Anti-Oxidative Stress and Anti-Inflammatory Effects of Thai Shallot (*Allium Ascalonicum* L.) Extracts in Human Monocytic (U937) Cells

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Oxidative stress is involved in many physiological and pathological phenomena, including human infection and inflammation. Chronic inflammation activates inflammatory cells to produce high concentration of oxidants and free radicals such as superoxide anions, nitric oxide, hydrogen peroxide, and hydroxyl radicals. These species can damage DNA, RNA, lipids, and proteins, leading to increased mutations and altered functions of enzymes and proteins and contributing to the multistage carcinogenesis process[1)]. H_2O_2, especially acts as a pro-oxidant and inflammatory modulator of oxidative stress and the inflammation process in monocytic U937 cell line. Lipopolysacharride (LPS) is also a modulator of the inflammatory process with the producition of nitric oxide (NO)[2)] and a tendency to progress to cancer.[1)] Most challenging studies about the protective and inhibitory effects of different medicinal plants on the oxidative stress and inflammation processes *in vitro* or *in vivo* models such as gingko, tomato, or antioxidant compounds like N-acetylcysteine (NAC). However, onion and garlic in the *Allium* family which demonstrate high antioxidant, anti-inflammatory and anti-cancer activities, have their usefulness in various diseases such as respiratory and nervous system diseases. Compounds in garlic extract: such as S-allyl cysteine (SAC) and S-allyl-mercaptocystein (SAMC) show antioxidant activity[3)] and glutathione detoxification[4)]. They also show anti-inflammatory effect by regulating the expression of nuclear factor kappa B (NF-B) in T lymphocytes[5)]. Onion extracts and quercetin can induce the GSH synthesis[6)]. Moreover, the diallyl disulfide in water- insoluble garlic extract inhibited the proliferation of various cancer cell lines[7)]. Shallot (*Allium ascalonicum* L.) is also classified in the *Allium* family and contains different sulfur and phenolic compounds. There have not been any reports about the activities of Thai shallot on oxidative stress and inflammation. This study was to evaluate the antioxidant activity of Thai shallot extracts on total intracellular GSH synthesis and peroxide formation in a monocytic cell line before and after gamma irradiation or H_2O_2 treatment. The anti-inflammatory effect of using lipopolysaccharide (LPS) stimulation followed by determination of the level of nitric oxide production in medium, was also studied.

Materials and Methods

Chemicals and Materials; RPMI 1640, fetal bovine serum (FBS), penicillin-streptomycin were purchased from GIBCO-BRL (Rockville, MD, USA), Nicotinamide adenine dinucleotide phosphate (NADPH), reduced glutathione (GSH), GSH reductase, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), xylenol orange (o-cresosulfonapthalein-

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3,3-bis sodium methyliminodiacetate)), glunidine hydrochrolide, DMSO (dimethyl sulfoxide), MTT (3,[4,4-dimethyl-2-thianolyl]-2,5-diphenyl-tetrazolium bromide), perchloric acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Diallyl disulfide (DADS) was supplied by Aldrich Chemical Co., Gillingham, Dorset UK). N-acetylcysteine (NAC) and H_2O_2 were obtained from Fluka Chemika (Switzerland) and Carlo Erba (Rodna) respectively. Lipopolysaccharide (LPS) and Griess reagent were kindly donated from Dr. Usanee's Laboratory, Department of Biochemistry, Faculty of Medicine, Chiang Mai University. U937 (monocytic cell lines) were provided by Department of Biological Sciences, Macquarie University, Sydney and Department of Immunology, Faculty of Associated Medical Sciences, Chiang Mai University. Tissue culture vessels were supplied by Department of Biological Sciences, Macquarie University, NSW, Sydney.

Preparation of crude shallot extract. Shallot bulbs from local agricultural farms (approximately one-month old after harvesting) and garlic bulbs from a local market in Chiang Mai were purchased. Three lyophilized preparations after mortar pressing and extracting with water or hexane (1:1. w/v) homogenized with an electrical blender and filtered. The mixture from the hexane-extract was settled overnight before filtering. The hexane layer was separated and hexane was then evaporated off before freeze-drying. From this working extract, solution was freshly prepared by reconstituting three lyophilized materials with de-ionized water and DMSO, and then centrifuged at 14,000 rpm for 5 min.

Monocytic cell culture (U937) treatment. Human monocytic cell line (U937) was grown in RPMI 1640 culture medium supplemented with 10% (v/v) FBS, penicillin (100 units/ml), and 100 μg /ml streptomycin at 37℃ in a 5% CO_2 in 95% air humidified incubator. The toxicity of shallot extracts after incubation for 24 hr was determined with 3[4,4-dimethyl-2-thianollyl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. The effect of shallot extracts at 50-250 μg/ml in U937 cells was studied by incubation for 24 hrs. and total intracellular glutathione (tGSH), total intracellular peroxide levels were evaluated. The action of shallot extracts on GSH synthesis in U937 cells was demonstrated by comparison with inhibitor of a GSH synthetase, butyl sulfoxide (BSO), and a precursor of GSH synthesis, N-acetylcysteine (NAC).

The antioxidant activity was studied by gamma irradiation of the cells with a Co^{60} source; afterwards cells were treated with shallot, garlic extracts, and NAC for 24 hrs. The level of tGSH was evaluated. Another model used H_2O_2 (200 μM) in the absence and presence of shallot extracts at 125-500 μg/ml, mixed in cells for 24 h and the level of total peroxide formation was evaluated.

The anti-inflammatory activity of shallot and garlic extracts was demonstrated by co-treatment of the cells with and without LPS (1 μg/ml) for 24 hrs. The levels of nitrate in the medium, intracellular peroxide and total glutathione were evaluated.

Total intracellular GSH and total peroxide were determined with Tietze's method[8] and guanidine HCl-xylenol orange (FOX) method[9]. Nitrite production in the medium was determined with Griess reagent[10].

Cell viability and toxicity assay. Toxicity of extracts and cell viability were evaluated with 3,[4,4-dimethyl-2 thianolyl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, after U937 cells (3,000 cells) in RPMI-1640 medium were seeded into 96-well microculture plates kept overnight at 37℃ in a 5% CO_2 atmosphere and shallot or garlic extracts (50-250 μg/ml) were added and the plates were incubated

overnight at 37℃ in a 5% CO_2 atmosphere. Cells were centrifuged (5000 rpm. for 5 min.) and media were replaced with new completed RPMI-1640 medium. MTT solution (5 mg/ml final concentration) was mixed in and incubation for 3-4 hr, then the reaction with DMSO and absorbance was measurement at 590 um with a spectrophotometer. The obtained values were compared with those of the control incubated with vehicle only, water or DMSO.

Total intracellular glutathione (GSH) determination. A method modified from Tietze's protocol[8] was used for determination of total intracellular GSH in U937 cells. After cells were exposed to the extracts at 50, 125, or 200 μg/ml levels for 24 hrs. at 37℃, and 5% CO_2, the cells were washed with phosphate buffer solution (pH 7.4) and centrifuged (500x g). The cells were lysed with 5% (v/v) perchloric acid. Lysates (50 μl) of the supernatant fraction of each sample were mixed with a buffer solution of 100 mM sodium phosphate, 5 mM EDTA (pH 7.4), 4 mg NADH/ml, DTNB, and 50 units/ml glutathione reductase. The rate of change of absorbance at 412 nm/min was monitored with a spectrophotometer every 10 sec. for 180 sec. The concentration of total intracellular glutathione in samples was determined from the standard calibration curve from reduced GSH (Sigma) and represented in pmol/10^4 viable cells.

Total intracellular peroxide evaluation. The cellular peroxide level was measured after precipitating cells with 2 M cold PCA and dissolving in 0.6 M guanidine HCl. Five mM of xylenol orange and a ferrous solution of 110 mM PCA were added, then incubated at room temperature for 30 min. and the color developed was read at 560 nm. The concentration of peroxide was calculated using molar absorptivity (36,000 M^{-1}, cm^{-1}). The data represented the yield of total intracellular peroxide in 5X10^5 cells.

NO_2 measurement.

The production of nitric oxide (NO) was assessed as the accumulation of NO_2 in the culture supernatant, using a colorimetric reaction with Griess reagent. Briefly, after stimulation for 24 hrs. with LPS (1 μg/ml), with and without shallot extracts, the culture supernatants were collected and mixed with equal volumes of Griess reagent [0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide, and 5% H_3PO_4 for 10 min. The absorbance at 550 nm was measured in an automated plate reader. The NO_2 concentration was determined from a sodium nitrite standard curve (0-50 μM).

Statistical analysis. Data was presented as mean ± SD and ANOVA for statistic analysis with $p< 0.05$ significant level.

Results

Cell survival

From triplicates of the MTT assay, the percentage of cell survival with the addition of shallot extracts at 250, 150 and 50 μg/ml in U937 cells were 80, 90 and 95% respectively (Fig 1.a). With garlic extracts, they were 70%, 80% and 95% respectively (Fig.1.b).

Shallot extracts affects total glutathione and hydroperoxide formation in the U937 cells

After incubation of U937 cells with shallot extracts at 50, 125 and 250 μg/ml for 24 hrs., the total intracellular glutathione were significantly increased in shallot extract (crude) and water extraction, compared to hexane-extracted shallot and cell control (Fig 2.a) ($p<0.05$). This activity was clearly seen after incubating the cells with shallot extract for 6 hrs. or until 24 hrs. The shallot extract from crude also reduced total intracellular peroxide formation compared to cell control or cell treated garlic extracts. This phenomenon was similar to the effect of N-acetylcysteine (NAC). (Fig 2.b).

Mechanism of shallot action involving on GSH synthesis

This study confirmed the mechanism of

induction GSH synthesis by crushed shallot extract by using N-acetylcysteine (NAC), that is a precursor in GSH synthesis and BSO that is an inhibitor of GSH synthetase (Fig 3). As a preliminary, it was found that NAC at 3.2, 6.4, and 12.8 mol/l dose-dependently increased GSH content (235.62 ± 8.1, 250.0 ± 34, and 308.12 ± 1.72 pmol/10^4 viable cells) compared with the control (235.00 ± 8.16 pmol/10^4 viable cells). GSH concentration was reduced by dose-response reduction of GSH synthesis by BSO (100, 200, and 400 μM) (observed in 235.00 ± 8.16 to 82.45 ± 5.7, 52.82 ± 1.53, and 7.03 ± 1.23 pmol/10^4 viable cells respectively) (Data not shown).

In addition, this study also tested the divergent effects between crude shallot extract (250 μg/ml) and NAC (6.4 mol/L) and BSO (200 μM) on GSH synthesis. It was found that the cells incubated with crude shallot extract plus NAC had slightly higher activity compared with only crude shallot extract (343.56 ± 7.16 and 323.23 ± 6.34 pmol/10^4 viable cells), and with the addition of NAC and crushed shallot extract. Both showed a significantly higher activity compared with cell controls (225.67 ± 7.81 pmol/10^4 viable cells) ($p<0.05$). The tGSH level in the cells treated with BSO and shallot extract (20.78 ± 3.45 pmol/10^4 viable cells) (Fig 3) was reduced significantly compared with cell controls ($P<0.01$).

Protective effect of gamma-irradiated shallot extract.

The shallot extract (crude) at 125 μg/ml had a significant protective activity on intracellular GSH depletion (141.44 ± 16.37 pmol/10^4 viable cells) from hydroxyl radical-generated by Co^{60} irradiation for 20 min., compared with non-irradiated cells, irradiated cells, 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)($p<0.05$).

Effect of shallot extract on H_2O_2-induced intracellular peroxide formation

Using monocytic cells ($5x10^5$ cells) stimulated with H_2O_2 (100-200 mM) as a model of the inflammation process, total intracellular hydroperoxide production was responded to H_2O_2 stimulation (100 μM) after incubation for 24 hrs. (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). Shallot crude extract showed activity in anti-hydroperoxide formation with dose response (125-500 μg/ml) compared to H_2O_2-stimulated U937 cells and the activity was similar to NAC (6.4 mol/l). (Fig 5)

Lipopolysaccharide (LPS) and nitric oxide formation

The activity of LPS (1 μg/ml) on nitric oxide (NO) production in RPMI-1640 medium of U937 cells ($5x10^5$ cells) was detected as nitrate formation after incubation at 37℃ in 5% CO_2 for 24 hrs. Crude extract of shallot at 250 μg/ml inhibited nitric oxide formation in the cells, compared with shallot extracts using water and by using hexane with NAC (6.4 mol/l) (Fig.6.a) with a time-dependent manner (5-24 h) (Fig 6.b). Moreover, crude shallot extract also protected the total glutathione in the cell more efficiently than other extracts (Fig 6.c)

Discussion

There is a lot of evidence involving high potential capacity of aged garlic extract (AGE) on controling inflammation and bacterial growth. These include the inhibition of cancer cell progression by the active sulfur compound: allyl sulfide or S-allyl cysteine[3]. Some evidence showed that aged garlic extract (AGE) exhibited a scavenging activity on H_2O_2[4] and a protective effect on lipid peroxidation in low density lipoprotein (LDL) by chelating with copper ion (Cu^{2+})[11].

From our previous studies, Thai shallot extracts (by using hexane) showed a higher antioxidant

capacity by scavenging ABTS cation radicals compared with garlic extracts and garlic capsule commercials. Moreover, shallot extracts also protected the liposome from auto-oxidation at 37℃[12]. In addition, it had protected protein and amino acids from gamma-irradiation and had a high potential protection of glutathione from destruction protein hydroperoxide *in vitro*[13].

Oxidative stress and glutathione

Intracellular GSH redox homeostasis is strictly regulated to govern cell metabolism and protect cells against oxidative stress. Increasing GSH is for detoxification of peroxides such as hydrogen peroxide and lipid peroxides[14]. Glutathione elevation protects mechanism against hydroxyl radical-induced protein oxidation in rat brain from Fe^{2+}/H_2O_2 stimulation[15].

Shallot and GSH synthesis.

This study demonstrated that shallot from crude and water extraction increased the total intracellular glutathione in the U937 cells with dose response (Fig 2.a) and reduced the total intracellular hydroperoxide (Fig 2.b).

The rationale that shallot extracts can reduce peroxide and increase tGSH in U937 cells can be explained from previous evidence which state that the important compounds in the *Allium* family (including onion, garlic or shallot) are various, but were mainly phenolic compounds with free hydroxyl group (-OH)[16]. In addition, furostane, saponins, quercetin, isorhametin and various glycosides are present in shallot (*Allium ascalnicum* L.)[17]. Moreover, it has been reported that the flavonoid compounds in extract, quercetin, kaempferol and apigenin increased reporter gene activity driven by the γ-glutamylcysteine synthetase heavy subunit (GCSh) promoter. Thus they can stimulate GSH synthesis in the cells[6]. In this study, we added a precursor of GSH synthesis, N-acetylcysteine (NAC) or standard diallyl disulfide (DADS), into the cell mixtures and found that NAC induced tGSH in the U937 cells (Fig 2.b). Possibly, it is a cystiene donating compound which is a cellular precursor of GSH, and interacts directly with oxidants[18]. A previous report showed that lipid peroxide (LOOH) acted as a pro-oxidant in U937 cells and caused depletion of GSH and inactivation of antioxidant enzymes, consequently leading to a decrease in survival and oxidative damage of DNA[19]. NAC strongly inhibited ricin-induced apoptotic cell death in U937 cells and prevented a decrease in cellular glutathione[20]. Shallot extract may act as a precursor of GSH, like NAC. To prove the co-activity of shallot extract with a precursor of GSH synthesis like NAC (6.4 mol/l), a specific inhibitor of glutathione biosynthesis in the U937 cell glutathionine sulfoximine (BSO) (200 μg/ml) compared with only shallot extract and cell control (Fig 3.a). This study indicated that the activity of shallot extract on GSH synthesis was through the GSH synthetase in the U937 cell.

Antioxidant capacity of shallot extract was demonstrated by incubating the cells with the extracts before irradiation. Theoretically, gamma-irradiation from Co^{60} will increase the hydroxyl radical and hydrogen peroxide in medium or aqueous part of the cells. Both radicals react with lipid or protein to form lipid or protein hydroperoxides using the equations below[21]

$$PrH + OH^{\bullet} \longrightarrow Pr^{\bullet} + H_2O$$
$$Pr^{\bullet} + O_2 \longrightarrow PrOO^{\bullet}$$
$$PrOO^{-} + e^{-} \longrightarrow PrOO^{-}$$
$$PrOO^{-} + H^{+} \longrightarrow PrOOH$$
$$PrOO^{\bullet} + PrH \longrightarrow PrOOH + Pr^{\bullet}$$

In this study, the shallot extract could inhibit tGSH depletion and total hydroperoxide formation in the cells (Fig 4). The active compounds of shallot extract either containing phenolic or sulfur group were expected to trap the hydroxyl radical or hydroperoxide directly[12].

Shallot extract and anti-oxidative property.

This study used H_2O_2 as a stimulant and a pro-oxidant in cells, includes human monocytic

cells. Hydrogen peroxide is capable of damaging proteins and unsaturated fatty acids[22]. Preliminary experiments showed the influence of H_2O_2 on hydroperoxide formation in U937 cells with dose response between 100 and 200 μM after incubation at 37℃ in a 5% CO_2 atmosphere for 24 hrs. It was found that the inhibitory activity of Thai shallot from crude extract on hydroperoxide formation was dose dependent (125-500 μg/ml) (Fig 5), similar to the activity of NAC.

Another study showed that H_2O_2 induced the inflammation process in various cell lines including monocytic cell line like U937. Meyer and colleagues[23] showed that certain transcription factors of the NF-kB/rel family can be activated not only by receptor-targeted ligands but also by direct application of oxidizing agents such as H_2O_2 or ionizing radiation[24]. Subsequently, several other protein kinase cascades and transcription factors have been discovered to possess redox-sensitive elements. NF-kB is activated upon phosphorylation of an inhibitory subunit (IkB). H_2O_2, acting as a second messenger to stimulate protein kinase cascades coupled to inflammatory gene expression, is synthesized endogenously in certain cell types as a response to activation by specific cytokines or growth factors[25].

H_2O_2 formed during gamma-glutamyl transpeptidase (GGT) ectoenzymatic activity appears to stimulate and/or maintain U937 cell proliferation, which is inhibited by antioxidant treatment[26]. Thus, Thai shallot not only inhibited total hydroperoxide formation but also scavenged H_2O_2, likely depressing the proliferation of leukemia cells (U937).

Shallot extract and anti-inflammation.

Chronic inflammation is associated with increased risk of human cancer at various sites from inflammatory cells such as monocytic cells[1]. Many enzymes such as nitric oxide synthase, myeloperoxidase or eosinophil peroxidase produce high concentrations of diverse free radicals as nitric oxide, nitroxyl, nitrogen dioxide, and hydrogen peroxide. They can damage DNA, RNA, lipid, and protein by nitration, oxidation, chlorination, and bromination reactions, leading to increased mutations and altered functions of enzymes and proteins involving multi-stage carcinogenic processes. LPS-stimulated U937 increase the release of cytokine and interleukin-10[27].

The activity of Thai shallot extracts on the inflammatory process was studied by using an inflammatory stimulator; endotoxin lipopolysaccharide (LPS). LPS can elicit inflammatory reaction both *in vitro* and *in vivo*[28]. Nitric oxide (NO) is produced from a guanido group of L-arginine by three NO synthase isotypes[29]. Inducible NOS (iNOS) is particularly typically expressed in response to proinflammatory stimuli[30]. Though NO produced in inflammatory cells such as T-cells or macrophages is a free radical, it is not a strong oxidant and will be converted to more reactive species such as peroxynitrite and nitrogen oxides (NOx) by reactions with oxygen *via* a strong nitrodating agent (N_2O_3). N_2O_3 deaminates various DNA bases and can react with secondary amines to form caricinogenic N-nitrosamines[1]. As a result, the high to low inhibitory activity of shallot extract on NO production was observed in crude, water-, and hexane-extractions respectively. (Fig 6.a). The shallot extract had a maximum activity at 5 hrs then gradually decreased until 24 h compared with LPS-stimulated cells and control cell (Fig 6.b). In addition, shallot extract (crude) was able to inhibit intracellular hydroperoxide formation (data not shown) and induce total glutathione synthesis in the U937 cells (Fig 6.C)

Earlier evidence show that antioxidant and anti-inflammatory activities of garlic on various cells lines were caused by different sulfur compounds. Garlic extract composed of S-allyl

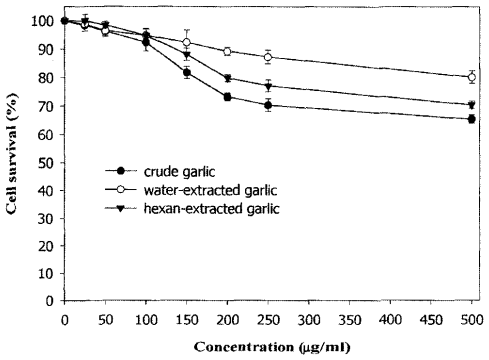

Figure 1.a

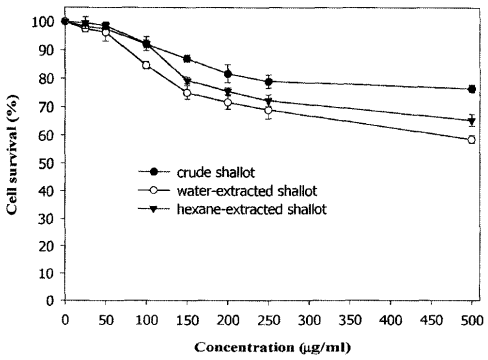

Figure 1.b

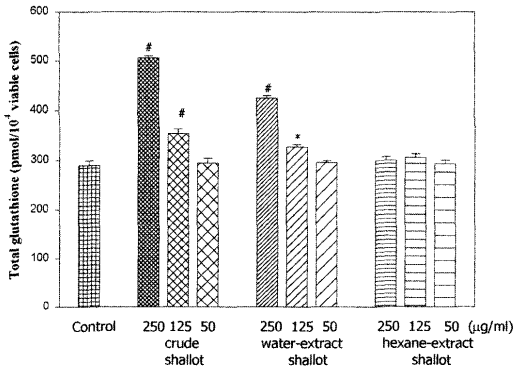

Figure 2.a

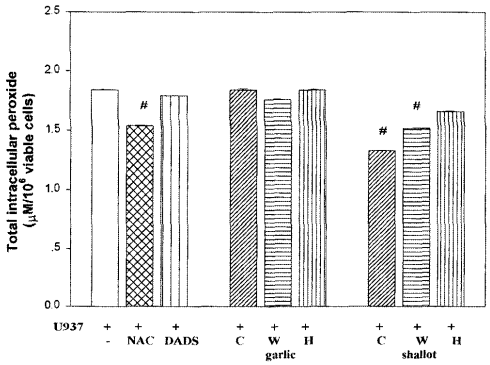

Figure 2.b

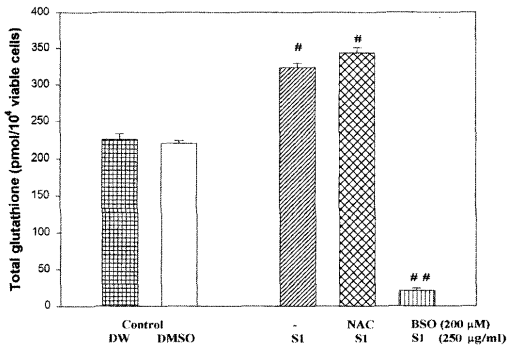

Figure 3.

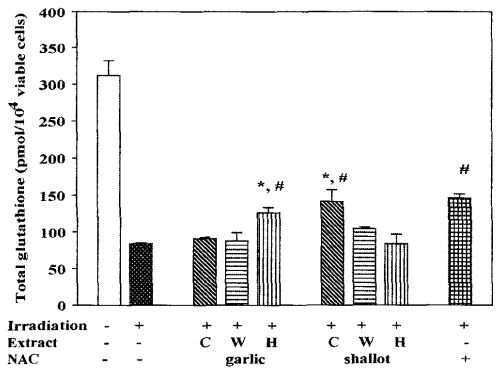

Figure 4.

cysteine inhibited NO production in peritoneal macrophages[31]. S-allyl cysteine (SAC) inhibited NF-kB activation in human T-lymphocytes when induced by H_2O_2[32]. Diallyl disulfide containing allyl and sulfur at 100 and 500 μM showed a significant growth inhibition of human cancer cell lines of lung (A549) and skin (SK MEL-2)[7].

Here, we can conclude that Thai shallot from a crude preparation has an antioxidant and anti-inflammatory activity involving an important intracellular antioxidant, glutathione (GSH) synthesis that controls the intracellular peroxide and nitric oxide formation.

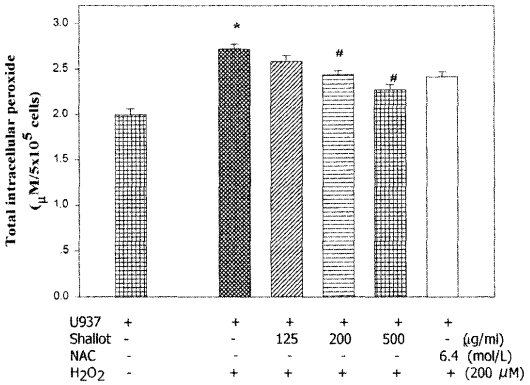

Figure 5.

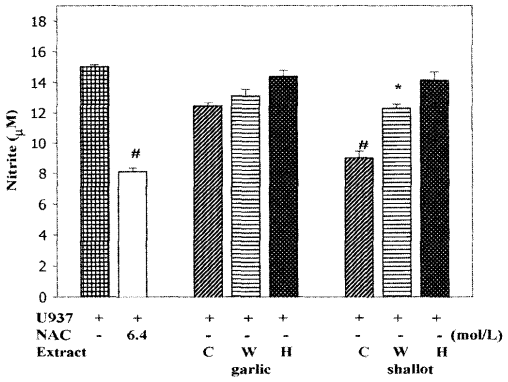

Figure 6.a

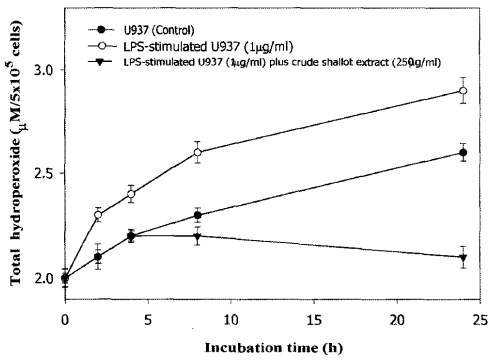

Figure 6.b

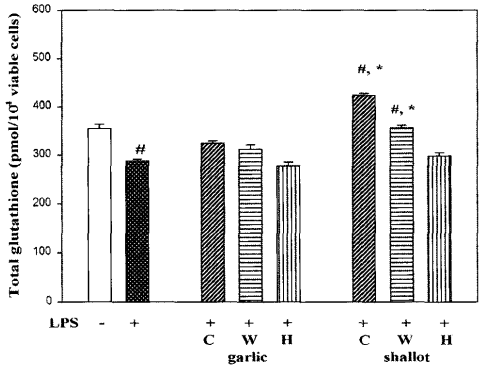

Figure 6.c

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Figure captures

Figure 1. The percent of cell survival of U937 cell line after incubation with garlic (a) or shallot (b) extracts at 25-500 μg/ml prepared using crude, water-, and hexane-extraction at 37℃, 5%CO_2, 24 h. Each point represents mean ± SD from triplicates and three repeated experiments.

Figure 2. (a) 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 hrs. at 37℃, 5% CO_2. (b) Total intracellular hydroperoxide or peroxide formation in U937 (between cells untreated and treated with 6.4 mol/l N-acetylcysteine (NAC), 250 μM diallyl disulfude (DADS), garlic and shallot extracts at 125 μg/ml. Each bar represents the mean ± SD from five samples; $^{\#}$, $p < 0.01$ and *, $p < 0.05$ compared with cell control.

Figure 3. The co-activity of crushed shallot extract (S1) with N-acetylcysteine (NAC) at 6.4 mol/L (precursor of GSH synthesis), BSO at 200 μmol/L (inhibitor of GSH synthetase) and only crushed shallot extract at 250 μg/ml on total GSH synthesis in U937 cell line after incubation at 37℃, 5%CO_2 for 24 h. Each bar represents the mean ± SD of total glutathione (pmol/104 viable cells), $^{\#}$, $p< 0.05$ compared with cell control (DW&DMSO), $^{\#\#}$, $p< 0.01$ compared with cell control, S1 only and S1 plus NAC.

Figure 4. The protective activity of 250 μg/ml shallot extracts (C = crude, W = water extraction, and H = hexane extraction) compared with garlic and NAC (6.4 mol/L) after incubation with cells for 24 hr and gamma-irradiation (65Gy/min) for 20 min. Each bar represents the mean ± SD from five samples; *, $p< 0.05$ compared with irradiated control cells, $^{\#}$, $p< 0.05$ compared with all garlic extracts or water-, hexane extracted shallot.

Figure 5. Total intracellular peroxide formation in U937 cell lines from H_2O_2 (100 μM), and inhibitory activity of crude shallot extract at 125-500 μg/ml compared with N-acetylcysteine (NAC) at 6.4 mol/L after incubation at 37℃,

5% CO_2 for 24 h. Each bar represents mean ± SD from five samples; *, $p< 0.05$ compared with H_2O_2-untreated cells, and #, $p< 0.05$ compared with H_2O_2 -treated cells.

Figure 6. (a) The inducible activity of lipopolysaccharide (LPS) at 1 μg/ml on nitric oxide production in RPMI-1640 medium by U937 cells compared with LPS-treated cells, and LPS-treated cells plus NAC (6.4 mol/L), garlic, and shallot extracts (C = crude, W = water, H = hexane extraction) at 250 μg/ml, (b) the kinetic response of nitric oxide production at 0-24 hrs. during incubation at 37℃, and (c) the total intracellular glutathione response after incubation the shallot and garlic extracts in LPS-stimulated U937 for 24 hrs. Each point represents the mean ± SD from triplicates, and twice-repeated experiments, * $p< 0.05$ compared with cells in presence hexane-shallot extract, # $p< 0.05$ compared with LPS-stimulated cells.