Relationships between Chemical Compositions of Shallot Extracts and Antioxidant Activity

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Abstract

A shallot (*Allium ascalonicum* L.) is the same genus as an onion (*Allium cepa* L.) and contain similar chemical compositions. Shallots exhibit an antioxidant and antibacterial activity, which can inhibit some cancer cell lines. This study investigated quercetin equivalent contents and an antioxidant activity of shallot extracts. Shallot bulbs were extracted with water and 20% ethanol, and the remaining shallot bulbs were macerated in those two solvents for 1 month. The antioxidant activity was determined using DPPH radical scavenging assay. The chemical compositions of shallot extracts were characterized using HPLC analysis and HPTLC densitometry. With the HPLC analysis, the result showed the order of eluted flavonol glucosides in shallot extracts corresponded to the results of previous studies. Quercetin monoglucosides and quercetin diglucoside were the main compounds found in shallot extracts while free quercetin was mostly found in shallot extracts after 1 month. The HPTLC result showed that no alliin was found in all shallot extracts. The 20% ethanolic shallot extract through 24-hour moisture removal and 1-month maceration exhibited the highest antioxidant activity. Water and 20% ethanol did not give significantly different yields. In conclusion, the extraction of shallot bulbs in water and 20%ethanol produced similar compositions as found in extracts with other solvents. When fresh shallot bulbs were extracted at a cold temperature, thiosulfinate compounds were rarely detected. Further studies were recommended to determine the activity of shallot extracts and other biological activities.

Keywords**:** *Allium ascalonicum L., antioxidant activity, flavonol glucosides, isorhamnetin, quercetin*

บทคัดย่อ

หอมแดงเป็นพืชที่อยู่ในสกุลเดียวกับหอมหัวใหญ่และมีสารเคมีที่เป็นส่วนประกอบคล้ายคลึงกัน หอมแดงมีฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ยับยั้งเชื้อแบคทีเรีย และยับยั้งเซลล์มะเร็งเพาะเลี้ยงบางชนิด การศึกษานี้ตรวจสอบปริมาณสารเคอเซตินและฤทธิ์ต้านอนุมูลอิสระของสารสกัดหอมแดง วิธีดำเนินการวิจัย หอมแดงสกัดด้วยน้ำและ 20% เอทานอล และกากนำมาแช่สกัดต่อในตัวทำละลายทั้งสองเป็นระยะเวลา 1 เดือน การศึกษาฤทธิ์ต้านอนุมูลอิสระใช้วิธีทดสอบ DPPH เทคนิค HPLC และ HPTLC densitometry ใช้ตรวจสอบสารเคมีที่เป็นส่วนประกอบในสารสกัดหอมแดง ผลการวิจัย การวิเคราะห์ด้วย HPLC ตรวจสอบพบสารกลุ่มฟลาโวนอลกลูโคไซด์ในสารสกัดหอมแดงซึ่งมีลำดับการแยกออกมาเหมือนกับผลการศึกษาที่มีรายงาน สารเคอเซตินไมโนกลูโคไซด์ และเคอเซตินไดกลูโคไซด์พบเป็นส่วนใหญ่ในสารสกัดหอมแดง ในขณะที่เคอเซตินพบในสารสกัดหอมแดง 1 เดือน ผลการตรวจสอบด้วย HPTLC ไม่พบสารอะลิอินในสารสกัดหอมแดงทุกตัวอย่าง สารสกัดหอมแดงด้วยการแช่หมักใน 20% เอทานอลระยะเวลา 1 เดือน ที่นำหอมแดงมาลดความชื้นออกเป็นระยะเวลา 24 ชั่วโมงแสดงฤทธิ์ต้านอนุมูลอิสระสูงสุด ผลการสกัดด้วยน้ำและ 20%เอทานอลให้ร้อยละของผลผลิตไม่แตกต่างกัน ข้อสรุป การสกัดหอมแดงที่อุณหภูมิต่ำด้วยน้ำและ 20%เอทานอล พบสารเคมีที่คล้ายคลึงกับการสกัดด้วยตัวทำละลายอื่น แม้จะสกัดหัวหอมแดงที่อุณหภูมิต่ำก็ยังตรวจไม่พบสารประกอบกลุ่มไธโอซัลฟิเนต การศึกษาต่อไปควรมีการตรวจสอบฤทธิ์ของสารสกัดหอมแดง และประเมินฤทธิ์ทางชีวภาพอื่นๆ

คำสำคัญ: *หอมแดง ฤทธิ์ต้านอนุมูลอิสระ ฟลาโวนอลกลูโคไซด์ ไอซอรแรมเนติน เคอเซติน*

1. Introduction

Allium is the largest genus of the Alliaceae (Amaryllidaceae) family including garlics, *Allium sativum* L.; onion, *Allium cepa* L.; and shallot, *Allium ascalonicum* L. A lot of research studied about onions, garlics, and shallots. Volatile organosulfur compounds or thiosulfinates, mainly disulfides and trisulfides, were the characteristics of pungent taste of onions and garlics (Block, 1992). The other polar compounds were saponins and flavonoids (Bonaccorsi *et al.*, 2005; Lanzotti, 2006). These compounds performed some biological activities i.e. antioxidant, antibacterial, antifungal activities (Lee *et al.*, 2014; Rose *et al.*, 2005; Roldán-Marín *et al.*, 2009; and Whitmore and Naidu, 2000). Therefore they were used for

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both prevention and therapy and prescribed as hypoglycemic, hypotensive, hypocholesterolemic, antiatherosclerotic and antithrombotic agents. Flavonoid quercetin, isorhamnetin-3,4′-diglucosides and phenolic compounds were previously found in shallots. Traces of isorhamnetin 3′-diglucosides, free quercetin and ascalonicosides were also detected (Block, Putman and Zhao, 1992 and Bonaccorsi *et al.*, 2005; *et al.*, 2002). Shallot bulbs exhibited antioxidant (Leelarungrayub *et al.* 2006), antimicrobial activities (Amin and Kapadnis, 2005; Chaisawadi *et al.*, 2005; Kyu Hang Kyung, 2012), and antifungal activities (Mahmoudabadi and Nasery, 2009). Aqueous extracts exhibited anti-inflammatory and anticancer activities in Jurkat and K562 cell lines (Mohammadi-Motlagh, Mostafaie and Mansouri 2011).

Previous research reported solvent extraction of onions and shallots using a series of solvent of increasing polaritie,s for example, hexane, dichloromethane, and methanol (Leelarungrayub *et al.* 2006; Seyfi *et al.*, 2010). Isolation of crude extracts was proceeded when they were suspended in water and then extracted with butanol or alternatively extracted with acetone or partitioned with ethyl acetate. GC MS (Block, Putman and Zhao, 1992; Mondy *et al.*, 2002), RP-HPLC (Crozier *et al.*, 1997; Bonaccorsi *et al.*, 2005; Cheng *et al*., 2012), LC MS/MS (Bonaccorsi *et al.*, 2008), ^{1}H-NMR and ^{13}C NMR (Fattorusso *et al.*, 2002; Soininen *et al*., 2014) was used to identify those compounds in shallot extracts. This study aimed to optimize extraction in a cold temperature to preserve chemical compositions and evaluate their activities.

2. Objectives

The objective of the study was to investigate quercetin contents and antioxidant activities of fresh shallot extracts compared with one-month maceration.

3. Materials and 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 ^{o}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 remaining shallots were macerated in those two solvents for 1 month. The sample solutions were centrifuged at 4,000 rpm, 4 ^{o}C and filtered. After that, the samples were dried using a lyophilizer.

HPTLC densitometry

HPTLC was performed on a HPTLC plate Kieselgel 60 F254 (Merck, Germany) 10 x 10 cm. Standard and sample solutions were spotted 3 µL each using an automatic spotter Linomat (CAMAG, Switzerland). Mobile phase was butyl alcohol : n-propanol : glacial acetic acid : water (3 : 1 : 1 :1) (The United State Pharmacopoeia, 2008). The developing distance was 8 cm, the band width was 4 mm, and the developing time was about 1 hour. The plate was detected at the wavelengths of 254 and 366 nm. Ninhydrin solution (0.2 %w/v) was used as the spraying reagent. Alliin (Sigma), diallyl disulfide, DADS (Sigma) and quercetin hydrate (Acros Organics) were used as standards.

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 ^{o}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 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 flavonol glucosides was recorded. Quercetin equivalence was calculated based on a standard curve of standard quercetin hydrate (0.625 – 30 µg/mL).

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DPPH radical scavenging assay

DPPH solution was prepared at a concentration of $2x10^{-4}$ M in ethanol. The shallot extracts were solubilized in 80% ethanol at various concentrations (0 -1.5 mg/mL). Both 100 μL of the sample solutions and 100 μL of the DPPH solution were added into a 96-well plate and mixed well. After leaving it at a room temperature for 30 minutes, absorbance was measured at the 517 nm wavelength using a Biorad® microplate reader. The assessment of the antioxidant activities was expressed in IC_{50} value and compared with quercetin hydrate. Each measurement was conducted 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 post hoc test at a significance level of *p*-values < 0.05. IBM SPSS software version 21.0 was used for all statistical analyses.

4. Results and Discussion

HPTLC densitometry was a rapid method to screen and identify some chemical compounds in shallot extracts. HPTLC was smaller in a particle size than a regular TLC plate resulting in a sharp band. The R_f value of alliin was 0.5-0.53 slightly higher than the value (R_f = 0.3) mentioned in the USP garlic monograph. HPTLC fingerprint showed a similar pattern between shallot extracts in water and in 20% ethanol (Figures 1 and 2). However, the standard alliin turned light brown, not dark violet, after being sprayed with ninhydrin because the standard solution was diluted. The spot of DADS was not detected in the wavelengths and after being sprayed with ninhydrin. The light yellow spot of quercetin hydrate was detected at R_f = 0.88 at 366 nm. Alliin was not detected in all samples. Similarily, it was not supposed to be found in shallot extracts (Bladt & Wagner, 1996). All samples showed 10-11 bands elution and could be separated into three zones: R_f 0-0.3, light brown to orange-pink spots (not clearly separated), R_f 0.31-0.50, purple to violet spots, and R_f 0.51-0.75, light violet to light yellow and brown after being sprayed with ninhydrin. The R_f 0.55 and was clearly seen at 366 nm, and this bands were found to be bigger in size in shallot extracts after 1-month maceration, both in water and 20% ethanol.

In this study, quercetin hydrate was used as a standard which was equivalent to quercetin aglycone (MW=302) (Figure 3A). The small peak in the shallot extract chromatogram eluted at RT 14.8-14.9 minute was (6) quercetin aglycone. The eluted peak at this retention time also showed corresponding maximum absorption wavelengths in UV spectra as shown in Figure 4. The HPLC chromatograms of water and 20%ethanolic shallot extracts were showed in Figure 5. The HPLC chromatograms showed similar profiles although that sample was extracted with methanol (Bonaccorsi *et al.*, 2005; Bonaccorsi *et al.*, 2008). Our preliminary study identified the eluted peaks in shallot extracts using ESI-MS in a negative mode in a corresponding order compared with the results reported by Bonaccorsi *et al.*, (2008). The eluted peaks at RT 9.1 - 9.3 minute were (1) quercetin 3,4′-diglucoside (MW=626) and (2) isorhamnetin 3,4′-diglucoside (MW=640). The small peak at RT 10.3-10.4 minute was (3) quercetin 3′-glucoside (MW=464). In addition, the eluted peaks at RT 11.6 – 11.8 and 12.2 -12.4 minute were (4) quercetin-4′-glucoside (MW=464) and (5) isorhamnetin-4′-glucoside (MW=478), respectively. After 1-month maceration in the refrigerator, the order of eluted peaks was the same. However, the peak area of quercetin aglycone was highly increased. The small peak eluted at RT 18.6 was corresponding to (7) isorhamnetin (m/z=315), and this peak slightly increased (Figure 6).

Quercetin equivalent content was calculated from the peak area of this quercetin aglycone peak. The standard curve of the standard quercetin hydrate was showed in Figure 3B. Quercetin contents in water and 20%ethanolic shallot extracts were not significantly different (Table 1). Interestingly, quercetin equivalent contents of shallot extracts for 1 month increased significantly different from both water and 20% ethanolic extract at the beginning (*p*-value < 0.05). Average quercetin equivalent contents showed a correlation with DPPH, average IC_{50} values (Pearson correlation, *p*-value < 0.05). There was some evidence that reported the correlation between total phenoloic compounds and flavonoid contents with antioxidant activities (Cheng *et al.*, 2012). Applying a low temperature (5 ^{o}C) and a high pressure (400 MPa) triggered

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better onion extraction and increased approximately 33% quercetin-4′glucoside contents which maintained antioxidant activities compared with untreated onions (Roldán-Marín *et al*., 2009). Processing shallots to make shallot powder could maintain nutrient values and anti-oxidative properties of shallots (Liang *et al*., 2012).

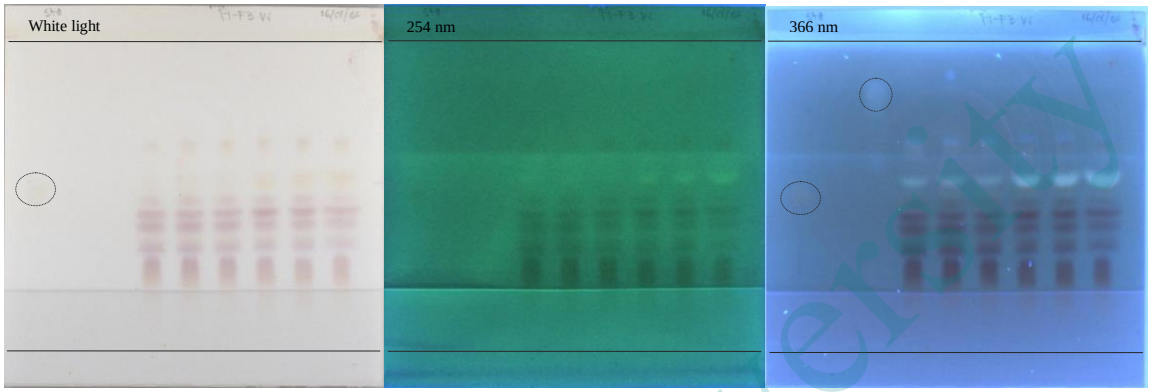

Figure 1 HPTLC of standard alliin, DADS, quercetin hydrate, shallot extracts: L(12), L(24), L(48) in water and in water after 1 month (left to right)

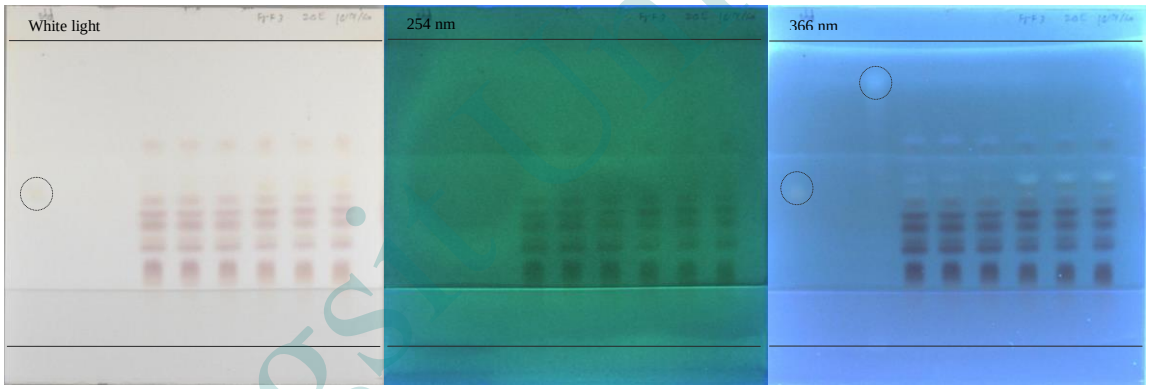

Figure 2 HPTLC of standard alliin, DADS, quercetin hydrate, shallot extracts: L(12), L(24), L(48) in 20%ethanol and in 20%ethanol after 1 month (left to right)

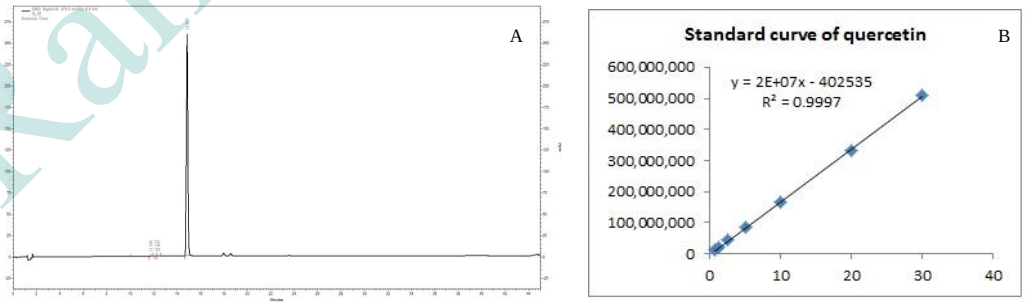

Figure 3 A. HPLC chromatograms of quercetin hydrate 20 µg/mL B. Standard curve of quercetin hydrate

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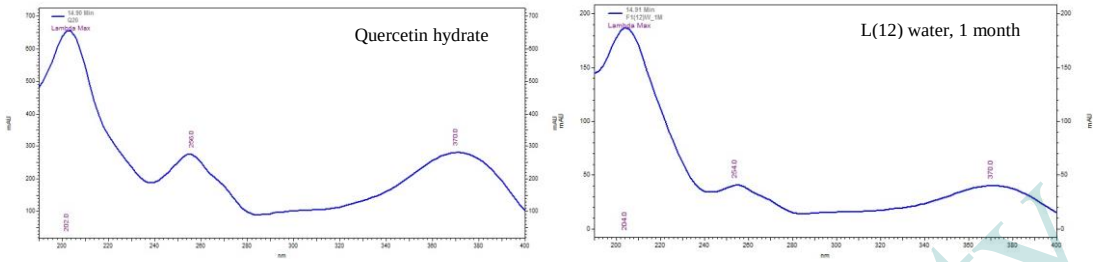

Figure 4 UV spectra of quercetin hydrate (20 µg/mL) and shallot extract in water for 1 month (10 mg/mL)

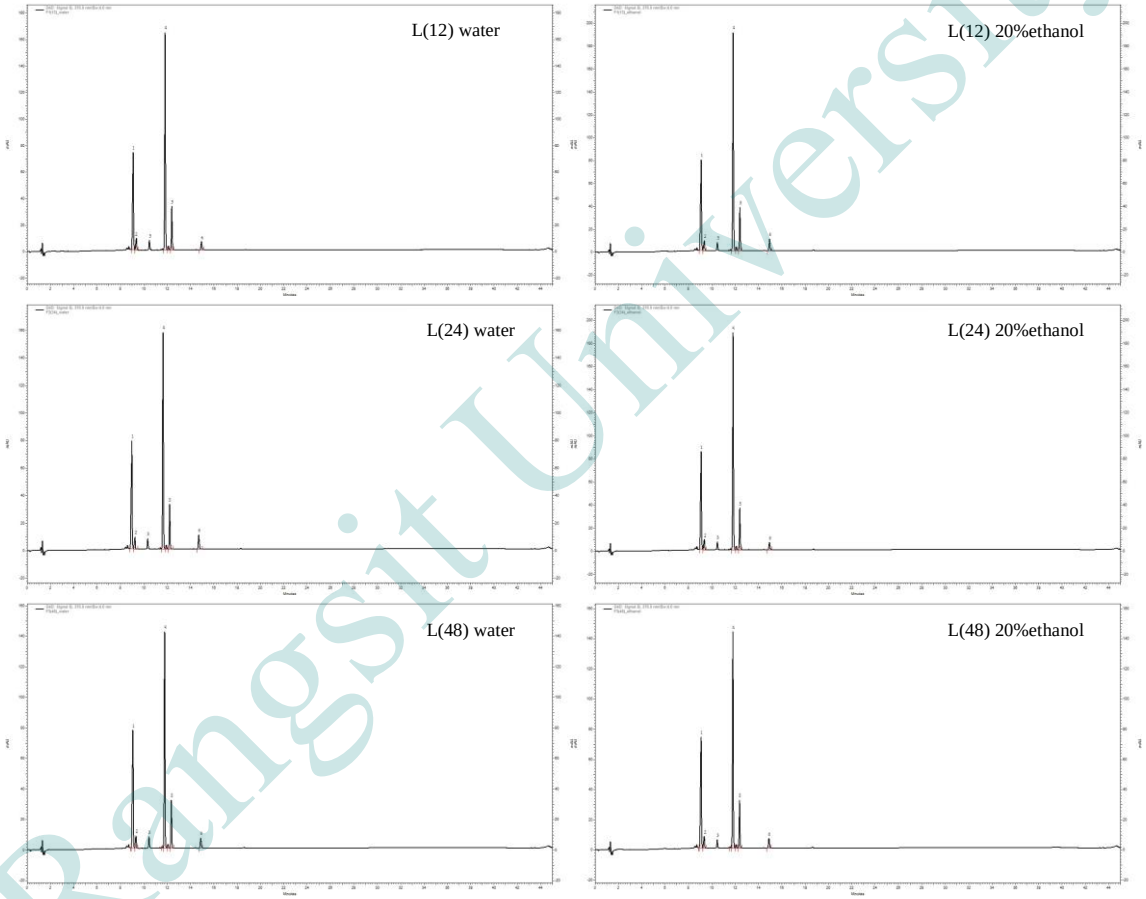

Figure 5 HPLC chromatograms of shallot extracts in water (left) and 20% ethanol (right)

Organosulfur compounds, i.e. allicin, diallyl sulfides, and diallyl trisulfides, were commonly found in onion extracts with supercritical carbon dioxide extraction while they were not detected in onion oil obtained from steam distillation (Sinha *et al.*, 1992). In this study, maceration in water and 20% ethanol at 4^{o}C was prepared, and organosulfur compounds were not detected. Flavonoids and phenolic compounds were reported to their relevance to antioxidant activities (Cheng *et al.*, 2012; Leelarungrayub *et al.*, 2006). In this study, flavonol glucosides were found in shallot extracts. However, they were different in patterns from 1-month shallot extracts. Quercetin 4′-glucoside, quercetin 3,4′-diglucosides, and isorhamnetin 4′-diglucosides, respectively, were the most abundant components found in shallot extracts and isorhamnetin 3,4′-diglucosides, quercetin 3′-diglucosides, and quercetin aglycone were minor components (Figure 5). This result was similar to that of southern Italian red onions (Bonaccorsi *et al.*, 2005). However, in 1-month shallot extracts, quercetin aglycone was the major component whereas quercetin 4′-glucoside and isorhamnetin 4′-diglucosides were minor ones. In addition, isorhamnetin was also detected. The HPLC

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analysis also revealed that quercetin 4′- glucoside comprised 50.79-54.87% of total flavonol glucosides detected in this study (Table 2). In 1-month shallot extracts, quercetin aglycone was about 64.14-84.83% of total flavonol glucosides. An increase in quercetin aglycone contents was consistent with enzyme fermented onions causing an increase in quercetin contents and resulting in a high antioxidant activity (Yang *et al.*, 2012). This may be due to a break in a glycoside linkage.

Moisture in fresh shallots was removed when they were placed in three lyophilized times for 12, 24 and 48 hours each. The moisture contents in fresh shallots before extraction were 33.17, 26.56, and 16.14 %w/w, respectively. Shallot extracts in water were obtained in the range of 7.50 – 9.40 %w/w while %yields from 20% ethanol extraction were in the range of 8.47 – 10.02 %w/w (Table 1). After 1-month maceration in a cold temperature, %yields of shallot extracts in water slightly increased (1.15-1.65 fold), but those extracts in 20%ethanol slightly decreased (1.40-1.42 fold). %Yields of water and 20%ethanol extraction in a cold temperature were not significantly different. Shallot extracts showed a relatively low antioxidant activity. The antioxidant activities of these two solvents and different moisture contents in fresh shallots were not significantly different. However, the antioxidant activity of shallot extracts for 1 month significantly increased, and the average IC_{50} values ± SEM were 10.97±2.46 & 2.27±0.10 mg/mL at the beginning and after 1 month, respectively. In addition, lyophilized shallots of which moisture was removed yielded shallot powder with a little change in color. This process can help preserve shallots for a long term and remain anti-oxidative properties due to the presence of flavone and polyphenolic compounds (Liang *et al*., 2012).

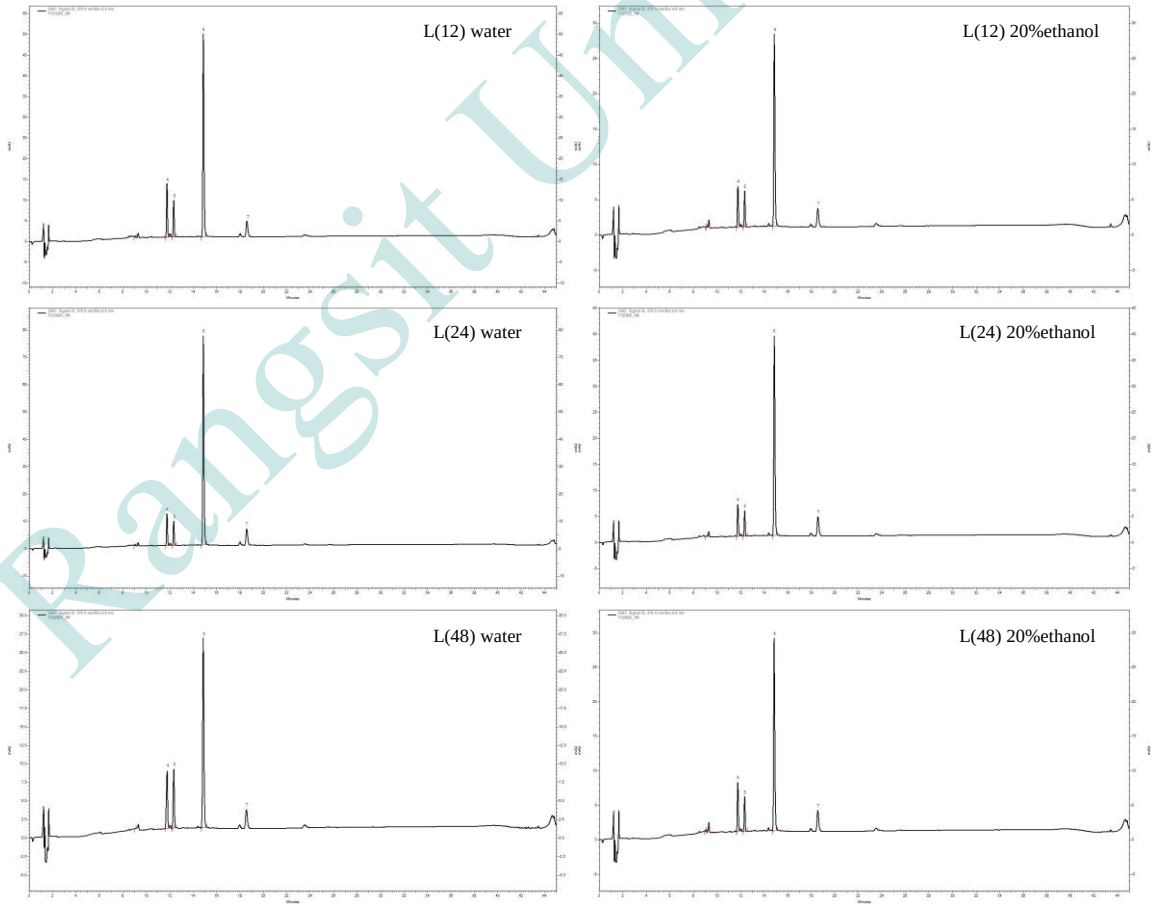

Figure 6 HPLC chromatograms of shallot extracts in water (left) and 20% ethanol (right) for 1 month

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5. Conclusion

Water and 20% ethanol extractions in a cold temperature did not give significantly different yields and chemical compositions of shallot extracts. Quercetin monoglucoside was the major compound detected in shallot extracts while quercetin aglycone was found to increase, and it could be detected in 1 month shallot extracts. The antioxidant activity of 1-month shallot extracts significantly increased although it was much lower than quercetin positive control. However, there was a statistical correlation between the quercetin content and the antioxidant activity. In addition, this inferred that a break in the glycosidic bond resulting in an increase in quercetin contents and subsequently affecting the antioxidant activity. The results suggest more investigations and biological evaluations.

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

Shallot extracts	Beginning			After 1 month maceration		
	%Yield	Quercetin content (mg/kg) [1]	DPPH, IC_{50} (mg/mL) [1]	%Yield	Quercetin content (mg/kg) [1]	DPPH, IC_{50} (mg/mL) [1]
Water						
L(12)	7.50	26.94 ± 1.15	7.85 ± 0.21	11.62	234.00 ± 1.21*	2.16 ± 0.24*
L(24)	8.29	40.15 ± 0.53	22.92 ± 3.92	13.76	354.58 ± 1.13*	2.68 ± 0.72*
L(48)	9.40	27.63 ± 0.10	8.26 ± 2.21	10.84	138.01 ± 0.69*	2.09 ± 0.15*
20%Ethanol						
L(12)	8.47	37.77 ± 0.98	11.42 ± 0.14	5.93	131.67 ± 0.02*	2.36 ± 0.43*
L(24)	9.77	25.28 ± 0.03	7.66 ± 0.06	6.97	182.92 ± 0.28*	1.96 ± 0.14*
L(48)	10.02	27.25 ± 0.05	7.70 ± 1.34	7.03	136.11 ± 0.68*	2.35 ± 0.27*
Quercetin (µg/mL)			1.55 ± 0.04			1.79 ± 0.04

[1]Quercetin content and IC_{50} values are means of samples ± SD * p-value < 0.05

Table 2 Average percent peak area of quercetin-4′-glucoside and quercetin aglycone in shallot extracts

Shallot extracts	%Peak area			
	Beginning		After 1 month maceration	
	Quercetin-4′-glucoside	Quercetin aglycone	Quercetin-4′-glucoside	Quercetin aglycone
Water				
L(12)	53.51±1.25	2.65±0.19	16.05±0.00	72.97±0.02
L(24)	51.11±0.05	3.99±0.07	10.75±0.57	84.83±4.52
L(48)	50.79±0.72	2.74±0.12	18.65±0.32	64.14±0.41
20%Ethanol				
L(12)	54.87±0.02	3.63±0.04	13.33±0.02	75.39±0.02
L(24)	54.78±0.00	2.42±0.01	10.87±0.01	80.90±0.00
L(48)	51.53±0.02	2.76±0.02	15.81±0.02	73.57±0.00

6. Acknowledgements

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