Effects of different extraction methods on total phenolic content and antioxidant activity in soybean cultivars

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Abstract. Soybean secondary metabolites particularly phenolic compounds act as chemical defence against biotic stress such as pathogen infection. Functional properties of these compounds have also been investigated. This study aimed to determine the effects of particle size and extraction methods on total flavonoid, phenolic contents as well as antioxidant activity in soybean seeds. This study also investigated the total phenolic contents and antioxidant activity of Indonesian soybean cultivars using the optimized extraction method. Soybean flour of ≤ 177 µm as many as 0.5 g was selected for extraction with 50% acetone for estimation of total phenolic and flavonoid contents and with 80% ethanol for antioxidant activity. Treatments of twice extraction either shaking followed by maceration or ultrasound-assisted extraction followed by maceration could be used to extract the secondary metabolite contents in soybean seeds. Flavonoid, phenolic contents and antioxidant activity of twenty soybean cultivars ranged from 0.23 to 0.44 mg CE/g, from 3.70 to 5.22 mg GAE/g, and from 4.97 to 9.04 µmol TE/g, respectively. A simple extraction with small amount of soybean flour such as investigated in this present study is effective to extract secondary metabolites especially when the availability of samples is limited such as breeding materials or soybean germplasm.

Keywords : Extraction methods, phenolic content, antioxidant activity, soybean

1. Introduction

Plants naturally produce phenolic compounds during their normal growth. These chemicals are also synthesized when the plants are stressed by biotic as well as abiotic factors such as pathogen infection and radiation of ultra violet. Phenolic compounds also serve as molecular signals in symbiotic process between plant and microorganisms [1-6]. Legumes contain excess amount of phenolics, for example phenolic contents in soybean seeds (*Glycine max* (L.) Merr.) are estimated between 3.04 and 5.71 mg catechin equivalent/g [7-8]. Leguminous seeds are rich in flavonoids, phenolic acids, and procyanidins [9]. Saponins and triterpenoids are also constituent of soybean phytochemical compounds [10-11].

Production of phenolic compounds by seeds and roots at early stage of growth such as during germination has significant contribution to combat infection from several soil borne pathogen and root eating insects [12]. Soil borne pathogens such as *Rhizoctonia solani*, *Macrophomina phaseolina*, *Sclerotinia sclerotiorum* and *Phytophthora sojae* are inhibited by phenolic compounds. Foliar diseases such as *Phakopsora pachyrhizi* are also inhibited by these compounds [13-15]. These chemicals also

function to protect leaves, fruits and seeds from pathogen infection at the later stage of growth [13, 16-18].

The structures of phenolics influence their activity. Phenolic compounds in leguminous plants such as cajanin, medicarpin, glyceolin, rotenone, coumestrol, phaseolin, isoflavonoid, and flavonoid have properties as phytoanticipins (constitutively formed) and phytoalexins (induced) as well as nematicidal against soil borne pathogens and insect pests [12]. Interaction between crops and plant pathogens is related to phenylpropanoids. These organic compounds also have toxic properties or inhibition to plant pathogens either as phytoanticipins or phytoalexins [14].

Extraction of phenolic compounds from plant materials are one of the crucial steps which influences the yield of the whole process before estimation of its contents either spectrophotometric or chromatographic determination was conducted [19-25]. Solid-liquid extraction (SLE) techniques which can be divided into traditional and recent methods have been widely performed in the laboratories for specific purposes [26]. Traditional methods including maceration, percolation, soxhlet (reflux) extraction, sonication, and turbo-extraction (high speed mixing) [26-27] are still used in many laboratories where modern equipments to perform recent methods have not been available yet. Information of different extraction methods to estimate secondary metabolite contents in soybean seed is still limited. The objective of this study was to investigate the effect of particle size and different traditional extraction methods on the extraction efficiency of total flavonoid, phenolic contents as well as antioxidant activity in soybean seeds. This study also investigated the contents of those secondary metabolites in selected Indonesian soybean cultivars.

2. Materials and methods

2.1. Sample preparation

Soybean seeds were ground with a grinder to obtain soybean flour. The flour was gradually sieved and grouped into three different particle sizes, i.e. 211 – 297 μm, 178 – 210 μm, and ≤ 177 μm. The flour was stored at 2-8 ^{o}C in vacuum sealed plastic bags prior to be analyzed. Moisture content was measured to convert each sample into dry weight basis.

2.2. Sample extraction

The flour (0.1, 0.5, 1.0, and 2.5 g) grouped into three particle sizes (210 – 297 μm, 177 – 209 μm, and ≤ 177 μm), in total of 12 combinations, was extracted with suitable solvents. Phenolic and flavonoid compounds were extracted with 50% acetone and antioxidant activity was extracted with 80% ethanol (Table 1). Extraction solvent (1:10 w/v) was poured into capped glass tubes containing soybean flour and the tubes were placed on a shaker. The sample was macerated for 18 h after shaking. The supernatant was collected after centrifugation. The second extraction was conducted with the same procedure. The supernatant obtained from the second extraction was combined with the first extraction..

Different extractions were carried out as follows: (1) proportion of sample and organic solvent was 1:20 (w/v). The sample was placed on an orbital shaker at 150 rpm for 2 h followed by maceration for 18 h. Supernatant was collected in an amber vial after centrifugation, (2) proportion of sample and organic solvent was 1:10 (w/v). The sample was placed on an orbital shaker at 150 rpm for 2 h prior to maceration for 18 h. The supernatant was collected. The second extraction was performed with the same procedure. Combined supernatant was pooled in an amber vial, (3) proportion of sample and organic solvent was 1:20 (w/v). The sample was macerated for 20 h. The supernatant was collected, (4) proportion of sample and organic solvent was 1:10 (w/v). The sample was macerated for 20 h and the supernatant was collected. The second extraction with the same procedure was conducted. The combined supernatant was pooled, (5) proportion of sample and organic solvent was 1:20 (w/v). Ultrasound-assisted extraction for 5 min was conducted and the sample was macerated for 20 h. The supernatant was collected, and (6) proportion of sample and organic solvent was 1:10 (w/v). Ultrasound-assisted extraction for 10 min was performed and the sample was macerated for 20 h. The

second extraction with the same procedure was conducted. The combined supernatant was pooled. In short, all treatments were summarized in Table 2.

2.3. Determination of total flavonoid content

The extract for flavonoid determination was added in distilled water (1:5 v/v) in a glass tube. The extract was mixed thoroughly. As many as 150 µL of 5% $NaNO_2$ was added. The mixed solution was vortexed. Incubation was performed for 6 min. Additional of 5 min incubation was conducted after 300 µL of aluminium chloride was reacted. Then, sodium hydroxide (1M, 1000 µL) was added in the solution. The final volume was brought to 5000 µL with distilled water. The solution was mixed thoroughly. Absorbance values of the solution were recorded using a spectrophotometer at 510 nm. Catechin equivalents per gram of the sample (mg CE/g) were used to express total flavonoid contents [28-29].

2.4. Determination of total phenolic content

Folin Ciocalteu's reagent was utilized to estimate total phenolic content in soybean extract [29-31]. The soybean extract for phenolic determination in distilled water (1:60 v/v) was reacted with Folin-Ciocalteu's reagent (250 µL). Then, sodium carbonate (750 µL) was added. Incubation for 8 min was conducted after the solution was thoroughly mixed. Distilled water (950 µL) was added and the mixture was incubated in the dark room for 2 h. The values of absorbance were recorded at 765 nm using a spectrophotometer. Contents of total phenolic were expressed as gallic acid equivalents per gram of sample (mg GAE/g).

2.5. DPPH free radical scavenging activity

A solution of 1 mM methanolic DPPH was used to estimate antioxidant activity [32-33]. In DPPH solution, soybean extract (1:19 v/v) was reacted. To allow complete reaction, incubation for 30 min in the dark room was conducted after mixing. Absorbance values of each sample (A_{sample}) and control ($A_{control}$) were read using a spectrophotometer at 515 nm. Antioxidant activity of the samples as represented by percent discoloration was calculated using the following formula: percent discoloration = $[1-(A_{sample}/A_{control})]$ x 100%. Antioxidant activity of each sample was expressed as micromoles of Trolox equivalent per gram of sample (µmol TE/g).

2.6. Total flavonoid, phenolic contents, and antioxidant activity in soybean cultivars

Soybean seeds of twenty cultivars were finely ground separately as described in the sample preparation and the flour was extracted for quantification of total flavonoids, phenolics as well as antioxidant activity as described above. A completely randomized design repeated three times was used in this study. The difference among treatments was determined with the least significant different.

3. Results and discussion

3.1. Sample quantity and particle size

The proportion of sample to extraction solvent of all treatments is 1:10 (w/v) with twice extraction. The only difference is the quantity and particle size of the samples. The purpose of this study was to determine the minimum amount of sample in combination with the particle size could be employed to extract secondary metabolites in soybean flour for practical reasons with respect to environmental and economic considerations. The use of limited amount of working samples and solvents for extraction is an effective approach to reduce chemical disposal, specifically when the chemical waste is strictly regulated. Minimal amount of solvents is one of the alternatives in which more environmental friendly procedure can be conducted [34]. The use of 0.1, 0.5, 1.0, and 2.5 g of samples with different particle sizes showed no significant effect on total flavonoid content (0.52 to 0.59 mg CE/g) (Table 1). However, there was a slight difference in total phenolic contents (2.30 to 2.82 mg GAE/g). Small

quantity of sample with bigger particle size showed least amount of phenolic content as shown in treatment 1.

The same amount of phenolic content was also observed in treatment 7 and 10 in which bigger particle size was used. The use of different quantity and particle size of samples showed different antioxidant activity. The same results as observed in total phenolic content, the use of bigger particle size of the sample resulted lesser DPPH scavenging activity. Similar to this finding, extract obtained from smaller particle size of wheat brand showed higher secondary metabolites particularly phenolic acid, flavonoid, anthocyanin, as well as carotenoid contents compared to those from coarse particle samples because of the increase of accessible surface area [35]. Ultra-fine grinding of wheat brand also resulted the increase of antioxidant capacity [36]. Considering contents of the secondary metabolites in soybean extract and the value of standard deviation, treatment of 6 was selected for further optimization.

Table 1. Total flavonoid and phenolic contents as well as antioxidant activity derived from different particle size of soybean flour

Treatment	Combination	TFC (mg CE/g)	TPC (mg GAE/g)	AA (µmol TE/g)
1	0.1 g, 210 – 297 µm	0.52 ± 0.01 ^{a}	2.43 ± 0.08 ^{c}	3.423 ± 0.10 ^{f}
2	0.1 g, 177 – 209 µm	0.52 ± 0.02 ^{a}	2.81 ± 0.03 ^{a}	4.37 ± 0.04 ^{a}
3	0.1 g, ≤ 177 µm	0.59 ± 0.04 ^{a}	2.82 ± 0.19 ^{a}	4.18 ± 0.11 ^{bc}
4	0.5 g, 210 – 297 µm	0.53 ± 0.01 ^{a}	2.35 ± 0.04 ^{c}	3.32 ± 0.14 ^{f}
5	0.5 g, 177 – 209 µm	0.53 ± 0.01 ^{a}	2.61 ± 0.02 ^{b}	4.14 ± 0.08 ^{bc}
6	0.5 g, ≤ 177 µm	0.55 ± 0.01 ^{a}	2.72 ± 0.06 ^{ab}	4.13 ± 0.02 ^{bc}
7	1.0 g, 210 – 297 µm	0.53 ± 0.02 ^{a}	2.30 ± 0.03 ^{c}	3.64 ± 0.26 ^{e}
8	1.0 g, 177 – 209 µm	0.53 ± 0.01 ^{a}	2.64 ± 0.07 ^{b}	4.20 ± 0.02 ^{abc}
9	1.0 g, ≤ 177 µm	0.54 ± 0.01 ^{a}	2.63 ± 0.04 ^{b}	4.17 ± 0.07 ^{bc}
10	2.5 g, 210 – 297 µm	0.54 ± 0.04 ^{a}	2.37 ± 0.09 ^{c}	3.83 ± 0.08 ^{d}
11	2.5 g, 177 – 209 µm	0.52 ± 0.03 ^{a}	2.61 ± 0.01 ^{b}	4.32 ± 0.03 ^{ab}
12	2.5 g, ≤ 177 µm	0.53 ± 0.05 ^{a}	2.61 ± 0.08 ^{b}	4.29 ± 0.06 ^{ab}

The numbers followed by the same letter in the same column were not significantly different based on the LSD test ($p < 0.05$)
TFC = total flavonoid content, TPC = total phenolic content, AA = DPPH scavenging activity

3.2. Traditional extraction methods

Extraction of natural products is a crucial step prior to isolation, identification and the use of secondary metabolites for further purposes and there is no specific and standard method has been established to perform the extraction [37]. A significant variation of total flavonoid content was observed in different extraction methods (Table 2). Total flavonoid content of maceration treatments (C and D treatments, 0.32 and 0.41 mg CE/g) showed lesser amount compared to those of other treatments. Unlike total flavonoid content, maceration only was not significantly influenced the total phenolic content and antioxidant activity.

Number of extraction influenced the secondary metabolite contents. Twice extraction provided more secondary metabolite contents than those in single extraction. With twice extraction, total phenolic content and DPPH scavenging activity were in the range from 2.82 to 2.83 mg GAE/g and 3.87 to 4.01 µmol TE/g, respectively. The treatments of twice extraction either shaking followed by maceration or ultrasound-assisted extraction followed by maceration (B and F treatments) could be chosen to extract secondary metabolite contents in soybean seeds. The use of ultrasonication influenced the amount of plant secondary metabolites was also reported by Rostagno *et al*.[38]. They

found that ultrasonication extracted the highest amount of isoflavones when compared to soxhlet, superctitical-CO_2 and ultrasonication extractions. The advantage of ultrasonication to extract the secondary metabolites is to enhance plant cell disruption, to facilitate solvent penetration as well as mass transfer. This treatment therefore, provides more efficiency in the extraction [39-40].

Table 2. Effect of different extractions on total flavonoid, phenolic contents and antioxidant activity of soybean flour

Treatment	Extraction	TFC (mg CE/g)	TPC (mg GAE/g)	AA (µmol TE/g)
A	1x extraction, shaking 2 h + maceration 18 h	0.46 ± 0.02 [b]	2.57 ± 0.06 [b]	3.67 ± 0.05 [b]
B	2x extraction, shaking 2 h + maceration 18 h	0.54 ± 0.02 [a]	2.82 ± 0.06 [a]	4.01 ± 0.17 [a]
C	1x extraction, maceration 20 h	0.32 ± 0.01 [d]	2.58 ± 0.01 [b]	3.56 ± 0.06 [b]
D	2x extraction, maceration 20 h	0.41 ± 0.02 [c]	2.83 ± 0.08 [a]	3.87 ± 0.05 [a]
E	1x extraction, UAE 5 min + maceration 20 h	0.51 ± 0.02 [a]	2.52 ± 0.02 [b]	3.63 ± 0.05 [b]
F	2x extraction, UAE 5 min + maceration 20 h	0.52 ± 0.04 [a]	2.83 ± 0.08 [a]	3.94 ± 0.11 [a]

The numbers followed by the same letter in the same column were not significantly different based on the LSD test ($p < 0.05$)
TFC = total flavonoid content, TPC = total phenolic content, AA = DPPH scavenging activity, UAE = ultrasound-assisted extraction

3.3. Secondary metabolites in soybean cultivars

3.3.1. Total flavonoid content in soybean cultivars. There were variations of flavonoid contents in all yellow soybean seeds. The contents of total flavonoid were from 0.23 to 0.43 mg CE/g (Figure 1). Dering and Gema had the highest flavonoid contents among other soybean cultivars. The range of total flavonoid contens from 0.22 to 0.45 mg CE/g in yellow soybeans obtained from germplasm collection was also reported in previous study [31]. Nevertheless, the contents of flavonoids were slightly lower than flavonoid contents in yellow soybeans (0.25 to 0.50 mg CE/g) as investigated by previous researchers [29].

The difference in extraction methods can be one of the reasons in the difference of total flavonoid contents. Yellow soybeans contain high isoflavones which contribute to major compounds of flavonoids [41-42]. Isoflavone variation of all genotypes in this present study was of interest should be investigated further to determine its contribution to the total flavonoid content. Besides isoflavones, other compounds especially carotenoids and luteins contribute to total flavonoids in yellow soybeans [43-44]. It has been comprehensively reported that other factors also influence the concentration and distribution of secondary metabolites particularly environmental factors [11, 45].

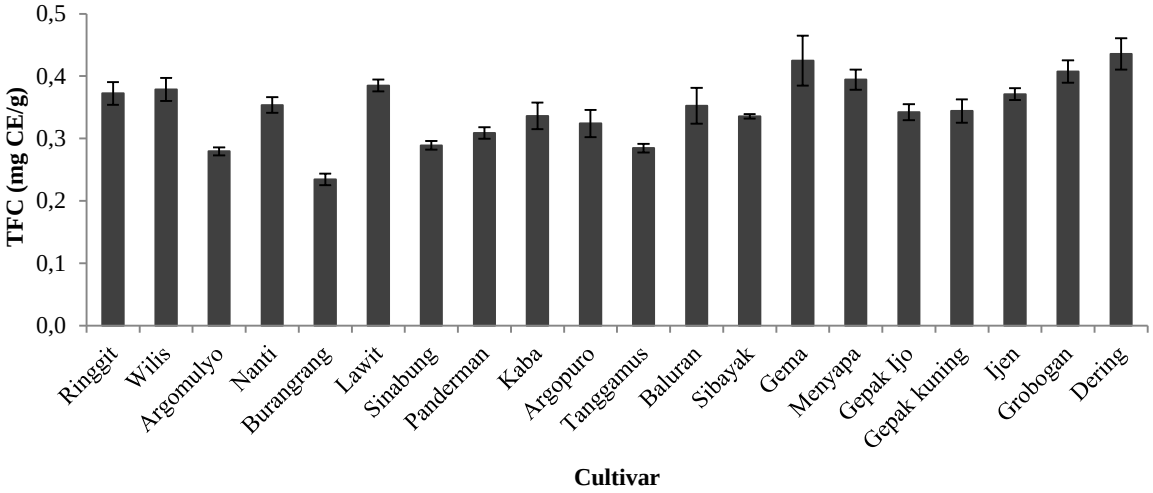

Figure 1. Total flavonoid content of twenty soybean cultivars. Error bars represent standard deviation from measurements in triplicate.

3.3.2. Total phenolic content in soybean cultivars. Similar as observed in total flavonoid contents, total phenolic contents of all soybean cultivars also showed variations (Figure 2). Interestingly, Dering cultivar which had the highest flavonoid contents also expressed the highest phenolic contents (5.22 mg GAE/g), however, similar high content of phenolic was not found in Gema cultivar (4.53 mg GAE/g). The range of phenolic contents of all soybean cultivars was from 3.70 to 5.22 mg GAE/g. This range was similar range of total phenolic contents in yellow soybean germplasm (3.49 to 5.42 mg GAE/g) as stated in a previous report [31]. Nevertheless, phenolic contents of yellow soybeans extracted from Indian genotypes (1.06 – 1.54 mg GAE/g) were lower than phenolics in Indonesian genotypes [44]. Again, different extraction as well as quantification methods used may influence quantity of phenolic contents besides genotypic variation as reported by previous researchers [42].

Seed coat, embryogenic axis and cotyledon contain phenolic compounds, however, the proportion of phenolics in the seed coat was dominant [31, 46]. Estimation of phenolic contents among different seed coat color, i.e. light yellow, yellow, green, and black soybeans has been studied and black seed coat color soybean expressed the highest phenolic contents [29, 31, 44, 46-47]. Approximately three times higher in phenolic contents is observed in black soybean than in yellow soybeans [31].

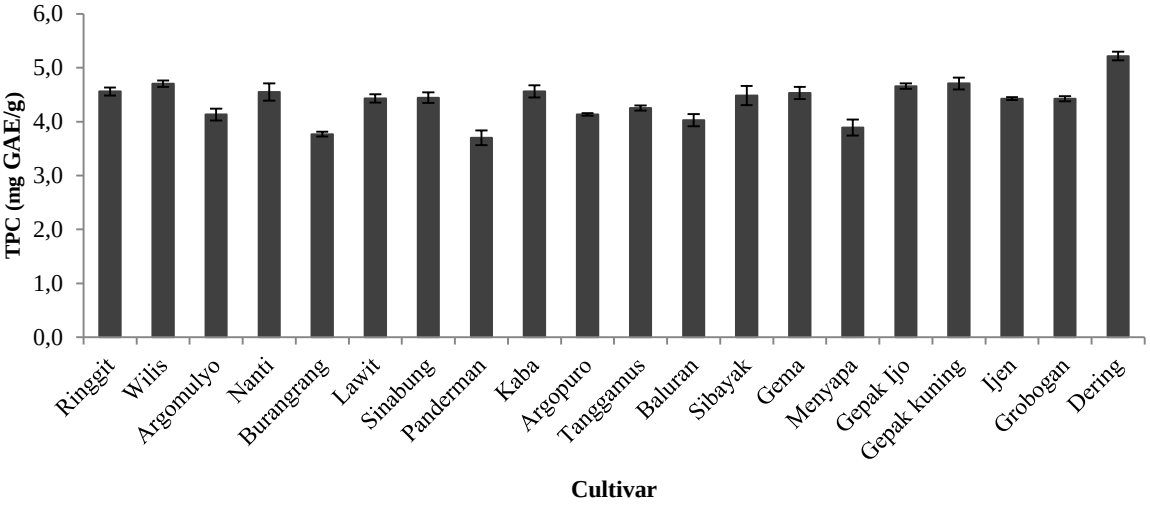

Figure 2. Total phenolic content of twenty soybean cultivars. Error bars represent standard deviation from measurements in triplicate.

3.3.3. DPPH scavenging activity of soybean cultivars. Variations among soybean cultivars were also observed in antioxidant activity (Figure 3). There was approximately one and a half-fold variation of antioxidant activity from the lowest to the highest. Grobogan which had the second highest of flavonoid content had the highest antioxidant activity. Even though total phenolics were similar contents to those in Xu and Chang study [29], DPPH scavenging activity was about 3-fold higher. This finding supports a previous study [31], in which antioxidant activity was observed approximately 3.5 times higher.

In yellow soybeans, most of antioxidant activity may be predominantly supported by isoflavones and ferulic acid in cotyledons and embryo [42]. As comparison with black seed coat color soybeans, antioxidant activity of these soybeans is mostly concentrated in its seed coat. Anthocyanins are compounds contribute to high antioxidant activity as previously observed [41]. Nevertheless, isoflavones in yellow soybeans have weaker antioxidant activity than that of procyanidins and anthocyanins in dark seed coat color soybean [46, 48].

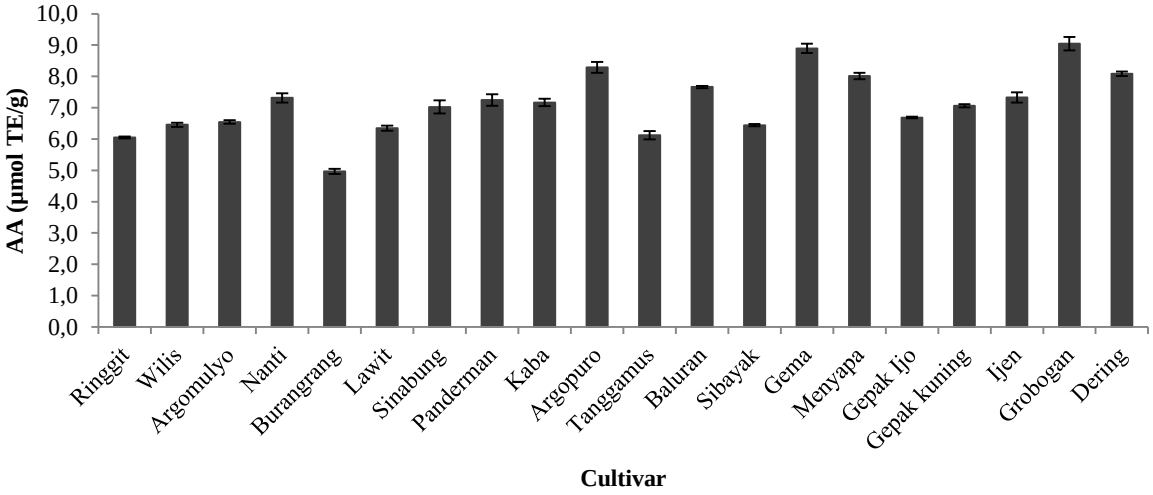

Figure 3. Antioxidant activity (AA) of twenty soybean cultivars. Error bars represent standard deviation from measurements in triplicate.

A linear correlation ($P < 0.05$) of total flavonoid and phenolic contents ($r = 0.56$) as well as total flavonoid content and antioxidant activity ($r = 0.65$) was investigated in this study. However, no significant correlation ($P > 0.05$, $r = 0.16$) was found in total phenolic content and antioxidant activity. A positive correlation among total flavonoid and phenolic contents confirmed the previous finding [31] when observing yellow soybeans. Flavonoids are one of the large groups of phenolic compounds, therefore, this significant correlation is not surprising [46]. In addition, isoflavones, one of the sub classes of flavonoids also contribute to high antioxidant activity [49]. Nevertheless, this recent result was not in line with a study conducted by previous researchers [29] in which the three parameters were correlated. Yusnawan [31] suggested to conduct other antioxidant capacity assays to obtain better understanding of correlations of the three parameters.

Phenolics in plants particularly simple phenols, lignins, and flavonoids are chemical compounds effective to inhibit fungal infection. Inhibition of hydrolytic enzymes produced by fungal pathogens triggers plant resistant to the fungal infection [15]. However, in healthy seeds for instance, total isoflavonoids are higher than seeds infected by *Phomopsis longicolla*. The amount is 5-fold higher than the diseased seeds. The same compounds are also detected twice higher in healthy seeds than in diseased seeds by *Cercospora kikuchii* [50]. Even though there is different content of flavonoids in healthy and diseased seeds, the authors suggested that the changes may be not only necessarily caused by the pathogen infections. In fact, other factors both environmental and genotipic factors such as sample condition and storage period prior to analysis may influence the chemical contents. In addition, agronomic condition, moisture, region, light, temperature and cultivars also influenced the compounds [45, 50-52]. However, the estimation of secondary metabolite contents in soybean seeds as investigated in the present study could be of value as initial information in terms of variations in chemical defenses against seed diseases among cultivars. Higher amount of secondary metabolites in certain soybean cultivars compared to the other cultivars whether may be related to more resistant to plant pathogen infection needs further studies.

In conclusion, this study revealed that extraction of fine particle sizes of soybean flour produced higher amount of flavonoid and phenolic contents as well as antioxidant activity compared to the use of coarse particles. Small amount of the sample and simple extractions either shaking followed by maceration or ultrasound-assisted extraction followed by maceration repeated twice in each treatment could be conducted to extract secondary metabolite contents. This approach was valuable to determine

secondary metabolite variations particularly when a large number of samples will be analyzed with limited quantity of the samples such as breeding materials or soybean germplasm.

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