CHAPTER III

RESULTS

3.1. Water content

Water contents of shallot and garlic bulbs were 83.79±0.6 % and 37.87±0.6% respectively. The lyophilized solids expressed as fractions of the weight of the fresh bulbs subjected to crude, water and hexane extraction were 6.42±0.95%, 8.68±0.56%, and 2.78±0.25%, respectively in shallot; and 7.29±0.85%, 17.6±0.68%, and 6.84±0.62%, respectively in garlic. It was found that more than 95% of antioxidant activities of the extracts contained in the lyophilized preparation compared with the whole mixture or supernatant after dissolving the lyophilized extract with water or hexane.

3.2. Antioxidant content and activity

Three standard antoxidants, gallic acid, Trolox and BHT were used to compare with the activities of shallot and garlic extracts. The results showed that the percentage of reduction of ABTS cation radical at 743 nm was rapidly decreased and generally reached a plateau in approximately 60 minutes. Antioxidant activity from percentage of reduction (%) of standard BHT was increased and related to the concentrations at 0.075-0.3 μM (Fig. 24).

Prolonged and slow absorbance change was found in shallot, garlic extracts or commercial garlic products that reached a plateau at least 13 hr (Fig. 25-27). The percentage of reduction in hexane-extracted shallot showed higher percentage than other extracts (Fig. 25), including in garlic extract at 200 and 500 μg/ml (Fig. 25).

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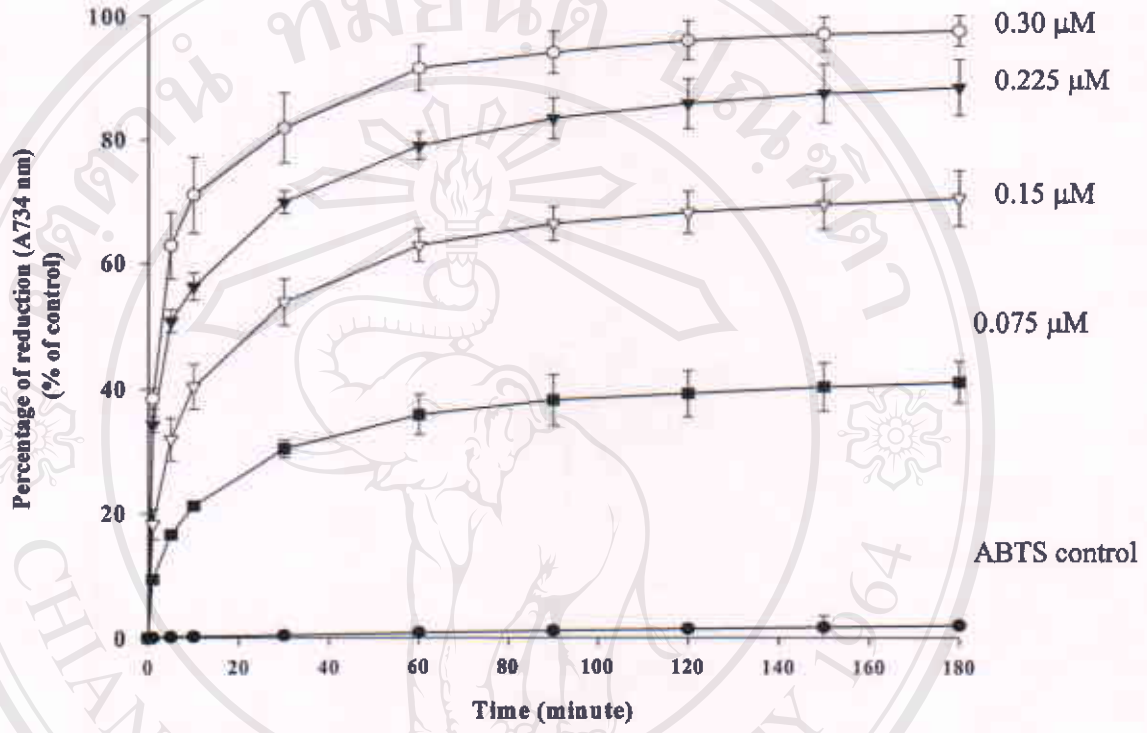

Figure 24. The antioxidant activity of standard BHT at different concentrations

The percentage of reducing the ABTS cation radicals at 7 mM after addition of standard BHT at various concentrations (0.075-0.3 µM) that read at 734 nm and calculated the percentage by compared to ABTS control. Each point represents the mean and standard deviation from triplicate samples.

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For shallot and garlic extracts (200 and 500 µg/ml), the reactions required 13 to 15 hours to reach a plateau (Fig. 25 and Fig. 26).

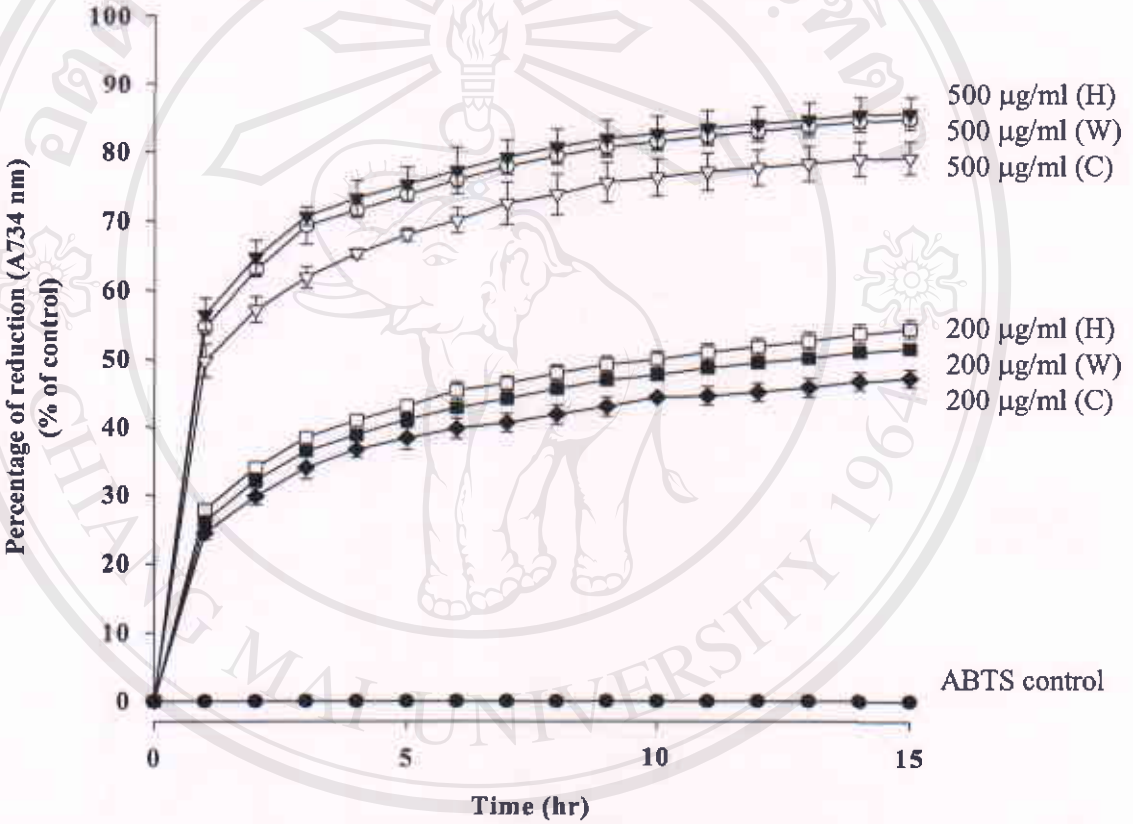

Figure 25. The antioxidant activity of shallot extracts. The percentage of reduction of the ABTS cation radicals (7 mM) by crude (C), water (W) and hexane (H) extracts of shallot. The percentage was calculated by comparing to ABTS control. Each point represents the mean and standard deviation from triplicate samples.

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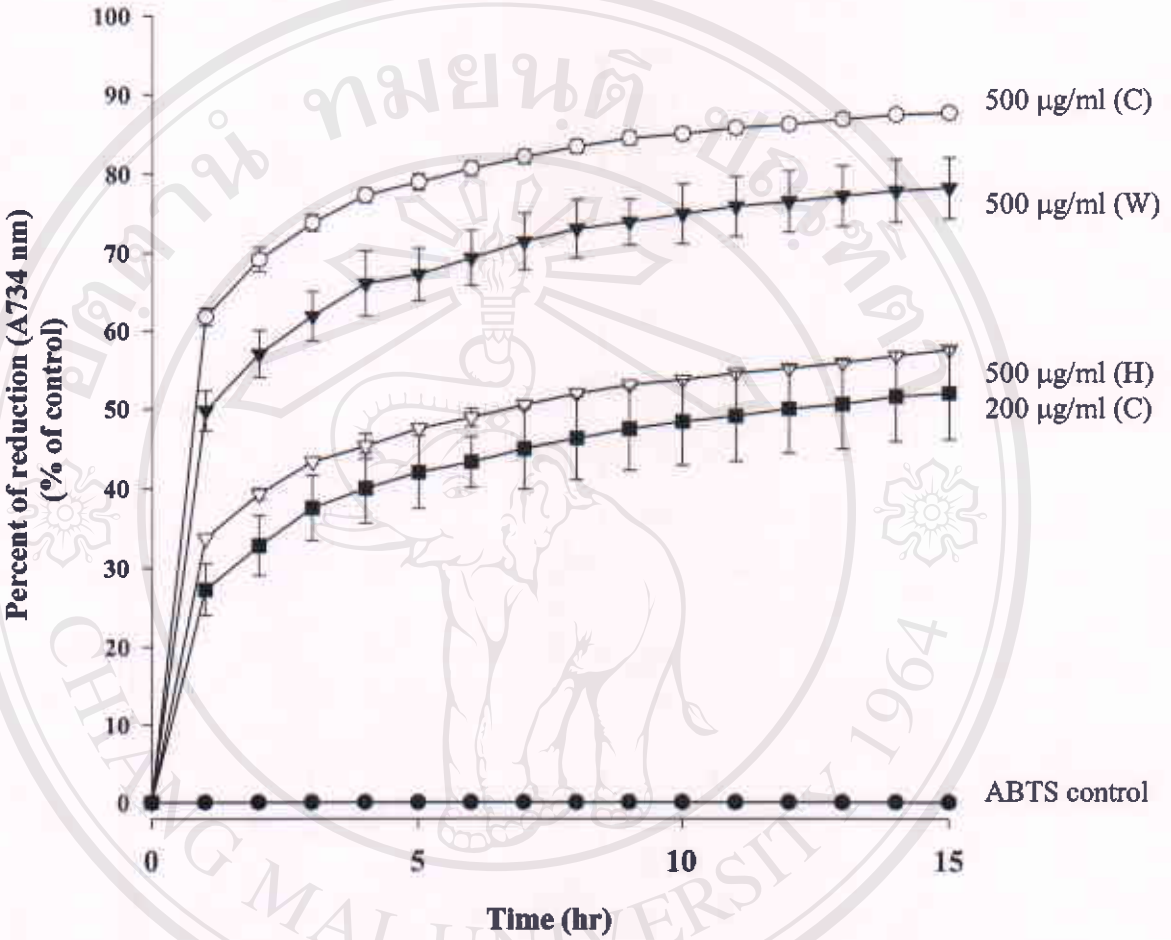

Figure 26. The antioxidant activity of garlic extracts. The percentage of reduction of the ABTS cation radicals (7 mM) by crude (C), water (W) and hexane (H) extracts of garlic. The percentage of reduction was calculated by comparing to the ABTS control. Each point represents the mean and standard deviation from triplicate samples.

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Two commercial garlic products, Immunytop from Thailand and Kyolic from New Zealand, were compared to garlic and shallot extracts (Fig. 27). The percentage of ATBS radical reduction by these two products were lower than garlic and shallot extracts.

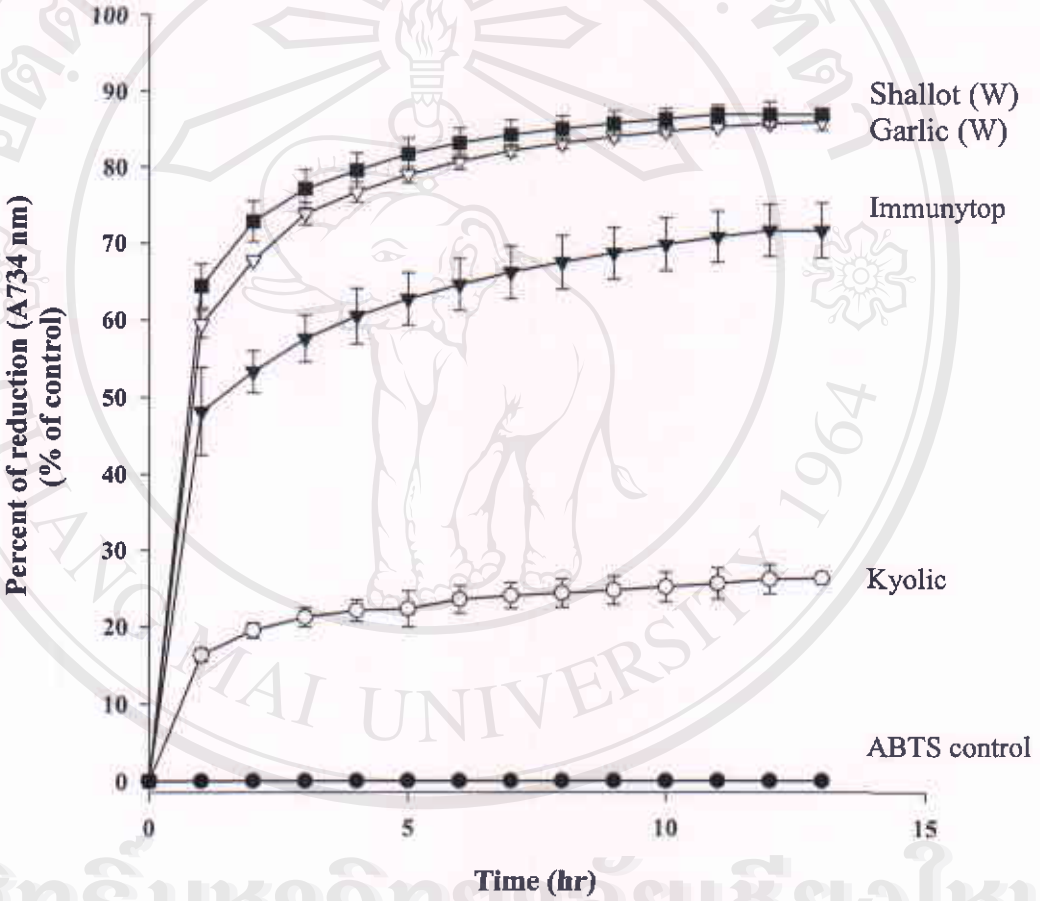

Figure 27. The antioxidant activities of commercial garlic products. The comparative percentage of reduction (A734 nm) between commercial garlic products Immunytop (Thailand) and Kyolic® aged garlic extract (New Zealand), water extract of garlic (W), and water extract of shallot (W) at 500 μg/ml. Each point represents the mean and standard deviation from triplicate samples.

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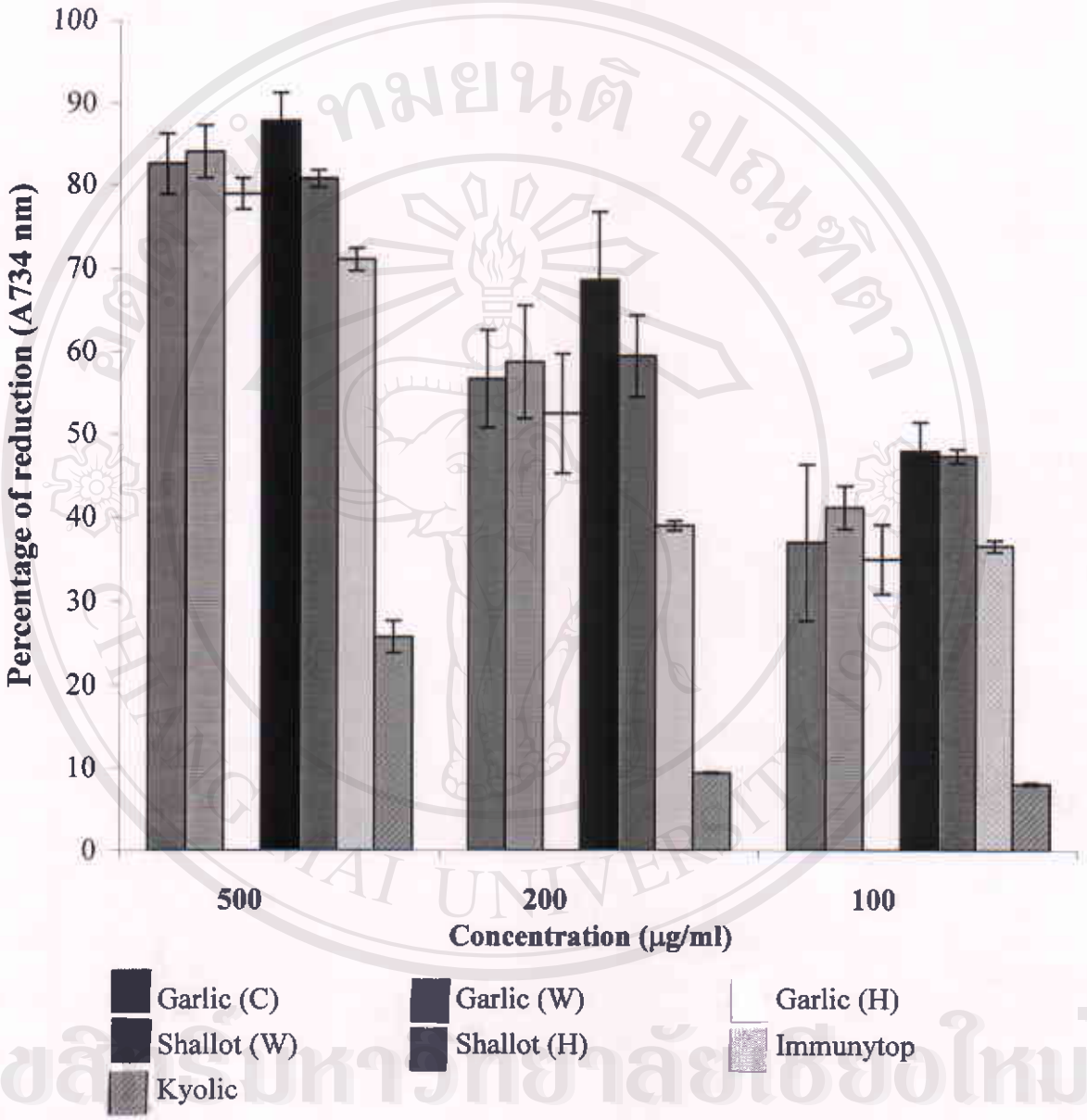

Figure 28. Comparison of antioxidant activities between shallot, garlic extracts and commercial garlic products. The percentage of reduction (A734 nm) of shallot, garlic, and commercial products from Thailand (Immunytop) and New Zealand (Kyolic) at 500, 200, and 100 µg/ml. Mean and standard deviation were calculated from triplicates trials. (Note: *p<0.01 compared with all extracts in each concentration, C = crude, W = distilled water, H = hexane).

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At 100, 200, and 500 μg/ml, shallot extracted with distilled water showed slightly higher percentage of ABTS radical reduction (47.78±3.43 %, 68.47±8.12 %, and 87.78±3.40 %, respectively) than garlic extracted with distilled water (40.98±2.56 %, 58.61±6.86 %, and 84.02±3.23 %, respectively), crude (36.31±9.27 %, 56.57±5.91 %, and 82.57±3.67 %, respectively), and was also higher than the garlic extracted with hexane (34.89±4.09 %, 52.35±7.21 %, and 78.89±1.85 %, respectively) (Fig.28).

Hexane extracts of shallot had a higher reducing percentage (47.15±0.83 %, 59.26±4.95 %, and 80.69±1.03 %, respectively) than hexane extracts of garlic, but not significantly different. At such concentrations, water extracts of shallot had significantly higher reducing activity than kyolic (8.06±0.15 %, 9.4±0.10 %, and 25.77±1.90 % at 100, 200 and 500 μg/ml, respectively), and significantly higher than Immunytop at 200 and 500 μg/ml (36.41±0.67, 38.37±0.55, and 71.00±1.32 %, respectively).

Initial rates of reduction of the $ABTS^{\bullet+}$ radicals

The initial rates of reduction (ΔA/min) of other antioxidants (Trolox, BHT and gallic acid) are shown in Figure 29. The results showed the linearity ($r > 0.99$) between initial rate of reduction (ΔA/min) and BHT concentration.

The initial rates of reduction were compared at various concentrations, 200, 500, and 1000 μg/ml. This initial rate of reduction at 200 and 500 μg/ml showed the potential significant difference in the distilled water extract of shallot (0.052±0.0012, and 0.115±0.0047, respectively), compared with extract of garlic crude (0.024±0.0021, and 0.056±0.0026, respectively), water extract (0.032±0.0017, and 0.065±0.0006, respectively), and hexane (0.027±0.0029, and 0.064±0.0051, respectively). The hexane extracts of shallot also had a significant higher initial rate of reduction (0.050±0.0065, and 0.111±0.0048, respectively) compared with hexane extracts of garlic significantly (Fig. 30).

Shallot extracts from both distilled water and hexane demonstrated a significantly higher initial rate than Kyolic (0.025±0.0029, 0.012±0.0014, and 0.005±0.0026, respectively) at 500, 200, and 100 μg/ml.

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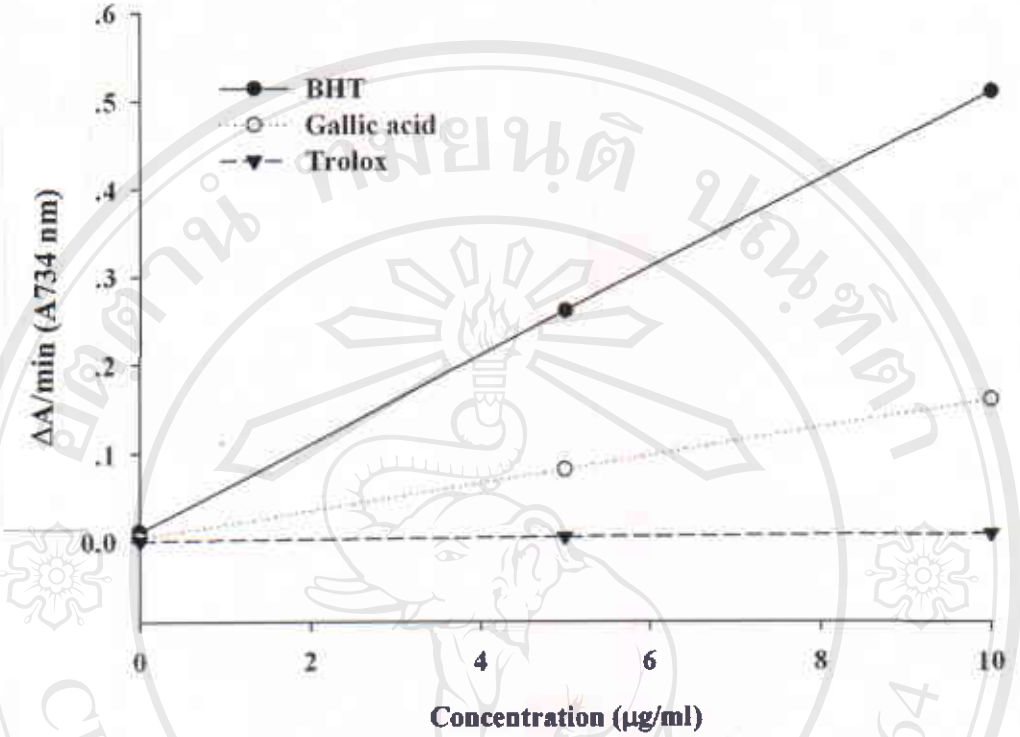

Figure 29. The initial rates of $ABTS^{\bullet+}$ reduction by standard BHT, gallic acid, and Trolox. The initial rates of $ABTS^{\bullet+}$ reduction (ΔA/min)) of standard antioxidants BHT, Trolox and gallic acid at 0-10 μg/ml was evaluated with kinetic program. Each point represents the mean and standard deviation from triplicate samples.

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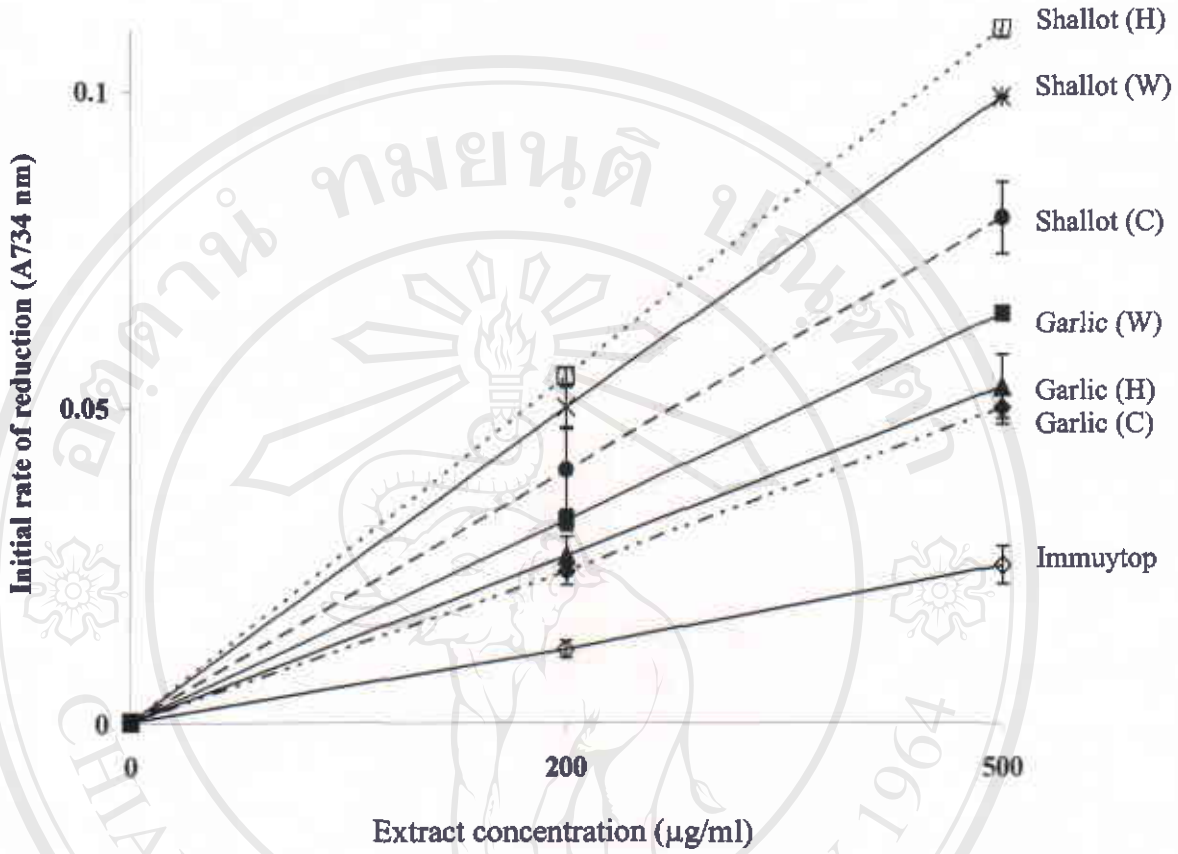

Figure 30. The initial rates of reduction by shallot and garlic extracts. The initial rates of reduction (A734 nm) of shallot and garlic extracts (C = crude, W = water, H = hexane extaction) at 50, 100, 200, and 500 µg/ml were recorded. Initial rate of reduction between 0.05 and 0.1 from all extracts were preferred to compare the initial rates at 0.05 and 0.1 of BHT, Trolox, or gallic acid. Each point represents the mean and standard deviation from triplicates samples.

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The antioxidant content in shallot and garlic extracts were analyzed by comparing the initial rate of reduction (ΔA/min) at 0.1 ΔA/min between extracts and standard antioxidant; BHT, gallic acid, and Trolox. The initial rate of reduction (ΔA/min) at 0.1 ΔA/min of BHT, gallic acid and Trolox were 0.0019 mg, 0.616 mg and 0.115 mg, respectively.

Comparison of the weight of each extract to the standard antioxidants BHT, gallic acid, Trolox at 1 mg (Table 4) indicated that hexane extract of shallot had the lowest necessary amount of dry weight, or highest amount of antioxidant (Table 4).

Table 4. Comparison of the antioxidant content in shallot, garlic, and commercial garlic products. The amount of shallot and garlic extracts (mg) that yielded an equal initial rate to 1 mg of BHT, gallic acid and Trolox. Each value was calculated from triplicate trials of initial rate of reduction (ΔA/min) from each extract.

	BHT	Gallic acid	Trolox
Crude garlic (mg)	292±2.3	4340±23	1531±23
Distilled water extract of garlic (mg)	278±2.2	4143±22	1462±22
Hexane extract of garlic (mg)	165±3.1	2457±31	867±31
Crude shallot (mg)	142±1.9	2123±19	749±19
Distilled water extract of shallot (mg)	121±2.2	1806±22	673±22
Hexane extract of shallot (mg)	85±1.5	1272±15	449±15
Immunytop (mg)	158±2.5	2349±25	829±25
Kyolic (mg)	540±2.4	8037±24	2836±24

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3.3. Effect of shallot and garlic extracts on scavenging H_2O_2

The results showed the antioxidant activity of shallot extract on scavenging the H_2O_2 at 16 μM directly. Hexane extract of shallot showed the highest activity on scavenging H_2O_2 compared with garlic extracts either at 100 or 200 μg/ml (Fig. 31). This activity also demonstrated with dose response from 20-200 μg/ml in shallot and

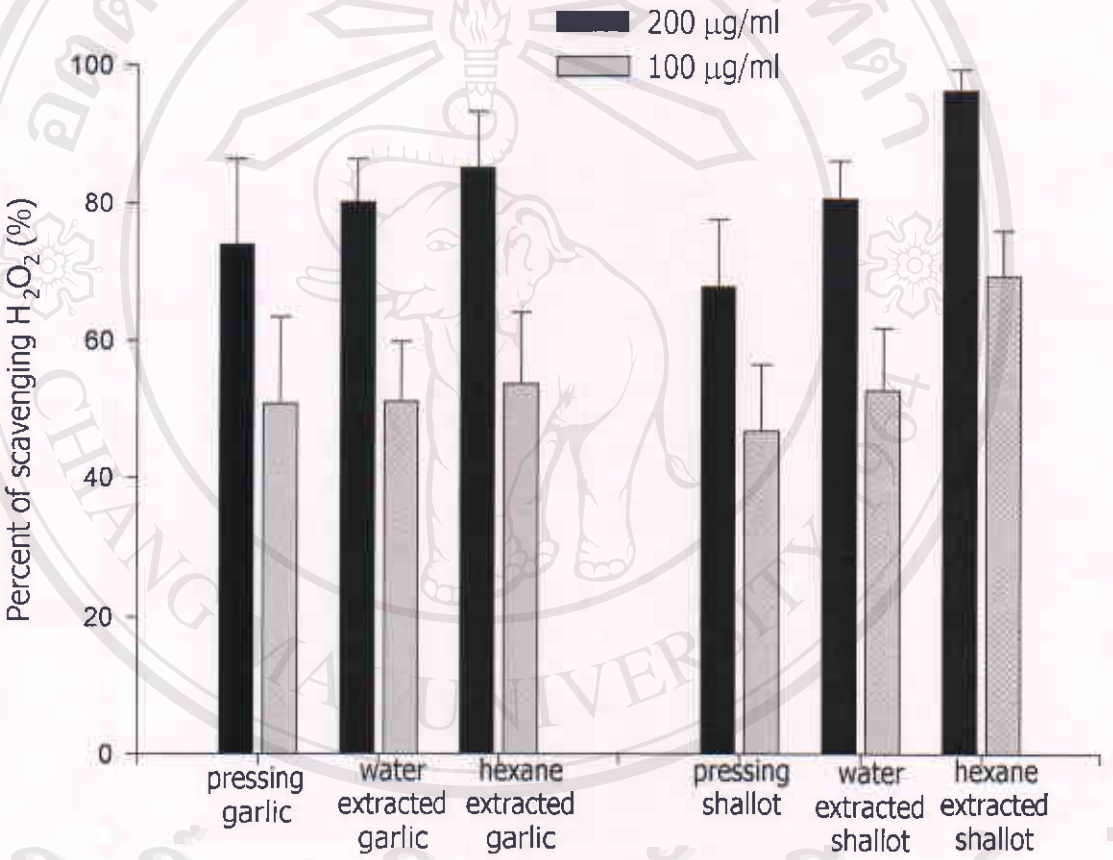

Figure 31. The antioxidant capacity on scavenging H_2O_2 between shallot and garlic extracts at 100 and 200 μg/ml. Extracts at 200 and 100 μg/ml were incubated in 16 μM H_2O_2, 10 U/ml of peroxidase and 0.1% ABTS solution were then added. The absorbance at 734 nm was read. Each bar represents the mean±SD from 10 repeated trials (p<0.05) compared at the same concentration.

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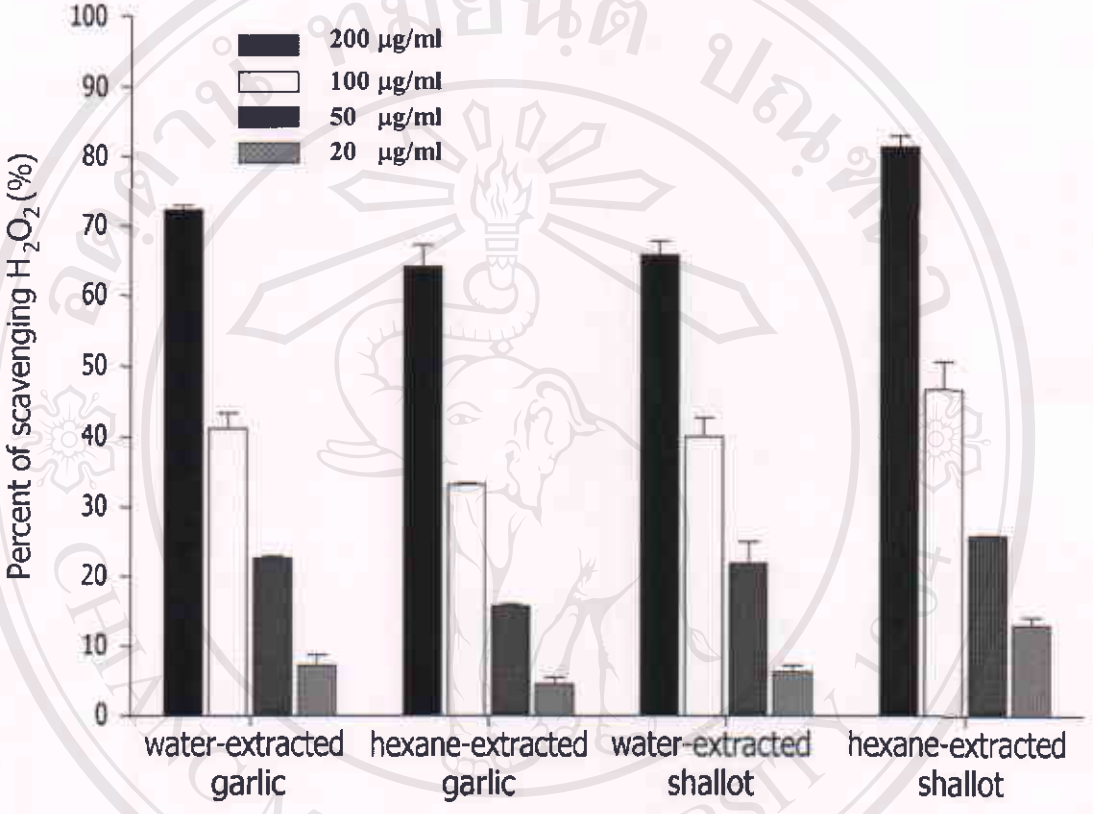

Figure 32. The antioxidant capacity on scavenging H_2O_2 between shallot and garlic extracts at 20-200 µg/ml. Extracts at 20, 50, 100, and 200 µg/ml were incubated in 16 µM H_2O_2, then 10 U/ml of peroxidase and 0.1%ABTS solution were then added. The absorbance at 734 nm was read. Each bar represents the mean±SD from 10 repeated trials (p<0.05) compared at the same concentration.

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3.4. Effects of shallot and garlic extracts on protein and lipid hydroperoxide

3.4.1. Effect on protein hydroperoxide formation

Shallot or garlic extracts at 0.5 and 1.0 mg/ml reduced the BSA hydroperoxide resulted from gamma irradiation (Fig. 33). All extracts gaven lower percentage of BSA hydroperoxide compared with control (BSA alone) after the irradiation. Shallot extract with distilled water had a higher percentage of BSA hydroperoxide than garlic from distilled water, but a lower percentage than shallot and garlic from hexane.

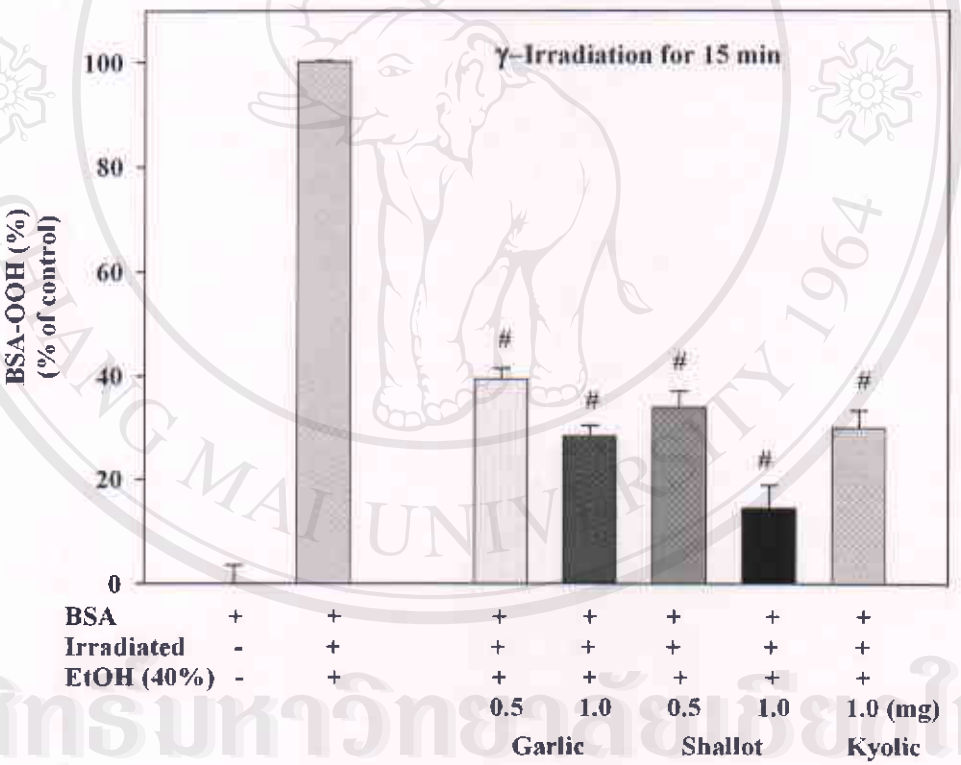

Figure 33. Effect of shallot and garlic extracts on inhibition of the protein hydroperoxide formation. The percentage of BSA-hydroperoxide (%) in mixtures of garlic or shallot extracts and BSA (5 mg/ml) after being irradiated for 15 minutes as detected by tri-iodide method. Each measurement was calculated from triplicate samples. (Note EtOH= ethanol, BSA = bovine serum albumin, and commercial garlic product from New Zealand, Kyolic, $^{\#}p < 0.01$ compared with BSA control present with EtOH and irradiation)

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For scavenging activity of shallot extract showed higher in water extract either at 30 or 120 minutes, but was not different from other extracts (Fig. 34) compared to the BSA-OOH control. NAC at 0.26 mM was also used as a referent standard and showed the positive antioxidant activity significantly ($p< 0.05$) on scavenging BSA-OOH in system.

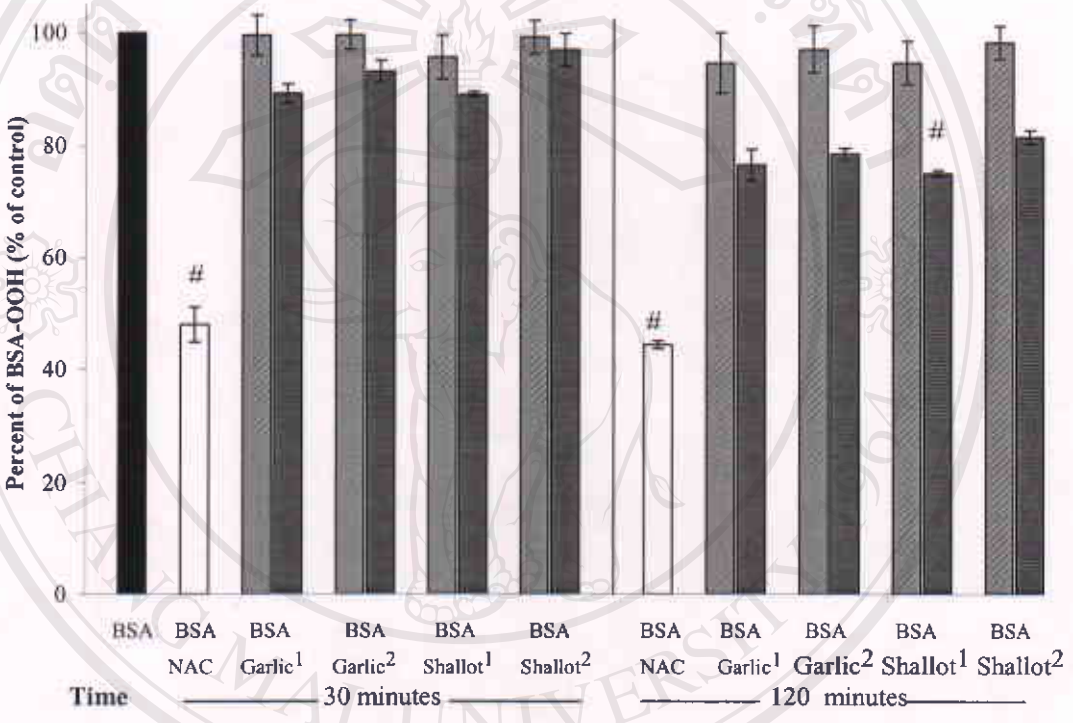

Figure 34. Effect of shallot and garlic extracts on reduction of the protein hydroperoxide. The percentage of BSA-hydroperoxide remaining after irradiation for 15 minutes addition of garlic or shallot extracts (1 = distilled water extraction, and 2 = hexane extraction) at 0.5 mg/ml ( ) and 1.0 mg/ml ( ) for 30 and 120 minutes of incubation, compared with BSA control (100%) ( ) and N-acetylcysteine (NAC) at 0.26 mM ( ). The mean and standard deviation was calculated from triplicate trials ($^{\#}p<0.01$ compared with BSA control and other extracts at the same concentration).

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Shallot activity on scavenging BSA-OOH responded in a dose dependent manner by comparing the percentage of remaining of BSA-OOH in extract-treated BSA-OOH. Result showed that the percentage of remaining of BSA-OOH reduced from 100% to 83% after ebing incubated with shallot extract at 0-1 mg/ml and still reduced until 2.1 mg/ml (Fig. 35).

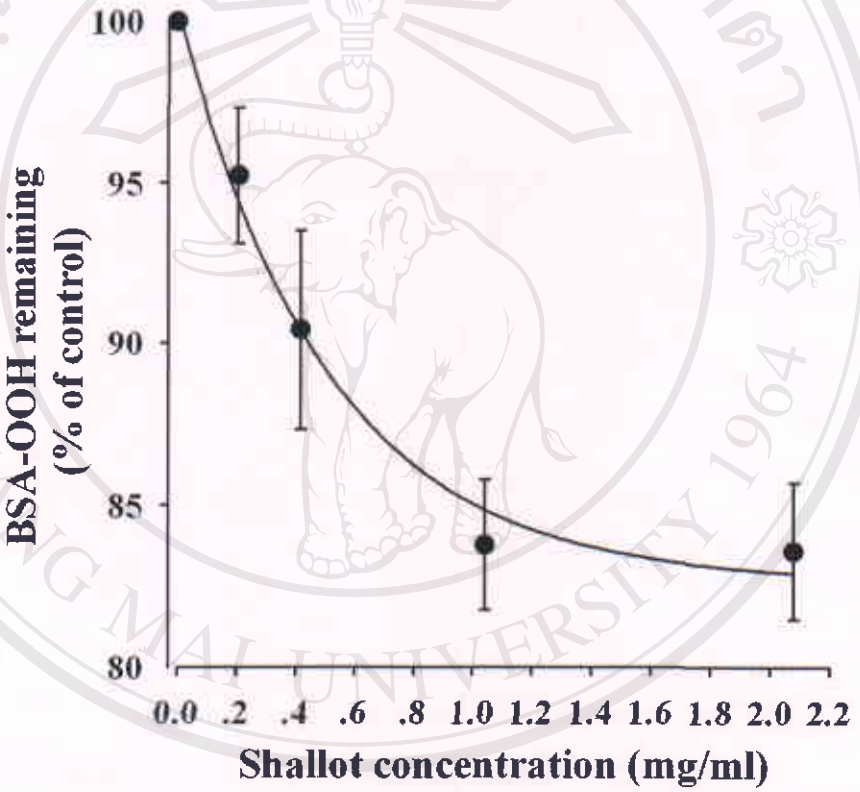

Figure 35. Effect of water extract of shallot at various concentrations on reduction of the protein hydroperoxide Shallot extracts at 0-2.1 mg/ml were incubated for 20 min in protein hydroperoxide generated by γ-irradiation for 10 minutes. Each point represents the mean and standard deviation of the percentage of remaining the BSA-hydroperoxide from triplicate samples compared to untreated BSA-OOH.

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3.4.2. Effect on lipid hydroperoxide formation

Shallot extract from hexane at 24 μg/ml inhibited the lipid hydroperoxide from irradiated linoleic acid in ethanol compared with all garlic extracts or shallot extracts both crude or water extraction (Fig. 36).

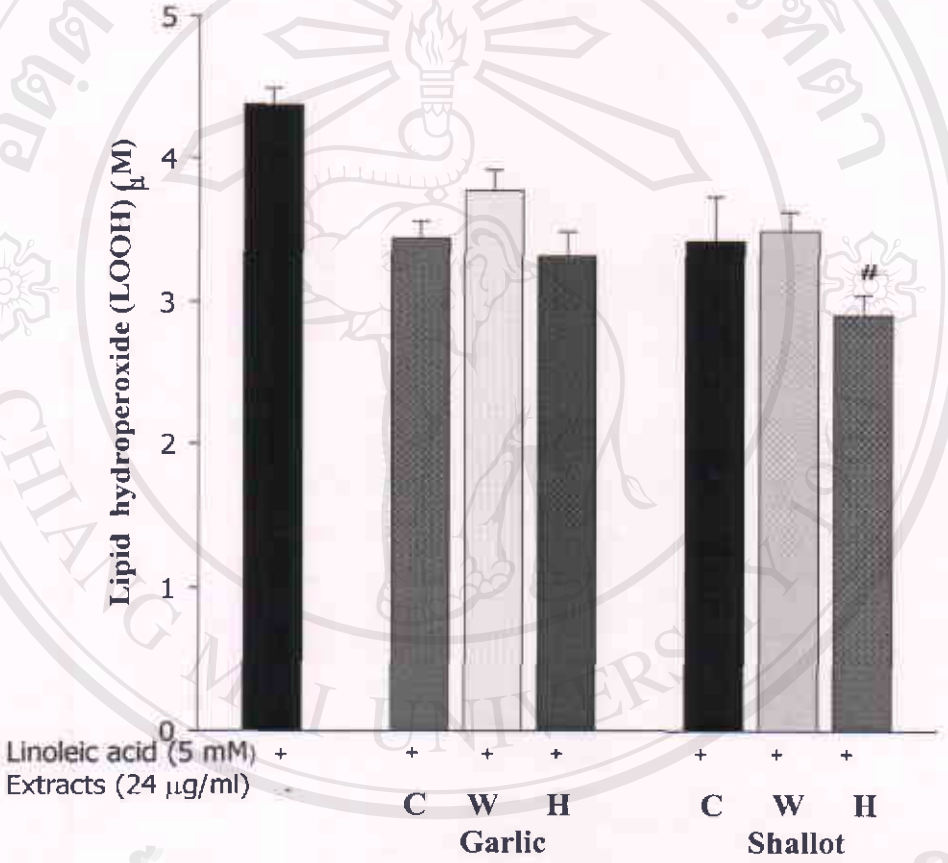

Figure 36. Effect of shallot and garlic extracts on inhibition of the lipid hydroperoxide formation. The level of lipid hydroperoxide formation was determined from irradiated ethanol-water (1:1, *v*:*v*) solution in linoleic acid (5 mM) for 10 minutes (C = crude, W = water, H = hexane). The mean and standard deviation from triplicate samples are shown ($^{\#}p$ <0.01 compared with control).

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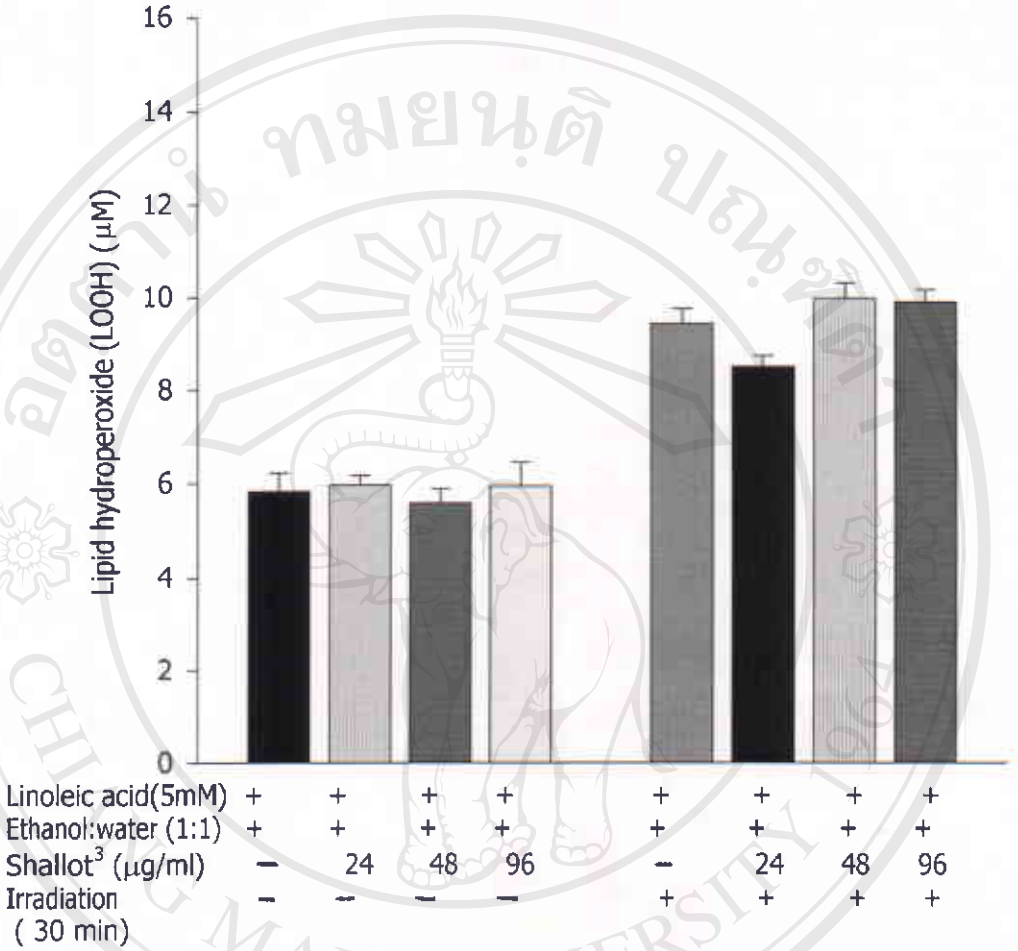

Figure 37. Effect of hexane extract of shallot on inhibition of the lipid hydroperoxide formation. The level of lipid hydroperoxide formation was determined from ethanolic:water (1:1, *v*:*v*) solution in linoleic acid (5 mM) for 30 minutes in irradiated and non-irradiated hexane extract of shallot (Shallot[3]) at 24, 48, 96 μg/ml. The mean and standard deviations from triplicate samples are shown ($^{\#}p$ <0.01 compared with control).

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Lipid hydroperoxide from irradiated liposomes

The activity of shallot extracts in liposome is shown in Figure 38. All extracts at 0.5 or 1.0 mg/ml gave significantly a lower percentage of remaining lipid hydroperoxide compared with control (liposome alone).

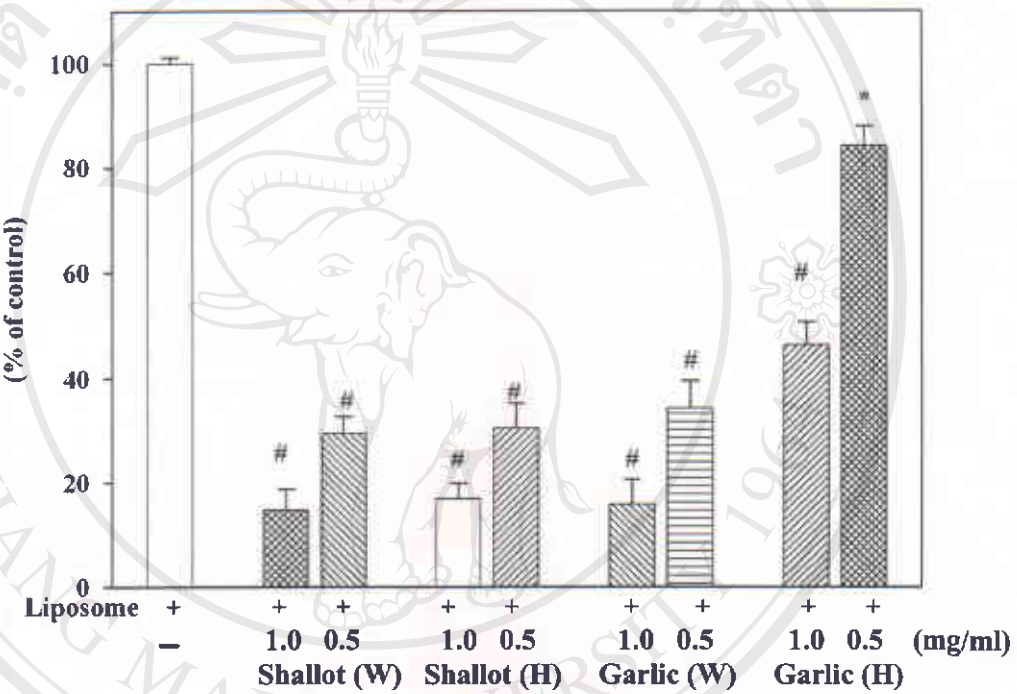

Figure 38. Effect of shallot and garlic extracts on reduction of the lipid hydroperoxide. The percentage of remaining of lipid hydroperoxide (LOOH) from *liposome* treated with garlic or shallot extracts at 1.0 mg/ml and 0.5 mg/ml as compared to control, liposome alone (100%) after irradiation for 10 min. Mean and standard deviations from triplicate samples are shown. (Note: W = distilled water, H = hexane extraction. $^{\#}p < 0.01$, $^{*}p < 0.05$ compared with liposome control)

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Inhibition in liposomes from auto-oxidation.

The activity of garlic and shallot extracts on inhibition of lipid peroxidation in liposome from auto-oxidation is shown in Figure 39.

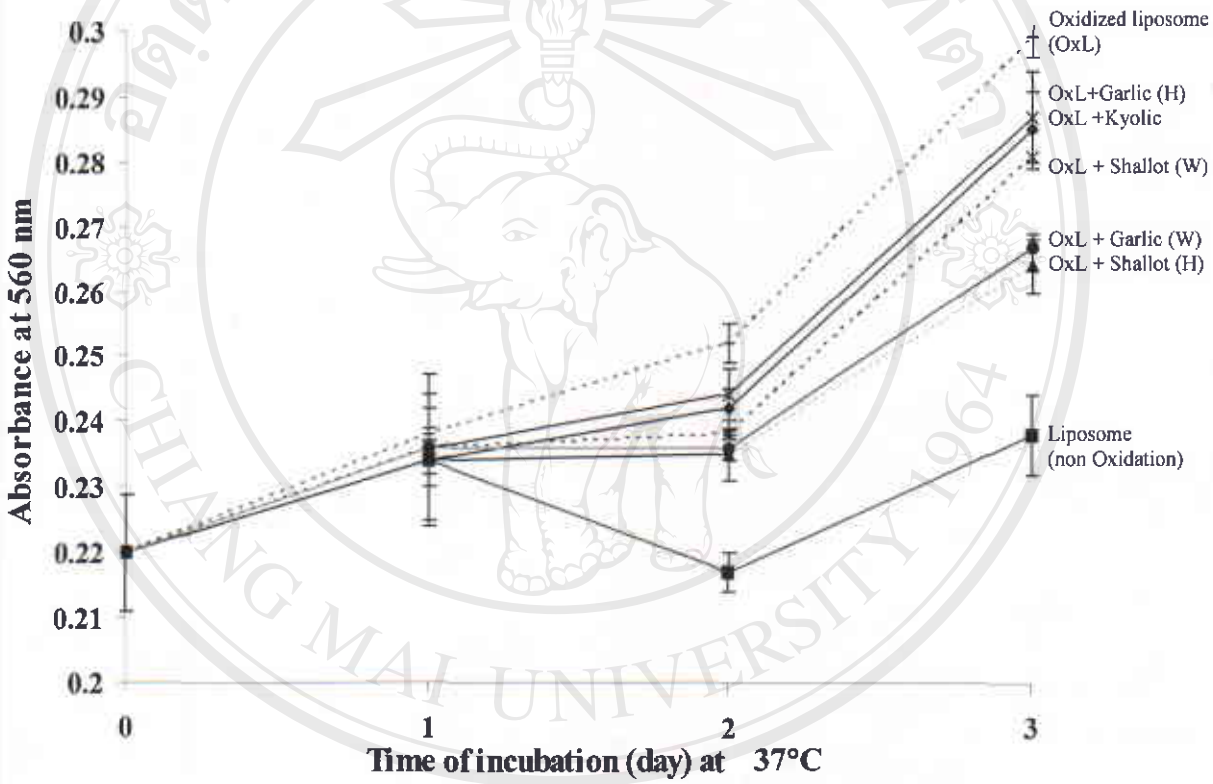

Figure 39. Effect of shallot and garlic extracts, and commercial garlic products on inhibition of the lipid hydroperoxide from auto-oxidation. The inhibition of lipid hydroperoxide formation in shallot and garlic extracts (W = water, H = hexane) at 1 mg/ml in liposome (15 mg/ml) from auto-oxidation (OxL) at 37°C for three days. The lipid hydroperoxide formation was detected with tri-iodide assay at 560 nm. Each point represents the mean from triplicate samples.

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3.5. Effects of shallot extract on protection the proline and GSH from irradiation or lysozyme hydroperoxide

3.5.1. Effect of shallot extract on proline hydroperoxide

Water extract of shallot at 0.5 and 1.0 mg/ml were able to prevent proline hydroperoxide formation after irradiation for 15 minutes ($p<0.05$ at 0.5 mg/ml and $p<0.01$ at 1.0 mg/ml) compared with controls (Fig. 40). Moreover, the Thai shallot at 0.25, 0.5 and 1.0 mg/ml significantly reduced proline hydroperoxide (20 mM) after irradiation at 30 or 120 seconds, compared with the absence of shallot in the control ($p<0.05$ at 0.25 and 0.5 mg/ml and $p<0.01$ at 1.0 mg/ml) (Fig. 41).

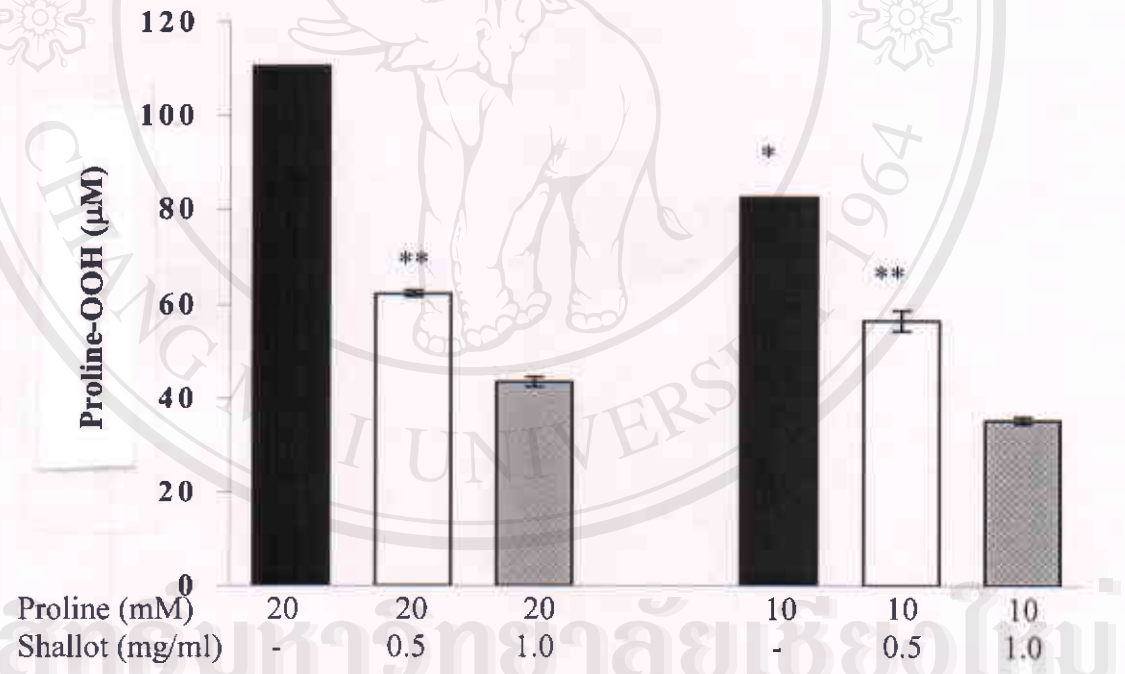

Figure 40. Effect of shallot extract on inhibition the proline hydroperoxide formation. The inhibition and reduction effects of distilled water extract of shallot on proline hydroperoxide from 20 min irradiation (a) incubated proline (20 and 10 mM) and shallot extract (0.5 or 1.0 mg/ml) before irradiation. Each bar is the mean and standard deviation from triplicate samples (*$p<0.05$, **$p<0.01$ compared with untreated proline).

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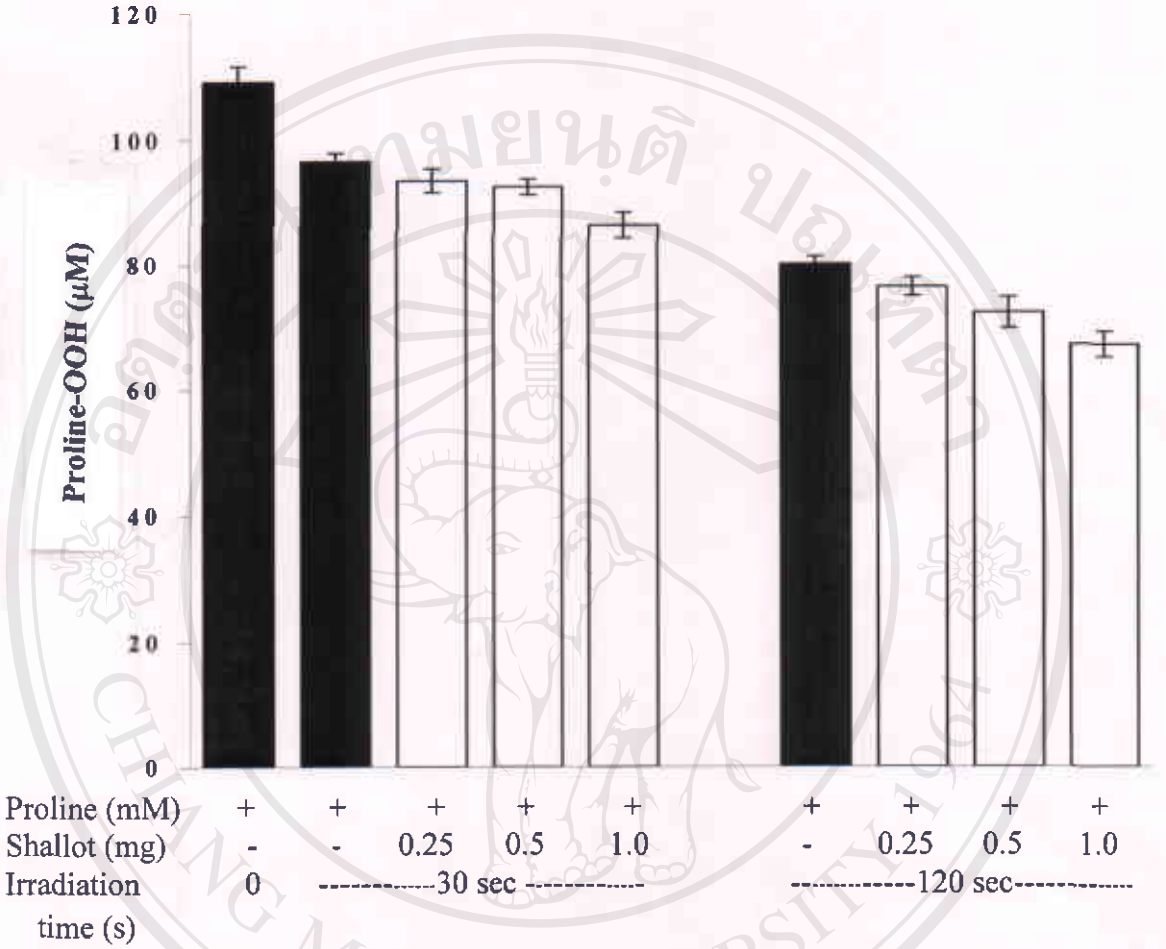

Figure 41. Effect of shallot extract on reduction the proline hydroperoxide. The reduction effect of distilled water extract of shallot at 0.25-1.0 mg/ml on proline hydroperoxide from hydroxyl radical generated by 15-minutes irradiation showed at 30 and 120 seconds after adding extracts. Each bar represents the mean and standard deviation from triplicate samples.

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3.5.2. Effect of shallot extract on protection of GSH from irradiation and lysozyme hydroperoxide

Irradiation of lysozyme produced protein hydroperoxide and directly affected GSH (20 mM). Irradiation with a ^{60}Co source was able to reduce GSH in a time-dependent manner (Fig. 42). Shallot extract (0.05-1.0 mg/ml) protected the GSH (20 mM) from lysozyme hydroperoxide and irradiation depending on the dosage (0.05-1.0 mg/ml) (Fig.43).

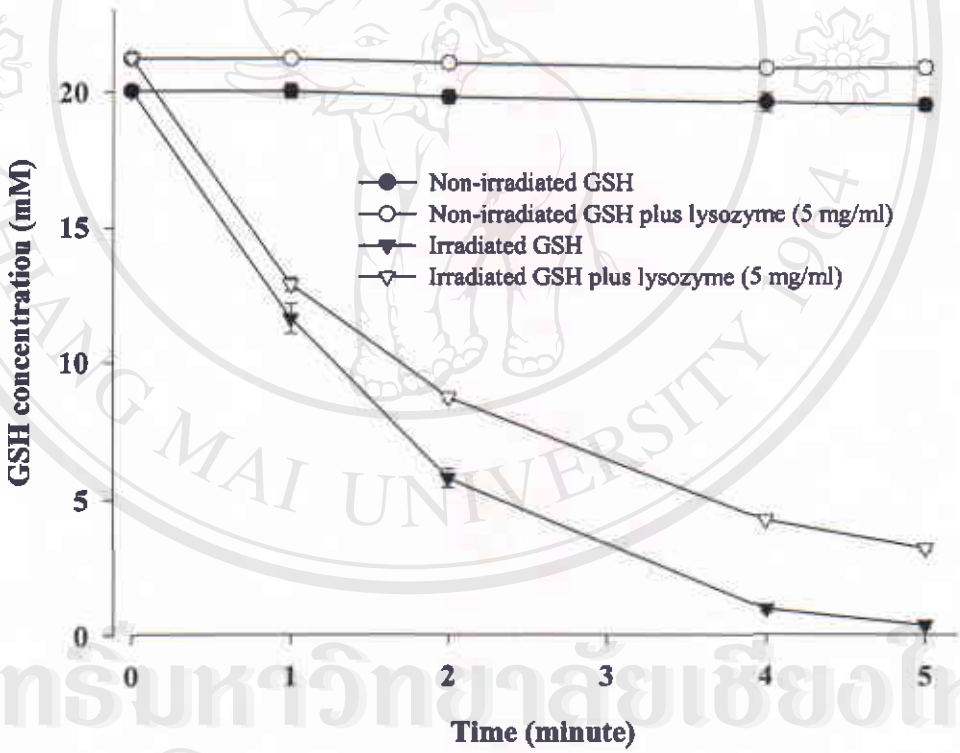

Figure 42. Effects of γ-irradiation on lysozyme and GSH mixture. The GSH levels from non-irradiation and irradiation of GSH (20 mM) plus lysozyme (5 mg/ml) for 5 minutes. Each point represents the mean and standard deviation from triplicate samples.

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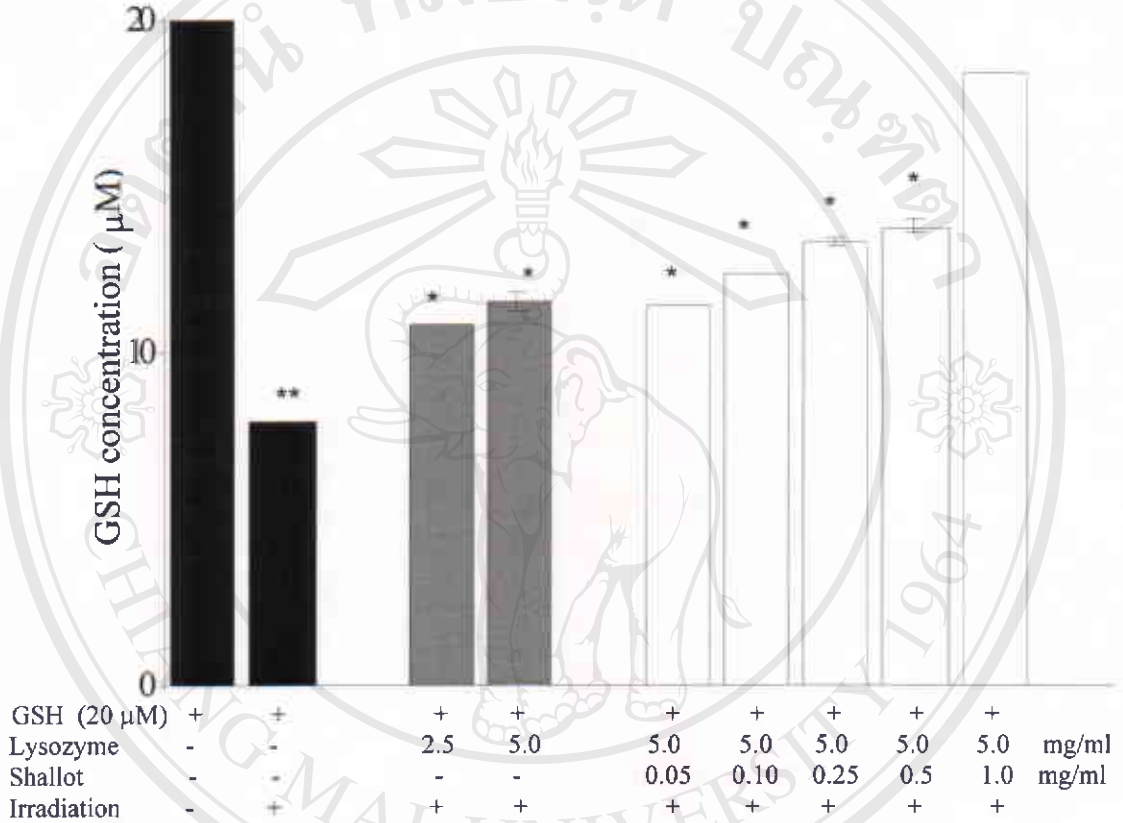

Figure 43. Effect of shallot extract at various concentrations on protection of GSH depletion from irradiated lysozyme. The levels of glutathione after irradiation mixtures of GSH (20 mM) and/or lysozyme (5 mg/ml) in the presence or absence of shallot extract (0.05 – 1.0 mg/ml) for 90 seconds. Each bar represents the mean and standard deviation from triplicate samples. (*p<0.05, **p <0.01 compared with untreated GSH control).

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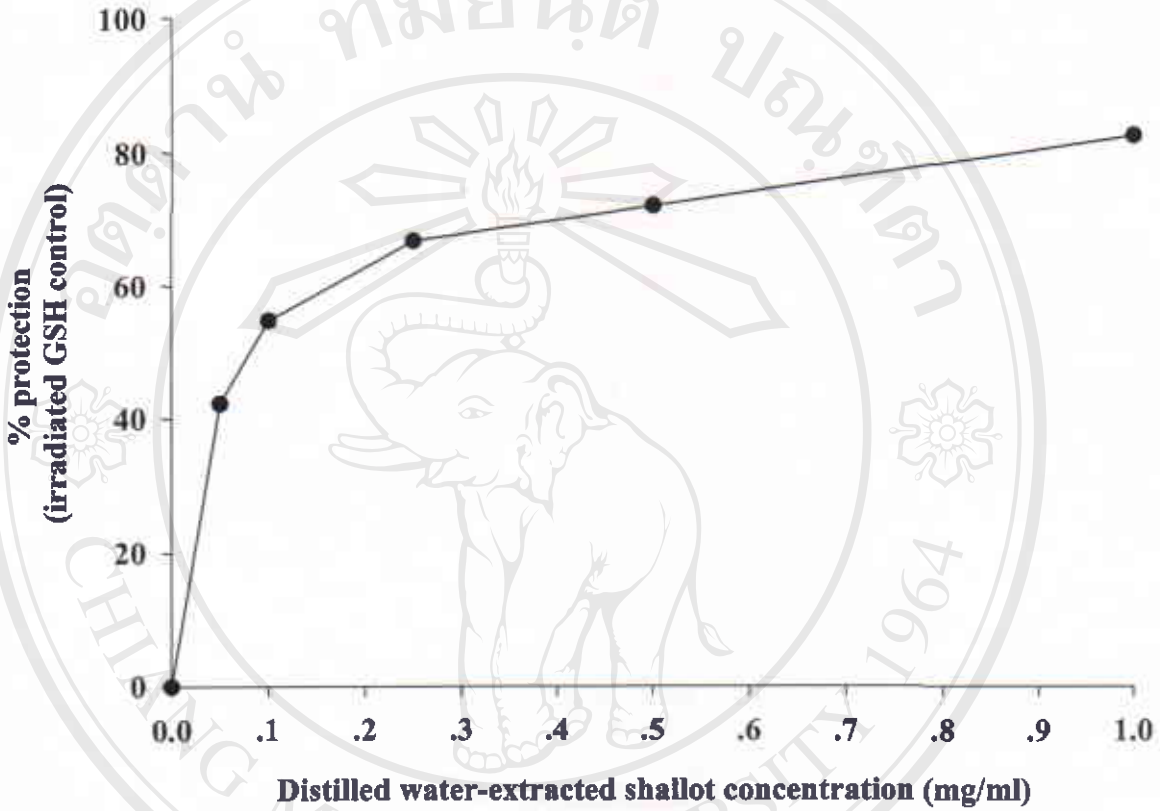

Figure 44. Effect of water extract of shallot on protection of GSH from irradiation. The percentage of protected glutathione, that was destroyed by irradiated lysozyme radicals in distilled water-extract shallot at 0.0-1.0 mg/ml, compared with no protection (irradiated GSH alone). Each point represents the mean and standard deviation from triplicate trials.

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3.6. Protective effects of shallot and garlic extracts on lipid peroxidation and glutathione depletion in AAPH-oxidized erythrocytes

Erythrocytes treated with 10 mM AAPH and incubated at 37°C for 6 h showed an increase in lipid peroxidation and a decrease in glutathione level. Shallot extracts (hexane extraction) at concentration of 100 and 200 μg/ml, significantly inhibited the lipid peroxidation (Fig. 46) and GSH depletion (Fig. 45).

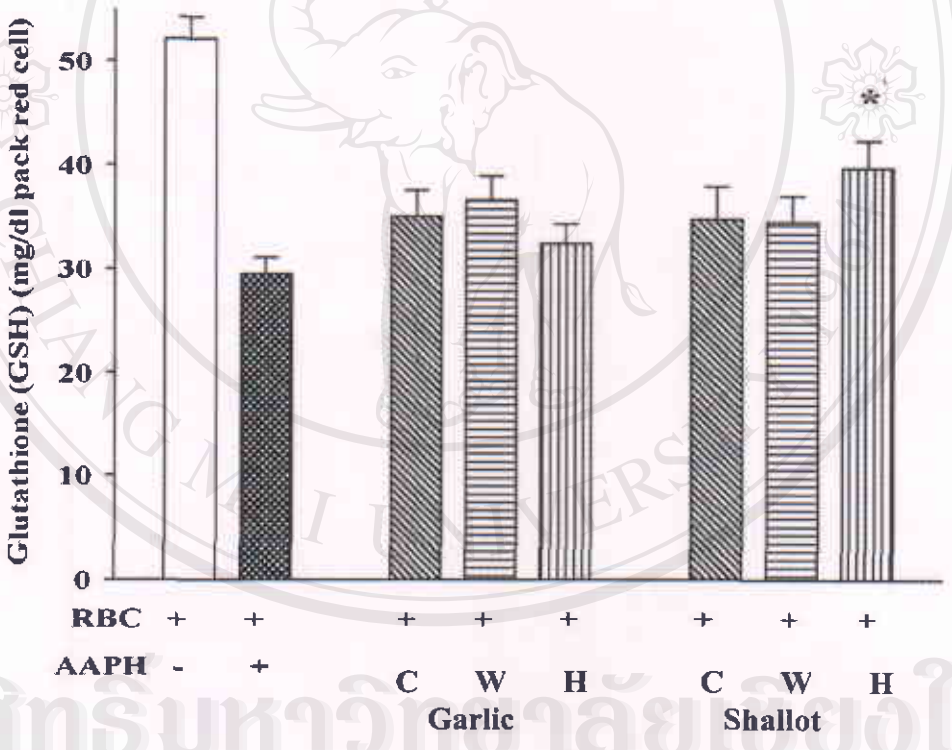

Figure 45. Effect of shallot and garlic extracts on protection of the GSH from AAPH oxidation in red blood cells. Red blood cells were treated with 200 μg/ml of all extracts (C = crude, W = water, H = hexane) in present of 10 mM AAPH at 37°C for 6 hr. Erythrocyte GSH was detected with DTNB reagent. Each bar represents the mean and standard deviation from five samples. (*p< 0.05 compared with other shallot and garlic extracts).

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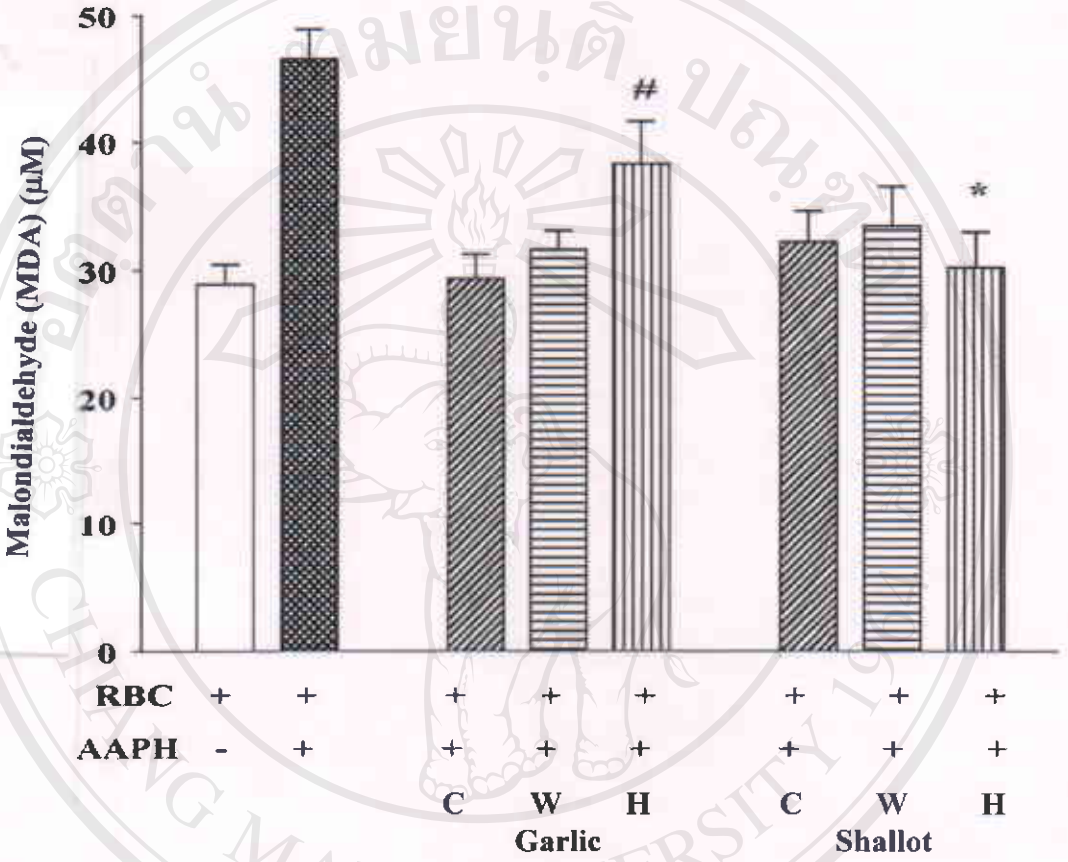

Figure 46. Effect of shallot and garlic extracts on lipid peroxidation in red blood cells. Whole blood was treated with 200 μg/ml of all extracts (C = crude, W = water, H =- hexane) in present of 10 mM AAPH at 37°C for 6 hr. The plasma was separated for measurement of malondialdehyde concentration with TBARs assay. Each bar represents the mean and standard deviation from five samples. ($^{*}p< 0.01$ and $^{\#}p< 0.05$ compared with control RBC treated with AAPH treatment).

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3.7. Bioactive compounds.

3.7.1. Phenolic compounds

Shallot extracts contained total phenolics more than garlic extracts and the garlic commercial products (Immunytop and Kyolic). Kyolic had one least amount of phenolic compounds (Fig. 47).

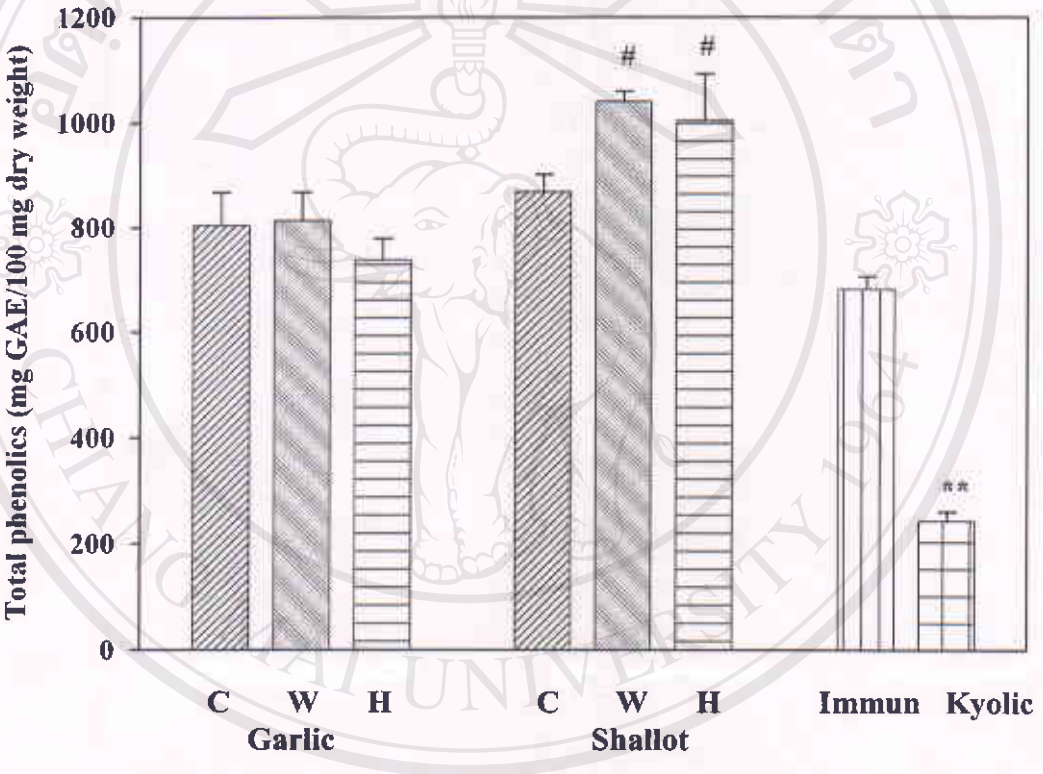

Figure 47. The total phenolic content in the garlic and shallot extracts. Ten mg of each extracts (C = crude, W = water extraction, H = hexane extraction) was hydrolyzed in 1.2 M of HCl and 50% MeOH by heating at 80°C for 2 h. Supernatant (0.1 ml) was taken and added 0.1 ml of Folin-Ciocalteau reagent and 0.5 ml of 20% Na_2CO_3. After incubation at room temperature for 20 minutes, absorbance was read at 765 nm. Total phenolics in 100 mg of dry extract was calculated by comparing to the gallic acid (mg) and presents in gallic acid equivalent (GAE). Each bar represents the mean±SD from five repeated analyses ($^{\#}p<0.05$ compared with garlic (W), and $^{**}p<0.01$ compared with shallot (W).

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Table 5. The total phenolics in shallot, garlic, and onion extracts. The one kilogram of onion, garlic, and shallot prepared as crude, water-, and hexane- extraction were analyzed for total phenolics with Folin-Ciocalteau reagent and reported in terms of gallic acid equivalent (GAE) in mg/kg concentration.

		Total phenolics (mg GAE/kg extract)
Onion	Crude	2,283±16
	Water	3,325±69
	Hexane	8,734±258
Garlic	Crude	4,102±193
	Water	3,804±77
	Hexane	5,477±33
Shallot	Crude	4,086±84
	Water	4,599±88
	Hexane	5,413±62
Immunytop		2,649±66
Kyolics		1,029±84

The result in table 5, showed that the lowest amount of phenolics was found in Kyolic (1,029±84 GAE mg/kg) and Immunytop (2,649±66 GAE mg/kg), and the highest in hexane extract of onion (8,734±258 GAE mg/kg). Comparative results between garlic and shallot showed that hexane extract of shallot (5,413±62 GAE mg/kg) contained total phenolics equal to hexane extract of garlic (5,477±33 GAE mg/kg). Comparison to the total phenolics in water extract showed that shallot contained more (4,599±88 GAE mg/kg) than that of garlic (3,804±77 GAE mg/kg) ($p<0.01$). In the crude extract, shallot (4,086±84 GAE mg/kg), contained nearly the same amount of phenolics as the garlic extract (4,102±193 GAE mg/kg).

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3.7.2. Organosulfur compounds

The standard diallyl disulfide showed mono-, di-, and trisulfide peaks at 5.6, 6.4, and 8.2 min, respectively (Fig. 48). Doses of each diallyl sulfide were correspondent to a concentration of standard diallyl disulfide compounds (See appendix H).

Figure 48. HPLC chromatogram of organosulfur compounds in the standard diallyl disulfide (DADS). The chromatogram showed three dominant peaks of diallyl disulfides (DADS), which were mono-sulfide (5.6 min), di-sulfide (6.4 min), and tri-sulfide (8.2 min). The signal was read at 240 nm. The elution was performed at with a mixture of acetonitrile:water:tetrahydrofuran (70:27:3, *v*:*v*:*v*) at 1.0 ml/min (0.10 AUFS).

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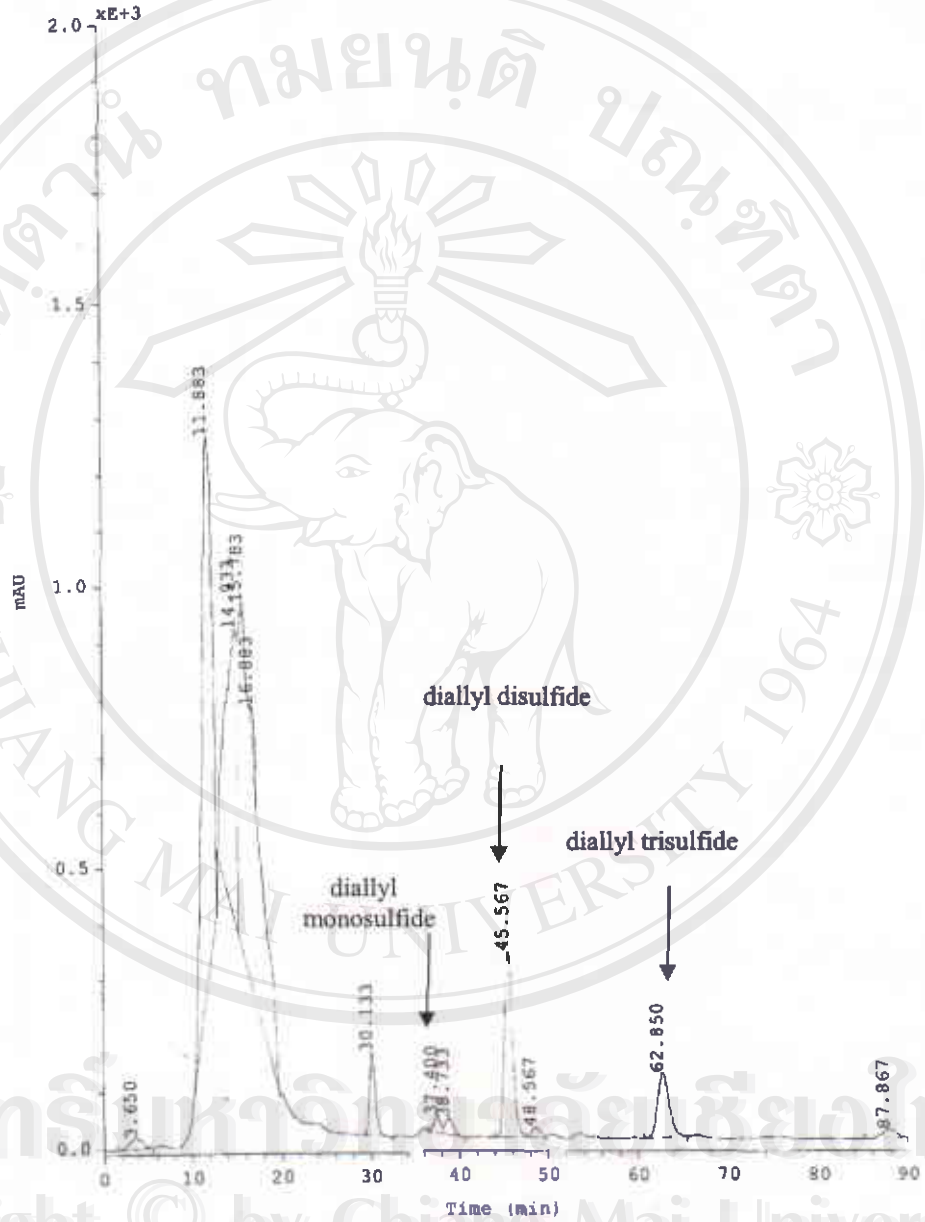

Figure 49. HPLC chromatogram of organosulfur compounds in the hexane extract of garlic. The chromatogram of diallyl sulfide from hexane extract of garlic showed three peaks of diallyl sulfides, which were diallyl mono-sulfide (38.733 min), di-sulfide (45.567 min), and tri-sulfide (62.85). The signal was read at 240 nm. The elution was performed with a mixture of acetonitrile:water:tetrahydrofuran (70:27:3, *v:v:v*) at 1.0 ml/min (0.10 AUFS).

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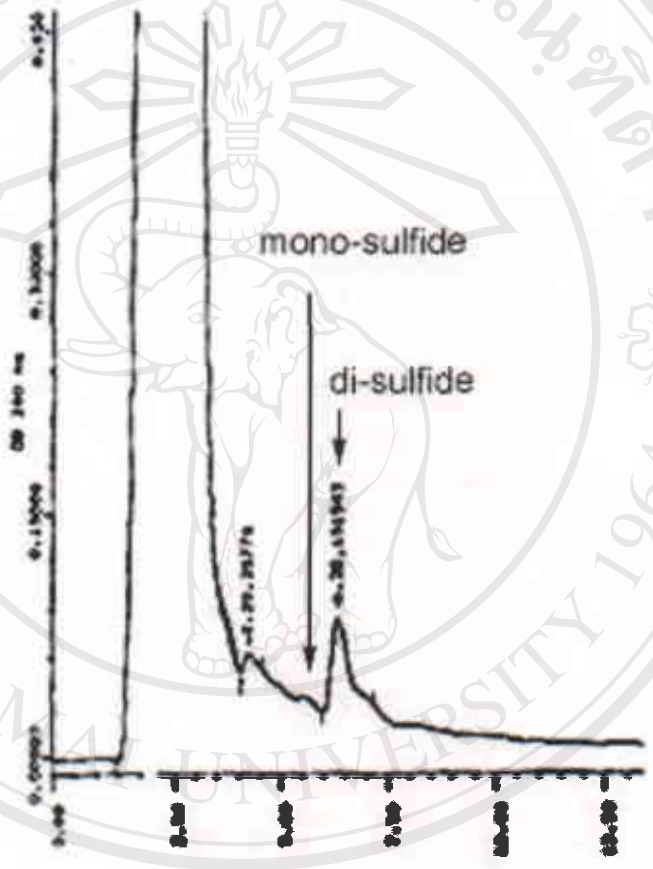

Figure 50. HPLC chromatogram of organosulfur compounds in the hexane extract of onion. The chromatogram of diallyl sulfide showed dominant peak of diallyl disulfide at 6.38 min. The signal was read at 240 nm. The signal was read at 240 nm. The elution was performed with a mixture of acetonitrile:water: tetrahydrofuran (70:27:3, *v:v:v*) at 1.0 ml/min (0.10 AUFS).

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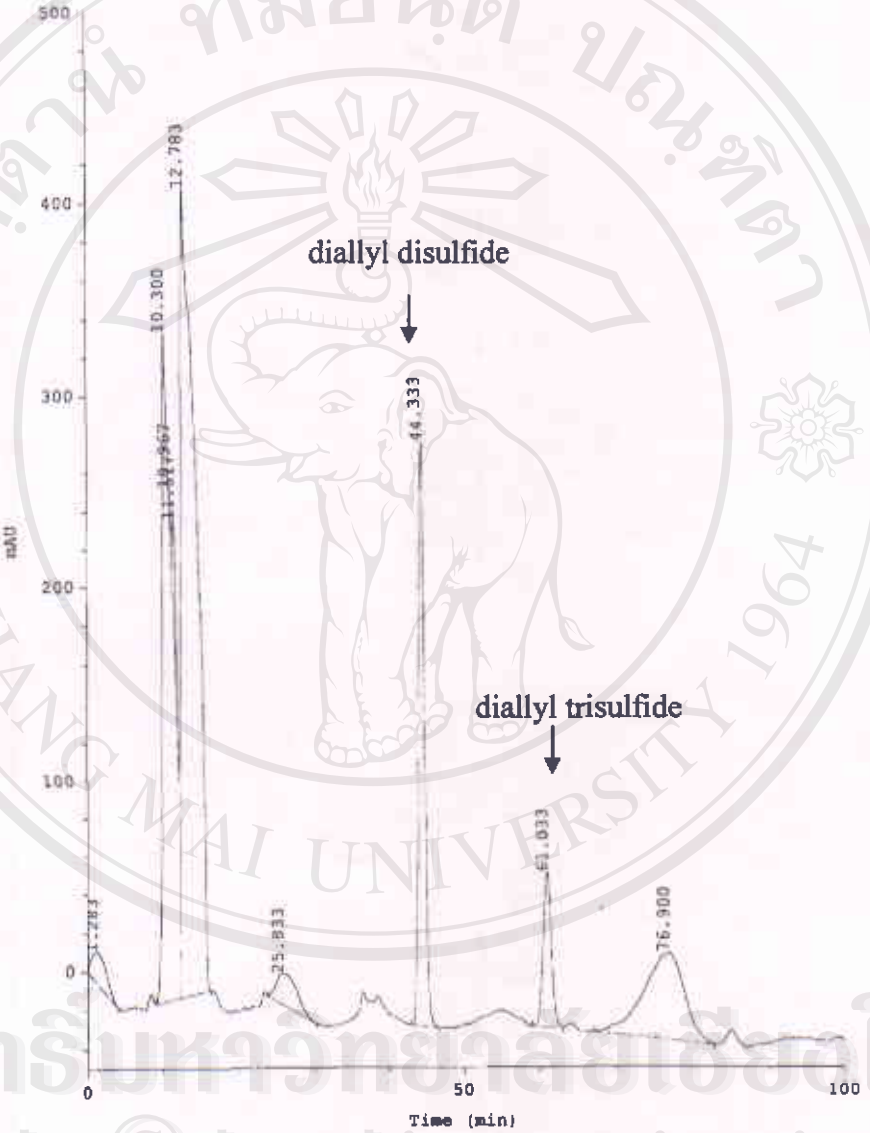

Figure 51. HPLC chromatogram of organosulfur compounds in the hexane extract of shallot. The chromatogram showed two dominant peaks of diallyl di-sulfide (44.33 min) and tri-sulfide (61.033 min). The signal was read at 240 nm. The signal was read at 240 nm. The elution was performed with a mixture of acetonitrile:water:tetrahydrofuran (70:27:3, *v:v:v*) at 1.0 ml/min (0.10 AUFS).

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Table 6. Diallyl sulfides in shallot , garlic, and onion extracts. Comparison of the concentration of diallyl sulfide compounds in standard diallyl sulfide (DADS), onion, garlic, and shallot extracts from hexane preparation at 1 gram extract.

	Total concentration (μg/g extract)	**Sulfur compounds**	**Concentration (μg/ml)**
Onion	1,864±32.3	Diallyl mono-sulfide	55.92±2.25
		Diallyl di-sulfide	1,808.08±10.25
		Diallyl tri-sulfide	-
		Diallyl tetra-sulfide	-
Garlic	283.5±28.12	Diallyl mono-sulfide	158.13±5.25
		Diallyl di-sulfide	29.88±2.31
		Diallyl tri-sulfide	95.45±5.54
		Diallyl tetra-sulfide	-
Shallot	325.50±42.17	Diallyl mono-sulfide	-
		Diallyl di-sulfide	248.38±6.25
		Diallyl tri-sulfide	77.09±4.25
		Diallyl tetra-sulfide	-

Table 5, showed the dominated concentration of diallyl disulfide (248.38±6.25 μg/1 g extract) in shallot extract and higher than that in garlic extract (29.88±2.31 μg/ 1 g extract).

The onion, garlic and shallot lyophilized extracts that were prepared by crude and water extract did not yield peaks of allyl sulfide, whereas lyophilized extracts from hexane extraction showed the mono-, di-, tri-sulfides peaks in garlic, onion, and shallot.

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3.7.3. Allyl-2 propenethiosulfinate (Allicin)

Futuremore, standard immunytop contained allicin which was shown by HPLC chromatogram (Fig. 52). Fresh onion and garlic juices also contained allicin (Fig. 53).

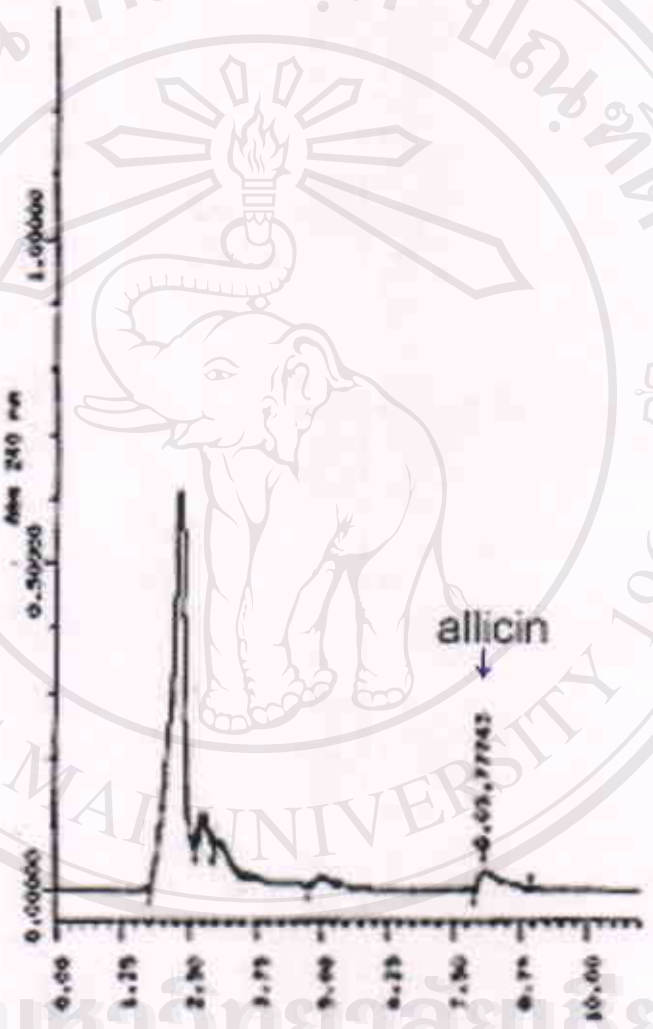

Figure 52. HPLC chromatogram of allicin in commercial garlic product (Immunytop). The chromatogram of allicin (allyl-2-propenethiosulfinate) was obtained from 20 µl of Immunytop (a stock solution at 40 mg/ml). The peak of allicin was eluted at 8.05 min. The signal was read at 240 nm. The elution was performed with 50% methanol at 1.0 ml/min (0.10 AUFS).

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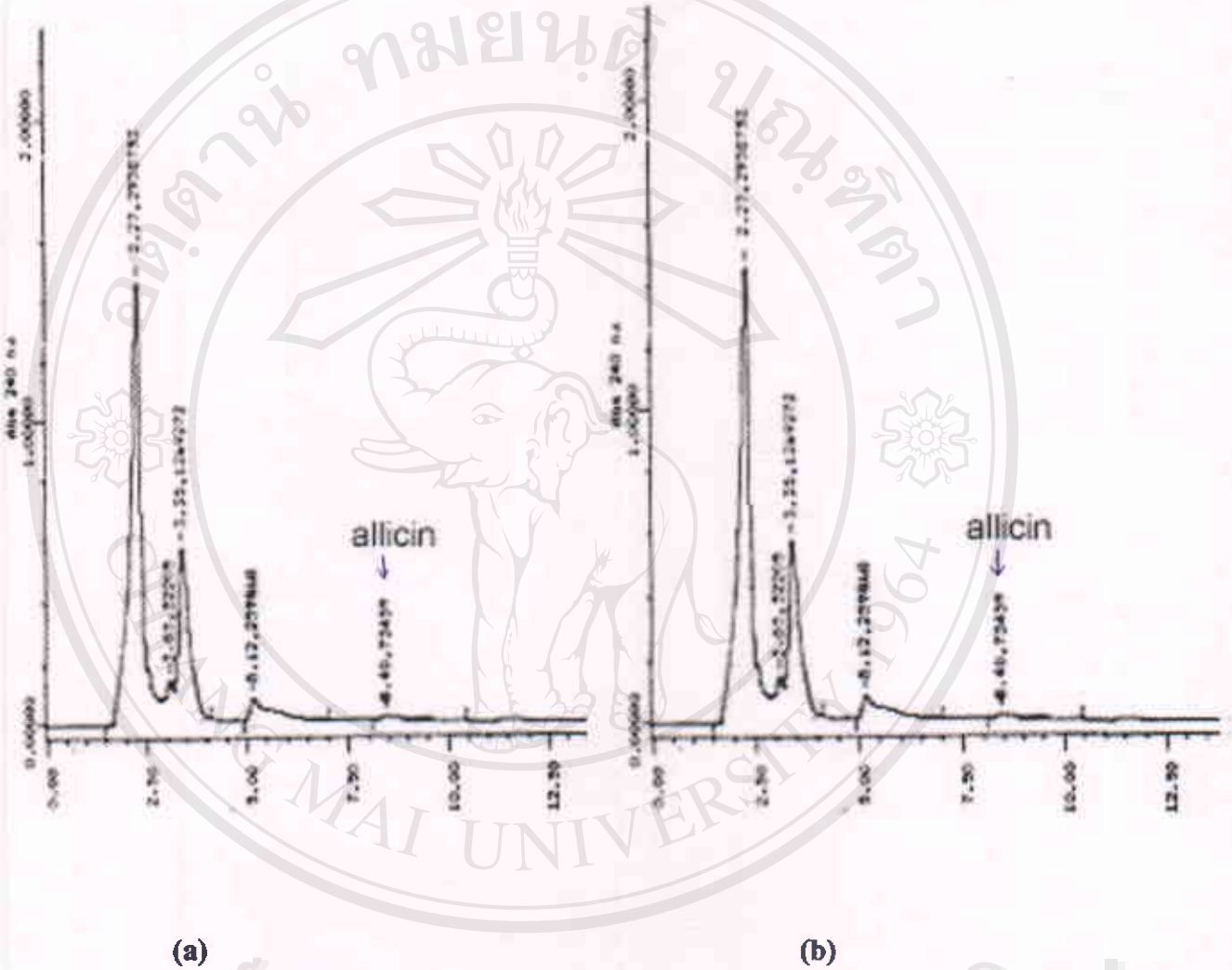

(a) (b)

Figure 53. HPLC chromatograms of allicin in fresh onion and garlic. The chromatogram of allicin (allyl-2 propenethiosulfinate) peak from fresh onion juice (a) and garlic (b) were at 8.55 and 8.68 min. The signal was read at 240 nm. The elution was performed with 50% methanol at 1.0 ml/min (0.10 AUFS).

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Table 7. Comparison of the *allicin contents between onion, garlic, and shallot* extracts. The allyl-2 propenethiosulfinate (allicin) in onion, shallot, and garlic compared with commercial garlic preparations: Immunytop and Kyolic.

	Extractant	Mean±S.D
Onion extracts (1g)	Crude	1,909±373
	Water	1,628±108
	Hexane	ND
Garlic extracts (1g)	Crude	ND
	Water	ND
	Hexane	ND
Shallot extracts (1 g)	Crude	ND
	Water	ND
	Hexane	ND
Immunytop (1 mg)		9±2
Kyolics (1 mg)		ND

ND = non detectable

In table 7, this showed the contents of allyl-2 propenethiosulfinates (allicin) in the extracts that were found only in fresh onion and garlic juices and onion extracts comparisions with the allicin peaks from commercial garlic preparasions (Immunytop, Thailand) (3,400 μg/370 mg per capsule) (shown in Table 7). Here, the peak of allicin eluted at 8.05 min and similar to the one assayed by Roson and colleagues (Rosen *et al*, 2001) that acetonitrile plus water (30:70, *v:v*) was used a mobile phase and the detection was done at 195 nm. Allicin was present in onion crude (1,909±373 μg/g of lyophilized extract) as well as water extract (1,628±108 μg/g lyophilized powder), but was not found in any shallot or garlic extracts.

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3.7.4. The relative antioxidant capacity of shallot extracts and their bioactive compounds

Regression analysis of antioxidant activity as a function of the amount of bioactive compound showed stronger correlation between initial rate of reduction and total phenolic content (Fig. 54).

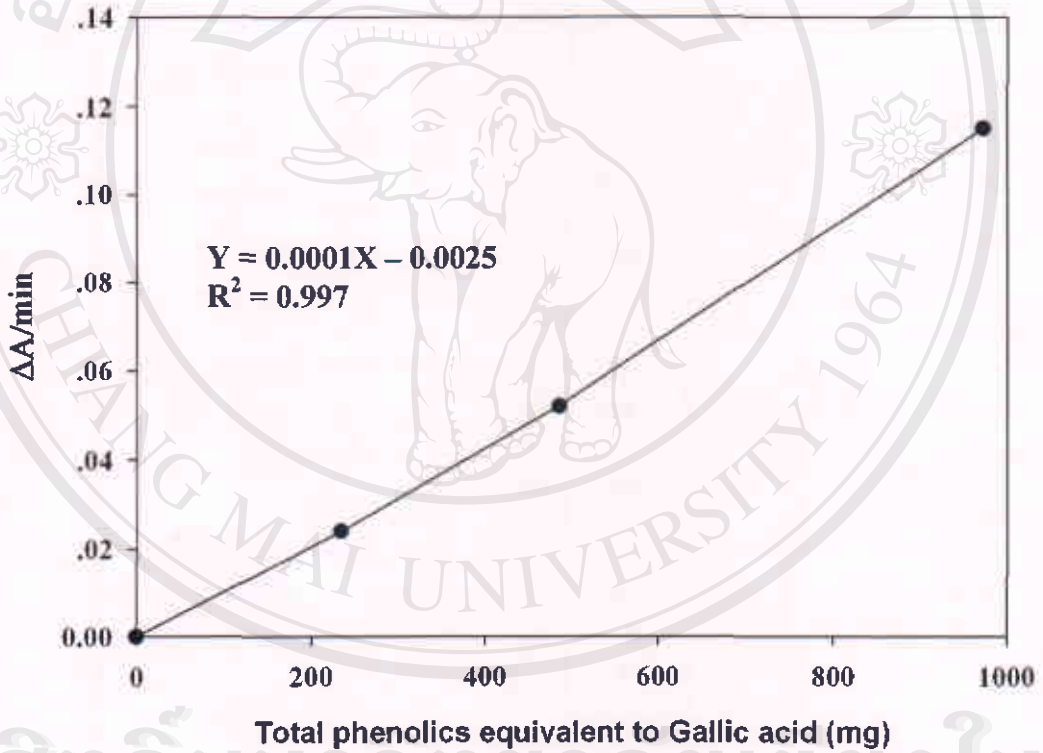

Figure 54. The correlation between antioxidant capacity and total phenolics. Linear regression of the initial rate of reduction of ABTS radical cation as a function of total phenolic content in water extract of shallot (R^2= 0.997). The initial rate of reduction at 743 nm was evaluated and total phenolics were determined using a Folin-Ciocalteau reagent. Each point represents the mean and standard deviation from triplicate samples.

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3.8. Effects of shallot and garlic extracts on oxidative stress in U937 cell line

3.8.1. Effects of shallot and garlic extracts on cell survival

The percentage of cell survival in MTT assay with addition of shallot extracts at 250, 150 and 50 μg/ml in U937 cells were 80, 90 and 95%, respectively (Fig. 56). With garlic extracts, they were 70%, 80% and 95%, respectively (Fig. 55).

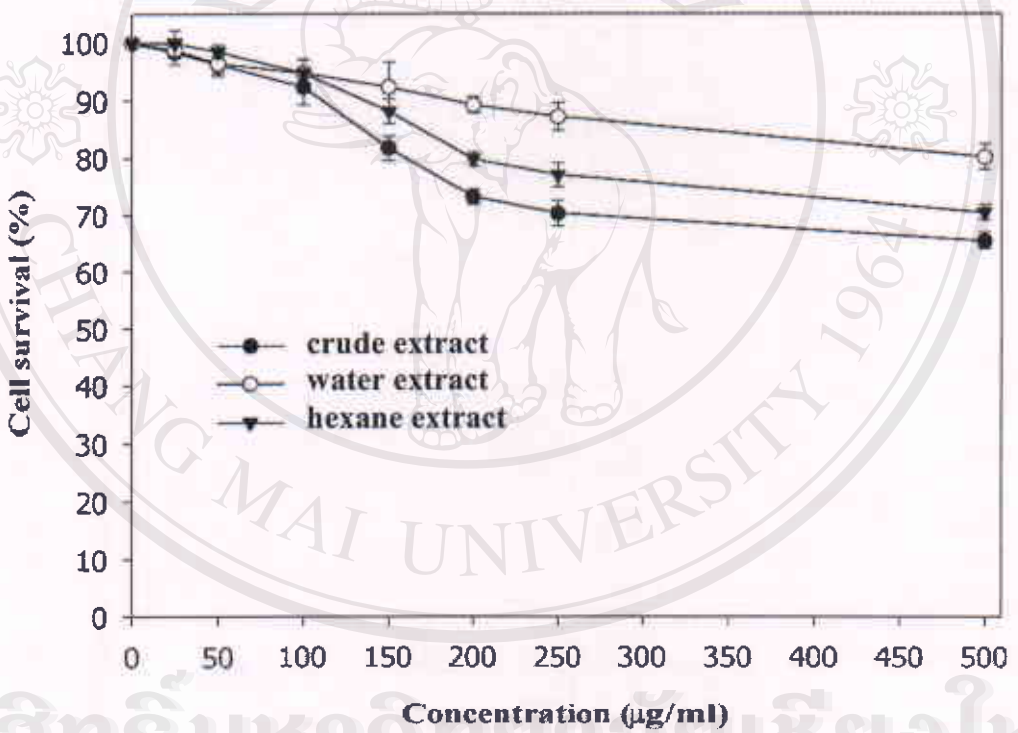

Figure 55. Effect of garlic extracts on cell survival in U937 cells. The percentage of cell survival of U937 cell line after incubation with garlic extracts at 25-500 μg/ml prepared by crude, water-, and hexane-extraction at 37°C, 5%CO_2 for 24 h. The percentage of cell survival was determined using MTT assay. Each point represents the mean and standard deviation from three replicate trials.

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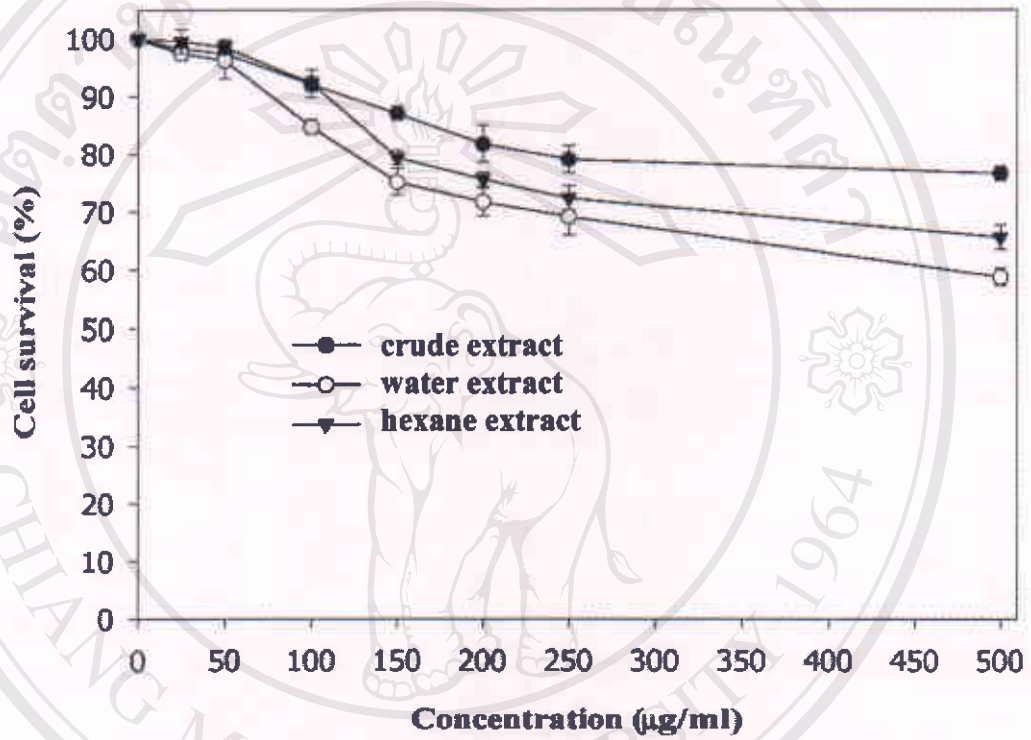

Figure 56. Effect of shallot extracts on cell survival in U937 cells. The percentage of cell survival of U937 cell line after incubation with shallot extracts at 25-500 µg/ml prepared by crude, water-, and hexane-extraction at 37°C, 5%CO_2 for 24 hr. The percentage of cell survival was determined using MTT assay. Each point represents the mean and standard deviation from triplicate trials.

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3.8.2. Effects of shallot and garlic extracts on total intracellular glutathione levels

After incubation of U937 cells with shallot extracts at 50, 125 and 250 μg/ml for 24 h, the total intracellular glutathione levels were significantly increased in shallot extract (crude) and water extraction compared to hexane-extracted shallot and cell control ($p<0.05$) (Fig. 57).

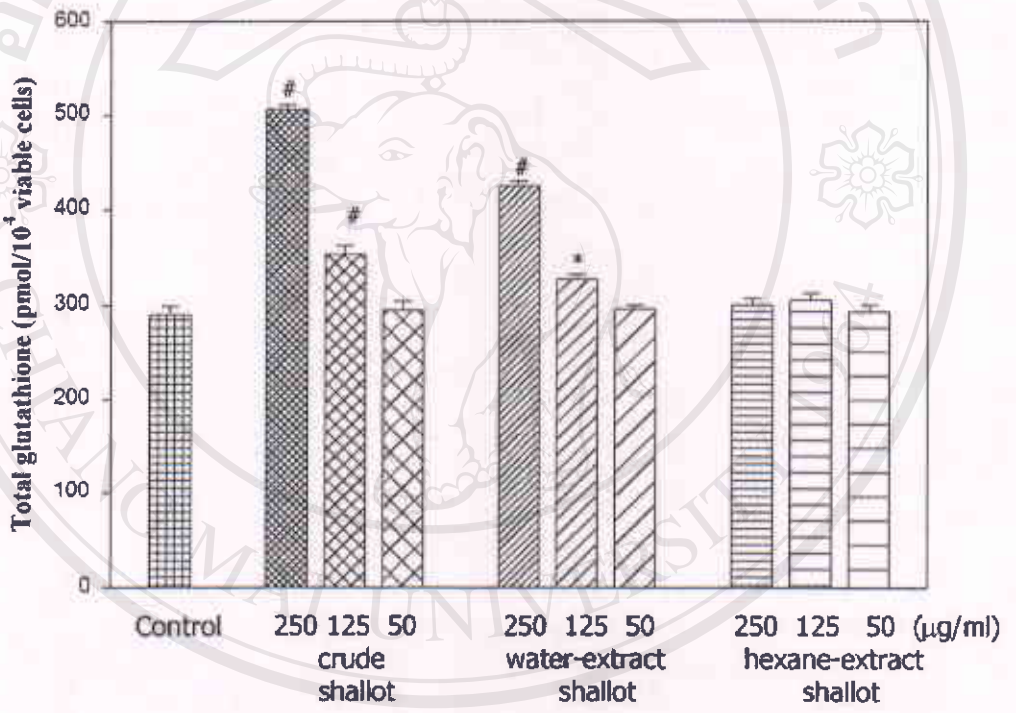

Figure 57. Effect of shallot extracts on total glutathione levels in U937 cells. The activity of shallot extracts from crude, water-, and hexane-extraction on total glutathione in U937 cell at 50-250 μg/ml after incubation for 24 hr at 37°C, 5% CO_2. The level of total glutathione in cells was determined using glutathione-reductase cycling method. Each bar shows the mean and standard deviation from five samples. ($^{\#}p < 0.01$ and $^{*}p < 0.05$ compared with cell control).

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3.8.3. Effects of shallot and garlic extracts on total intracellular peroxide levels

This activity was clearly seen after incubating the U937 cells with shallot extract for 6 h or 24 h. The crude extract of shallot also reduced total intracellular peroxide formation compared to cell control or cell treated with garlic extracts (Fig. 58).

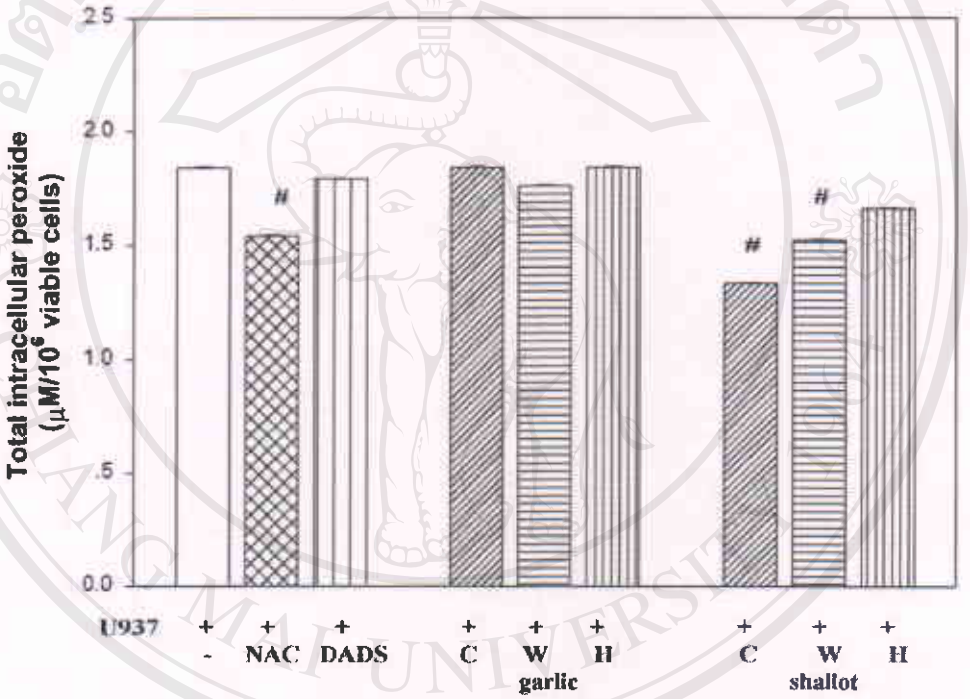

Figure 58. Effect of shallot and garlic extracts, NAC, and DADS on total peroxide levels in U937 cells. Total intracellular hydroperoxide or peroxide formation in U937 cells untreated or treated with 6.4 mM N-acetylcysteine (NAC), 250 μM diallyl disulfude (DADS), garlic and shallot extracts at 125 μg/ml after incubation for 24 h at 37°C, 5% CO_2. The level of total glutathione in cells was determined using glutathione-reductase cycling method. Each bar shows the mean and standard deviation from five samples. ($^{\#}p < 0.01$ and $^{*}p < 0.05$ compared with cell control).

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3.9. Anti-oxidative property of shallot extract

3.9.1 Irradiation

The shallot extract (hexane extraction) at 125 μg/ml protected the intracellular GSH depletion (141.44±16.37 pmol/10^4 viable cells) from hydroxyl radical-generated by ^{60}Co irradiation for 20 min as compared to non-irradiated cells, irradiated cells, (hexane extraction) garlic extract-treated cells and NAC-treated cells. (311.90±13.45, 84.03±1.26, 126.45±7.17, and 146.11±5.67 pmol/10^4 viable cells, respectively) (p< 0.05) (Fig. 59).

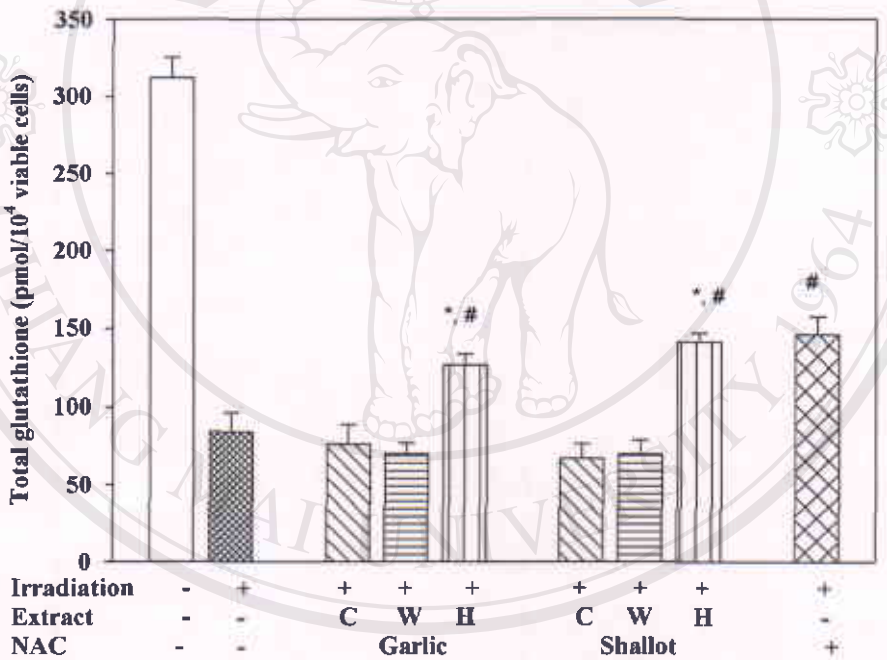

Figure 59. Protective effect of shallot and garlic extracts, and NAC on total glutathione level after γ-irradiation. The protective effect of shallot extracts (C = crude, W = water extraction, and H = hexane extraction) compared with garlic at 250 μg/ml and NAC (6.4 mM) after incubation with cells for 24 hr and gamma-irradiation (65Gy/min) for 20 min. The level of total glutathione in cells was determined using glutathione-reductase cycling method. Each bar represents the mean and standard deviation from five trials. (*p< 0.05 compared with irradiated control cells, #p< 0.05 compared with all garlic extracts or water-, hexane extract of shallot).

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3.9.2. H_2O_2 activation

Oxidation of monocytic cells ($5x10^5$ cells) with H_2O_2 (100-200 μM) was used as a model of the inflammation process. The total intracellular hydroperoxide production after H_2O_2 incubation for 24 h (2.67±0.045 and 2.72±0.05 μM/$5x10^5$ viable cells) compared with non-stimulated cells (2.2±0.034 μM/$5x10^5$ viable cells) is shown in Figure 60. Crude extract of shallot showed clear evidence of hydroperoxide formation at dosages of 125-500 μg/ml compared to H_2O_2-stimulated U937 cells, and the activity was similar to NAC (6.4 mM).

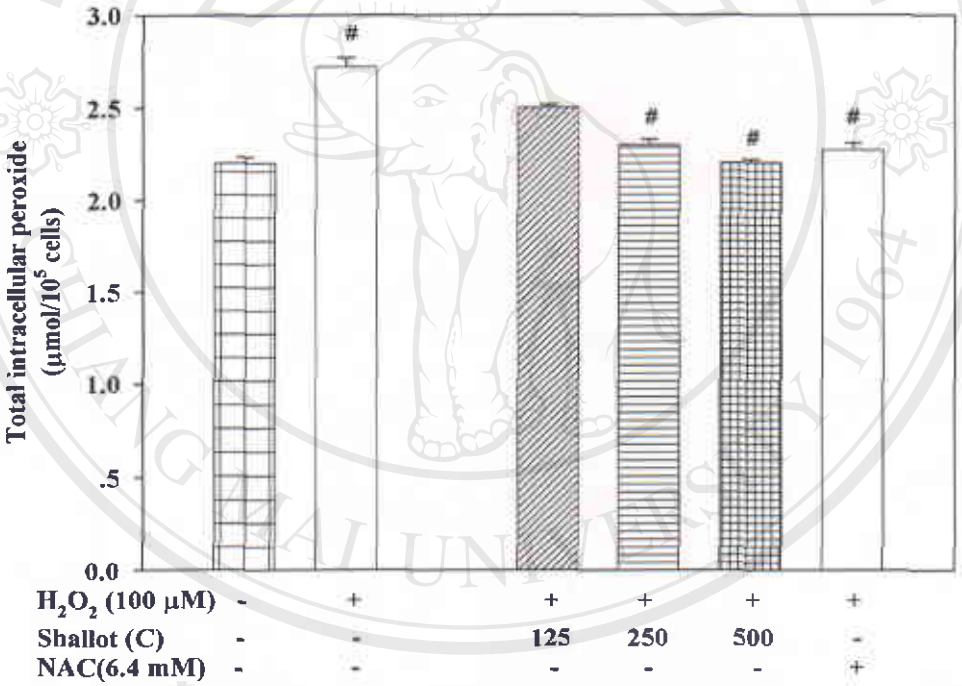

Figure 60. Inhibitory effect of shallot extract and NAC on total peroxide in U937 cells after H_2O_2 oxidation. Total intracellular peroxide formation in U937 cell lines from H_2O_2 stimulation (100 μM), and inhibitory activity of crude shallot extract at 125-500 μg/ml compared with N-acetylcysteine (NAC) at 6.4 mM after incubation at 37°C, 5%CO_2 for 24 h. The level of peroxide was determined using FOX method. Each bar represents the mean and standard deviation from five samples. ($^{\#}p< 0.05$ compared with H_2O_2 –treated cells).

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3.10. Effects of diallyl disulfide (DADS) on glutathione levels in U937 cells

This study confirmed the mechanism of induction GSH synthesis by crude shallot extract using N-acetylcysteine (NAC) as the precursor of GSH synthesis and BSO as an inhibitor of GSH synthetase. NAC at 3.2, 6.4, and 12.8 mM dose-dependently increased GSH content (235.62±8.1, 250.0±34, and 308.12±1.72 pmol/10^4 viable cells) compared with cell control (235.00±8.16 pmol/10^4 viable cells). GSH concentration was reduced by BSO (100, 200, and 400 μM) with dose response (235.00±8.16, 82.45±5.7, 52.82±1.53, and 7.03±1.23 pmol/10^4 viable cells respectively).

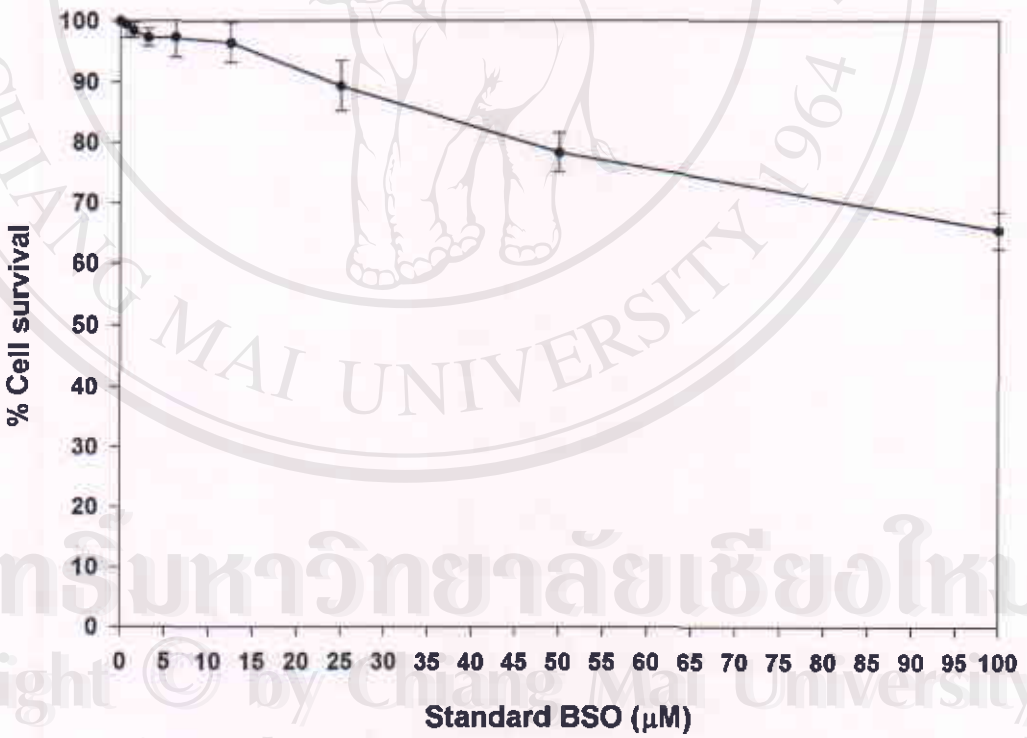

Figure 61. Effect of BSO on cell survival in U937 cells. U937 cells were treated with BSO at 0-100 μM at 37°C, 5%CO_2 for 24 h. The cell survival was determined using MTT assay. Each point shows the mean and standard deviation from triplicate samples.

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The BSO at 20 μM decreased GSH level by inhibition of GSH synthesis in the cells with time response at 3, 6, 12, and 24 hr (Fig. 62). Tests of the activity of extracts and standard diallyl disulfide on GSH synthesis via regulator GSH synthetase with specific enzyme inhibitor BSO at 20 μM showed total GSH depletion of more than 50% after incubation with BSO for 12 hr and 80% at 24 hr (Fig. 62).

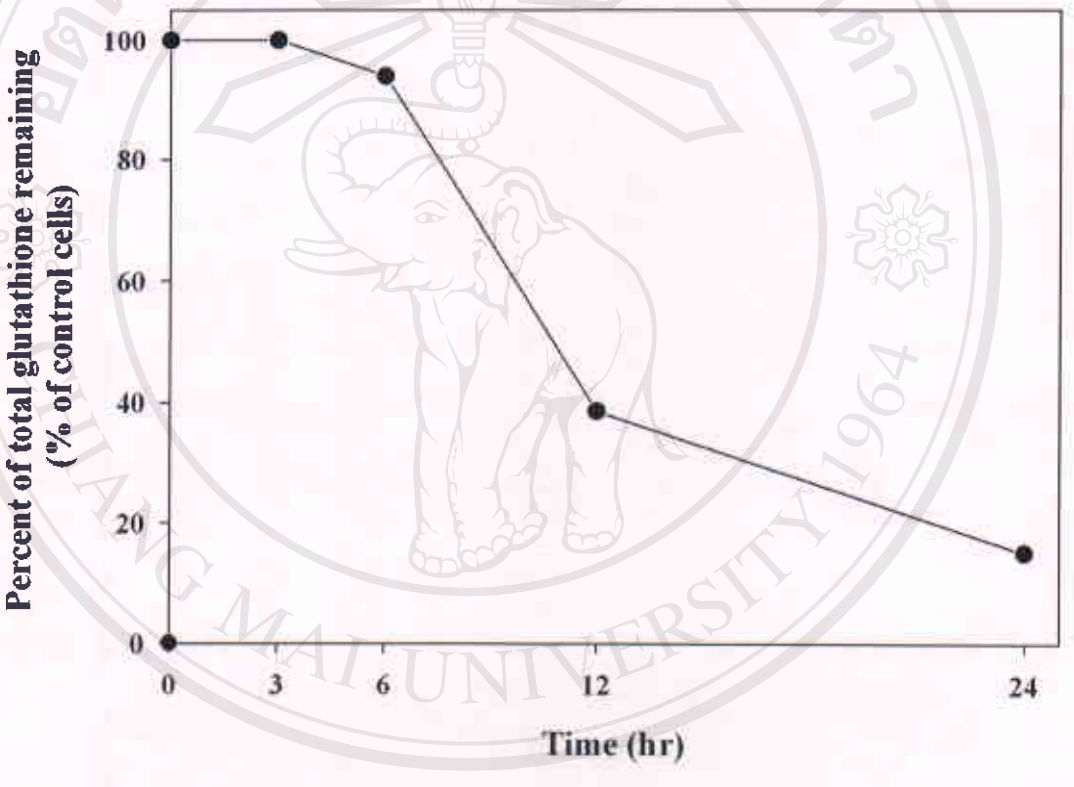

Figure 62. Inhibitory effect of BSO on total glutathione levels in U937 cells. U937 cells were treated with 20 μM BSO and the percent remaining of total glutathione in cells was followed up at 3, 6, 12, and 24 hr compared with untreated cells. The total glutathione was determined using glutathione reductase-cycling method. Each point shows the mean and standard deviation from triplicate samples.

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The BSO effect on reducing the GSH level in the cell was inhibited by standard diallyl disulfide DADS (1-12 μM) and standard NAC (10-40 mM) (Fig. 63).

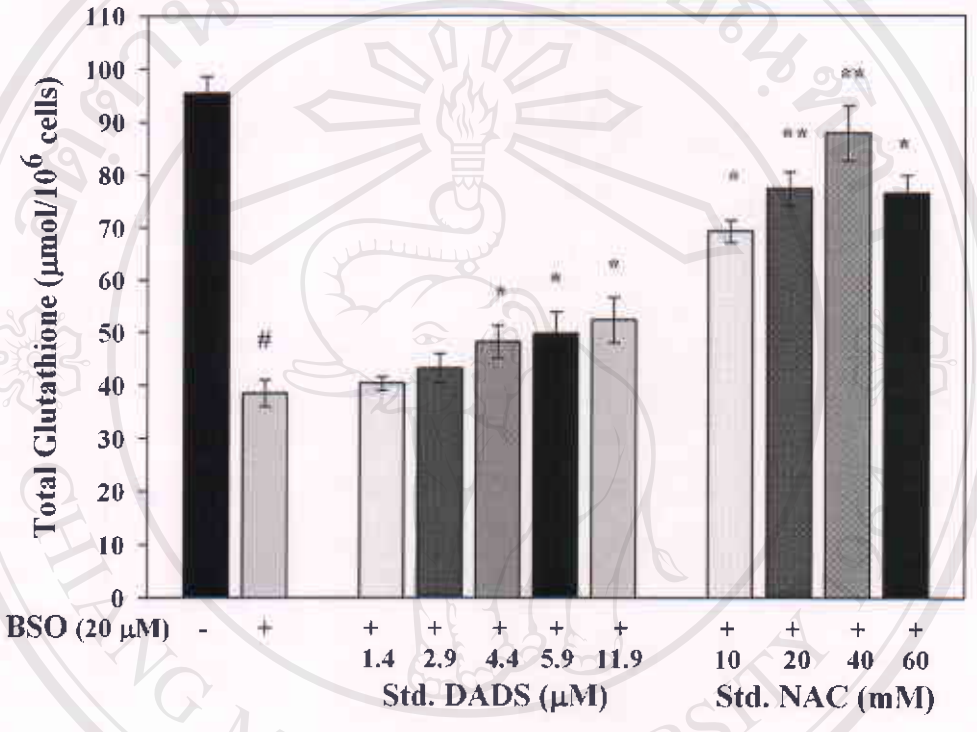

Figure 63. Effect of standard diallyl disulfide (DADS) and NAC on total glutathione levels in U937 cells. Standard DADS at 1.4-11.9 μM and standard NAC at 10-60 mM were incubated in the BSO-treated cells (10^6 cells). Total glutathione level in cells was assayed using glutathione reductase-cycling method after incubation for 24 hr at 37°C, 5% CO_2. Each bar shows the mean and standard deviation from triplicate trials. (*p< 0.05, ** p<0.01 compared with BSO-treated control cells).

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From previous results, DADS was identified by HPLC and showed the concentration of DADS in shallot and garlic extracts are shown in Table 6. The concentration of shallot extract at 238 μg/ml was equal to the standard DADS at 10.8 μM. The effect of shallot and garlic extracts on total glutathione levels was studied by incubation of the standard DADS and the extracts at equivalent concentrations in the cells for 24 hr (Fig. 64).

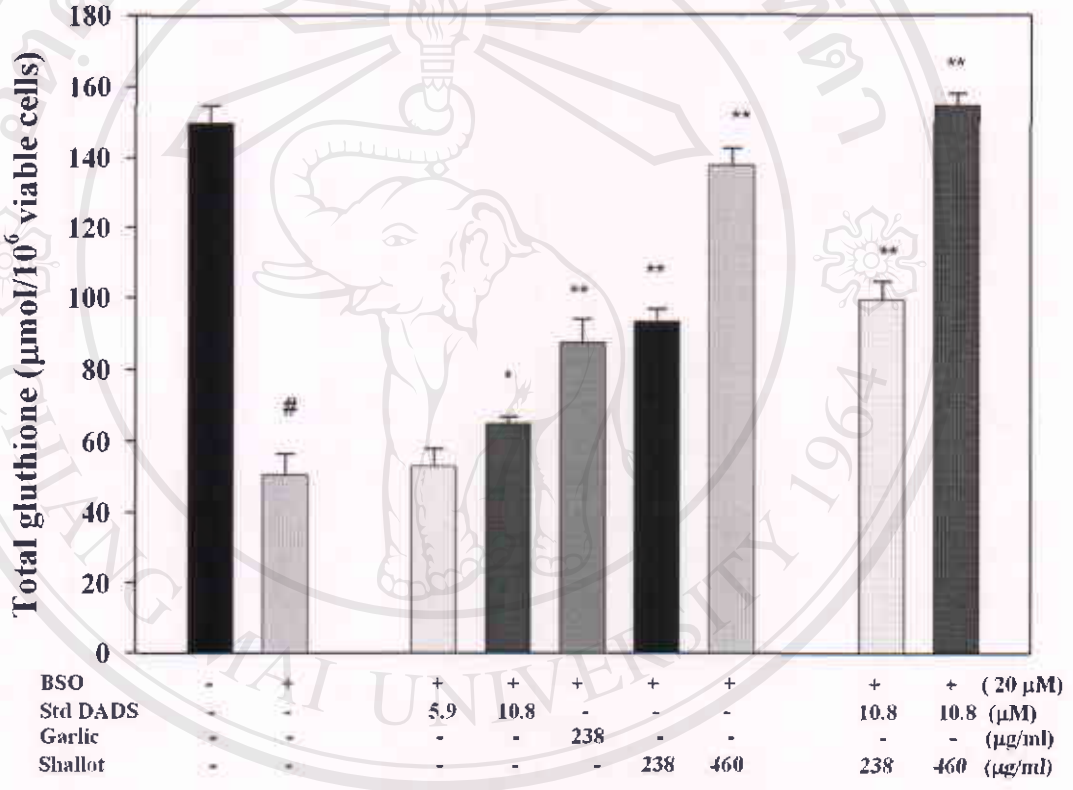

Figure 64. Effect of shallot and garlic extracts, and standad DADS on total glutathione levels in BSO-treated U937 cells. The level of total intracellular glutathoine in U937 cells after incubation with BSO (20 μM) and standard DADS (5.9 and 10.8 μM), shallot and garlic extracts (238 μg/ml that gave concentration equivalent to standard DADS at 10.8 μM) for 24 hr at 37°C, 5% CO_2. Total glutathione level in cells was assayed using glutathione reductase-cycling method. Each bar represents the mean and standard deviation of five samples. ($^{\#}p< 0.05$ compared with cell control, $^{*}p< 0.05$, $^{**}p< 0.01$ compared with BSO-treated cell control).

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In addition, this study tested the divergent effects between crude shallot extract (250 μg/ml) and NAC (6.4 mM) and BSO (200 μM) on GSH level. The crude shallot extract added with NAC gave slightly higher level of GSH compared with only crude shallot extract (343.56±7.16 and 323.23±6.34 pmol/10^4 viable cells, respectively), cells treated with BSO and shallot extract (20.78±3.45 pmol/10^4 viable cells) or cell controls (225.67±7.81 pmol/10^4 viable cells) (p<0.05) (Fig. 65).

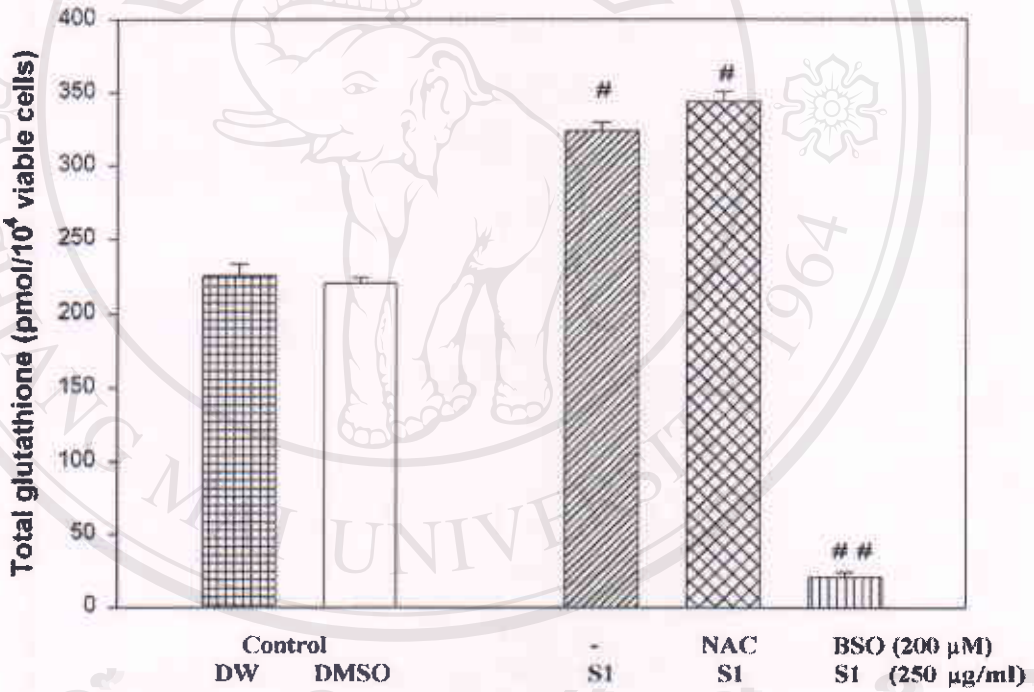

Figure 65. Effect of shallot extract and co-treated shallot with NAC on total glutathione levels in BSO-treated U937 cells. Shallot extract (S1 = crude extract) at 250 μg/ml and NAC (6.4 mM) were treated in cells that added BSO at 20 μM for 24 hr at 37°C, 5% CO_2. Total glutathione level in cells was assayed using glutathione reductase-cycling method. Each bar shows the mean and standard deviation from triplicate trials. ($^{\#}p$< 0.01 compared with cell control (DW&DMSO), $^{\#\ \#}p$<0.01 compared with cell control, S1 only and S1 plus NAC).

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3.11. Effects of shallot extracts containing DADS on GSH level against oxidative stress and inflammation in cell lines.

3.11.1. The activity of shallot and garlic extracts against oxidative stress (H_2O_2 at 200 μM) in U937 cells

The results showed the ability of garlic and shallot extracts to restore the level of GSH from H_2O_2 oxidation.

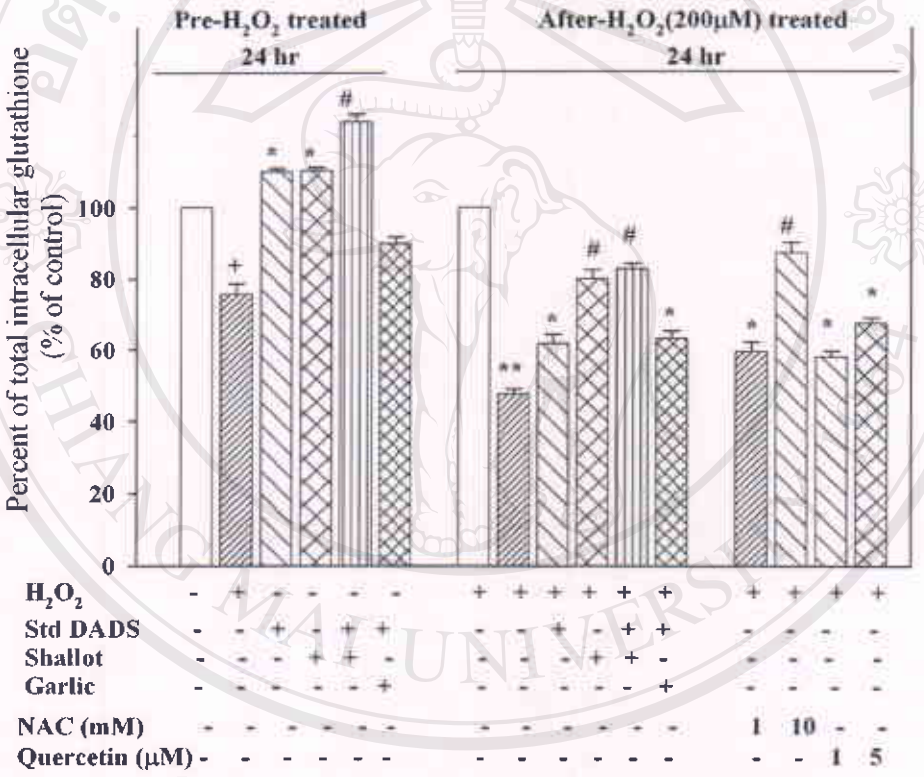

Figure 66. Effect of shallot and garlic extracts, standard DADS, and quercetin on total glutathione levels in H_2O_2-oxidized U937 cells. U937 cells were pretreated with standard DADS (10.8 μM), shallot or garlic extracts (238 μg/ml equal to 10.8 μM of standard DADS), NAC (1 and 10 mM), and quercetin (1 and 5 μM) and then treated with H_2O_2 (200 μM) for 24 hr 37°C, 5% CO_2. Total glutathione level in cells was assayed using glutathione reductase-cycling method. Each bar shows the mean and standard deviation from triplicate trials. (+$p< 0.05$, ** $p< 0.01$ compared with H_2O_2 untreated cell control, * $p< 0.05$ and # $p< 0.01$ compared with H_2O_2 treated cell).

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Figure 67 showed the percentage of GSH in U937 cells after being treated with standard diallyl disulfide (DADS) (20 μM alone), hexane extract of shallot alone or combined with DADS and extracts or NAC. There was a significant increase in total GSH level in U937 cells that were treated with DADS and shallot extract, compared to shallot or DADS alone.

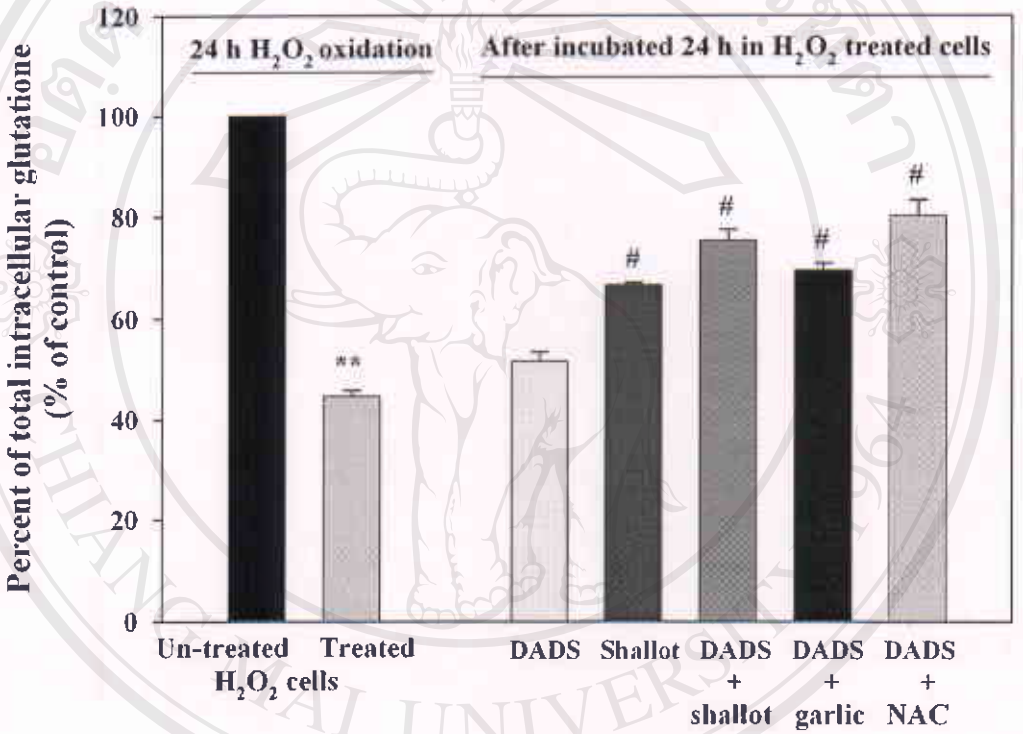

Figure 67. Effect of the co- treatment between shallot or garlic extracts and standard DADS or NAC after pre-tread cells with H_2O_2. After depletion of the total intracellular glutathione in cells with H_2O_2 (200 μM), cells were separated to treat with standard DADS (20 μM), hexane extract of garlic (20 μM equivalent to standard DADS), or NAC (20 mM), and co-treatment between shallot, garlic, or NAC with standard DADS. After incubation for 24 h at 37°C, 5% CO_2. Total glutathione level in cells was assayed using glutathione reductase-cycling method. Each bar shows the mean and standard deviation from triplicate trials. (**$p< 0.01$ compared with un-treated H_2O_2 cells, $^{\#}p<0.01$ compared with DADS-treated alone).

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3.11.2. The activity against the inflammation in U937 and J774.2 cell lines

This study showed the activity of shallot extracts (crude, water or hexane) on LPS-activated U937 cells, as well as hexane shallot extract that contained DADS on LPS-activated J774.2 cells.

3.11.2.1. U937 cells

Shallot extracts (crude-, water-extraction) reproduced GSH in U937 cells after LPS treatement compared to LPS treatment alone, $p<0.05$, as shown in Figure 68.

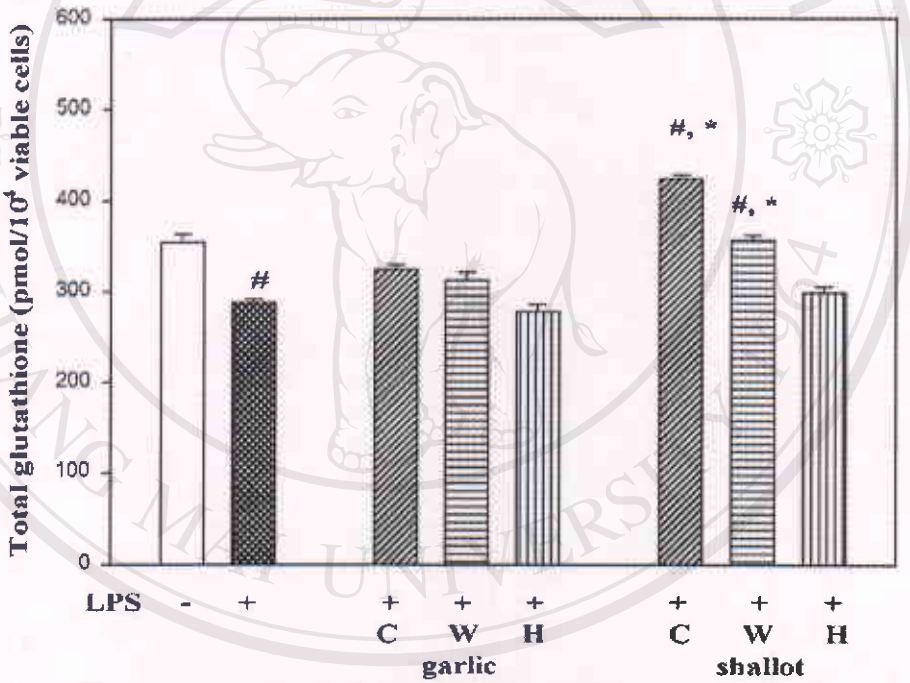

Figure 68. Effect of garlic and shallot extracts on total glutathione in LPS-activated U937 cells. After incubation with shallot and garlic extracts (C = crude, W = water, H = hexane) at 250 μg/ml, U937 cells that activated with LPS at 1 μg/ml for 24 h at 37°C, 5% CO_2, were assayed for total glutathione levels using glutathione reductase-cycling method. Each point shows the mean and standard deviation from triplicate samples, and two independent experiments. (*$p< 0.05$ compared with cells treated with hexane extract of shallot, # $p< 0.05$ compared with LPS-stimulated cells).

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Nitric oxide (NO) production (Fig. 69) in U937 cells ($5x10^5$ cells) was detected as nitrate formation after incubation at 37°C, 5% CO_2 for 24 h. Crude extract shallot at 250 μg/ml increased total glutathione levels and inhibited nitric oxide formation in the cells, compared with shallot extracts from water and hexane extract, and with NAC (6.4 mM) (Fig. 69).

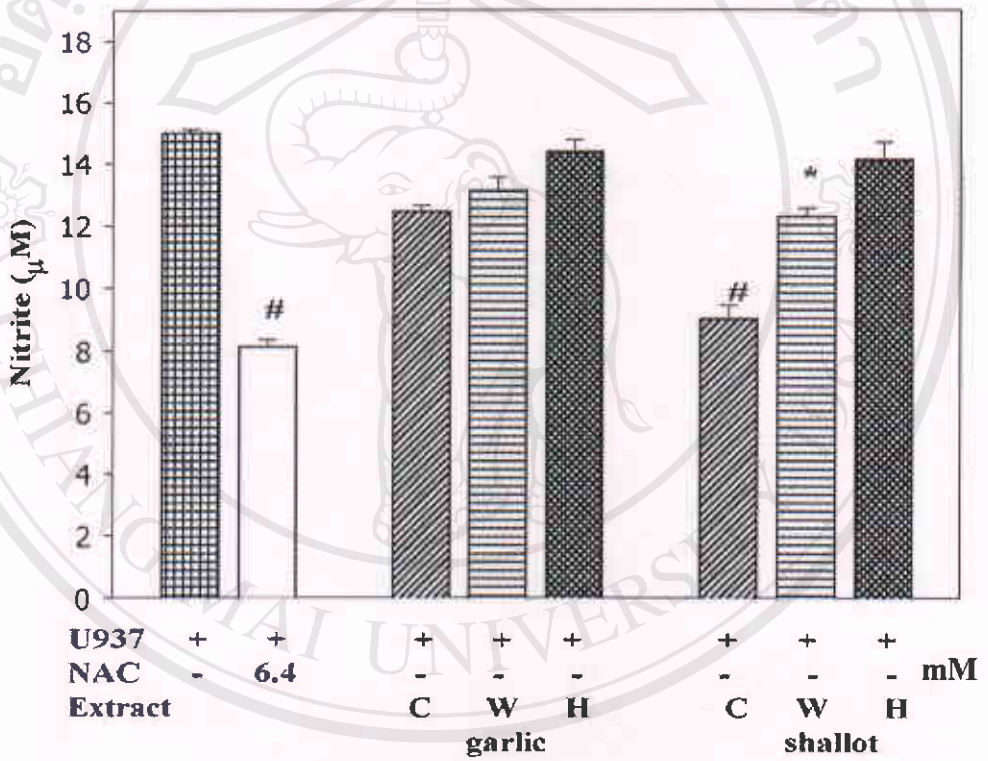

Figure 69. Effect of shallot and garlic extracts, and NAC on nitrite levels in LPS-activated U937 cells. Garlic and shallot extracts (C = crude, W = water, H = hexane) at 250 μg/ml and N-acetylcysteine (NAC) at 6.4 mM were incubated with U937 cells that were activated with LPS (1 μg/ml) for 24 h at 37°C, 5% CO_2. Nitrite level was determined using Griess reagent. Each bar shows the mean and standard deviation from triplicate trials. (* $p< 0.05$, # $p< 0.01$ compared with cell control).

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Moreover, Figure 70 showed the activity of shallot extract at 250 μg/ml on inhibition the peroxide formation in LPS-activated cells after incubation for 24 h at 37°C. 5%CO_2. The level of peroxide was reduced after incubation for 5 h at least, compared to non-activated cell or LPS-activated cell control (Fig. 70).

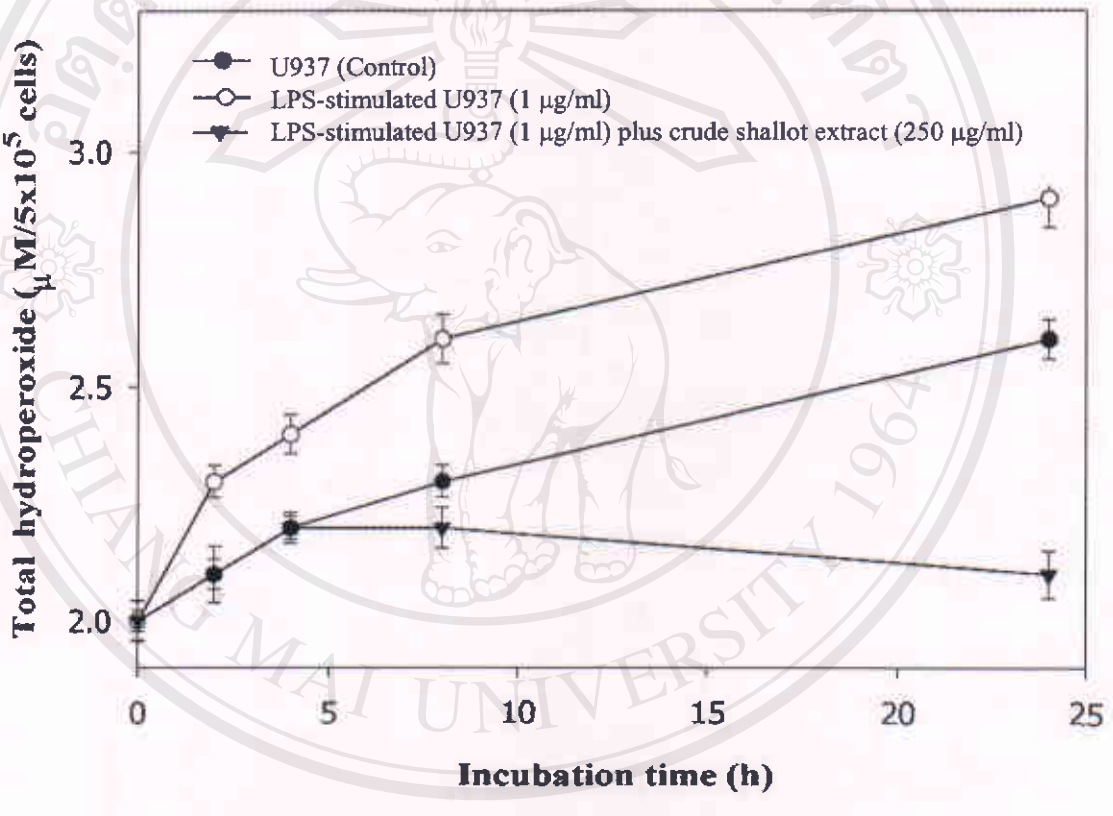

Figure 70. Inhibitory effect of crude shallot extract on total hydroperoxide in U937 cells. The effect of crude shallot extract at 250 μg/ml on inhibition the hydroperoxide formation in U937 cells activated with LPS (1 μg/ml) for 24 h, compared with LPS-inactivated and activated U937 cells. Total hydroperoxide was determined using FOX method. Each bar shows the mean and standard deviation from triplicate trials.

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3.11.2.2 . J774.2 cell.

Figure 71 showed the activity of LPS (1 μg/ml) on nitric oxide (NO) production in DMEM medium (10^6 cells) after incubation with J774.2 cells at 37°C, 5% CO_2 for 24 hr. Hexane extract of shallot at equal amount to standard DADS at 20 μM inhibited nitric oxide formation in the cells, compared with hexane extract of garlic and standard DADS at 20 μM.

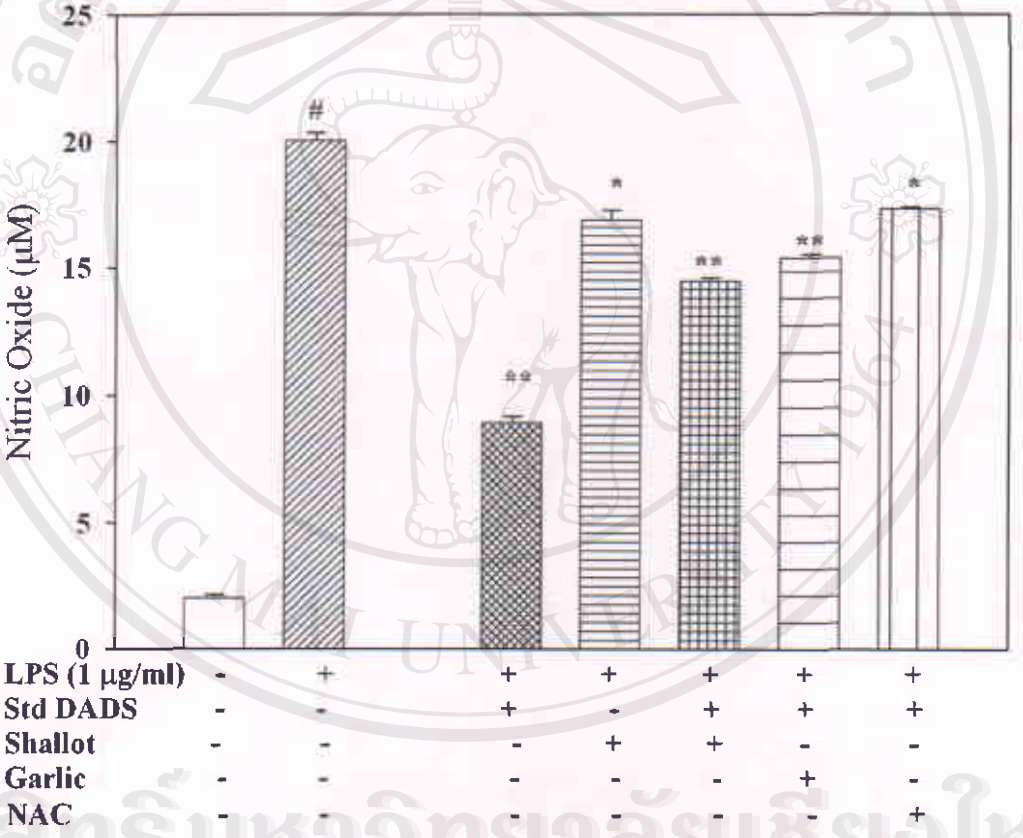

Figure 71. Effect of shallot and garlic extracts, standard DADS, and NAC on nitric oxide production in LPS-activated J774.2 cells. Shallot and garlic extracts at 238 μg/ml, 20 μM standard DADS, 20 mM NAC were treated in J774.2 (10^6 cells) in combination with LPS (1 μg/ml) for 24 h at 37°C, 5%CO_2. The level of nitric oxide was determined using Griess reagent. Each bar shows the mean and standard deviation from triplicate trials. ($^{\#}p<0.05$, compared with non-activated LPS, $^{**}p<0.01$ compared with LPS-activated cells).

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