ฤทธิ์ในการต้านอนุมูลอิสระของสารสกัดหอมแดง น้ำมันไพลและขมิ้นในหลอดทดลอง

ANTIOXIDANT ACTIVITY OF SHALLOT EXTRACT AND PLAI AND TURMERIC OILS IN VITRO

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บทคัดย่อ

จุดประสงค์ของการศึกษางานวิจัยนี้ เพื่อทำการศึกษาฤทธิ์ในการต้านอนุมูลอิสระ และองค์ประกอบสำคัญทางเคมีของสารสกัดหอมแดง น้ำมันไพล และน้ำมันขมิ้นในหลอดทดลอง วิธีการศึกษา ขั้นแรกเตรียมสารสกัดหอมแดงโดยใช้เฮกเซนเป็นตัวทำละลาย นำไประเหิดเฮกเซนออก จากนั้นนำไประเหยให้เป็นผง ส่วนน้ำมันไพลและขมิ้นนำไปสกัดน้ำมันกับชุดเครื่องกลั่นน้ำมันแบบไอน้ำ ทำการทดสอบฤทธิ์ในการสารต้านอนุมูลอิสระใช้วิธี Diphenylpicrylhydrazyl (DPPH) radical scavenging สำหรับการศึกษาส่วนประกอบของฟีนอลิกโดยรวม ด้วยวิธี Folin-Cioaclteu reagent ส่วนการศึกษาสารองค์ประกอบสำคัญทางเคมีในกลุ่มซัลไฟด์ของหอมแดงจะวิเคราะห์โดยเครื่อง High Liquid Chromatography (HPLC) น้ำมันไพลและขมิ้นจะนำมาวิเคราะห์หาสารสำคัญโดยใช้วิธี Gas chromatrography-mass spectrometry (GC-MS) ผลการศึกษาพบว่า ประสิทธิภาพการทำงานของสารต้านอนุมูลในน้ำมันขมิ้น และหอมแดง อยู่ในระดับปานกลาง แต่น้ำมันไพลอยู่ในระดับสูง เมื่อเทียบกับสารมาตรฐานTrolox สำหรับปริมาณสารประกอบฟีนอลิกทั้งหมดในสารสกัดที่ 1 มิลลิกรัม น้ำมันไพลมีปริมาณฟีนอลิกมากกว่าน้ำมันขมิ้นและสารสกัดหอมแดงตามลำดับ การวิเคราะห์สารสกัดหอมแดงโดย HPLC พบว่ามีปริมาณซัลไฟด์ชนิด Diallyl disulfide (DADS) มากที่สุด และยังพบกลุ่ม Tri- and Tetra-allyl disulfides องค์ประกอบสำคัญทางเคมีของน้ำมันไพลมี 3 สาระสำคัญ คือ Sabinene, Terpinen-4-ol และ (E)-I- (3,4-dimethyoxyphenyl) butadiene น้ำมันขมิ้นมีสารองค์ประกอบสำคัญทางเคมีที่พบมากคือ ar-turmerone, alpha-turmerone และ zingiberene จากการศึกษาสารสกัดหอมแดง พบว่าสามารถกระตุ้นการสร้างกลูตาไทโอนในเซลล์ชนิด Monocyte cells (U937) ได้ โดยเพิ่มขึ้นตามปริมาณของสารสกัดหอมแดง เช่นเดียวกับสารมาตรฐาน DADS.

คำสำคัญ: สารต้านอนุมูลอิสระ, หอมแดง, ไพล, ขมิ้น, กลูตาไทโอน

ABSTRACT

The aim of this study was to evaluate the antioxidant activity and active compounds of shallot extract, plai and turmeric oils in vitro. Methods: shallot extract was prepared by extraction with hexane and evaporation followed by lyophilization. Oils of turmeric and plai were extracted by steam distillator. Antioxidant activity was evaluated using the diphenylpicrylhydrazyl (DPPH) radical scavenging method, and total phenolic content was tested with a Folin-Cioaclteu reagent. For dominant active compounds of sulfide groups in shallot were analyzed by high liquid chromatography (HPLC), whereas plai and turmeric oils were identified by gas chromatrography-mass spectrometry (GC-MS). The results showed moderate antioxidant activity in turmeric oil and shallot extract, but the significant highest activity was plai oil as well as standard trolox. The total phenolic content at 1 mg in plai oil was more than in turmeric oil and shallot extract. In addition, shallot extract showed dominant peaks of diallyl disulfide (DADS), tri- and tetra-allyl disulfides. Three major compounds; sabinene, terpinen-4-ol and (E)-I-(3,4-dimethyoxyphenyl) butadiene, were represented in plai oil, whereas three dominant peaks of ar-turmerone, alpha-turmerone, and zingiberene were shown in tumeric oil. Moreover, shallot extract, especially, significantly increased the level of intracelluar glutathione in monocyte cells (U937) as well as standard DADS.

Keywords: Anti-oxidant, Shallot, Plai, Tumoric, Glutathione

Introduction

Many Thai natural plants have given benefit to antioxidants and anti-inflammation. Free radicals are a species of atoms or molecules that contain an unpaired electron such as superoxide anion, hydrogen peroxide, hydroxyl radicals, and nitric oxide. Excessive free radical production in the human body is related both directly and indirectly to disease progression in biological component destruction, physiology, and pathological and inflammation phenomena, i.e., damaged lipid protein or DNA. Damaged DNA, RNA or lipid protein leads to increased mutations that alert enzyme and protein functions, and contribute to the multistage

carcinogenesis process. The most challenging studies of shallot (*Allium ascalonicum* L.) (Leelarungrayub et al, 2006) have been on antioxidant properties that are major bioactive compounds such as different phenolic compounds and flavonoids, including sulfur compound, which rather improves antioxidant glutathoine. Sulfur compound may be a precursor of the glutathione (GSH) generation. Plai, *Zingiber cassumunar* Roxb., is a Thai herb that helps to relieve pain, swelling, anti-neuralgic, anti-inflammatory, antioxidant, antitoxic, anti-viral and immunity problems as well as nausea, respiratory problems, muscle spasm or strain and ligament sprain. Plai contains many active chemical compounds such as terpinen-4-ol, sabinene, (E)-l-(3,4-dimethoxyphenyl) butadiene (DMPBD), alpha -pinenene,cassumunin A, cassumunin B and curcumin (Wasuwat 1989). Each of these has anti-inflammatory activity, and turmeric (*Curcuma longa* L.) has health benefits (Leela et al, 2002). The volatile oil fraction of turmeric has demonstrated significant anti-inflammatory activity in a variety of experimental models. The yellow or orange pigment of turmeric, namely curcumin, is even more potent than its volatile oil. Curcumin is thought to be the primary pharmacological agent in turmeric, and an anti-inflammatory agent for effective treatment of inflammatory bowel disease; rheumatoid arthritis; cystic fibrosis; cancer prevention, cell growth and metastases; and childhood leukemia. The above evidence shows the interesting benefits of shallot extract and turmeric and plai oil, and their antioxidant and anti-inflammatory activities, but few studies have researched the activity and sulfur compounds in shallot extract.

Objectives

This study aimed to measure the antioxidant activity and active compounds in shallot extract, and plai and turmeric oil, and moreover, study the shallot activity on GSH synthesis in human cells.

Methods

Essential oil extraction and shallot extract preparation

Rhizomes of plai and tueric from natural community grwoing that aged more than 2 years were collected for distillation from locally grown plai and turmeric

in Chiang Mai province, Thailand. Pure oils of plai and tumeric were extracted by steam distillator before the experiments. Hexane extracts from shallot that growed naturally and no anti-insect substance used were prepared by crushing the bulbs in a mortar, with the volume of hexane being the same as the weight of the bulbs, and left to settle overnight before filtering. The hexane layer was then separated and the hexane evaporated before freeze-drying the clear solution. All extracts were freeze dried by lyophilization and stored while dry at room temperature in the dark.

Antioxidant assays

Diphenylpicrylhydrazyl (DPPH) scavenging activity was preliminary tested by following the protocol of Thitilertdecha et al (2010). An aliquot of essential oil (75 µL) was mixed with 3.0 mL of DPPH (Sigma-Aldrich) solution (1.5 mg/mL in methanol). After 30 min of incubation in the dark at room temperature, an absorbance decrease at 515 nm was recorded. The control sample was prepared using ethanol instead of essential oil (blank sample). Trolox (300 µg/mL of stock solution) (Sigma-Aldrich), a stable antioxidant, was used as a positive control. The radical-scavenging activities of the samples were expressed as percentage inhibition of DPPH radicals.

Phenolic compounds

The assay was based on the method of Gao et al (2000). Samples containing 10 mg of each extract were hydrolyzed in 1.2 M of HCL and 50% MeOH by heating at 80 C for 2 hr. After centrifugation at 18,000g., 0.1-ml portions of the supernatants were mixed with 0.1 ml of the Folin-Ciocal-teau reagent and 0.5 mL of 20 % Na_2CO_3 and allowed to stand in the dark for 20 min. Absorbance was measured at 765 nm with gallic acid as a standard, and the total phenolic content was calculated as milligrams of gallic acid equivalent per kilogram of dry weight of extract.

Active compound analysis

The three allylsulfides in shallot extract were analyzed with high liquid chromatography (HPLC) using modified Lawson's protocol (1991). The lyophilized extract was dissolved in ethanol at 100 mg/ml. After passing through 0.45 um poly tetrafluoroethylene (PTFE) membrane (LIDA,Kenosha, WI,USA), a 20 µl sample was injected into rheodyne and the individual peaks were analyzed by HPLC on a reverse phase C18 column (Phenomenex, MA USA) (250 mm x 4.60 mm, 5 µm) using a

mixture of acetonitrite, water, tetrahydrofuran (70:27:3,*v:v:v*) and methanol (50%) as the mobile phases, with a 1.0-mL/min flow rate. Standard diallyl disulfide (DADS) (80%) was purchased from the Aldrich Company (Milwaukee WI, USA). Each peak of allyl disulfides was separately identified at 240 nm.

Major active compound of plai and turmeric oils was analyzed by gas chromatrography-mass spectrometry (GC/MS) at the Science and Technology Service Center, Faculty of Science, Chiang Mai University. The Agilent GC/MS system consisted of GC6850 and a 5973 MSD, which was installed with a HP-MS 5 column *(*30 m x 0.25 mm ID x 0.25 um film thickness*)*. The helium gas carrier was controlled with a 1.0 ml/ min flow rate. The interface and ion source temperatures were 150 oC and 230 ^{o}C, respectively. The program for GC analysis was set up with 250 ^{o}C of Inlet via a 20 ul split ratio (200:I), and the oven temperature was set up at 80 ^{o}C for 10 min and increased 10 ^{o}C every minute to 240 ^{o}C within 14 min. The mass spectrum of each compound in the essential oil was compared to a standard mass spectrum in an MS library (Wiley7n.l).

Antioxidant activity in monocyte cells (U937)

For cell viability and the toxicity assay, toxicity of extracts and cell viability were evaluated with a 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, and kept overnight at 37 ^{o}C in a 5% CO_2 atmosphere, shallot (50-250 µg/ml) was added and the plates were incubated overnight at 37 ^{o}C in a 5% CO_2 atmosphere. Cells were centrifuged (5,000 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 reacted with DMSO, and absorbance was measured at 590 nm with a spectrophotometer. The values obtained were compared with those of the DMSO or control incubated with the vehicle only.

Total intracellular GSH determination was a method modified from Beutler's protocol (1963), and used to determine total intracellular GSH in U937 cells. After cells were exposed to the extracts at 50,125, or 250 µg/ml levels for 24 h. at 37 ^{o}C, and 5% CO_2, they were washed with phosphate buffer solution (pH 7.4) and

centrifuged (500x g). They 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/mL of NADH, DTNB, and 50 units/ml of glutathione reductase. The change rate of absorbance at 412 nm/min was monitored with a spectrophotometer every 10 sec for 180 sec. The concentration of total intracellular GSH in the samples was determined from the standard calibration curve from reduced GSH (Sigma) and represented in pmol/10^4 viable cells.

Statistic analysis

All data showed in a mean ± SEM, and One-way ANOVA was used to statistical analysis ($p = 0.05$)

Results

Plai and turmeric oils

From the plai and turmeric oils prepared by steam distillator, 500 g of plai and turmeric rhizomes produced 3.0 and 0.31 mL of pure oils, and moreover, their density was 930 ± 25 mg/mL and 870 ± 19 mg/mL. The characteristic of plai and turmeric oil was colorless.

Antioxidant activity and total phenolic content

From the three samples; shallot extract, and turmeric and plai oils were tested with DPPH radical scavenging. Results showed moderate inhibitory activity in shallot extract [1.25 mg/mL (2.03%), 2.5 mg/mL (3.00 %), 5.0 mg/mL (3.49%, 10 mg/mL (8.36%), and 20 mg/mL (18.53%)] and turmeric oil [8.7 mg/mL (3.93%), 17.4 mg/mL (6.94%), 34.8 mg/mL (10.26%), 68.2 mg/mL (16.37%), and 139.2 (25.64 %)]. Whereas, Plai oil showed very high activity [9.3 mg/mL (28.33%), 18.6 mg/mL (46.83%), 37.2 (70.25%), 74.4 (85.59%), and 148.8 mg/mL (92.45%)] when compared to standard trolox [39.06 µmol/L). After inhibitory activity comparison at 25 %, plai oil had the significant best activity (8.21± 1.12 mg/mL) when compared to shallot extract (26.98 ± 0.98 mg/mL) and turmeric oil (135.69 ± 1.06 mg/mL), whereas the standard trolox had activity at 114.89 ± 1.21 mmol/L on DPPH radical scavenging (Table 1).

Table 1. Inhibitory activity of DPPH radical at 25 % scavenging

Compounds	Activity
Trolox (Sigma)	114.89 ± 1.21 mmol/L
Plai oil	8.21 ± 1.12 mg **
Shallot extract	26.98 ± 0.98 mg *
Turmeric oil	135.69 ± 1.06 mg

** $p < 0.01$ and * $p < 0.05$ compared to all extracts

When comparing between the three samples at 1 mg; Plai oil had the highest yield of total phenolic content (123.15 ± 11.5 µg gallic acid), when compared to the turmeric oil (49.33 ± 21.2 µg gallic acid), and shallot extract (1.84 ± 1.56 µg gallic acid).

Active compounds

From HPLC analysis, standard diallyldisulfide (DADS) showed the four peaks of sulfur compounds; mono- (15.57%), di- (65.50%), tri- (17.56%), and tetra-allyl disulfides (1.37%) at 3.47, 3.84, 4.64, and 5.73 min, respectively (Figure 1.a). Shallot extract that was extracted with hexane showed a dominant peak of DADS at 3.82 min and the yield was highest when compared to tri-, and tetra-allyl disulfides. Thus, the concentration of DADS in shallot extract at 1 mg was equal to 3.74 µmol/L (Figure 1.b).

From the HPLC analysis, shallot extract contained the dominant diallyl disulfide (DADS) of 3.73 ± 0.98 µmol/L (RT = 3.82 min), including tri-allyl disulfide (RT = 4.72 min), and tetra-allyl disulfide (RT = 5.57), when compared to the standard peaks of DADS (RT = 3.84 min), tri-allyl disulfide (RT= 4.64 min), and tetra-allyl disulfide (RT = 5.73 min), respectively.

Pure plai oil showed a dominant peak of terpinene -4-ol, sabinene, and DMPBD (Figure 2.a), whereas, turmeric oil showed the dominant peak of ar-turmerone (62.3%), alpha-turmerone (35.2%), and zingiberene (10.34%) (Figure 2.b).

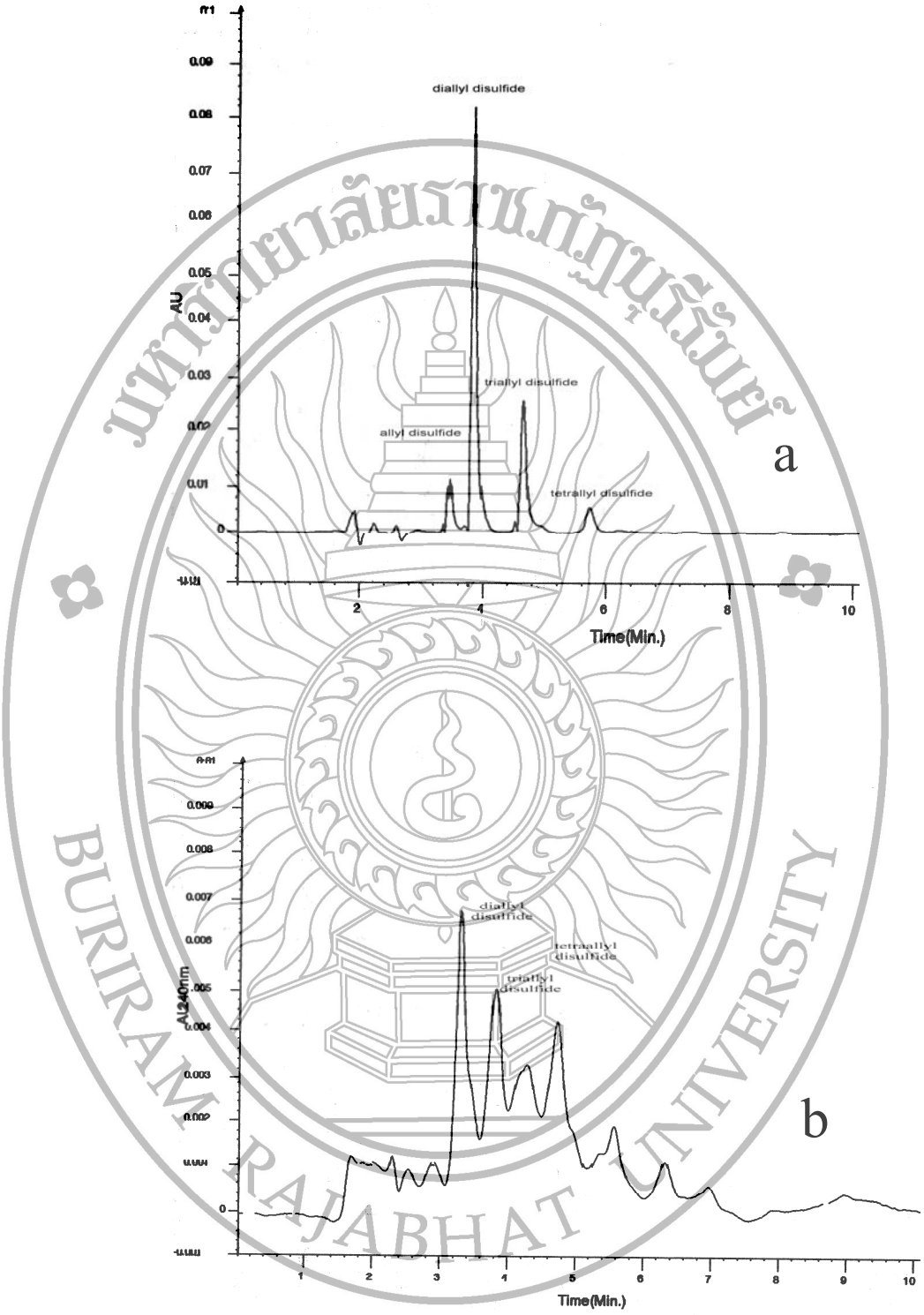

Figure 1. Chromatogram of HPLC profile of standard diallyl disulfide (DADS) (a), and shallot extract at 20 mg/mL (b).

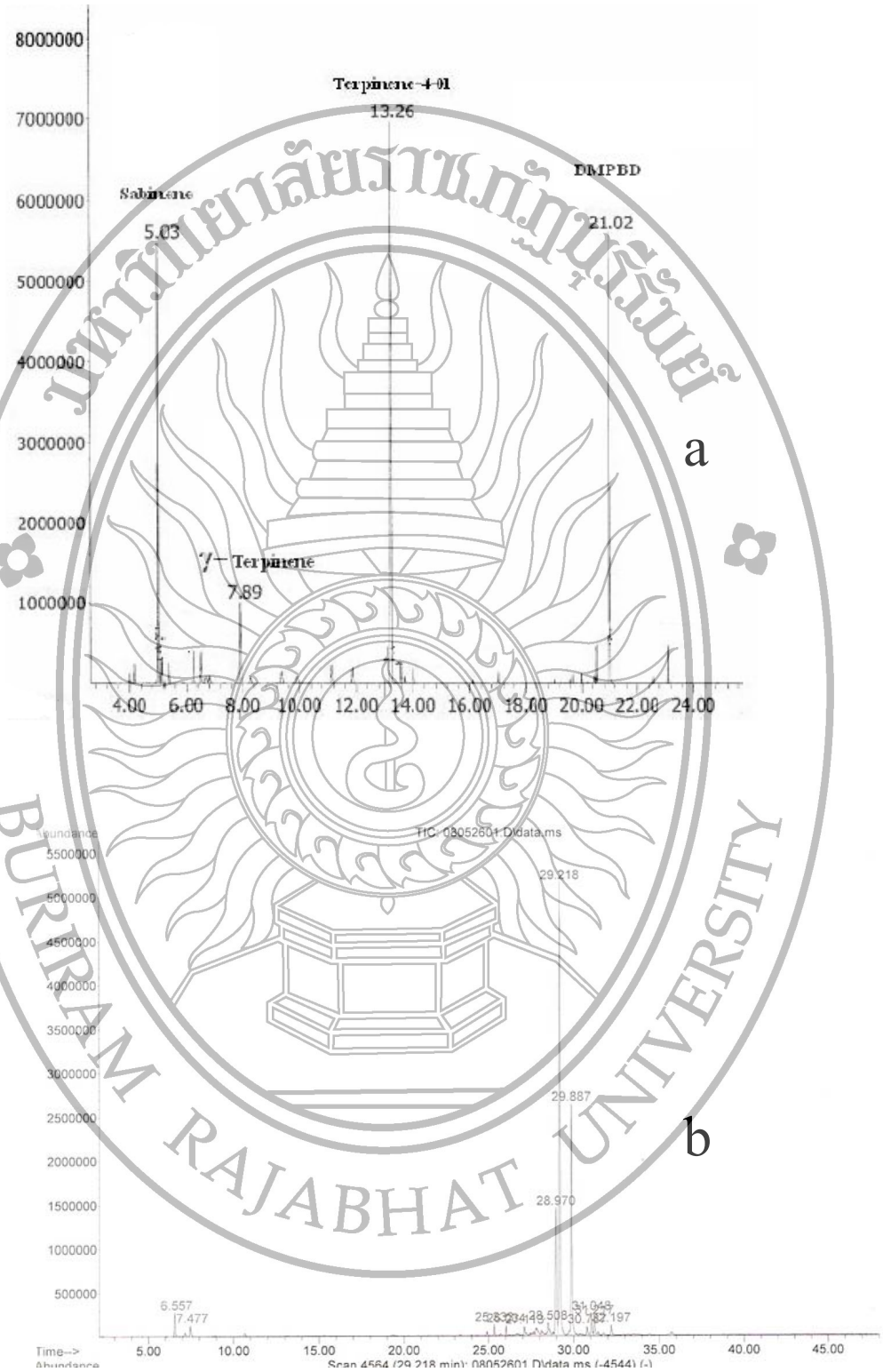

Figure 2. Chromatogram of GC-MS profiles from plai oil (above) and turmeric oils (b).

Toxicity and Antioxidant activity in monocyte cells

The toxicity of shallot extract was prohibited in U937 cells, if the concentration was less than 250 µg/mL (cell death < 20%). From the experiment (Figure 3), the intracellular GSH of cell control (299 ± 56 pmol/10^4 cell viable cells) was maintained for 24 h after treatment. After treatment with shallot extract at 50, 125 and 250 µg/mL, the GSH level improved slightly (320 ± 23, 360 ± 17, and 450 ± 15 pmol/10^4 cell viable cells). In standard DADS at 2,5 and 5 µmol/L, results showed that GSH also increased to 378 ± 12 and 465 ± 23 pmol/10^4 cell viable cells. At 125 and 250 µg/ml of shallot extract and 2.5 and 5 µmol/L of standard DADS were significant difference from the cell control and cell treated with shallot extract at 50 µug/ml.

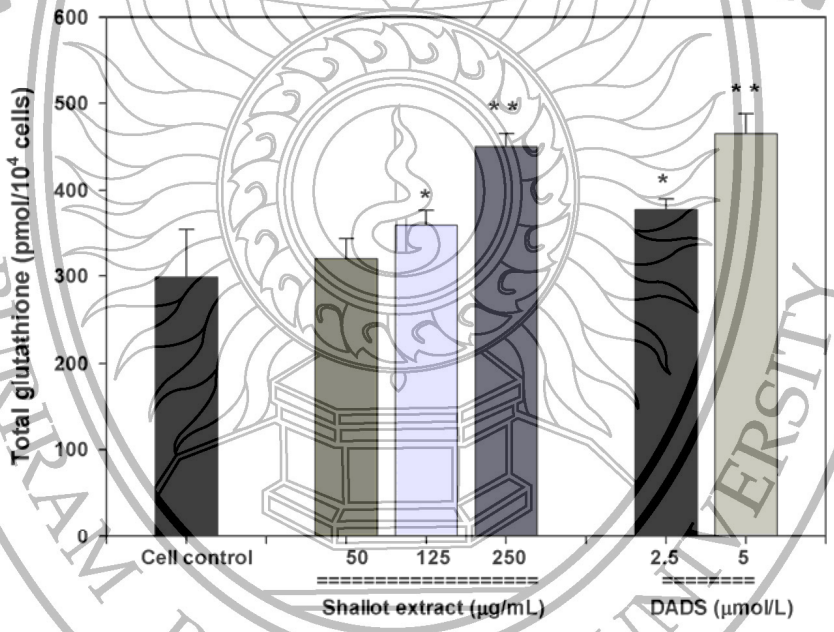

Figure 3. Total intracellular glutathione in cells (U937) after treatment with shallot extract at 50, 125, and 250 µg/mL, and standard DADS at 2.5 and 5 µmol/L.** $p < 0.01$ and * $p < 0.05$ compared to cellcontrol and cell treated with shallot extract at 50 µg/ml.

Discussion

From this study, the antioxidant activity of plai and turmeric oil was found from steam distillation, according to a previous study by Leelarungrayub et al (2009), which showed that essential oil of plai indicated antioxidant and anti-inflammatory activities in vitro and in the cell line. Moreover, active compounds in plai oil were terpinene-4-ol (18.79%), sabinene (48.17%), and DMPBD (48.17%) from GC-MS. It was also proved that Plai oil was active in the inhibition of COX-II and iNOS expression in PMA/LPS U937 cells (Singthong et al., 2010). Turmeric oil showed less antioxidant activity than plai oil. The active compounds were ar-turmerone (62.3%), alpha-turmerone (35.2%), and zingiberene (10.34%), consecutively, which was the same as in previous studies (Sacchetti et al, 2005; Leela et al., 2002). The ar-turmorone shows mainly the antiplatelet activity, whereas, curcumin has potential antioxidant activity (Jain et al, 2007). In this study, we assaysed the total phenolic contain in all extracts that plai oil had the highest yield (123.15 ± 11.5 µg gallic acid), when compared to the turmeric oil (49.33 ± 21.2 µg gallic acid), and shallot extract (1.84 ± 1.56 µg gallic acid). It related to the DPPH radical scavenging activity. Previous data showed that phenolics as phenols or flavonoids were important for direct scavenging the free radical and inhibited lipid peroxidation (Stangl et al, 2007). Shallot extract from hexane extraction showed the dominant peak of diallyl disulfide (DADS); the same as in a previous study by Leelarungrayub et al (2004; 2006), who found that Thai shallot bulbs from hexane extraction were antioxidant against gamma-radiation in U937 cells, and protective against toxicity from cyclosporineA in a rat model (Wongmekiat et al, 2008). Moreover, this study proved that shallot extract from hexane that contained DADS was a precursor of GSH within the cells (Leelarungrayub et al, 2006). Previously evidence refered that DADS induced GSH within cells, by sulfur precursor of GSH synthesis by de novo pathway (Robert et al., 2001), or in human hepatocyte (Sheen et al., 1999). Thus, dominant activity of shallot extract contained sulfides, and plai or tumeric oils with anti-inflamatory activity were very interesting, possible used in an anti-oxidant with glutathione inducer, but more studies in another cell lines, or mice model for accuracy results.

Conclusion

In this study found interesting antioxidants and active compounds in Thai shallot (diallyl-disulfide), plai (terpinene-4-ol, sabinene, and DMPBD), and tumeric (ar-turmerone, alpha-turmerone, and zingiberene). Moreover, DADS in shallot extract also had a potential activity on activation the GSH within the cell line. Thus, mixture of shallot, plai and tumeric oil is possiblely performed to an anti-oxidant with GSH activator product that has anti-skin wrinkles and regeneration activity on the skin.

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