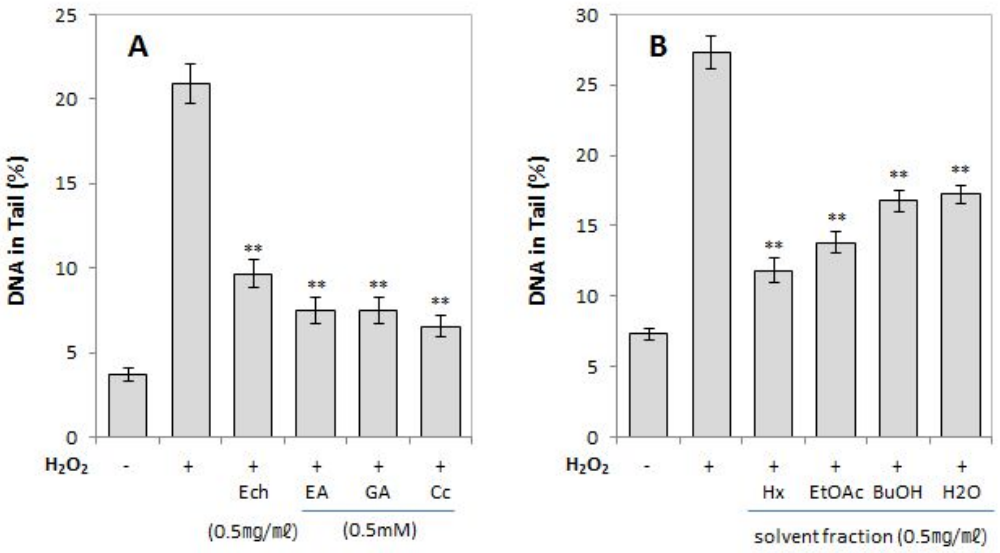


Figure 2-4. Protective effects of Ech, its solvent fractions, and the major polyphenols in Ech against H_2O_2-induced DNA damage in lymphocyte

Values are mean ± SE. Ech: 80% ethanol extract of chestnut inner shell. EA: ellagic acid, GA: gallic acid, Cc: (+)-catechin. Hx: *n*-hexane fraction of Ech, EtOAc: ethyl acetate fraction of Ech, BuOH: butanol fraction of Ech, H_2O: water fraction of Ech. *, $P< 0.05$; **, $P< 0.01$

4. Angiotensin converting enzyme (ACE) inhibition activity

For the ACE activity evaluation, we used fresh tissue ACE directly obtained from lung tissue of Sprague Dawley male rat, and the ACE activity is determined by measuring hippuric aicd (HA) released from hippuryl-L- histidyl-L-leucine (HHL) in incubation medium. The ACE activity assay method was validated with the reference ACE inhibitors captopril.

The ACE inhibition activities of the test substances are shown on Figure 2-5 as the percentage of HA production. Captopril, which is well known as ACE inhibitor, was significantly decreased HA productions by ACE in a dose-dependent manner ($P<$ 0.01). The inhibitory activities of captopril on ACE were 45.8, 74.2, and 84.7% at the concentration of 0.1, 1, and 100 μM, respectively (Figure 2-5A). Among the main polyphenols, only ellagic acid decreased HA production by ACE, and the inhibitory activity was 19.3% at the concentration of 100 μM. The others including Ech did not affect on inhibition of ACE activity. Solvent fractions of chestnut inner shell ethanol were also no effect of ACE activity (Figure 2-5B).

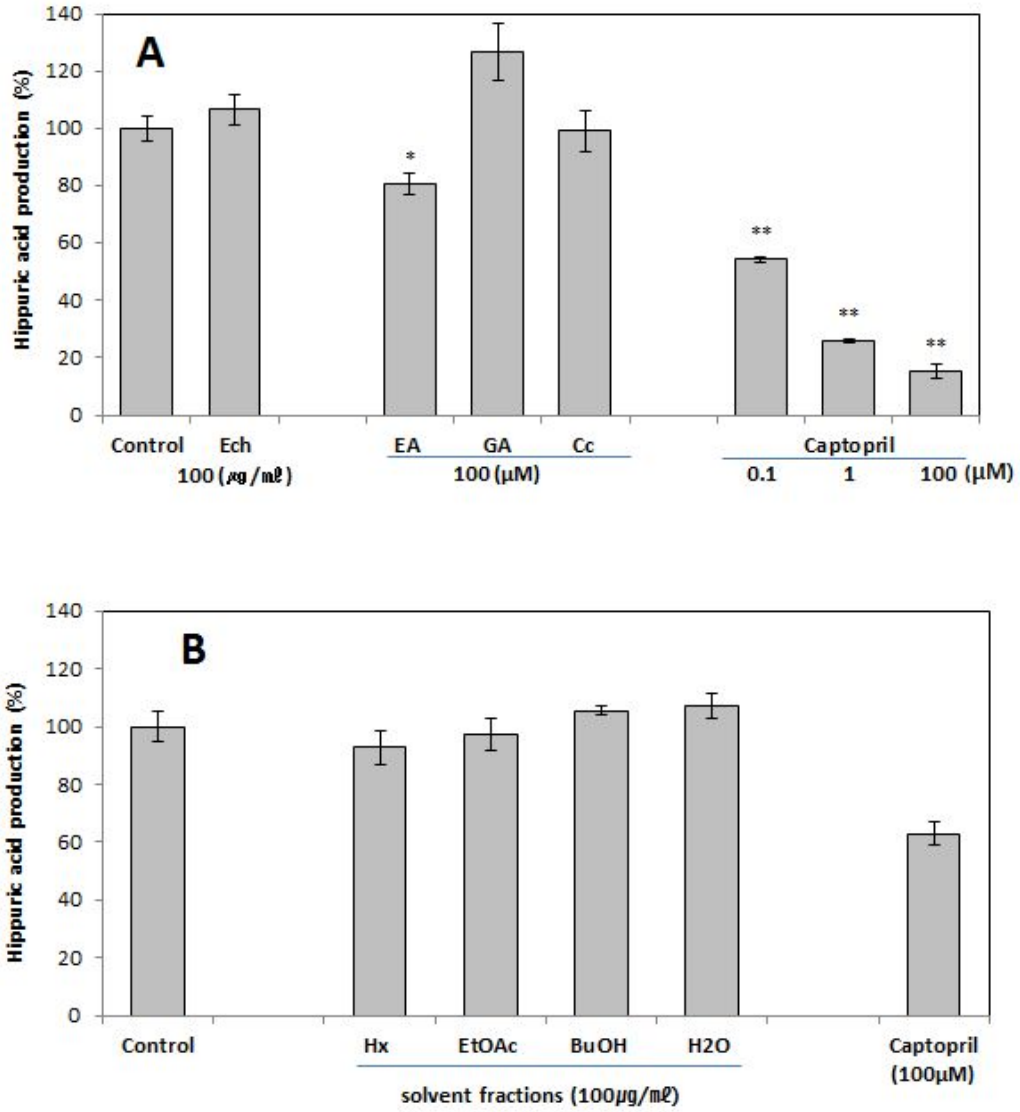


Figure 2-5. Effects of Ech, its solvent fractions, and the major polyphenols in Ech on angiotensin converting enzyme (ACE) activity

Values are mean ± SE. ACE activiey expressed as the percentage of HA (hippuric acid) production released from HHL (hippuryl-L-histidyl-L-leucine, 2mM/mg lung tissue) using 0.2 mg lung tissue. Ech: 80% ethanol extract of chestnut inner shell. EA: ellagic acid, GA: gallic acid, Cc: (+)-catechin. Hx: *n*-hexane fraction of Ech, EtOAc: ethyl acetate fraction of Ech, BuOH: butanol fraction of Ech, H_2O: water fraction of Ech. *, $P< 0.05$; **, $P< 0.01$

5. Whole blood platelet aggregation

Whole blood platelet aggregation results are shown in Figure 2-6. Comparison of Ech (100 μg/mℓ) and the main polyphenols (100 μM), maximum of platelet aggregation did not decreased by treated the test substance. Initial slope of platelet aggregation slightly decreased by Ech and (+)-catechin, but no statistical difference (Figure 2-6A and B).

Among Ech and solvent fractions of chestnut inner shell ethanol extract, only *n*-hexane fraction reduced maximum of platelet aggregation, whereas initial slope of platelet aggregation decreased by treated all test substances except H_2O fraction. Especially, the *n*-hexane and butanol fractions were significantly decreased 24.4% ($P< 0.01$) and 17.6% ($P< 0.05$) of initial slope of platelet aggregation at the concentration of 100μg/mℓ, respectively.

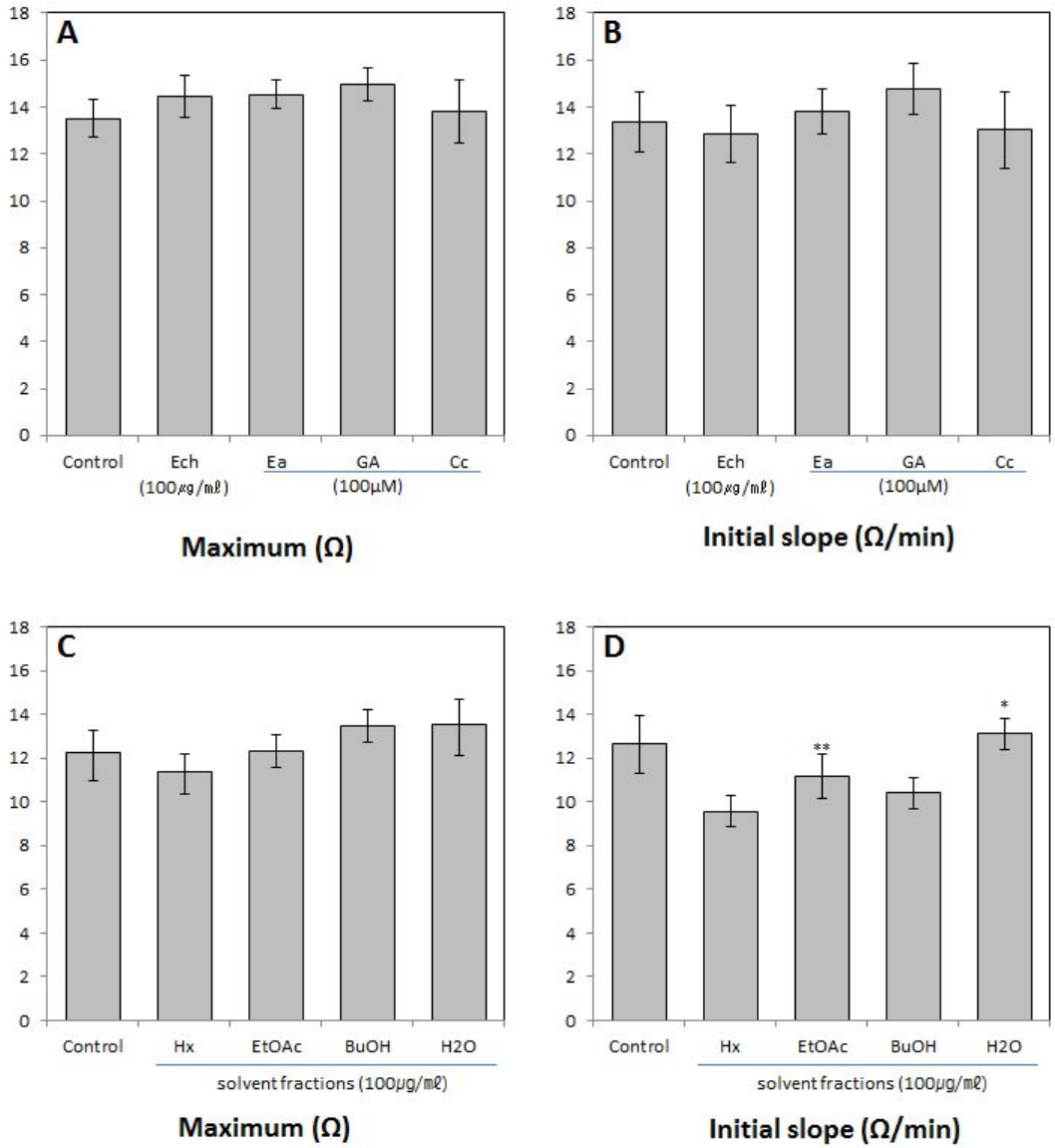


Figure 2-6. Effects of Ech, its solvent fractions, and the major polyphenols in Ech on platelet aggregation

Values are mean ± SE of 5 Sprague Dawley rat. Adenosine diphosphate (ADP, 1μM) was added to initiate aggregation, and three readings of impedance changes were averaged for each sample. Maximum aggregation in ohm at the point where platelet aggregate is dissociated. Initial slope is the ohm change for the first one minute of

aggregation. Ech: 80% ethanol extract of chestnut inner shell. EA: ellagic acid, GA: gallic acid, Cc: (+)-catechin. Hx: *n*-hexane fraction of Ech, EtOAc: ethyl acetate fraction of Ech, BuOH: butanol fraction of Ech, H_2O: water fraction of Ech. *, $P< 0.05$; **, $P< 0.01$

6. Effects on NO production in LPS-stimulated RAW 264.7 cells

LPS-induced production of NO from macrophages occurs in the inflammatory response. Nitrite levels, as measured with Griess reagent, are used as a measure of NO production due to the short half-life ofNO.

LPS treatment significantly increased the concentrations of NO. As shown in Figure 2-6, the *n*-hexane fraction (Hx) of chestnut inner shell ethanol extract exhibited the highest inhibition activity on LPS-induced production of NO in RAW 264.7 cells. The inhibition activities of *n*-hexane fraction on NO production were 21.7, 29.9, and 80.9% at the concentration of 25, 50, and 100 μg/mℓ, respectively (Figure 2-7B). The ethyl acetate fraction (EtOAc) also reduced LPS-induced NO production in a dose-dependent manner: 11.4, 15.9, and 30.8% at 25, 50, and 100 μg/mℓ, respectively (Figure 2-7C), but Ech and the polyphenols did not. The cytotoxic effect of the test substance was evaluated in the presence or absence of LPS using the LDH assay (Figure 2-7A). The test substances did not affect the cytotoxic of RAW 264.7 cells at the concentrations used to inhibit NO. Thus, the inhibitory effects were not attributable to cytotoxic effects.

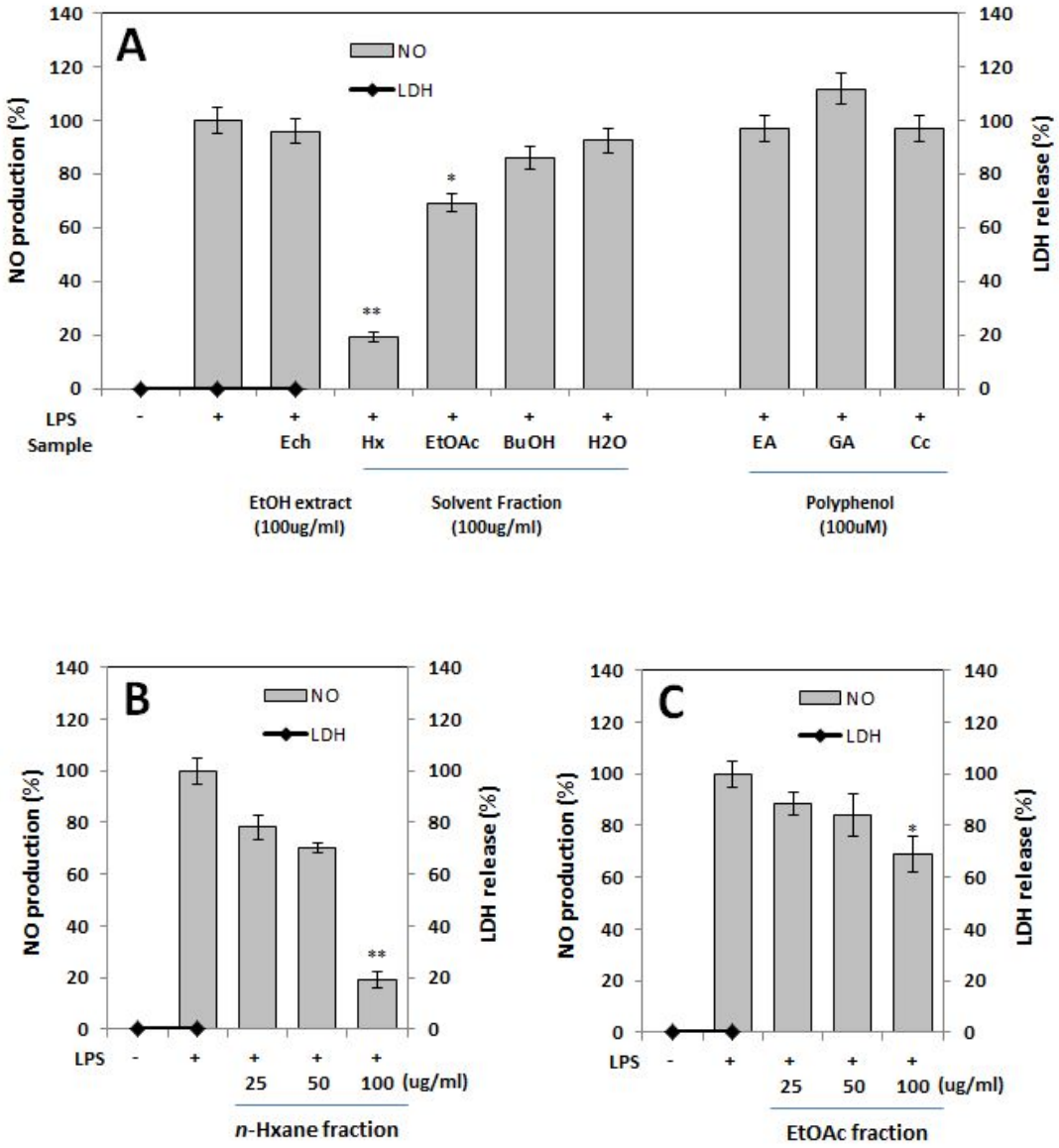


Figure 2-7. Effects of Ech, its solvent fractions, and the major polyphenols in Ech on nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells

The production of nitric oxide was assayed in culture medium of cells stimulate with LPS (1 μg/mℓ) for 24 h in the presence of the test substances. Cytotoxicity was determined using the LDH method. Values are the mean ±

SE of triplicate experiments. *,$P<0.05$ and **,$P<0.01$ indicate significant differences from the LPS-stimulated group. Ech: 80% ethanol extract of chestnut inner shell. EA: ellagic acid, GA: gallic acid, Cc: (+)-catechin. Hx: *n*-hexane fraction of Ech, EtOAc: ethyl acetate fraction of Ech, BuOH: butanol fraction of Ech, H_2O: water fraction of Ech.

7. Effects on PGE_2 production in LPS-stimulated RAW 264.7 cells

Prostaglandin E_2 (PGE_2) is an inflammatory mediator produced from the conversion of arachidonic acid by cyclooxygenase. In a variety of inflammatory cells, including macrophages, cyclooxygenase-2 (COX-2) is induced by cytokines and other activators, such as LPS, resulting in the release of a large amount of PGE_2 at inflammatory sites. LPS can stimulate PGE_2 release from RAW 264.7 macrophages.LPS (1 μg/mℓ) treatment for 24 h increased PGE_2 levels in the culture medium.Only 100 μg/mℓ of the *n*-hexane fraction significantly reduced this LPS-induced PGE_2 production (Figure 2-8). The others did not affect PGE_2 production.

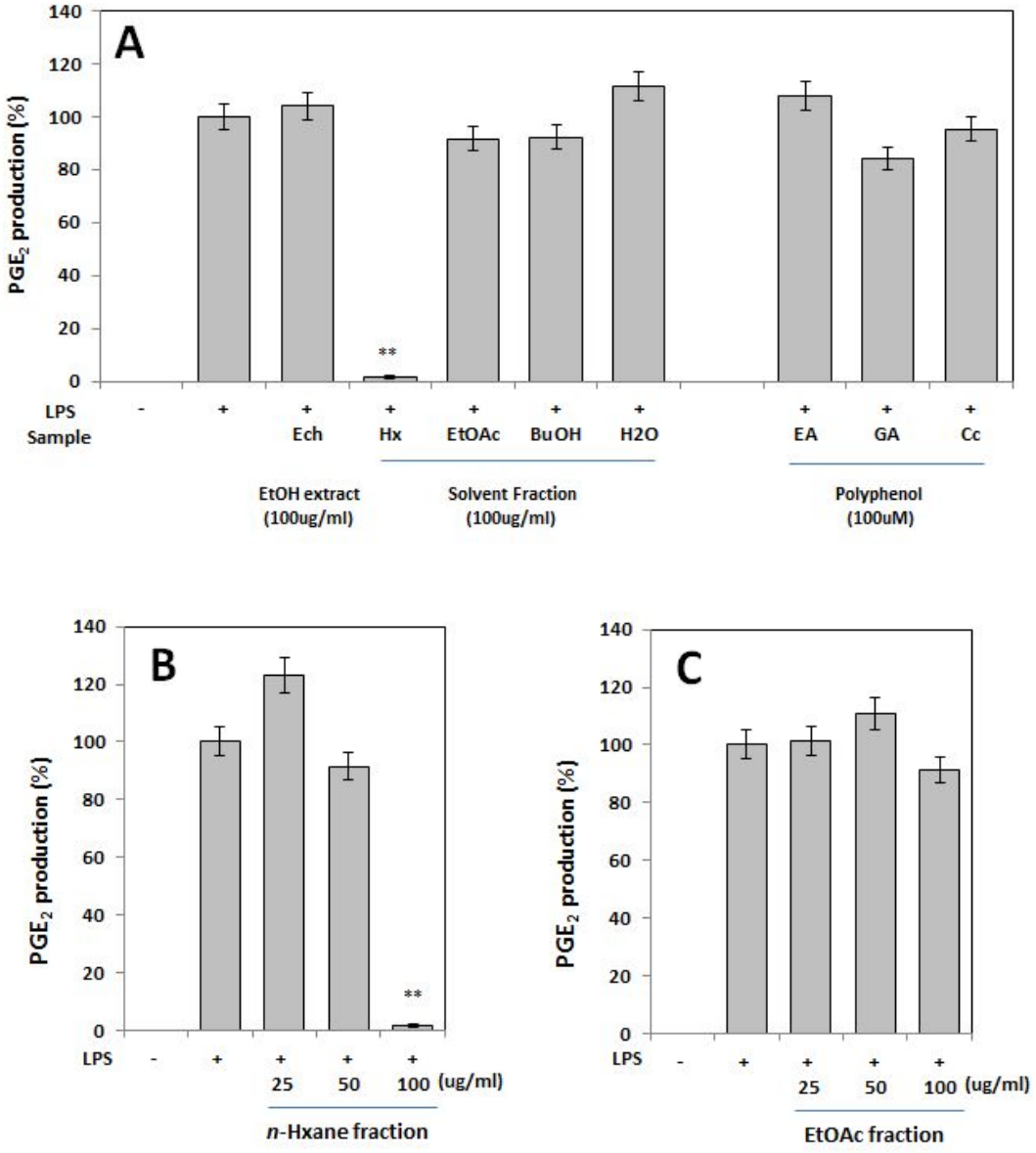


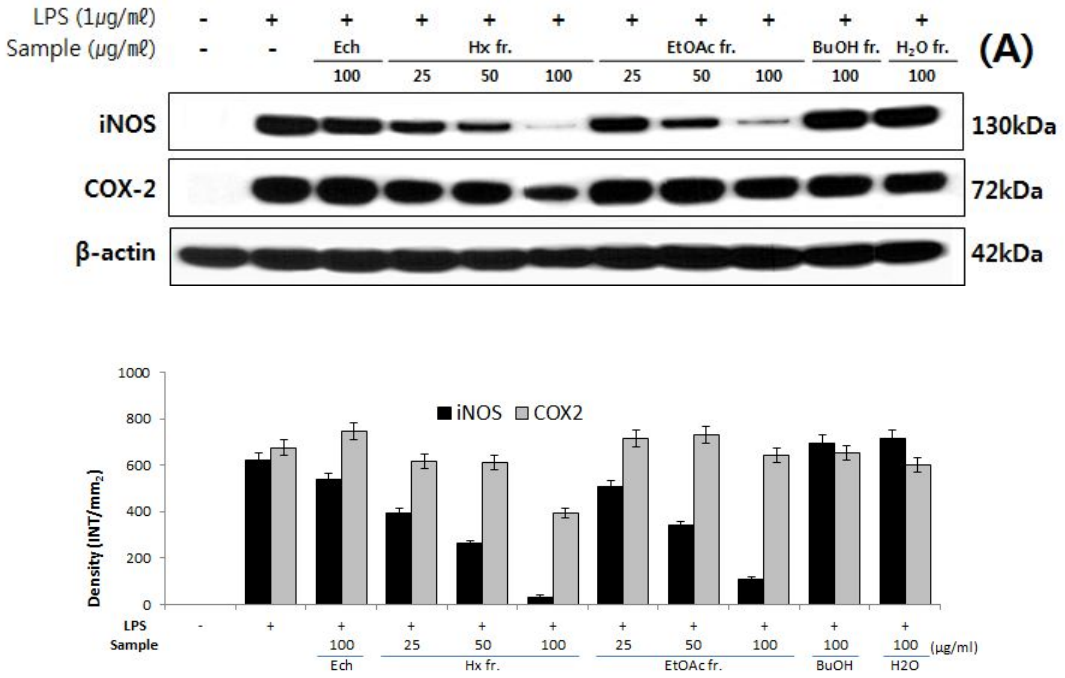
Figure 2-8. Effects of Ech, its solvent fractions, and the major polyphenols in Ech on prostaglandin E_2 (PGE_2) production in LPS-stimulated RAW 264.7 cells

Cells (1.8×10^5 cells/mℓ) were stimulated with LPS (1 μg/mℓ) for 24 h in the presence of the test substances. Supernatants were collected and the PGE_2 concentration in the supernatants was determined by ELISA. Values

are the mean ± SE of triplicate experiments. *,$P<0.05$ and **,$P<0.01$ indicate significant differences from the LPS-stimulated group. Ech: 80% ethanol extract of chestnut inner shell. EA: ellagic acid, GA: gallic acid, Cc: (+)-catechin. Hx: *n*-hexane fraction of Ech, EtOAc: ethyl acetate fraction of Ech, BuOH: butanol fraction of Ech, H_2O: water fraction of Ech

8. Effects on protein levels of iNOS and COX-2 in LPS-stimulated RAW 264.7cells

Inflammatory processes are mediated by multiple molecular mechanisms. Two of the most prominent are the production of NO by iNOS and the formation of prostaglandins by COX-2.[123)]To investigate the anti- inflammatory activity, we tested the effects of the test substances on LPS-induced iNOS and COX-2 protein up-regulation in RAW 264.7 cells by Western blotting analysis. The expression levels of iNOS and COX-2 were strongly induced by LPS. The *n*-hexane and ethyl acetate fraction inhibited iNOS protein levels in a dose-dependent manner, whereas COX-2 protein levels decreased by 100 μg/mℓ of *n*-hexane fraction (Figure 2-9A). Gallic acid and (+)-catechin slightly inhibited COX-2 protein levels, but did not affect iNOS protein levels (Figure 2-9B).



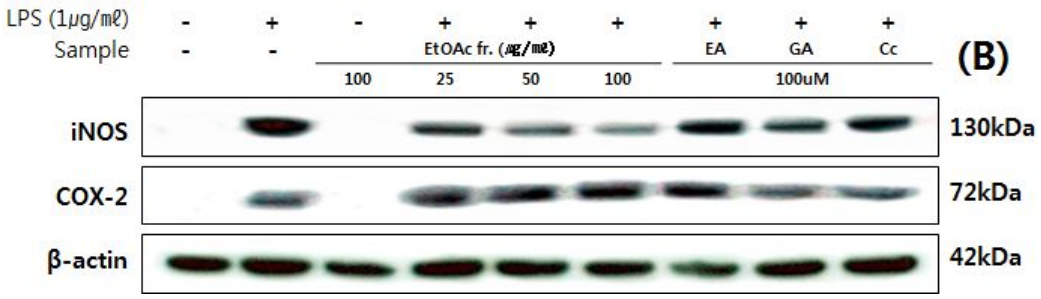


Figure 2-9. Effects of Ech, its solvent fractions, and the major polyphenols in Ech on protein levels of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells

Cells (1.8×10^5 cells/mℓ) were stimulated with LPS (1 μg/mℓ) for 24 h in the presence of the test substances. The levels of iNOS and COX-2 were determined using immunoblotting method. Ech: 80% ethanol extract of chestnut inner shell. EA: ellagic acid, GA: gallic acid, Cc: (+)-catechin. Hx: *n*-hexane fraction of Ech, EtOAc: ethyl acetate fraction of Ech, BuOH: butanol fraction of Ech, H_2O: water fraction of Ech.

9. Effects on the production of pro-inflammatory cytokines in LPS- stimulated RAW 264.7 cells

TNF-α, IL-1β, and IL-6 play important roles in the immune response to many inflammatory stimuli. LPS (1 μg/mℓ) stimulation of RAW 264.7 cells for 24 h increased TNF-α, IL-1β, and IL-6 levels in supernatants, as measured by ELISA. TNF-α levels treated with 100 μg/mℓ of *n*-hexane fraction were significantly decreased as compared to the LPS group. But the others of solvent fractions did not affect on TNF-α levels, whereas the three polyphenols reduced TNF-α levels (Figure 2-10). All of the solvent fractions and gallic acid significantly inhibited IL-1β production. Furthermore, the *n*-hexane and ethyl acetate fractions dose- dependently suppressed IL-1β levels (Figure 2-11). IL-6 levels exhibited lower in the supernatant from the cells treated with 100μg/mℓ of the *n*-hexane and ethyl acetate fraction than the LPS group (Figure 2-12).

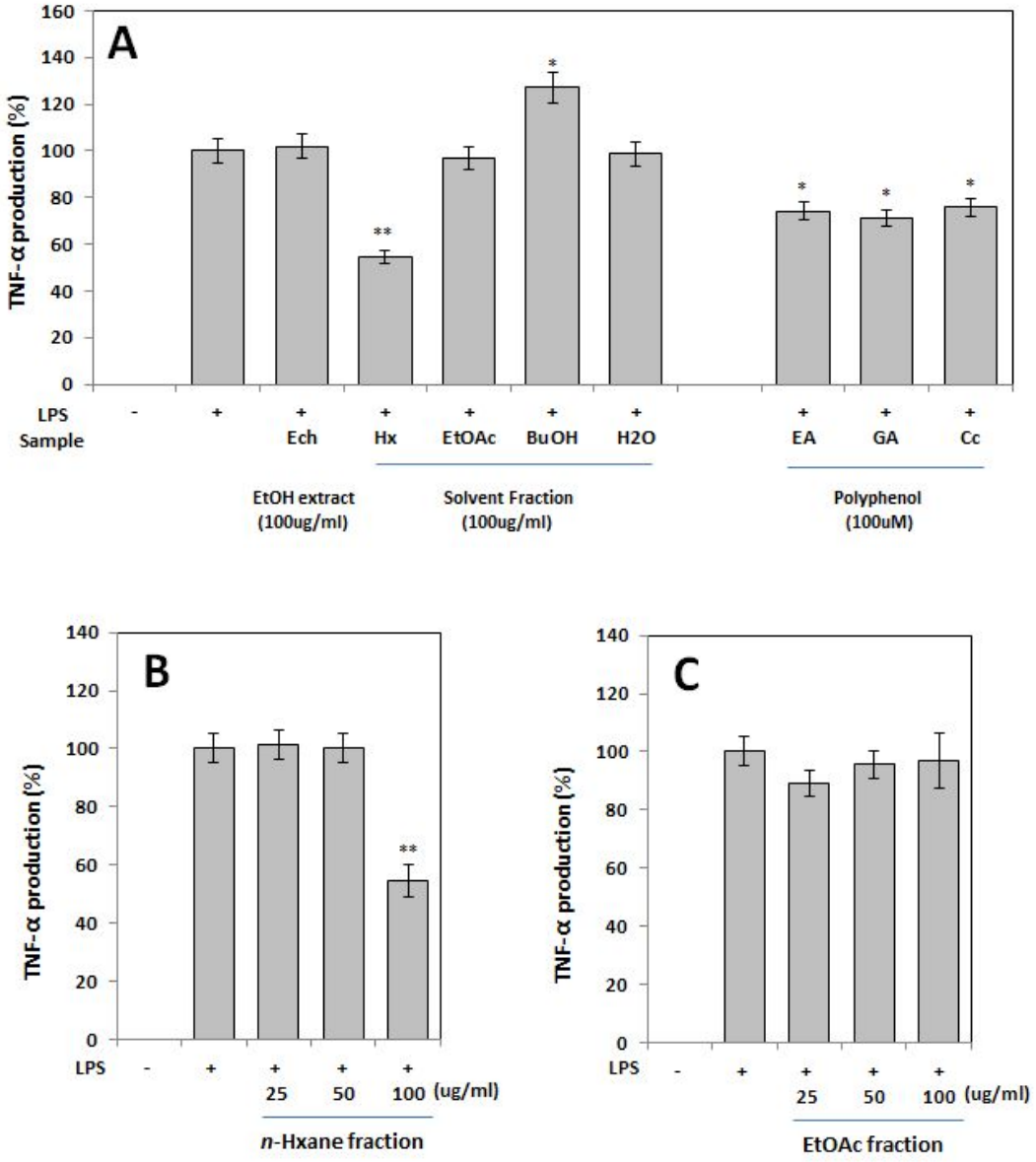


Figure 2-10. Effects of Ech, its solvent fractions, and the major polyphenols in Ech on TNF-α production in LPS-stimulated RAW 264.7 cells

Cells (1.8×10^5 cells/mℓ) were stimulated with LPS (1 μg/mℓ) for 24 h in the presence of the test substances. Supernatants were collected, and the TNF-α concentration in the supernatants was determined by ELISA. Values are the mean ± SE of triplicate experiments. **,P<0.05* and ***,P<0.01* indicate significant differences from the LPS-stimulated group. Ech: 80% ethanol extract of chestnut inner shell. EA: ellagic acid, GA: gallic acid, Cc: (+)-catechin. Hx: *n*-hexane fraction of Ech, EtOAc: ethyl acetate fraction of Ech, BuOH: butanol fraction of Ech,

H_2O: water fraction of Ech

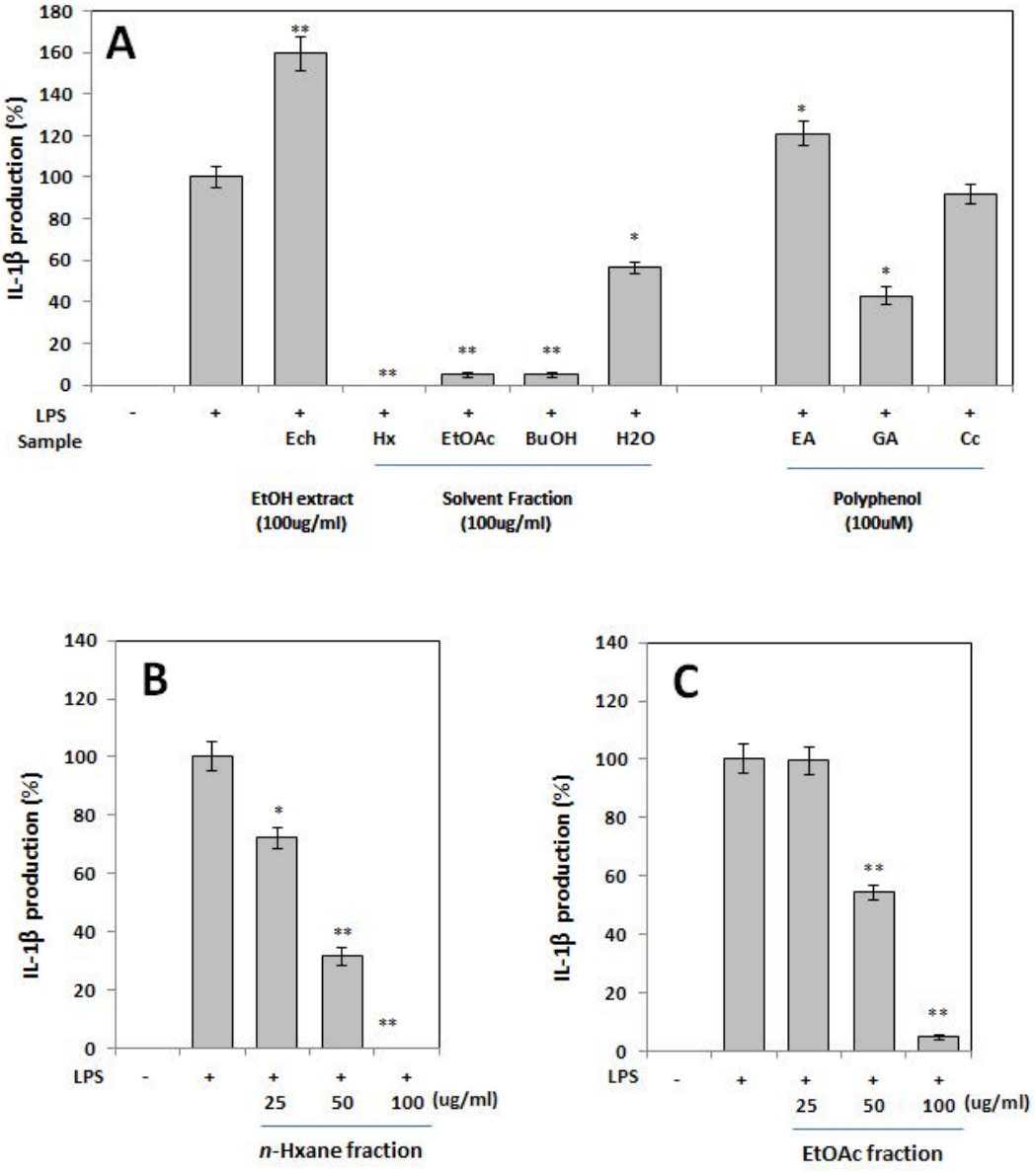


Figure 2-11. Effects of Ech, its solvent fractions, and the major polyphenols in Ech on IL-1β production in LPS-stimulated RAW 264.7 cells

Cells (1.8×10^5 cells/mℓ) were stimulated with LPS (1 μg/mℓ) for 24 h in the presence of the test substances. Supernatants were collected, and the IL-1β concentration in the supernatants was determined by ELISA. Values are the mean ± SE of triplicate experiments. **,P<0.05* and ***,P<0.01* indicate significant differences from the LPS-stimulated group. Ech: 80% ethanol extract of chestnut inner shell. EA: ellagic acid, GA: gallic acid, Cc: (+)-catechin. Hx: *n*-hexane fraction of Ech, EtOAc: ethyl acetate fraction of Ech, BuOH: butanol fraction of Ech, H_2O: water fraction of Ech

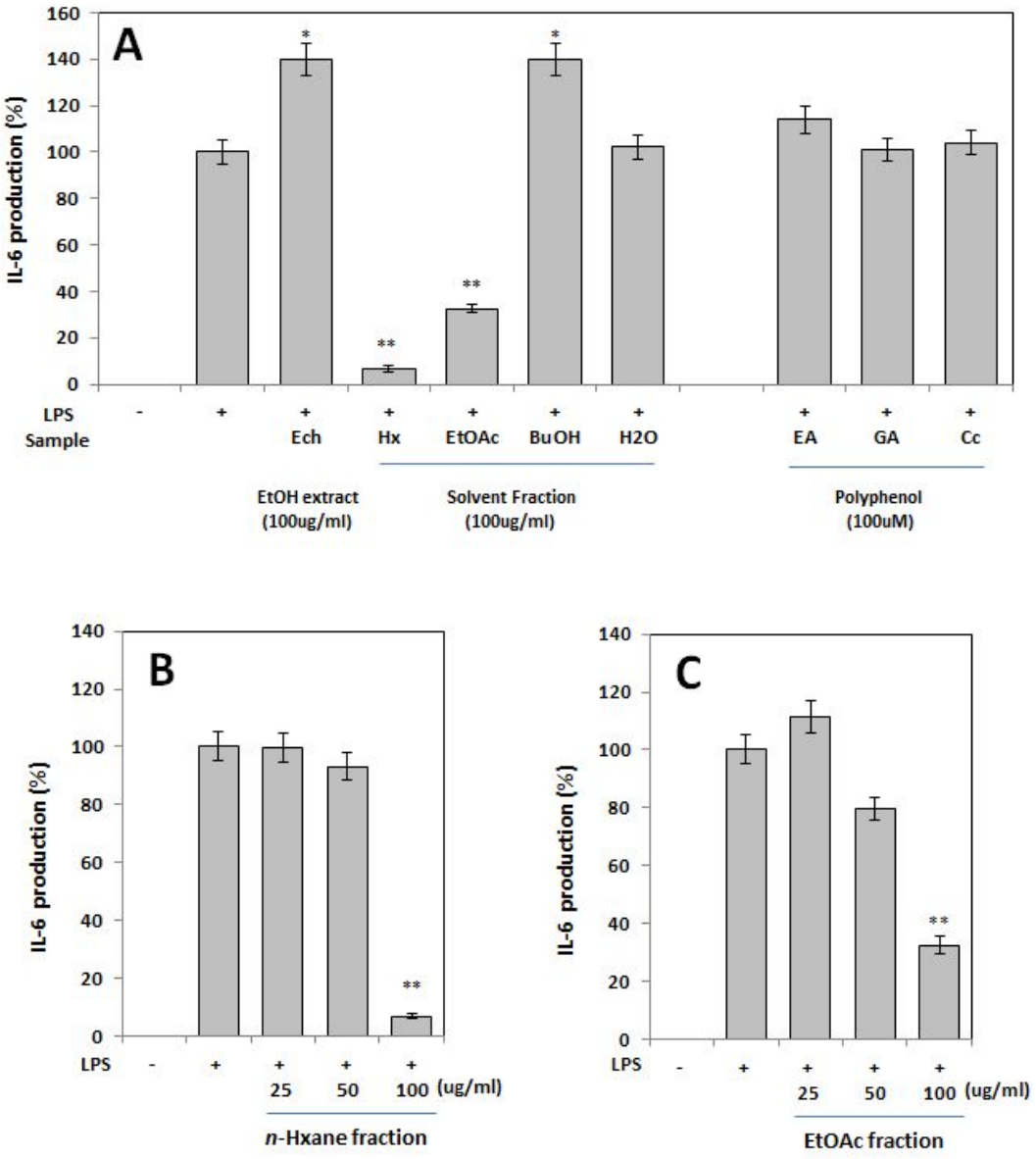


Figure 2-12. Effects of Ech, its solvent fractions, and the major polyphenols in Ech on IL-6 production in LPS-stimulated RAW 264.7 cells

Cells (1.8×10^5 cells/mℓ) were stimulated with LPS (1 μg/mℓ) for 24 h in the presence of the test substances. Supernatants were collected, and the IL-6 concentration in the supernatants was determined by ELISA. Values are the mean ± SE of triplicate experiments. **,P<0.05* and ***,P<0.01* indicate significant differences from the LPS-stimulated group. Ech: 80% ethanol extract of chestnut inner shell. EA: ellagic acid, GA: gallic acid, Cc: (+)-catechin. Hx: *n*-hexane fraction of Ech, EtOAc: ethyl acetate fraction of Ech, BuOH: butanol fraction of Ech, H_2O: water fraction of Ech

DISCUSSION

Chestnut inner shell, which is common food industrial byproduct,contains relatively high amount of tannins, which are well-known phenolic antioxidants. Although it has already been demonstrated that chestnut inner shell contain phenolic compound, little is known about its extract's antioxidant potential. To our knowledge, there are no reports describing any protective effects against CVD related risk factors. This study was performed to evaluate the effect of chestnut inner shell extract (Ech) on the CVD related parameters, such as antioxidative activity, inhibitory effects of platelet aggregation, ACE activity, and inflammatory mediators, including protective effect of oxidative DNA damage. Furthermore, the effects of its solvent fractions (*n*-hexane, EtoAC, BuOH, and H_2O fractions) and the major polyphenols (ellagic acid, gallic acid, and (+)-catechin) also were evaluated.

Oxidative stress have been accepted as important contributors to the development of atherosclerosis and hence, cardiovascular morbidity and mortality.[124)]Oxidative stress has been identified as critical in most of the key steps in the pathophysiology of atherosclerosis and acute thrombotic events, including dyslipidemia leading to atheroma formation, the oxidation of LDL, endothelial dysfunction, plaque rupture, myocardial ischemic injury.[125)]This study observed that the scavenging effects of Ech

and its polyphenols against both DPPH and hydroxyl radicals increased with the concentration. Ellagic acid was most potent, while the potency of Ech was similar to that of (+)-catechin. Among the solvent fractions of Ech, the BuOH fraction showed strong antioxidative activity, to follow the EtOAc fraction. Recent study have demonstrated that the predominant components in Korean chestnut inner shell were catechin, followed by gallic acid and coumarin by HPLC detection.[126]Kwon *et al*.[127]reported that ellagic acid and gallic acid were major phenolic acid of Korean chestnut husk, and ethyl acetate fraction of chestnut husk showed strong antioxidative activity, together with detection of gallic acid in EtOAc fraction. Considering these reports, high antioxidative properties of Ech, and BuOH and EtOAc fractions may attributed to the presence of phenolic compounds, such as gallic acid, (+)-catechin and ellagic acid.

In addition to the free radical scavenging activity, all of the test substances also showed the protective activities against oxidative DNA damage by comet assay. The cellular mechanism to reduce DNA damage is not clear at present. But, it is thought that Ech and solvent fractions along with the test polyphenols as antioxidants may protect DNA strand break induced by oxygen free radicals. It is remarkable that the DNA damage protective properties of Ech was higher than these of solvent fractions. Natural extracts with proven antioxidant activity usually contain compounds with a phenolic moiety, for example, cumarins, flavonoids, tocopherols and catechins. Organic acids, carotenoids, protein and tannins can also be present and act as antioxidants or have a synergistic effect with phenolic compounds.[128]Therefore, this result also suggested that the extracts including various phenolic compounds can

have better antioxidant activities against DNA damage than their fractions by synergistic effect.

The angiotensinIconverting enzyme (ACE) is a highly glycosylated zinc dipeptidyl-carboxypeptidase that plays an important role in the rennin-angiotensin system (RAS), where the latter regulates the arterial blood pressure and the electrolyte balance in mammals.[46]ACE catalyzes the degradation of angiotensin I to angiotensin II, a potent vasoconstrictor, by removing the carboxyl terminal dipeptide, His-Leu.[47]The inhibition of ACE activity is a "therapeutic approach" for the treatment of hypertension and associated coronary diseases in humans.[129]Currently, several synthetic drugs that act as ACE inhibitors have been synthesized and are used to treat arterial hypertension in humans, such as Captopril and Enalapril. Nevertheless, the identification of natural sources that act as ACE inhibitors had also been reported. In present study, only ellagic acid (100 μM) decreased hippuric acid (HA) production by ACE, whereas the others including Ech did not. Ranilla *et al.*[130]reported the correlation between the total phenolic content levels and antioxidant capacities of plant extracts and inhibitory activities against α-glucosidase, lipase, ACE or α-amylase, and observed that a moderately positive correlation between the ACE-inhibitory activities of aqueous peppers extracts and their total phenolic content (R^2 = 0.61), but not their antioxidant activities. The same authors reported no correlation between total phenolic and ACE-inhibitory activities for aqueous extracts of commonly used medicinal plants in Latin America. Eriz *et al.*[131]demonstrated that not only the proanthocyanidin concentration but also the structural differences influence the inhibition of ACE activity *in vitro*. On the other hand, previous studies had shown that the tannins could lower the basal blood

pressure within 10 minutes.[132)133)]However, the complexity of the blood pressure regulatory mechanisms means that the lowering of the blood pressure by the tannins cannot directly substantiate the inhibitory activity of endogenous ACE. Therefore, to understand the influence of this class of compounds on blood pressure or ACE activity, more information by *in vivo* and *invitro* tests is needed.

Decreased platelet aggregation is frequently associated with low incidence and prevalence of CVD. Drugs that attenuate platelet aggregability, such as aspirin, prostacyclin, or the nitric oxide donor nitroprusside, are protective for ischemic heart disease.[134)]In present study,it was observed that *n*-hexane and BuOH fractionssignificantly decreased initial slope of platelet aggregation, and the extract of chestnut inner shell and (+)-catechinslightly decreased initial slope of platelet. Several *in vitro* studies showed that polyphenols such as resveratrol, quercetin and catechin inhibit platelet aggregation.[135)~137)]De Lange *et al*.[138)]also reported that the polyphenol -rich grape extract strongly inhibit ADP-induced platelet aggregation and the main flavonoids present in this extract are catechin, epicatechin, gallic acid, and quercetin, which are also found in the chestnut inner shell. Oh *et al.*[139)]reported that the results of polyphenol compound of solvent fractions of chestnut inner shell extract analysis, ellagic acid, naringenin, gallic acid, flavonol were included in the butanol extracts.

Inflammatory process also plays an important role in all stages of atherosclerosis, including increasing plaque vulnerability to rupture.[140)]Atherosclerotic plaques from unstable symptomatic patients exhibit significant infiltration by leukocytes, which secrete matrix-degrading enzymes and thrombogenic substances, resulting in plaque disruption and local thrombosis.[38)]Accordingly, for the primary prevention of CVD,

interest in finding natural anti-oxidants or anti-inflammatory materials has increased greatly.

There are many mediators of inflammation, example of which are some cytokines, nitric oxide (NO), and prostaglandins (PGs). Nitric oxide (NO) is involved in a variety of cellular processes, including vasodilation,[141] host defense,[142] and cell proliferation,[143] yet high levels of NO can be pathogenic. NO is generated by three isoforms of nitric oxide synthase: eNOS (endothelial NOS), nNOS (neuronal NOS), and iNOS (inducible NOS). The genes encoding eNOS and nNOS are constitutively expressed at low levels, while iNOS expression is dormant in resting cells, but can be induced to 10-fold higher levels than eNOS by various inflammatory agents such as IFN-γ and lipopolysaccharide (LPS).[142] High levels of NO generated by iNOS are thought to contribute to pathology in inflammatory diseases such as atherosclerosis,[144][145] multiple sclerosis,[146] diabetes, and arthritis.[147]

Prostaglandin (PG) biosynthesis has been implicated in the pathophysiology of cardiovascular processes and a variety of inflammatory diseases.[148] The rate-limiting enzyme in the biosynthesis of PGs is prostaglandin-H_2-synthase, or cyclooxygenase (COX). Two COX isoforms have been identified, referred to as COX-1 and COX-2. In contrast to COX-1, a constitutively expressed enzyme, COX-2 is induced in response to growth factors, cytokines, and LPS.[149~151] Several studies have demonstrated the presence of COX-2 in atherosclerotic plaques, mainly co-localizing with macrophages.[152~154] It has been proposed that COX-2-mediated PG production by activated macrophages may promote atherosclerosis through a number of mechanisms, including stimulated chemotaxis, induction of vascular permeability, propagation of the inflammatory cytokine cascade, stimulation of smooth muscle cell

migration and proliferation, and synthesis of extracellular matrix.[155]PGE_2, which sensitizes nociceptors, prostacyclin (PGI_2) and perhaps thromboxane (Tx) A_2 mediate pain and inflammation.[156]Thus, agents that inhibit the production of these inflammatory mediators have been earlier considered as potential candidates for anti-inflammatory drugs. The results of this study showed that *n*-hexane and EtOAc fractions of chestnut inner shell extract inhibited NO production inhibited by suppressing iNOS expression in LPS-stimulated RAW 264.7 cells. Additionally, the *n*-hexane fraction reduced PGE_2 production and COX-2 expression.

Previous studies have shown that iNOS expression is stimulated by pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α, which contribute to multiple organ failure and tissue damage.[157]This study observed that *n*-hexane fraction of chestnut inner shell extract resulted in reduction of LPS-induced TNF-α, IL-1β, and IL-6 production. This indicates that the inhibitory activity of *n*-hexane fraction against LPS-induced NO production is associated with the inhibition of iNOS, COX-2, and pro-inflammatory cytokine production. In the other test substances of this study, although IL-6 production was suppressed by treated EtOAc fraction, IL-1β production was inhibited by all solvent fractions. All of test major polyphenols, which contained in chestnut inner shell, inhibited TNF-α production in LPS-stimulated RAW 264.7 cells. Further, gallic acid reduced even if IL-1β production.

There is now evidence that IL-1β has pro-atherogenic properties.[158]In apolipoprotein-E deficient mice lacking IL-1β, the severity of atherosclerosis is lowered as estimated by the atherosclerotic lesion size.[159]The pro-atherogenic effects of IL-1β are caused by increased endothelial adhesion, increased vascular

permeability, activation of macrophages, smooth muscle and endothelium cell proliferation, and protease induced plaque rupture.[160)]

TNF-α, another pro-inflammatory cytokine, plays a causative role in triggering and in the perpetuation of atherosclerosis.[161)]This is corroborated by findings that increased TNF-α levels predict an increased mortality risk in heart failure patients and is associated with the severity of heart failure.[162)163)]TNF-α exhibits pro-atherogenic effects through the following mechanisms: increments in ROS production, which results in endothelial dysfunctions[164)]; enhanced vascular permeability, depressed myocardial contractility, and induction of a prothrombotic state.[165)]

IL-6 is not really classified as a typically pro-atherogenic compound because this cytokine has anti- and pro-atherogenic effects.[166)]Nevertheless, IL-6 may contribute to atherosclerosis via atherosclerotic plaque formation and plaque destabilisation.[167)168)] Danesh *et al.*[169)]examined the association between subchronic increased IL-6 levels and the risk for CAD as defined by non fatal myocardial infarction and fatal CAD.

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말이나 글로는 그 감사한 마음을 다 전하지 못하겠지만, 학부를 포함해 근 20년 동안 저를 지도해 주신 강정숙 교수님께 진심으로 감사드립니다. 교수님 덕분에 학문의 길로 들어설 수 있었고, 교수님과 함께 할 수 있어서 부족한 제가 여기까지 올 수 있었던 것 같습니다. 또한, 바쁘신 가운데도 미흡한 저의 논문을 다듬어 주시고, 심사해 주신 전유진 교수님, 이남호 교수님, 권인숙 교수님, 김소미 교수님께도 깊은 감사를 드립니다.좋은 강의와 가르침으로 부족한 저의 학문적 범위를 넓혀 주시고, 여러모로 격려해 주신 윤창훈 교수님, 고양숙 교수님, 양양한 교수님, 신동범 교수님, 채인숙 교수님, 박성수 교수님, 강창희 교수님, 주홍구 교수님께도 감사드립니다.

나의 길었던 실험실 생활을 즐겁게 해준 실험실 식구들… , 늘 관심 갖고 격려해 준 정애 언니, 응원을 아끼지 않았던 후배 양희, 깐깐한 선배를 잘 견디며 실험 때마다 열심히 해준 자영, 혜진, 지미, 힘들었던 박사과정을 함께여서 견딜 수 있게 해준 성희에게도 고마운 마음을 전합니다. 한 사무실에서 일하며, 많은 이해와 양보를 해주고, 마음의 힘이 되어 주었던 은주, 현지, 그리고 자기 일보다 더 잘 챙겨주고, 아낌없는 도움을 주었던 혜숙에게도 이제서야 고맙다는 말을 전합니다.

새롭게 배워가면서 data를 만들어야 했던 in vitro 실험을 위해 많은 도움을 주신 제주생물종다양성연구소 정용환 소장님과 윤원종 박사님께 깊은 감사를 드리며, 바쁜 실험실 생활에도 늘 밝은 얼굴로 도와 주었던 민철, 혜미, 길남, 그리고

전유진 교수님 연구실 식구들에게도 고마운 마음을 전합니다. 언제나 연구실 문을 활짝 열어주시고, 많은 조언과 격려를 아끼지 않으셨던 전유진 교수님, 교수님이 계셨기에 박사 과정 동안 무모한 용기를 부려볼 수도 있었고, 더 든든했던 것 같습니다. 다시 한번 감사드립니다.

석사 시절부터 한결 같은 믿음으로 지켜봐 주고, 조언과 위로를 해주었던 미희언니와 충훈오빠, 민성오빠에게도 이 지면을 빌어 감사 드리며, 늘 관심 갖고 옆에서 응원해 주신 송현주 교수님, 고민조 약사님, 한수민 약사님께도 감사드립니다.

부족한 며느리를 늘 자랑스러워해 주시고, 사랑으로 대해주시는 시부모님, 바쁘다는 이유로 뭐 하나 제대로 챙기지 못하는 저를 이해해 주시고, 배려해 주시는 형님들, 관심과 격려를 아끼지 않으시는 시누이, 시아주버님들께도 죄송한 마음과 감사한 마음을 전합니다.

바쁜 나를 이해해 주고, 조카까지 봐주며, 마음 속으로 응원해 주신 형부와 지현언니, 동생 민주, 윤혜, 권우와 제부들에게도 고마움을 전하며, 언제나 나의 든든한 힘이 되어주신 아버지, 직장 생활에 공부까지 하는 딸이 안쓰러워 아픈 어깨로 반찬에, 국까지 해다주시던 우리 엄마, 끝없는 믿음으로 큰 격려가 되어주신 친정 부모님께 사랑한다는 말과 함께 감사의 마음을 전합니다.

마지막으로 결혼 생활 8년동안 나보다 애들을 더 잘 챙기며, 내가 공부에 전념할 수 있도록 배려를 아끼지 않았던, 나의 든든한 신랑 기철오빠와 많은 시간을 같이 보내주지 못했는데도 부족한 엄마를 늘 사랑한다고 말해 주며, 뭔지도 모르는 논문을 도와준다던 큰딸 나은이, 작은 딸 채은이에게 미안한 마음과 함께, 정말 고맙고, 사랑한다는 말을 전합니다.

지난 시간동안 끝없는 믿음으로 지켜봐 주시고, 격려가 되어주신 이 많은 분들이 계셨기에 이러한 결실을 맺을 수 있었던 것 같습니다. 이러한 믿음이 헛되지 않도록 앞으로도 더 열심히 살아가리라 다짐하면서 제게 도움을 주신 모든 분들께 다시 한번 감사의 마음을 전합니다.