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TOTAL FLAVONOID CONTENT AND ANTIOXIDANT ACTIVITY IN LEAVES OF TEN CULTIVATED *SONCHUS ARVENSIS* L. ACCESSIONS

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Abstract

Sonchus arvensis L., as source of herbal medicine, is mostly collected from nature and rarely cultivated. In cultivated condition, the quality of *S. arvensis* might increase. The qualities of 10 accessions *of S. arvensis*, especially total flavonoid content and antioxidant activity, were investigated under cultivation condition. The field experiment was conducted from February to August 2016 at an organic experimental farm of IPB University , Bogor, Indonesia (6°30'-6°45' S, 106°30'-106°45' E). The experiment used randomized complete block design with 3 replications. Single factor of treatment was used, namely the accessions, comprised of 10 accessions originated from different areas in Java island. The results showed that the phytochemical contents (total flavonoid content, antioxidant activity, phenylalanine ammonia-lyase (PAL) activity, vitamin C and anthocyanin) differed among accessions. Banjararum accession seemed to be best accessions among 10 cultivated *S. arvensis* accessions, as indicated by the highest flavonoid content and the highest PAL activity. Sumbersekar accession had the highest antioxidant activity and also the highest vitamin C content. Cibadak accession had the lowest content of anthocyanin and vitamin C, and PAL activity. Banjararum accession showed big potential to develop as flavonoid oriented variety near future. However, further study is required to reconfirm those results stability in different seasons and locations.

Key words: cultivated environment, organic, PAL activity, perennial sowthistle, *tempuyung*.

Introduction

Sonchus arvensis L. is known as perennial sowthistle or tempuyung in Indonesia. The leaves of *S. arvensis* can be used by Indonesian people as a source of traditional medicine and have been shown to possess an antiurolithiasis (Hidayati *et al*., 2009; Dhianawaty *et al*., 2012), anthelmintic and antibacterial activities (Wadekar *et al*., 2012; Xia *et al*., 2011). Those activities might relate to the abundance beneficial bioactive compounds in the form of phenolic compounds (Khan, 2012). Phenolic compounds are produced through the phenylpropanoids pathway with precursors of aromatic amino acids from the shikimic acid pathway. Phenylalanine is one of the three aromatic amino acid compounds and precursors of the phenolic compounds. Phenylalanine ammonia-lyase (PAL, EC 4.3.1.24) is a regulatory enzyme of the secondary metabolism compound biosynthesis in plants that catalyzes the primary reaction of reverse deamination of L-phenylalanine amino acid to the trans-cinnamic acid (Boudet, 2007; Vogt, 2010) and the activity of PAL has a direct effect on flavonoid formation (Ghasemzadeh and Ghasemzadeh, 2011, Nadernejad *et al*., 2013). The relationship between PAL activity and total phenolic has been widely reported for example in ginger (Ghazemzadeh *et al*., 2010) and corn (Gholizadeh, 2011).

Flavonoid is one of phenolic compounds which has antioxidants activities in scavenging free radical (Ghasemzadeh *et al*., 2010). The concentration and chemical structure of flavonoids affects antioxidant activity based on the ability to donate hydrogen atoms or through their ability to chelate metals (Miceli *et al*., 2009). Several studies have shown that high total flavonoid

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content increases antioxidant activity (Karimi *et al.*, 2012; Praven *et al.*, 2007; Ghazemzadeh *et al.*, 2012). Related to antioxidant activity, flavonol, as one of flavonoid subgroup, is also known can reduce the risk of heart disease, cancer, gastrointestinal, neurological, liver, atherosclerosis, obesity, and allergies (Boudet, 2007; Mouradov and Spangenberg, 2014).

As source of herbal medicine, *S. arvensis*is mainly collected from nature and it is rarely cultivated. It is rather difficult to control the quantity and the quality of phytochemical contents when the medicinal plants are collected from nature. It is caused by the wide variability of environmental factors occurred in the nature. Rohaeti *et al*., (2011) proved that there is a variation of flavonoids content in *S. arvensis* leaves taken from Tawangmangu, Cimanggu and Leuwilian. Raisawati *et al*., (2018), who studied *S. arvensis* originated from 10 different sites, also showed the variance of total flavonoid contents and antioxidant activity (IC_{50}) where they ranged from 0.51 to 0.85 mg EQ g^{-1} and 238.29 to 825.90 µg mL^{-1}, respectively.

The cultivation of plant allows the presence of adjustable supporting environmental condition to support optimal plant growth and production. Previous studies showed that the cultivation of *S. arvensis* using manure could improve plant growth and yield (Surat *et al*., 2008 Nurhayati *et al*., 2013; Wardani and Melati, 2014). However, there is still limited report dealing with the phytochemical quality, especially total flavonoid content and its antioxidant activity, of *S. arvensis* when this plant is cultivated as compared to those from nature conditions. Therefore, this study aimed to evaluate the total flavonoid content and antioxidant activity of 10 *S. arvensis* accessions cultivated at the experimental field away from the originated sites.

Materials and Methods

Experimental conditions and plant material

The field experiment was conducted from February to June 2016 at the organic experimental farm, IPB University, Bogor, Indonesia (6°30'-6°45' S, 106°30'-106°45' E) at 190 m above sea level. This location categorized as a type-A climate zone, based on Schmidt-Ferguson theory with an average monthly rainfall, temperature, humidity and light intensity was about 539.3 mm, 26.3°C, 85.5% and 304.5 cal cm min^{-1}, respectively (MCGA, 2016).

The experiment was arranged in a randomized completely block design with single factor namely *S. arvensis* accessions as treatments and three replications. There were 10 *S. arvensis* accessions used in present experiment and they were originated from different locations, i.e. Dramaga, Cibadak, Cicurug, and Lembang accessions from West Java Province; Matesih, Sekipan, Tawangmangu accessions from Central Java Province; Batu, Banjararum and Sumbersekar accessions from East Java Province.

Cultivation procedure

One week before planting, the land was applied with the rates per hectare of 20 tons chicken manure, 1 ton rice-hull ash, and 1 ton lime. There was no chemical fertilizer was added. The seeds, previously collected from those 10 locations, were sown in seedling tray filled with mixture of rice-hull charcoal and chicken manure at a ratio of 1:1 (w/w). At eight weeks after sowing, 150 healthy seedlings of each accession were transplanted into 3 trial plots (3m × 2m) with 30cm × 40cm planting distance. There were 50 plants in each plot. Weeding was done manually once a month, no chemical pesticide was used for plant protection.

Chemical analysis procedure: total flavonoid content

For chemical analysis, the samples were prepared from composite leaves taken from 24 plants in the middle part of the plot. Leaves were harvested at 8 weeks after transplanting. All harvested leaves were pooled within the same experimental plot. The composite leaves were weighed, washed with water, drained and then oven dried at 40°C for 3 days. Dried samples were ground into powder. As much as 5 g powder was soaked with alcohol 96% of 50 mL and shaken for six hours, then incubated in room temperature for 24 hours. The filtrate was separated from the residue by filtering and these steps were repeated two times. The residue was then added with the solvent extraction. The filtrates from each extraction were mixed, then dried with a rotary evaporator.

The total flavonoids content was determined with the aluminum chloride method followed Chang *et al*., (2002). Each plant extracts (0.5ml of 1:10g.ml^{-1}) in methanol were separately mixed with methanol (1.5 ml), 10% aluminum chloride (0.1 ml), 1 M potassium acetate (0.1 ml) and distilled water (2.8 ml), then mixed using vortex. It remained at room temperature for 30 min, the absorbance was measured at 415 nm with a Shimadzu UV-12808 UV-VIS spectrophotometer. The calibration curve was prepared with quercetin solutions at concentrations 0 to 200 mg mL^{-1} in ethanol. Total flavonoid content was expressed as mg equivalent quercetin per g dry weight.

Chemical analysis procedure: antioxidant activity

(IC_{50})

The determination of free radical-scavenging activity of the extracts was determined from the stable 1.1-diphenyl-2-picrylhydrazyl radical (DPPH) content referred to Pourmorad *et al*., (2006). The test of *antioxidant activity* used samples as total flavonoid analysis. Briefly, different concentrations of each sample extract were added, at an equal volume, to a methanolic solution of DPPH (100 µM). After 15 minutes at room temperature, the absorbance was recorded at 517 nm. Ascorbic acid was used as positive controls. DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tech Instruments, Inc; Winooski, VT, USA). The percentage of DPPH discoloration was calculated with the following equation:

= [(ADPPH - AS)/ADPPH] × 100,

where AS was the absorbance of the solution containing the sample, and ADPPH was the absorbance of the DPPH solution. IC_{50} values denoted the concentration of sample required to scavenge 50% of DPPH free radicals.

Chemical analysis procedure: protein and PAL

For protein and PAL analysis, one biggest fresh leaf (7 weeks after transplanting) was used. Collected fresh leaves (0.05 g) of *S. arvensis* were added with buffer extracts (500 ìl; 100 mmolL^{-1}Tris-HCl, pH 7.5; 1 mmolL^{-1} EDTA; 5 mmol L^{-1} $MgCl_2$, 0.05% Triton X-100; 2.5 mmolL^{-1} dithiothreitol) and crushed in a mortar by adding 500 ìl of buffer, then centrifuged with speed of 14000 rpm.

The protein content was estimated by following Waterborg and Matthews method (2002). Reagent A: 7 mM K-Na Tartrate. $4H_2O$, 0.81 M Na_2CO_3 in 500 mL NaOH 1 N, H_2O until 1 L. Reagent B: 70 mM K-Na Tartrate.$4H_2O$; 40 mM $CuSO_4.5H_2O$ in 10 mL NaOH 1 N. Reagen C:1 mL Folin-Ciocalteau dissolved with 15 mL H_2O. 1 mL soluble extract B (0.1 soluble extract sample A + 4.9 mL distilled water) + 0.9 mL reagent A was shaken with the vortex, incubated (50°C, 10 minutes used water bath); remained at room temperature was added with 0.1 mL of reagent B, shaken and incubated at room temperature for 10 minutes. This solution was then added with 3 mL reagent C (50°C, 10 minutes used water bath) and remained at room temperature for 30 minutes. The absorbance was measured at 650 nm using Shimadzu UV-1280 UV Vis spectrophotometer. The calibration curve was prepared with Bovine Serum Albumin Standard (BSA) at concentrations of 0 to 400 g mL^{-1}. Total protein content was calculated as milligrams Bovine Serum Albumin equivalents per gram fresh weight (mg EBSA g^{-1} FW).

PAL activity was determined using a modified method of Dangcham *et al*., (2008). The sample mixture comprised of 0.1 mL of enzyme extract and 2.4 mL of l-phenylalanine solution (10 g L^{-1}Tris–buffer, pH 8.5). The sample was incubated in a water bath at 37°C for 1 hour and added with 0.5 mL of 5 mol L^{-1} HCl. The sample was assayed for PAL activity at 290 nm. PAL activity was expressed as micromoles of trans-cinnamic acid per mg of protein.

Chemical analysis procedure: vitamin C

The measurement of *vitamin C* content referred to Kurniawati *et al*., (2010). The sample was prepared from 5g fresh leaves taken from two leaves (from the same plant) collected from middle part of trial plot. The 5 g fresh leaves was put into a 100 mL measuring cylinder, added with water and shaken. The homogenized solution was filtered. The filtrate was then added with an indicator of 1% amylum solution and titrated with 0.01 N iodium.

Chemical analysis procedure: leaf pigments

The concentrations of anthocyanin, chlorophyll a, chlorophyll b and carotenoid, as leaf pigments, were determined with Sims and Gamon method (2002). The single fresh fully expanded leaf at 7 weeks after transplanting) was used for measuring the leaf pigment content. Fresh leaves were placed in a mortar, grounded and then added with 2 mL of the extraction solvent (85% acetone, 15% Tris, adjusted to pH 8 with HCl), and centrifuged at 12000 rpm for 3 min. A supernatant (1 mL) was removed into test tube and then diluted into 3 mL and then vortexed. The absorbance was determined at 537, 663, 647 and 470 nm by using Shimadzu UV-1280 UV Vis spectrophotometer. The pigment content was determined by using following equations:

(a) Total Anthocyanins = (0.08173 * A537) – (0.00697 * A647) – (0.002228 * A663);

(b) Chlorophyll a = 0.01373 * A663 – 0.000897 *A537) (0.003046 * A647);

(c) Chlorophyll b = (0.02405 * A647) – (0.004305 * A537) – (0.005507 A663);

(d) Total Chlorophyll = Chlorophyll a + Chlorophyll b

(e) Total Carotenoids = (A470 (17.1*(CHla+CHlb)-9.479* Anthocyanin))/119.26

Statistical analysis

Data were analyzed with one-way ANOVA to identify significant differences between accessions, by using Minitab 16. The significant differences were further analyzed with the Tukey test at α=0.05. Pearson

correlation was performed to find correlation between variables. The principal component analysis (PCA) was used to plot a two-dimensional diagram of the dispersal among 10 tested accessions.

Results

Total flavonoid content among 10 cultivated *S. arvensis* accessions was significantly comparable ($P<0.05$), as showed in Fig. 1. The highest total flavonoid content was found in Banjararum accession, but it was not significantly different to those from Tawangmangu and Lembang. In opposite, the lowest total flavonoid content was found in Sumbersekar accession that was not significantly different to Cibadak accession. Compared to Sumbersekar, Banjararum accession had 150% higher flavonoid content.

The value of IC50 was used to indicate the level of antioxidant activity, with an assumption that the lower the IC50 value, the higher the antioxidant activity. The lowest IC50, indicated the highest antioxidant activity,

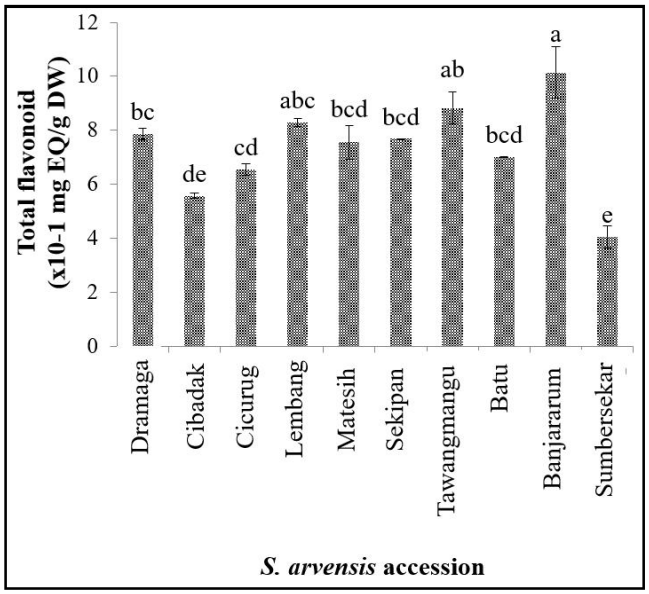

Fig. 1: Total flavonoid content of 10 cultivated *S. arvensis* accessions. The bars showed standard error. The same letter was not significantly different among accessions based on Tukey test at $\alpha=0.05$.

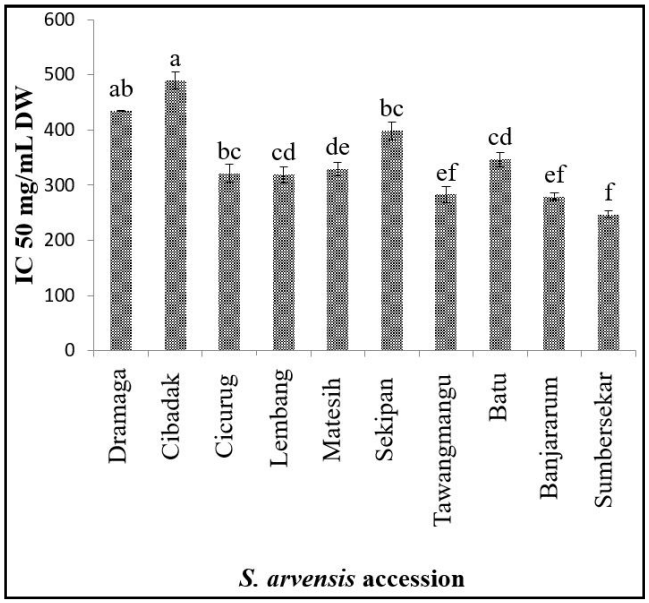

Fig. 2: Antioxidant activity of 10 cultivated *S. arvensis* accessions. The bars showed standard error. The same letter was not significantly different among accessions based on Tukey test at $\alpha=0.05$.

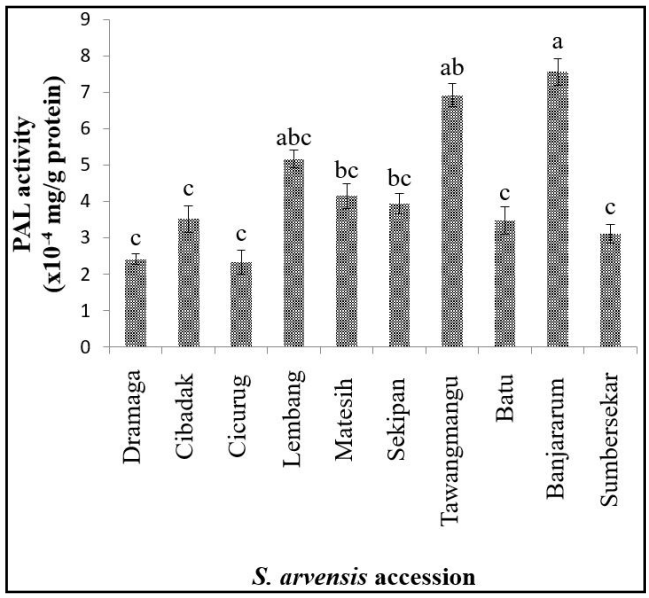

Fig. 3: Phenylalanine ammonia-lyase (PAL) activity of 10 cultivated *S. arvensis* accessions.

Note: The bars showed standard error. The same letter was not significantly different among accessions based on Tukey test at $\alpha=0.05$.

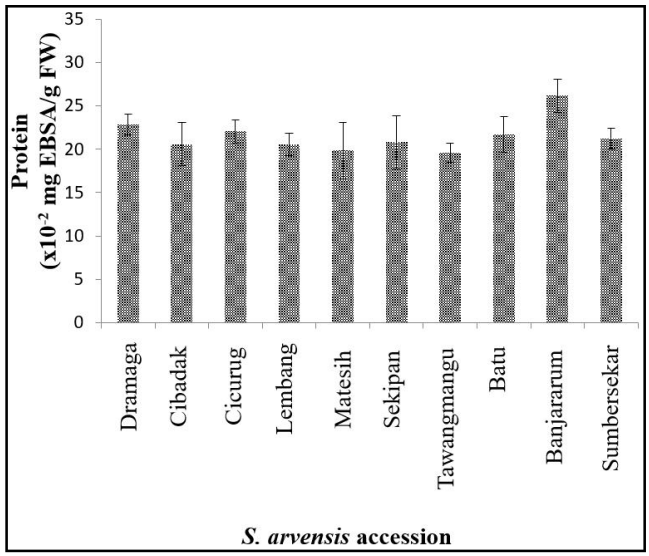

Fig. 4: Protein content of 10 cultivated *S. arvensis* accessions. The bars showed standard error. The same letter was not significantly different among accessions based on Tukey test at $\alpha=0.05$.

was observed in Sumbersekar accession, however, it was not significantly different to those of Banjararum and Tawangmangu. In contrast, the lowest antioxidant activity was observed in Cibadak that was not significantly different to Dramaga accession. Antioxidant activity of Sumbersekar accession had 99% higher antioxidant activity than Cibadak accession Fig. 2.

The 10 accessions show different phenylalanine ammonia-lyase (PAL) activity ($P<0.05$). The highest value of PAL activity was observed on Banjararum accession, but it was not significantly different to Tawangmangu and Lembang accession, whereas the lowest PAL activity was observed on Cicurug accession but it was not significantly different to Dramaga, Cibadak, Batu and Sumersekar accessions. Compared to Cicurug accession, PAL activity of Banjararum accession was 224% higher than PAL activity of Cibadak accession Fig. 3.

There was no significant different of protein content among 10 cultivated *S. arvensis* accessions Fig. 4. However, there was a tendency that Banjararum showed the highest protein content, while the lowest value was observed in Tawangmangu accession. Banjararum accession had 36% protein content than Tawangmangu accession.

The vitamin C contents were significantly comparable among accessions ($p<0.05$) Fig. 5 where Sumbersekar accession had the highest vitamin C content and significantly different to Cicurug which had the lowest vitamin C accession. Vitamin C of Sumbersekar accession was 57% higher than Cicurug accession.

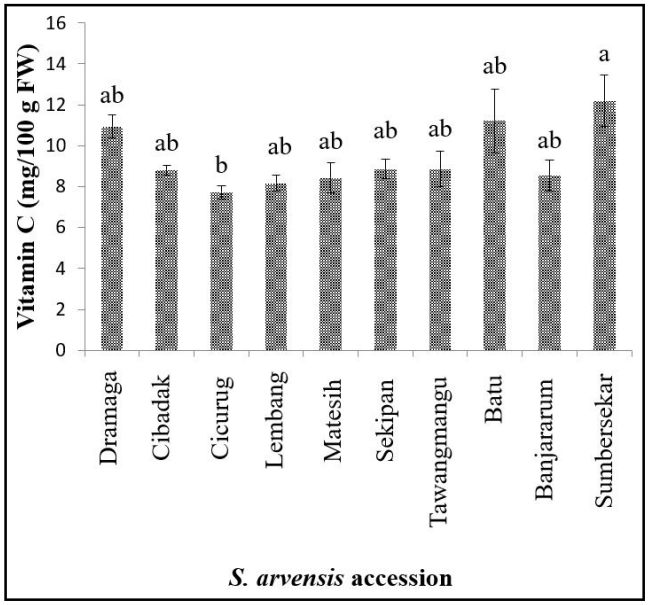

Fig. 5: Vitamin C content of 10 cultivated *S. arvensis* accessions.

Note: The bars showed standard error. The same letter was not significantly different among accessions based on Tukey test at $\alpha=0.05$.

Among leaf pigments, only the total anthocyanin differed among accessions (Table 1). Total chlorophyll and total carotenoids of 10 cultivated *S. arvensis* accessions varied from 1.53-1.82 $mg.g^{-1}$ WB and 0.35-0.39 $mg.g^{-1}$ WB, respectively. Batu accession contained the highest total anthocyanin but it was not significantly different to Cicurug accession. In the contrary, the lowest total anthocyanin was found in Cibadak accession. Total anthocyanin of Batu accession was 200% higher than Cibadak accession.

Correlation analysis showed that there was a positive and significant correlation between total flavonoid content and PAL activity. The coefficient of those positive correlation was 0.728 ($P<0.05$) indicated the strong enough relationship between two variables. Significant positive correlation was also found between total chlorophyll and total carotenoid. The coefficient of those positive correlation was 0.945 ($P<0.05$) indicated the very strong relationship of both variables (Table 2). For the rest, it was not showed any significant relationship.

Principle Component Analysis (PCA) was applied to explain the variance observed trough a two-dimensional map. This PCA could only explaine 59.4% of total variance (35.0% from PC1 + 24.4% from PC2) of all observed variables. This PCA separated into 4 quadrants, namely quadrant I, II, III and IV. The first quadrant (Q1) comprised of Sumbersekar and Cicurug accesions and this zone was indicated by the high vitamin C and IC50 level. The second quadrant (Q2) was a group for Batu, Sekipan and Tawangmangu accessions and this quadrant was indicated by the high total chlorophyll, total carotenoid and total anthocyanin. In the third quadrant (Q3), there were Cibadak and Dramaga accessions. Last quadrant or Q4 consisted of Matesih, Lembang and Banjararum accession and this zone was indicated by the high PAL activity, total flavonoid and protein content Fig. 6.

Discussion

Total flavonoid (TFL) content varied among 10 cultivated *S. arvensis* accessions. The present experiment, where 10 accessions of *S. arvensis* were cultivated in the same location, also showed higher value of total flavonoid compared to those from previous study reported by Raisawati *et al*., (2018) who evaluated the TFL from similar accessions collected from their original site (or growth by nature instead of cultivation). Moreover, total flavonoids of Banjararum (the highest TFL in present experiment) was 42% higher compared to those from collected from nature. The TFL of Banjararum accession

was also higher than TFL reported by Rafi *et al*., (2012). Additionally, our highest TFL results in *S. arvensis* was also higher than other species within the same genus, i.e. *S. asper* (Jimoh *et al*., 2011). This finding Banjararum accesion of *S. arvensis* could improve the phytochemical content under cultivated condition.

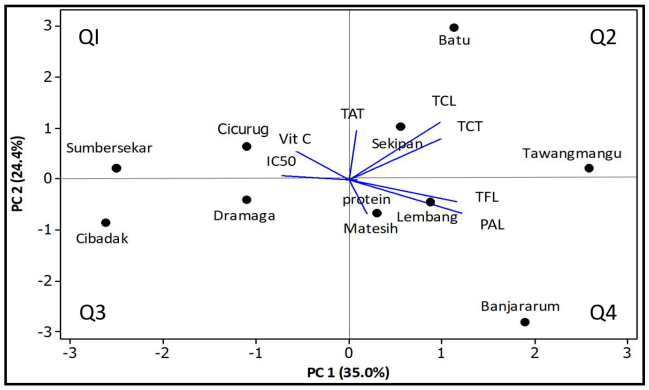

Fig. 6: Principal component analysis (PCA) of all observed variables of 10 cultivated *S. arvensis* accession.

Note: PAL, phenylalanine ammonia-lyase; TFL, total flavonoid; TCL, total chlorophyll; TCT, total carotenoids; TAT, total anthocyanin; Vit. C, Vitamin C.

Our finding showed a variance of bioactive compounds content among accessions and the difference in bioactive compounds content was also shown in *Centella asiatica* (Gbolahan *et al*., 2016), and *Picrorhiza kurrooa* (Kumar *et al*., 2012). Flavonoid is a subclass of secondary metabolites that belongs to phenolic compound class. The level of phenolic accumulation and the composition of phenolic pool varied beetwen organisms, tissues, developmental stages and also affected by the environmental condition (Winkel-Shirley, 2002). In addition, the genetic factor could also influence the secondary metabolite production of the plant (Khan 2012).

The antioxidant activity, expressed in IC50, also differed among 10 accessions *S. arvensis*. As previously mentioned, the lower the IC50 value, the higher the antioxidant activity (Miceli *et al*., 2009). In general, the high antioxidant activity associated with the high phenolic compound (Wu *et al*., 2011). Banjararum and Tawangmangu accessions had high antioxidant activity as well as high flavonoid content. It was likely that antioxidant activity in Banjararum and Tawangmangu accessions was contributed by the flavonoid compound. This finding was in agreement with previous study on *S. arvensis* by Khan (2012). However, Sumbersekar accession showed different relation between antioxidant activity and total flavonoid content, where this accession had the highest antioxidant activity but it had the lowest total flavonoid content. The high level of antioxidant activity of Sumbersekar accession could be related to its high content of vitamin C, anthocyanin and carotenoid rather than flavonoid. The antioxidant activity could be determined by various types of phytochemical compounds, not only by the flavonoid content. Previous study by Rafi *et al*., (2012) in six medicinal plants showed that antioxidant activity was not contributed only by the flavonoid.

Compared to previous report by Raisawati *et al*., (2018) who studied the same accessions collected from their original site, our finding showed lower antioxidant activity in Dramaga, Lembang, Matesih, Tawangmangu, Batu, Banjararum and Sumbersekar accessions, but higher antioxidant activity in Cibadak, Cicurug, Sekipan, Batu and Banjararum accessions. Those differences might be associated with the adaptability of certain accessions with the new cultivation environment. This is in line with

Table 1: Leaf pigment content in the form of total chlorophyll (TCL), total carotenoids (TCT) and total anthocyanin (TAT) of 10 cultivated *S. arvensis* accessions.

Accession	Total Chlorophyll(mg.g^{-1} WB)	Total Carotenoids(mg.g^{-1} WB)	Total Anthocyanin(mg.g^{-1} WB)
Dramaga	1.58±0.078	0.36±0.022	0.04±0.004 b
Cibadak	1.53±0.145	0.35±0.033	0.03±0.007 b
Cicurug	1.63±0.046	0.36±0.010	0.08±0.009 a
Lembang	1.61±0.166	0.36±0.028	0.05±0.006 b
Matesih	1.57±0.285	0.36±0.051	0.04±0.005 b
Sekipan	1.72±0.296	0.39±0.067	0.04±0.005 b
Tawangmangu	1.75±0.111	0.38±0.019	0.04±0.007 b
Batu	1.82±0.244	0.39±0.040	0.09±0.009 a
Banjararum	1.54±0.120	0.35±0.020	0.04±0.004 b
Sumbersekar	1.53±0.145	0.35±0.027	0.05±0.004 b

Note: WB, Wet basis. The same letter was not significantly different among accessions based on Tukey test at α=0.05.

Table 2: Pearson correlation coefficient among observed variables on 10 cultivated *S. arvensis* accessions.

Variable	TFL	IC 50	PAL	Protein	Vit. C	TCL	TCT	TAT
IC 50	-0.13							
PAL	0.73**	-0.44						
Protein	0.39	-0.13	0.23					
Vit. C	-0.49	-0.07	-0.37	0.06				
TCL	0.23	-0.06	0.07	-0.28	0.06			
TCT	0.36	-0.05	0.21	-0.24	0.01	0.95**		
TAT	-0.18	-0.26	-0.35	0.13	0.10	0.48	0.28	

Note: PAL, phenylalanine ammonia-lyase; TFL, total flavonoid; TCL, total chlorophyll; TCT, Total carotenoids; TAT, total anthocyanin; Vit. C, Vitamin C.

Andarwulan *et al*., (2010) who mentioned that environmental condition and also culture practice could also contribute to the level of phytochemical compound and subsequently antioxidant activity.

Correlation analysis showed the positive and significant relationship between total flavonoid and PAL activity. It indicated that the increase of PAL activity possibly induced the production of flavonoids. This result was in line with Nadernejad *et al*., (2013) who reported a correlation between PAL activity and total phenolics in leaves of three Pistachio cultivars. The highest of PAL activity and total flavonoids in Banjararum that was not significantly different to Tawangmangu and Lembang accessions could be preliminary information that those three cultivars would be more resistant to the stresses than other. Since PAL activity was an important variable that should be investigated during the stress period to obtain a better understanding on how resistance the plant facing the stress (Hura *et al*., 2008). Our study showed the high possibility of the positive role of flavonoids (as member phenolic compounds) in plant protection. It seems that better resistance of *S. arvensis* accessions could be preliminary characterized by the increase in PAL activity and total flavonoids content that acted as the antioxidant of reactive oxygen species generated under stress influence.

Conclusion

There were significant differences in phytochemical content among accessions of *S. arvensis*. Among 10 cultivated *S. arvensis* accessions, Banjararum showed the highest flavonoid content and the highest PAL activity. Sumbersekar accession had the highest antioxidant activity, as indicated by the lowest IC50, and also the highest vitamin C content. The accession of Banjararum showed big potential to develop as flavonoid oriented variety near future, however, further study was required to reconfirm those results stability in different season and location.

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