# CHAPTER IV

# DISCUSSION

## 4.1. Total antioxidant capacity (TAC) in shallot extracts

ABTS cation radical ($ABTS^{\bullet+}$) generated by persulfate is a target organic radical for testing the antioxidant capacity in this study. This method was simple and easy to assay without interference of any compound in system as $H_2O_2$-metmyoglobin ABTS protocol (Miller, 1998). Generation of the nitrogen centered radical of ABTS radical cation by $H_2O_2$ and peroxidase in metmyoglobin is very complex and $H_2O_2$ residue in system may be interaction to sample test to give positive result. Increasing of blue-green color of ABTS was assayed with kinetic program and area under curve was applied to calculate the antioxidant capacity. Re and co-worker (1991) has been developed the procedure for determining the antioxidant capacity using ABTS and oxidation with ammonium persulfate. Simple protocol is performed by production of the stable ABTS before adding the sample test. The antioxidant capacity will be calculated from the decreasing of absorbance at 734 nm with non-kinetic program. The antioxidant capacity of sample can be determined from the percentage of reduction or initial rate of reduction as mentioned in this study. Total antioxidant capacities of shallot and garlic extracts were determined and compared to standard antioxidants; Trolox, BHT, and gallic acid (Re *et al*., 1991). In this study, it was found that water and hexane shallot extracts had the highest initial rates of reduction, whereas the commercial aged garlic extract (AGE) powder showed a high initial rate on Immunytop (Thailand) and low initial rate on Kyolic (New Zealand). In further experiment designed to examine the activity of shallot extracts on scavenging $H_2O_2$, it was found that hexane extract of shallot showed the highest activity at 100 or 200 μg/ml. Previous study showed that AGE was able to scavenge $H_2O_2$ (Prasad *et al.*, 1996) more efficient than garlic extracts. Allicin, SAC, and diallyl polysulfide in garlic extracts were able to scavenge free radical directly (Imai *et al*., 1994).

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## 4.2. Effects of shallot extracts on protein and lipid hydroperoxide

Proteins and lipids may interact with hydroxyl radicals before either RNA or DNA. Protein in blood serum has also been shown to be oxidized by hydroxyl radicals generated by gamma irradiation (Gebicki, 1997). Therefore, the activity of shallot and garlic extracts on protein hydroperoxide formation by gamma irradiation was considered. The bovine serum albumin (BSA) was used as a target protein by Gebicki and co-workers (1993). This study used irradiated BSA at 5 mg/ml for 15 minutes to produce the hydroperoxide about 84.0-96.6 μM. The decomposition of hydroperoxide proceeded quite slowly. It has been found that the hydroperoxide in control showed very slow decomposition, down to 76% from 100% within 2 hours. The activities of shallot and garlic extracts inhibited protein hydroperoxide in BSA, as well as lipid hydroperoxide formation in linoleic acid solution after irradiation. The shallot extract also showed scavenging activity on lipid and protein hydroperoxide after irradiation in liposome or BSA from auto-oxidation. Moreover, in this study, proline was used to be a target amino acid for producing proline hydroperoxide because of its presence in BSA, hemolgobin and myoglobin could be oxidized by $HO^{\bullet}$ (Dean *et al*., 1997). From preliminary study, standard antioxidant; N-acetylcysteine (NAC) at 0.26 mM and gallic acid at 0.5 mM reduced the BSA-OOH formation from gamma irradiation (1446.8 Gy) distinctly after incubation for 15 min (50.47% of control) and 120 min (44.19% of control), respectively. Gebicki and co-worker (1993) showed that glutathione (GSH) was reduced to 66% by BSA-OOH generated by irradiation (1000 Gy).

Liposome and linoleic acid were used to study the lipid hydroperoxide formation. Lipid hydroperoxide formation, 40% ethanol solution was irradiated and produced hydroxyethyl radicals ($CH_3{}^{\bullet}CHOH$), and oxidized linoleic acid to generate the hydroperoxide (Navasumrit *et al*., 2000).

The result of this study gave similar result as previous studies in aged garlic extract (AGE) that showed protective activity on lipid peroxidation formation in the various models, as on LDL from $Cu_2SO_4$, in erythrocyte suspension (5% hematocrit) from tert-butyl hydroperoxide (tert-BuOOH) at 0.6 mM, or on liposome from $FeSO_4$, AAPH and AMVN stimulation (Gebicki, 1999). The results showed the reducing

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activity of all extracts at high concentration (1.0 mg/ml) from liposome auto-oxidation during incubation at 37°C for 3 days. All extracts inhibited lipid hydroperoxide formation.

## 4.3. Effects of shallot extracts on protection the GSH in human erythrocyte from AAPH oxidation.

The human erythrocyte was used as a model and oxidized with organic thermal oxidant, AAPH, is a water-soluble azo compound. AAPH radical attacked to the erythrocyte membrane.

Although there was evidence showing the high potential capacity of aged garlic extract (AGE) to control inflammation, bacterial growth, and inhibit cancer cell progression by active sulfur compound, allyl sulfide or S-allyl cysteine (Imai *et al.*, 1994). It also scavenged $H_2O_2$ (Prasad *et al.*, 1996), and protected lipid peroxidation formation on low density lipoprotein (LDL) by chelating the copper ion ($Cu^{2+}$) (Dillon *et al.*, 2003).

This result showed the ability of shallot extract on protection of the erythrocyte hemolysis as in the previous study in age garlic extract (AGE) (Moriguchi *et al.*, 2001). Moreover, the polyphenols in green tea such as epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG), and gallic acid (GA) were able to suppress the erythrocyte hemolysis from AAPH-oxidation. (Lanping *et al.*, 2000). This study showed that shallot extract protected the lipid peroxidation and glutathione depletion in AAPH-oxidized erythrocyte.

These results supported the previous data on concerning the shallot extracts and garlic extracts to prevent protein and amino acid hydroperoxide formation from irradiation and glutathione decomposition by protein hydroperoxide *in vitro*. GSH depletion is not only important in human erythrocytes for controlling thiol proteins and reducing free radicals, but also protects against hydroxyl free radical-induced protein oxidation in the brain (Pocernich *et al.*, 2000).

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## 4.4. Bioactive compounds

Garlic has been shown to contain compounds that have potential benefit for health including steroidal glycosides (Matsuura *et al*., 1998), lectin (Kaku *et al*., 1992), prostaglandins, fructan, pectin, essential oil, adenosine, vitamins B-1, B-2, B-6, C and E, biotin, nicotinic acid, fatty acids, glycolipids, phospholipids, anthocyanins, flavonoids, phenolics and amino acids, (Fenwick and Hanley, 1985).

Total phenolics in water extract of shallot was also higher (4,599±88 GAE mg/kg) and slightly than in hexane extraction (5,413±62 GAE mg/kg), both extracts were also higher than in water extract of garlic (3,804±77 GAE mg/kg). Crude extract of shallot (4,086±84 GAE mg/kg) contained the lowest amount of total phenolics, similar to in the garlic extract (4,102±193 GAE mg/kg) (Table 5).

Garlic extract contains various sulfur compound both polar and non-polar compound (Yin *et al*., 2002). This study identified four diallyl sulfides using HPLC from the retention time as follows diallyl monosulfide (4.62 min), a main diallyl disulfide (major peak at 5.15 min and minor at 8.58 min) and tri-sulfide at 6.6 min, respectively. The total concentration of only dominant diallyl disulfide (DADS) in hexane extract of shallot was 325.5±42.17 μg per gram of extract, compared with hexane extract of garlic (283.5±28.12 μg). A previous study showed that garlic produced from steam distillation contained mono-, di- and tri-sulfides (Lawson *et al*., 1991) as was found in the hexane garlic extract in this study. The large onion was used to comparing the sulfur compounds that showed very high levels of diallyl disulfide (1864.48±3.23 μg). Yin and co-worker (2002) indicated that organosulfur compounds had antioxidant property via their reducing power and interactions with biomembranes.

S-allycysteine (SAC), also known as allicin is odorless compound with antioxidant capacity (Ide *et al*., 1997). It can inhibit cancer progression (Amagase and Milner, 1993), protect the liver from toxins (Nakagawa *et al*., 1998), and reduce cholesterol (Yeh and Yeh, 1994).

Diallyl disulfide (DADS) is an oil-soluble organosulfur compound that inhibits the proliferation of human tumor cells; HCT-15, A549 (lung), and SK MEL-2 (skin), compared with water-soluble compound S-allyl cysteine (SAC) by controlling

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the calcium homeostasis (Sundaram *et al*., 1996). Diallyl disulfide and diallyl sulfide appeared to be the bioactive components in garlic that exert the anticarcinogenic effects (Sumiyoshi *et al*., 1989; Fukushima *et al*., 1997). These allylic compounds stimulates glutathione S-transferase activity in the liver by detoxifying carcinogens (Hirsch *et al*, 2000). Moreover, organosulfur compounds in garlic extract scavenge free radical and inhibit lipid peroxidation (Sumiyoshi *et al*., 1989; Imai *et al*., 1994).

This study found allicin in fresh onion and garlic juices, but not in Kyolic (commercial garlic from NZ), shallot or garlic extract (crude and water extraction). This was presumably because allicin is unstable and decomposes rapidly (Amagase *et al*., 2001).

Prasad and co-workers (1995) showed that allicin in garlic was able to decrease OH adducted products due to scavenging of OH radicals and not by scavenging of the formed OH adducted products (2,3-dihydroxybenzoic acid; DHBA).

Nuutila and coworkers (2003) measured the total phenolics in edible part of giant onion (845±104 mg GAE/kg), yellow onion (1,550±90.5 mg GAE/kg), red onion (2,075±147.0 mg GAE/kg) and garlic (115±12.9 mg GAE/kg) (Finnish giant). They also measured various quercetin contents in onion (7 mg to 83 g per 1 kg of lyophilized plant extract), especially in edible portions of different onions, such as giant onion (85.00±12.42 mg/kg), red spring onion (1,274.00±375 mg/kg), yellow onion (1,080±41 mg/kg), and red onion (1,926.00±266.00 mg/kg). Moreover, phenolic compounds such as allicin had antioxidant activity (Ide and Lau, 1997), as did N-fructoxyl glutatmate, N-fructoxyl arginine (O'Brien and Gillies, 1998) and selenium, as well as organosulfur compounds.

Previous data showed total phenolics in 80% methanol extract of onion and red onion were 2.5±0.1 and 3.0±0.1 mg GAE/g dry weight from lyophilization, respectively (Kahkonen *et al*., 1999) comparable to the yield from onion extracts in this study. The flavonoids that contain multiple hydroxyl substitutions showed anti-peroxyl radical activities stronger than Trolox (Cao *et al*., 1997).

Aged garlic extract (AGE) showed an antioxidative effect on scavenging superoxide ions and reduced lipid peroxide formation in a cell free assay, and was able to chelate copper ions (Dillon *et al*., 2003). Shobana and co-workers (2000)

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showed that the high potential antioxidant of onion extract was related to flavonoids; quercetin by inhibition the lipid peroxidation of linoleic acid. Moreover, AGE has ability on protection of DNA damage (Boyle *et al*., 2000), reduction of cardiovascular disease (Janssen *et al*., 1998), inhibition of the growth of transformed-tumorigenic cells (ras/3T3 and H359), prevention of the neoplastic transformation of NIH/3T3 cells, (Leighton *et al*., 1992) and reduction of carcinogenic activity (Steniner, 1997). But no evidences demonstrates the comparative activities of garlic and shallot extracts.

This study investigated the relative antioxidant abilities of onion and garlic extracts. Onion showed the highest antioxidant activity, and contained high levels of total phenolics in crude and water extracts. Garlic extract showed less antioxidant activity than onion extract. The hexane extract of Thai shallot showed high antioxidant activity as well as in the hexane extract of onion. The compounds in hexane extract of shallot were composed of total phenolics, and diallyl disulfide compound at a lower concentration than in onion extract as shown in Table 5 and 6.

Previous reports indicated that the active compounds in the onion group were phenolic substances. The antioxidant capacity of plants corresponded to the amount of total phenolics. Red onion (*Allium cepa* L.) had a high concentration at 300 mg GAE from 100 mg of dry extract. The result of this study was consistent with the study of Terrance (1992). who found that the flavonol level in the edible portions of leeks, shallots, green onions, garlic, and onions were inconsistency (less than 0.03 to more than 1 g/kg). Shallots has high concentration of total flavonols, more than 800 mg/kg in each of 5 independent samples. Onions varied widely in flavonoid content more than 1000 mg/kg. This study confirmed that the total phenolic content in hexane extract of onion (8734±258 GAE mg/kg) compared with crude (2283±16 GAE mg/kg), and water extraction (332±69 GAE mg/kg).

Lucienne and Annick (1967) found that the level of flavonoid compounds in alcohol extracts of the external brown, paper-like layers of the bulb of the shallot were quercetol and the glucosides of spireoside and the 3,4'-diglucoside. The 7,4′-diglucoside and quercetol are found in onion. The total amount of flavonoids was also higher in shallot than in onion (20% compared to 8%). In shallot the concentration of

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total flavonoids was highest in the outer layers and decreased toward the center of the bulb (20% to 1%).

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**Correlation of total phenolic substances and antioxidant activity**

Shallot had the highest reducing property and total amount of phenolic substances. Therefore, the correlation between total phenolic substance and reducing property at three concentrations, 100, 200 and 500 μg/ml was tested. Xiaolin and co-workers (2001) showed that there was a positive correlation between antioxidant activity and water-soluble total phenol content in 38 fruits and vegetables gathered from South of China. The correlation between the antioxidant activity and total water soluble- phenolic content of all samples was r = 0.47 and correlations of antioxidant activity to anthocyanin, chlorophyll and flavonoid were r = 0.58, 0.59 and 0.81 respectively. This study showed a positive correlation between total water-soluble phenolic compounds and reducing capacity in shallot extracts.

The correlation between total phenolics and initial rate of reduction in garlic extracts were as followed; crude, (r = 1.00), water (r = 0.999), and hexane extraction (r = 0.995). In shallot extracts these were: crude (r = 0.999), and hexane extraction (r = 0.980). The correlation between the total phenolic compounds and percentage of reduction, either in aged garlic extract (AGE) or in commercials garlic extracts (Immunytop (Thailand) or Kyolic (New Zealand) was not found in this study.

## 4.5. Effects of shallot extracts on oxidative stress and inflammation in U937 cells

It was suggested that aged garlic extract (AGE) can control inflammation, and bacterial growth. This includes the inhibition of cancer cell progression by active sulfur compounds, allyl sulfide or S-allyl cysteine (Imai *et al*., 1994). Some studies have shown that aged garlic extract (AGE) is able to scavenge $H_2O_2$ (Prasad *et al*., 1996) and prevent lipid peroxidation in low density lipoprotein (LDL) by chelating copper ion ($Cu^{2+}$) (Dillon *et al*., 2003).

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Thai shallot extracts (hexane extraction) demonstrated a higher antioxidant capacity by scavenging ABTS cation radicals compared with garlic extracts and aged garlic extract (AGE) capsules. Moreover, shallot extracts also protected the liposomes from auto-oxidation at 37°C. In addition, they protected proteins and amino acid from damage via gamma-irradiation and glutathione from destruction protein hydroperoxide *in vitro*.

## 4.6. Oxidative stress and glutathione

Intracellular GSH homeostasis regulates cell metabolism and protects cells against oxidative stress. Increasing GSH levels leads to deoxification of peroxides such as hydrogen peroxide and lipid peroxides (Rahman and MacNee, 2000). Glutathione elevation protects against hydroxyl radical-induced protein oxidation in the rat brain from $Fe^{2+}/H_2O_2$ stimulation (Pocernich *et al*., 2000) .

## 4.7. Shallot and glutathione synthesis.

The study demonstrated that crude and water extracts of shallot increased the total intracellular glutathione in the U937 cells and reduced the total intracellular hydroperoxide.

The reason that shallot extracts could reduce the peroxide and increased total GSH in the U937 cells is that the important compounds in plants of the Allium family such as onion, garlic or shallot are mainly phenolic compounds with free hydroxyl group (–OH) (Cao *et al*., 1997). In addition, furostane, saponins, quercetin, isorhametin and various glycosides are present in shallots (Fatorusso *et al*., 2002). Moreover, it has been reported that the flavonoid compounds in extract, quercetin, kaempferol and apigenin increased the antioxidant gene activity driven by the γ-glutamylcysteine synthetase heavy subunit (GCSh) promoter. Thus, they can stimulate GSH synthesis in cells (Myhrstad *et al*., 2002). In this study, a precursor of GSH synthesis, N-acetylcysteine (NAC) or standard diallyl disulfide (DADS), were added into the cell mixtures. The results showed that NAC increased the total GSH in the U937 cells because it is a sulfur donating compound to produce GSH, and also interacts directly with oxidants (Olsson *et al*., 1988).

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A previous evidence 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 (Tatsuya *et al*., 1999). NAC strongly inhibited ricin-induced apoptotic cell death in U937 cells and prevented the decrease in cellular glutathione (Tatsuya *et al*., 1999). Shallot extracts contain a precursor of GSH similar to NAC. Co-activity of shallot extract with a precursor of GSH synthesis, NAC (6.4 mM), a specific inhibitor of glutathione biosynthesis, and buthionine sulfoximine (BSO) (200 μM) were studied the U937 cell, compared with only shallot extract and cell control. It was shown that activity of shallot extract on GSH synthesis involves the GSH synthetase activity in the U937 cell. This study showed that hexane-extracted shallot can induce GSH synthesis at higher rate than in the cell control after depletion of GSH within the cells.

The antioxidant capacity of shallot extract was demonstrated by incubating the cells with the extracts before irradiation. Theoretically, gamma-irradiation from $^{60}Co$ will increase the amount of hydroxyl radical and hydrogen peroxide in medium or the aqueous part of the cells. Both hydroxyl radicals and react $H_2O_2$ with lipid or protein to form lipid or protein hydroperoxides (Gieseg *et al*., 2000).

In this study, the shallot extract inhibited depletion of total GSH and total hydroperoxide formation in the cells. The active compounds of shallot extract might be phenolic or sulfur group which were apparently able to trap hydroxyl radicals or hydroperoxide directly.

## 4.8. Shallot extract and anti-oxidative property

This study used $H_2O_2$ as a stimulant and a pro-oxidant in the cells, including human monocytic cells. Hydrogen peroxide was capable of damaging proteins and unsaturated fatty acids. The results showed the influence of $H_2O_2$ on hydroperoxide formation in U937 cells. It was found that the inhibitory activity of Thai shallot crude extract on hydroperoxide formation was dose dependent similar to the activity of NAC (Fig. 60).

Our results were similar to those of the previous study of Geng and Lau (1997) in which pre-incubation of endothelial cells with AGE or SAC prevented the intracellular GSH depletion from Ox-LDL, and the previous report that AGE

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modulated the GSH redox cycle by maintaining intracellular GSH levels (Pinto and Rivlin, 2001). Moreover, AGE and SAC minimized intracellular oxidative stress by removing peroxides or preventing their formation in endothelial cells (Ide and Lau 2001). This study supported the previous reports that allium compounds are possibly related to inhibition of carcinogenesis by induction of GSH synthesis (Pinto and Rivlin, 2001). GSH can detoxify carcinogens, serve as an intracellular antioxidant (protecting cell membranes and intracellular components from damage by free radicals) and assist in the regulation of DNA synthesis. GSH has not only functions as a cosubstrate for the family of GST enzymes necessary for conjugation of GSH to electrophiles and thus blocking DNA-adduct formation, but also serves as a reductant for glutathione peroxidase (Perchellet *et al*., 1986). This selenoenzyme protects cells against lipid peroxidation by using GSH to reduce organic peroxides to non reaction hydroxyl fatty acid.

Other studies have shown that $H_2O_2$ induced the inflammation process in various cell lines including monocytic cell line (U937). Meyer and colleagues (1992) showed that certain transcription factors of the NF-κB/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 (Schreck *et al*., 1991). Subsequently, several other protein kinase cascades and transcription factors have been discovered to possess redox-sensitive elements. NF-κB is activated upon phosphorylation of an inhibitory subunit (IκB). $H_2O_2$, acts 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 (Bae *et al*., 1997).

$H_2O_2$ is formed during gamma-glutamyl transpeptidase (GGT) activity, it is importance for normal cell growth and maintains U937 cell proliferation, which is inhibited by antioxidant treatment (Bello *et al*., 1999). Thus Thai shallot not only inhibited total hydroperoxide formation but also scavenged $H_2O_2$, which might suppress the proliferation of U937 cells.

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## 4.9. Shallot extract and anti-inflammation

Chronic inflammation is associated with increased risk of human cancer. Many enzymes such as nitric oxide synthase, myeloperoxidase or eosinophil peroxidase produce high concentrations of free radicals such as nitric oxide, nitroxyl, nitrogen dioxide, and hydrogen peroxide. These free radicals can damage DNA, RNA, lipid, and protein by nitration, oxidation, chlorination, and bromination, leading to increased mutation and altered function of enzymes and proteins involving the mutli-stage carcinogeneesis processes.

The activity of Thai shallot extracts on the inflammatory process was studied by using inflammatory stimulator endotoxin lipopolysaccharide (LPS). LPS can elicit inflammatory reaction both *in vitro* and *vivo* (Rylander, 1997). LPS-stimulated U937 cells increased the release of cytokine and interleukin-10 (Cheng *et al*., 2003). Nitric oxide (NO) is produced from a guanido group of L-arginine by three NO synthase isotypes (Moncada *et al*., 1989). Inducible NOS (iNOS) is particularly expressed in response to proinflammatory stimuli (Stuerhr and Marleta, 1985). Though NO produced in inflammatory cells such as T-cell 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 coupling between oxygen and a strong nitrodating agent ($N_2O_3$). $N_2O_3$ deaminates DNA bases and can react with secondary amines to form caricinogenic N-nitrosamines (Ohshima *et al*., 2003). The high to low inhibitory activity of shallot extract on NO production was observed in crude, water-, and hexane extracts, respectively. The shallot extract has maximum activity at 5 h, then gradually decreased the hydroperoxide formation in the cells until 24 h compared with LPS-stimulated cells and control cells (Fig. 70). In addition, shallot extract (crude) was able to inhibit intracellular hydroperoxide formation and induce total glutathione synthesis in the U937 cells. This study emplyed a J774.2 cell line that was able to produce NO after stimulation with LPS that confirmed to the previous study in LPS-activated U937 cell line. The results showed that hexane extract of shallot in which contented DADS inhibited NO production in U937 and J774.2 cell lines.

Earlier work had shown that antioxidant and anti-inflammatory activities of garlic on various cells lines were from different sulfur compounds. Garlic extract composed of S-allyl cysteine inhibited the NO production in peritoneal macrophages

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(Kim *et al*., 2001). S-allyl cysteine (SAC) inhibited NF-κB activation in human T-lymphocytes induced by $H_2O_2$ (Geng *et al*., 1997). Diallyl disulfide containing allyl and sulfur at 100 and 500 μM inhibited cancer cell growth either cancer cell line of lung (A549) or skin (SK MEL-2) (Sundaram *et al*., 1996).

Borek and co-workers (2001) reported that antioxidant activity of aged garlic extract acts via enhancement of glutathione synthesis. AGE increases cellular glutathione in a variety of cells, including those in normal liver and mammary tissue (Liu *et al*., 1992). Regarding to the ability of garlic to increase glutathione peroxidase and other ROS scavenging enzymes (Wei and Lau, 1998), it is able to prevent the formation of ROS that will damage DNA, lipid and protein.

Thus, Thai shallot in the form of crude extract and hexane preparations that contained total phenolics and DADS have antioxidant and anti-inflammatory activities that involved an important intracellular antioxidant, glutathione (GSH), which controls intracellular peroxide and nitric oxide formation.

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