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INVESTIGATION OF TOTAL PHENOLIC, TOTAL FLAVONOID, ANTIOXIDANT AND ALLYL ISOTHIOCYANATE CONTENT IN THE DIFFERENT ORGANS OF *WASABI JAPONICA* GROWN IN AN ORGANIC SYSTEM

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Abstract

Background: This study was carried out to investigate the total polyphenol (TP), total flavonoid (TF), antioxidative effect and allyl isothyocyanate (ITC) content in different organs of wasabi plant grown in an organic system.
Materials and Methods: Invitro study of methanol and boiled water extracts of wasabi were conducted by analyzing the 1-1- diphenyl-2-picryl hydrozyl (DPPH) radial scavenging activity, metal chelating activity and total antioxidant capacity in a comparative manner. Result: The result revealed that methanol extract showed higher TP in flower (3644 mg TAE/100 g dw), leaf (3201 mg TAE/100 g dw) and fruit (3025 mg TAE/100 g dw) as compared to the boiled water extract. Similarly, TF content was also higher in methanol extracts of flower (1152 mg QE/100 g dw) and leaf (325 mg QE/100 g dw), however, the other parts showed ignorable value.
Results: Results of antioxidant activity were found at different magnitude of potency. The methanol extract of different parts of wasabi exhibited higher activity in total antioxidant capacity and DPPH radical scavenging assay as compared to water extract. In metal chelating assay, the boiled water extracts of leaf showed higher (76.9%) activity, followed by fruit (68.8%) and flower (62.8%). Ally ITC detected by gas chromatography was present in all of the tissues of wasabi plant but the content was found to be varied in different tissues.
Conclusions: Overall, this study will allow consumers and processors to understand the possibility for medical application of wasabi plant by knowing the level of total polyphenol distribution, Ally ITC content and antioxidant property distributed in different parts and tissues.Key words: Allyl ITC, antioxidant, flavonoid, polyphenol, *Wasabi japonica.*

Introduction

In every parts of the world, effect of intensive farming has resulted in deteriorating soil tilth and organic matter content. Increased use of agro-chemical caused environmental pollution and thus effects on crop production and reflects on human health. According to the IFOAM definition, “Organic agriculture is a production system that sustains the health of soils, ecosystem and people. It relies on ecological processes, biodiversity and cycles adapted to local conditions, rather than the use of inputs with adverse effects. Organic agriculture combines tradition, innovation and science to benefit the shared environment and promote fair relationships and a good quality of life for all involved”.

Wasabi is a member of cruciferous family, used extensively in Japanese cuisine to garnish traditional foods like Sashimi and Sushi. Its rhizome is used as a condiment and has an extremely strong flavor. The characteristic order and strong pungent smell of wasabi is due to allyl isothiocyanate (Allyl ITC) (Kojima et al., 1973). Allyl ITC is a major constituent found in the different parts of wasabi (Sultana et al., 2000) and apart from their use in food, they are reported to have antimicrobial (Kinae et al., 2000), anti-gastric (Ono et al., 1998), and antioxidant (Lee, 2008) activities.

In contest to South Korea, several researches have been performed on wasabi, especially in cultivation method, agronomy, isothiocyanate contents (Lee et al., 1997; Choi et al., 2011, Byeon et al., 2002 and 2001) and its pharmaceutical value (Ogawa et al., 2010). However, there are no detail reports regarding total polyphenol content, bioactivity and allyl ITC content from each part and tissues of wasabi plant grown in organic system. Therefore, the objective of this study was to investigate the allyl ITC content in different parts and tissues of wasabi grown in organic system and also to compare its bioactivity (antioxidant activity) in methanol and boiled water extracts.

Materials and Methods

Chemicals

Analytical grade organic solvents (dichloromethane, methanol, ethanol, acetonitrile,) used for the extraction of wasabi and detection in HPLC were purchased from Merck KGaA Darmstadt, Germany. Tannic acid, Quercitin and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) purchased from Sigma Chemical Co. (St. Louis, Mo). Folin-Ciocalteu’s reagent was purchased from Wako Pure Chemicals, Japan. The pure compounds allylisothiocyanate was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents used were of analytical grade.

Collection of wasabi plant and Sample preparation

Daruma variety of wasabi plant was grown in an organic system (using animal dongs as a fertilizer and no use of pesticides in any case) at Samcheok (37°27'00"N and 127°46'59.88"E), Kangwon do province, South Korea. After 18 months of cultivation, the fruiting plants were harvested in the month of July 2013. The harvested plants were washed thoroughly and separated into different plant parts (rhizome, leaf, petiole, roots, flower, floral stalk and fruits). Rhizomes of the wasabi plants used in this study ranged from 63 to 65 gram. Each rhizome was divided into three subgroups by sectioning the rhizomes into three equal portions by length and leveled upper, middle and lower part. Further, each part of rhizome was sliced

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longitudinally to separate the tissues into epidermis, cortex and pith fractions. The roots were also separated according to their sizes into two subgroups (big and small hairy root).

Preparation of wasabi extracts

Samples were oven dried at 50 °C and powdered using mixture grinder. Five grams of powder was mixed with 250 mL of methanol and kept for two days in a shaker at room temperature. For activity comparison, same amount of wasabi powder was boiled at 100 °C for 2 hr. The extracts were filtered and dried using a vacuum rotatory evaporator (Eyela digital water bath SB-1000, Tokyo, Rikakikai Co., Ltd. Japan) in a 40 °C water bath. Dried samples were weighed and used for TP, TF and antioxidant analysis. For Allyl ITC analysis, fresh tissues of wasabi parts were homogenized individually using mixture blender following the protocol of Sultana et al., (2003b) with minor modification. In brief, 3 grams of finely ground rhizome was mixed with 10 mL of distilled water and 10 mL of dichloromethane. The samples were mixed thoroughly for an hr at room temperature. The solvent and aqueous phases were separated by centrifugation at 12000 g. The solvent extract of different parts of wasabi was used for allyl ITC content in fresh weight (fw) basis.

Estimation of total polyphenol and total flavonoid content

Total polyphenol (TP) content of wasabi samples were determined by the Folin-Ciocalteu assay. In brief, a sample aliquot of 1 mL of extract (1 mg/ mL) was added to a test tube containing 1 mL of phenol reagent (1 M) and 0.4 mL of Na_2CO_3 (10%). The volume was increased by adding 2.6 mL of distilled water and the solution was vortexed and left for 3 min for reaction. A reagent blank was prepared using distilled water. The absorbance was measured at 725 nm after incubation for 1 hr at room temperature. The TP was calculated from a calibration curve (R^2=0.999) using tannic acid as a standard and expressed as tannic acid equivalents (TAE) in mg per 100 g dry weight (dw). Total flavonoid (TF) content was determined using the protocol of Eom et al., (2008). Briefly, an aliquot of 1 mL of the sample (1 mg/ mL) was mixed with 0.1 mL of aluminum nitrate (10%) and 0.1 mL of potassium acetate (1 M). To the mixture, 3.8 mL of methanol was added to make the total volume 5 mL. The mixture was vortexed and the absorbance was measured after 40 min at 415 nm using a spectrophotometer (UV, 1800 Shimadzu, Japan). The TF was calculated from a calibration curve (R^2=0.999) using quercetin equivalents (QE) in mg per 100 g dry weight.

DPPH radical scavenging assay

DPPH radical scavenging activity of the wasabi sample extracts (boiled water and methanol) were determined (Bracca et al., 2003). Briefly, 1 mL of each of extract at the concentration of 1 mg/mL was added to 3 mL of DPPH (0.15 mM) solution. The mixtures were shaken vigorously and left to stand at room temperature in the dark for 30 min. The change in absorbance was measured at 517 nm using a spectrophotometer (UV, 1800 Shimadzu, Japan) and the inhibition percentage of the extracts were calculated against a blank.

Radical scavenging activity (%) = $(A_0 - A_1)/A_0 \times 100$

where, A_0 and A_1 were the absorbance of the control and the test sample, respectively.

Metal chelating assay and the estimation of total antioxidant capacity

The wasabi extracts were analyzed for the metal chelating activity according to the procedure of Dinis et al., (1994) with slight modification. Briefly, 0.25 mL of the sample extracts at the concentration of 1 mg/mL was mixed with 0.05 ml of 1 mM $FeCl_2$ followed by the addition of 0.1 mL of 2.5 mM ferrozine, vortexed and kept for 10 min. For blank, the sample extracts were replaced by distilled water. Finally, before measuring the absorbance in spectrophotometer at 562 nm, the total volume was made to 2 mL with the addition of 1.6 mL of methanol or water. The ability of extracts to chelate ferrous ions was calculated as follows:

Chelating effect (%) = $(1 - A_{sample}/A_{control}) \times 100$

Total antioxidant activity was determined according to the protocol of Prieto et al., (1999) with minor modification. Briefly, a sample aliquot of 1 mL extract (1 mg/ mL) was added to a tube containing 3 mL of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) making the total volume 4 mL. The tubes were covered with aluminum foil and incubated at 95 °C for 90 min. The absorbance was measured at 695 nm against a blank after the sample was cooled. Higher absorbance of the reaction mixture indicated greater antioxidant capacity of the sample.

Gas chromatography analysis and calibration

Dichloromethane extracts (1 uL) were injected onto a Hewlett- Packard (HP) 6890 capillary column HP-5 (30 m x 0.32 mm x 0.25 um) gas chromatograph fitted with a flame ionization detector (FID). The inlet and detector temperatures were 150 °C and 280 °C, respectively. The split mode ratio was 50:1. Separations were performed under the following temperature program: 50–150 °C at 5 °C /min, 100–250 °C at 10 °C/min, then at 250 °C for 2 min. Peaks areas were recorded and calculated using HP Chemstation software.

Statistical analysis

All data were expressed as the mean value ± standard deviation (SD) of each experimental group (n=3). The results were processed using Excel 2003 (Microsoft, Redmond, WA, USA).

Results and Discussion

Total Polyphenol (TP) and Total Flavonoid (TF) content in wasabi

Phenolic compounds play an important role as antioxidants or free radical terminators (Miliauskas et al., 2004) therefore, it is worthy to

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analyze polyphenolic content in the plant parts. Fig. 1 shows the distribution of total polyphenol in methanol and boiled water extracts of wasabi plant parts. TP values in the methanol extract of the rhizome parts and tissues were in the range of 510~778 mg TAE/100 g dw, whereas those in boiled water extract was within the range of 340~464 mg TAE/100 g dw. A comparison among different parts of the plant showed that methanol extracts of flower, leaf and fruit possess higher TP content with 3644, 3201 and 3025 mg TAE/100 g dw respectively. The floral stalk and petiole also showed TP content of 1227 and 1005 mg TAE/100 g dw respectively. Among root samples, small hairy root possessed slightly higher (887 mg TAE/100 g) TP than big root (696 mg TAE/100 g). The TP content in boiled water extracts of different plant parts was in decreasing order as follows: fruit (2221)> leaf (1923)> flower (1510)> petiole (747)> flower stalk (701)> small root (619)> big root (505 mg TAE/100 g dw)> rhizome parts.

Quantitative estimation of TF (Fig. 2) revealed that methanol extract of flower possessed higher (1152 mg QE/100 g dw) flavonoid content followed by leaf (325 mg QE/100 g dw) and fruit (64 mg QE/100 g dw). In boiled water extract, leaf showed higher flavonoid (98 mg TAE/100 g dw) content followed by flower (64 mg TAE/100 g dw) and fruit (51 mg TAE/100 g dw). In case of rhizome, petiole and floral stalk, the flavonoid content was not detected either of methanol or boiled water extracts.

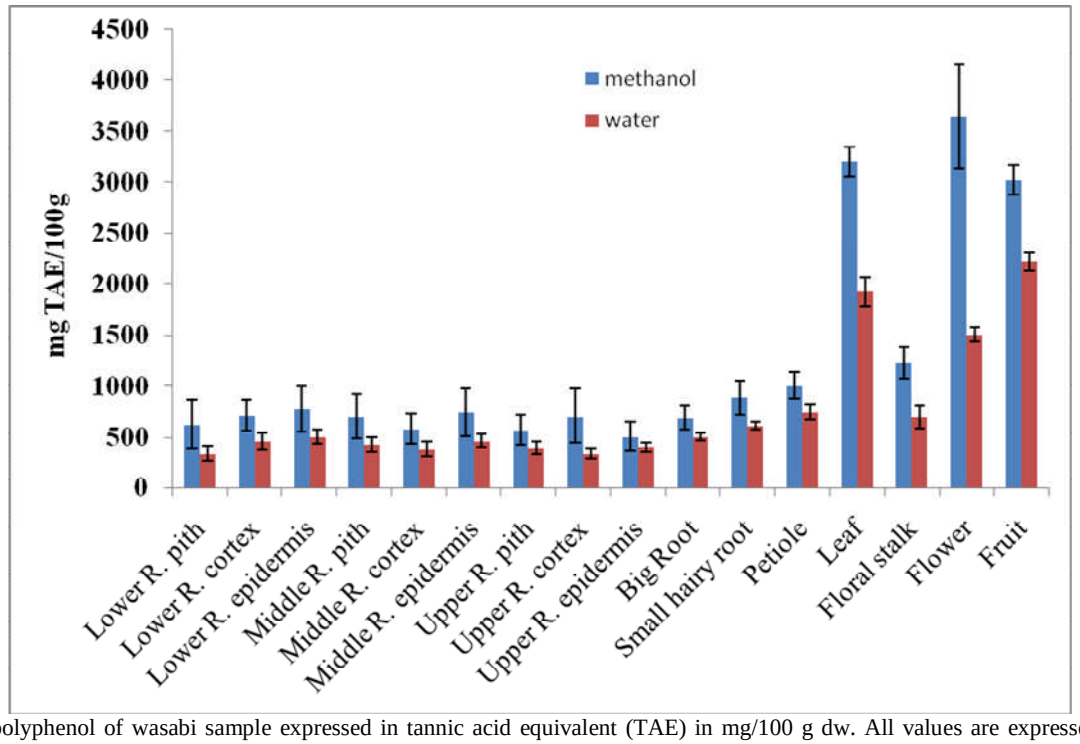

Figure 1: Total polyphenol of wasabi sample expressed in tannic acid equivalent (TAE) in mg/100 g dw. All values are expressed as the mean ± standard deviation (n=3).

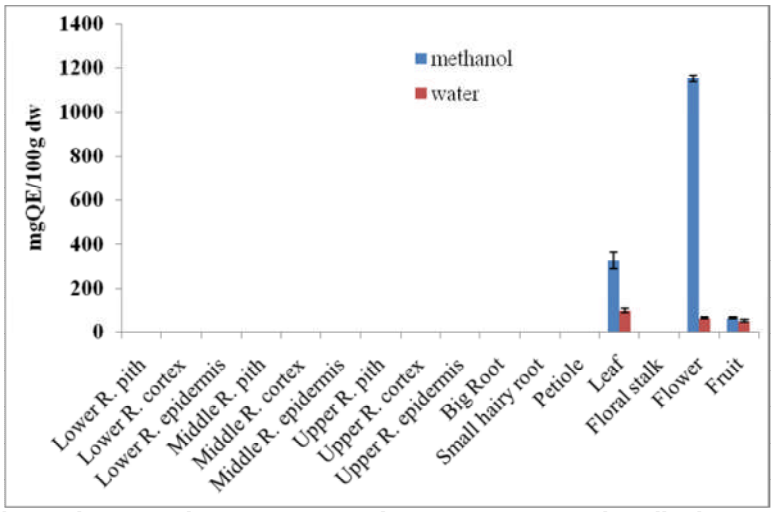

Figure 2: Total flavonoid of wasabi sample expressed in quercetin equivalent (QE) in mg/100 g dw. All values are expressed as the mean ± standard deviation (n=3).

Changes in DPPH radical scavenging activity

The antioxidant activity of methanol and boiled water extracts of wasabi plant parts was evaluated according to their ability to scavenge free radicals by using DPPH assays (Fig 3). According to our result, methanol extracts were slightly higher in inhibition activity compared to the boiled

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water extract except in the fruit. Methanol extracts of the leaf scavenged 57.5% followed by flower with 53.2% of inhibition. The water extract of fruit and leaf also showed higher scavenging activity with 53.1% and 52%. However, the methanol extract of fruit scavenged 20.7%. Among the rhizome parts, methanol extracts of lower parts and their tissues (epidermis, cortex and pith) exhibited slightly higher scavenging activity than those of water extracts. The DPPH radical scavenging activity of middle and upper parts of rhizome were not different, where the values ranged from 4.06~7.2% of inhibition in methanol extracts. Similarly, the water extracts of rhizome parts and tissue showed weak inhibition of DPPH free radical ranging from 3.0~5.6%. Among the root samples, methanol and water extracts of small hairy root showed 1.9 and 1.7 fold higher inhibition respectively than those of big root. This implies that small hairy root contains higher antioxidant compound than bigger one. The petiole and floral stalk showed weaker activity with 10.8 and 11.1 % in methanol and 4.1 and 9.7 % in water extract respectively.

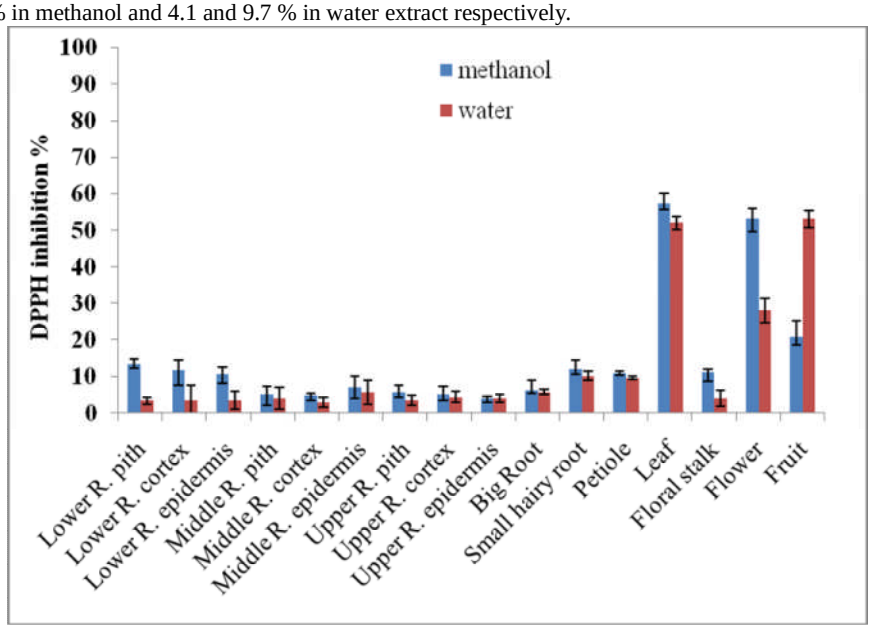

Figure 3: DPPH free radical scavenging activity of wasabi sample. All the samples were assayed at the concentration of 1 mg/mL for comparison. Absorbance was taken at 517 nm. Results are expressed as the mean ± standard deviation (n=3).

Metal chelating property and total antioxidant capacity

Metal chelating capacity is considered as one of the antioxidant mechanisms, since it reduces the concentration of catalyzing transition metal in lipid peroxidation (Diplock et al., 1997). In the assay, Ferrozine can quantitatively form complex with Fe+. In the presence of wasabi extracts (methanol and boiled water), the complex formation is disrupted by reducing its red color (Fig. 4). The highest chelating activity was expressed by boiled water extract of leaf (76.9 %) followed by fruit (68.9%), flower (62.9%) and floral stalk (38.5%). In case of root, methanol extract of small hairy root showed slightly higher (37.1%) chelating activity than big root (33.3%). Water extracts of both small hairy root (11.8%) and big root (6.6%) showed poor activity. The methanol extract of rhizome parts showed higher activity, ranged between 29.3 and 37.5% as compared to boiled water extract which ranged from 2.7 to 4.9%.

Total antioxidant capacity was determined based on the reduction of Mo (**VI**) to Mo (**V**) by the extract that leads to the formation of a green phosphate/Mo (**V**) complex at acidic pH. In this study, both methanol and water extracts were found to exhibit effective total antioxidant capacity (Fig. 5). Methanol extracts from wasabi parts showed stronger antioxidant capacity in comparison to boiled water extracts. The higher the absorbance value the higher is the antioxidant capacity. The order of antioxidant capacity of methanol extract was as follows: rhizome (1.66~1.72)> small hairy root (1.71)> big root (1.33)> flower (0.88)> leaf (0.90)> fruit (0.82)> floral stalk (0.74)> petiole (0.55). In case of boiled water extract, the activity decreased in the order; leaf (0.54)> flower (0.49)> fruit (0.46)> petiole (0.33)> floral stalk (0.32)> small hairy root (0.31)> big root (0.23)> rhizome (0.22~0.27).

Ally isothiocyanate distribution among wasabi plant parts

The concentration of allyl ITC varied among the plant parts and also among the tissues of the rhizomes (Table 1 and 2, Fig. 6). The allyl ITC concentration in different parts was found to be decreased in following order: rhizome> small hairy root> big root> fruit> floral stalk> leaf> petiole. Similarly, the allyl ITC level was varied among different rhizome parts (lower> middle> upper) and tissues (epidermis> cortex> pith). The epidermis tissue of lower part of rhizome was found to have highest level of allyl ITC with 125 mg/100 g fw basis. The allyl ITC level in different rhizome parts was found to be decreased in following order; lower (104 mg/100 g)> middle (99 mg/100 g)> upper part (65 mg/100 g) in fw basis. Average allyl ITC content of the whole rhizome was 89 mg/100 g fw and that of the root was 79 mg/100 g fw. Fruits and floral stem showed almost similar level of allyl ITC content with 57 and 54 mg/100g fw, respectively. Leaves showed 2.9 fold higher levels of ITC than petiole.

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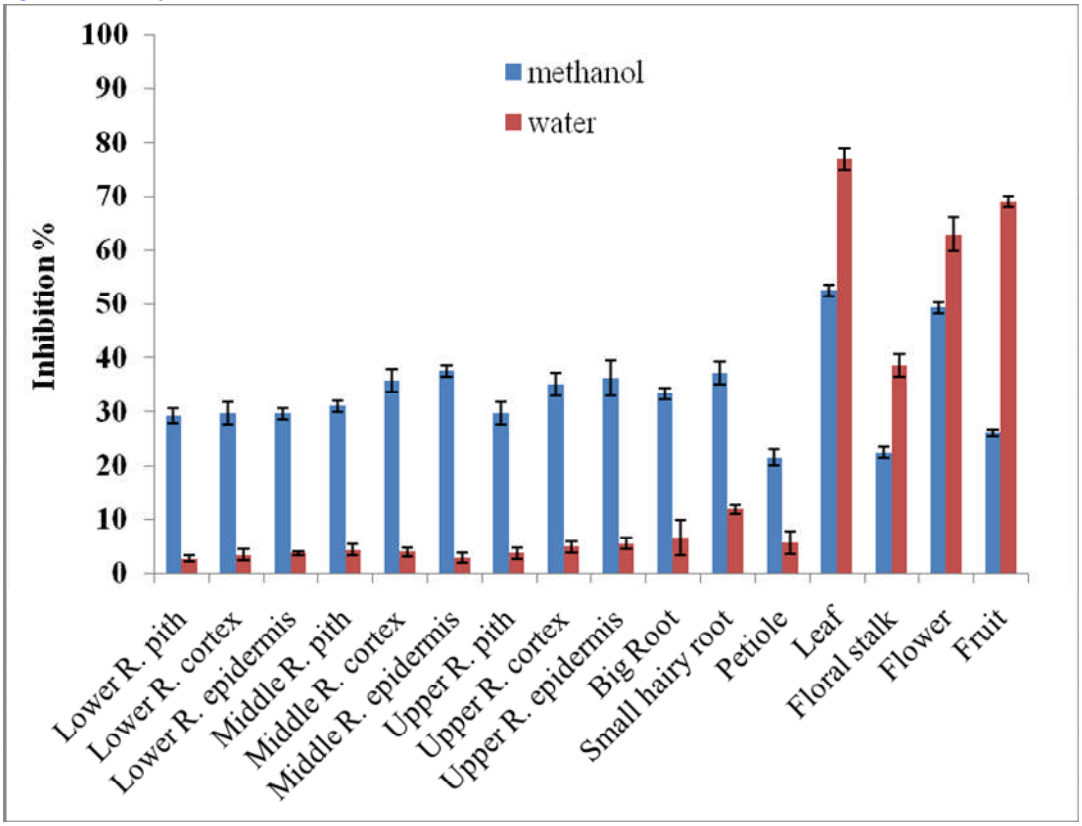

Figufe 4: Metal chelating activity of wasabi sample. All the samples were used at the concentration of 1 mg/mL and analyzed for comparison. Absorbance was taken at 562 nm. Each value is expressed as the mean ± standard deviation (n=3).

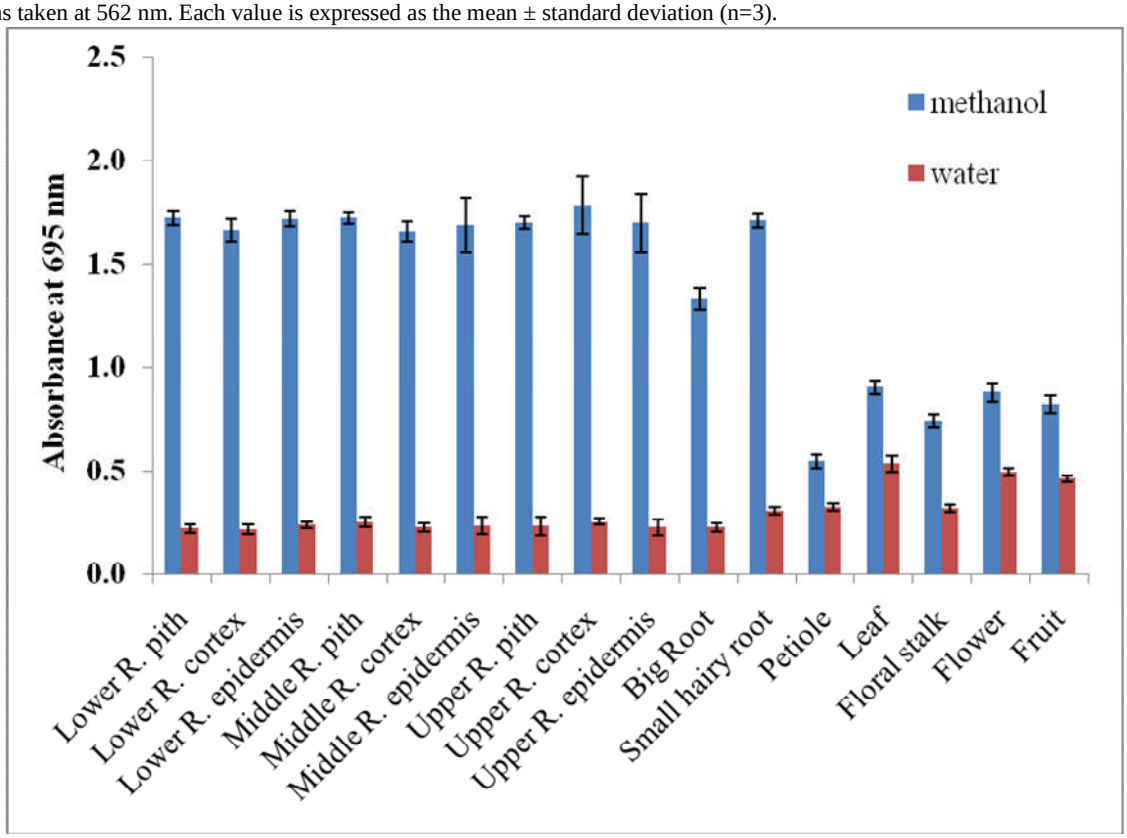

Figure 5: Total antioxidant capacity wasabi sample. All the samples were used at the concentration of 1 mg/mL and analyzed. Absorbance was taken at 695 nm. Each value is expressed as the mean ± standard deviation (n=3).

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Table 1: Difference of allyl isothiocyanate content in upper, middle and lower rhizome parts and in different tissues (epidermis, cortex and pith). The content is expressed in mg/100 g of fresh weight.

	Allyl isothiocyanate content in mg/100 g fresh weight				
	Upper	Middle	Lower	Average	Total content in rhizome
epidermis	**70.77**	**115.94**	**125.50**	**104.07**	
cortex	**66.00**	**103.39**	**96.17**	**88.52**	**89.33**
pith	**58.92**	**79.18**	**91.15**	**75.41**	

Table 2: Distribution of allyl isothiocyanate in different parts of wasabi plant. The content is expressed in mg/100 g of fresh weight.

Allyl isothiocyanate content in mg/100 g fresh weight						
Rhizome	Small hairy root	Big root	Leaf	Petiole	Floral stem	Fruit
89.33	**80.63**	**78.48**	**19.64**	**6.69**	**54.72**	**57.98**

Discussion

This study quantified the total polyphenol, total flavonoid, antioxidant activity and allyl ITC content in methanol and boiled water extracts of wasabi plant parts. In this research, the higher value of total polyphenol in methanol extracts from all plant parts and total flavonoid from leaf, flower and fruits of wasabi may be due to the fact that methanol is less polar solvent than water and able to release more polyphenol compounds from the tissues. The higher phenolic content also reflects to increase in antioxidant (DPPH radical and total antioxidant capacity) activity (Lapomic et al., 2005). According to our result, methanol extracts were slightly higher in DPPH radical inhibition activity as compared to the boiled water extract except in the fruit (Fig. 1). However, according to previous report, water (cold) extract of rhizome was superior in DPPH radical scavenging activity over methanol extract with the IC50 value of 989 and 1200 ug/mL respectively (Ryu et al., 2007). Similarly, Lee (2008) also found water (cold) extract has higher value (IC50 value 558 ug/mL) of DPPH inhibition as compared to ethanol extract (IC50 value 644 ug/mL). This anomaly in the data with the previous findings in antioxidant activity and also the lower value of phenolic and flavonoid compound in boiled water extract could be due to the activity of polyphenol oxidase which degrades polyphenols in boiled water extracts (Zhang et al., 2001; Al-Mustafa et al., 2008). In metal chelating assay the boiled water extract of leaf, flower, fruit, and floral stalk exhibited higher activity than methanol extract. This increased activity might be due to the formation of millard products or thermal effect where high molecular phenolics converts to active low molecular compounds by breaking covalent bonds of phenolic compounds (Eom et al., 2008, Ghimeray et al., 2012, Xu et al., 2007).

In allyl ITC quantification, allyl ITC yield varied greatly between the parts (upper, middle and lower) and inner and outer tissues of the rhizomes. The outer layers of rhizome i.e. epidermis tissue gave higher levels of allyl ITC than the cortex and pith tissues. This result is in agreement with the findings of Sultana et al., (2003a) in which they reported that the epidermis and cortex tissues of wasabi rhizome grown in either soil or water contain higher level of allyl ITC. Similarly, our result also in accordance with the findings of Lee et al., (1997) where reported that the 'daruma' variety of wasabi plant grown in Chuncheon area of 'Kangwon do' province of Korea content higher ally ITC level in rhizome (1.33 mg/g), followed by root (1.05 mg/g), leaf (0.29 mg/g) and petiole (0.07 mg/g fw). They also reported higher level allyl ITC content in the lower parts (1.25 mg/g fw) of wasabi rhizome which is almost similar with our findings (Table 1). However, according to Byeon et al., (2002), the rhizome of same (daruma) variety of wasabi grown in the same region of Korea showed slightly lower value (0.63 mg/g fw) than that found in our findings (0.89 mg/g). These discrepancies in allyl ITC content may be due to the difference in cultivation method, topography, location, soil, rhizome size, age and maturity, which caused an alteration in phenolic composition and isothiocyanate content (Sultana et al., 2003b). Especially during the agriculture method, Sultana et al., (2002) found that S and N are important nutrient in both organic and inorganic fertilizers that vary the level of total isothiocyanate yield in rhizome and other parts of wasabi plant.

In conclusion, data from this study will allow consumers and processors to understand the level of total polyphenol distribution, Ally ITC content and antioxidant property distributed in different parts or tissues of wasabi plant grown in an organic system. The study has also showed higher possibility for medical application of boiled water extracts of leaf and reproductive parts of wasabi due to their efficient antioxidant properties. Furthermore, this research also highlighted the high possible use of wasabi plant organs like small hairy roots, floral stalk and petiole which are usually neglected by the consumers.

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Conflict of Interest: None of the authors of the above manuscript has declared any conflict of interest.

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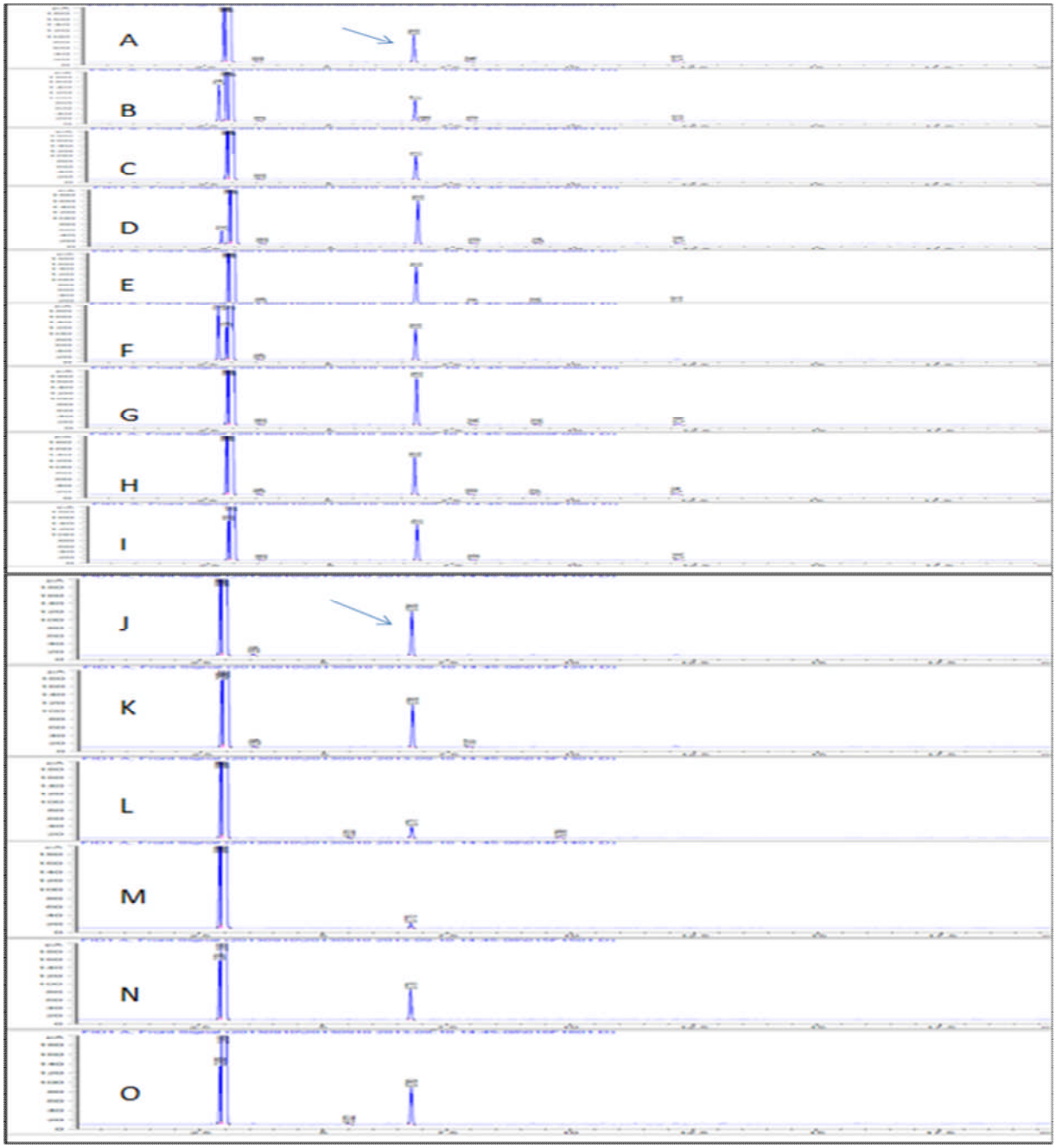

Figure 6: GC chromatogram of allyl isothiocyanate (denoted by arrow with retention time 6.78) content in rhizome upper (epidermis A, cortex B, pith C), middle (epidermis D, cortex E, pith F) and lower (epidermis G, cortex H, pith I) parts. J, K, L, M, N and O represent small hairy root, big root, leaf, leaf petiole, floral stalk and fruit of wasabi plant respectively.

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