CHAPTER III

RESULTS

1. Extraction of antioxidative compound from tamarind seed-coat powder.

The tamarind seed coat was powdered and extracted successively into five fractions according to the solvents used, 70% ethanol (Fraction 1), chloroform (Fraction 2), water (Fraction 3), ethyl acetate (Fraction 4), methanol (Fraction 5). Each fraction was determined for the antioxidant activity to find out the fraction containing the greatest antioxidant activity. Figure 11 shows antioxidative effect of each fraction using ABTS/H_2O_2/WRP system and compared to the solvent used. The result shows that the ethanol fraction (Fraction 1) has the highest inhibition of ABTS radical generation, followed by those of the aqueous fraction (Fraction 3) and the methanol fraction (Fraction 5). The ethyl acetate fraction and the chloroform fraction have fair inhibitory effect.

The specific antioxidant activity is based on dry weight of each fraction was also determined. Figure 12 reveals that % inhibition per microgram of the extract in methanol fraction has the highest antioxidant activity. The extract in water (aqueous) fraction exhibits slightly lower antioxidant activity than that of methanol fraction, but comparable to that in the ethyl acetate fraction. The antioxidant activities of the extract in ethanol and chloroform fraction were quite low when calculated by weight. The result indicates that the extract was more purified by stepwise extraction. However, the dried residue of the methanol fraction, had the greatest antioxidant activity, but could not be re-dissolved in methanol, while the water and ethyl acetate fractions could. The water fraction of tamarind seed coat powder was evaporated at 60-80 °C in a vacuum rotating evaporator and then incubated at 60 °C in a ventilated oven until the residue was obtained and completely dried. The dry residues, re-dissolved in methanol. In this study, therefore the methanol solution of dry residues from the water fraction was used in all the following experiments.

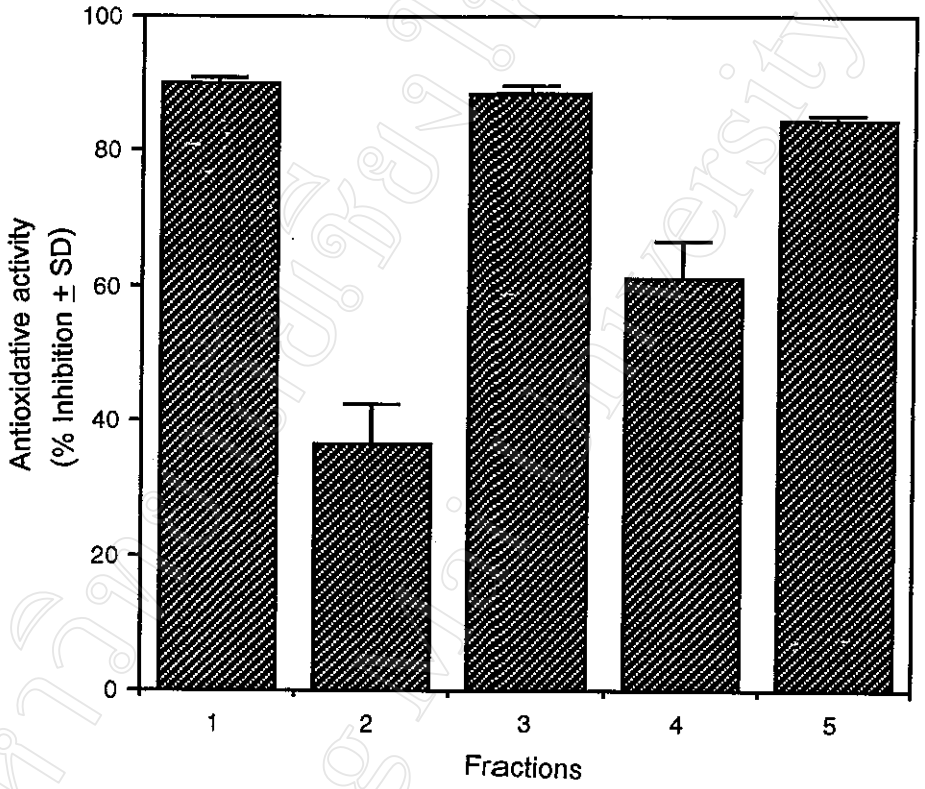


Figure 11: Antioxidative effects of fractions extracted from tamarind seed coat as determined by ABTS/ H_2O_2/WRP method. Each solvent used in different fraction has no antioxidative effect. Fractions: 1;ethanol, 2;chloroform, 3;water, 4;ethyl acetate, 5;methanol.

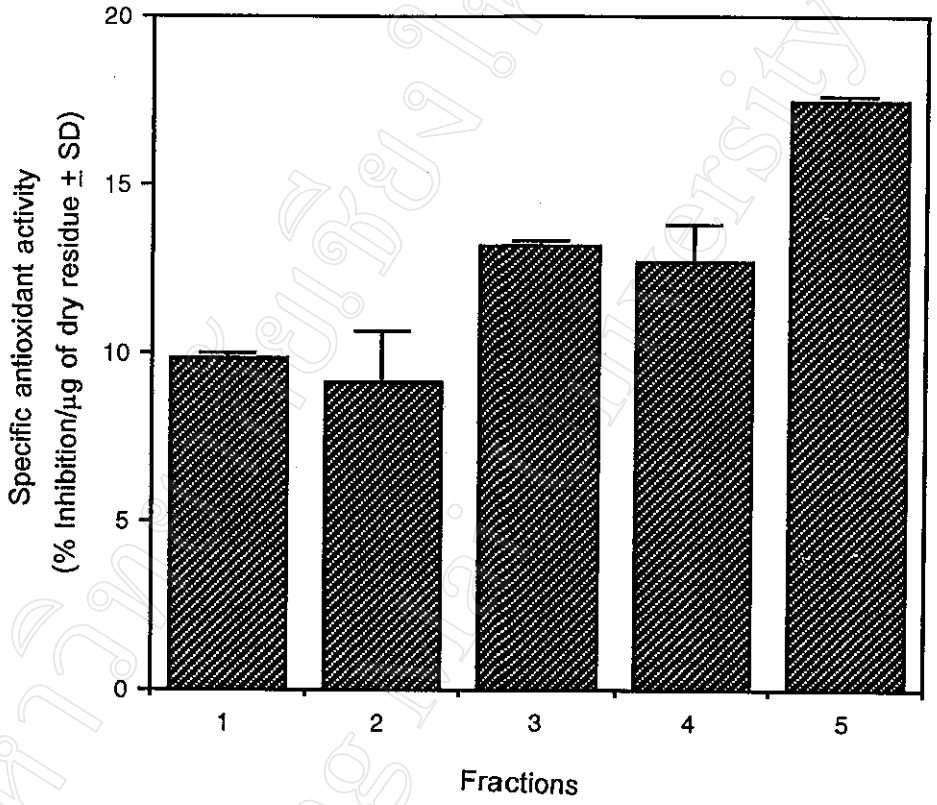


Figure 12: Specific antioxidant activity of fractions . The fractions extracted from tamarind seed coat and the inhibition rate was determined by ABTS/ H_2O_2/WRP reaction and expressed as % inhibition per 1 µg dry residue in each fraction. Solvents used in each fraction, 1:ethanol; 2:chloroform; 3:water; 4:ethyl acetate; 5:methanol.

2. Characteristics of the tamarind seed-coat extract.

Since the extract possessed the physical properties likely to be in a condensed tannin group. It has a distinct astringent taste, and after hydrolysis with 1 % HCl, the dark brown-colored extract is changed to red- or pink-colored pigment which is the typical characteristic of flavonoid anthocyanidin released from the extract. The result of acid hydrolysis is shown in TLC chromatogram (Figure 13). It indicates that the hydrolyzed extract could be specially separated by this system into six spots from origin to solvent front. As shown in the TLC chromatogram, the result indicates that solely heating, at 50 ° C and 100 °C, could only discompose the complex molecule separating two spots at origin and solvent front. The extract contains at least six distinct components that each has different Rf values as shown in Table 3.

The hydrolyzed extracts at various times were assessed the antioxidant activity and the result is shown in Figure 14. The result indicates that decomposed extract slightly decreases antioxidant capacity, but after hydrolysis taking more long time the antioxidative inhibition gradually enhances until at 120 minutes of heating, its level is a little higher than that of the non-hydrolyzed extract, indicating that mild acid condition of hydrolysis possibly changed some parts of the extract structure and contributed to its antioxidant activity. In addition, the extract is heat stable (Figure 15) at various temperatures: at 25 °C, 50 °C, 100 °C and 140 °C. It was found that all % inhibition rates were clearly constant at 100 °C but slightly decreased at 140 °C.

In this study, chemical screening tests specific to condensed tannin and absorption spectra were examined by comparing the extract with a condensed tannin, particularly a so-called proanthocyanidin or oligomeric proanthocyanidin (OPC) obtained from two sources, one is a commercial tablet extracted from pine bark and the another is powder compounds from grape seeds. The specific chemical reactions shown in Table 4 indicated that the extract exhibited the positive results and contained compounds similar to proanthocyanidins from both sources. To further the identification, their ultraviolet absorption and IR spectra were determined. They were all dissolved in methanol. Figure 16 suggests that OPC extracted from grape seeds showing a spectrum at 289.20 nm contains only OPC. It seems likely to be a purified compound. Whereas the extract

showed two spectra peaks at 281.00 and 212.50 nm and OPC derived from pine bark in a tablet form expressed at 281.50 and 208.00 nm, indicating their impurity at 212.50 and 208.00 nm, respectively. The spectra slightly shifted probably because of its impurity or even resolution error of the spectrometer.

The result obtained from IR spectra (Figure 17) indicated that the extract contains compounds similar to those from grape seeds, although it is slightly different from the spectra derived from the extract of pine bark containing OPC. Since the OPC commercial extract of pine bark composed of starch and other materials to be formed as a film-coated tablet, whereas the dry powder extract of OPC from grape seeds was contained in a capsule in which it more resembles the extract from tamarind seed coat.

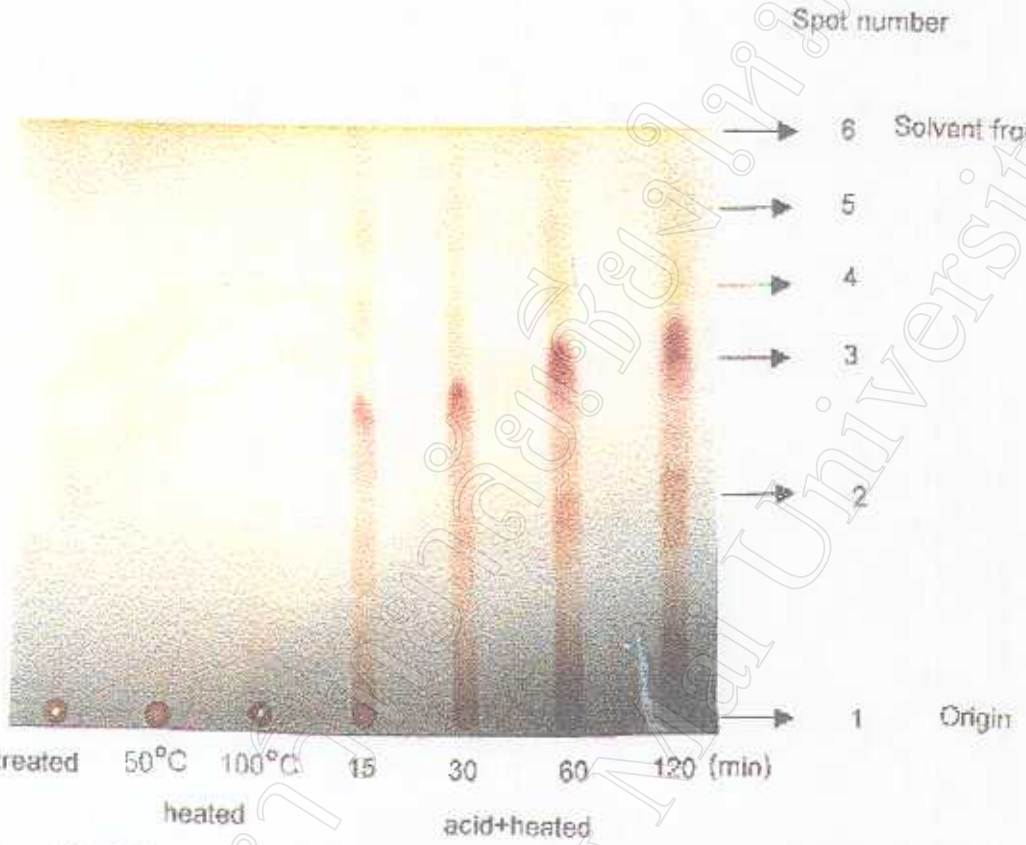


Figure 13: TLC chromatogram of the hydrolyzed extract. The extract, 1.0 mg/ml. was hydrolyzed by heating in 1 % HCl at 100 °C and various periods : 15, 30, 60, 120 minutes. The control extracts had no acid but heated at 50 °C, 100 °C and the untreated extract are also shown on TLC chromatogram. The developing system of TLC is chloroform: methanol: acetic acid (7:2:1).

Table 3: Rf values of components in the hydrolyzed extract from tamarind seed coat separated on TLC chromatogram.

Spot number	Comments	Rf
1	origin, dark brown-colored pigment	0
2	red-brown colored pigment	0.37
3	dense, violet-colored pigment	0.62
4	brown pink-colored pigment	0.74
5	trace, pale brown-colored pigment	0.86
6	solvent front, pale yellow-colored pigment	1.0

Note: Data from Figure 13

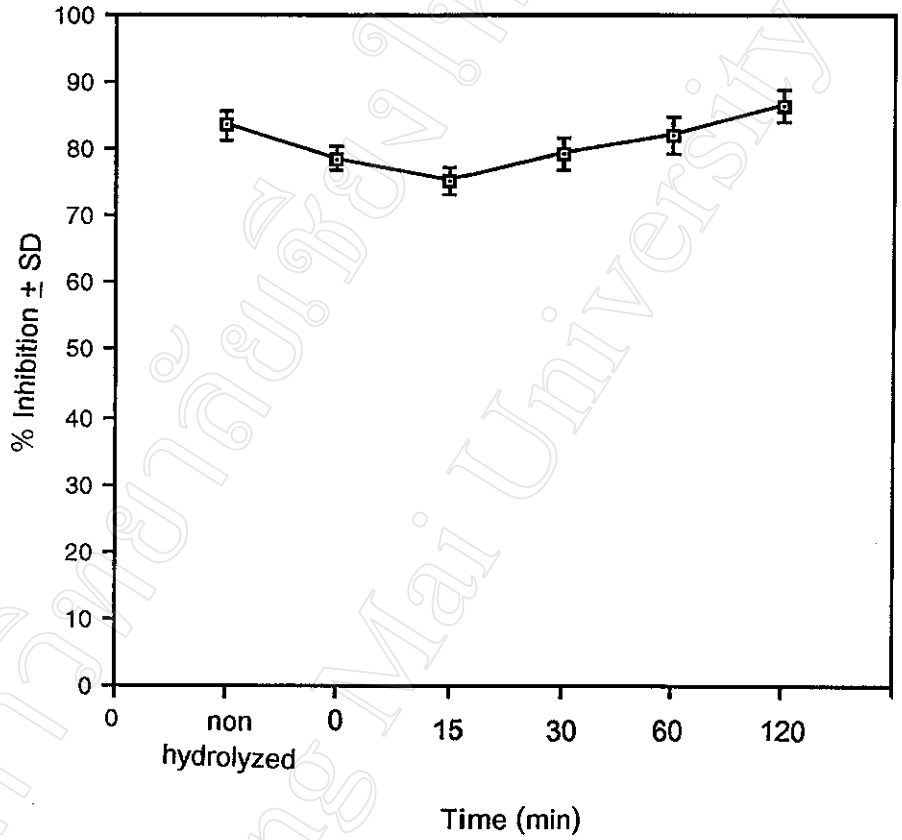


Figure 14: Antioxidative effect of the hydrolyzed extract: The extract, 4.93 μg/ml, was hydrolyzed by heating in 1 % HCl at 100 °C and various periods: 15, 30, 60, 120 minutes and their antioxidative activities were determined by ABTS/ H_2O_2/WRP method.

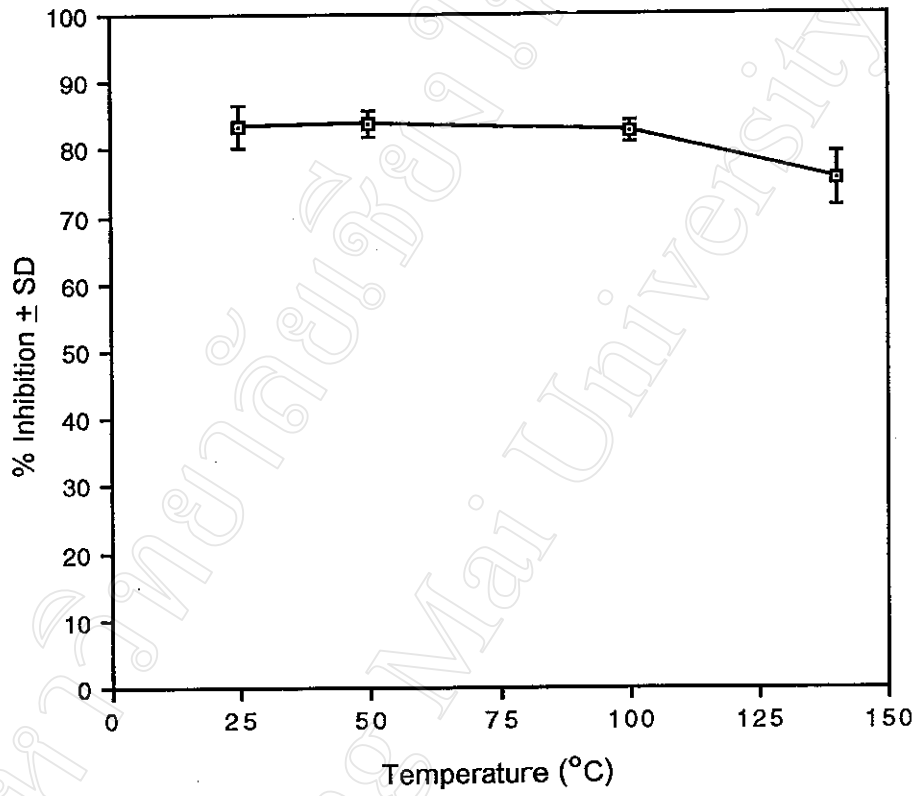


Figure 15: Heat stability of the extract. Dry samples of the extracts were pre-incubated at 25°C, 50°C, 100°C and 140°C respectively, for 1 hour, then the antioxidant activity of the samples dissolved in methanol (4.93 μg/ml) was determined by ABTS/ H_2O_2/WRP method.

Table 4: Chemical screening tests for the presence of condensed tannin in the extract from tamarind seed coat compared to oligomeric proanthocyanidin (OPC), commercial componds extracted from grape seed and pine bark.

Chemical Tests	Positive results	OPC Grape seed	OPC Pine bark	Extract Tamarind seed coat
1.$FeCl_3$ reaction	Green or brown-green colored precipitates.	+	+	+
2.Bromine reagent reaction	Buff- colored precipitates	+	+	+
3.Formaldehyde-HCl reaction	Red/pink colored, undissolved precipitates	+	+	+
4.Boiling with 1%HCl	Red/pink colored precipitates	+	+	+

Note: + stands for positive result

(The methodology of chemical tests are from reference 99)

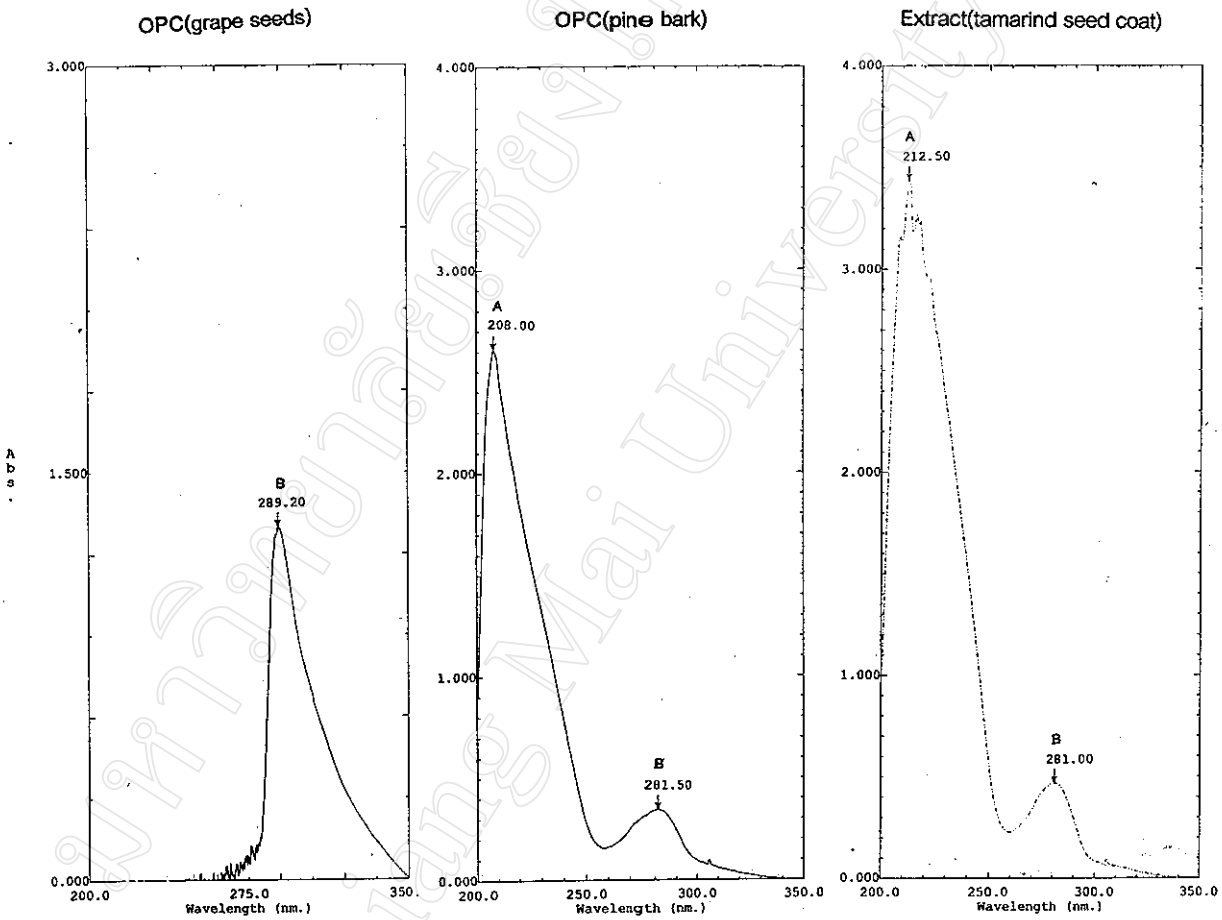


Figure 16: Comparison of UV absorption spectra of the extract from tamarind seed coat and known OPCs from pine-bark and from grape seeds in methanol.

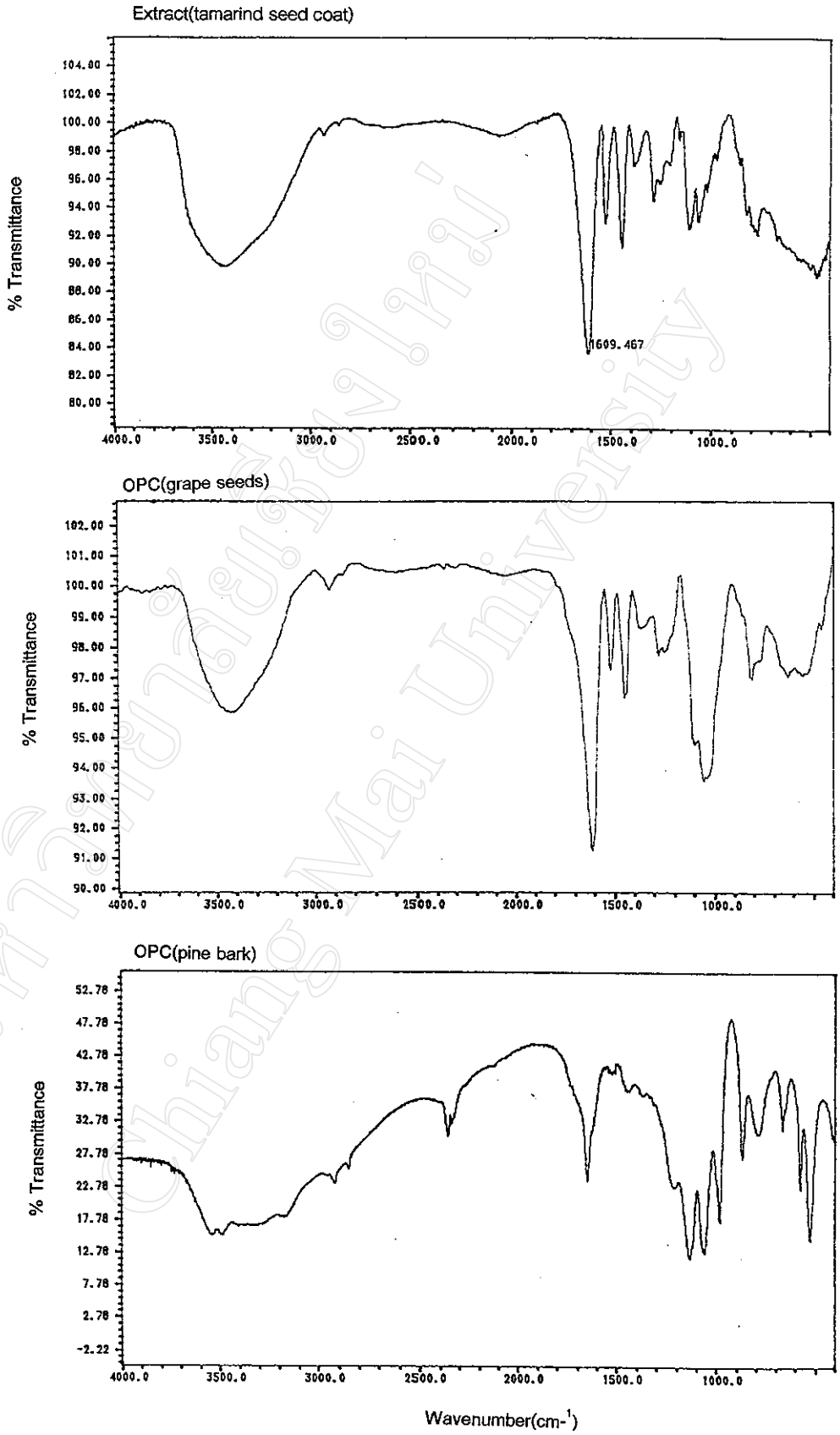


Figure 17: Comparison of IR spectra (KBr) of the extract from tamarind seed coat and known OPCs from pine-bark and from grape seeds.

3. Antioxidant properties of the dry residue extracted from tamarind seed coats.

The extract, 4.93 µg/ml, was determined for the antioxidant activity by ABTS/H_2O_2/WRP method which generates peroxyl radicals as described in the Materials and Methods. Figure 18 shows that the dry extract could inhibit formation of the oxidized ABTS compared with the blank control. This result also implies that the active antioxidant compound is stable at temperature as high as 60-80°C.

The experiment was also designed to investigate whether the extract was a direct inhibitor of peroxidase (WRP) or it was a scavenger of specific reactive oxygen species. The results shown in Figure 19 indicates that the dose-dependent experiments were performed in the concentration range of 2.46-9.85 µg/ml in four free-oxygen generation systems; (a) ABTS/H_2O_2/WRP system giving peroxyl radicals by enzymatic reaction, (b) ABTS/H_2O_2/metmyoglobin system giving peroxyl radicals by non-enzymatic system, (c) ABTS/H_2O_2/$FeCl_3$ system producing hydroxyl radicals and (d) NBT/HPX/XOD system generating superoxide anion. The results indicated that the extract possess inhibitory effect in a proper dose response to all reactive-oxygen species used: peroxyl radical, hydroxyl radical, supperoxide anion. A similar inhibitory pattern of the extract to peroxyl radicals producing by both enzymatic and non-enzymatic reactions indicateed that the inhibitory effect is due to inhibition of free radicals but not peroxidase.

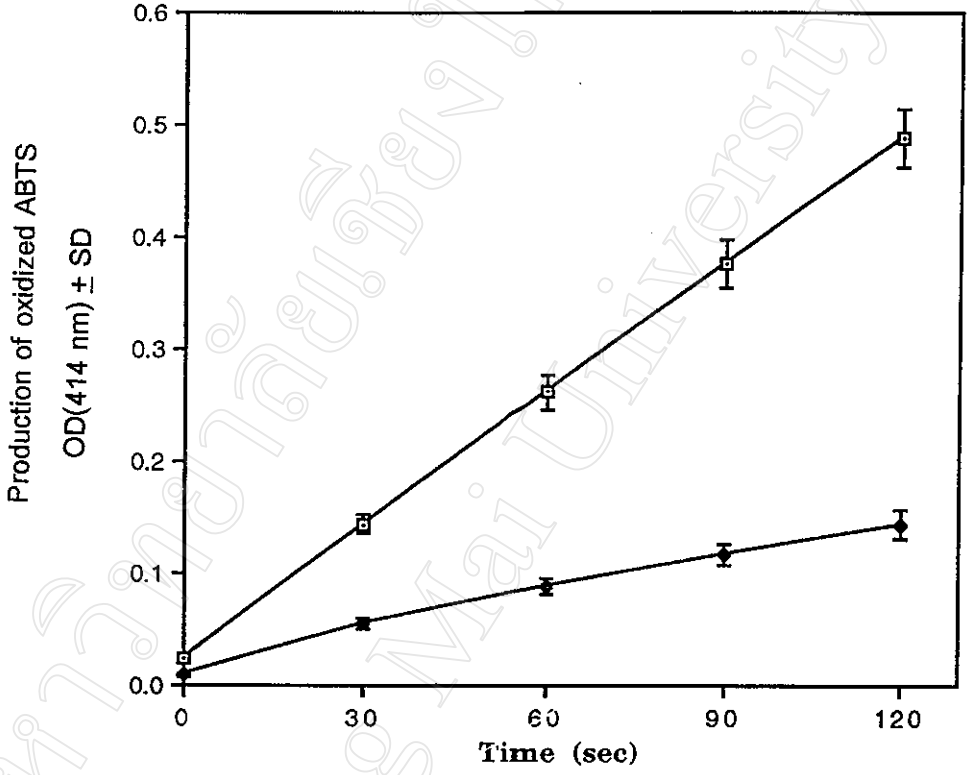


Figure 18: Antioxidant activity of the residue extract of tamarind seed coat from water fraction. The activity was determined by ABTS/ H_2O_2/WRP method. —□—:negative control, —◆—:dry extract at 4.92 μg/ml.

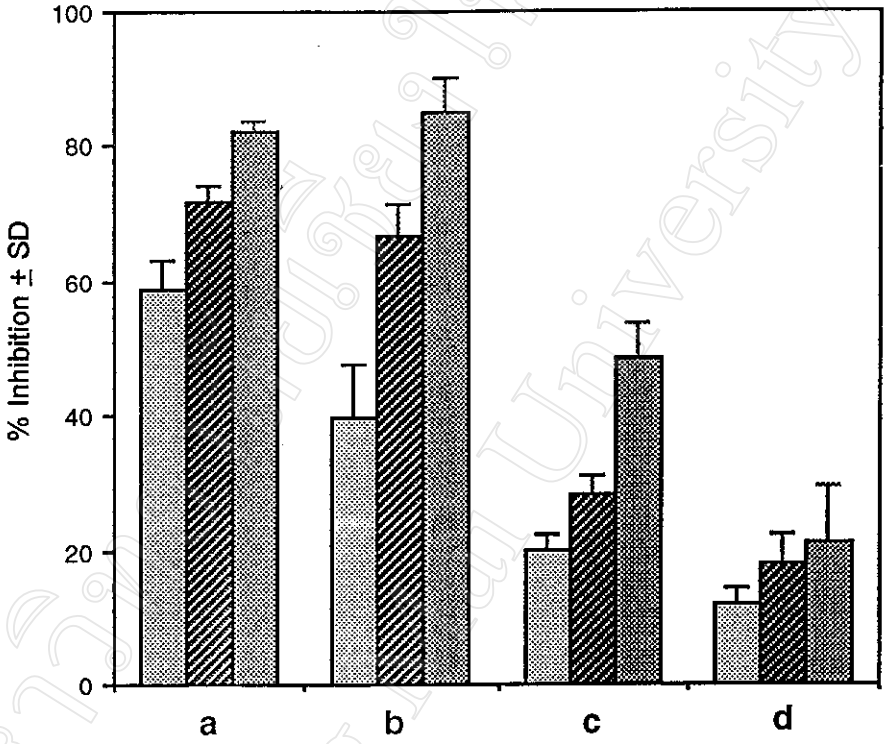


Figure 19: Dose dependence and specific anti-peroxyl radical effect of the extract. Various concentrations of the extract were determined for the antioxidant activity in 4 systems generating reactive-oxygen radicals: (a)ABTS/H_2O_2/WRP; **(b)**ABTS/ H_2O_2/Metmyoglobin; **(c)** ABTS/$_2O_2$/$FeCl_3$; (d)NBT/HPX/XOD. Concentrations of the extract: ▨ :2.46 μg/ml, ▨ :4.93 μg/ml, ▨ :9.85 μg/ml.

4. Antioxidant activity of the extract in comparison to standard antioxidants.

The antioxidant capacity of the extract was compared with those of four pure antioxidants, i.e. vitamin E, a well known antioxidant with high potential free radical scavenger; curcumin, an effective polyphenolic antioxidant in term of iron-chelator; Trolox, a commercial, water-soluble analog of vitamin E; L-ascorbic acid or vitamin C, an natural antioxidant in water-soluble phase. Methanol used as the solvent did not show any antioxidant property. In Figure 20.1 the profile of antioxidant property of vitamin E was fairly different from that of the extract in ABTS/H_2O_2/WRP system. Vitamin E had two different rates of reaction in which it showed lag time in the first period but gradually increased at the same time. In comparison to tamarind seed coat extract, curcumin and its solvent, 4% dimethyl sulfoxide (DMSO), at a certain concentration of curcumin used and 4%DMSO did not show antioxidative effect to peroxyl radicals produced in this assay system.

The antioxidant patterns of Trolox and L-ascorbic acid differed from that of the crude extract. Since both gave a particular lag time in the first period of reaction time (Figure 20.2), thereafter, the rate of oxidized ABTS production was similar to that of the blank control. These results suggest that the extract could scavenge free radicals but in a way of antioxidant mechanism differing from vitamin E, Trolox and L- ascorbic acid.

However, the result in Figure 20.3 tested in the Fenton reaction (ABTS/H_2O_2/$FeCl_3$) system, the extract and curcumin exhibited a similar antioxidant effect; whereas the antioxidative activity of vitamin E was slightly different by which the reaction rate just like the rate in the first system. The result indicated that the extract may also have a property of iron-chelator resembling curcumin. The same as in peroxyl generating system, 4% DMSO clearly did not have antioxidant effect on the hydroxyl radicals.

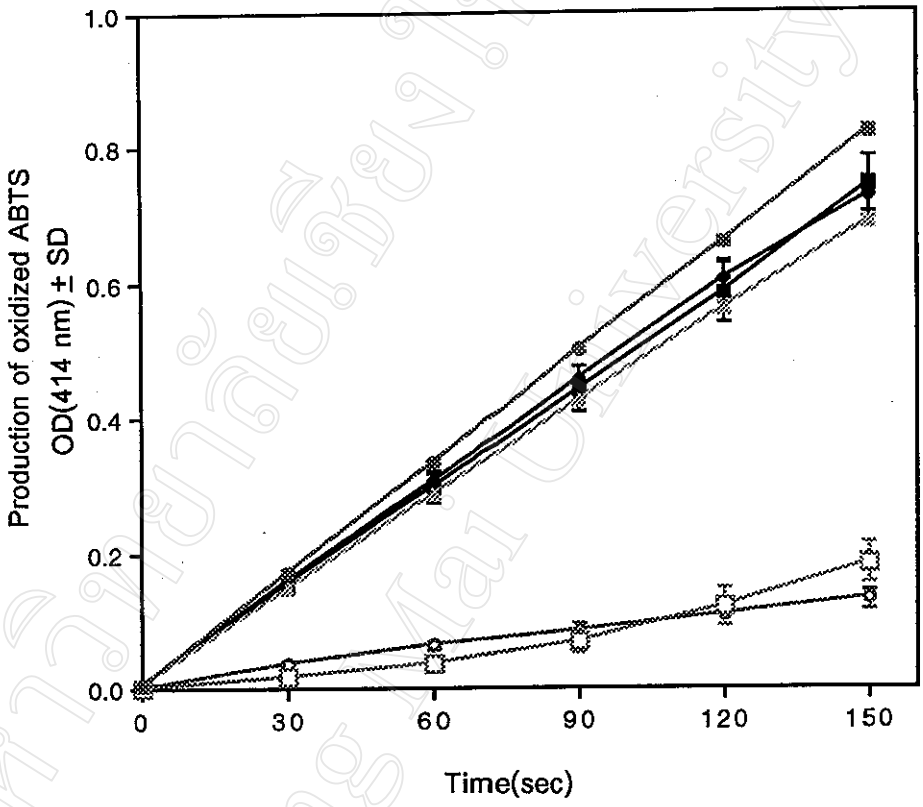
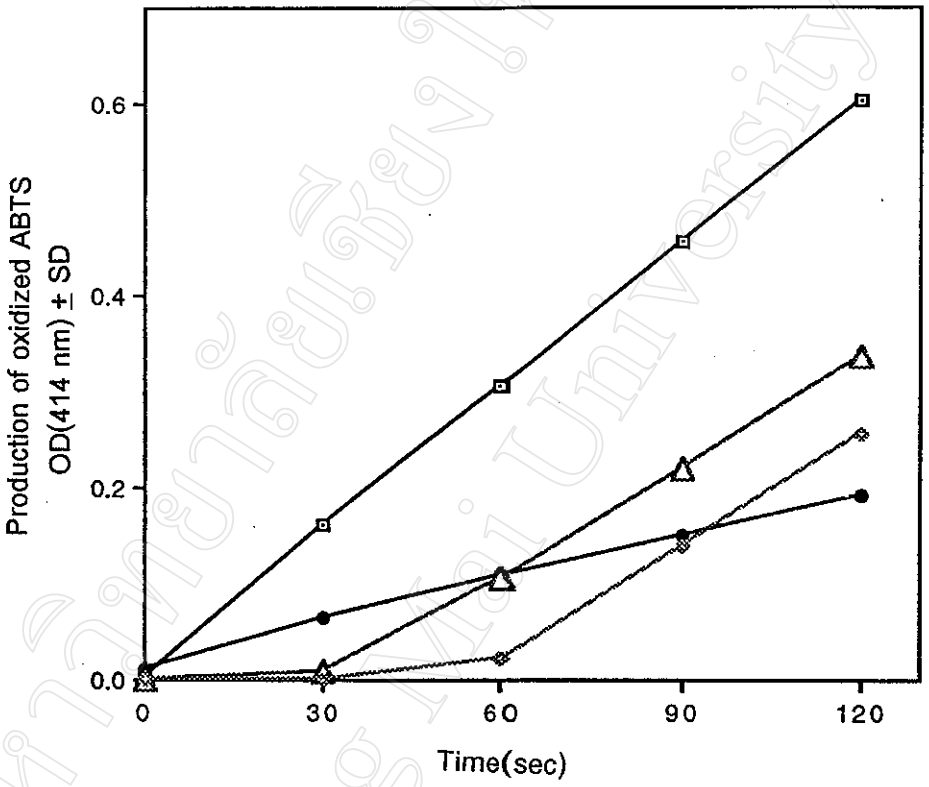


Figure 20.1: Antioxidant activity of the extract against peroxyl radicals in comparison to vitamin E and curcumin. The activity was assayed by ABTS/ H_2O_2/WRP method generating peroxyl radicals. —●— :blank control; —■— :methanol; —▨— :4%DMSO; —◈— :9.85 µg/ml curcumin;—◇—:4.93 µg/ml extract;—□—:4.24 µg/ml vitamin E.



Figfure 20.2: Antioxidant activity of the extract against peroxyl radicals in comparison to Trolox and vitamin C. The activities were assayed by ABTS/ H_2O_2/WRP method generating peroxyl radicals. —●—:2.46 μg/ml extract; —△—:2.46 μg/ml Trolox; —◇—:2.46 μg/ml vitamin C. —□—:control.

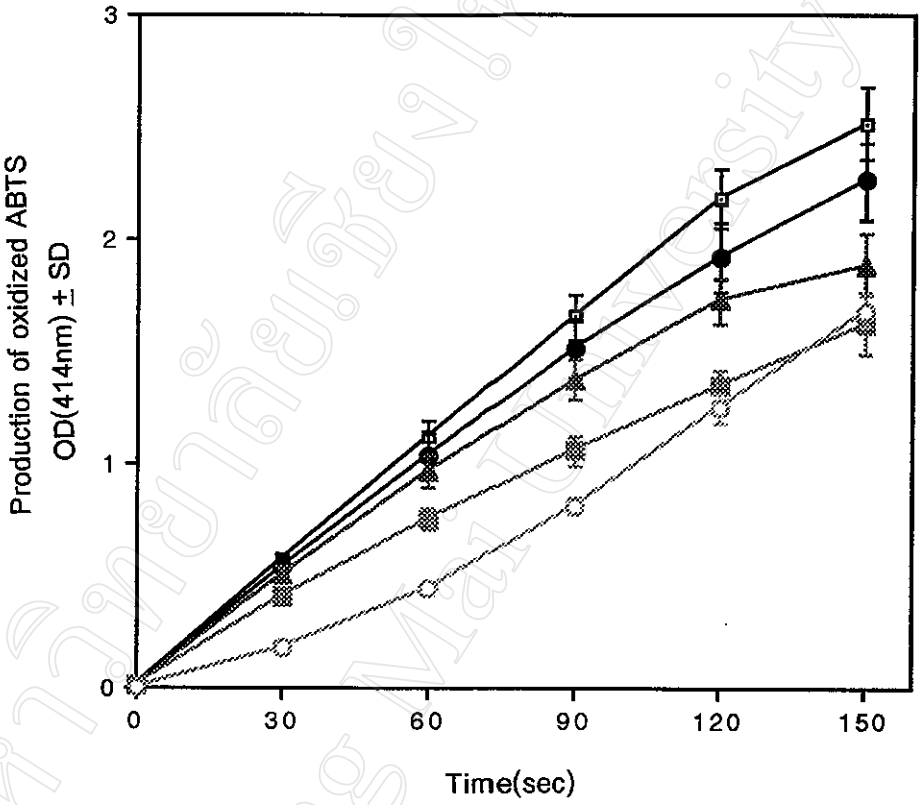


Figure 20.3: Antioxidant activity of the extract against hydroxyl radicals in comparison to vitamin E and curcumin. The activity was assayed by ABTS/ H_2O_2/ $FeCl_3$ method generating hydroxyl radicals. —●—:blank control;—▫—:4%DMSO; —▲—:9.85μg/ml curcumin;—▩—:4.93 μg/ml extract;—◇—:4.24 μg/ml vitamin E.

5. Prevention of lipid peroxidation in erythrocyte membrane by the extract.

The protective effect of the extract on lipid peroxidation was studied in erythrocyte membrane exposed to oxygen radical-generating system. The oxidants system used in this experiment was 10% tert-butylperoxide and $FeCl_3$, which catalysed the generation of organic peroxyl radicals. The extract was studied in comparing to standard antioxidants, vitamin E and curcumin that both are reactive antioxidants in lipid-soluble phase. A product of lipid peroxidation is malondialdehyde (MDA) which could be determined by TBARS (Thiobarbituric acid reactive substances) reaction. The Figure 21.1 showed that the extract could prevent lipid peroxidation of the erythrocyte membranes in dose-dependent fashion. The result of the tamarind seed coat extract, preventing lipid peroxidation of red cell membranes, was similar to those observed from vitamin E and curcumin which were assayed by the same system as shown in Figure 21.2 and 21.3, respectively. A negative control, unoxidized erythrocyte membranes showed small amount of MDA in TBARS reaction and the values were discounted from those of the treated groups.

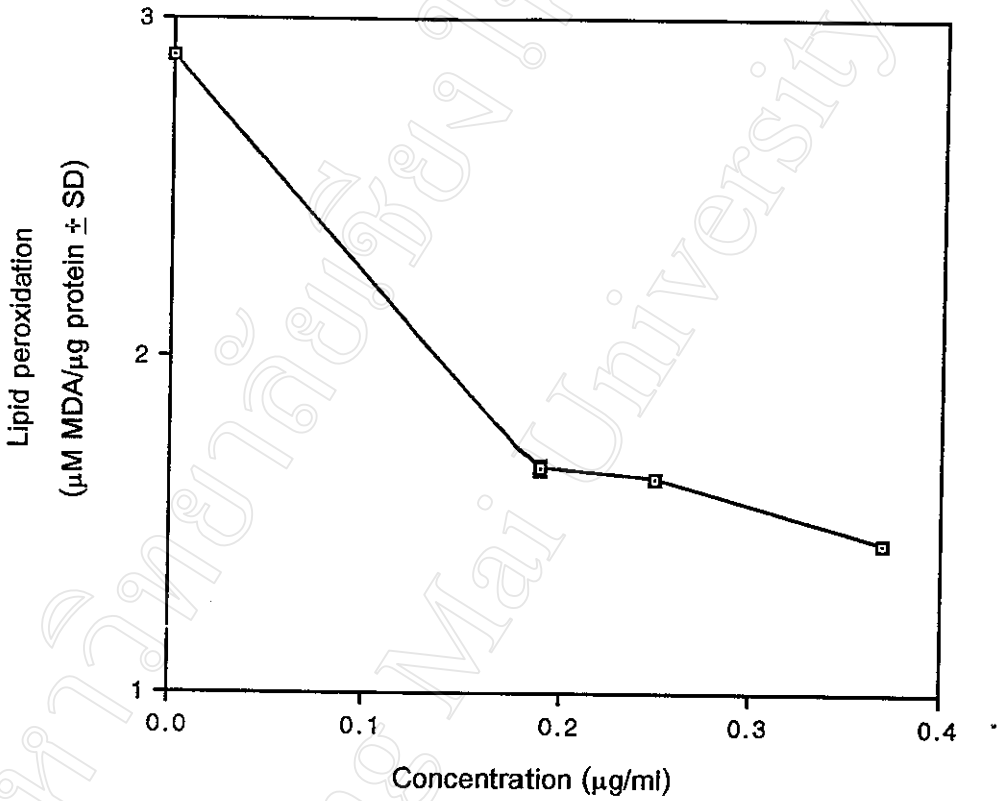


Figure 21.1: Prevention of lipid peroxidation of the extract. RBC membranes (4 mg/ml protein) were pre-incubated by with 0.19, 0.25, and 0.37 μg/ml of the extract before adding tert-butylperoxide and $FeCl_3$. The pre-incubated membrane preparations were then determined for MDA by TBARS reaction.

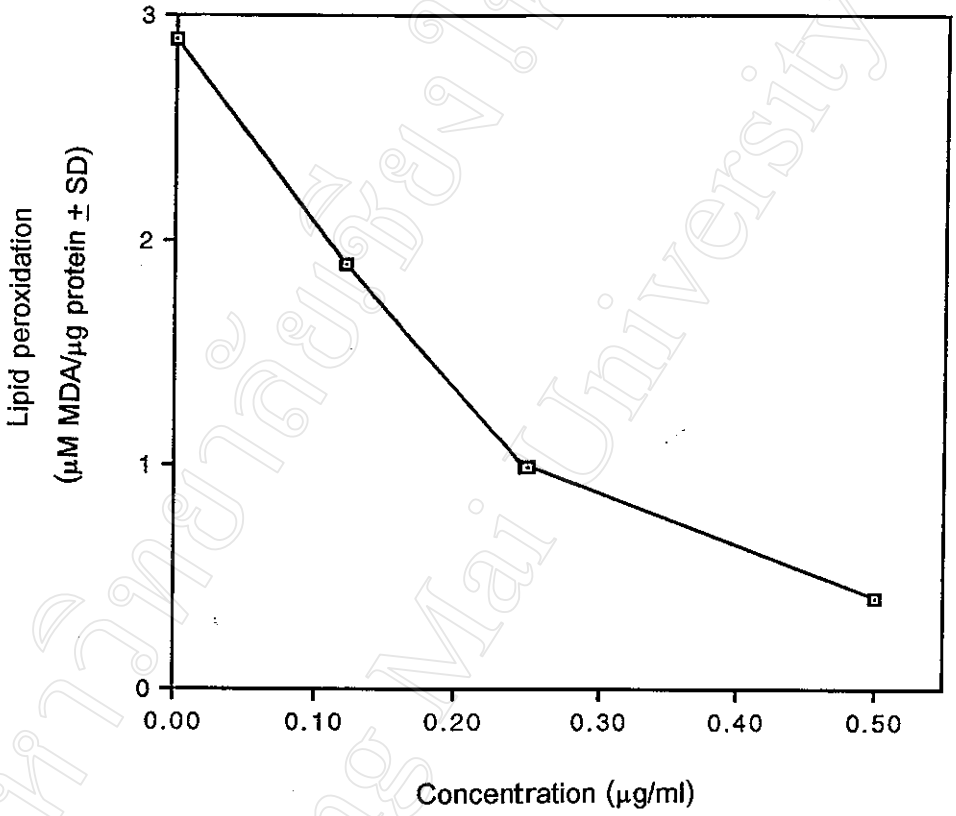


Figure 21.2: Prevention of lipid peroxidation of curcumin. RBC membranes (4 mg/ml protein) were pre-incubated by with 0.12, 0.25, and 0.50 μg/ml of curcumin before adding tert-butylperoxide and $FeCl_3$. The pre-incubated membrane preparations were then determined for MDA by TBARS reaction.

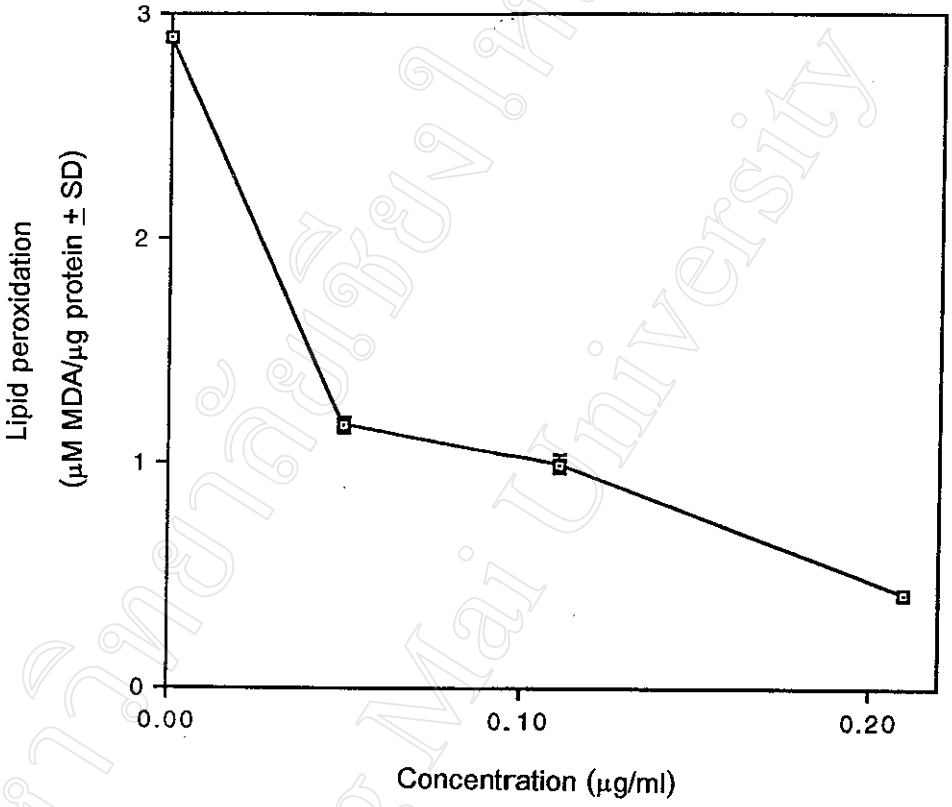


Figure 21.3: Prevention of lipid peroxidation of vitamin E. RBC membranes (4 mg/ml protein) were pre-incubated by with 0.05, 0.11, and 0.21 μg/ml of vitamin E before adding tert-butylperoxide and $FeCl_3$. The pre-incubated membrane preparations were then determined for MDA by TBARS reaction.

6. Protection of Ca^{2+}-ATPase activity in erythrocyte membrane by the extract.

The Ca^{2+}-ATPase activity of calmodulin-deficeint erythrocyte membranes were determined by coupled enzyme assay under both absence (basal activity) and presence (stimulated activity) of calmodulin. The calmodulin response of the enzyme was calculated from calmodulin-stimulating activity to the basal activity. The membranes were pre-incubated with the extract in the similar method of lipid peroxidation experiment. The result was showed in Figure 22. In normal, membranes with treatment of both antioxidant and oxidant, the enzyme showed about 1.55 folds response to calmodulin stimulation. When oxidized, the enzyme lost its response to calmodulin. The tamarind seed coat extract could recover the Ca^{2+}-ATPase activity responding to calmodulin stimulation at 1.71 folds. This indicates that the extract, at 0.19 µg/ml, can effectively protect the Ca^{2+}-ATPase on RBC membrane from oxidative damage by free-oxygen radicals.

Curcumin, at 0.50 µg/ml, gave the calmodulin response at 1.92 folds. Therefore the result suggests that curcumin also prevented the damage of the enzyme on RBC membrane under oxidative condition.

Vitamin E, 0.21 µg/ml, has a protective effect on the plasma membrane Ca^{2+}-ATPase from oxidative damage as well, though the calmodulin response is 1.17 folds which is lower than those observed in the experiments using curcumin and the tamarind seed coat extract.

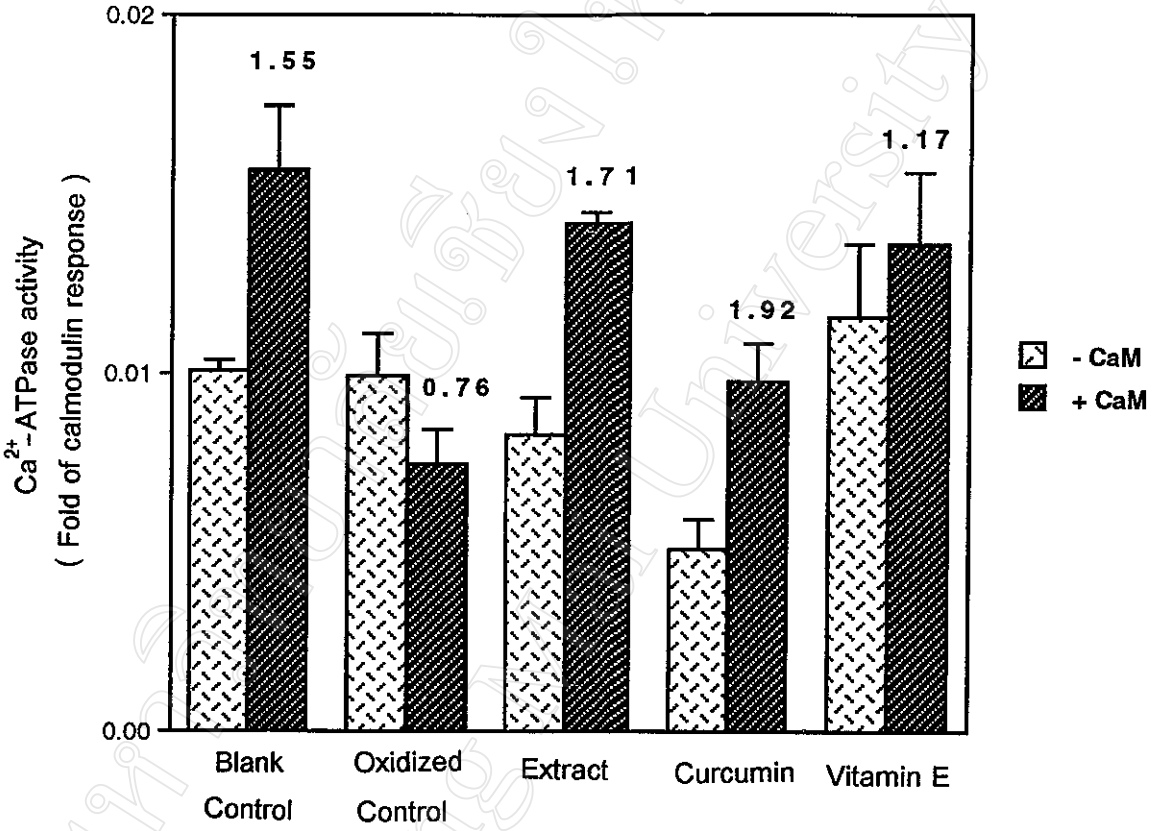


Figure 22: Ca^{2+}-ATPase activity protection of the extract compared to curcumin and vitamin E. RBC membranes were pre-incubated with the antioxidants and subsequently added with oxidants, tert-butylperoxide and $FeCl_3$. The pre-incubated membrane preparations were determined for the Ca^{2+}-ATPase activity by the coupled enzyme method in absence and presence of calmodulin. Fold of calmodulin response is shown over each bar-graph.