ANTIOXIDANT, ANTIMICROBIAL, TYROSINASE ENZYME INHIBITION AND CHEMICAL COMPOSITIONS OF SHALLOT EXTRACTS

Laksana Charoenchai¹,*, Sukanya Settharaksa¹, Lukman Sueree¹, Thaniya Wunnakup¹, Thidarat Phetmanee², Thitiya Lukkunaprasit², Duangdeun Meksuriyen³

¹ Sino-Thai Traditional Medicine Research Center, Faculty of Pharmacy, Rangsit University / 52/347 Pahonyothin St. Soi 87, Muang Pathum, Pathum Thani, Thailand. 12000.
² Department of Pharmacology, Faculty of Pharmacy, Rangsit University / 52/347 Pahonyothin St. Soi 87, Muang Pathum, Pathum Thani, Thailand. 12000.
³ Innovation Center, Faculty of Pharmacy, Rangsit University / 52/347 Pahonyothin St. Soi 87, Muang Pathum, Pathum Thani, Thailand. 12000.

* Corresponding author: E-mail: laksana.c@rsu.ac.th

Abstract: Shallot (Allium ascalonicum L.) was in the same genus as garlic and onion. These plants exhibit some activities; however, more studies need to be conducted to evaluate the activities of ethanolic shallot extracts. The objectives of this study were to prepare shallot extracts, evaluate their safety, and determine their antimicrobial, antioxidant and anti-tyrosinase activities. Shallot bulbs were extracted with water and 20-95% ethanol concentration. The antioxidant activity was determined using DPPH radical scavenging assay and anti-tyrosinase activity was evaluated using enzyme assay technique. Disc agar diffusion method and broth micro-dilution method were used to determine antimicrobial properties of shallot extracts against S. aureaus, S. epidermis and P. acne. The toxicity of the extracts was assessed using MTT assay on primary human dermal fibroblast cells. Chemical compositions of shallot extracts were examined using HPLC analysis. The results showed that 20% ethanolic shallot extract exhibited the highest quercetin equivalent and the highest antioxidant activity. In contrast, 80% ethanolic shallot extracts showed the highest tyrosinase inhibition. Shallot extracts were not toxic to HDF cells at the concentration lower than 20 mg/mL. They did not have antimicrobial activity against tested bacteria in this study. HPLC analysis showed elution pattern of flavonol glucosides similar to methanolic onion and shallot extracts. This result suggests that ethanol concentration may involve in the chemical compositions of shallot extract and resulting in various antioxidant activity. Some flavonol glucosides may be responsible for the activities of shallot extracts. However, antimicrobial and anti-tyrosinase enzyme activity may relate to other substances in shallot extracts.

Keywords: Allium ascalonicum L., antimicrobial, antioxidant, anti-tyrosinase, shallot
**INTRODUCTION**

Shallot (*Allium ascalonicum* L.) was a plant in the Amaryllidaceae family and was the same genus as garlic (*Allium sativum* L.) and onions (*Allium cepa* L.). They were extensively used as spices in oriental cuisine. It is known that inhaling the vapor of freshly crushed shallot bulbs could alleviate nasal congestion from common cold in Thai traditional medicine. There are several studies reported some activities of shallot bulbs; for example, antioxidant antimicrobial activities. Hexane extract of shallot bulbs reduced ABTS•⁺ radical in antioxidant assay in higher extent than water extracts and bulb pressing (Leelarungrayub et al., 2006). Shallots also exhibited the highest total antioxidant activity with 45.5±2.1 μmol of vitamin C equivalent/g onion (Yang et al., 2004). Aqueous extracts (50%) of shallot showed inhibition zone (30-50 mm) against *Pseudomonas aeroginosa* (Ashrafi, 2004). In opposite, crude juice of shallots were tested in an agar diffusion assay and showed an inhibitory effect against gram negative bacteria (Dankert et al., 1979). Water extract of shallot bulbs showed highly inhibition of *Bacillus subtilis*, *Aspergillus niger*, *Staphylococcus aureus*, and *Salmonella typhi* with MIC of 38, 62.5, 75 and 78.1 μg/mL, respectively (Amin et al., 2009). Recently, aqueous shallot extract was found to produce anticancer activity on K562, Wehi164 and Jurkat cell lines with low cytotoxic to HUVECs normal cell lines (Mohammadi-Motlagh et al., 2011). This research focused on exploring the activities of shallot extracts.

Many methods were used for preparation of shallot extracts. Different Allium species contain different kinds and levels of chemical compositions. Solvent extraction method yields the extracts with various activities; for example, water extraction of fresh bulbs (Amin and Kapadnis, 2005; Chaisawadi et al., 2005) and solvent (ethanol, methanol or hexane) extraction by maceration or sonication (Seyfi et al., 2010; Leelarungrayub et al., 2006). The chemical compounds in shallots show some similarities to those of onion and garlic. They were flavonol glucosides, phenolic, tannins, and other organosulfur compounds (Bonaccorsi et al., 2005; Bonaccorsi et al., 2008; Lanzotti, 2006). Quercetin glucosides are mostly compounds found in shallots. Isorhamnetin glucosides were reported as the specific compounds in shallot bulbs (Bonaccorsi et al., 2005). Various dialk(en)yl sulfides were observed in crushed or pressed bulbs including steam distillation oil. Therefore, extraction process was the key factor to obtain the extract with high potency.

In the present study, shallot extracts was prepared by maceration in water and different concentrations of ethanol. Then these extracts were evaluated for their antimicrobial activities against some gram-positive bacteria, antioxidant activity and tyrosinase enzyme inhibition. It was determined cytotoxicity to human dermal fibroblast cell also.
MATERIALS AND METHODS

Preparation of shallot extracts
Fresh shallots were bought from local market Pathum Thani province Thailand. Fresh shallot bulbs were removed from the outer shell and were crushed using an electric blender. Shallots (500 g, each) were extracted in water and 20% to 95% ethanol with 1:2 ratio weight (g) by volume (mL) for three times. Then they were put in the water bath at 50 °C for 4 hours. The sample solutions were combined, filtered through muslin cloth and centrifuged at 4000 rpm for 10 minutes. Ethanol in the filtrates was removed using a rotary evaporator. After that, the samples were dried using a lyophilizer.

HPLC analysis
HPLC analysis was performed on the Agilent 1260 HPLC system with EZchrome software. Poroshell C18 column (2.1 x 150 mm, 4 µm) at 30 °C was used as a stationary phase, and the mobile phase composition was 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) in gradient elution. The mobile phase was eluted from 5 20 %B (0-5 min), 20 30 %B (5-10 min), 30 35 %B (10-20 min), 35 50 %B (20-35 min), 50 95 %B (35-38 min) and stayed at 95%B, then back to 5%B and equilibrated for 4 minutes, total run time in 45 minutes modified from Bonaccorsi et al., 2005. The flow rate was 0.3 mL/min and the injection volume was 10 µL. Quercetin equivalent content in each sample was calculated related to the standard curve of quercetin hydrate (0.625-30 µg/mL). The shallot extract was dissolved in 80% methanol and prepared at the concentration of 10 mg/mL, then filtered through nylon syringe filter 0.45 µ before injection to HPLC.

MTT assay
Primary human dermal fibroblast cells (HDF) were used for cytotoxicity determination. HDF were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were incubated in 37 °C and 5% CO₂. Cells were seeded at a density of 5 x 10^3 cell/well into a 96-well plate for 24 hours. The viability of cells following shallot extracts treatment (0-30 mg/mL) for another 24 hours was tested by MTT assay. Then the medium was replaced by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (0.4 mg/mL) and incubated at 37 °C for 4 hours. The formazan was formed and dissolved in DMSO. The intensity of the formazan product was measured at 570 nm using a Biorad® microplate reader. Percentage of cell viability and IC₅₀ of cytotoxicity were calculated and compared with a control group.

DPPH radical scavenging assay
The shallot extracts were solubilized in 80% ethanol at various concentrations (0 - 3 mg/mL). Quercetin hydrate solution was prepared at the concentration of 0.5-200 µg/mL. DPPH solution was prepared in ethanol at the concentration of 200 µM. Both 100 µL of the sample solution and 100 µL of the DPPH solution were added into 96-well plate and mixed well. After leaving in the dark and at room temperature for 30 minutes, absorbance was measured at the 517 nm wavelength using a Biorad® microplate reader. The assessment of the antioxidant activity was expressed in IC₅₀ value and compared with quercetin hydrate. Each measurement was performed in triplicate and IC₅₀ values are means of samples ± SEM.
**Tyrosinase inhibition assay**

Tyrosinase inhibition assay was modified from the method of Uchida et al., 2014. Briefly, 20 µL of shallot extracts at the concentration of 5 mg/mL was added into a 96-well plate and mixed with 115 µL of phosphate buffer (50 mM, pH 6.8). Then 40 µL of L-dopa (0.9 mg/mL in phosphate buffer) was added. Finally, 25 µL of mushroom tyrosinase (403.05 U/mL in phosphate buffer) was added and incubated for 10 minutes at room temperature. Kojic acid (20 µM) was used as a positive control. Dopachrome content was determined spectrophotometrically at 450 nm. Percentage of tyrosinase inhibition was calculated. Each measurement was performed in triplicate and %inhibition is mean of samples ± SEM.

**Antimicrobial tests**

Shallot extracts were determined antimicrobial activity against *Staphylococcus aureus* (TISTR 1466), *Staphylococcus epidermidis* (TISTR 518) and *Propionibacterium acnes* (DMST 14916) using disc agar diffusion method. The microbes were prepared at the concentration of 1.5 x 10⁸ CFU/mL (equivalent to 0.5 McFarland Number) and were streak on the MHA plate for *S. aureus*, *S. epidermidis* and on a BHI agar plate for *P. acnes*, respectively. Shallot extracts were prepared in 1,000 µg/disc each, put on the plate and incubated at 37 °C for 18-24 hour (*S. aureus*, *S. epidermidis*) and 24-48 hour (*P. acnes*). Inhibition zone was observed compared with clindamycin (10 µg/disc) as a positive control.

Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) were evaluated using broth microdilution method. Shallot extracts were prepared at the concentration of 102.40 mg/mL and diluted to 1-1,024 µg/mL. The extract solution was added into 96 well plate 20 µL each (n=3). The microbes were grown in culture medium at the concentration of 1.5 x 10⁸ CFU/mL (equivalent to 0.5 McFarland Number) and were added into 96 well plate 100 µL each to give a final concentration of 10⁶ CFU/mL. After incubating at 37 °C for 18-24 hour (*S. aureus*, *S. epidermidis*) and 24-48 hour (*P. acnes*), MIC value was observed. Then the microbes were streaked on MHA plate and BHI agar plate to determine MBC value.

**Statistical Analysis**

Data were obtained from three independent experiments and presented as means ± standard error of mean (SEM). Statistical analysis was performed using Probit, one-way ANOVA and post hoc test at a significance level of *p*-values < 0.05. IBM SPSS 21.0 was used for all statistical analyses.

**RESULTS AND DISCUSSION**

Percentage yield of shallot extracts was in the range of 8.68-11.80 %w/w as showed in Table 1. Quercetin equivalent content was quantitative analysis by HPLC. The content of quercetin equivalent in all shallot extracts was calculated based on quercetin hydrate in mg/kg fresh weight. It was a representative of flavonol glucosides in shallot extracts. Quercetin equivalent of these extracts was 14.22-116.52 mg/kg fresh weight. In the previous study by Bonaccorsi et al., 2008 reported flavonol glucosides values in mg/kg fresh weight of French shallot and Italian shallot in the range of 5.2-605 and 0.8-572 mg/kg fresh weight,
respectively. Increasing ethanol concentration in the extraction process decreased quercetin equivalent of ethanolic shallot extracts. The quercetin equivalent of 20% ethanolic shallot extract was significantly different from 60%, 80% and 95% ethanolic extracts ($p$-value < 0.05). Moreover, ethanol concentration was significantly correlated with quercetin content (Pearson correlation, $p < 0.05$).

**HPLC analysis chemical compositions of shallot extracts**

All shallot extracts showed similar eluted peak pattern to methanolic onion extracts that was reported by Bonaccorsi et al., 2008. Quercetin aglycone peak of shallot extracts was eluted as a corresponding retention time (14.89 - 14.92 minutes) to standard quercetin hydrate (14.92 minutes) as shown in Figure 1. Since all shallot extracts showed the same elution profile in HPLC, some HPLC chromatograms were presented in Figure 1 and 2. UV spectra of shallot extracts at RT 14.90 showed a corresponding spectra pattern and maximum absorption wavelength to that of quercetin hydrate as shown in Figure 3. Seven flavonol glucoside compounds were separated and eluted in order: (1) quercetin 3,4'-diglucosides, (2) isorhamnetin 3,4'-diglucosides, (3) quercetin 3'-glucoside, (4) quercetin 4'-glucoside, (5) isorhamnetin 4'-glucoside, (6) quercetin aglycone and (7) isorhamnetin aglycone in HPLC analysis. The molecular ions were identified by ESI-MS/MS and corresponding to their molecular weights (supplement data).

The eluted peak corresponding to quercetin aglycone decreased when increasing ethanol concentration in shallot extract while the eluted peak corresponding to quercetin diglucoside slightly increased. Isohamsnetin monoglucoside which was characteristics of shallot extract was mainly found in these water and ethanolic shallot extracts too. Isohamsnetin diglucosides and isorhamnetin aglycone were found in small contents and was rarely seen in high ethanolic shallot extracts. In addition, quercetin 4'-glucoside was mostly observed in all shallot extracts in simialr to methanolic onion extracts (Bonaccorsi et al., 2005; 2008).

Organosulfides including disulfides, trisulfides and cyclic polysulfides were identified from hydroditilled and solvent (diethyl ether) extracted oil of Allium cepa L. Aggregatum by GC-MS/FID (Tocmo et al., 2014). These organosulfides were benefical as antimicrobial and cardiovascular health. In this study no organosulfides were observed because the method of extraction was ethanolic solvent extraction.

**Table 1** Percent yield, quercetin equivalent, toxicity and activities of shallot extracts

<table>
<thead>
<tr>
<th>Samples</th>
<th>%Yield</th>
<th>Quercetin equivalent (mg/kg)</th>
<th>DPPH, IC$_{50}$ (mg/mL)</th>
<th>%Tyrosinase Inhibition</th>
<th>MTT, IC$_{50}$ (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water extract</td>
<td>11.80</td>
<td>83.21 ± 5.69</td>
<td>2.85 ± 0.10</td>
<td>14.63 ± 2.63</td>
<td>24.84 ± 1.05</td>
</tr>
<tr>
<td>20% ethanolic extract</td>
<td>8.68</td>
<td>116.52 ± 22.46*</td>
<td>2.18 ± 0.07</td>
<td>16.20 ± 2.70</td>
<td>24.06 ± 1.05</td>
</tr>
<tr>
<td>40% ethanolic extract</td>
<td>11.41</td>
<td>60.36 ± 0.46</td>
<td>3.23 ± 0.32</td>
<td>23.73 ± 2.90*</td>
<td>14.76 ± 1.07</td>
</tr>
<tr>
<td>60% ethanolic extract</td>
<td>11.06</td>
<td>25.20 ± 0.59</td>
<td>3.91 ± 0.29</td>
<td>16.86 ± 1.40</td>
<td>17.19 ± 1.07</td>
</tr>
<tr>
<td>80% ethanolic extract</td>
<td>11.35</td>
<td>14.22 ± 0.22</td>
<td>3.19 ± 0.69</td>
<td>29.27 ± 3.08*</td>
<td>18.16 ± 1.07</td>
</tr>
<tr>
<td>95% ethanolic extract</td>
<td>11.43</td>
<td>23.06 ± 0.36</td>
<td>4.37 ± 0.17</td>
<td>23.60 ± 2.33*</td>
<td>18.47 ± 1.07</td>
</tr>
<tr>
<td>Quercetin hydrate(ug/mL)</td>
<td>-</td>
<td>-</td>
<td>3.16 ± 0.21</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kojic acid (20 µM)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

Quercetin equivalent : * $p$-value < 0.05 compared with 60%, 80%, and 95% ethanolic extract

%Tyrosinase inhibition : * $p$-value < 0.05 compared with water, 20% and 60% ethanolic extracts
**Figure 1** HPLC chromatograms of quercetin hydrate (7.5 µg/mL) and water shallot extract

**Figure 2** HPLC chromatograms of (A) 20% and (B) 80% ethanolic shallot extracts

**Figure 3** UV spectra of (A) quercetin hydrate and (B) 20% ethanolic shallot extract
Figure 1S HPLC chromatograms of (A) water and (B) (F) 20, 40, 60, 80 and 95% ethanolic shallot extracts, respectively
Figure 2S  ESI-MS/MS (negative mode) of flavonol glucosides eluted from shallot water extract
**Cytotoxic effects of shallot extract on HDF cells**

HDF cells were used to represent as a model of normal human cells. The results showed that IC50 values of all shallot extracts were not significantly different as shown in Table 1. As the results shown in Figure 4, all extracts at 5 mg/mL did not affect to cell viability of HDF when compared with control group. The extracts that showed the significant cytotoxic effects at the concentration of 10 mg/mL were including 40% and 60% ethanolic extracts. For the other extracts, they possessed the cytotoxic effects at the concentration of 20 mg/mL. Therefore, the concentration of the extracts for other study was 5 mg/mL.

![Figure 4](image)

**Figure 4** The effect of shallot extracts on HDF cell viability. Values are means of three independent triplicate samples ± SEM. *p*-value < 0.05 versus non-treated control

**Effect of shallot extracts on the antioxidative effects**

The results showed that IC50 value of 20% ethanolic extract exhibited the highest antioxidant activity even though its activity was much lower than quercetin hydrate. Between groups the antioxidant activities were not statistically significant. From these results, the antioxidative effect of shallot extracts was related to the amount of quercetin in the extracts. This finding consistented with previous studies that quercetin exhibits antioxidant activity (Rice-Evans et al., 1996) and the extract from onion with high quercetin-4’-glucoside content possessed high antioxidation activity (Roldán-Marín et al., 2009). Moreover, fermented onion with enzyme extract from soybean paste fungi, Aspergillus kawachii was found to increase quercetin-3-O-β-D-glucoside and quercetin content which resulted in raising DPPH radical scavenging effect with 79.06±1.48% compared with 18.24±2.10% of untreated onion extract at the same 100 μg/mL concentration (Yang et al., 2012).

**Effect of shallot extracts on tyrosinase inhibition effect**

Tyrosinase inhibition effect of shallot extracts was investigated by mushroom tyrosinase inhibition assay. Shallot extracts with 40%, 80% and 95% ethanol at a concentration of 5 mg/mL significantly inhibited tyrosinase activity. Especially, shallot extract with 80% ethanol exhibited the highest percentage of tyrosinase inhibition as shown in Table 1. However, their activities were lower than kojic acid which used as a positive control.
In this study the tyrosinase inhibitory effect seemed not to relate to quercetin content although quercetin 4′-O-β-D-glucopyranoside from methanolic extract of *Allium cepa* dried skin, showed tyrosinase inhibitory activity with IC₅₀ 52.7 μM (Arung et al, 2011). From previous studies, there were other compounds in *Allium* species that exhibit tyrosinase inhibitory activity. Volatile flavor containing dimethyl sulfides in onion oil competitively inhibited mushroom tyrosinase (Perez-Gilabert and Garcia-Carmona, 2001). An indene derivative having the substituent of the hydroxyl and alkyl group of *Allium Cepa* possessed tyrosinase inhibition effects (Jang et al, 2008).

**Effect of shallot extracts on the antimicrobial tests**

The results showed that there were no inhibition zone for all shallot extracts against tested bacteria compared with clindamycin (3.00±0.00 cm, 2.92±0.00 cm and 4.80±0.00 cm for *S. aureus*, *S. epidermidis*, and *P. acnes*, respectively). In agreement with agar disc diffusion method, MIC and MBC of all shallot extracts were higher than 1,024 μg/mL, while clindamycin inhibited these bacteria with high potency with MIC and MBC less than 0.5 μg/mL. Those shallot water extract which inhibited some bateria was prepared at room temperature (Amin and Kapis, 2005) but in this study it was macerated at 50°C. In addition, thermal processing condition of *Allium cepa* L. aggregatum reduced organosulfides while freeze drying retained the majority of these compounds (Tocmo et al., 2014).

**CONCLUSION**

Quercetin aglycone and other flavonol glucosides of ethanolic shallot extracts were clearly separated by HPLC analysis. Ethanol concentration showed significantly correlated to quercetin content. Quercetin equivalent also related to antioxidant activity of shallot extracts but not their anti-tyrosinase activities. Since shallot extracts were not toxic to HDF cells, it will be further studied tyrosinase inhibitory effect in cell culture model.

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