CHAPTER II

MATERIALS AND METHODS

2.1. Equipments, chemicals, reagents preparation are described in Appendix A-B.

2.2. Preparation of glassware and plastic ware

All glassware used in free radical experiments was soaked in detergent overnight before rinsing with water, then soaked in nitric acid at 80°C for several hours. After cooling, the glassware was thoroughly rinsed with water purified in a-4-stage Milli Q (Sydney) apparatus equipped with a 0.2 μM filter, and dried in 180°C oven. For cell culture experiments, the glassware was soaked in detergent, then rinsed in tap water and distilled water before drying. The glassware was sterilized by autoclaving at 121°C, 100 KPa for 20 min. All eppendorf tubes and pipette tips used in cell culture experiments were autoclaved at 121°C, 100 kPa for 20 min.

2.3. Preparation of plant extracts

Shallot bulbs were obtained from local agriculture farms and garlic raw bulbs from local markets. Shallot and garlic bulbs were weighed and combined with distilled water or hexane in equal volume (1000 mg: 1000 ml of solvent), then homogenated with an electrical motor. The mixture in distilled water solvent was filtered freshly and freeze-dried. The hexane mixture was soaked overnight and filtered, the hexane layer was separated by evaporation. The non-polar solution left after evaporation was processed by freezed-drying. Other preparation was from pressing and squeezing juice from the bulbs directly. The mixed suspension was filtered, freezed-dried and then lyophilized.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

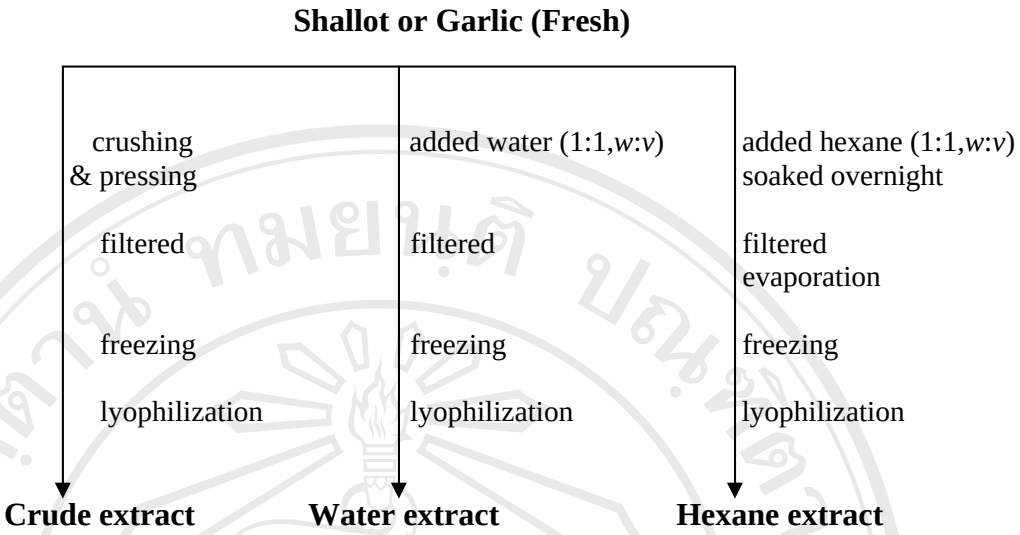

Figure 15. Protocol for preparation of shallot and garlic extracts.

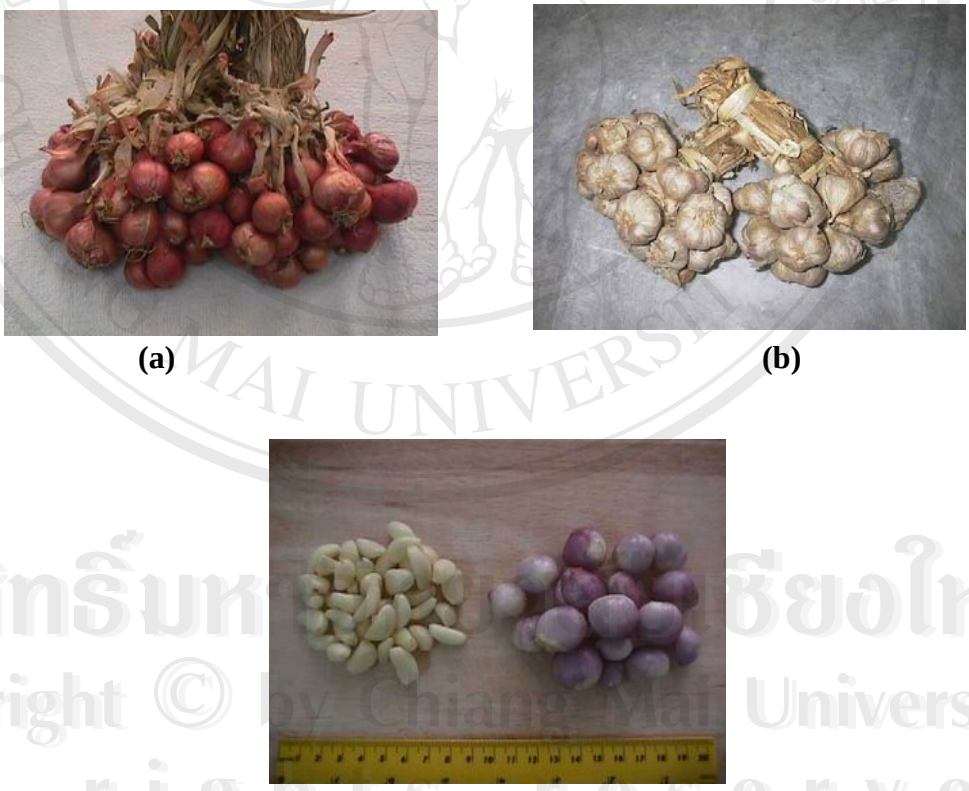

Figure 16. Fresh shallot and garlic bulbs. Shallot (a) and garlic (b) bulbs were brought from local market in Chiang Mai province and peeled the bulbs before extraction (c).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

(a) **(b)**

Figure 17. The procedures of crushing and pressing of shallot bulbs. Naked bulbs were compressed, pressed (a) and filtered (b) to collect the juice for freezing and lyophilization.

(a) **(b)** **(c)**

Figure 18. The procedures of extraction of the shallot and garlic with water or hexane. The extract was prepared by blending (a), then kept over night (b) and separated in a funnel glass (c).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

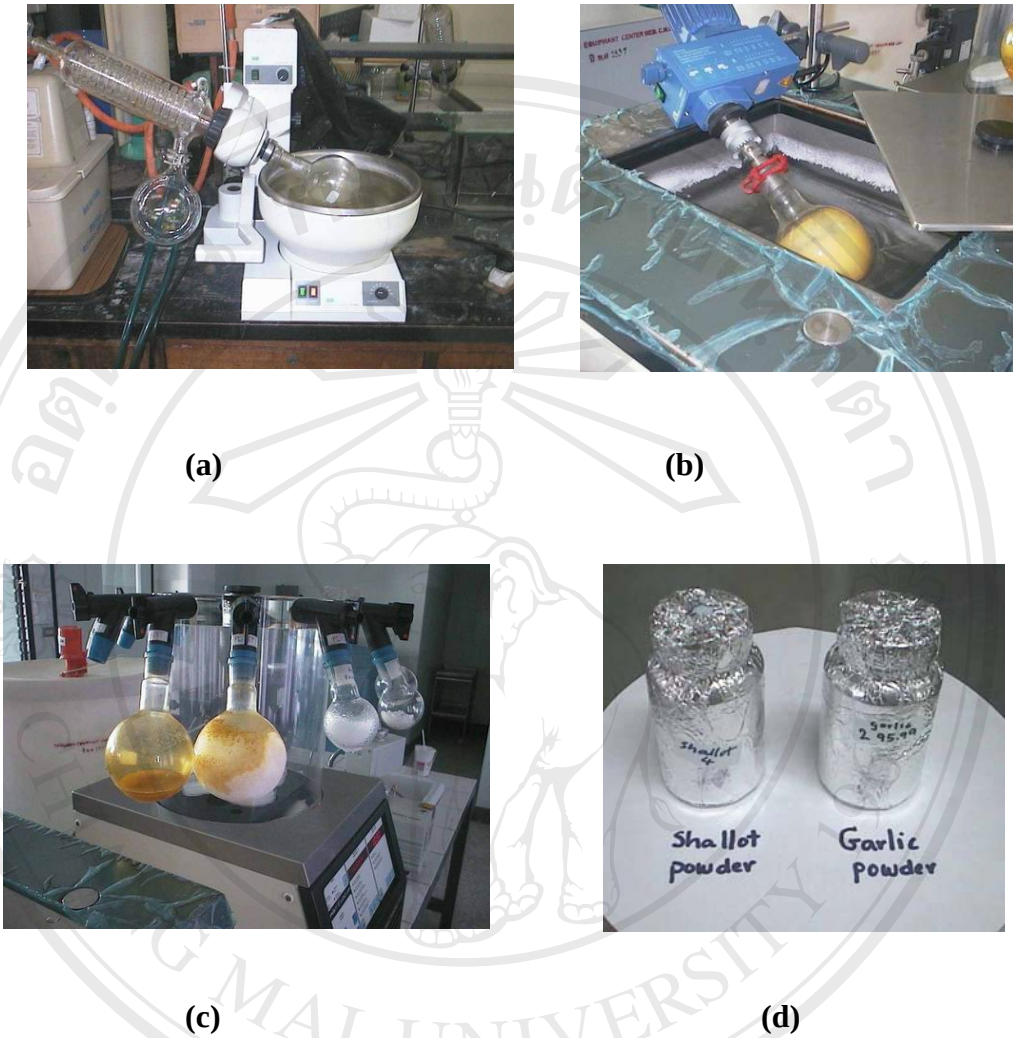

Figure 19. The procedures of powder preparation after extraction. Evaporation of the hexane extract (a) and freezing (b), before lyophilization (c). The extract was kept in dark with anti-moisture (d).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

2.4. Methods for the antioxidant capacity assay.

2.4.1. ABTS decolorization assay. (Re *et al*., 1999)

Principle: ABTS was dissolved in distilled water to a concentration of 7 mM. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. Because ABTS and potassium persulfate react stoichiometrically at a ratio of 1:0.5, these were conditions for incomplete oxidation of the ABTS. Oxidation of the ABTS occurred immediately, but the absorbance was not maximal and stable until after more than 6 h incubation. The radical was stable in this form for more than two days when stored in the dark at room temperature.

Method: For the study of the shallot extracts, the $ABTS^{\bullet+}$ solution was diluted with dH_2O to give an absorbance of 0.70 ± 0.2 at 734 nm. Stock solution of extracts (20 mg/ml) in distilled water or DMSO was diluted before introduction into the assay. The final concentration of extract was 1.0-0.l25 mg/ml and the final volume was 1 ml. All determination was carried out at least three times, on the standard and samples. The change in absorbance was recorded. The percentage of reduction of the ABTS absorbance at 734 nm was calculated using the formula.

$$\frac{\text{(Initial absorbance before adding extract – Final absorbance after adding extract)X100}}{\text{Initial absorbance before adding extract}}$$

The initial rate of a reduction of A734 nm with a 3 seconds lag, 20 seconds to 1 minute, and Factor = 1 was monitored and recorded with a kinetic spectrophotometer (Shimadzu, UV-1220 model). The data represented the ΔA/min. The antioxidant content in each extract was calculated and compared with standard antioxidant, Trolox, BHT, and gallic acid at 1 mg.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

2.4.2. Hydrogen peroxide scavenging assay

Principle: The scavenging effects of AGE and Shallot on H_2O_2 were determined according to the method of Jha (Jha *et al.,* 1984). The hydrogen peroxide was activated by peroxidase, generating a hydroxyl radical that modulated the ABTS non radical to ABTS cation radical. The peak absorption of ABTS radical was at 734 nm.

Method: The H_2O_2 (0.003% or 16 μM) mixed with 0.1 ml of different concentrations of extracts (0.125-1.0 mg/ml). Then 0.6 ml of 0.1% ABTS and 0.1 ml of 10 U/ml peroxidase, was added to 2.4 ml of 0.1 mM phosphate buffer (pH 6.0). The solution was then incubated at 37°C for 15 min. Absorbancy at 734 nm was measured in the Spectronic 2000 spectrophotometer. The data represented the percentage of hydrogen peroxide scavenging by shallot, garlic, as compared with control.

2.5. Methods for evaluation of the protein/amino acid and lipid hydroperoxide

The activity of shallot extracts on protein or lipid hydroperoxide formation was studied by using bovine albumin serum (BSA), liposome, and linoleic acid as models.

1: The activity on inhibitory on protein hydroperoxide formation was evaluated by mixing the extracts with BSA (5 mg/ml) before irradiation (60 Gy/minute). The level of protein hydroperoxide was determined via the tri-iodide method. Proline hydroperoxide (20 mM) was produced from irradiation (500 Gy) because previous studies (Gebicki, 1993) had shown a high level of peroxide (about 20 μM)

2: The activity on reduction or scavenging of protein hydroperoxide was evaluated by adding extracts in irradiated BSA and following the lost of peroxide by the tri-iodide method.

3: The inhibitory activity on lipid hydroperoxide formation was shown by mixing the extracts with liposome and allowing it to generate peroxide by auto-

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

oxidation at 37°C for 3 days. The level of lipid hydroperoxide was determined by the tri-iodide method.

4: The activity on reduction of lipid hydroperoxide was demonstrated by adding extracts after irradiation (60 Gy/min) the linoleic acid with ethanol (50%, *v*:*v*), and following the loss of peroxide by the FOX method.

Liposomes were prepared by evaporating 250 mg of soybean lipid and 25 mg of phosphate chloroform solution, followed by addition of degassed water and sonication under argon until the suspension shown only faint turbidity. The final lipid concentration was 15 mg/ml and the liposomes were stored at –20°C in 1.5 ml microcentrifuge tubes.

Liposomes were diluted in water (45 μl from stock liposome to yield a final volume of 2400 μl in water). The shallot extratcts were prepared in 20 mg/ml and mixed in liposome solution with final concentrations at 0.5 and 1.0 mg/ml. After irradiation with ^{60}Co source for 10 minutes, catalase was added. After incubating for 10 minutes, the methanol-FOX assay was used to determine the lipid hydroperoxide following the method of Tweeddale and Gebicki (2000)**.** Briefly, Irradiated liposome with shallot extract (700 μl) was mixed with 750 μl of chloroform containing 20% BHT, following by vigorous mixing and centrifugation at 14,000 rpm for 5 min. 250 μl of chloroform layer was incubated with 1,495 μl of methanol, 105 μl of perchloric acid (2.0 M), and 100 μl of xylenol orange). After vortexing 50 μl ferrous solution (5.0 mM) was added, and the mixture was incubated at room temperature in the dark for 30 minutes. The absorbance was then read at 560 nm. The percentage of remaining lipid hydroperoxide was calculated from control, oxidized liposome alone (100%).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

2.5.1. Tri-iodide assay (Simpson *et al*., 1992)

Principle: The tri-iodide assay or Iodometric assay is a method for detecting hydroperoxide. Hydroperoxide can oxidize iodide (I^-) to iodine (I_2), and at high I^- concentration, the I_2 will combines with I^- to form I_3^- at equilibrium.

$$ROOH + 2\,H^+ + 3I^- \longrightarrow ROH + H_2O + I_3^-$$

peak at 358 nm, with an excitation coefficient of 30,000 $M^{-1}cm^{-1}$. As can be seen from the two equations, the stoichiometry between hydroperoxide and I_3^- is 1:1. Hydroperoxide concentration can therefore be easily calculated via the Beer-Lambert law.

Concentration = absorbance / molar extinction coefficient

The assay is simple, fast and very sensitive. It can measure the level of hydroperoxide to a concentration of 1 μM with good reproducibility, but care must be taken to prevent interfering reactions of excess I^- with molecular oxygen. This is achieved by use of argon or oxygen-free nitrogen to provide a fully anaerobic system.

Figure 20. The equipment setting in an anaerobic system of tri-iodide assay. Ten percentage of potassium iodide in acetic acid was prepared in Oxford pipettor, and filling in a capped cuvette. The yellow color was produced and read at 358 nm after incubating in water bath at 50°C for 15 min, and intensity of color was correlated to the hydroperoxide concentration.

Method: Fifty percent solution of acetic acid in the Oxford pipettor was prepared and oxygen-free nitrogen or argon was bubbled through the solution for 5-10 min. Potassium iodide (10%, *w*:*v*) was added. Potassium iodide/acid solution was put into the cuvette while needle was still inside, then the needle was removed before the cap was onto cuvette. The absorbance at 358 nm was initially recorded. Sample working by two models of this study was designed as the incubation of BSA with shallot extracts (crude, water- or hexane extraction) before irradiation (protective study) or after irradiation (scavenging study) for 10-15 minutes (56.63 Gy/min). Irradiated BSA (0.5 ml) was added to the cuvette and the air was removed by using oxygen-free nitrogen gas through solution about 10 sec. Stopper was put on and the cuvette was incubated at 50°C for 15 min. Then cuvette was placed in cold water bath

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

for 2 min, the absorbance at 358 nm was finally read. The yield of hydroperoxide was calculated by using coefficient (30,000 M^{-1}. cm^{-1}).

2.5.2. Methanol-perchloric acid-xylenol orange (FOX) assay

(Gay and Gebicki, 2003)

Principle: The method for detecting hydroperoxide (-OOH) by xylenol orange is based on the complex of Fe^{3+}-XO (yellow color). The hydroperoxide changes the Fe^{2+} form to Fe^{3+} by binding to the xylenol orange. This complex is stable in acidic condition for more than 30 min after incubation at room temperature

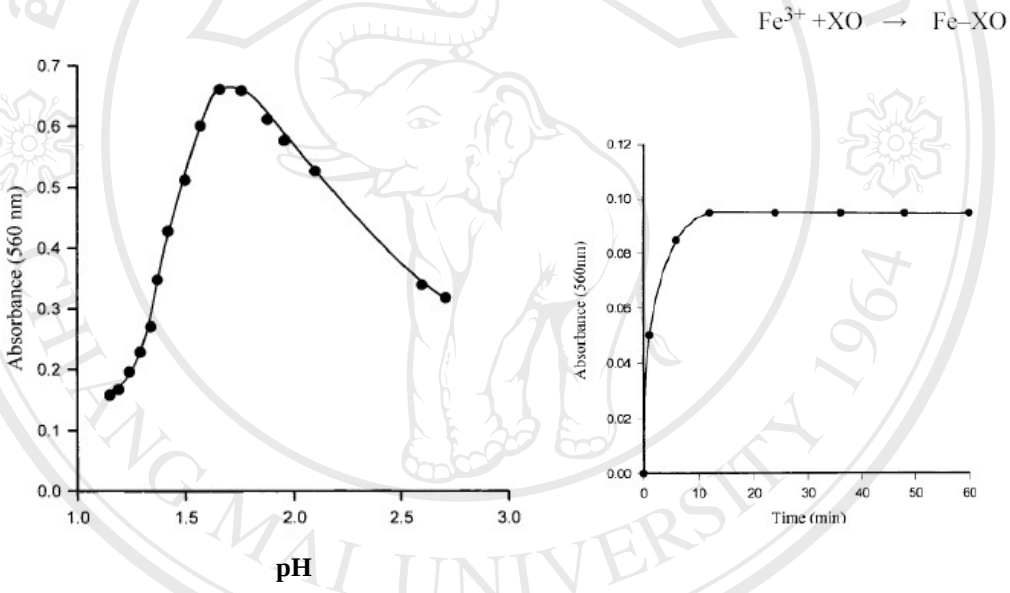

Figure 21. The absorbance of Fe^{3+}-XO complex at pH condition and time incubation. The maximum absorbance of complex showed at 1.5-2.0 in acidic condition (left), and stable plateau after 30 min incubation (right).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

Method: This technique modified is from Gay and Gebicki's method (2003) for measuring the protective (before irradiation) and scavenging activity (after irradiation) of each extract (crude, water- and hexane extraction) and standard antioxidants (NAC). In the assay of lipid hydroperoxide (LOOH), extracts were mixed with irradiated liposomes at indicated conditions. The chloroform containing 20% BHT was added, mixed vigorously and centrifuged at 14,000 rpm for 5 minutes. Chloroform layer was taken, then mixed with methanol, perchloric acid (2.0 M) and

xylenol orange (5.0 mM). Afterwards, ferrous solution (5.0 mM) was added, and allowed to stand at room temperature in the dark for 30 minutes. The absorbance was measured at 560 nm. The data represented the percentage of LOOH of each extract compared with control liposome without any extract.

2.6. Evaluation the activity of GSH from protein hydroperoxide and depletion from AAPH in human erythrocytes.

2.6.1. Protective effect on GSH from lysozyme hydroperoxide from irradiation

Principle: This study demonstrated the activity of shallot on protection of the GSH from protein hydroperoxide. The main protein (lysozyme) was selected to generate the hydroperoxide.

$$\text{Protein (Lysozyme)} \longrightarrow \text{Pro-OO}^{\bullet}$$

$$\text{Pro-OO}^{\bullet} + \text{GSH} \longrightarrow \text{Pro-OOH} + \text{GSSG}$$

Method: Briefly, supernatant of the shallot extract at 0.1-1.0 mg/ml was mixed with 0.02 M Na_2HPO_4 buffer (pH 8.0), lysozyme (2.5 or 5.0 mg/ml), and 20 mM GSH. Then, a mixture was irradiated with ^{60}Co source for 90 seconds. Ellman's reagent (25 mM) was added after incubation and irradiated mixture with catalase for 10 minutes. The glutathione concentration was calculated with standard reduced glutathione during 5-20 mM at 412 nm with the spectrophotometer (Shimadzu, UV-1200). The results yielded the glutathione concentration and the percentage of protection compared with irradiated glutathione control.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

2.6.2. Protective effect on depletion of GSH in human erythrocyte from AAPH.

2.6.2.1. Human erythrocytes

Heparinized blood was provided from healthy subjects. The character of blood was as followed hemoglobin 14.1 (10-16 g/dl), hematocrits 42 (40-50%), white blood cell (WBC) 5700 (5000-10000/cu.mm), ESR (erythrocyte sedimentary rate 6: 0-15 mm/hr), mean corpuscular volume (MCV) 89 (80-95 fL), mean corpuscular hemoglobin (MCH) 29.2 (27-32 pg), mean cell hemoglobin concentration (MCHC) 32.8 g/dl (32-36 g/dl), Hemoglobin typing (HbA2=2.5%, HbF=0.6%), and G-6-PD negative.

2.6.2.2. AAPH-oxidized RBC

One mL sample of blood, either untreated or treated with 1 ml of (10 mM) AAPH in the presence or absence of extract at 1 mg/ml was incubated for up to 6 hours at 37°C. The control was an equivalent volume of isotonic buffer solution, which did not significantly change the contents of total glutathihone and thobarbituic acid-reactive substances (TBARs) in RBCs within 6 hours (data not shown). After incubation at 37°C for 4 h, and being centrifuged at 3,000 rpm for 5 min, total glutathione (GSH) level in erythrocyte and malondialdehdye (MDA) in plasma were detected with DTNB (Beutler *et al*., 1963) and TBARs (Chirico *et al*., 1994) methods, respectively.

2.6.2.3. Total GSH determination

Erythrocyte (0.4 ml) was mixed with 1.6 ml of distilled water and 3.0 ml of precipitant solution. The supernatant was separated by filtering with Whatman No.1. One milliter of clear supernatant was added to 4.0 ml of phosphate buffer (pH 7.4) and 0.5 ml of DTNB reagent. The absorbance was detected at 412 nm within 5 min. The total glutathione concentration was measured compared to standard GSH (Sigma) at 20–100 mg/ml.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

2.6.2.4. Malondialdehyde (MDA) determination

One ml of plasma was mixed with 0.75 ml of H_3PO_4 (0.44 M), and stored at room temperature for 10 min, then 0.25 ml of TBA (0.6%) were added, and the sample was incubated in a water bath (90^oC) for 30 min. A pink color was developed and detected at 532 nm after stopping reaction with a cool water. The MDA concentration was compared with standard malonaldehyde (bis) acetate.

2.7. Analysis of bioactive compounds.

Principle of HPLC: A high performance liquid chromatography (HPLC) system is developed with high powerful analytic, excellent resolution with high speed and sensitivity for separating the types of compounds not amenable to analysis by gas chromatography (GC). A HPLC system is basically composed of a pump, injector, a column, and detector. Separation is normally performed on a stainless stell column packed with small particles (3-10 μM dimeter) of silica gel or resin under high pressure. There are two types of separation mechanisms employed in HPLC techniques, adsorption and reverse phase chromatography. Revere phase chromatograph has formed widely applications in the area of biochemical separation and purification. It involves an interaction between a saturated hydrocarbon, and the non-polar portion of the solute molecule. The most common mobile phase used in reversed phase system is a mixture of water and methanol, or another solvents; tetrahydrofuran, acetonitrile, or dioxane. The quantity of compound for separation depends on capability of its interaction with the stationary phase. An interested sample compound is usually less polar than a mobile phase, a polar portion in the mobile phase (water) push the component away to the low-polar phase (methanol phase). These component eluted from the column are detected by a detector and the detector's output signal is recorded by a data processor. The retention time of the peak is employed for qualitative identification in comparison with standard sample, while the peak area or peak height is necessary for quantitative determination of interested component.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

2.7.1. Organosulfide compounds analysis with HPLC

The lyophilized extracts from crude and water extraction were re-dissolved in water, and the hexane extract was dissolved in 100% DMSO at 20 mg/ml. After centrifugation at 14000 rpm for 10 min, all supernatants were filtered with 0.45 μM PTFE. The allyl sulfides and allicin in onion, garlic, and shallot extracts were detected by HPLC that was modified from the method of Lawson (1991).

An acetonitrile: water: tetrahydrofuran (70/27/3, *v*:*v*:*v*) mixture was used as a HPLC mobile phase. The HPLC chromatogram of standard allyl disulfides showed peaks of diallyl sulfide (4.62 min), diallyl disulfide (5.15 and 8.58 min), and diallyl trisulfide (6.6 min) by elution at 1.0 ml/min flow rate. A UV detector was monitored at 240 nm. The amount of each allyl sulfide compound was calculated aginst its standard calibration curve and expressed in μg/ml of lyophilized extract.

The thiosulfonate in lyophilized extracts was identified and separated using a mobile phase of 50% methanol (Lawson *et al.*, 1991). The allicin peak was eluted at approximately 8-10 min by elution with 1.0 ml/min flow rate at UV 240 nm. The concentration of allicin in extracts was calculated in the same way as above using a commercial garlic product (Immunytop), which contained a high amount of allicin (3,400 μg in 370 mg capsule) as standard compound to configure the calibration curve. The yield of allicin was expressed in the μg per 1 gram of extract.

2.7.2. Total phenolic assay (Singletion and Rossi, 1965)

Principle: This is a sensitive and quantitative method, independent of the degree of polymerization. Reaction of phenolics, alkaline, Folin-Cioaclteu reagent, and heat yields a blue colored product (Absorbance at 755 nm). FC reagent is an oxidizing agent comprised of heteropolyphosphotungstate-molybdate. The blue colored product is a mixture of the 1-, 2-, 4-, and 6-electron reduction products in the tungstate series $P_2W_{18}O_{62}^{-7}$ to $H_4P_2W_{18}O_{62}^{-8}$ and the 2-, 4-, and 6-electron reduction products in the molybdate series $H_2P_2Mo_{18}O_{62}^{-6}$ to $H_6P_2Mo_{18}O_{62}^{-6}$.

Method: The amount of total phenolics was determined using Folin-Ciocalteu reagent. After redissolving the lyophilized extracts in distilled water or DMSO, the solutions were centrifuged at 14,000 rpm for 10 min at 4°C. One hundred

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

microliters of extract (three replicates) were centrifuged at 12,000 rpm for 5 min and mixed with 100 µl of Folin-Ciocalteu's phenol reagent and 500 µl of 20% Na_2CO_3. The mixtures were shaken, 200 µl of Na_2CO_3 was added , and the solution was centrifuged at 14,000 rpm for 3 min and allowed to stand for 20 min in the dark at room temperature. The absorbance was read at 745 nm which yielded the maximum peak from spectrum scanning with UV-1601 PC Spectrophotometer (Shimadzu, Co, LTD, Kyoto, Japan). The standard curve was plotted using gallic acid. The amount of total phenolics was expressed as yield of each lyophilized extract (kg) equivalent to 1 mg of gallic acid (GAE).

2.8. Evaluation the activities of shallot extracts in U937 cell line

The activity of shallot on protection from oxidative stress in a human monocytic U937 cell line was studied via detection of intracellular glutathione and hydroperoxide formation.

2.8.1. U937 cell line

U937 cells were grown as a suspension in RPMI 1640 containing 2 mM L-glutamine, 10% (*v*:*v*) heat-inactivated fetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were subcultured at 1x 10^5 viable cells/ml every 2 to 4 days, and the cultures were maintained in 25-cm^2 tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2.

2.8.2. Cell culture experiments

Cell culture treatments were carried out in a sterile laminar flow hood. The cells were routinely cultured in 25-cm^2 flasks with vented caps. All cell cultures were in exponential phase when harvested. Cell concentrations were determined by counting the cells using a Neubauer improved haemocytometer under a microscope. Prior to counting, the cell suspensions were dispersed by pipetting. After counting the cell number, the required amount of cell culture was centrifuged in 50 ml tubes at 500xg for 5 min. The cells were subsequently washed with warm PBS; resuspended in PBS or RPMI 1640. The cells were used in three experiments. The cytotoxicity of shallot and garlic extracts on U937 cells was assayed using the MTT assay (Fig. 22.a)

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

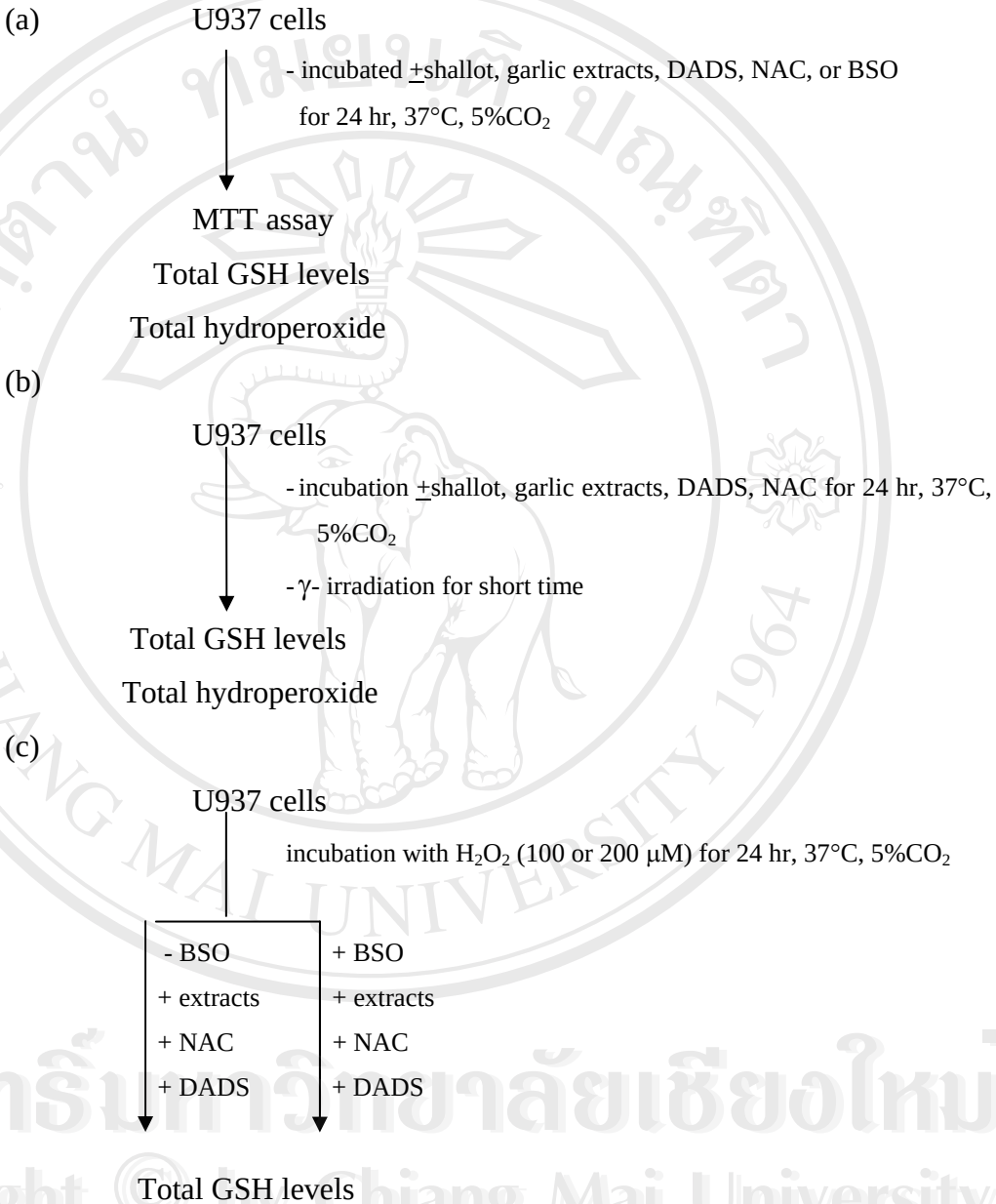

Figure 22. Protocols for studying the total intracellular GSH in U937 cells. Effects of shallot extracts were studied in 3 steps; (a) cell survival assay, (b) oxidation with γ- irradiation, and (c) GSH synthesis in cells.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

1. After treating cells with extracts, standard antioxidant or standard diallyl disulfide for 24 hr, the cells were irradiated with ^{60}CO gamma irradiation for 10-20 min (65 Gy/min). The total intracellular glutathione level was determined (Fig. 22.b)

2. The cells were pretreated with various extracts or standard DADS for 24 hr before oxidation with H_2O_2 at 100 or 200 μM. The levels of total glutathione were assayed (Fig. 22.c)

3. The mechanism of extract action on U937 cell glutathione synthesis and degadration was studied by depletion GSH by either BSO (50 μM) or H_2O_2 (200 μM) and then followed by GSH re-synthesis by adding extracts and incubating for 24 hr. The regained GSH was detected between the cells treated with extracts, compared with a low concentration of standard DAS (10-20 μM) or NAC (10-60 mM) (Fig. 22.c)

4. LPS-activation in U937 (monocyte cell line) and J774.2 (macrophage cell line) were selected as a model of inflammatory action studying using treated cells with the highest concentration of LPS at 1 μg/ml for 24 hr. The levels of nitric oxide production were evaluated in the medium, as well as the levels of total intracellular glutathione and total intracellular peroxide production.

2.8.3. MTT cell viability assay

Principle: The MTT assay provides an indirect measurement of mammalian cell survival and proliferation, based on the ability of mitochondria to convert the pale yellow substrate MTT into a dark blue formazan product that is measured spectrophometrically (Mosmann, 1983). Live growing cells have competent mitochondrial function and will actively convert MTT to formazan. Conversely dead or dying cells are unable to convert MTT to formazan.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

Method: The cells (3,000 cells/ 100 μl) were placed in the wells of a 96-well plate and incubated overnight at 37°C, 5% CO_2. Various concentrations of 0-500 μg/ml of extracts (100 μl) were added and incubated overnight at 37°C, 5% CO_2. The plates were centrifuged at 5,000 xg for 5 min, the old medium was removed, and 200 μl of new completed RPMI-1640 medium was added. Then 50 μl of MTT solution

(5 mg/ml) (final concentration) was added, the solution was incubated for 3-4 hr at 37°C, 5% CO_2. The plates were centrifuged at 5000 x g for 5 min, the supernatant removed, and DMSO (200 μl) added. After short incubation for 10 min and mixing, the absorbance at 590 nm was recorded.

Notes;

1. The cell controls were prepared by adding distilled water or DMSO (0.5% final volume).
2. The MTT assay were repeated three times; the results show the mean and standard deviation of cell survival (%) using following formula:

$$\frac{[\text{OD (extract-treated cells)} - \text{OD (control)}] \times 100}{\text{OD (control)}}$$

2.8.4. γ–Irradiation

In the beginning of the study, cells (3.5×10^5 cells/ml) in PBS were irradiated in 5 ml test tubes at room temperature, using ^{60}Co source with an average dose of 17 Gy/min. Accuracy of dose rates was measured by a Fricke dosimeter (O'Donnell & Sanger, 1970) and recalculated when necessary from standard decay tables. The cultures were saturated with O_2 to prevent the cells from becoming anaerobic. In a later experiment, cells ($1\text{-}2 \times 10^6$/ml) were irradiated in 60-mm petri dishes, and the depth of the medium kept within the range of 2-5 mm. There are three reasons for this stage; (1) A new ^{60}Co source with a total dose rate of 65 Gy/min has been used; (2) G-PCA-FOX assay has been developed, with the sensitivity about 5 times greater than the TCA-FOX used in the earlier phase; (3) Using petri dishes increased the surface area of medium which helps oxygen diffuse to the cells, increasing the yields of peroxides.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

2.9. Evaluation of the oxidative stress and inflammation in U937 cells of glutathione, total peroxide and nitric oxide system

The effect of Thai shallot extracts on inhibition of inflammation in U937 cells by H_2O_2 and lipopolysaccharide (LPS) was studied.

(1) Cells were pre- or post-treated with H_2O_2 plus shallot extract and incubated for 24 hr at 37°C, 5% CO_2. The levels of total intracellular GSH and total peroxide formation were determined via the GSH- reductase cycling and FOX methods.

(2) U937 cell was pre-treated with BSO (inhibitor of GSH synthetase) at 100 μM (cell survival more than 80 percent) and the GSH re-synthesized was determined.

(3) U937 Cells were treated with LPS (1 μg/ml) plus shallot extracts and incubated for 24 hr at 37°C, 5% CO_2. The nitric oxide, total intracellular GSH, and peroxide were determined by Griess reagent, GSH-reductase cycling and FOX methods, respectively.

2.10. Evaluation of the anti-oxidative stress and anti-inflammation of hexane extracts of shallot and garlic in U937 and J774.2 cells on glutathione synthesis and degradation.

(1) U937 cells were pretreated with H_2O_2 (200 μM) until intracellular total GSH depletion, then the shallot and garlic extracts from hexane extraction were added and incubated for 24 hr at 5%CO_2, 37°C.

(2) J774.2 cells were stimulated with LPS (1 μg/ml) and co-treated with extracts for 24 hr to evaluated the nitric oxide in the medium.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

2.10.1. Glutathione (GSH)-reductase cycling assay

Principle: GSH assay was performed by the DTNB reductase recycling method described by Tietze (1969).

$$\text{GSH} + \text{DTNB} \longrightarrow \text{TNB}$$

$$\text{GSSG} + \text{NADPH} \xrightarrow{\text{GSH Rx}} \text{GSH}$$

The increase in the absorbance at 412 nm was recorded for 1 min. Different concentrations of GSH were used to generate a standard curve, and the results were represented as the amount of GSH (pmol) per 10^4 viable cells.

Method: After cell treatment, the cells ($3.5x\ 10^4$ cells/ml) were separated by centrifugation at 500 x g for 5 min at 4°C, and washed with PBS (pH 7.4) once. The cells were precipitated with 3% PCA in a cold box for 5 minutes, and the clear supernatant was centrifuged (14,000 rpm for 5 min) at 4°C. The 20 µl of supernatant from centrifugation (500 x g) was mixed in 3% PCA (30 µl), phosphate–EDTA buffer (pH 7.5) (800 µl), H_3PO_4 solution (23 µl). The solution was gently mixed and 100 µl of NADPH and 50 µl of DTNB were added. Five µl of GSH reductase (5,000 unit/ml) were added, the tube was covered with paraffin before mixing. The change in absorbance at 412 nm was read within 3 min (kinetic program; read every 10 s, 18 times at 412 nm). The change in absorbance at 412 per min (ΔA/min) was recorded. The total intracellular glutathione was calculated by using a comparison with ΔA/min of standard glutathione (pmol) in 10^4 viable cells.

2.10.2. Perchloric acid -ferric-xylenol orange assay (Gay and Gebicki, 2003)

Method: Cells were precipitated with 5% PCA and centrifuged. The cell pellet was re-suspended with 745 µl of 6 M Guanidine hydrochloroide. The hydroperoxide was determined, by adding 40 µl of 0.5 M PCA, 10 µl of 5 mM ferrous ammonium sulphate and 25 µl of 5 mM xylenol orange and keeping the mixture in the dark for 30 min. The yellow color change was detected at 560 nm. The total protein hydroperoxide was calculated using a coefficiency of BSA-hydroperoixde ($\varepsilon = 36{,}000\ M^{-1}cm^{-1}$) (Gebicki, 1999). The percentage of scavenging of protein hydroperoxides was compared between the shallot extracts and other extracts (garlic extracts, commercial garlic) or standard NAC.

2.10.3. Nitric oxide assay (Promega protocol, 2005)

Principle: The Griess reagent system is based on the chemical reaction between sulfanilamide and N-(1-naphthlyl) ethylenediamine dihydrochloride (NED) and nitrate (NO_2^-) under acidic (phosphoric acid) conditions. Sulfanilamide reacts

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved

with nitrate to form sulfanilamide cation, in which NED is trapped to produce azo complex molecule that yield the maximal absorption between 550-650 nm (Fig. 23).

Method: One hundred μl of RPMI1640 were separated from cells by centrifugation at 3,000 rpm for 5 min at 4°C and placed into 1.5 ml micro-centrifuged tube. One hundred μl of Griess reagent that contained sulfanilamide and NED were added and the solution was kept in the dark for 20 min. The absorbance at 550 nm was recorded and the concentration of sodium nitrite ($NaNO_3$) calculated by comparison with a standard curve (Appendix). The sodium nitrite ($NaNO_3$) was used as a standard curve at 0-20 μM.

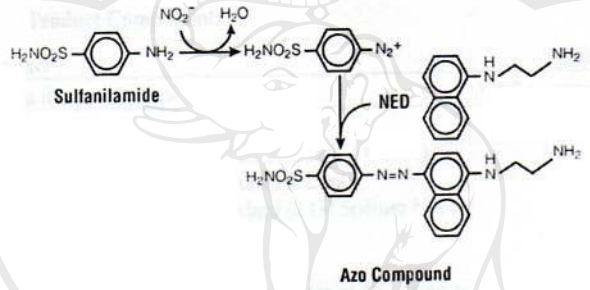

Figure 23. Azo compound formation in Griess reagent. Sulfanilamide captured nitrate to form stable nitrite and mixed with NED to produce the stable azo compound that detected at 550 nm.

2.11. Statistical analysis

All results were given as mean ± (SD). Comparative data were analyzed with mutivariables as post-hoc tests using Bonfferni adjusted significance values. Statistical analyses were performed using SPSS.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright © by Chiang Mai University
All rights reserved