Extraction treatments affect total flavonoid and phenolic contents of cowpea (*Vigna unguiculata* L. Walp.)

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Abstract. Plant secondary metabolites exist during normal plant growth. The production increases remarkably when the plants are challenged by environmental stress. Quantification of these metabolites is affected by extraction solvents and preparation treatments. This study aims to select extraction solvents and treatments which are able to extract high total flavonoid and phenolic contents in cowpea seeds. Acetone at concentrations of 70-80% produced higher total flavonoid and phenolic contents of 10.37–11.93 mg CE/g and 18.20–20.20 mg GAE/g in two cowpea cultivars. Antioxidant activities were in the range from 115.9 to 126.1 umol TE/g. Extraction of cowpea seeds using 70% acidified acetone produced a similar amount of total phenolic contents to those of 70-80% acetone. Traditional extraction treatments of shaking and maceration extracted a similar amount of the secondary metabolites. These simple extraction methods, therefore, could be suggested to extract flavonoid and phenolic contents as well as antioxidant activity in cowpea where access to modern types of equipment is limited.

1. Introduction

Cowpea is one of the underutilized legumes which has drought and heat tolerant properties. This legume is rich in polyphenols and some of which are not found in other legumes [1]. Variations in the seed coat color of cowpea are observed in cultivars and germplasms including breeding lines and landraces. These bioactive compounds are mostly present in the seed coats [2].

Polyphenolic compounds increase remarkably during interaction with biotic stress. Interactions with plant pathogen during infection, symbiotic microorganism, pests and herbivore eating have been reported to markedly increase these compounds. Similarly, abiotic stress such as ultraviolet radiation also triggers the increase of polyphenolic compounds [2-4].

Several major polyphenols in cowpea are phenolic acid derivatives, ranging from 148 – 1176 ug/g and flavonol glycosides, ranging from 27-1060 ug/g [1]. Anthocyanins, flavanol, tannins, sphingolipids and fatty acids are also found in this legume [1-2]. Trace amounts of delphinidin, catechin glucoside, catechin and epicatechin are observed in some accessions, which contribute to variation among the accessions [2].

Determination of polyphenolic compounds in various legumes particularly in seeds has been conducted to estimate either total phenolic content or individual phenolic compound [5-6]. The choice
of extraction methods including extraction solvents would significantly influence the amount of secondary metabolite compounds. According to Azmir et al. [7], techniques for extraction and sample preparation are the main concern of secondary metabolite experiments since these steps influence the final results. This study, therefore, aimed to extract total flavonoid and phenolic contents as well as antioxidant capacity using different organic solvents and traditional extraction treatments in two cowpea cultivars.

2. Materials and Methods

2.1. Cowpea sample preparation
Two different seed coat color of cowpea, i.e. KT 4 (brown seed coat) and KT 5 (red seed coat) cultivars were finely ground to obtain small particle sizes. The flour was sieved to obtain ≤ 80 mesh of particle size. The powder was stored in sealed plastic bags at 4 °C prior to being extracted.

2.2. Cowpea flour extraction with organic solvents
Three different solvents were used to extract secondary metabolites in cowpea. Acetone, ethanol and methanol with different concentrations of 50%, 70% and 80% were used as performed by Yusnawan et al. [8] to extract flavonoid and phenolic contents in mungbean. Briefly, cowpea flour was extracted in the organic solvents (1:10 w/v). The solution was macerated for 18 h after being shaken for 2 h. The extraction was conducted twice and the supernatant was pooled in an amber vial and stored at 4°C. Quantification of total flavonoid and phenolic contents in the supernatant were conducted. Antioxidant activity was also determined using the same supernatant. The suitable extraction solvent was used for further experiments.

2.3. Effect of different preparation treatments on total flavonoid and phenolic contents in cowpea
Two different treatments of shaking and maceration for extraction were conducted to extract the secondary metabolites in cowpea. A number of extractions was combined with those two treatments as follows: (1) cowpea flour in an organic solvent (1:20 w/v) was shaken for 18 h. The supernatant was collected after centrifugation, (2) cowpea flour in the organic solvent (1:10 w/v) was shaken for 18 h. The supernatant was taken after centrifugation. This process was repeated twice and the second supernatant was combined with the first extraction, (3) cowpea flour in the organic solvent (1:20 w/v) was macerated for 18 h. The supernatant was collected after centrifugation, (4) cowpea flour in the organic solvent (1:10 w/v) was macerated for 18 h. The supernatant was taken after centrifugation. This process was repeated twice and the second supernatant was combined with the first extraction. All supernatants were determined in terms of total flavonoid and phenolic contents as well as antioxidant capacity.

2.4. Determination of total flavonoid content
Total flavonoid content was estimated as conducted by Lee et al. [9] with slight modification. Briefly, the supernatant was diluted in distilled water. The solution of sodium nitrite (1:20 v/v) was added and thoroughly mixed. Aluminum chloride was added after incubation for a few minutes. Sodium hydroxide and distilled water were added to the solution. Catechin was used as a standard and the content of total flavonoid was measured using a spectrophotometer.

2.5. Determination of total phenolic content
Folin Ciocalteu’s reagent was used to estimate total phenolic content in cowpea extract according to a modification method by Kim et al. [10]. After dilution of cowpea extract in distilled water, 250 uL of the Folin Ciocalteu’s reagent was added. The solution was added with 750 uL of sodium carbonate and finally was added with distilled water. Gallic acid was used as a standard. Total phenolic content was estimated using a spectrophotometer.
2.6. Determination of antioxidant capacity
Antioxidant capacity was measured by reacting cowpea extract with ethanolic DPPH solution as described by Yusnawan et al. [8]. After reacted with the DPPH solution, incubation for 30 min was conducted in the darkroom. The change of color then was measured with a spectrophotometer. Antioxidant activity as represented by inhibition percentage was calculated as \[1 - \frac{(A_{\text{sample}})}{A_{\text{control}}}\] x 100%, where \(A_{\text{sample}}\) was absorbance value of the sample and \(A_{\text{control}}\) was absorbance value of the control. Antioxidant activity was expressed as Trolox equivalent.

3. Results and Discussion

3.1. Effect of extraction solvents on total flavonoid, phenolic contents and antioxidant capacity
Different organic solvents and concentrations for cowpea extraction significantly affected quantities of total flavonoid and total phenolic as well as antioxidant activity (Figure 1, 2, 3). Among three extraction solvents, acetone was the suitable solvent to extract those secondary metabolites than methanol and ethanol. Acetone at concentrations of 70-80% were able to extract flavonoid contents from 11.26 to 11.93 mg CE/g of KT 4 and 10.37 to 10.50 mg CE/g of KT 5 cultivars, which were more than four folds than those of solvent counterparts.

Similar findings were also reported by Kumar et al. [11], Zhang et al. [12], Yusnawan [13]. Higher flavonoid and phenolic contents were achieved when mung bean flour were extracted with acetone [8]. Antioxidant components from yellow, green, and black soybean were extracted using aqueous acetone at a concentration of 70% [11].

![Figure 1. Total flavonoid contents of two cowpea cultivars. Bars represent standard deviation. Solvent 1 = 50% acetone, 2 = 70% acetone, 3 = 80% acetone, 4 = 70% acidified acetone, 5 = 50% methanol, 6 = 70% methanol, 7 = 80% methanol, 8 = 70% acidified methanol, 9 = 50% ethanol, 10 = 70% ethanol, 11 = 80% ethanol, and 12 = 70% acidified ethanol. KT 4 = KT 4 cowpea cultivar, KT 5 = KT 5 cowpea cultivar.](image-url)

Extraction of flavonoid in cowpea cultivars both in KT 4 and KT 5 with acidified acetone reduced the flavonoid content (Figure 1). This result was not the same when this solvent was used to extract phenolics and antioxidant components. The use of this solvent gave the same amount of phenolics and antioxidants as the use of 70-80 acetone (Figure 2, 3), which were 18.37-19.87, 18.20-20.20, and 19.97-
20.90 mg GAE/g for 70% acetone, 80% acetone, and 70% acidified acetone for KT 4 and KT 5, respectively. Several studies used solvents with additional of acetic acid to extract secondary metabolites in samples. Acetic acid added to the solvent aimed to hydrolyze the sample as reported by Özcan and Özkan [14].

Two different seed colours of cowpea contributed to variations in total phenolic and flavonoid contents extracted with the same organic solvents. KT 4 cultivar consistently exhibited high flavonoid content than that of KT 5 cultivar after extraction with all solvents (Figure 1). Flavonoids are mainly found in seed coat than in cotyledon [15]. Interestingly, phenolic contents in KT 5 were the highest when this cultivar was extracted with acetone at concentrations of 70-80% Figure 2. Acetone-water solvent was more efficient in the breaking of polyphenol-protein complexes than other organic solvents such as methanol and ethanol [16]. However, with the same solvents, antioxidant capacities of KT 4 cultivar were higher Figure 3. Extraction of phenolic contents in various coloured legumes showed that these legumes have more phenolic contents than uncoloured legumes [17]. In the present study, different solvents and concentrations influenced total phenolic contents Figure 2.
Figure 3. Antioxidant activity of two cowpea cultivars. Bars represent standard deviation. Solvent 1 = 50% acetone, 2 = 70% acetone, 3 = 80% acetone, 4 = 70% acidified acetone, 5 = 50% methanol, 6 = 70% methanol, 7 = 80% methanol, 8 = 70% acidified methanol, 9 = 50% ethanol, 10 = 70% ethanol, 11 = 80% ethanol, 12 = 70% acidified ethanol. KT 4 = KT 4 cowpea cultivar, KT 5 = KT 5 cowpea cultivar.

3.2. Total flavonoid, phenolic contents and antioxidant capacity of cowpea affected by different preparation treatments

Two traditional extraction methods were conducted to extract flavonoid and phenolic contents in two cultivars of cowpea. In general, total flavonoid and phenolic contents obtained from treatments of maceration both single and twice extractions were quite similar amounts (9.37-11.98 mg CE/g for flavonoid contents, 19.73-24.53 mg GAE/g for phenolic contents). The same trend was also observed in treatments of shaking of single and twice extractions (9.13-11.25 mg CE/g for flavonoid contents, 18.85-23.82 mg GAE/g for phenolic contents) for both cowpea cultivars (Figure 4).

The secondary metabolite contents in KT 4 cultivar were consistently higher than those in KT 5. The trend of antioxidant capacity of these two cultivars were also similar, KT 4 had higher antioxidant activity than that of KT 5. Coloured legumes contained more secondary metabolites [17] such as observed in KT 4 cultivar. Traditional extractions have been using in many laboratories for years since limitation to the access of modern equipments, although require more solvents and time [18]. Ultrasonic assisted extraction in combination with maceration was also used to extract secondary metabolites in soybean and mung bean [8, 19].
Figure 4. (a) Total flavonoid content, (b) total phenolic content, (c) antioxidant capacity of two cowpea cultivars affected by different treatments. Bars represent standard deviation. Treatment 1 = shaking, single extraction, 2 = shaking, twice extraction, 3 = maceration, single extraction, 4 = maceration, twice extraction. KT 4 = KT 4 cowpea cultivar, KT 5 = KT 5 cowpea cultivar.

Although traditional extraction approaches are considered not economic for some reasons such as consuming more solvent and longer time to obtain working extract [18], these approaches such as conducted in this current study have been using in many laboratories where access to advanced types of equipment is limited. This extraction therefore could be used in laboratories where modern types of equipment for extraction are not available. Research on the determination of total flavonoid and phenolic compounds are valuable to be investigated since these compounds are essential to protect the emerging seeds against environmental stress [17]. Investigation of these secondary metabolites using more cowpea cultivars would be further studied to observe the variations among the cultivars.

4. Conclusions
The total flavonoid, phenolic contents and antioxidant capacity in cowpea extracts were affected by extraction solvents. Acetone extracted these flavonoid and phenolic contents approximately four times higher than those of methanol and ethanol. The organic solvent containing acetone-water mixture was more efficient in the breaking of polyphenol-protein than other organic solvents, therefore exhibiting more secondary metabolites. Extraction with 70-80% acetone both shaking and maceration could be used to simplify extraction for total flavonoid and phenolic contents as well as antioxidant capacity.
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