CHAPTER V

CONCLUSIONS

Conclusions

The conclusion of the present study is summarized as follows.

Acetone/water (7:3, v/v) was shown to be a suitable solvent for the extraction of tamarind seed husk (TaSH), yielding the higher content of polyphenolic compounds including flavonoids as compared with ethanol/water (7:3, v/v). The extract from TaSH with acetone/water (7:3, v/v), which was called TA, had potent antioxidant activity on scavenging free radicals, inhibiting free radical formation, changing the redox state of iron and chelating ferric ion. TA enhanced the autooxidation of ferrous ion and formed ferric-TA complex resulting in the inhibition of the hydroxyl radical production by Fenton reaction.

TA was demonstrated to contain flavonoids by color reactions such as cyanidin reaction and vanillin method, UV and IR spectra. TA was shown to increase the absorbance of neutral red used in viability assay of both RGC-5 and HT-22 normal cells. The effect of TA on increasing cell viability seemed not to be caused by cell proliferation. It was proposed that neutral red assay was not suitable for determining cell viability in the presence of TA.

TA showed the weaker cytotoxic activities than tannic acid against cancer cells (GLC4 human small lung carcinoma cell line and K562 human erythromyelogenous leukemia cell line).

TA was further purified by silica gel column chromatography to give the acetone fraction (aTAQ) and methanol fraction (mTAQ). aTAQ seemed to contain the several kinds of polyphenols and flavonoids except polymeric proanthocyanidins. aTAQ was shown to inhibit H_2O_2-induced hemolysis more effectively than mTAQ. The protective mechanism of aTAQ on erythrocytes was considered to be the inhibition of ROS generation by H_2O_2, accompanied by the decrease in metHb formation and the increase in GSH level.

TA, aTAQ and mTAQ inhibited the plasmid DNA oxidative damage induced by Fenton reactant (hydrogen peroxide with ferrous sulfate), in both single-strand and double-strand DNA. The results suggested that the potential antioxidant activity of TA, aTAQ and mTAQ related to their protective effects on DNA damage.

Tannins in TaSH were first confirmed to belong to condensed tannins (polymeric proanthocyanidins), which did not contain the galloylated derivatives and had a higher content of prodelphinidins in B ring than grape seed extract (GE).

The simple and efficient method to produce the extract (TE) with the very high content of polymeric proanthocyanidins (94%) from TaSH by one pot extraction using ethanol/water (3:2, v/v) at 60°C was established for the first time. The extraction method can be very useful for manufacturing polymeric proanthocyanidins products.

Antioxidant activities of polymeric proanthocyanidins from TE and grape seed extract (GE) were investigated by ABTS and DPPH method. The IC_{50} values of polymeric PA of TE and GE were 4.2±0.2 and 3.2±0.2 μg/ml (DPPH assay), 6.2±0.3 and 6.0±0.1 μg /ml (ABTS assay), respectively. The differences in the chemical structures between polymeric proanthocyanidins from TE and those of GE had no significant effect on their antioxidant activities.

Discussions

1. Solvents used for extracting polyphenols in TaSH

The solvents for extracting phenolic compounds are so important that the properties of the extract from TaSH were determined by the extraction process. Cork and Krockenberger (1991) reported that aqueous acetone was better than aqueous methanol for condensed tannins and total phenolic compounds. Moreover, Kallithraka (1995) reported that acetone/ water (7:3, v/v) was the best solvent for extraction of phenolic compounds especially procyanidins from grape seeds. In this study, yield and antioxidant activity of TaSH extracted by aqueous acetone (TA) were higher than those by aqueous ethanol at room temperature (Table 5). However, yield of TA extracted by acetone/ water (7:3, v/v) at room temperature was lower than TE extracted by ethanol/water (3:2, v/v) at 60°C. These results showed that aqueous acetone was better than aqueous ethanol. Phenolic content in TA dissolved in PBS was

about half of the phenolic content dissolved in ethanol (Table 4), which suggested that TA contained both water-soluble and water-insoluble phenolic compounds.

2. Quanlitative and quantitative analysis of condensed tannins in TA

The chemical screening helps to reveal the chemical nature of the constituents of TA and predominates over the others. Oligomeric proanthocyanidins was the main compounds in GSE. All chemical screening of TA revealed the presence of phenolic in form of flavonoids compounds especially condensed tannin without flavanonol and flavanonol-3 glycoside. The presence of condensed tannins in TaSH supported the results that reported by Pumthong (1999) and Sudjaroen, et al. (2005). Tamarind pericarp, but not tamarind seeds, was reported to contain low amount of taxifolin which was flavanonol compounds (Sudjaroen, et al., 2005).

To quantitative determination of condensed tannin, the acid butanol method and vanillin method were determined. The amount of condensed tannins in TA was more than in GSE. The average degree of polymerization of the condensed tannins in TA was essentially the same as the level of polymerization in GSE.

3. TA purification using Sephadex LH20 column chromatography

Sephadex LH20 was classically used to fractionate proanthocyanidins (flavanols or flavan 3-ol) on the basis of molecular size (Jerez, et al., 2007). However, size-based separation is not achieved on any Sephadex for condensed tannin (Hangerman, 1998). According the report of Hangerman (1998), Sephadex LH20 absorbed tannins in alcohol, and released them in aqueous acetone. The red brown extract of TA contained numerous mobile phenolic compounds which had antioxidant activity when examined by thin-layer chromatography and visualized by DPPH (Figure 17b). On the other hand, the tannin remained at or near the origin in all the usual solvent system of TLC (Strumeyer and Malin, 1975). The tannin was found to be very tightly bound to the support substances of TLC and column chromatography (using silica gel, cellulose and polyamide) and could not be recovered (Strumeyer and Malin, 1975). However, by utilizing Sephadex LH-20, it was possible to both separate the tannins from non-tannin compounds and recover approximately 90% of the original material applied to the column (Strumeyer and Malin, 1975). According to Lea (1978), the polymeric proanthocyanidins remained at the origin of TLC plate when using the toluene: acetone: formic (6:6:1, v/v) as the mobile phase. The main

compounds in aTAS should be polymeric proanthocyanidins (condensed tannin). The compounds in TaSH, which were reported by Tsuda, et al. (1994), such as hydroxyl-3', 4'-dihydroxyacetophenone, methyl 3, 4 –dihydroxybenzoate, 3,4-dihydroxyphenyl acetate and epicatechin, should be eluted in eTAS or mTAS. This solvent eluting system from Sephadex LH20 was a suitable system to separate polymeric proanthocynidins (eluting with acetone/water) out of the other molecules (eluting with aqueous ethanol and aqueous methanol). The antioxidant activities of fractions (eTAS, mTAS and aTAS) from this solvent system of Sephadex LH20 column chromatography were determined in further experiments.

4. Chemicals characterization of flavonoids and condensed tannins in TA

The similarity of tamarind seed husk extracted with aqueous acetone (TA) and grape seed extract (GSE) on TLC, IR and HPLC chromatograms was clear. The GSE mainly contained with oligomeric proanthocyanidins, however they were not the pure compounds. Grape seeds are a particularly rich source of proanthocyanidins, and only the procyanidin-type of proanthocyanidins has been detected in the seeds (Fuleki and Silva, 1997; Santos-Buelga, et al., 1995). According to Waterhouse and Walzem (1998), a few monomeric flavanols have been detected, but other flavonoid compounds such as anthocyanins and flavonols are not found in the seeds. Prieur, et al. (1994) found that 55% of the procyanidin extracted from grape seeds consisted of more than five monomer units and determined that their mean degree of polymerization ranged from and from 2.4 to 16.7. Thus, the proanthocyanidins from grape seeds contain procyanidin oligomers and polymers.

From the results of IR (Figure 20-27), TA may contain with flavonoids and the compounds with carbonyl group such as galloyl group or flavonols or flavanone or flavones or other non-flavonoid phenolic compounds. Sudjaroen, et al. (2005) reported that tamarind pericarp contained flavanone (narigenin and eriodictyol) and flavones (apigenin). The aTAS may contain the flavonoids without carbonyl group such as flavan-3-ol (proanthocyanidins).

TLC and HPLC chromatograms of hydrolysate aTAS (Figure 19 and 29) revealed many small degradation products of condensed tannin after hydrolysis. The acid hydrolysis was not specific cleavage resulting in many degradation products.

Many of major peaks in hydrolysate of TA were considered to be flavonoids compounds according to their UV spectrums (see detail in Appendix B) and other (chemical tests, TLC and IR spectrums). Non-flavonoids phenolic compounds were also found in small amounts (see the detail in Appendix B) thus TA could be rich source of flavonoids. Catechin was not mainly detected as a degradation product of condensed tannin in aTAS according to HPLC profile (Figure 32).

5. Antioxidant activities of flavonoids from TA and their mechanisms

The decrease in absorbance of ABTS radicals after adding with antioxidant compounds at a fixed time can be used as an index of total antioxidant capacity. While the kinetic determination of ABTS radical formation after adding with antioxidant compounds can estimate the antioxidant mechanism (Strube, et al., 1997). The antioxidative mechanisms of TA were both inhibitor of free radical formation and scavenger of free radicals using kinetic method of ABTS/Metmyoglobin/H_2O_2. However, using this kinetic method antioxidant capacity may be over estimated, due to both a scavenger effect and an effect on the rate of ABTS oxidation (Strube, et al., 1997). To distinguish between these effects, a post-addition assay was used in which the sample is added when the formation of radicals is stable (Strube, et al., 1997). TA was proved to display the mix action of scavenger and inhibitory effects on free radicals.

For primary conclusion, the crude acetone extract of TaSH (TA) was found to have a potent radical scavenging action on both the ABTS and DPPH methods. According to kinetic determination of ABTS formation, the flavonoids in TA were proved to be both free radical scavenger and inhibitor of free radicals formation. The condensed tannins in aTAS exhibited greater antioxidant potentials than mixtures of tannins and non-tannins in mTAS and non-tannins compounds in eTAS, respectively using both ABTS and DPPH methods.

The protective properties of flavonoids against oxidative stress have been reported to be structure-dependent. The free radical scavenging of flavonoids depends on: their H donating properties, primarily due to a catechol group on the B ring; metal chelation and prevention of catalysis of free radical reactions (Rice-Evans, et al., 1996). The iron chelating property of flavonoids in TA will be investigated in the next experiment.

6. Chelation and enhancing autooxidation of iron by TA flavonoids

The principal hypothesis associated with the biological effects of flavonoids is linked to their radical-scavenging properties. However, in addition to directly quenching ROS and free radicals, flavonoids could chelate iron to prevent their participation in Fenton-type reactions, which lead to the formation of free radicals (Rice-Evans, et al., 1995). Due to the importance of metal chelation for the characterization of antioxidant behavior of compounds, the ability of TA to chelate ferric ion was investigated.

The chelation of flavonoids could be performed by means of ultraviolet-visible absorption spectroscopy, analyzing the bathochromic shifts of band I at 300-550 nm and band II at 240-285 nm which characterize the flavonoid spectra. The spectroscopic studies by Chvátalová, et al. (2008) indicated that only phenolic acids containing the 3,4-dihydroxy (catechol) substitution in aromatic ring formed the complexes with ferric ions. Addition of ferric ions to phenolic acid with catechol moiety produced a strong bathochromic shift. For example, interactions of caffeic acid with ferric ions induced bathochromic shift in band from 311 nm to 341 nm accompanying by the formation of new broad band around 590 nm (Chvátalová, et al., 2008).

At a physiological pH, most of the catecholic compounds form 2:1 complexes with Fe^{3+} (Moridani, et al., 2003). Generally, the 2:1 ratio iron: flavonoid mixture was used for the spectrophotometric study, to ensure that the observed spectral changes were mainly due to an interaction between iron and flavonoids. Since the number of flavonoids in TA was unknown, the various concentration of ferric iron was used to confirm the iron-flavonoids complexes formation.

Interactions of ferric ions with the flavonoids in TA produced bathochromic shifts of both band I (ring B of flavonoids) and band II (ring A of flavonoids). The changes in the flavonoid spectra of TA that occurred when Fe^{3+} was added to TA indicated the formation of a flavonoid: Fe^{3+} complex. Information from Khokhar, et al. (2003) shows that the following functional groups of flavonoids are important for Fe-binding: (1) ortho-dihydroxyl group, e.g. 3'-4' dihydroxy groups (ring B) and 7-8 dihydroxy groups (ring A); (2) the presence of 5-OH and/or 3-OH in conjuction with a C4 keto group (cf. quercetin), and (3) a large number of OH group.

Binding of iron to the flavonoid in TA may suppress the accessibility of the iron to oxygen molecules. Preferential ligand binding to iron will change the redox potential for converting the ferrous ion to ferric ion and thereby inhibit oxidative damage (Khokhar, et al., 2003). However, the ability of putative antioxidants to chelate iron should be balanced with the redox state of iron.

The natural polyphenolics can be classified according to the antioxidant effect using iron redox reaction into two groups: flavonoids enhanced the autooxidation of ferrous ion, resulting in the inhibition of the hydroxyl radical production and non-flavonoid polyphenolics can reduce iron and form ferrous-polyphenol complexes that are inert to oxygen molecule (Yoshino and Murakami, 1998). Polyphenols can also exert antioxidant activity by enhancing the autooxidation of ferrous to ferric ion (Yoshino and Murakami, 1998). This prevents radical propagation since Fe^{2+} is required to react with superoxide to form H_2O_2, and to further convert H_2O_2 to hydroxyl radical and hydroxide ion. The carboxylate group and catechol substitution instead of galloyl moidty facilitated the ferrous ion oxidation more effectively (Chvátalová, et al., 2008).

The TA at 20 μg/ml induced autooxidation of Fe^{2+} which was the same mechanism as flavonoids (Figure 39). The antioxidant mechanisms of TA for inhibiting free radicals formation was due to increasing the rate of ferrous (Fe^{2+}) autooxidation and chelated with ferric (Fe^{3+}) iron. Moridani *et al.* (2003) reported that the Fe^{3+} complexes of flavonoids (polyphenols) were much more effective than the uncomplexed flavonoids in protecting irolated rat hepatocytes against hypoxia-reoxygenation injury. These complexes also readily scavenged superoxide radials (Moridani, et al., 2003). So TA-Fe^{3+} complexes may be the effective compounds to protect cells in the biological systems.

7. Effects of TA flavonoids on cell viability

The effect of TA flavonoids on cell culture was determined using neutral red assay. The principle of the neutral red assay is based on the ability of viable cells to incorporate and bind the dye in the lysosomes. Cell survival is calculated from the change in absorbance at 570 nm. The disadvantage of neutral red assay has been reported that neutral red uptake have been induced by osmotic swelling agents such as polyols (Oliver, et al., 1995). The apparent increasing percentage of cell survival in

this study cannot be explainable. It was proposed that neutral red assay might not be suitable to determine cell viability in the presence of TA containing flavonoids.

Ye, et al. (1999) and Bagchi, et al. (2002) have reported that grape seed proanthocyanidin extract enhanced the growth and viability of the normal cells whereas it exhibited cytotoxity towards cancer cells using MTT assay. Recently, the artifacts in cells-base studies have reported that many of flavonoids and condensed tannin interfered with MTT assay (Wisman, et al., 2008). Many studies of the biological effects of polyphenols in cell culture have been affected by their ability to oxidize in culture media, and awareness of this problem can avoid erroneous claims (Halliwell, 2008). The interfering effects of some polyphenols on cell viability determined by neutral red uptake ability are not clearly understood. So far, only false positive results of polyols on cell viability by neutral red have been reported (Olivier, et al., 1995). This artifact of the measurement may be the one explanation of why the assessment of the flavonoids effect on cell uptake and cell function *in vitro* cell culture models does not fully reflect the cell uptake and cell function *in vivo*.

The artifacts in cells-based studies resulting from the reactivity of polyphenols in MTT assay was investigated by Wisman, et al. (2008). Many of phenolic compounds including proanthocyanidin, catachin and quercetin can react with MTT because of their potent redox activity (Wisman, et al., 2008). At the low concentration of TA which is the mixture of polyphenols and proanthocyanidins could react with MTT in the same way to give the increasing of absorbance at 570 nm and give no precision result. However, the high concentrations of TA can calculate the IC_{50} of TA comparing with tannic acid (hydrolysable tannins) as shown in Table 11. TA had the weaker cytotoxicity effects on both cancer cells than tannic acid. TA may not be the effective compound for anti-cancer activity.

8. Preparation of small molecular-weight polyphenols from TA

Some preliminary experiments of TA on cells gave the false positive results such as the anti-platelet aggregation method (data not shown) and cells survival methods. The false positive results couldn't conclude why it happened. However it was possible that the high molecular weight of condensed tannin in TA intervened with the experiment due to the tannin-protein interactions. So the other purification method, TaSH, was planned to eliminate the high polymer of condensed tannin.

Chunmei, et al. (2010) found that silica gel could not be used to fractionate condensed tannin, presumably because of its high molecular weight. The high polymer of condensed tannin should not be eluted with this solvent system using silica gel column chromatography. HPLC using eluting solvent system 2 was employed to separate and identify phenolic compounds at 280 nm according to Soong and Barlow (2006). It was possible that aTAQ and mTAQ contained many small polyphenols and flavonoids including oligomeric proanthocyanidins. However, HPLC chromatograms of aTAQ (Figure 47) and mTAQ (Figure 48) showed many unidentified peaks. So the identification of each peak needs more standard compounds.

The Folin reaction is based on the reduction of phosphomolybdic acid by phenol in aqueous alkali solution. This method was used to determine the total phenolic groups (polyphenols). Povichit, et al. (2010) reported the content of polyphenols and the DPPH radical scavenging activity of the extract prepared by extracting TaSH with 95% aqueous ethanol. The total polyphenolic content in aTAQ and mTAQ shown in Table 8 was virtually in good agreement but the IC_{50} value was about 15 times stronger in comparison with those by Povichit, et al., (2010).

9. Effects of small molecular-weight polyphenols from TA on hemolysis and their mechanisms

The erythrocytes are affected by the continuous oxidative insults by being exposed to endogenous and exogenous ROS. Although erythrocytes contain an extensive antioxidant defense system, the oxidative damage of the membrane proteins and lipids has negative effects on normal erythrocytes. The major source of intracellular ROS in the erythrocytes is the autoxidation of oxyhemoglobin, which generates superoxide and, through dismutation, produces H_2O_2 (Mista, et al., 1972).

Many experimental methods for studying the relation between erythrocytes and the oxidative insults have been reported, and in particular the oxidative agents to induce the oxidative damages to erythrocytes and the enzyme inhibitors for the defense system of erythrocytes have been studied. The oxidative agents were H_2O_2 (Ajila and Rao 2008; Tedesco, et al.,2001; Tedesco, et al., 2000), tert-butyl hydroperoxide, β-cyclodextrin or methyl-β-D-cyclodextrin (inducer of cholesterol depletion) (López-Revuelta, et al., 2005; López-Revuelta, et al., 2006),

phenylhydrazine (PHZ; inducer of metHb and Heinz body formation and mild GSH depletion, releaser of free iron), diethyl maleate (DEM; inducer of GSH reduction), divicine, isouramil, copper sulfate and ascorbic acid (inducer of lipid peroxydation) (Chaudhuri, et al., 2007), sodium nitrite, ferrous sulfate and adenosine 5-phosphate (inducer of mild lipid peroxydation) (Nagababu, et al., 2003). The enzyme inhibitors were sodium azide (catalase inhibitor) and iodoacetamide (glutathione peroxydase inhibitor) because these enzymes are considered to play a dominant role in the defense system (Nagababu, et al., 2003; Tedesco, et al., 2001).

Ferrali, et al. (1992) reported the effect of diethyl maleate (DEM) and phenyl hydrazine (PHZ) on iron release, metHb formations, GSH level, MDA level, and hemolysis using mice erythrocytes. Many experimental conditions have been used to study on the protective agents for blood cell hemolysis. Many of them used H_2O_2 in combination with sodium azide (NaN_2) to inhibit catalase in erythrocytes (Nagababu, et al., 2003; Tedesco, et al., 2001; Gringberg, et al., 1997; Ko, et al., 1997). Ko, et al. used 10mM H_2O_2 with 1mM NaN_2 and about 45-65% of considerable hemolysis was caused (Ko, et al., 1997). The hemolysis assays using H_2O_2 alone have also been reported. The examples of the assay conditions which were reported in the journal were listed in Table 2 (Chapter 2). As described in the Table 2, the very wide range of H_2O_2 concentration was used, and even at the very high concentration of H_2O_2, the low hemolysis have been reported, which may be due to the strong defense system of erythrocytes. Under such conditions, the percent inhibition of hemolysis by taking the hemolysis caused by H_2O_2 as 100% have been shown, but the percent of hemolysis caused by H_2O_2 alone has not always been shown. In our experiment, the hemolysis was low. As shown in Table 10, the increase in hemolysis caused H_2O_2 alone, however, was statistically significant.

With respect to the studies on the interaction between antioxidant and erythrocytes, the various parameters have been reported. They are ROS (DCFDA method) and methemoglobin (Tedesco, et al., 2001; Tedesco, et al., 2000), lipid peroxydation [MDA (malondialdehyde as MDA-2-thiobarbituric adid)] (Ajila and Rao 2008; Chaudhuri, et al., 2007), erythrocyte membrane protein by SDS-PAGE and morphological observation (Ajila and Rao 2008), TBARS, cholesterol and phospholipid (López-Revuelta, et al., 2005), catalase, GSHPX (glutathione peroxidase),

glutathione reductase, glutathione-S-transferase, superoxide dismutase, glucose-6-phosphate dehydrogenase, reduced glutathione, and oxidized glutathione (Sangeetha, et al., 2005), anisotropy, microviscosity and osmotic fragility (Chaudhuri, et al., 2007), the heme degradation products measured by flow cytometric analysis (Nagababu, et al., 2003), NADH (β-nicotinamide adenine dinucleotide) (Galati, et al., 2002), and etc. Among these parameters, ROS levels during the oxidative insult process caused the major damage to RBCs including membrane lipid peroxidation. In the present study, in order to confirm and characterize the protective effect of aTAQ and mTAQ on RBCs hemolysis, we measured three different parameters, ROS concentration, glutathione concentration, and metHb production, which relate to hemolysis and oxidative damage.

aTAQ showed the inhibitory effect of H_2O_2 on hemolysis, ROS, GSH and metHb level. The activity of aTAQ on hemolysis, ROS, GSH, and metHb was confirmed at the concentration of 0.05-0.5 μg/ml of aTAQ under the given experimental conditions. The facts suggested that the protective effect of aTAQ was limited. The limited effect of antioxidant on erythrocytes protection against the oxidative stress has been reported (Ajila, et al., 2008; Chaudhuri, et al., 2007; Tedesco, et al., 2001; Tedesco, et al., 2000; Youdim, et al., 2000) Therefore a study aimed at achieving the sufficient protection of erythrocytes against oxidative stress is being awaited, and the antioxidants will be one of the important agents by using them with other appropriate agents to achieve the adequate protective activity.

10. Effects of small molecular-weight polyphenols from TA on DNA damage

Hydrogen peroxide combining with ferrous iron, called as Fenton reactant, could damage DNA due to hydroxyl radical formation. The ability of TA to protect pUc18 plasmid DNA from Fenton reactant-induced single-strand and double-strand breaks were investigated. Although, Suksomtip, et al. (2008) reported the inhibition effect of methanolic extract from TaSH on Fenton reactant - induced DNA damage, the acetone extract of TaSH (TA) has not been reported yet. There were only two fractions of extracts from TA using silica gel quick column chromatography, acetone fraction and methanol fraction (aTAQ and mTAQ), showing the high antioxidant activity.

In an earlier study (Moriwaki, et al., 2008), the interaction between DNA and metal ion was influenced on DNA oxidative stress, and a site-specific mechanism is involved in the formation of the base adducts, while the formation of single-strand breaks is more likely to involve generation of hydroxyl radicals in solution. The concentration up to 0.24 mM of Fe^{2+} with 5 mM H_2O_2 enhancing single strand breaks of plasmid pBR322 DNA could be observed by previous study of Ambroz, et al., (2001).

In this study, crude (TA), acetone (aTAQ) and methanol (mTAQ) fractions of tamarind seed husk (TaSH) as well as grape seed extract (GSE) can clearly inhibit Fenton reactants -induced supercoiled DNA strand scissions in both single strand and double strand scissions (Figure 55-57). The ability to protect DNA of flavonoids seems related with the iron chelating ability of each flavonoids (Melidou, et al., 2005). The possible mechanism explaining the protective effect of TA against DNA scission was the free radicals scavenging and iron chelating activity. This protective effect of flavonoids from TA, aTAQ and mTAQ on bacterial plasmid DNA oxidative damage still needs further experiments on human cells such as comet assay and micronucleus assay.

11. Preparation of polymeric proanthocyanidins from TE and GE by Sephadex LH20 column chromatography

The next experiment was designed to purify polymeric proanthocynidins in TE (crude ethanolic extract of tamarind seed husk) comparing with GE (crude ethanolic extract of grape seed). Sudjaroen, et al. (2005) reported that the content of total phenolic compounds and polymeric tannins in tamarind seed was 0.654% and 2% (dry weight), respectively, though they described the polymeric tannins as unidentified. As shown in Table 12, the content of polymeric proanthocyanidins in fresh TaSH reached about 39.1% (w/w), which was about 13 times higher than that in fresh grape seeds. The content of polymeric proanthocyanidins in TE prepared by one pot extraction with ethanol/water (3:2, v/v) at 60°C for 24 hours was very high (about 94%, w/w). The fact that TaSH contained the very large amount of polymeric proanthocyanidins and that TE with very high content could be simply and efficiently prepared by one pot extraction using aqueous ethanol and heating at 60°C was first demonstrated in the present study.

Sephadex LH20 purified samples and the crude extracts were analyzed by HPLC eluting with solvent system 3 according to the analytical condition reported by Weber, et al. (2007). A number of alcohol/water and acetonitrile/water mixtures with and without the addition of acid, were investigated as diluents and extraction solvents for flavonoids. Weber, et al. (2007) reported that the use of acetonitrile/water as the sample diluents led to both peak fronting and peak tailing of catechin and epicatechin. Subsequent investigations proved that methanol/water (1:1, v/v) was the most suitable solvent for dilution of grape seed extracts.

12. HPLC/UV analysis of polymeric proanthocyanidins from aTES and aGES

The HPLC chromatograms of aTES and aGES were almost the same (Figure 59). The main peak of all the samples shows the same retention time around 60 min though the main peak of TE is sharper than that of GE. The pattern of the HPLC chromatogram main peak is similar to that reported by Weber, et al. (2007) though the retention time, which was 57 min for polymeric proanthocyanidins, was a little different, probably due to the use of the different HPLC column and analytical conditions. Then the main peak was considered to be polymeric proanthocyanidins as reported previously (Peng, et al., 2001; Weber, et al., 2007). This was further confirmed by ^{13}C-NMR spectroscopy (see below).

Czochanska, et al. (1980) reported that the polymeric proanthocyanidins consisting of procyanidin (PC) alone showed more intensive absorbance at 270-280 nm than the polymeric proanthocyanidins consisting of prodelphinidins (PD) alone. The slope for GE was larger than that for TE, which suggested the higher content of procyanidin (PC) chromophore in GE than that in TE. This was also confirmed by ^{13}C-NMR spectroscopy (see below).

The main problems that have affected our experiment are the property of tannin, which is polymer of proantrocyanidins, and the complexity of the compound. It is impossible to collect the amount of one pure compound of polymeric proanthocyanidins because they have difference kinds of monomer, branching, linkage and number of subunits. It is well known that Sephadex LH20 is used for the separation of both condensed and hydrolysable tannins from the simple phenolic compounds. Peng, et al. (2000) reported the purification of polymeric proanthocyanidins

in GSE with LH20, and they used the purified polymeric proanthocyanidins as a standard for HPLC analysis.

13. Structural characterization of polymeric proanthocyanidins in aTES

The structural characterization of polymeric tannins in TSH was performed mainly by comparing and analyzing the ^{13}C-NMR spectra of polymeric proanthocyanidins obtained by purifying the TE and GE with Sephadex LH20 because the chemical structures of proanthocyanidins (monomers, oligomers and polymers) of grape seeds had been established through a lot of studies in the past. Czochanska, et al. (1980) reported the pioneering study on ^{13}C-NMR spectroscopy relating to polymeric proanthocyanidins of *Actinidia chimensis* (leaf), *Aexculus hippocastanum* (fruit), *Cydonia oblonga*(fruit), *Gravillea robusta* (fruit and leaf), *Ribes nigrum*, *Vaccinium corymbosum*(fruit), *Vitis vinifera*(fruit) and so on. They introduced the ^{13}C-NMR analytical method to study the ratio of procyanidin (PC to PD), the stereochemistry of the heterocyclic ring of the monomer units, the structures of the chain-terminating flavan-3-ol units, and the number-average molecular weight. Since the study by Czochanska, et al. (1980), ^{13}C-NMR spectroscopy by the proton-decoupled, inverse-gated sequence method has been employed to study the chemical structure of polymeric proanthocyanidins of many natural sources such as grape skin (Kennedy, et al., 2001), black spruce neeedle (Lorenz and Preston, 2002), pear juice (Es-Safi, et al., 2006), *Lithocarpus glaber* leaves (Zhang and Lin, 2008).

The detailed chemical structure of aTES was further characterized by ^{13}C-NMR according to the method of Czochanska, et al. (1980) as follows:

1. The ratio of PC and PD: The signals near 145 ppm are typical of the presence of PD units, and the ratio of PC to PD was estimated by the relative ratio of the peak area at 144 ppm (C-3' and C-4' of PC) and 145 ppm (C-3' and C-5' of PD). The PC/PD ratios were calculated using the expanded chart of ^{13}C-NMR spectra (data not shown). The ratio of PC to PD of polymeric proanthocyanidins of aTES was estimated to be 40:60. In the case of polymeric proanthocyanidins of aGES, the estimation of PC to PD was not exactly accurate because C-3" and C-5" of galloyl group overlapped the signals of 143-144 ppm (Kennedy and Jones, 2001), but the approximate value of PC/PD was calculated to be 80:20. Accordingly, the higher

content of PD units of polymeric proanthocyanidins of aTES than that of aGES was confirmed.

2. Galloyl group: As for aGES, the signals for galloyl group were confirmed. They were the signals at 110 ppm (C-2" and C-6" of galloyl group) [12] and 138 ppm (C-4" of galloyl group) [9]. These signals were not observed in aTES, showing that galloyl group was not virtually contained in aTES.

3. Degree of polymerization (DP): DP of aTES was estimated to be about 7 from the ratio of extension C-3 to terminal C-3.

4 The stereochemistry of the heterocyclic ring of extension units: According to Czochanska *et al.* (1980), the ^{13}C chemical shifts of C-2 (cis 2, 3) and C-2 (trans 2, 3) should be 77 ppm and 84 ppm, respectively. In our experiment, trans 2, 3 peak was not observed in both aTES and aGES. Therefore, the stereostructure (flavan C-2 and C-3 positions of extension units) of aTES was considered to be all cis 2, 3 form. The DP of polymeric proanthocyanidins of aTES estimated by ^{13}C-NMR was in good agreement with that by vanillin assay. The differences in the chemical structures between polymeric proanthocyanidins of aTES and aGES had no effect on the antioxidant activities.

Recommendations

In the present study, all the experiments used the extracts of TaSH for demonstrating the antioxidant activity by ABTS and DPPH method, chelating activity by spectroscopic method and autoxidation of ferrous methods. The further purification of polyphenolic compounds in aTAQ and mTAQ and their identification should be performed, and the purified compounds should be used for investigating their activities.

The chelating activity should be confirmed by using more sophisticated methods. The UV spectroscopy measurements in the chelating activity test give only the indirect evidence for complex formation between flavonoids and metal ion. The direct evidence for flavonoids/transition metal complexation should be performed by means of electrospray ionization mass spectrometry (ESI-MS).

The inconsistencies of the colorimetric assay for cell viability depend on cell types, chemical structures of polyphenolic compounds, and assay systems. For more

evidence and confirmation relating to the effect of different types of polyphenol compounds on cell viability *in vitro*, further studies should be planned. The new assay methods to minimize the interference due to redox-active compounds should be proposed in the future.

The antioxidant compounds in young TaSH were interesting and should be further studied because the seed husk from young tamarind showed a higher antioxidant activity.

The catalase inhibition of erythrocytes by preincubation with sodium azide should be investigated to study the hemolysis under a higher percent hemolysis of positive control. The *in vivo* hemolysis study is needed for the evaluation under physiological condition because *in vitro* study may be different from *in vivo*.

The oligomeric proanthocyanidins of grape seed and other natural sources have been reported to exhibit a variety of biological activities *in vitro* and *in vivo*, including antiulcer (Saito, et al., 1998), antibacterial (Kusuda, et al., 2006), anticancer (Kim, et al., 2005), and the preventive properties in relation to the development of multiple human diseases, such as atherosclerosis (Yamakoshi, et al., 1999), and cardiovascular diseases (Facino, et al., 1999). However, little is currently known about the pharmacokinetics of proanthocyanidins in humans. Therefore, for the purpose of developing health products using TaSH, *in vitro* and *in vivo* biological activities and pharmaco-kinetics of the products are waited upon.

The detailed chemical structure of polymeric tannins from TaSH should be elucidated by further studies on gel permeation chromatography and phloroglucinol or thiolysis reaction to confirm the constituent units and interflavonoid linkage. The polymeric proanthocyanidins in TaSH can be used for the wider applications.

Oligomeric proanthocyanidins are more interesting than polymeric proanthocyanidins especially for *in vivo* applications. Proanthocyanidins with the lower molecular weight have been also known as a long-acting antioxidant which remains in the plasma and tissues for up to 7-10 days and exerts the antioxidant properties (Bagchi, et al., 2000). The conversion from polymeric proanthocyanidins to oligomeric proanthocyanidins, however, has not been satisfactorily achieved. Groenewoud, et al., (1986) reported the microbial degradation of polymeric proanthocyanidins using caecal bacteria and Spencer, et al. (2000) reported the

physiological degradation of polymeric proanthocyanidins under the low gastric pH, suggesting the feasibility of the conversion to oligomeric proanthocyanidins with lower molecular-weight.