Conference Paper

Comparison of the Main Bioactive Compounds and Antioxidant Activity from Garlic Water-soluble and Garlic Oil

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Abstract

Garlic is a natural source which has abundant organosulfur constituents. Garlic is divided into water-soluble organosulfur constituents mainly SAC (S-allylcystein), NAC (N-acetylcysteine) and oil soluble organosulfur constituents such as DATS (diallyl trisulfide), DADS (diallyl disulfide), DAS (diallyl sulfide). The aim of this research was to compare the bioactive constituents and antioxidant activity between garlic water-soluble and garlic oil. Garlic water-soluble constituents were identified by Liquid Chromatography–Mass Spectrometry (LC-MS) and five constituents were found, namely N-acetylcysteine (NAC), cysteinyl-alanine, phenol-2-2-benzoxazolyl and two unknown constituents. The GC-MS chromatogram also showed three main constituents present in garlic oil as diallyldisulphide (DADS), diallyltrisulphide (DATS) and D-limonene. Interestingly, garlic water-soluble extract had higher antioxidant activity 70 % ± 0.02 % in comparison with garlic oil 58 % ± 0.07 %. This study conducts a novel preparation of garlic water-soluble for enhancing antioxidant properties on garlic novel preparation.

Keywords: antioxidant activity; garlic oil; garlic water-soluble; organosulfur constituents.

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1. Introduction

Humans are often exposed to stressful environmental factors such as radiations, chemicals, and stress resulting free radicals in cells. The abundance of free radical in cells leads to disrupted normal cellular metabolism [1]. Numerous studies revealed that antioxidants can eliminate harmful free radicals converting to neutral [2–6].

Garlic is regarded as the most common medical agent containing antioxidant activity [3, 5, 7]. Garlic acts as an exogenous antioxidant for neutralizing free radicals and helps prevent some diseases. It is a natural source which has abundant organosulfur constituents. Organosulfur has beneficial health effects particularly, inhibiting Reactive Oxygen Species (ROS) leading to oxidative stress [8].

Vitamin, proteins, lipids, trace elements Se, flavonoids and at least 33 different organosulfur compounds are identified on garlic [9, 10]. Organosulfur compounds are divided into water soluble organosulfur constituents mainly SAC (S-allylcystein), NAC (N-acetylcysteine [3, 11] and oil soluble organsulfur constituents such as DATS (diallyl trisulfide), DADS (diallyl disulfide), DAS (diallyl sulfide). NAC has a nucleophile and a –SH residue donor [11] for counteracting harmful molecules free radicals notably ROS.

Presence of NAC in garlic water-soluble may complete bioactive compound which has antioxidant activity in garlic water-soluble preparations. This study initially conducts a novel preparation of garlic water-soluble for enhancing antioxidant properties. Heating and aging were used in novel preparation methods of garlic water-soluble. The present study was designed to obtain maximum efficacy of garlic water-soluble in comparison with garlic ol.

2. Materials and Methods

2.1. Preparation of Garlic Water-soluble

The amount of 15 g fresh garlic bulbs were obtained from local market in Neipu, Pingtung Taiwan. Garlic water-soluble constituents were prepared with slight modification by [11]. Briefly the garlic bulbs (*Allium sativum* L.) were divided into separate cloves.The cloves were peeled and chopped into small cubes (5 mm). The minced garlic was shaked with 150 mL of distilled water (dd H_2O) and heated for 15 min at 65 °C by hotplate stirrer (Thermo Scientific, USA) and incubated for 48 h at 37 °C.

Water soluble garlic was removed and dried by freeze-drying (FD, LGJ-10, USA) with plate temperature at 45 °C and absolute pressure at 10 Pa combined with vacuum drying (VD, DZG-6050, USA) for 5 h. The amount of 350 mg of dried garlic powders were stored at –20 °C.

2.2. Preparation of Garlic Oil

Fresh garlic bulbs were obtained from local market in Neipu, Pingtung Taiwan. The garlic bulbs were described previously. In brief, 300 g of chopped garlic were dissolved in 800 mL destilated water (dd H_2O) and extracted for 5 h with water extraction. The garlic extract was centrifuged (Centrifuge 5810 R amp version) at 5 000 rpm (1 rpm = 1/60 Hz) for 30 min. Supernatan was removed and garlic oil stored at 4 °C.

2.3. Identification of garlic oil constituent by GC-MS

The garlic oil constituents was identified using GC-MS based on comparison of their retention times (RT) and mass spectra which was processed as described [11]. Briefly, GC/MS/MS analysis was conducted in Department of Biological Science and Technology, NPUST. The instrument are described below : Agilent 7890 GC system Water Quattro Micro GC/MS/MS : Triple quadrupole Mass Spectrometer with Column : DB-5MS, 30 m, ID : 0.25 mm, Film thickness : 0.25 µm, Initial temperature : 60 °C ; Hold time 1 min, temperature ramp rate : 7.5 °C · min^{-1} final temperature : 180 °C, second temperature ramp rate : 50 °C · min^{-1}, injection temperature : 250 °C, Injection volumes : 1 µL, Injection mode : split (10:1), Screen range : m/z 50 to 300, Ionization Mode : E1+, Solvent Delay : 5.0 min.

Moreover, diluted samples (1/1 000 in hexana, v/v) of 1.0 were injected manually then was performed three independent times. The relative percentage was measured depend on the individual peak area of the total identified constituent peak area.

2.4. Identification of Garlic Water-soluble Constituent by LC-MS/MS

Garlic water-soluble constituent was performed using a Thermo LCQ DECA XP MAX system with an electrospray ionization (ESI) source (Thermo Scientific Inc., USA) equipped with an autosampler, a surveyor 2000 quaternary pump. Garlic Water-soluble powder (1 µg · $µl^{-1}$) was loaded onto a Biobasic C18 column with diameter 150 mm × 2.1 mm, particle size 5 µm. Peak identification in samples was carried out by comparing retention times with NAC (N-Acetyl L-Cysteine) and SMC (S-Methyl-L-cysteine) as standards.

Elution gradients previously were described by [12] with slightly modification were performed with solvent A (5 % acetonitrile and 0.2 % formic acid) and solvent B (95 % acetonitrile and 0.2 % formic acid) using gradient with A and B as follows: 0 min 100 % A, 3 min 85 % A, 18 min 75 % A, 28 min 60 % A, 35 min 20 % A, 40 min 100 % A. The flow rate was 200 µL· min^{-1}. The injection volume was 35 µL.

Conditions for analysis were as follows: spray voltage, 4.0 KV; sheath gas flow rate, 50 arbitrary units; auxiliary gas flow rate, 3.0 arbitrary units; capillary temperature, 300 °C; capillary voltage, 20 V. The MS scan and MS/MS raw data were scanned using Thermo-Xcalibur^{TM} data acquisition over a range of m/z 50 to 300.

2.5. Antioxidant activity of garlic using DPPH assay

1.1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity assay was carried out for the evaluation of the antioxidant activity. The hydrogen atom or electron donating ability of garlic oil, garlic water-soluble constituents powder and BHT (Butylated-hydroxytoluene) as standard was determined by bleaching of purple colored methanol solution of DPPH.

The diluted working solution of garlic oil and water-soluble garlic powder were prepared in methanol and distilled water. Methods of DPPH radical scavenging assay following [13]. Working solution samples at a final concentration were (5 000, 4 000, 3 000, 2 000, 1 000) $\mu g \cdot mL^{-1}$ of garlic water-soluble and garlic oil. DPPH was prepared at concentration of 1 mM in absolute methanol. Sample was mixed with 25 µL of DPPH in a 96-well and incubated at room temperature for 30 min and kept in dark.

The solution was measured using microplate reader (Biorad Model 680 Microplate Reader) at 517 nm. The DPPH radical scavenging capacity was calculated as follows:

$$\% \text{ Inhibition of DPPH activity} = (A - B/A) \times 100\ \% \quad (1)$$

A = Absorbance control (DMSO)
B = Absorbance sampel

3. Results

3.1. Identification of Garlic Water-soluble Constituent by LC-MS

LC-MS/MS thermo LCQ DECA XP MAX system with an electrospray ionization (ESI) source was used to identify individual compound in natural source based on polarity and involatile compounds [12, 14]. Therefore, this study provided LC-MS/MS for identifying several constituents in garlic water-soluble. LC–MS chromatogram of garlic water-soluble constituent fraction were performed using solvent A (5 % acetonitrile and 0.2 % formic acid) and solvent B (95 % acetonitrile and 0.2 % formic acid) using gradient with A and B followed : 0 min 100 % A, 3 min 85 % A, 18 min 75 % A, 28 min 60 % A, 35 min 20 % A, 40 min 100 % A. NAC and SAC commercial were used as standards.

LC-MS chromatogram showed five peaks present in garlic water-soluble accompanied five constituents with m/z 79.14; 163.91; 212.22; 198.12; 212.06, respectively. Several data bank, likely NIST (National Institute of Standards and Technology) and HMDB (Human Metaboloma Data Bank) was used to evaluate five constituents upon five peaks with different retention time (tR).

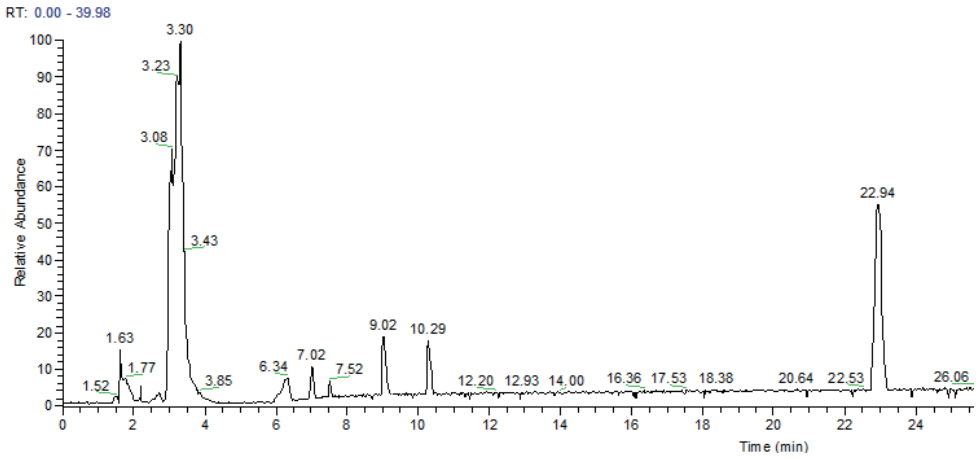
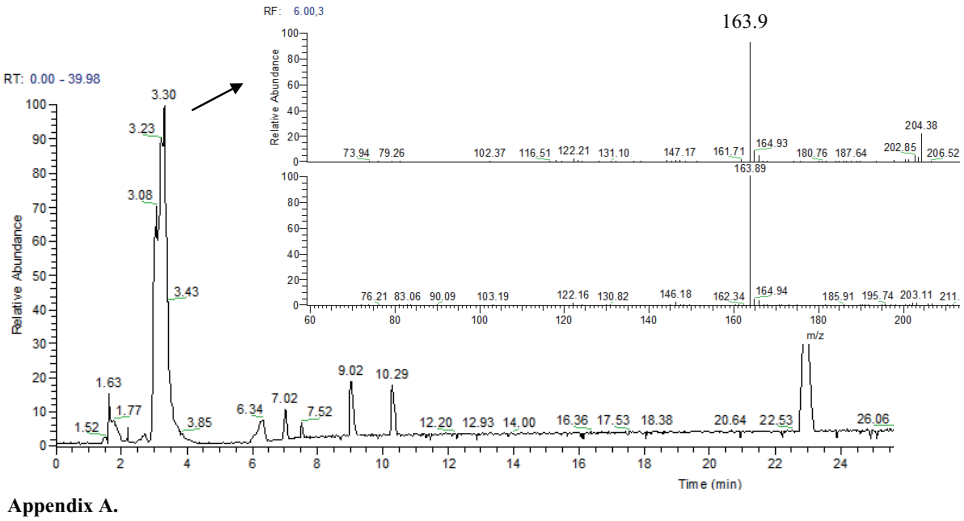


Figure 1: The presence of garlic water-soluble constituents in LC-MS were observed in the full LC–MS chromatogram with (m/z) = 50 to 300 for 28 min.

Peak	t_R (min)	$(M+H^+)$ (m/z)	Molecular Weight (M/W)	Relative Abudance (%)	Constituent
1	1.65	79.14	78.14	1	Unknown
2	3.3	163.91	162.91	60	NAC
3	9.02	212.22	211.22	6	Unknown
4	10.29	198.12	197.12	3	Cysteinyl-Alanine Phenol, 2-(2-
5	22.04	212.06	211.06	30	benzoxazolyl)

TABLE 1: Chromatographic of garlic water-soluble extract.



Appendix A.

Figure 2: The presence of NAC in garlic water-soluble extract with m/z 163.91 and tR = 3.30 min.

On most occasions, identified peaks in LC-MS chromatogram is used commercial compounds which was establish and also used data bank. Present study was provided NAC commercial as standard to compare with major peak m/z 163.91 and tR = 3.30 min (peak no.2). NAC standard was injected together with garlic water-soluble

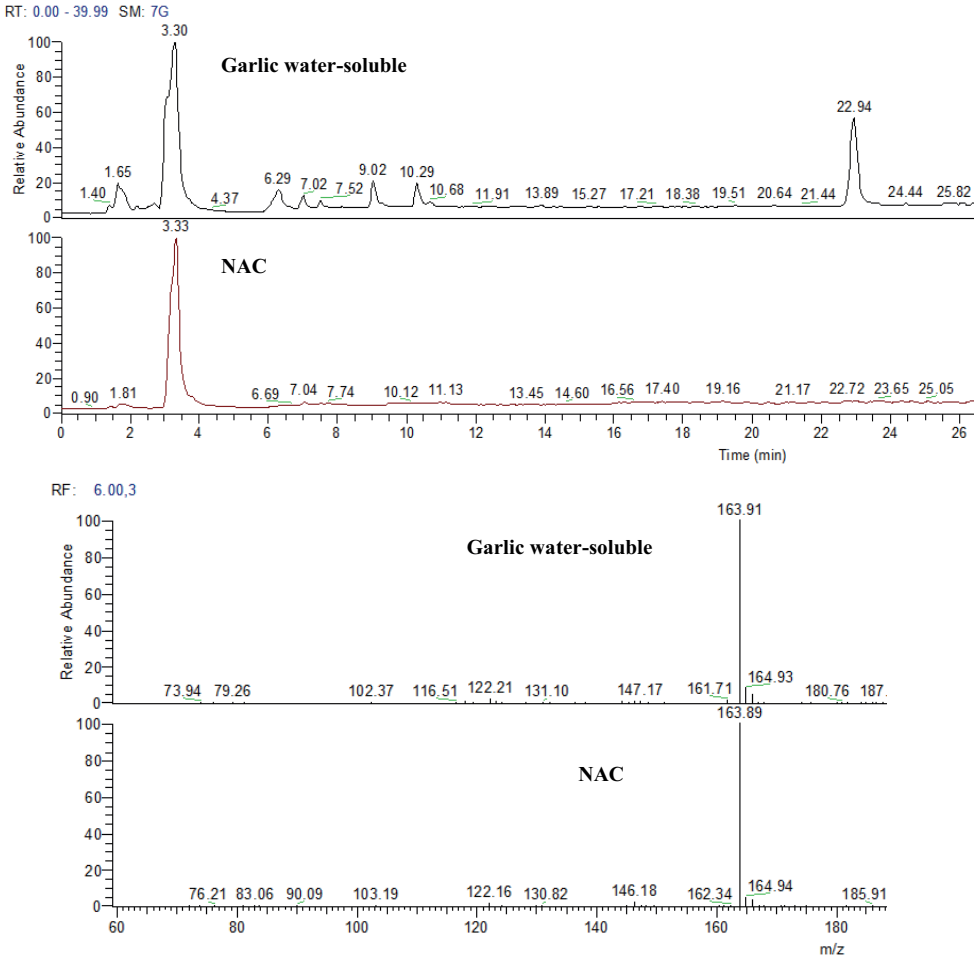


Figure 3: Peak no.2 as NAC compare with NAC standard (peak no.2: m/z = 163.91; t_R = 3.30 min). Presence in garlic water-soluble compare NAC standard (m/z = 163.89; t_R = 3.33 min) thus was established as NAC (N-allylcysteine). .

followed elution gradient by [12] with slight modification. Fig. 3 showed that LC-MS chromatogram between NAC and garlic water-soluble.

Investigation of the other constituents were presented in garlic water-soluble, valid data bank (NIST and HMD) were used. Consequently the data of SAC and SAMC (S-mercaptoallylcysteine) which are commonly observed garlic water-soluble constituents, did not match with the others peaks in garlic water-soluble samples. Accordingly, several constituents were injected and compared with data bank

The preparation methods of garlic water-soluble such as cruching, heating at 65 °C, incubating 48 h, freezing, and dehydration using freeze-dried showed five constituents presence in LC-MS chromatogram. The intact garlic bulb consist of S-amino acids notably cysteine and methione (traces) [15]. Due to oxidation of gamma glutamyl-S-allylcysteine, N-acetylcysteine $[C_5H_9NO_3S+H]^+$ and cysteinyl-alanine $[C_6H_{12}NO_3S+H]^+$ were detected at lower retention times. The hydrophilic compounds in garlic extract were eluted at lower retention times [14]. Phenol, 2-(2-benzoxazolyl) $[C_{22}H_{27}NO_2+H]^+$,

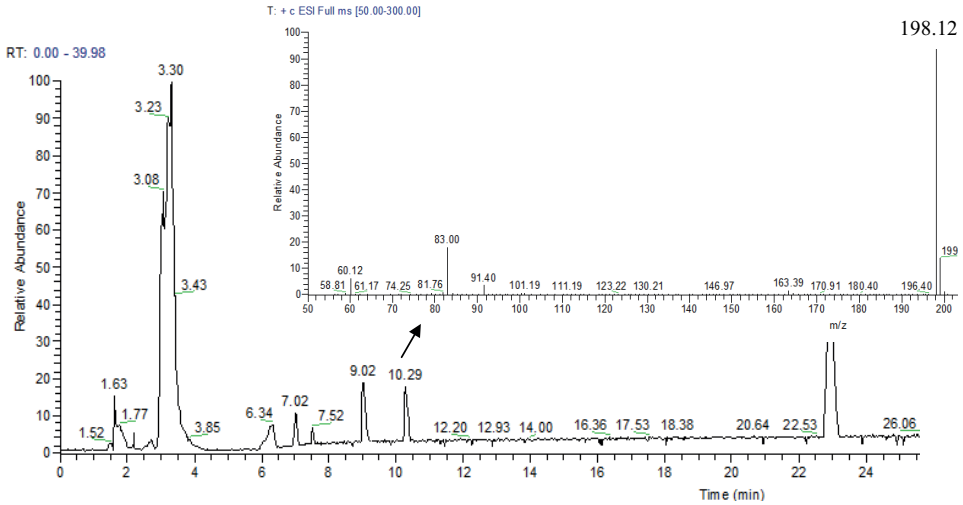


Figure 4: Peak no.4 as cysteinyl-alanine (m/z = 198.12; MW = 197.12; t_R = 10.29 min) was identified as cysteinyl-alanine (MW = 197.12) by comparing with the information in data bank NIST and HMD.

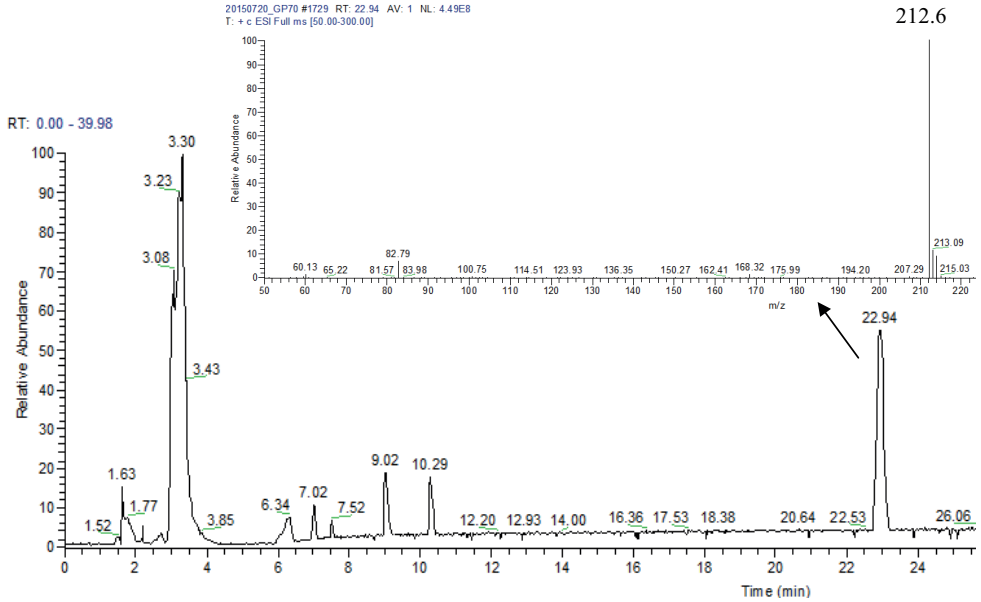


Figure 5: Peak no.5 as phenol, 2-(2-benzoxazolyl) (m/z = 212.06; MW = 211.06; t_R = 22.04 min) upon data bank NIST and HMD.

were produced during the garlic extraction likely chopping and heating thus degrade phenolic constituents in intact garlic. Fortunately, SAC and SAMC were absent in garlic water-soluble identified as LC-MS chromatogram when SAC and SAMC standards was injected, described in Fig. 6.

3.2. Analysis of Garlic Oil Constituents by GC-MS

Thiosulfinates (TS) compounds are abundant in garlic oil. Consequently disruption of garlic bulb, the formation of thiosulfinates are converted into allicin by allinase activity.

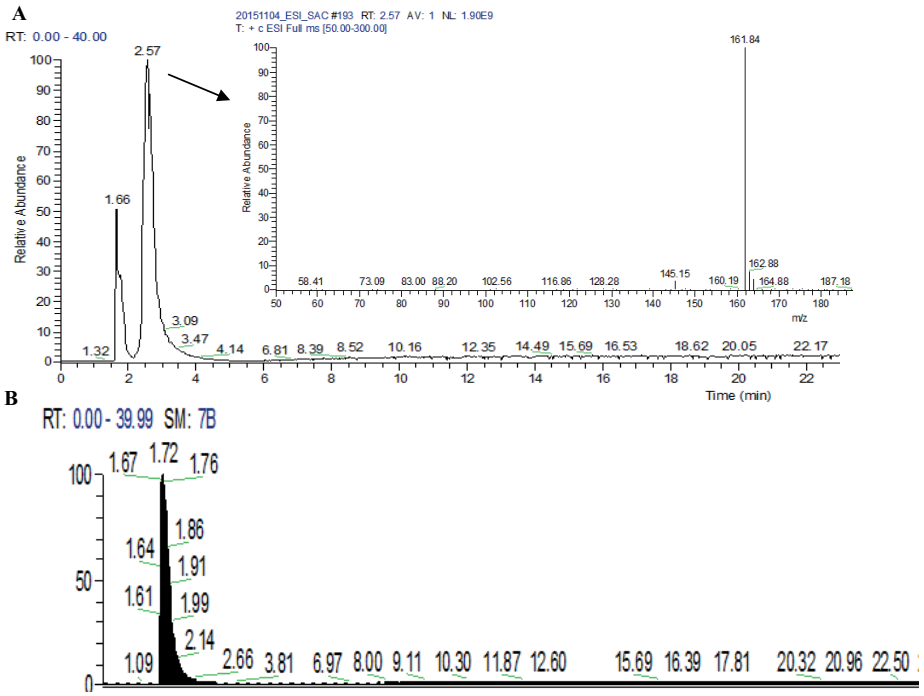


Figure 6: (A) The presence of SAC and SAMC with m/z = 161.84; MW = 160.84; t_R = 2.57 min accompanied with (B) SAMC at m/z = 194.02; MW = 193.02; t_R = 1.72.

No	Constituent	Relative Area (%)	Mode of Identification
1	D-limonene	14.71	RT, MS
2	diallyl disulfide (DADS)	53.81	RT, MS
3	diallyl trisulfide (DATS)	31.49	RT, MS

Table 2: Chemical constituents of garlic oil by GC-MS.

Present study provided, garlic oil upon water extraction at 122 °C. High temperature of extraction gained many compounds having biological activities.

Diallyl disulphide (DATS), diallyl trisulphide (DATS), and D-limonene as a organosulfur volatiles constituents were presented in garlic oil. The organosulfur volatile constitutes were determined using GC-MS. GC-MS is a combination of two different analytical techniques, Gas chromatography (GC) and mass spectrofotometry (MS), is used to analyze complex organis and biochemical mixtures [16] oil was identified by GC-MS and the detailed constituents were presented in Table 2 below.

RT, identification based on retention time; MS, identification based on comparison of mass spectra

Based on retention time and mass spectra by GC-MS analyze, three mains constituents present in garlic oil likely D-limonene (14.71 %), diallyl disulfide (DADS) (53.81 %), and diallyl trisulfide (DATS) (31.49 %). Additionally, DADS was the majority constituent in GC-MS chromatogram. It is possible to obseve that DADS is accompanied with DATS present in the heated samples of garlic [15].

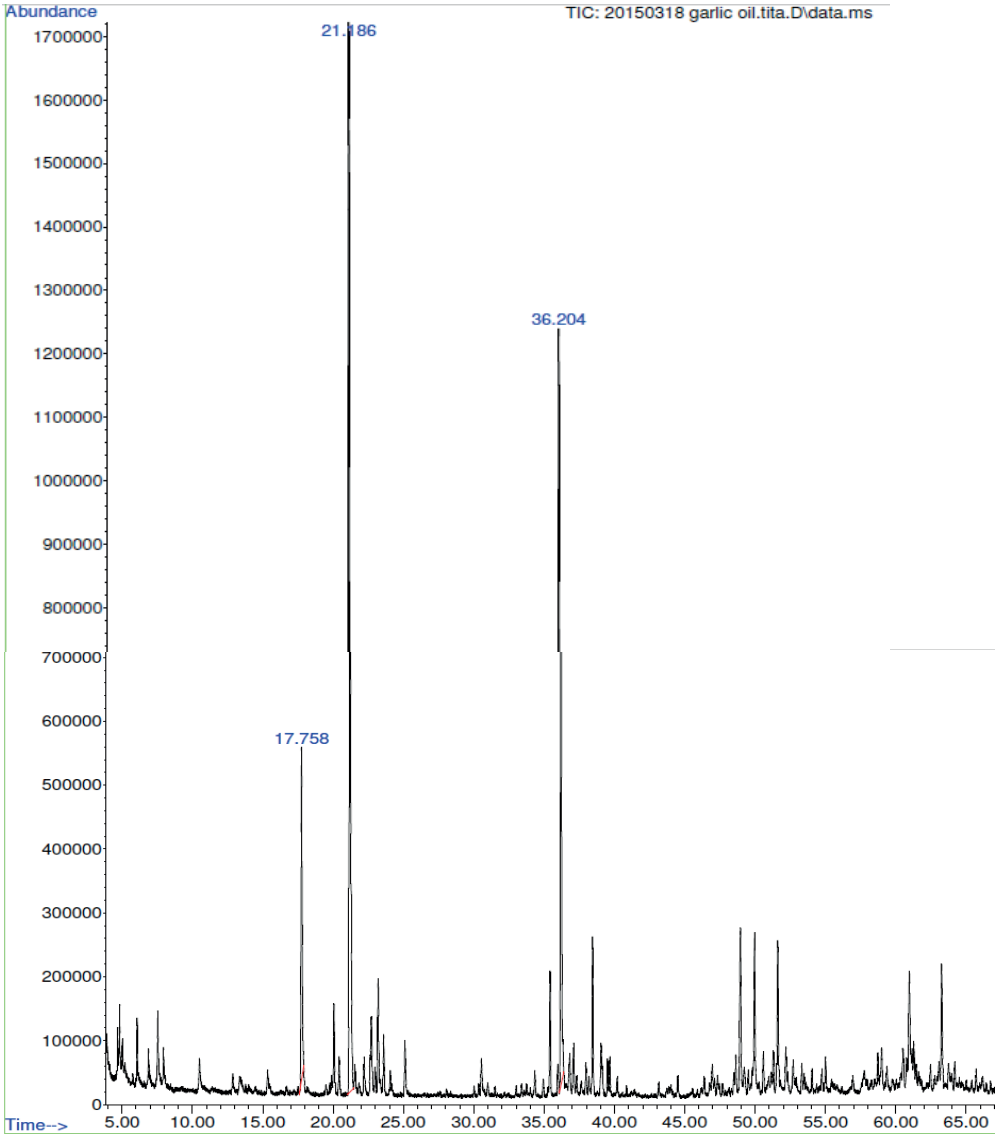


Figure 7: The presence of garlic oil constituents in GC-MS. GC-MS chromatogram of garlic oil showed Diallyl disulfide (peak 2), Diallyl trisulfide (peak 3), and D-limonene (peak 1) were quantified at 53.81 %, 31.49 %, and 14.71 %, respectively.

3.3. Antioxidant Capacity of Garlic by DPPH assay

To evaluate antioxidant activity of both garlic oil and garlic water-soluble, DPPH radical scavenging assay was conducted. According on previous reports that DADS, DAS, and DATS which are garlic oil constituents from garlic extraction have the capability of suppress low density lipoproteins oxidation in vitro [17, 18]). Moreover, SAC, SAMC, and NAC as garlic water-soluble constituents possess anti-oxidative [2, 11, 17]. Reference [18] reported that garlic contains polyphenols compounds which directly correlate with antioxidant activity in vitro.

Garlic has abundant organosulfur compounds, flavonoids, proteins, and polyphenols act as high antioxidant activity [7, 17]. Percentage of DPPH scavenging increased as

Sample	Radical scavenging activity (%)				
	1 000 μg · mL^{-1}	2 000 μg · mL^{-1}	3 000 μg · mL^{-1}	4 000 μg · mL^{-1}	5 000 μg · mL^{-1}
Garlic oil	40 ± 0.031	42 ± 0.022	47 ± 0.03	52 ± 0.03	58 ± 0.04
Garlic water soluble	46 ± 0.04	50 ± 0.037	57 ± 0.03	63 ± 0.03	69 ± 0.04

Table 3: Antioxidant activity of garlic constituents were described in various concentration of garlicwater-soluble and garlic oil with BHT as positive control.

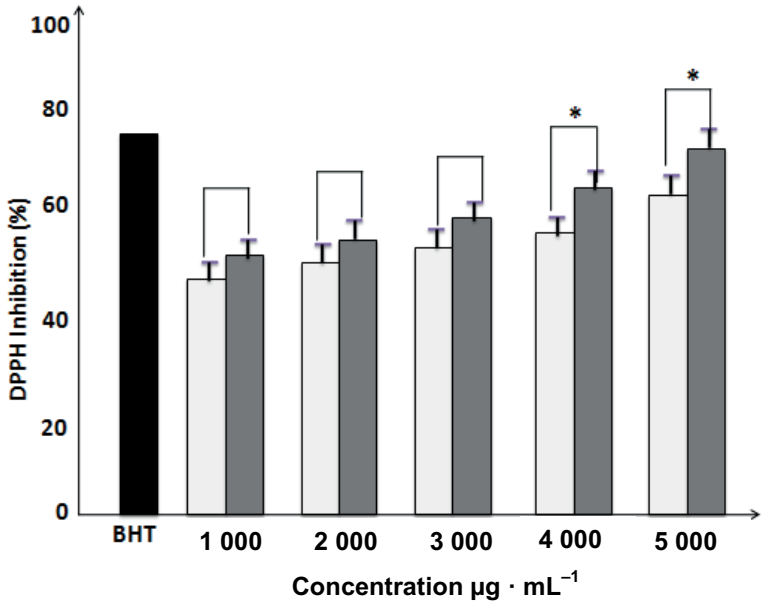


Figure 8: Antioxidant activity of garlic constituents using DPPH assay. Data showed five different concentrations (1 000 μg · mL^{-1} to 5 000 μg · mL^{-1}) of garlic water-soluble and garlic oil. The highest dose showed statistically significant difference between garlic oil and garlic water-soluble by student's t test at *$p < 0.05$.

concomitant enhanced doses of both garlic oil and garlic water-soluble. The highest dose of both garlic water-soluble and garlic oil induced highest % of DPPH inhibition which was significantly different (t test, *$p < 0.05$).

This results based on reduction of radical DPPH by presence of hydrogen donating antioxidant. Radical scavenging potential was expressed as EC50 value, which represents the sample concentration at which 50 % of the DPPH radical scavenged. The results were measured at 517 nm appearing as a deep violet colour. Garlic water-soluble constituents showed significantly higher antioxidant activity than garlic oil. These results agree with previous reports that garlic organosulfur constituents act as hydrogen sulfide donors counteracting radical DPPH [19]. Garlic water-soluble has –SH residue donor and phenolic compounds have capability of reducing DPPH radicals. To investigate the DPPH scavenging assay various constituents presented between garlic oil and garlic water-soluble, different commercials compounds likely NAC, SAC, SAMC are well known as garlic water-soluble compounds and DADS, DATS which are garlic oil compounds.

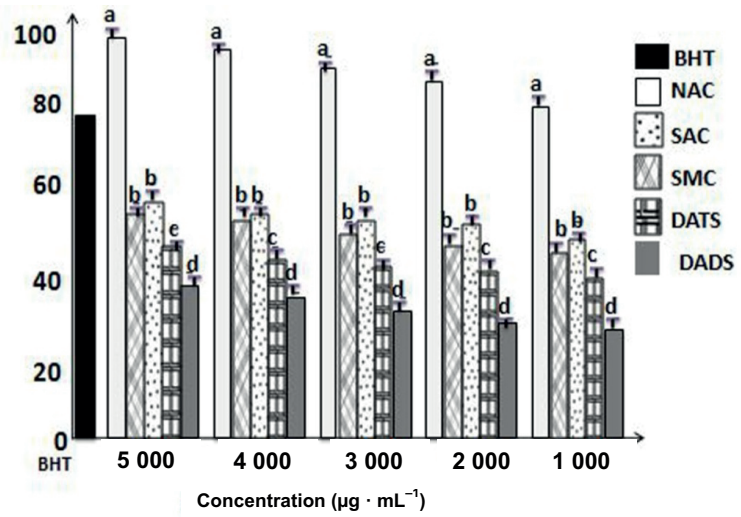


Figure 9: Antioxidant activity of commercial garlic constituents. Various commercial garlic constituents with different concentrations .

Sample	Radical scavenging activity (%)				
	1 000 μg · mL^{-1}	**2 000** μg · mL^{-1}	**3 000** μg · mL^{1}	**4 000** μg · mL^{-1}	**5 000** μg · mL^{-1}
NAC	96 ± 0.172	92 ± 0.183	89 ± 0.312	81 ± 0.172	77 ± 0.254
SMC	50 ± 0.372	46 ± 0.172	40 ± 0.389	37 ± 0.49	34 ± 0.472
SAC	58 ± 0.012	55 ± 0.072	50 ± 0.145	48 ± 0.076	45 ± 0.077
DATS	46 ± 0.04	44 ± 0.022	42 ± 0.016	40 ± 0.026	38 ± 0.004
DADS	38 ± 0.372	35 ± 0.282	30 ± 0.37	28 ± 0.472	25 ± 0.32

Table 4: Antioxidant activity of commercial constituents of garlic water-soluble and garlic oil.

(1 000 μg · mL^{-1} to 5 000 μg · mL^{-1}) of each compounds. Experiment was performed three times independently as mean ± S.D a-bp < 0.05 significantly different compared to each compounds (μg · mL^{-1})

The ability of scavenging DPPH radical of commercial constituents showed that NAC (N-Acetyl L-Cysteine) has the highest capability of neutralized the radicals of DPPH at variant concentration. Increasing doses of compounds could enhanced the antioxidant activity each commercial compounds. NAC has the highest antioxidant activity was followed SAC, SAMC, DATS, and DADS. It proven that NAC which is one of garlic water-soluble constituents [7, 11] is a potent agent as bioactive constituents present in garlic water-soluble which has highest antioxidant activity compared with the other garlic compounds.

4. Discussion

Garlic is considered as natural source which has ability for prevent and detoxify cells upon endogenous and exogenous free radicals. Cutting and crushing garlic, produce

hundreds of organosulfur constituents. Organosulfur constituents are divided into two major constituents particularly water-soluble and oil constituents [4, 5, 7]. This present study provides LC-MS Thermo LCQ DECA XP MAX system with an electrospray ionization (ESI) source determining the presence water-soluble constituents in garlic. Three constituents were identified as N-Acetyl L-Cysteine (NAC), cysteinyl alanine, phenol 2-2 benzoaxolyl, and two constituents unknown. Previous studies revealed that S-ally-cysteine (SAC), N-Acetyl L-Cysteine (NAC) and S-mercaptocysteine (SAMC) presence in garlic water-soluble constituents [2, 7, 8, 17, 20].

Polysaccarides, proteins, enzymes, amino acids, gamma-glutamyl-S-allyl-cysteine (GSAC), and alliin are present in intact garlic [8, 20]. Owing cutting, crushing, heating, aging, and dehydration in garlic, NAC is well known as water-soluble constituent, the highest antioxidant activity among the organosulfur constituents is achieved.

Conversely, S-ally-cysteine (SAC) and S-mercaptocysteine (SAMC) are not present due to heated at 65 °C cause gamma-glutamyltranspeptidase completely inactivate [7]. SAC is generated by the enzymatic hydrolysis of gamma-glutamyl-S-allyl-cysteine (GSAC) by gamma-glutamyl transpeptidase [5]. Phenol and cysteine alanine are exist probably due to protein hydrolysis and biodegradation of phenolic compounds in intact garlic bulbs [20]. On the other hand, more than half of the alliin is lost during the dehydration [21] and alliinase presumably inactivate during heated at 65 °C.

Present study also analyzed garlic oil constituents which are determine using mass spectra by GC-MS. Three main constituents are present as D-limonene (14.71 %), diallyl disulfide (DADS) (53.81 %), and diallyl trisulfide (DATS) (31.49 %). Numerous studies have revealed that there are more than 200 constituents identified from garlic, such as vitamins, proteins, lipid, trace elements Se, flavonloids and at least 33 different organosulfur constituents [9, 10, 17]. Garlic bulbs consist of 10 mg $\cdot$ g^{-1} fresh weight of allinase, allinase in bundle sheath cells and alliin in the storage cells. More than 60 s crushed garlid caused convert allin into allicin [10] then decompose into various sulfur constituents mainly DADS and DATS [15].

D-limonene (14.71 %) also present in garlic, this compound is one of the most common terpenes in nature. It is a major constituent in several citrus oils (orange, lemon, mandarin, lime, and grapefruit). Various phytochemichal changes in garlic such as flavor, colour, and nutrient content by processing method, including heat treatment, fermentation, and soaking with solvent in certain period [7].

1.1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity assay was carried out evaluating antioxidant activity. The hydrogen atom or electron donating ability of garlic oil, garlic water-soluble constituents and BHT (Butylated hydroxytoluene) as standard was determined by bleaching of purple colored methanol solution of DPPH.

Garlic organosulfur constituents have been widely investigated regarding their therapeutic applications acting as hydrogen sulfide donors or mediators in pharmaceutical

studies [19]. The presence of DADS and DATS in garlic oil are related to increase antioxidant activity which exert antioxidant by breaking the free radical chain through the donation of hydrogen atoms neutralize DPPH radicals [7].

Nevertheless, garlic water-soluble has higher antioxidant activity than garlic oil in various concentrations. Consequently, garlic water-soluble consist of NAC, a derivative of the amino acid L-cysteine which the highest antioxidant activity among the organosulfur compounds. NAC has a nucleophile and a -SH residue donor to counteract free radicals in cells leads to oxidative stress [2, 7, 11, 21].

According on the LC-MS results, NAC accompanied with phenol 2-2 benzoaxolyl which also has ability to scavenge DPPH radicals. Previous studies elucidated that garlic has stable organosulfur compounds, flavonoid, and polyphenols which possessed powerful antioxidant properties [14, 17, 18]. Phenolic compounds are more effective antioxidants than non phenolic compound such as allyl sulfide. Bae et al. also explained that garlic heated at over 65 °C might acts as DPPH radical scavenging activity and reducing power largely [7]

5. Conclusion

According on these results, both of garlic water soluble and garlic oil have potent as exogenous antioxidant. Garlic water-soluble constituents were identified by Liquid Chromatography–Mass Spectrometry (LC-MS) and five constituents were found, namely N-acetylcysteine (NAC), cysteinyl-alanine, phenol-2-2-benzoxazolyl and two unknown constituents. The GC-MS chromatogram also showed three main constituents present in garlic oil as diallyldisulphide (DADS), diallyltrisulphide (DATS) and D-limonene. The presence of N-allyl cystein (NAC), cysteinyl alanine and phenol 2-2 benzoazolyl in garlic water-soluble using heating and aging preparation, probably act as strong antioxidant activity 70 % ± 0.02 % compared with garlic oil 58 % ± 0.07 %. This present study established a novel production process of garlic water-soluble and provided a further research basis to explore the effective in vitro antioxidant from garlic water-soluble. In addition, future studies need to focus on the bioavailability of each compound and its in vivo activity.

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