CHAPTER IV
DISCUSSION

This study has demonstrated that the extract of tamarind seed coat in aqueous fraction contains the polyphenolic flavonoid which acts as a scavenger of oxygen radicals generated in aqueous phase, and can protect lipid peroxidation and Ca^{2+}-ATPase *in vitro* from oxidation in the isolated erythrocyte membrane. Several kinds of polyphenolic flavonoids exhibit protective properties, such as anticarcinogenic and antimutagenic properties as well as their immune-stimulating, anti-allergic and anti-viral effects, it has been suggested that these components play a role as antioxidants inhibiting lipid peroxidation, low density lipoprotein (LDL) oxidation and scavenging oxygen radicals (51,82).

The polyphenols act as antioxidants by 2 effects, the first is by virtue of the hydrogen-donating capacity of their phenolic groups, and another property by their metal-chelating potential which may also play a role in the protection against iron- and copper-induced free radical reactions (83-84). The proposals of Bore et al, 1990 (85), concerning the chemical criteria for determining the radical scavenging functions of the flavonoids are as the followings.

i. The presence of the 3',4'-dihydroxy structure in the B ring;

ii. The presence of the 2,3-double bond in conjunction with the 4-oxo group in the C ring, which allows conjugation between the A and B ring, or electron delocalisation;

iii. The presence of a 3-hydroxyl group in C ring and a 5-hydroxyl group in the A ring for maximal radical scavenging potentials.

The results obtained from chemical reactions specific to condensed tannin shown in Table 4 reveal that the tamarind seed-coat extract is chemically identified as one of polyphenolic compounds and is called as oligomeric anthocyanidins (OPC) which was classified as a condensed tannin group of flavonoid. The extract and commercial OPC from both sources from pine bark and grape seeds exhibited similar positive tests of OPC. Moreover, the UV absorption spectra and IR spectra of the extract are

comparable with those of the OPC extracted from pine bark and grape seeds. The slight difference of their spectra between the extract and the OPC from both sources was due to presence of impurities of the extract.

The TLC chromatogram reveals that the complex extract composed of at least six components that each had distinct Rf values after hydrolysis in mild acid condition. Naturally, when proanthocyanidins are hydrolyzed by enzymes in plants, anthocyanidins, flavonoid compounds with red or blue color, will be derived, and that is logically why the precursor compounds are called "proanthocyanidins".

Since it has been reported that the antioxidant activity of OPC in lipid phase decreased with polymerization of proanthocyanidin, this is in contrast in the aqueous phase in which the antioxidative activity of OPC increased from its monomer to trimer and then decreased from trimer to tetramer (86). This data suggest that the active form of the extract properly is in oligomer form in a certain system. The present study also indicated that OPC in the extract could be due to rearrangement of its structure into an active oligomeric form. The results showed higher antioxidant capacity when it was hydrolyzed with mild acid for longer time. In addition, the extract as dry residue could be stable to heat up to 100°C, though its stability slightly decreased at 140°C.

Proanthocyanidin possesses oligomer of polyphenolic structures which are consistent with all of the proposals of Bore and et al (85), it therefore becomes as a potent antioxidant. However, the results in this study indicated that the tamarind seed coat extract exhibited the inhibitory effect on peroxyl radicals, hydroxyl radicals and superoxide anions in dose dependent manner. The active substance isolated from tamarind seed coat has concomitant antioxidant activity of both as a free radical scavenger and may also play a role as iron-chelator.

Compared with vitamin E, Trolox and vitamin C, the tamarind seed coat extract decreased the absorbance of ABTS radical cations, by a different mechanism. Strube et al, 1997(87), reported that Trolox and vitamin C scavenged the ABTS radical acting by combining intermediate radicals showing lag time at the first period of the reaction, when the whole antioxidant was consumed, the reaction rate gradually increased. Some

flavonoids act by both scavenging ABTS radicals and inhibiting radical formation. Since in this study, the extract was assessed for the antioxidant activity by monitoring the inhibition rate of ABTS radical production in enzymatic (ABTS/H_2O_2/peroxidas) and non-enzymatic (ABTS/H_2O_2/metmyoglobin) systems, it was found that the extract showed the antioxidant capacity at the same level. The result suggested that the extract did not act directly on peroxidase activity, but may involve iron-chelating effect in heme rings of both peroxidase and metmyoglobin in which it inhibits free-radical formation.

Besides, the inhibition effect of the extract in neotetrazolium medthod using xanthine oxidase (XOD) to generate superoxide is not due to the enzyme inhibitor, because the previous study by Hatano et al, 1990 (88), reported that the inhibition of superoxide generation by tannins is due to their radical-scavenging activity, and not due to their inhibitory activity upon the enzyme.

The biomembrane of living cells may be mostly susceptible to free-radical attack due to its content of polyunsaturated fatty acids, resulting in lipid peroxidation. End products produced during the lipid peroxidation process, including MDA, are very active, and capable of cross-linking of membrane proteins by MDA may lead to the inactivation of important membrane-spanning proteins including the ion transport ATPase (89,90). Obviously, inhibition of the Ca^{2+}-pump ATPase would also lead to increased Ca^{2+} in the cell. Increased intracellular cause or contributes to the cell injury and death seen in a variety of pathological states (91). An increase of intracellular Ca^{2+} from 0.1 to 0.5 μM is sufficient to activate calpain, a proteinase capable of degrading to Ca^{2+}-ATPase (92). In addition high intracellular Ca^{2+} levels may also activate transglutaminases that are capable of cross-linking and thus inactivating a variety of intracellular proteins (93). Finally, Ca^{2+}-activated phospholipases, such as phospholipase A_2, may be important to contributing to the degradation of the plasma membrane by hydrolysis of membrane phospholipids (94).

The previous study (70) reported that the possible role of oxygen free-radical-induced proteolytic cleavage of the Ca^{2+}/ Mg^{2+}–ATPase in thalassemic erythrocytes and

oxygen free-radical-exposed erythrocytes resulting in the removal of the calmodulin-binding domain and the decoupling of both the ATPase and the Ca^{2+}translocation activities.

The present study demonstrates that the calmodulin-deficient erythrocyte membranes pre-incubated with the tamarind seed coat extract exposing to tert-butylperoxide and $FeCl_3$ could prevent lipid peroxidation in a dose-dependent manner, and protect Ca^{2+}-ATPase consequently observed by the response from stimulation of calmodulin. This may suggest that the extract can protect living cells from damage occurring from oxidative metabolism by inhibiting or reducing lipid peroxidation of polyunsaturated fatty acid and preventing loss of calmodulin-binding domain of Ca^{2+}-ATPase on cell membrane from attacking of ROS. It has been proposed that flavonoid located near the surface of phospholipid structures are ideally located for scavenging oxygen radicals generated in the aqueous phase (51). In doing so, the polyphenols act to produce an impervious layer (polyphenol-protein and/or polysaccharide complex) under hydrogen bonding and hydrophobic effects as the mechanisms mediating complexation (95). The efficacy of polyphenols binding to protein derives from the fact that polyphenols are multidentate ligands which are able to bind simultaneously (via different phenolic group) at more than one point to the protein surface. It indicates that molecular size results in the efficacy of binding increase in series of monomer units to di→ tri→ tetra→ penta.

Since the tamarind seed-coat extract consists of proanthocyanidin, in spite of containing other components, naturally its structure is an oligomer of polyphenolic compounds owning several hydroxyl groups. Thus, the antioxidative efficiency of the extract possessing the sizeable molecule increases by enhancing both binding protein or polysaccharide and scavenge ROS. Dauer et al, 1998 (96) reported that the mechanism of antimutagenic action of proanthocyanidins did not act as bioantimutagens, but rather as direct-acting desmutagens. The antimutagenic effect increased with an increasing degree of polymerization in the proanthocyanidins. Besides, some reports demonstrated that proanthocyanidin extract from grape seeds is

stronge scavenger of oxygen free radicals as compared to vitamin E and vitamin C (97), and exhibited a dose-dependent inhibition of lipid peroxidation and DNA fragmentation (98). However, in this study using high concentration of the extract, the TBARS reaction was interfered by red-colored anthocyanidins in which naturally they are derived from proanthocyanidin by heating with mild acid (41). And at high amount of the extract also results in diminishing Ca^{2+}-ATPase activity, the polyphenols binding to proteins on membrane possibly cause membrane loss of the fluidity and affect the biological activity of membrane-spanning proteins particularly membrane-bound enzymes. The data suggest that further purification of the extract and the purified antioxidative compounds are needed for the study.

The ROS-protective effect of the extract resembles to curcumin but not to that of vitamin E in the reaction protecting Ca^{2+}-ATPase on erythrocyte membrane, although the three compounds can prevent lipid peroxidation in a dose-dependent manner. Both the extract and curcumin are polyphenols, even though curcumin used is in a pure form. Vitamin E is a potent antioxidant inhibiting lipid peroxidation but could protect Ca^{2+}-ATPase activity but its inhibition is less than the extract and curcumin. There may be another factor or mechanism in addition to the inhibition of lipid peroxidation leading to the loss of calmodulin-binding domain of Ca^{2+}-ATPase.