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Flavonol Glucosides in Shallot Extracts and Tyrosinase Enzyme Inhibition

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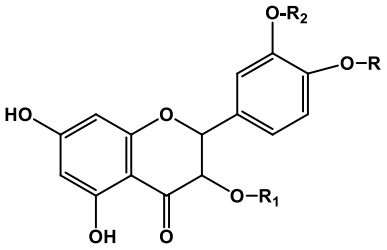
Introduction

Shallot (*Allium ascalonicum* L.) is one of the Allium genus which has been used as the condiment and medicinal applications. Its chemical compositions are similar to onion (*Allium sativum*) and garlic(*Allium cepa*). Chemical compositions in shallot are flavonol glucosides[1], saponins, and tannins including sulfur-containing compounds. Quercetin diglucosides and monoglucoside were mainly flavonol glucosides found in shallots. Quercetin and its glucosides commonly found in other fruits and vegetables also. Ascalonicoside A1/A2 and ascalonicoside B are saponins which are identified from *Allium ascalonicum* Hort[2]. The organosulfur compounds found in shallots composed of volatile and non-volatile compounds. Volatile organosulfur compounds are sulfur dioxide, dimethyl trisulfide, diallyl mono-, di and trisulfides, and allylmethyl di- and trisulfides[3]. Non-volatile organosulfur compounds such as *s*-alk(en)yl cysteine sulfoxides are stored in the cytoplasm of bulbs[3]. When plant tissues were disrupted (e.g. crushing, pressing, etc.), the organosulfur compounds can be subsequently converted to other sulfurous based compounds; for example, thiosulfinates. Thiosulfinates can undergo further dissociation or rearrangement to other volatile and non-volatile compounds. These organosulfur compounds represent a strong pungent smell of allium plants. Chemical structures of some flavonol glucosides are shown in Figure 1.



No.	Compounds	R	R_1	R_2
1	Quercetin-3,4′-diglucoside	β-Glu	β-Glu	H
2	Isorhamnetin-3,4′-diglucoside	β-Glu	β-Glu	CH_3
3	Quercetin-3-glucoside	H	β-Glu	H
4	Quercetin-4′-glucoside	β-Glu	H	H
5	Isorhamnetin-4′-glucoside	β-Glu	H	CH_3
6	Quercetin	H	H	H
7	Isorhamnetin	H	H	CH_3

Figure 1 Chemical structures of some flavonol glucosides found in shallot extracts

Both flavonol glucosides and organosulfur compounds are responsible for some bioactivity; for example, antioxidants[4], anti-microbials[5], anti-melanogenesis[6], and anti-inflammatory[4]. Quercetin was reported to inhibit mushroom tyrosinase activity in cell-based system[6,7]. Therefore, these bioactive compounds are interesting to be developed as food supplements and additives in the pharmaceutical industry. However, the method of extraction was important to maintain these bioactive compounds. In order to determine the effect of lyophilized times for preparation of shallot extracts, shallot bulbs were removed moisture before maceration in cold temperature. Water and 20%ethanol were used as solvents to avoid toxic residual left in the extracts. In this study focused on flavonol glucosides and their tyrosinase enzyme inhibition.

Methods

Preparation of shallot extracts

Shallots were bought from a local market in Thailand. Shallot bulbs were dried for 12, 24, and 48 hours. They were ground using a cold mortar. Then, they were macerated in water and 20% ethanol at 4 °C for 4 hours. The shallots (60-80 g, each) were extracted in a solvent with 1:1 ratio weight (g) by volume (mL) for two times. The sample solutions were centrifuged at 4,000 rpm, 4 °C and filtered. After that, the samples were dried using a lyophilizer.

HPLC analysis

All samples were analzed using HPLC on the Agilent 1260 HPLC system with EZchrome software. A stationary phase was Poroshell C18 column (2.1 x 150 mm, 4 µm) and controlled at 30 °C. The mobile phase composition was 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) in step 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 remained at 95%B, then back to 5%B and equilibrated for 4 minutes, with a total run time of 45 minutes modified from Bonaccorsi *et al.,* (2005). The flow rate was 0.3 mL/min, and the injection volume was 10 µL. Chromatogram was detected using a diode array at the wavelengths 260 and 370 nm. A peak area of each flavonol glucoside was recorded and calculated as relative area percent of total flavonol glucosides.

Tyrosinase inhibition assay

Mushroom tyrosinase inhibition assay was modified from the method of Uchida et al., 2014[8]. Shallot extracts were soluble in water at the concentration of 5 mg/mL and each 20 µL was added into a 96-well plate. The solution was 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.

Statistical analysis

Data were obtained from independent experiments and presented through means ± standard deviation (SD). Statistical analysis was performed using independent t-test, one-way ANOVA, and Pearson's correlation at a significance level of p-value < 0.05. IBM SPSS software version 21.0 was used for all statistical analyses.

Results

Shallot bulbs were removed moisture in three lyophilized times: 12, 24, and 48 hours. The moisture contents in fresh shallots before extraction were 33.17, 26.56, and 16.14 %w/w, respectively. Percent yields (%w/w) of shallot extracts in water (7.50-9.40%) were slightly less than that of shallot extracts in 20%ethanol (8.47-10.02%). In this study quercetin, isorhamnetin and their glucosides were eluted in order from HPLC analysis and ESI-MS/MS identification in our previous report[9]. The chemical structures of flavonol glucosides are shown in Figure 1. The average peak areas of each flavonol glucoside were not significantly different among solvents and lyophilized times except for quercetin-3-glucoside and isorhamnetin-3,4′-diglucosides (Table 1). The relative quercetin-3-glucoside contents were significantly different between the extracts in water and 20%ethanol and so as isorhamnetin-3,4′-diglucosides (Table 2). The relative quercetin-3-glucoside and isorhamnetin-3,4′-diglucosides contents of the extracts obtained from water maceration were higher than those obtained from 20% ethanol maceration. In addition, the relative isorhamnetin-4′-glucoside content was significantly different between 24 and 48 hours lyophilized times (Table 2). Percent tyrosinase inhibition was significantly different between the extract in water and 20% ethanol. Although the water shallot extract and 12 hours lyophilized time exhibited the highest tyrosinase inhibition, it was significantly lower potency (1.5 fold) than kojic acid (Figure 2).

Table 1 Percent yield, average area percent and tyrosinase enzyme inhibition

Shallot extracts	*Water*			*20%Ethanol*		
	L(12)	L(24)	L(48)	L(12)	L(24)	L(48)
%Yield (%w/w)	7.50	8.29	9.40	8.47	9.77	10.02
Quercetin-3,4′-diglucoside[1]	27.91±0.02	30.23±0.01	31.68±0.00	27.02±0.02	28.66±0.01	30.52±0.00
Isorhamnetin-3,4′-diglucoside[1]	3.24±0.01	3.18±0.01	3.15±0.00	2.85±0.00	2.88±0.00	3.10±0.00
Quercetin-3-glucoside[1]	2.83±0.00	2.81±0.01	2.89±0.00	2.48±0.01	2.35±0.00	2.25±0.00
Quercetin-4′-glucoside[1]	52.70±0.12	49.48±0.00	48.61±0.01	53.26±0.03	53.26±0.00	50.11±0.01
Isorhamnetin-4′-glucoside[1]	10.43±0.01	10.07±0.00	10.57±0.00	10.36±0.00	10.15±0.00	10.89±0.01
Quercetin aglycone[1]	2.69±0.13	3.97±0.00	2.83±0.00	3.62±0.03	2.41±0.01	2.79±0.00
Isorhamnetin[1]	0.20±0.00	0.27±0.01	0.27±0.00	0.41±0.01	0.29±0.01	0.34±0.00
%Tyrosinase inhibition[2]	39.17±1.92*	35.76±5.18*	32.74±1.06*	23.98±4.52*	22.56±5.43*	22.07±5.84*

[1] Calculated from %average peak area of total flavonol glucosides (N=2, duplicate)

[2] %Inhibitions are means of samples ± SD * p-value < 0.05 %Inhibition of Kojic avid (20 µM) = 59.35±4.19

Table 2 Statistical analysis

	Solvents	Water	20%Ethanol	Sig*
Quercetin-3-glucoside		2.84 ± 0.02	2.36 ± 0.07	0.002
Isorhamnetin-3,4′-diglucosides		3.19 ± 0.03	2.94 ± 0.08	0.041
Tyrosinase enzyme inhibition		35.89 ± 1.86	22.87 ± 0.57	0.003
	Lyophilized times	24 Hours	48 Hours	
Isorhamnetin-4′-glucoside		10.11 ± 0.14	10.73 ± 0.14	0.020

* *p*-value < 0.05

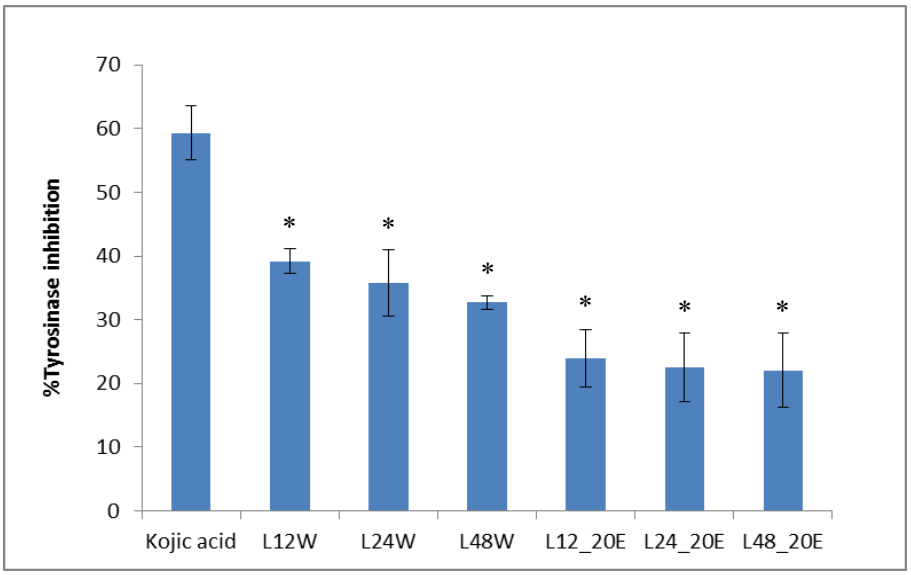


Figure 2 %Tyrosinase inhibition of shallot extracts in water and 20%ethanol and three lyophilized times compared with kojic acid (N=3, triplicate) * *p*-value < 0.05

Discussion

In this study lyophilize times did not give any significant difference on %yield and relative flavonol glucoside contents. Although fresh shallot bulbs were macerated in cold temperature, no organosulfur compounds were observed in all shallot extracts. Non-polar solvent may be more suitable for extraction of organosulfur compounds while polar solvent was acceptable for extraction of flavonol glucosides. HPLC analysis showed these flavonol glucosides in similar pattern to that of methanolic extracts of allium plants reported by Bonaccorsi P[1]. Since the HPLC profiles of all shallot extract were similar patterns, an example of HPLC chromatogram of water shallot extract was shown in Figure 3. Isorhamnetin aglycone peak was very tiny and rarely seen in un-zoom chromatogram.

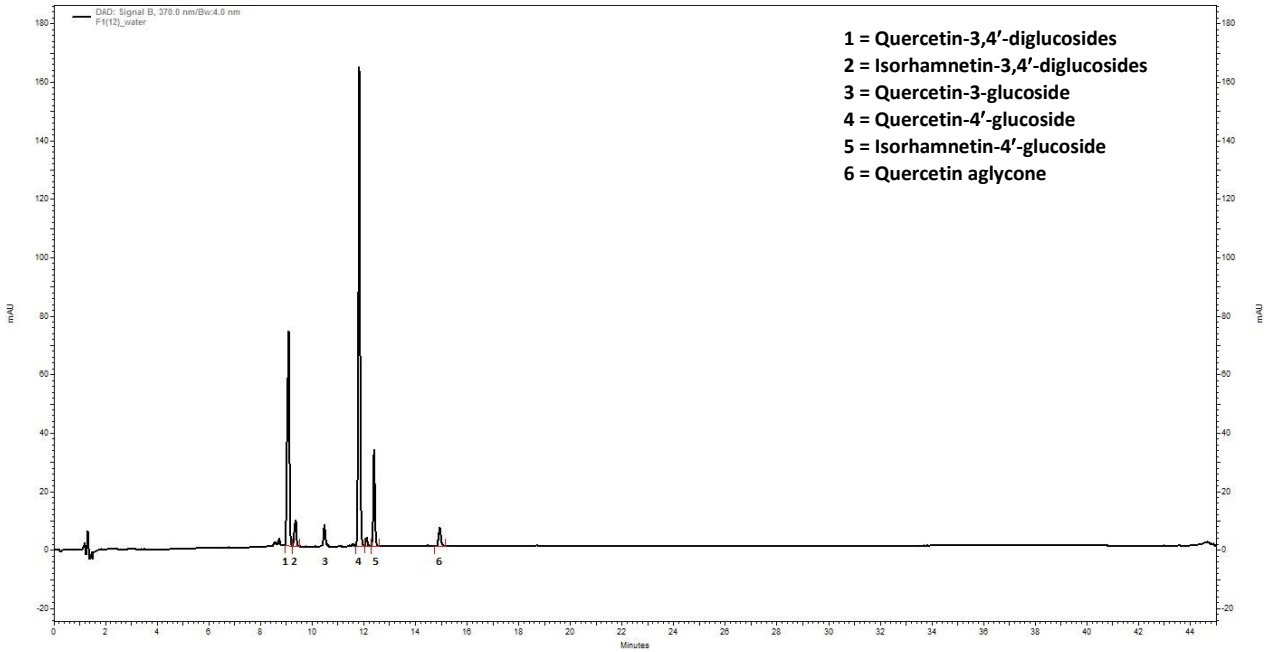


Figure 3 HPLC chromatogram of water shallot extract

Despite the relative quercetin-4′-glucoside content was the highest flavonol glucoside contents, Pearson's correlation showed that the relative quercetin-3-glucoside content significantly correlated with tyrosinase enzyme inhibition in this study. It is still not clear whether quercetin aglycone or its glucosides has affected on tyrosinase enzyme inhibition. Since the shallot extracts in this study were the crude extracts and tyrosinase enzyme inhibition assay was enzyme assay model, it needed further study of the activity in cell-based assay compared with the high concentrate of isolated flavonol glucosides. In comparison, the study of dried *Allium cepa* reported quercetin glucoside decreased anti-melanogenesis effect in melanoma cells. The isolated quercetin-3-glucoside (IC_{50} > 215 μM) and quercetin-4′-glucoside (IC_{50} = 130 μM) of the methanolic extract showed reducing melanin inhibition in B16 melanoma cells compared with quercetin (IC_{50} = 26.5 μM) while its glucosides maintained cell viability (82-88%)[10]. In addition, anti-melanogenesis effect of quercetin was concentration dependent, at the concentration of 10-20 μM melanin contents increased while the concentration more than 20 μM decreased melanin contents. High concentration (50-100 μM) of quercetin decreased melanin contents but showed cytotoxicity to cells[11]. However, natural quercetin extracts at the concentration of 10-500 μM increased melanin contents with less cell toxicity[11].

Conclusion

Flavonol glucoside contents and profiles were not significantly different among these shallot extracts. Quercetin-4′-glucoside was the main compound of flavonol glucosides and quercetin-3,4′-diglucosides was subsequently found in less content. Tyrosinase enzyme inhibition was significantly correlated with the relative quercetin-3-glucoside content with relatively low potency compared with kojic acid. Quercetin and its glucoside derivative may less effective as a whitening agent. High concentrate of quercetin content in shallot extract may be used as natural anti-melanogenesis.

Acknowledgements

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