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Characteristics of Pressurized Liquid Extracts from Natural Plants in Jeju

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This thesis has been examined and approved.

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Contents

Part II: Optimization of Extraction Conditions for Total phenolics from *Sapium japonicum* using a Pressurized Liquid Extractor

Part III: Characteristics of Pressurized Liquid Extracts from Natural Plants in Jeju

요 약

제주 자생식물 20종을 대상으로 고압용매 추출하여 총고형분 함량, 총페놀 함량, 통합적 항산화 능력, 세포독성 및 NO 생성 저해 활성, GC/MS에 의한 개별폴리페놀 성분을 정성・정량하였다.

(1) 제주 자생식물 20종을 대상으로 고압용매 추출 (추출용매 100% MeOH, 추출온도 40℃, 추출 압력 13.6 MPa, 추출 시간 10분) 하여 총고형분 함량, 총페놀 함량과 통합적 항산화 능력을 측정함으로써, 고압용매 추출에 의한 폴리페놀 성분의 추출 가능성 여부를 확인 하였다. 고압용매 추출물의 총고형분 추출수율은 붉나무, 말오줌때, 사방오리나무, 사람주나무, 팥배나무가 각각 21.8, 21.5, 21.1, 20.7, 20.1%로 가장 높았다. 총페놀 함량은 아그배나무가 68.3 mg GAE/g dry sample로 가장 높았고, 다음으로 사람주나무, 석위, 말오줌때가 각각 57.6, 56.6, 55.1 mg GAE/g를 나타내었다. 수용성 항산화 능력은 이질풀, 사람주나무, 산딸나무, 붉나무가 각각 598, 394, 293, 270 μmol ascorbic acid equivalents/g로 높았고, 지용성 항산화 능력은 백량금, 새우나무, 이질풀, 붉가시나무가 611, 314, 296, 242 μmol trolox equivalents/g로 높았다.

(2) 사람주나무를 대상으로 추출변수 (추출용매의 종류 및 농도, 추출횟수, 추출온도, 추출압력)를 달리하여 고압용매 추출하고 총고형분 함량과 총페놀 함량을 측정하여 고압용매 추출조건을 최적화하였다. 추출수율은 용매 종류 및 농도, 추출 횟수, 추출온도에 의하여 변화되었다. 추출용매에 따른 고압용매 추출물의 총고형분 추출수율은 H_2O, MeOH, EtOH가 각각 17.3, 17.9, 8.4%로 MeOH 추출물이 가장 높았고, 총페놀 함량도 H2O, MeOH, EtOH이 각각 48.8, 50.4, 27.2 mg GAE/g로 MeOH 추출물이 가장 높았다. 추출용매의 농도에 따른 고압용매 추출물의 총고형분 추출수율은 MeOH:H_2O (40:60, 60:40 v/v)와 EtOH:H_2O (40:60, 60:40 v/v)가 각각 24.4, 23.8, 23.6, 22.1%로 MeOH:H_2O (40:60, v/v) 추출물이 가장 높았고, 총페놀 함량은 MeOH:H_2O (40:60, 60:40 v/v) 와 EtOH:H_2O (40:60,

60:40 v/v) 추출물이 각각 85.0, 84.3, 90.6, 76.8 mg GAE/g로 EtOH:H_2O (40:60, v/v)추출물이 가장 높았다. 추출횟수가 증가할수록 총고형분 추출수율과 총페놀 함량은 증가하였다. 추출온도에 따른 고압용매 추출물의 총페놀 함량은 추출온도가 40℃(97.4 mg GAE/g)에서 50℃ (108.3 mg GAE/g)으로 증가하였을 때 11% 증가하였으나, 그 이상의 추출온도에서는 변화가없었다. 추출압력 증가에 따른 고압용매 추출물의 총고형분 추출수율과 총페놀 함량은 추출압력에 관계없이 변화가 없었다. 고압용매 최적추출조건은 추출용매와 농도 EtOH:H_2O (40:60, v/v), 추출횟수 2회, 추출온도 40℃, 추출압력 10.2 MPa 이었다.

(3) 고압용매 최적추출조건에서 제주 자생식물 20종을 추출하여 항산화 활성, 세포독성 및 NO 생성 저해 활성, 폴리페놀 성분을 동정·정량하여 식품산업에 응용할 천연항산화소재로서의 가능성을 검정하였다. 고압용매 추출물의 추출수율은 이삭여뀌가 28.5%로 가장 높았으며, 사람주나무, 귀룽나무, 말오줌때가 각각 27.3, 25.8, 25.2%를 나타내었다. 총페놀 함량은 새우나무, 사람주나무, 이질풀, 짚신나물, 석위가 각각 105.4, 105.1, 104.4, 92.2, 90.6, 90.5 mg GAE/g로 가장 높았다. 수용성 항산화 능력은 이질풀과 석위가 976, 948 μmol ascorbic acid equivalents/g로 가장 높았고, 지용성 항산화 능력은 백량금과 이질풀이 945, 520 μmol trolox equivalents/g로 가장 높았다. GC/MS에 의하여 폴리페놀 성분을 동정한 결과 8개의 폴리페놀 피크를 얻었는데, gallic acid와 catechin이 가장 많이 함유되어 있었다. 폴리페놀 전체 함량은 catechin이 많이 함유된 새우나무와 gallic acid가 많이 함유된 사람주나무가 2,970 ppm, 2,963 ppm로 가장 높았다. 세포독성을 고려한 NO 생성 저해 활성인 선택지수는 사람주나무, 사방오리나무, 산딸나무, 아그배나무가 각각 4.5, 3.4, 2.3, 2.2로 자생식물 추출물 중 가장 높았다. 자금우, 된장풀, 아그배나무, 귀룽나무, 붉나무 추출물은 HS-68 세포에 대한 독성이 없었다.

List of Figures

List of Tables

INTRODUCTION

Free radicals are produced in oxidation processes that are essential to most living organisms for the production of energy to fuel biological processes (Soares et al., 2009). However, the excessive production of free radicals, such as superoxide radicals (O_2^{-} ・), hydroxyl radicals (・OH), and peroxyl radicals (ROO ・), and the unbalanced mechanisms of antioxidant protection have been associated with carcinogenesis, coronary heart disease, and many other health issues related to advancing age (Borneo et al., 2009; Slusarczyk et al., 2009).

Almost all organisms are well protected against free radical damage by oxidative enzymes such as superoxide dismutase and catalase or chemical compounds such as α-tocopherol, ascorbic acid, carotenoids, and polyphenolic compounds. However, these systems are frequently insufficient to totally prevent diseases and accelerated ageing (Soares et al., 2009). Since antioxidants terminate directly reactive oxygen species mediated oxidative reactions, they may be used as a method of preventing aging-associated diseases and health problems (Borneo et al., 2009).

Natural products with antioxidant activity may be used to help the human body to reduce oxidative damage (Soares et al., 2009). Therefore, there is a growing interest in natural substances exhibiting antioxidant properties that are supplied to human and animals as food components or as specific preventive pharmaceuticals (Slusarczyk et al., 2009). This has led to an accelerated research for the identification of natural resources and the isolation of active antioxidant molecules (Borneo et al., 2009).

The plant kingdom offers a wide range of natural antioxidants. However, there is still not enough information about the practical usefulness of them. In

the group of secondary plant metabolites, antioxidant phenolics are commonly found in various fruits, vegetables, herbs, cereals, sprouts, seeds, and edible mushrooms and they have been shown to provide a defence against oxidative stress from oxidizing agents and free radicals (Slusarczyk et al., 2009). A general recommendation to the public is to increase the intake of foods rich in antioxidant compounds due to their wellknown healthy effects (Borneo et al., 2009).

Phenolic compounds are a large class of secondary plant metabolites that are distributed widely in the plant kingdom and possess an aromatic ring with one or more hydroxyl substituents (Mukhopadhyay et al., 2006). Polyphenols, possessing two or more phenolic subunits, include flavonoids (anthocyanins, flavanols, flavonols, flavanones) and several classes of non-flavonoids (phenolic acids, stilbenes and complex molecules derived from them) (Chirinos et al., 2007). Phenolic compounds are known to exhibit various health benefits such as antioxidant, antiinflammatory, antihepatotoxic, antitumor, atherosclerosis, arthritis, diabetes, antimutagenic, anticarcinogenic, antithrombotic, vasodilatory activities and antimicrobial activities (Cook and Samman, 1996; Proestos et al., 2004; Rice-Evans et al., 1997; Tsao and Deng, 2004; Yoshimoto et al., 2001). Phenolic compounds also play an important role in the nutritional, organoleptic and commercial properties of agricultural foodstuffs, since they contribute to their sensory properties such as color, astringency, bitterness and flavor (Alonso-Salces et al., 2001).

The chemical structure of the phenolic compounds varies from simple phenolics to complex polymerics that may possess multiple hydroxyl groups conjugated to sugars, acids or alkyl groups. The polarities of phenolic compounds also vary significantly. Thus, extraction of phenolic compounds from plant matrices is complex and challenging (Luthria et al., 2008;

Mukhopadhyay et al., 2006).

Traditionally, the techniques employed in the extraction of phenolic compounds from fruits and vegetables involve the use of organic solvent mixtures including methanol, ethanol and acetone (Howard and Pandjaitan, 2008). These traditional extraction methods have several drawbacks. They are time consuming and laborious. They are also employed large amounts of organic solvents which are expensive and environmentally unfriendly (P′eres et al., 2006).

At present, there is a growing interest in developing new extraction methods of natural plants based on the use of supercritical fluids or small amounts of organic solvents. Among them, pressurized liquid extraction (PLE) is the most promising process.

PLE is an excellent alternative to conventional organic solvent extraction techniques that combines elevated temperature and pressures with liquid solvents to increase the extraction efficiency of phenolic compounds from natural plants. The use of PLE with water and ethanol offers the potential to minimize or eliminate the use of toxic solvents (Howard and Pandjaitanet, 2008). Since PLE is conducted at elevated pressures, it allows liquid extraction at temperatures above the boiling points of the solvent at atmospheric pressure, thereby improving analyte solubility and its desorption from the matrix (Rostagno et al., 2004). Pressure is also used to increase the contact between the extracting fluid and the sample, thus enabling rapid extractions (P′eres et al., 2006). Temperature is used to break the analyte-matrix bonds and modify the relative permittivity of the extracting fluid. This technique also allows the required volume of extraction solvent to be reduced and the extraction time to be shortened (Rostagno et al., 2004;

Palma et al., 2001).

The main reasons for the enhanced performance of PLE over other conventional extraction methods are the higher solubility of analytes in solvent and higher diffusion rate as a result of higher temperature. At higher temperatures, the strong solute-matrix interaction caused by van der Waals forces, hydrogen bonding and dipole attractions between solute molecules and active sites on the matrix are disrupted (Sae-Yun et al., 2006).

Light and oxygen in the air are two most important factors that facilitate degradation reaction of phenolic compounds. PLE offers the possibility of performing the extractions under an inert atmosphere and protected from light, which represents an attractive advantage since phenolic compounds are sensitive to these two factors (Rostagno et al., 2004; Palma et al., 2001).

There are several methods to measure antioxidative capacity. However, most techniques require long experimental times and high costs to determine antioxidative capacity of hydrophilic or lipophilic compound.

In photochemiluminescence (PCL) method, free radicals are generated photochemically by UV irradiation of a photosensitizer (dye) compound. These radicals are partially eliminated by reaction with antioxidants in the extracts. The remaining radicals are quantified by the measurement of the produced light as a result of the chemical reaction with a detection chemical, "luminol". The PCL assay is easy and rapid to perform, and does not requires high temperatures to generate radicals. It is suitable to measure the radical scavenging properties of single antioxidants as well as more complex systems in the nanomolar range (Besco et al., 2007; Shlesier et al., 2002; Popov and Lewin, 1994).

The objectives of this study were (1) to verify the applicability of PLE on the extraction of phenolic compounds from natural plants in Jeju, (2) to determine the optimal extraction conditions for total phenolics from *Sapium japonicum* as a model substrate using a pressurized liquid extractor, and (3) to characterize the extracts of plant materials at the optimized extraction condition.

Part I

Integral Antioxidative Capacity of Pressurized Liquid Extracts from Natural Plants in Jeju

1. ABSTRACT

Twenty natural plants collected from Jeju were extracted by pressurized organic solvent (100% MeOH, 40℃, 13.6 MPa, 10 min). Extraction yields of total soluble solids (TSS) and total phenolics (TP), and integral antioxidative capacity were evaluated. Extraction yields of TSS were higher as 21.8, 21.5, 21.1, 20.7, and 20.1% in *Rhus javanica, Euscaphis japonica*, *Alnus firma*, *Sapium japonicum,* and *Sorbus alnifolia*, respectively. Higher TP (mg GAE/g) were obtained from *Malus sieboldii* (68.3), *Sapium japonicum* (57.6), *Pyrrosia lingua* (56.6), and *Euscaphis japonica* (55.1). Integral antioxidative capacities of water-soluble substances were 598, 394, 293, and 270 μmol ascorbic acid equivalents/g in *Geranium thunbergii*, *Sapium japonicum*, *Cornus kousa,* and *Rhus javanica*, respectively. Integral antioxidative capacities of lipid-soluble substances were 611, 314, 296, and 242 μmol trolox equivalents/g in *Ardisia crenata*, *Ostrya japonica*, *Geranium thunbergii,* and *Quercus acuta*, respectively.

2. MATERIALS AND METHODS

2.1. Plant materials

Table 1 shows the scientific and traditional names of twenty natural plants collected in Jeju, Korea. The plants were washed, dried, grinded (Ika Work, Inc., USA), and passed through a standard sieve No. 30 (Chung Gye Sang Gong SA., Seoul, Korea). The samples were stored in freezer at -20℃ until needed.

Table 1. List of natural plants used for experiments

Scientific name	Traditional name	Part used
Agrimonia pilosa	Jibsinnamul	stem, leaves
Alnus firma	Sabangorinamu	leaves, branch
Ardisia crenata	Baegryanggum	leaves, branch
Ardisia japonica	Jakumwu	leaves, branch
Cornus kousa	Sanddalnamu	leaves, branch, fruit
Desmodium caudatum	Dounjangpul	leaves, branch
Euscaphis japonica	Malojumttaenamu	leaves, branch, fruit
Geranium thunbergii	Ijilpul	stem, leaves
Malus sieboldii	Agubaenamu	leaves, branch
Myrica rubra	Sogwuinamu	leaves, branch
Ostrya japonica	Saeunamu	leaves, branch
Persicaria filiformis	Isacyouggui	stem, leaves
Potentilla chinensis	Ddakjiggot	leaves, branch
Prunus padus	Kwuirungnamu	leaves, branch, fruit
Pyrrosia lingua	Sukwi	stem, leaves
Quercus acuta	Buggasinamu	leaves, branch
Rhus javanica	Bugnamu	leaves, branch
Sapium japonicum	Saramjunamu	leaves, branch
Sorbus alnifolia	Patbaenamu	leaves, branch, fruit
Stauntonia hexaphylla	Moulggul	leaves, branch

2.2. Pressurized liquid extraction

Dried powder (1 g) of natural plants was extracted using enhanced solvent extraction system (SFX 3560, Isco Inc., USA). The system was outfitted with two syringe pumps, one (B) for organic solvent (Model 260DX) and the other (A) for CO_2 (Model 100DX), and an variable restrictor (Fig. 1).

The extraction involved first placing an extraction cartridge (9 mL) with the sample inside the extraction chamber. Next, the supply valve was switched to the open position which allowed organic solvent (MeOH) from pump B to enter the extraction cartridge. The extraction chamber was then pressurized to the desired pressure. After attaining equilibrium (13.6 MPa, 40℃), a static extraction was initiated. After completion of the static period (3 min), the analyte valve was switched to the open position which allowed a certain volume of solvent (MeOH 10 mL) from pump B to extract the sample out of

the extraction cartridge at a flow rate of 1 mL/min. After completion of the dynamic extraction period (10 min), pump A which was filled with pressurized CO_2 flushed the remaining solvent out of the extraction chamber for 5 min. A 20 mL vial at ambient temperature was used to collect the extract. After completion of the CO_2 flush, both supply and analyte valves were closed and the system was vented. Extraction of the sample was carried out using three extraction steps, and the extracts were collected in one vial (Table 2). After the extraction solvent was evaporated in a rotary vacuum evaporator at 40℃, it was adjusted to 10 mL with neat MeOH and filtered through a 0.45 μm cellulose acetate filter (Advantec, Toyo Roshi Kaisha, Ltd., Japan). The extract was kept in the dark in a freezer (-20℃) before analysis. Each extraction and analysis was carried out in triplicate.

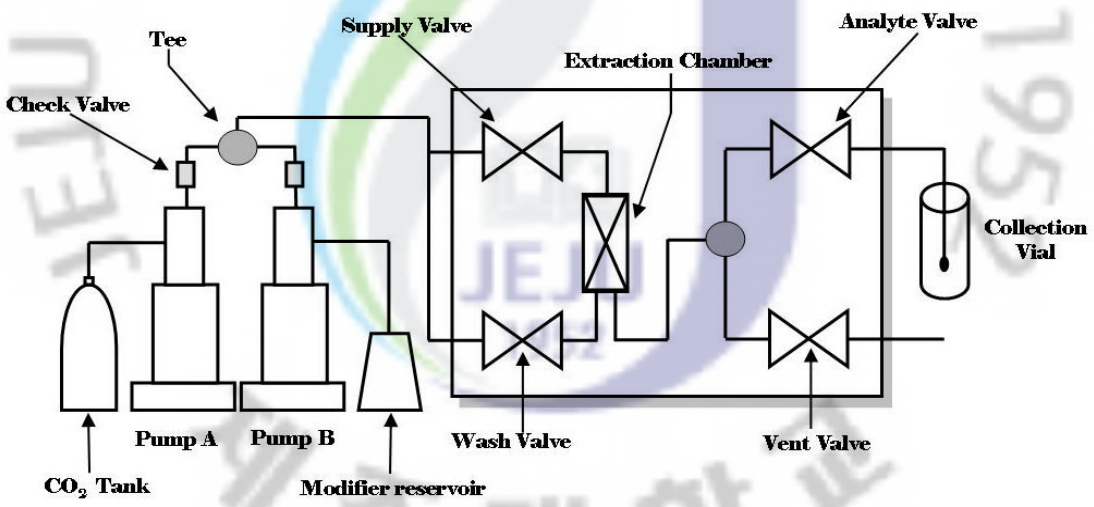

Fig. 1. Schematic diagram of pressurized liquid extraction system.

Table 2. PLE condition for extraction of natural plants in Jeju

Extraction pressure	13.6 MPa
Extraction temperature	40℃
Extraction solvent	100% MeOH
Collection trap	20 mL empty vial
Extraction cartridge volume	10 mL
Static extraction time	3 min
Dynamic extraction time	10 min
Flush solvent	CO_2
Flush time	5 min

2.3. Total solids assay

Total solid content was measured by drying method at 105℃. The extraction yield of total soluble solids (TSS) was calculated from the solid content in the extract based on that in the dried sample.

2.4. Total phenolics assay

Total phenolics (TP) content in the extract was determined by the Folin-Ciocalteu method (Peschel et al., 2006). The reaction mixture was composed of 0.1 mL extract, 7.9 mL distilled water, 0.5 mL of Folin-Ciocalteu's reagent (Fluka, Switzerland), and 1.5 mL of a 20% sodium carbonate solution (added 2 min after the Folin-Ciocalteu's reagent). After initial mixing, the flasks were allowed to stand for 2 hr. The optical density of the blue-coloured samples was measured at 765 nm using the UV/VIS spectrophotometer (Thermo Spectronic, NY, USA). Results were expressed in mg gallic acid equivalents per gram of dry sample (mg GAE/g) by comparison with a calibration curve built with standard gallic acid (Sigma, USA).

2.5. Integral antioxidative capacity assay

Integral antioxidative capacity of the extract was measured in the Photochem system (Analytik Jena AG, Jena, Germany) with the kits of antioxidative capacity of water-soluble substances (ACW) and of lipid-soluble substances (ACL) (Kranl et al., 2005; Besco et al., 2007; Chua et al., 2008).

2.5.1. ACW protocol

A 490 µL of reagent 1 (buffer solution, pH 10.5) and 10 µL H_2SO_4 were added to the reagent 4 containing ascorbic acid and mixed by vortex for 20~30 sec. This stock solution was then diluted 1:100 with reagent 1. A 10 µL of this working solution contain 1 nmol ascorbic acid as a calibration standard.

Integral antioxidative capacity of water-soluble substances was measured with the ACW kit. A 1.5 mL of reagent 1, 1 mL of reagent 2 (water), 25 µL of reagent 3 (photosensitizer) and 10 µL of the extract were mixed. Free radicals are generated photochemically by UV irradiation of a photosensitizer (dye) compound and are partially eliminated by reaction with antioxidants in the extracts. The remaining radicals are quantified by the measurement of the produced light as a result of the chemical reaction with a detection chemical, "luminol". The antioxidant potential of the extract was determined by comparing with the lag phase at different standard concentrations. Measurements were repeated triplicate. Results are expressed as µmol equivalents of ascorbic acid for each gram of the dried extract.

2.5.2. ACL protocol

A 500 µL of reagent 1 (MeOH) were added to the reagent 4 containing Trolox and mixed by vortex for 20~30 sec. This stock solution was then diluted 1:100 with reagent 1. A 10 µL of this working solution contain 1 nmol Trolox as a calibration standard.

Integral antioxidative capacity of lipid-soluble substances was measured with the ACL kit. A 2.3 mL of reagent 1, 200 μL of reagent 2 (buffer solution), 25 μL of reagent 3 (photosensitizer) and 10 μL of the extract were mixed. The remaining free radicals are quantified by the measurement of the produced light as a result of the chemical reaction with a detection chemical, "luminol". The antioxidant potential of the extract was determined by comparing with the area under the curve at different standard concentrations. Triplicate runs were made for each sample. Results are expressed as μmol equivalents of Trolox for each gram of the dried extract.

2.6. Statistical analysis

Statistical analyses were performed using SAS V.8.2 software (SAS Institute, Cary, NC, USA). Signification differences ($p<0.05$) among treatment means were determined by the Duncan's test.

3. RESULTS AND DISCUSSION

3.1. Total soluble solids

Table 3 shows the extraction yield of TSS of pressurized MeOH extracts from twenty natural plants in Jeju. TSS were higher as 21.8, 21.5, 21.1, 20.7, and 20.1% in *Rhus javanica*, *Euscaphis japonica*, *Alnus firma*, *Sapium japonicum* and *Sorbus alnifolia*, respectively. In addition, TSS were over 15% in *Malus sieboldii*, *Pyrrosia lingua*, *Ardisia crenata*, *Prunus padus*, and *Cornus kousa*. The lowest TSS was found in *Persicaria filiformis* as 8.2%.

Hyun et al. (2007) reported that TSS by organic solvent (MeOH) extraction at atmospheric pressure were slightly higher than our results in this study (*Ardisia japonica* (16.8%), *Cornus kousa* (18.4%), *Myrica rubra* (18.6%) and

Rhus javanica (19.9%)). However, that method is time consuming and use large amounts of organic solvents, which are expensive, and environmentally unfriendly. Thus, PLE will be an economic and efficient extraction process.

Table 3. Extraction yields of total soluble solids (TSS) and total phenolics (TP) of pressurized MeOH extracts from natural plants in Jeju

Plant species	Total soluble solids (%)	Total phenolics (mg GAE/g)	TP/TSS (%)
Agrimonia pilosa	13.8±0.2	36.5±0.4	26.4
Alnus firma	21.1±0.6	46.8±1.6	22.2
Ardisia crenata	16.4±0.7	36.3±1.6	22.1
Ardisia japonica	14.0±0.3	53.8±1.4	38.3
Cornus kousa	15.4±0.1	37.3±1.8	24.2
Desmodium caudatum	12.4±0.8	21.8±1.2	17.6
Euscaphis japonica	21.5±0.1	55.1±0.8	25.6
Geranium thunbergii	14.4±0.9	53.3±2.8	36.9
Malus sieboldii	16.9±0.3	68.3±1.9	40.4
Myrica rubra	12.6±0.5	36.1±0.4	28.6
Ostrya japonica	12.5±0.4	52.8±3.1	42.4
Persicaria filiformis	8.2±0.4	11.4±0.4	13.8
Potentilla chinensis	14.0±0.3	38.0±2.3	27.1
Prunus padus	15.9±0.4	47.0±2.0	29.6
Pyrrosia lingua	16.9±0.3	56.6±1.7	33.5
Quercus acuta	13.6±0.3	40.0±1.6	29.5
Rhus javanica	21.8±0.3	54.3±1.3	24.9
Sapium japonicum	20.7±0.4	57.6±3.2	27.9
Sorbus alnifolia	20.1±0.3	50.8±1.4	25.3
Stauntonia hexaphylla	11.0±0.2	16.1±0.6	14.6

3.2. Total phenolics

Table 3 also shows the TP content of pressurized MeOH extracts from twenty natural plants in Jeju. Higher TP were obtained from *Malus sieboldii* (68.3 mg GAE/g), *Sapium japonicum* (57.6), *Pyrrosia lingua* (56.6) and *Euscaphis japonica* (55.1). *Rhus javanica*, *Ardisia japonica*, *Geranium thunbergii*, and *Ostrya japonica* also showed more than 50 mg GAE/g.

Hyun et al. (2007) extracted TP by organic solvent (MeOH) extraction at

atmospheric pressure. TP contents were lower than the values exhibited in this study.

Luthria et al. (2006) reported that Black Bell cultivar of eggplant showed a great increase in extraction yields of TP by PLE compared with other extraction methods such as wrist shaker, rotary shaker, stirring, sonication, and reflux. The quantity of TP extracted from other extraction procedures ranged from 5% to 95% compared with PLE.

The ratio TP to TSS account for more than 30% in *Ostrya japonica* (42.4%), *Malus sieboldii* (40.4%), *Ardisia japonica* (38.3%), *Geranium thunbergii* (36.9%), and *Pyrrosia lingua* (33.5%), respectively.

3.3. Integral antioxidative capacity

Integral antioxidative (IAC) capacities of water- and lipid-soluble substances from twenty natural plants in Jeju were shown in Table 4. IAC of water-soluble substances were 598, 394, 293 and 270 μmol ascorbic acid equivalents/g in *Geranium thunbergii*, *Sapium japonicum*, *Cornus kousa* and *Rhus javanica*, respectively. IAC of lipid-soluble substances were 611, 314, 296 and 242 μmol trolox equivalents/g in *Ardisia crenata*, *Ostrya japonica*, *Geranium thunbergii* and *Quercus acuta*, respectively.

The extracts from *Geranium thunbergii* and *Sapium japonicum* showed higher antioxidative capacities both in water- and lipid-soluble substances, with higher TP as 359 and 270 mg GAE/g, respectively. The extracts from *Ostrya japonica* and *Malus sieboldii* also showed higher IAC of lipid-soluble substances with higher TP of 411 and 399 mg GAE/g, respectively. From those results, it revealed that the antioxidative capacities of the extracts from natural plants in Jeju closely correlated with their total phenolic contents.

Chang et al. (2001) reported that the antioxidant activity of plant materials is well correlated with their phenolic contents, and the antioxidant activities of phenolic compounds are due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers.

Besco et al. (2007) reported that the lipid and water-soluble antioxidant activities of the extraction from baobab products measured by PCL method ranged from 1.2 to 386.0 μmol of ascorbic acid/g and from 1.0 to 508.0 μmol of trolox/g, respectively. The antioxidant potentials of pressurized liquid extracts from natural plant in Jeju are much higher than those obtained for the extraction from baobab product.

Besco et al. (2007) reported that as the water soluble antioxidants, flavonoids, ascorbic acid, and amino acid were detected, while as the lipid soluble antioxidants, tocopherols, tocotrienols, and carotenoids were measured.

Table 4. Integral antioxidative capacity (IAC) of pressurized MeOH extracts from natural plants in Jeju

Plant species	IAC of water-soluble substances (Ascorbic acid, μmol/g)	IAC of lipid-soluble substances (Trolox, μmol/g)
Agrimonia pilosa	11.4±00.4	199.8±12.4
Alnus firma	112.6±03.5	198.0±09.1
Ardisia crenata	191.2±01.7	611.7±23.8
Ardisia japonica	246.1±00.6	190.4±06.2
Cornus kousa	293.2±26.0	219.2±11.5
Desmodium caudatum	56.9±03.9	147.5±12.7
Euscaphis japonica	230.7±34.2	177.0±01.1
Geranium thunbergii	598.7±10.9	296.3±26.8
Malus sieboldii	-	231.4±12.9
Myrica rubra	172.6±09.5	186.5±06.0
Ostrya japonica	-	314.0±20.6
Persicaria filiformis	76.0±03.1	227.4±17.3
Potentilla chinensis	115.3±05.4	148.9±09.2
Prunus padus	61.6±01.8	223.1±13.4
Pyrrosia lingua	62.1±02.9	202.9±10.9
Quercus acuta	62.1±03.3	242.1±04.9
Rhus javanica	270.1±16.4	208.2±11.3
Sapium japonicum	394.8±08.1	230.0±00.2
Sorbus alnifolia	114.5±07.6	213.4±05.1
Stauntonia hexaphylla	90.3±05.0	132.6±03.7

Part Ⅱ

Optimization of Extraction Conditions for Total phenolics from *Sapium japonicum* using a Pressurized Liquid Extractor

1. ABSTRACT

Sapium japonicum, a natural plant in Jeju, was extracted by a pressurized liquid. Operating parameters such as the type of solvent, the ratio of solvent to water, temperature, pressure, and number of extraction steps were investigated as the main variables that influence the extraction efficiencies of total soluble solids (TSS) and total phenolics (TP). TP contents were affected by the type of solvent, solvent-water ratio, extraction step and temperature. Higher extraction yields (17.9 and 17.3%) of TSS were obtained when MeOH and H_2O were used as the extraction solvents. MeOH extracted the highest level of TP as 50.4 mg GAE/g compared with 48.8 and 27.2 mg GAE/g with H_2O and EtOH, respectively. EtOH:H_2O (40:60, v/v) was found to be the best solvent for TP extraction as 90.3 mg GAE/g compared with 85.0, 84.3, and 76.8 mg GAE/g in MeOH:H_2O (40:60, 60:40, v/v) and EtOH:H_2O (60:40, v/v), respectively. TSS and TP were increased with the increase of the number of extraction steps. TP content was increased by 11% as the extraction temperature was increased from 40℃ (97.4 mg GAE/g) to 50℃ (108.3 mg GAE/g). Extraction pressure had no effect on the extraction efficiency. The optimum extraction conditions of TSS and TP were ; 40% EtOH, 2 extraction steps, temperature 50℃, and pressure 10.2 MPa.

2. MATERIALS AND METHODS

2.1. Plant materials

Sapium japonicum was used as the test material and the sample preparation

method was the same as in Part Ⅰ.

2.2. Pressurized liquid extraction

The extraction method was the same as in Part Ⅰ except the followings. All extraction were done using 9 mL high temperature crystalline polymer cartridges. This cartridge was filled with a 15~20 mesh of inert sea sand (Junsei Chemical Co., Ltd., Japan) between the sample (2 g and 5.2 g of sand at the bottom and top, respectively) to prevent the clogging of the system. End caps have molded in 2 μm frits. Extraction was carried out using two extraction steps with EtOH:H_2O (40:60, v/v) at 40℃ and 10.2 MPa for 10 min. Unless otherwise mentioned, each extraction was carried out under this default conditions in triplicate.

To study the influence of different extraction parameters on the extraction efficiency of TSS and TP, the type of solvent, the ratio of solvent to water, temperature and pressure, number of extraction steps were investigated.

2.2.1. Extraction solvent and ratio of solvent to water

Neat solvents (MeOH, EtOH, H_2O), as well as a systematic variation of MeOH and EtOH concentration in H_2O (20, 40, 60, 80%), were used for extraction of phenolic compounds from natural plants. The other extraction conditions were kept as described above.

2.2.2. Extraction steps

The number of extraction steps was varied between one and three. Natural plants were extracted with EtOH:H_2O (40:60, v/v) solvent mixture. Two extracts from each step were collected in the same vial.

2.2.3. Temperature

Temperature varied from 40 to 80℃ in increments of 20℃. Extractions were carried out with EtOH:H_2O (40:60, v/v) using two extraction steps.

2.2.4. Pressure

Extractions were conducted at four different pressures (10.2, 13.6, 17.0, and 20.4 MPa) with EtOH:H_2O (40:60, v/v) at 50℃ using two extraction steps.

2.3. Total solids assay

The method was the same as in Part Ⅰ.

2.4. Total phenolics assay

The method was the same as in Part Ⅰ except that the optical density of the blue-coloured sample was measured at 750 nm using the ELISA reader (Multiskan EX, Thermo Electrom Corp., Vantaa, Filand).

3. RESULTS AND DISCUSSION

3.1. Extraction solvent

The plant material is frequently extracted using the organic solvents with different polarity, and the extraction yield of the soluble solids is strongly depend on the properties of the solvent because polyphenolic compound may exist as free, conjugated and polymeric forms, and complexes with carbohydrate, protein or other plant components (Luthria, 2008).

Effect of neat solvents on the extraction yield of TSS from *Sapium japonicum* is shown in Fig. 2. The highest extraction yields of TSS were achieved with H_2O (17.3%) and MeOH (17.9%), while the lowest with EtOH (8.4%).

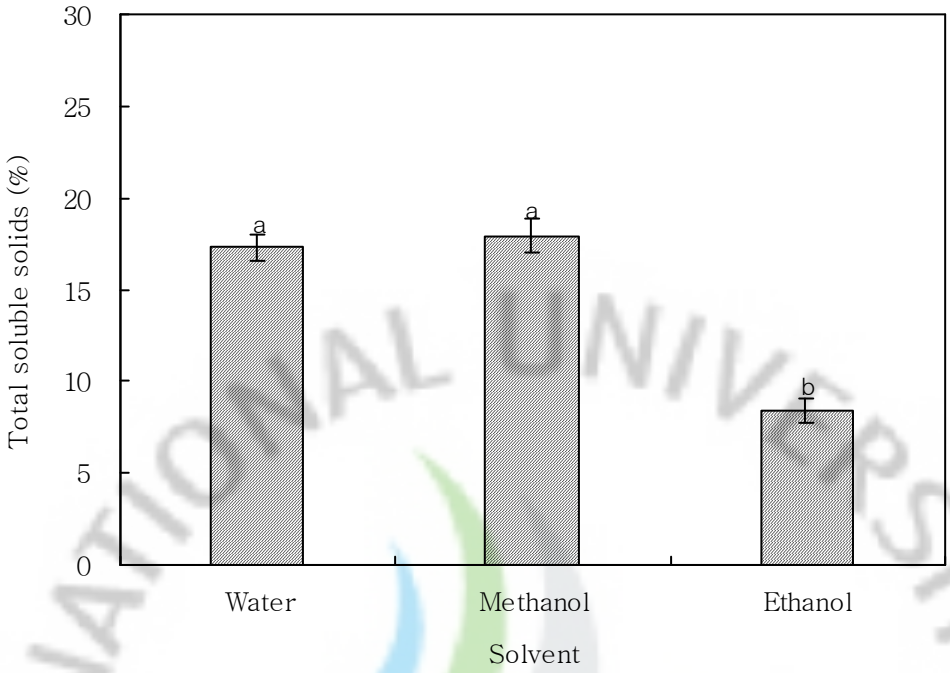

Fig. 2. Influence of neat solvents on the extraction yield of total soluble solids from *Sapium japonicum* (extraction pressure: 10.2 MPa, temperature: 40℃, no of extractions: 2). The same letters are not significantly different at 5% level by Duncan's multiple test.

Influence of neat solvents such as MeOH, EtOH, H_2O on the extraction efficiency of TP from *Sapium japonicum* is shown in Fig. 3. Use of neat MeOH resulted in the highest TP extraction yield (50.4 mg GAE/g) compared with EtOH (27.2) and H_2O (48.8).

As flavonoids and phenolic acids are more soluble in methanol than ethanol (Markham, 1982), it is reasonable to obtain a higher extraction yield when methanol is used as an extraction solvent as shown in Fig. 3. Chirinos et al. (2007) have also reported that MeOH gave the highest TP values for five solvents such as water, methanol, ethanol, acetone, and hexane from mashua

tubers.

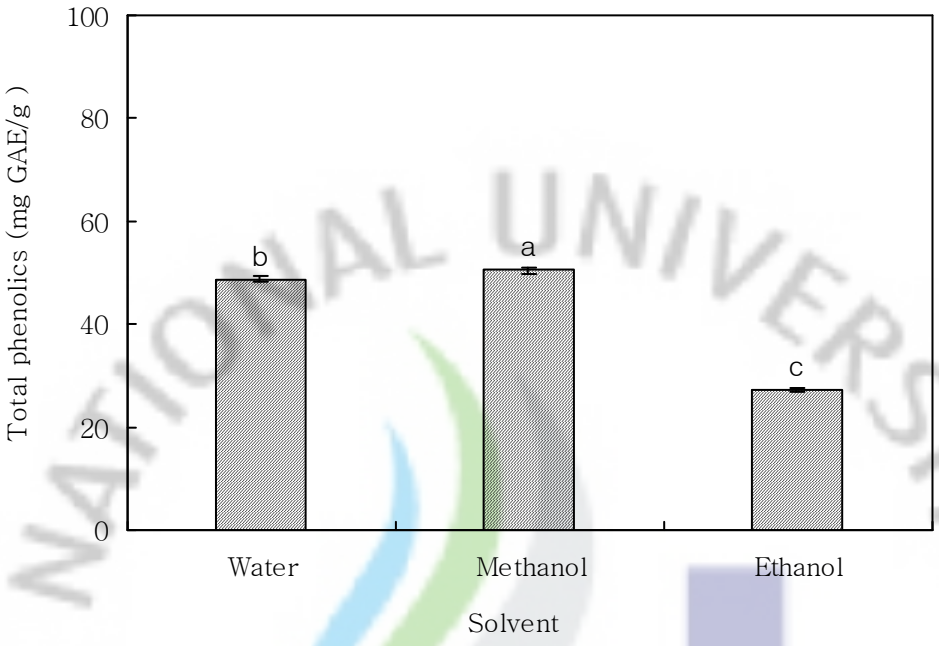

Fig. 3. Influence of neat solvents on the extraction efficiency of total phenolics from *Sapium japonicum* (extraction pressure: 10.2 MPa, temperature: 40℃, no. of extractions: 2). The same letters are not significantly different at 5% level by Duncan's multiple test.

3.2 Ratio of solvent to water

The extraction yields of TSS and TP as a function of solvent-water ratio were evaluated. The proportion of solvent in the extraction medium had a significant effect on the extraction yields of TSS and TP.

Fig. 4 shows the influence of solvent composition on the extraction yield of TSS from *Sapium japonicum*. The highest yield of TSS were obtained when extractions were carried out with MeOH:H_2O (40:60, v/v), EtOH:H_2O (40:60, v/v), and MeOH:H_2O (60:40, v/v) solvent mixtures.

Spigno et al. (2007) also reported higher extraction yield of total extract from grape seeds when 50% ethanol was used, and when ethanol concentration was higher than 75%, the extraction yield decreased.

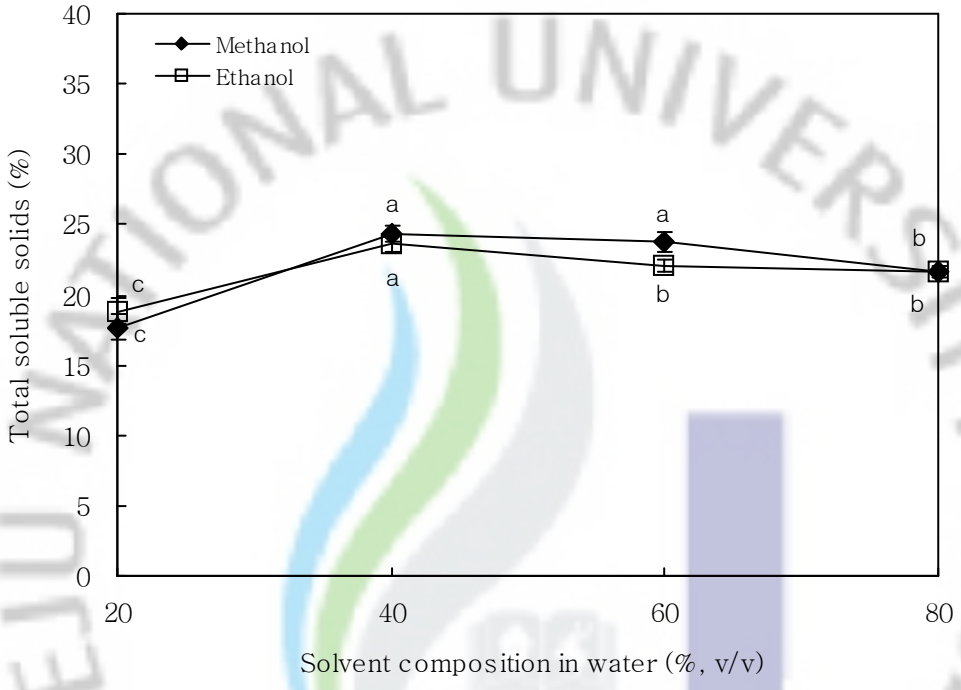

Fig. 4. Influence of solvent composition on the extraction yield of total soluble solids from *Sapium japonicum* (extraction pressure: 10.2 MPa, temperature: 40℃, no of extractions: 2). The same letters are not significantly different at 5% level by Duncan's multiple test.

Fig. 5 shows the variation in the extraction efficiency of TP with different MeOH:H_2O and EtOH:H_2O solvent mixtures. The highest TP (90.3 mg GAE/g) was obtained with EtOH:H_2O (40:60, v/v). The extraction efficiency of TP with MeOH:H_2O (40:60, v/v)(85.0 mg GAE/g) was similar to that with MeOH:H_2O (60:40, v/v)(84.3 mg GAE/g).

Jayaprakasha et al. (2008) also reported that 40~50% ethanol has a greater effectiveness in extracting polyphenolic compounds compared to pure ethanol which was probably due to the increased solubility of flavonoids, phenolic compounds, hydrolysable tannins and polysaccharides in the mixture of ethanol and water.

Alonso-Salces et al. (2001) also reported that mixture of methanol with water improved the extraction of polyphenols with several hydroxyl groups, such as glycosides which are hydrophilic than a pure alcoholic solvent.

The combination of water with other organic solvents contribute to the creation of a moderately polar medium (Chirinos et al., 2007).

Mukhopadhyay et al. (2006) reported that addition of water was found to swell the plant material and allow the solvent to penetrate to the solids matrix more easily. Thus, TP yields using solvent mixtures were higher those using neat solvents only.

When the extract was used for medicinal or ingestion purposes, pure EtOH or a mixture of EtOH and H_2O has typically been used due to the toxicity of MeOH.

Also due to the results obtained so far, EtOH:H_2O (40:60, v/v) solvent mixture was selected for further experiments.

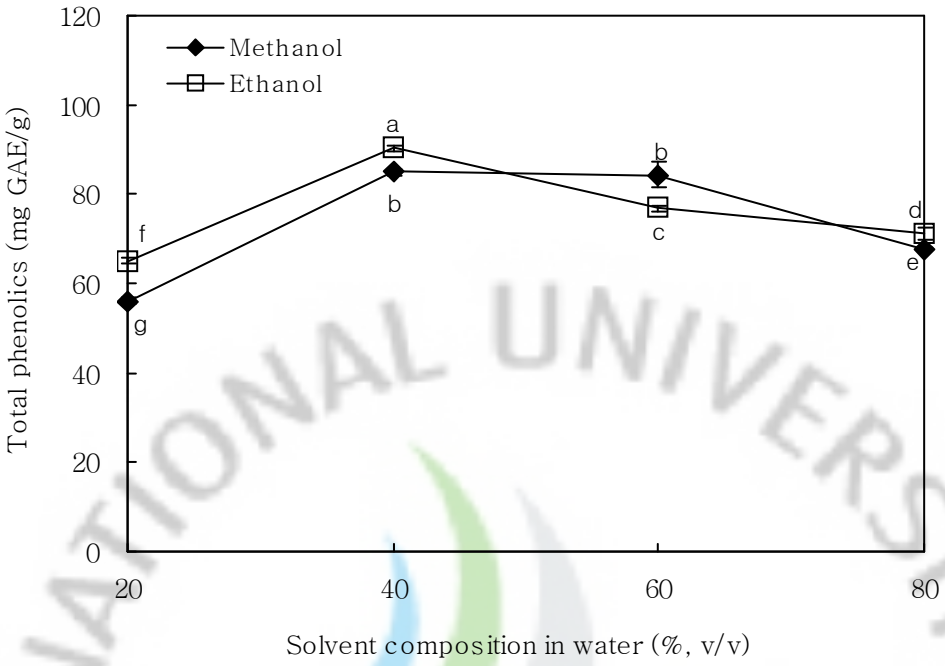

Fig. 5. Influence of solvent composition on the extraction efficiency of total phenolics from *Sapium japonicum* (extraction pressure: 10.2 MPa, temperature: 40℃, no. of extractions: 2). The same letters are not significantly different at 5% level by Duncan's multiple test.

3.3. Extraction steps

Fig. 6 shows the influence of extraction steps on the extraction yield of TSS from *Sapium japonicum* using EtOH:H_2O(40:60, v/v) as an extraction solvent. Higher extraction yield of TSS was obtained in more than one extraction step. However, two extraction steps showed the same yield as three extraction steps.

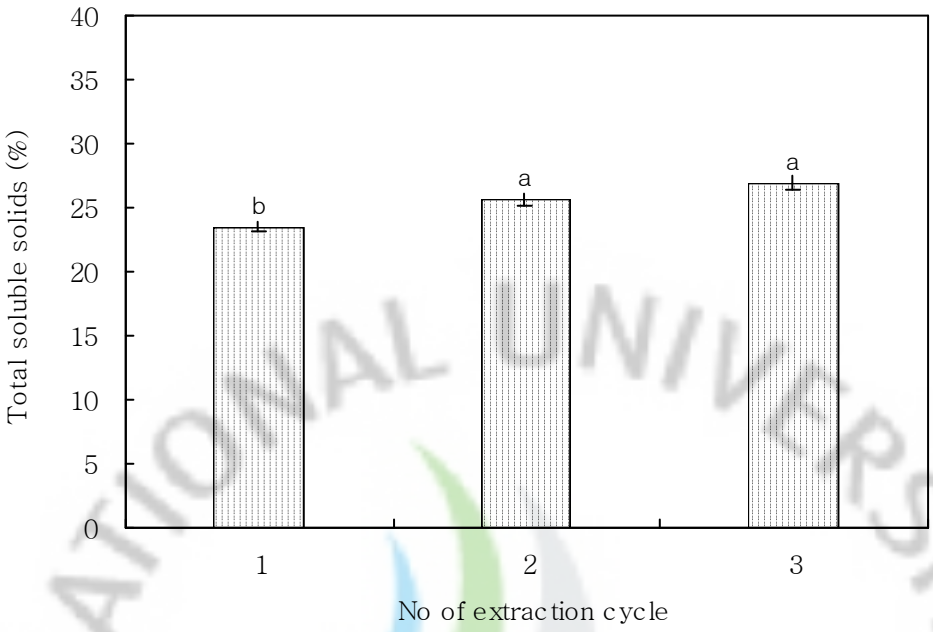

Fig. 6. Influence of extraction steps on the extraction yield of total soluble solids from *Sapium japonicum* (solvent composition: EtOH:H_2O(40:60, v/v), extraction pressure: 10.2 MPa, temperature: 40℃). The same letters are not significantly different at 5% level by Duncan's multiple test.

Influence of extraction steps on the extraction efficiency of TP from *Sapium japonicum* is shown in Fig. 7. In one, two and three extraction steps, 80.7, 88.8, and 97.0 mg GAE/g of TP were obtained, respectively.

Even though, the extraction yield of TP with two extraction steps was higher than that with three extraction steps, two extraction steps were chosen for optimizing additional PLE parameters for extracting TP from natural plants.

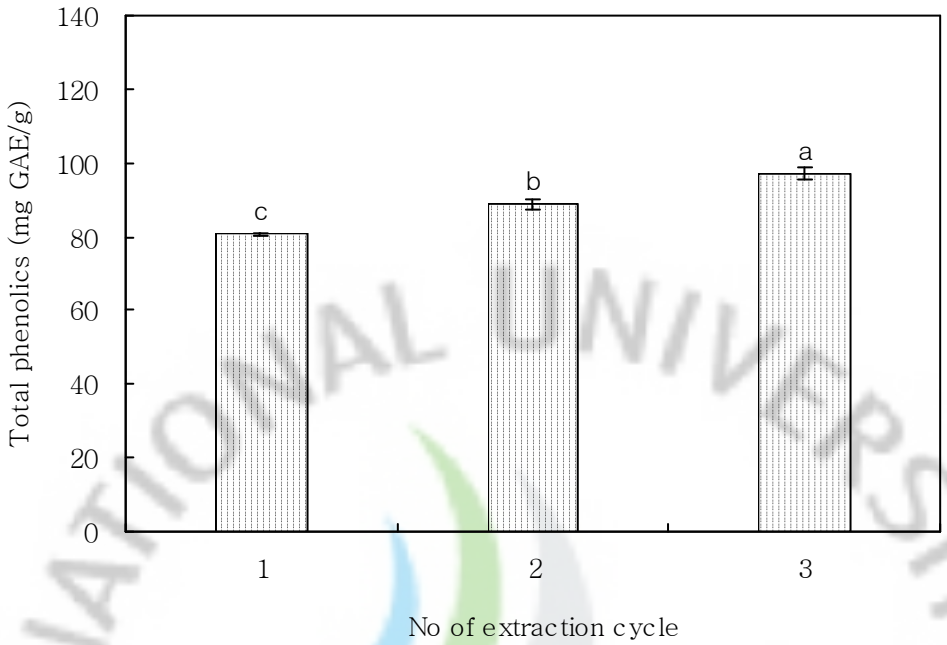

Fig. 7. Influence of extraction steps on the extraction efficiency of total phenolics from *Sapium japonicum* (solvent composition: EtOH:H_2O(40:60, v/v), extraction pressure: 10.2 MPa, temperature: 40℃). The same letters are not significantly different at 5% level by Duncan's multiple test.

3.4. Temperature effect

The influence of temperature on extraction efficiency was investigated since it impact the equilibrium solubility, mass transfer rate and the stability of phenolic compounds (Luthria, 2008).

Natural plant was extracted with EtOH:H_2O (40:60, v/v) solvent mixture at five different temperatures (40, 50, 60, 70, 80℃) and 10.2 MPa with two extraction steps.

Extraction temperature had very little effect on extraction yields of TSS as shown in Fig. 8.

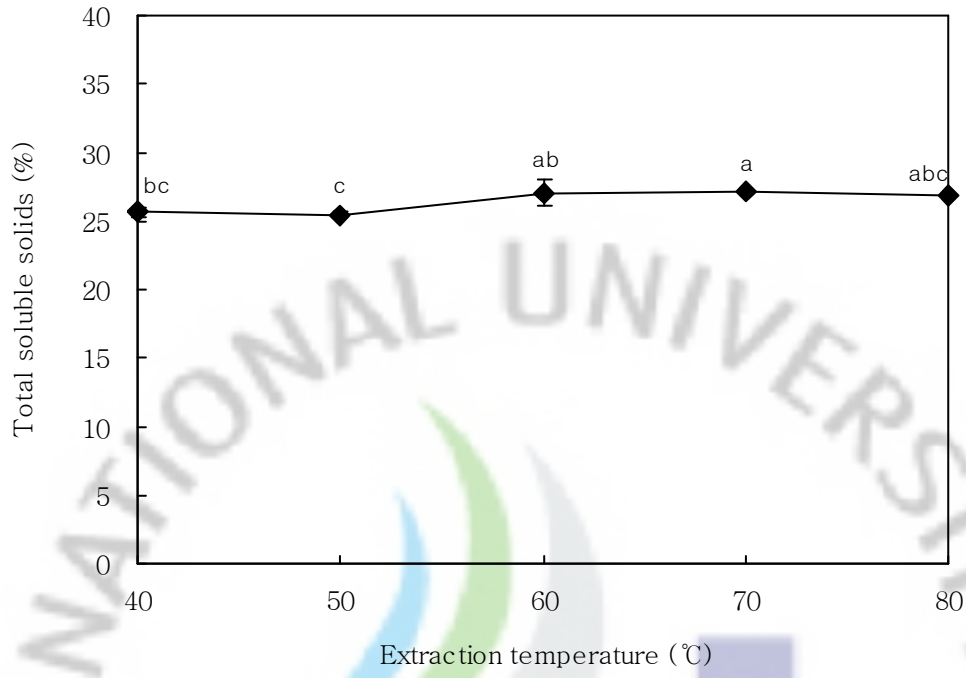

Fig. 8. Influence of temperature on the extraction yield of total soluble solids from *Sapium japonicum* (solvent composition: EtOH:H_2O(40:60, v/v), extraction pressure: 10.2 MPa, no. of extractions: 2). The same letters are not significantly different at 5% level by Duncan's multiple test.

Fig. 9 shows influence of temperature on the extraction efficiency of TP from *Sapium japonicum*. TP were increased by almost 11% as the temperature increased from 40℃ (97.4 mg GAE/g) to 50 ℃ (108.3 mg GAE/g).

Higher TP yield is due to the breakage of bonds between various phenolic and the plant matrix (Mukhopadhyay et al., 2006).

However, TP contents were remained almost constant over 50℃ such as at 60℃ (110.9 mg GAE/g), 70℃ (112.0) and 80℃ (113.4). Alonso-Salces et al. (2001) also reported that extraction yields of TP were slightly increased from

40℃ to 60℃ and decreased at higher temperatures. In those experiments over 50 ℃, the formation of black colored precipitates in the extracts was observed. This phenomena could be due to a possible degradation of polyphenols at high temperatures, caused by hydrolysis, internal redox reactions and polymerizations (Alonso-Salces et al., 2001).

Taking into accounts, 50℃ was selected as the optimum temperature.

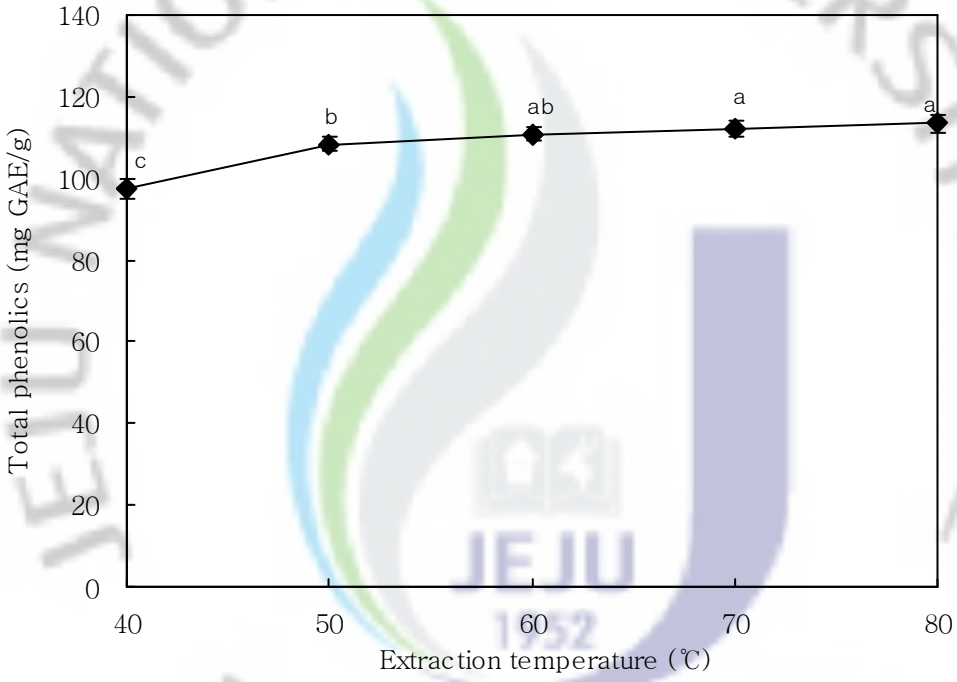

Fig. 9. Influence of temperature on the extraction efficiency of total phenolics from *Sapium japonicum* (solvent composition: EtOH:H_2O(40:60, v/v), extraction pressure: 10.2 MPa, no. of extractions: 2). The same letters are not significantly different at 5% level by Duncan's multiple test.

3.5. Pressure effect

The high pressure of PLE allows the use of temperature well above their atmospheric boiling point of the solvent and increases the diffusivity of the

extraction solvent with the plant matrix (Lou et al., 1997).

Extractions were carried out at four different pressures (10.2, 13.6, 17.0, 20.4 MPa). Other operating parameters were kept unchanged except the extraction temperature of 50℃.

Fig. 10 shows the influence of extraction pressure on the extraction yield of TSS from *Sapium japonicum*. Extraction pressure had no effect on the extraction yields of TSS. The extraction yields of TSS obtained at 10.2, 13.6, 17.0 and 20.4 MPa were 25.7, 26.6, 24.5 and 26.1%, respectively.

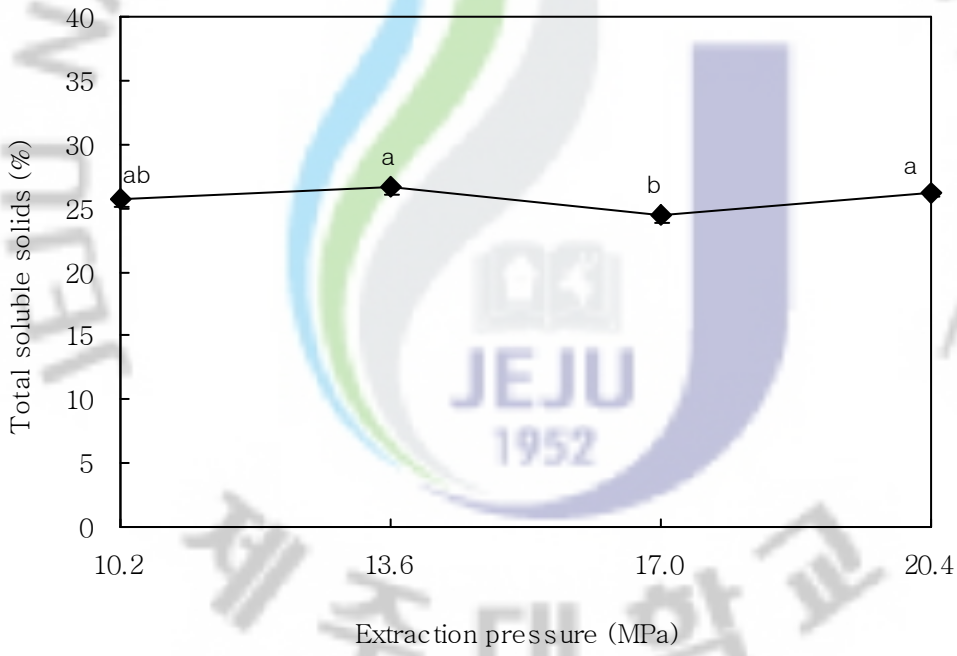

Fig. 10. Influence of pressure on the extraction yield of total soluble solids from *Sapium japonicum* (solvent composition: EtOH:H_2O(40:60, v/v), extraction temperature: 50℃, no. of extractions: 2). The same letters are not significantly different at 5% level by Duncan's multiple test.

Influence of extraction pressure on the extraction efficiency of TP from *Sapium japonicum* is shown in Fig. 11. An increase in pressure did not change the amount of TP. Values of TP obtained at 10.2, 13.6, 17.0 and 20.4 MPa were 103.1, 102.0, 96.3 and 102.1 mg GAE/g, respectively.

Similar results were also observed during extraction of phenolic compounds from black cohosh at 3.4, 6.8, 10.2 MPa (Mukhopadhyay et al., 2006) and from parsley at 6.8, 8.5, 10.2 MPa (Luthria., 2008). Actually the purpose of pressurizing the extraction chamber is to prevent the solvent from boiling at the extraction temperature and to ensure that the solvent remains in intimate contact with the sample (Alonso-Salces et al., 2001).

In conclusion, the optimized operating conditions for extraction of TP from natural plants in Jeju were : extraction solvent, EtOH:H_2O (40:60, v/v); temperature, 50℃; pressure, 10.2 MPa; and two extraction steps.

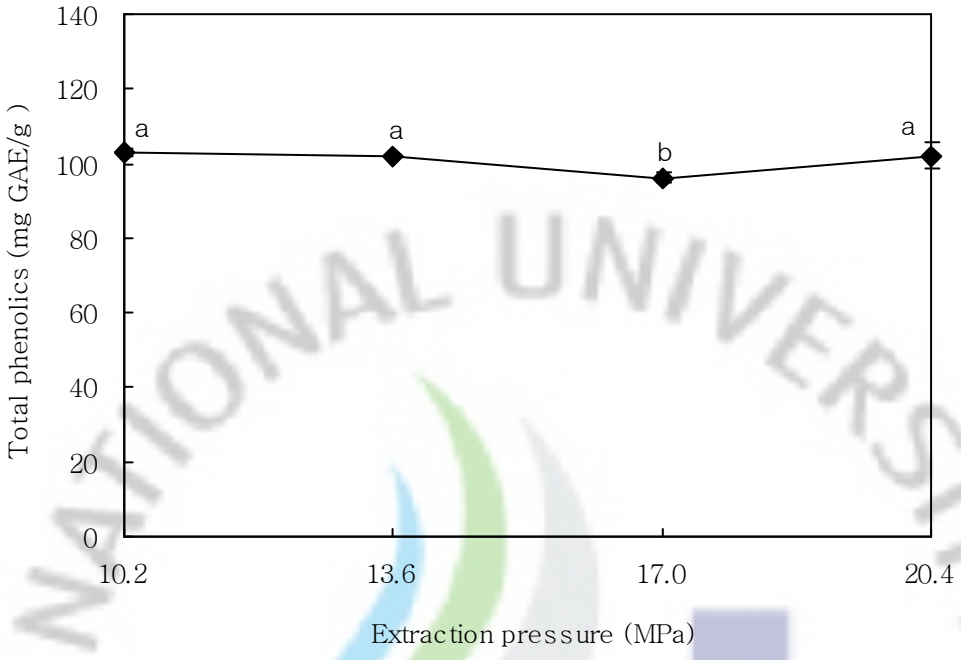

Fig. 11. Influence of the pressure on the extraction efficiency of total phenolics from *Sapium japonicum* (solvent composition: EtOH:H_2O(40:60, v/v), extraction temperature: 50℃, no. of extractions: 2). The same letters are not significantly different at 5% level by Duncan's multiple test.

Part Ⅲ

Characteristics of Pressurized Liquid Extracts from Natural Plants in Jeju

1. ABSTRACT

Twenty natural plants collected in Jeju were extracted at optimized pressurized liquid extraction condition (40% EtOH, 50℃, 13.6 MPa, 10 min). Extraction yields of total soluble solids (TSS) and total phenolics (TP), integral antioxidative capacity (IAC), individual phenolics by GC/MS, and cell toxicity and NO production inhibitory activity were evaluated. Extraction yield of TSS was the highest in *Persicaria filiformis* as 28.5% with the decreasing order of 27.3, 25.8 and 25.2 % in *Sapium japonicum*, and *Prunus padus*, respectively. *Ostrya japonica* and *Sapium japonicum* showed the highest TP (105.4 and 105.1 mg GAE/g), followed by *Geranium thunbergii* (104.4), *Agrimonia pilosa* (92.2), *Pyrrosia lingua* (90.6), and *Prunus padus* (90.5). IAC of water-soluble substances were higher as 976 and 948 μmol ascorbic acid equivalents/g in *Geranium thunbergii* and *Pyrrosia lingua*, respectively. IAC of lipid-soluble substances were 945 and 520 μmol trolox equivalents/g in *Ardisia crenata*, *Geranium thunbergii* and *Pyrrosia lingua*, respectively. Eight phenolic compounds were identified by GC/MS from the extracts of pressurized liquid extracts. Gallic acid and catechin were the predominant phenolics among the ones identified in the extracts of natural plants. The sum of the individual phenolics quantified by GC/MS were higher in *Ostrya japonica* and *Sapium japonicum* as 2,970 and 2,963 ppm. The extracts of *Sapium japonicum*, *Alnus firma*, *Cornus kousa,* and *Malus sieboldii* indicated NO production inhibitory activity with much less toxity. The extracts from *Ardisia japonica, Desmodium caudatum, Malus sieboldii, Prunus padus,* and *Rhus javanica* did not affect cell toxicity using HS-68 cells.

2. MATERIALS AND METHODS

2.1. Plant materials

The plant materials and the sample preparation method were the same as in Part Ⅰ.

2.2. Pressurized liquid extraction

The extraction method was the same as in Part Ⅱ except the following. The extraction of natural plants was carried out using two extraction steps with EtOH:H_2O (40:60, v/v) at 50℃ and 13.6 MPa for 10 min. The extraction pressure 13.6 MPa was chosen because the minimum operating pressure of the PLE machine used in this experiment was 10.2 MPa and at this pressure the flow rate of the solvent was slightly unstable. Each extraction was carried out in triplicate.

2.3. Total solids assay

The measurement method was the same as in Part Ⅰ.

2.4. Total phenolics assay

The assay method was the same as in Part Ⅱ.

2.5. Integral antioxidative capacity assay

The assay method was the same as in Part Ⅰ.

2.6. GC/MS analysis

Individual phenolics were qualified and quantified by GC/MS following the method described by Chiou et al. (2007).

Acid hydrolysis of the extract was performed as follows. This procedure was

performed to investigate the presence of conjugated forms of polyphenols, i.e. esters or glycosides, that would not be identified by GC/MS. An aqueous solution of hydrochloric acid (3 M, 0.25 mL) was added to 0.5 mL of the extract. The mixture was maintained at 80℃ for 1 hr. After cooling, 0.5 mL of potassium hydrogen phosphate (1 M) were added.

Each phenolic compounds was isolated by solids phase extraction(SPE) on an C8 cartridge (WAT036780, Waters, USA) (Soleas et al., 1997). The SPE cartridges were preconditioned with ethyl acetate (3 mL), MeOH (3 mL), and bi-distilled water (6 mL), and loaded with the acid-hydrolyzed sample (0.5 mL). The solvent in the cartridge was dried under reduced pressure, and each phenolics retained in the cartridge was eluted with ethyl acetate (3 mL). A 2 mL of the elute was evaporated in a rotary vacuum evaporator at 40℃ and the residue was redissolved in MeOH (0.5 mL). This was mixed with internal standard, 3-(4-hydroxy-phenyl)- 1-propanol (995 mg/L, 10 μL), evaporated to dryness in the rotary vacuum evaporator at 40℃, and derivatized by adding 250 μL BSTFA followed by incubation at 75℃ for 20 min. Each sample (1 μL) was injected into the gas chromatograph.

An Agilent (Wallborn, Germany) series GC 6890N coupled with a HP 5973 MS detector (EI, 70 eV), and a HP 7683 autosampler were used for qualitative and quantitative analysis of each phenolic compounds. Analysis of the sample was achieved using an HP-5 MS capillary column (30 m × 0.25 mm × 250 μm) at a split ratio 1:5. Helium was used as a carrier gas at a flow rate of 0.6 mL/min. The injector and transfer line temperature were set at 280℃ and 300℃, respectively. The oven temperature was held at 120℃ for 1 min, then increased to 220℃ at 5℃/min, then to 300℃ at 10℃/min and held for 10 min. A selective ion monitoring (SIM) GC/MS method was applied for the detection of target phenolic compounds.

Identification of chromatographic peaks was made by comparing the retention times and three fragment ions of each polyphenolic compounds with those of reference compounds, while quantification was carried out by using 3-(4-hydroxy-phenyl)-1-propanol as internal standard at target ion m/z 206 and qualifiers 191 and 179.

Target and qualifier ions for eight polyphenolic compounds were set as following; protocatechuic acid 193, 370, 355, syringic acid 327, 342, 312, p-coumaric acid 308, 293, 219, gallic acid 281, 458, 443, caffeic acid 396, 219, 381, catechin 368, 355, 179, chlorogenic acid 207, 345, 307, and quercetin 647, 559, 575.

2.7. Cell toxicity and nitrite assay

2.7.1. Cell culture

The RAW 264.7 murine macrophage and HS-68 normal skin fibroblast cell line were obtained from the Korea Cell Line Bank (Seoul, Korea). The cells were grown at 37℃ in DMEM and RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin in humidified atmosphere with 5% CO_2.

2.7.2. Cell toxicity assay

Cytotoxicity cell viability was determined by the MTT assay (Ferrari et al., 1990). Cells were seeded at a density of 5×10^4 and 1×10^3 cells/well in 96-well, flat-bottom culture plates in the presence or absence of the extract. Mitochondrial enzyme activity, an indirect measure of the number of viable respiring cells, was determined using the MTT reagent after 24 hr of the extract treatment. Absorbance was read using a μQuant microplate reader

(Bio-Tek Instrument, Winooski, VT, USA). The effect of the extract on cell viability was evaluated as the relative absorbance compared with that of the control culture.

2.7.3. Nitrite assay

The amount of nitrite, the end product of NO generation by activated macrophages, was determined by a colorimetric assay (Green et al., 1982). Briefly, a 100 μL of cell culture medium was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid) and incubated at room temperature for 10 min. The absorbance at 540 nm was read in a microplate reader. The nitrite concentration was determined by extrapolation from a sodium nitrite standard curve. The concentration of each extract that reduced the NO production by 50% with respect to the control (50% inhibition concentration, IC_{50}) was estimated. The selectivity index (SI) was determined as the ratio of TC_{50} (50% toxicity concentration) to IC_{50}.

3. RESULTS AND DISCUSSION

3.1. Total soluble solids

Table 5 shows the extraction yields of total soluble solids (TSS) from pressurized liquid extracts of twenty natural plants collected in Jeju. TSS was the highest in *Persicaria filiformis* as 28.5% with the decreasing order of 27.3, 25.8 and 25.2% in *Sapium japonicum*, *Prunus padus*, and *Euscaphis japonica,* respectively. In addition, TSS were over 20% in *Agrimonia pilosa*, *Alnus firma, Ardisia crenata*, *Cornus kousa*, *Ostrya japonica, Potentilla chinensis*, *Rhus javanica*, *Sorbus alnifolia,* and *Stauntonia hexaphylla.* All the extraction yields of TSS were 0.9 to 3.5 times higher than those from Part Ⅰ.

The ratios of TP to TSS were greatly higher in *Geranium thunbergii* (80.9%), *Pyrrosia lingua* (79.4%), and *Ostrya japonica* (50.3%).

Table 5. Extraction yields of total soluble solids (TSS) and total phenolics (TP) of pressurized liquid extracts at optimized extraction condition from natural plants collected in Jeju

Plant species	Total soluble solids (%)	Total phenolics (mg GAE/g)	TP/TSS (%)
Agrimonia pilosa	21.5±0.3	92.2±1.6	42.9
Alnus firma	23.0±0.2	75.8±1.3	33.0
Ardisia crenata	21.2±0.1	60.2±1.7	28.3
Ardisia japonica	19.2±0.1	83.2±0.3	43.3
Cornus kousa	23.1±0.4	83.6±0.7	36.1
Desmodium caudatum	13.4±0.1	33.5±1.2	24.9
Euscaphis japonica	25.2±0.1	81.8±1.2	32.5
Geranium thunbergii	12.9±0.0	104.4±2.4	80.9
Malus sieboldii	19.1±0.6	81.3±1.2	42.7
Myrica rubra	15.8±0.1	62.9±1.7	39.8
Ostrya japonica	20.9±0.2	105.4±1.1	50.3
Persicaria filiformis	28.5±0.1	36.9±0.4	12.9
Potentilla chinensis	21.3±0.8	78.4±2.3	36.9
Prunus padus	25.8±0.1	90.5±1.7	35.1
Pyrrosia lingua	11.4±0.0	90.6±0.9	79.4
Quercus acuta	17.0±0.3	68.4±2.6	40.2
Rhus javanica	24.5±0.3	79.7±0.5	32.5
Sapium japonicum	27.3±0.3	105.1±0.5	38.5
Sorbus alnifolia	23.6±0.4	76.6±1.5	32.5
Stauntonia hexaphylla	23.7±0.2	30.8±1.1	12.9

3.2. Total phenolics

Table 5 also shows the extraction yields of total phenolics (TP) from pressurized liquid extracts. Much higher concentrations of TP were observed in *Ostrya japonica* (105.4 mg GAE/g) and *Sapium japonicum* (105.1 mg GAE/g), followed by *Geranium thunbergii* (104.4), *Agrimonia pilosa* (92.2), *Pyrrosia lingua* (90.6), and *Prunus padus* (90.5). TP account for more than 80 mg GAE/g in *Cornus kousa*, *Ardisia japonica*, *Euscaphis japonica*, and

Malus sieboldii. TP contents at optimized PLE condition were 1.2 to 3.2 times higher than those from Part Ⅰ. *Geranium thunbergii* (80.9%) and *Pyrrosia lingua* (70.4%) had much higher ratios of TP to TSS.

3.3. Integral antioxidative capacity

Integral antioxidative capacities of water- and lipid-soluble substances from pressurized liquid extracts of twenty natural plants collected in Jeju were shown in Table 6. IAC of water-soluble substances were 976, 948, 493, 419, and 393 μmol ascorbic acid equivalents/g in *Geranium thunbergii*, *Pyrrosia lingua*, *Malus sieboldii*, *Sapium japonicum*, and *Ostrya japonica*, respectively. In addition, *Sorbus alnifolia*, *Euscaphis japonica*, *Cornus kousa* also showed more than 250 μmol ascorbic acid equivalents/g.

IAC of lipid-soluble substances were 945, 520 and 197 μmol trolox equivalents/g in *Ardisia crenata*, *Geranium thunbergii* and *Pyrrosia lingua*, respectively. *Ostrya japonica*, *Myrica rubra*, *Quercus acuta*, *Cornus kousa*, *Euscaphis japonica* were over 100 μmol trolox equivalents/g. The extracts from *Geranium thunbergii* and *Pyrrosia lingua* showed higher IAC of lipid-soluble substances with higher TP of 104.4 and 90.6 mg GAE/g, respectively.

Table 6. Integral antioxidative capacity (IAC) of pressurized liquid optimization extracts at optimized extraction condition from natural plants collected in Jeju

Plant species	IAC of water-soluble substances (Ascorbic acid, μmol/g)	IAC of lipid-soluble substances (Trolox, μmol/g)
Agrimonia pilosa	178.6±14.0	84.0±01.6
Alnus firma	121.2±02.4	78.4±04.8
Ardisia crenata	201.4±03.7	945.1±15.3
Ardisia japonica	191.8±03.5	70.1±04.8
Cornus kousa	263.9±14.7	103.5±01.5
Desmodium caudatum	46.7±02.8	33.5±00.7
Euscaphis japonica	269.9±17.6	102.8±03.1
Geranium thunbergii	976.4±14.4	520.1±04.5
Malus sieboldii	493.0±16.2	88.4±01.7
Myrica rubra	205.9±07.5	111.1±02.3
Ostrya japonica	393.5±28.2	127.5±02.9
Persicaria filiformis	51.6±02.7	34.8±00.1
Potentilla chinensis	163.4±04.3	89.6±04.3
Prunus padus	191.5±07.2	78.2±01.5
Pyrrosia lingua	948.9±23.2	197.1±07.3
Quercus acuta	136.6±06.5	104.0±00.2
Rhus javanica	195.5±07.3	97.5±00.3
Sapium japonicum	419.7±06.3	81.0±03.2
Sorbus alnifolia	273.3±03.2	88.9±02.3
Stauntonia hexaphylla	47.8±00.7	16.3±00.4

Figs. 12 and 13 show the linear correlations between TP/TSS and IAC. Linear correlation coefficients (R^2) between TP/TSS and ACW, and ACL were 0.7373, and 0.8281, respectively, demonstrating that TP contents gives good estimation of IAC. This kind of linear correlation has been obtained in many studies (Katalinic et al., 2006; Parejo et al., 2003; Silva et al., 2007).

The antioxidant activity of plant materials is well correlated with their content in phenolic compounds (Velioglu et al., 1998). However, in some cases, even though total polyphenol content was significantly low, IAC was high. This would suggest that either only some polyphenols actually act as

antioxidants, or there is a substantial contribution of non-phenolic compounds.

Phenolic compounds (flavonoid, phenolic acids) can play a major role in the antioxidant activity of plant materials. Nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids and amines), carotenoids, lignans and terpenes also possess antioxidative activity in suppressing the initiation or propagation of chain reaction (Hall and Cuppett, 1997)

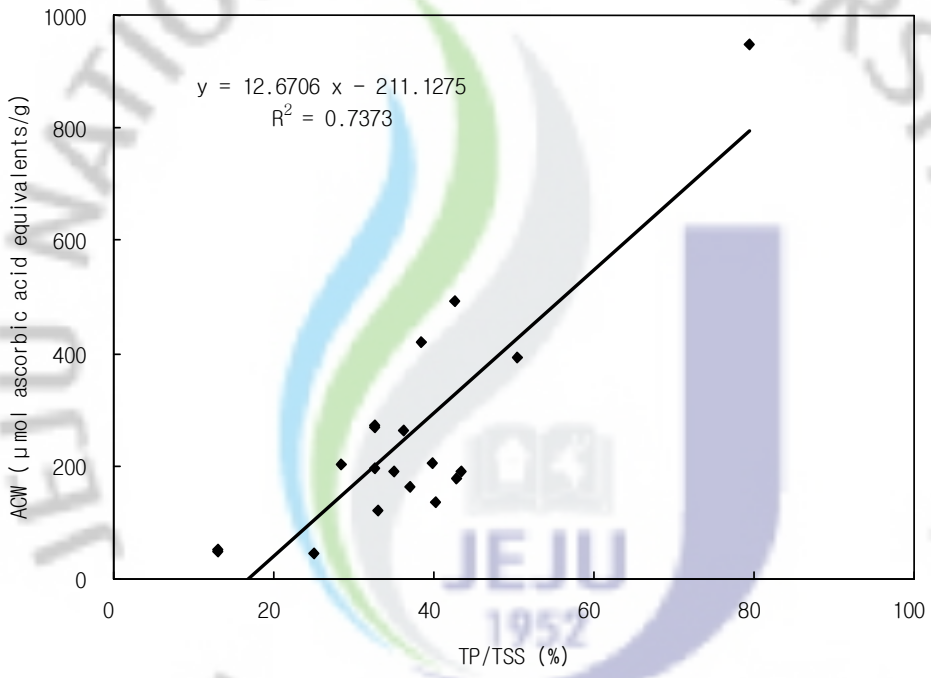

Fig. 12. Linear correlation of TP/TSS versus ACW.

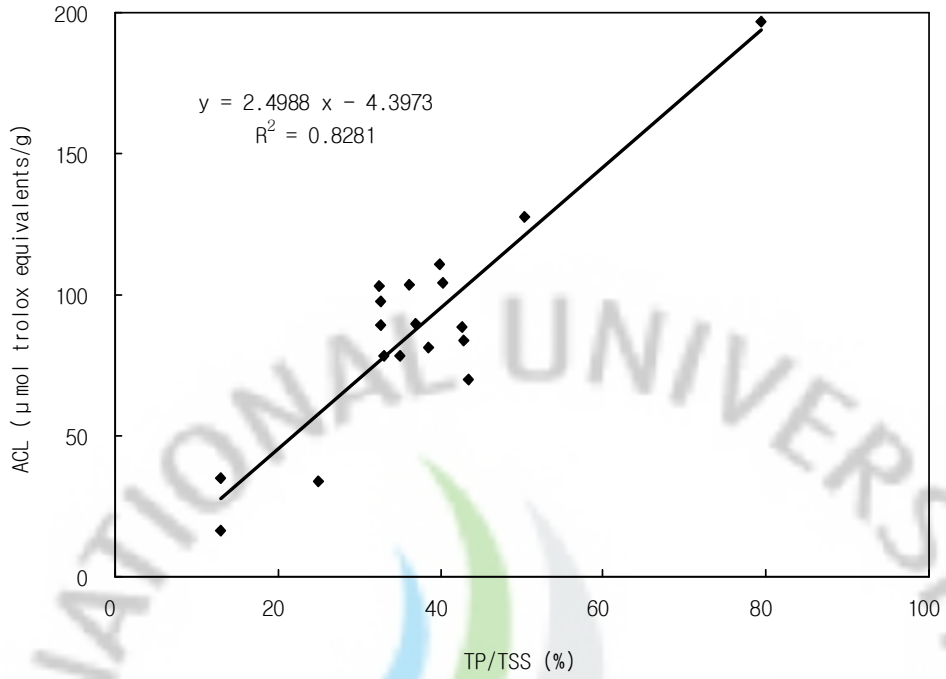

Fig. 13 Linear correlation of TP/TSS versus ACL.

3.4. Quantitative determination of individual phenolic compounds by GC/MS

Qualitative and quantitative determination of individual phenolic compounds was performed by GC/MS after SPE of PLE extracts as shown in Fig. 14 and Table 7. The SPE procedure was adopted since in PLE extracts several compounds, mainly carbohydrates, eluted as overlapping peaks to the ones of polyphenols. Moreover, this sample clean-up was followed to avoid sililation reagent consumption by carbohydrates.

A typical total ion chromatogram obtained for the extract of *Cornus kousa* is presented in Fig. 14. The identification of each phenolic compounds for which we had standards was carried out by comparison of their retention time and typical m/z valves with those obtained injecting standards in the same GC/MS condition.

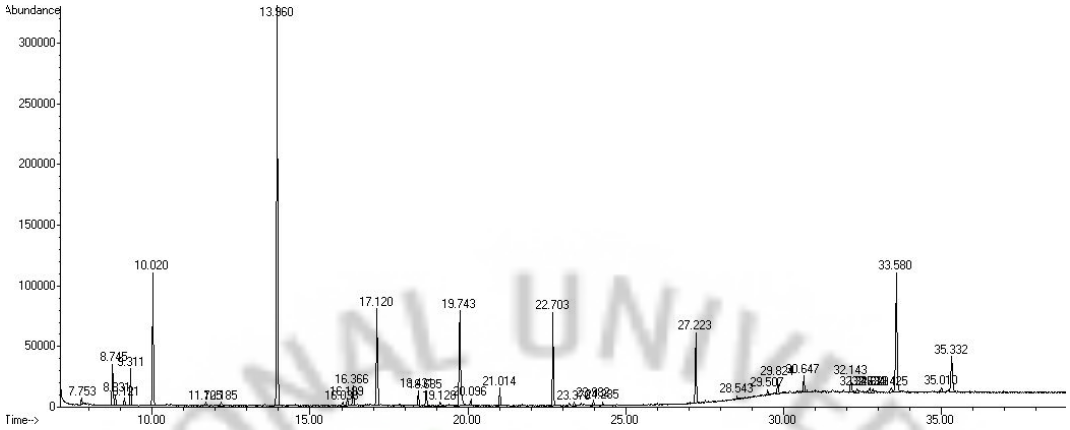

Fig. 14. Total ion chromatogram of pressurized liquid extract from *Cornus kousa*.

Eight phenolic compounds such as gallic acid, catechin, quercetin, caffeic acid, chlorogenic acid, protocatechuic acid, p-coumaric acid, and syringic acid were identified from the PLE extracts of natural plants collected in Jeju in this study. Gallic acid and catechin were the predominant polyphenols among the ones identified in the extracts of natural plants. Catechin was the highest in *Ostrya japonica* as 2,455 mg/100g. Gallic acid was the highest in *Geranium thunbergii* and *Sapium japonicum* as 2,312 and 2,211 mg/100g with higher TP as shown in Table 6. Protocatechuic acid was the highest in *Potentilla chinensis* as 35.4 mg/100g. Quercetin was also identified as the highest 276 mg/100g in *Cornus kousa*. Syringic acid was identified only in *Cornus kousa* (30.1 mg/100g). p-Coumaric acid was the highest in *Potentilla chinensis* as 32.5 mg/100g. Caffeic acid was the highest in *Pyrrosia lingua* as 268 mg/100g. Chlorogenic acid was identified as the highest in *Pyrrosia lingua* as 1,716 mg/100g. The sum of the individual phenolic compounds identified and quantified by GC/MS were ranged from 11 mg/100g to 2,970 mg/100g. *Ostrya japonica* and *Sapium japonicum* showed higher concentrations in the sum of

the individual phenolic compounds, as expected from the higher total phenolic content as shown in Table 6.

This result is consistent with IAC, since it is known that quercetin has about two time higher antioxidant activity with respect to other phenolic compounds (Rice-Evans et al., 1997).

Typical phenolic compound that possess antioxidant activity has been characterized as phenolic acid and flavonoid (Kahkonen et al., 1999). Phenolic acids have been implicated as natural antioxidants in fruits, vegetables, and other plant. For example, caffeic acid, ferulic acid, and vanillic acid are widely distributed in the plant kingdom (Larson, 1998).

Gallic acid is known by its antioxidant, anticarcinogenic inhibition and antifungal properties (Gali et al., 1991; Hayatsu et al., 1988; Perchellet et al., 1992; Sanchez-Moreno et al., 1998).

Quercetin is regularly consumed by humans as it is the major flavonoid found in human diet (Manach et al., 1998). This flavonoid is possessed antiviral and antiallergenic activities to inhibit platelet aggregation and the oxidation of low density lipoproteins, and to act as an anti-inflammatory agent (Formica and Regelson, 1995; Hertog and Hollman, 1996; Stavric, 1994)

Caffeic acid is known by antiinflammatory, antiviral, immunomodulatory properties, and suppress lipid peroxidation. This phenethyl ester is an active component of propolis from honeybee hives (Armutcu et al., 2007).

Catechins are extracted from plants and present in natural food and drinks, such as green tea or red wine. There are scavengers of reactive oxygen

species, and their resulting antioxidant properties are of great interest in dietetics and cosmetology. Furthermore, their antiviral and cancer inhibiting properties could have pharmaceutical applications (Yanagida et al., 2006; Tagliazucchi et al., 2005; Ishizu et al., 1999).

Chlorogenic acid is a major phenolic compound on coffee (Margret et al., 2001). Recent reports indicate that the compound is shown to be an antioxidant and anticarcinogenic, analgesic, antipyretic activities, antiinflammatory, and antifungal effect (Dos Santos et al., 2006; Feng et al., 2005; Huang et al., 1988).

Protocatechuic acid had a wide variety of biological activities including antioxidant, antitumor promotion effects and induced apoptosis in HL-60 human leukemia cells (Tseng et al., 1996, 2000). It has been revealed that PCA may be a candidate chemical for the treatment of oxidative stress-induced neurosdegenerative diseases such as Parkinson's disease (An et al., 2006; Guan et al., 2006; Liu et al., 2008).

p-Coumaric acid has interest as chemoprotectant and antioxidant. In addition, it is believed to reduce the risk of stomach cancer by reducing the formation of carcinogenic nitrosamines (Torres and Rosazza, 2001; Ferguson et al., 2005; Kikugawa et al., 1983).

Syringic acid is representative of phenolic carboxylic acid occurring in the soil. It is an antioxidant and also exhibits antiproliferative action (Kampa et al., 2004).

Table 7. Identification and quantification of individual phenolic compounds (mg/100g of dried plant) by GC/MS from pressurized liquid extracts of natural plants collected in Jeju

Polyphenols	Protocatechuic acid	Syringic acid	*p*-Coumaric acid	Gallic acid	Caffeic acid	Catechin	Chlorogenic acid	Quercetin	Total
RT(min)	16.9	18.4	19.1	19.7	22.7	30.2	32.8	33.6	
Agrimonia pilosa	19.4±0.4	-	12.6±0.7	14.4±0.0	-	1240.4±29.1	-	70.1±9.2	1356.9
Alnus firma	2.1±0.0	-	2.6±0.3	50.4±1.0	13.8±0.2	145.7±12.7	123.1±16.3	220.1±0.3	557.8
Ardisia crenata	1.9±0.1	-	-	26.8±0.2	-	-	-	-	28.7
Ardisia japonica	-	-	-	218.6±9.3	-	-	-	-	218.6
Cornus kousa	1.9±0.1	30.1±0.3	6.2±0.0	156.2±5.7	202.6±9.2	14.2±10.2	48.8±4.0	276.1±4.6	736.1
Desmodium caudatum	-	-	11.1±0.8	-	-	-	-	-	11.1
Euscaphis japonica	5.0±0.2	-	11.6±0.6	325.9±4.7	-	-	-	51.8±10.4	394.3
Geranium thunbergii	-	-	25.6±0.2	2312.6±10.8	10.6±0.4	-	-	-	2348.8
Malus sieboldii	9.0±0.7	-	2.3±0.0	4.2±0.1	2.8±0.6	573.7±4.0	173.3±13.1	154.4±12.9	919.7
Myrica rubra	-	-	-	134.1±4.7	-	-	-	-	134.1
Ostrya japonica	10.4±0.3	-	-	-	37.0±0.5	2455.4±60.7	251.8±66.7	216.2±13.6	2970.8
Persicaria filiformis	-	-	-	60.0±1.2	-	-	-	49.2±0.2	109.2
Potentilla chinensis	35.4±1.8	-	32.5±0.3	17.3±0.5	75.3±0.8	227.6±0.7	87.7±13.0	114.5±3.3	590.3
Prunus padus	11.9±0.1	-	4.2±0.1	1.7±0.1	100.6±1.5	483.8±9.7	-	185.5±2.6	787.7
Pyrrosia lingua	22.6±1.8	-	18.3±1.6	-	268.2±10.4	320.4±4.7	1716.9±14.9	-	2346.4
Quercus acuta	4.9±0.1	-	-	60.9±0.4	-	690.0±10.7	-	51.6±3.4	807.4
Rhus javanica	12.9±0.6	-	3.7±0.2	1517.8±92.1	-	58.6±3.1	-	175.4±20.1	1768.4
Sapium japonicum	6.9±0.5	-	-	2211.4±79.9	156.1±6.5	-	454.2±27.1	135.3±13.6	2963.9
Sorbus alnifolia	19.1±0.2	-	2.0±0.3	-	90.3±0.3	-	1239.9±1.6	52.8±5.5	1404.1
Stauntonia hexaphylla	-	-	-	-	5.4±0.0	-	24.0±11.4	26.0±0.7	55.4

3.5 Cell toxicity and LPS-induced NO production

Cell toxicity and the effects of LPS-induced NO production of pressurized liquid extracts from natural plants collected in Jeju using RAW 264.7 cells were shown in Table 8.

Most of the pressurized liquid extracts from natural plants collected in Jeju were not indicated the concentration producing 50% toxicity (TC_{50}) at the concentration used (3.9~500 μg/mL) with the exception of *Agrimonia pilosa, Alnus firma, Potentilla chinensis,* and *Quercus acuta* as TC_{50} values of 146, 426, 294, 445 μg/mL.

It is well known that iNOS from macrophages is predominantly responsible for the overproduction of NO in injured tissues and inflammatory processes (Wilson et al., 1996). Thus, we chose to utilize the well characterized murine macrophage cell line, RAW 264.7, to perform our studies. Treatment of cells with LPS (100 ng/ mL) induced nitrite accumulation in the culture medium during the 24 hr observation period, indicative of NO production. Simultaneous treatment with the extracts from natural plants and LPS significantly and dose-dependently decreased the production of NO. The pressurized liquid extracts from natural plants collected in Jeju showed dose dependent inhibitory effect on the LPS-induced NO production. The calculated IC_{50} values of the *Sapium japonicum*, *Alnus firma*, *Cornus kousa,* and *Malus sieboldii* were 109, 123, 219 and 219, respectively; the selectivity indices were >4.5, 3.9, >2.3, and >2.2, respectively. The extracts of *Sapium japonicum*, *Alnus firma*, *Cornus kousa,* and *Malus sieboldii* indicated NO production inhibitory activity with much less toxity.

Ohigashi et al. (1972) and Egawa et al. (1972) reported that *Sapium*

japonicum showed the highest NO production inhibitory activity. They isolated O-acetyl-12-O-n-deca-2,4,6-trienoyl phorobol and methyl 8 -hydroxy-5,6-octadienoate which showed anti- fish toxicity effect and antifungal effect.

Cell toxicities of pressurized liquid extracts from twenty natural plants collected in Jeju using HS-68 normal skin fibroblast cells were shown in Fig. 15-1 and 15-2. The pressurized liquid extracts did not affect cell toxicity at the concentration used (100-500 ㎍/mL) in *Ardisia japonica, Desmodium caudatum, Malus sieboldii, Prunus padus, Rhus javanica. Ardisia japonica* and *Rhus javanica* with higher TP contents. *Prunus padus* and *Rhus javanica* were indicated higher NO assay. In addition, *Malus sieboldii* also showed higher activity in TP, IAC, and NO assay. Hur et al. (2007) reported that *Malus sieboldii* is a species in the family Rosaceae. It is in the genus *Malus* in the Maloideae. *Malus* is mainly distributed in northeastern Asia. Especially, the genus *Malus pumila* Miller is economically important apple family.

Table 8. Cell toxicity and LPS-induced NO production of pressurized liquid extracts from natural plants collected in Jeju using RAW 264.7 cells

Plant species	TC_{50}[1] (μg/mL)	IC_{50}[2] (μg/mL)	Selectivity index[3]
Agrimonia pilosa	146.3±18.6	166.7±09.2	0.9
Alnus firma	426.5±31.7	123.5±04.1	3.9
Ardisia crenata	>500	>500	1
Ardisia japonica	>500	>500	1
Cornus kousa	>500	219.5±25.7	>2.3
Desmodium caudatum	>500	>500	1
Euscaphis japonica	>500	248.7±07.1	1
Geranium thunbergii	>500	>500	1
Malus sieboldii	>500	219.6±33.7	>2.2
Myrica rubra	>500	>500	1
Ostrya japonica	>500	318.4±50.6	>1.6
Persicaria filiformis	>500	>500	1
Potentilla chinensis	294.9±18.8	203.3±15.1	1.5
Prunus padus	>500	327.5±09.4	>1.5
Pyrrosia lingua	>500	>500	1
Quercus acuta	445.3±53.0	>500	>0.9
Rhus javanica	>500	337.5±31.7	>1.5
Sapium japonicum	>500	109.1±02.6	>4.5
Sorbus alnifolia	>500	>500	1
Stauntonia hexaphylla	>500	>500	1

[1] TC_{50} is the concentration producing 50% toxicity in RAW 264.7 cells.

[2] IC_{50} is the concentration producing 50% inhibition of NO production in RAW 264.7 cells.

[3] Selectivity Index = TC_{50} / IC_{50}

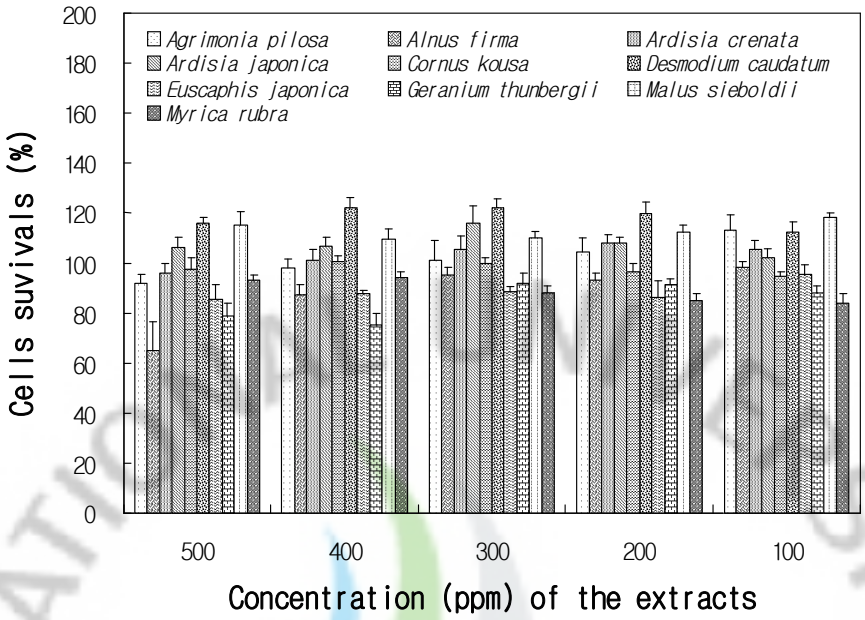

Fig. 15-1. Cell toxicity of pressurized liquid extracts from natural plants collected in Jeju using HS-68 normal skin fibroblast cells

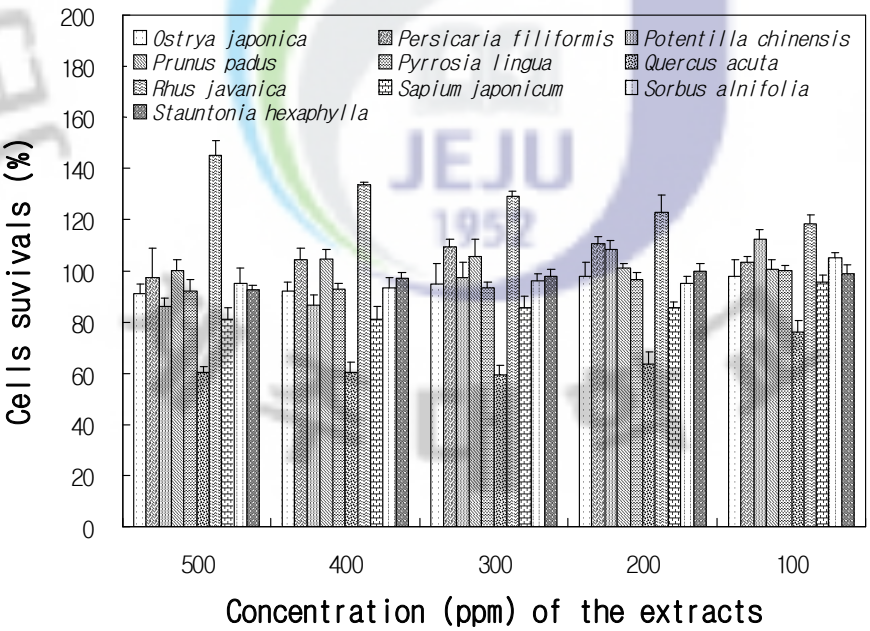

Fig. 15-2. Cell toxicity of pressurized liquid extracts from natural plants collected in Jeju using HS-68 normal skin fibroblast cells

SUMMARY

Twenty natural plants collected from Jeju were extracted by pressurized organic solvent. Extraction yields of total soluble solids (TSS) and total phenolics (TP), integral antioxidative capacity, individual phenolic compounds by GC/MS, and cell toxicity and NO production inhibitory activity were measured.

(1) Twenty natural plants collected from Jeju were extracted by pressurized organic solvent (100% MeOH, 40℃, 13.6 MPa, 10 min). Extraction yields of total soluble solids (TSS) and total phenolics (TP), and integral antioxidative capacity were evaluated. Extraction yields of TSS were higher as 21.8, 21.5, 21.1, 20.7, and 20.1% in *Rhus javanica, Euscaphis japonica*, *Alnus firma*, *Sapium japonicum,* and *Sorbus alnifolia*, respectively. Higher TP (mg GAE/g) were obtained from *Malus sieboldii* (68.3), *Sapium japonicum* (57.6), *Pyrrosia lingua* (56.6), and *Euscaphis japonica* (55.1). Integral antioxidative capacities of water-soluble substances were 598, 394, 293, and 270 μmol ascorbic acid equivalents/g in *Geranium thunbergii*, *Sapium japonicum*, *Cornus kousa,* and *Rhus javanica*, respectively. Integral antioxidative capacities of lipid-soluble substances were 611, 314, 296, and 242 μmol trolox equivalents/g in *Ardisia crenata*, *Ostrya japonica*, *Geranium thunbergii,* and *Quercus acuta*, respectively.

(2) *Sapium japonicum,* a natural plant in Jeju, was extracted by a pressurized liquid. Operating parameters such as the type of solvent, the ratio of solvent to water, temperature, pressure, and number of extraction steps were investigated as the main variables that influence the extraction efficiencies of total soluble solids (TSS) and total phenolics (TP). TP contents were affected

by the type of solvent, solvent-water ratio, extraction step and temperature. Higher extraction yields (17.9 and 17.3%) of TSS were obtained when MeOH and H_2O were used as the extraction solvents. MeOH extracted the highest level of TP as 50.4 mg GAE/g compared with 48.8 and 27.2 mg GAE/g with H_2O and EtOH, respectively. EtOH:H_2O (40:60, v/v) was found to be the best solvent for TP extraction as 90.3 mg GAE/g compared with 85.0, 84.3, and 76.8 mg GAE/g in MeOH:H_2O (40:60, 60:40, v/v) and EtOH:H_2O (60:40, v/v), respectively. TSS and TP were increased with the increase of the number of extraction steps. TP content was increased by 11% as the extraction temperature was increased from 40℃ (97.4 mg GAE/g) to 50℃ (108.3 mg GAE/g). Extraction pressure had no effect on the extraction efficiency. The optimum extraction conditions of TSS and TP were ; 40% EtOH, 2 extraction steps, temperature 50℃, and pressure 10.2 MPa.

(3) Twenty natural plants collected in Jeju were extracted at optimized pressurized liquid extraction condition (40% EtOH, 50℃, 13.6 MPa, 10 min). Extraction yields of total soluble solids (TSS) and total phenolics (TP), integral antioxidative capacity (IAC), individual phenolics by GC/MS, and cell toxicity and NO production inhibitory activity were evaluated. Extraction yield of TSS was the highest in *Persicaria filiformis* as 28.5% with the decreasing order of 27.3, 25.8 and 25.2 % in *Sapium japonicum*, and *Prunus padus*, respectively. *Ostrya japonica* and *Sapium japonicum* showed the highest TP (105.4 and 105.1 mg GAE/g), followed by *Geranium thunbergii* (104.4), *Agrimonia pilosa* (92.2), *Pyrrosia lingua* (90.6), and *Prunus padus* (90.5). IAC of water-soluble substances were higher as 976 and 948 μmol ascorbic acid equivalents/g in *Geranium thunbergii* and *Pyrrosia lingua*, respectively. IAC of lipid-soluble substances were 945 and 520 μmol trolox equivalents/g in *Ardisia crenata*, *Geranium thunbergii* and *Pyrrosia lingua*, respectively. Eight phenolic compounds were identified by GC/MS from the extracts of

pressurized liquid extracts. Gallic acid and catechin were the predominant phenolics among the ones identified in the extracts of natural plants. The sum of the individual phenolics quantified by GC/MS were higher in *Ostrya japonica* and *Sapium japonicum* as 2,970 and 2,963 ppm. The extracts of *Sapium japonicum*, *Alnus firma*, *Cornus kousa,* and *Malus sieboldii* indicated NO production inhibitory activity with much less toxity. The extracts from *Ardisia japonica, Desmodium caudatum, Malus sieboldii, Prunus padus,* and *Rhus javanica* did not affect cell toxicity using HS-68 cells.

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한 줄기 빛조차 보이지 않아 끝이 없을 것 같던 긴 터널을 빠져 나온 것 같습니다. 하지만, 아쉬움과 기쁨마음이 교차하는 가운데 한권의 석사논문에 마침표를 찍게 되었습니다. 이것이 끝이 아니라 시작이라는 마음가짐을 가지며 논문이 완성되기까지 많은 도움을 주신 분들께 이렇게 지면으로나마 감사의 마음을 전하고자 합니다.

먼저 부족함이 많은 저에게 끝없는 관심과 가르침을 주신 임상빈 교수님께 머리 숙여 깊은 감사를 드립니다. 논문이 나오기 까지 격려를 아끼지 않으신 김수현 교수님, 고영환 교수님, 송대진 교수님, 강영주 교수님, 하진환 교수님, 김광표 교수님께도 감사의 말을 전합니다.

항상 따뜻한 관심으로 저를 잘 보듬어 주셨던 좌미경 선생님께 감사드리고, 언제나 받기만 해서 죄스러운 마음만 드는 저를 걱정하고 격려해주셨던 선희언니, 성근오빠, 실험하는 동안 힘든 내색 하지 않고 옆에서 항상 힘이 되어 주었던 재성오빠, 그리고 분리공정 실험실 선배님, 후배님들께도 감사의 마음을 전합니다.

시료채취에 도움을 주시고 식물이라는 공통분모 하나로 좋은 인연을 만들어주신 김지훈 선생님, 바쁘신 와중에도 저에게 세포실험을 가르쳐주시고 논문을 쓰는 동안 조언을 주셨던 황준호 선생님께 감사드립니다. 2년 동안 같이 공부하며 서로를 격려 했던 대학원 선·후배님들께도 감사드립니다.

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