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## Antioxidant Activities and Total Phenolic Content in Germinated and Non-Germinated Legume Extracts Following Alkaline-Acid Hydrolysis

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**Abstract:** This study was aimed to determine and compare Total Phenolic Content (TPC) and Antioxidant Activities (AA) in germinated and non-germinated legume [peanut (*Arachis hypogea*) and soybean (*Glycine max*)] extracts prepared using alkaline-acid hydrolysis. Total phenolic content and antioxidant activities 1, 1-diphenyl-2-picrylhydrazyl free radical (DPPH) scavenging and reducing Ferric Ion Antioxidant Potential (FRAP) in extracts were determined spectrophotometrically. Total phenolic content in non-germinated peanut and soybean were 1090.58 and 888.08 mg GAE/100g dry weight, respectively. After germination, total phenolic content in peanut increased to reach 1151.33 mg GAE/100g dry weight; while soybean significantly decreased. In non-germinated peanut and soybean DPPH was 1084.99 and 507.59  $\mu\text{M TE}/100\text{g dry weight}$ ; and FRAP was 47966.67 and 23010.00  $\mu\text{M Fe}^{2+}$  equivalent/100g dry weight respectively. Following germination, DPPH and FRAP were decreased in peanuts but increased in soybeans. The process of germination has resulted to the increments of antioxidant activities in soybean and total phenolic content in peanut.

**Key words:** Germination, peanut, soybean, antioxidant activities, total phenolic content, acid-alkali hydrolysis

### INTRODUCTION

Legume plays an important role in human nutrition as sources of protein, vitamins and minerals (Maiti and Wesche-Ebeling, 2002). Peanut and soybean are the most popular and economically important legumes (Duffus and Slaughter, 1980). A part from protein, legume is a rich source of antioxidants. Unfortunately, less attention is being paid to antioxidant activity in legume as well as their total phenolic content. Germination process is a cheap and effective way to enhance the nutritional value and quality of legume. The antioxidant activities in raw legume can be further boost up after germination (López-Amorós *et al.*, 2006). Phenolic acids exist as free forms and insoluble bound complexes (Nardini *et al.*, 2002). Generally, bound phenolic acids are not extractable by organic solvents (Escarpa and Gonzalez, 2001; Mattila and Kumpulainen, 2002; Nardini and Ghiselli, 2004). Thus, alkaline-acid hydrolysis is required to release the bound phenolics from their cell wall polymers.

To date, studies on germinated legumes following different hydrolysis conditions or sequences are still lacking. Moreover, information on antioxidant activities and total phenolic content in germinated peanut and soybean is still scarce. Identification of antioxidant

activities and total phenolic content in germinated peanut and soybean will enable these products to be used as diet supplement, pharmaceutical ingredients and food additives.

Germinated peanut or soybean can be consumed or ingested in the form of food or can be powdered as medicine that may contribute to the medical or pharmaceutical field. But prior to that, the trend of increasing or decreasing of antioxidant activities and phenolics compound in legumes must be determined and confirmed by substantial research. There is an increasing interest towards the benefits of legume germination as well as its food products. Thus, there is a great possibility to commercialize the germinated legume products as it will be accepted and adopted in the future consumer's food practices.

This study was aimed to determine and compare total phenolic content and antioxidant activities in germinated and non-germinated legumes extracts prepared using alkaline-acid hydrolysis.

### MATERIALS AND METHODS

**Materials:** Peanut and soybean were purchased from the local market located in Batu 9, Cheras, Selangor, Malaysia. The samples were contained in

plastic sealed and stored in refrigerator at 4°C prior to germination process.

**Chemicals:** Ethanol, methanol, Folin-Ciocalteu reagent, anhydrous sodium carbonate (NaCO<sub>3</sub>), gallic acid, sodium hydroxide (NaOH), 1, 1-diphenyl-2-picrylhydrazyl free radical (DPPH), 6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acids (Trolox), hydrochloric acid (HCl), sodium hypochlorite (NaClO), glacial acetic acid (C<sub>2</sub>H<sub>4</sub>O<sub>2</sub>), 2, 4, 6-tripyridyl-s-triazine (TPTZ), sodium acetate trihydrate (C<sub>2</sub>H<sub>3</sub>NaO<sub>2</sub>•3H<sub>2</sub>O), hexane, ferrous sulfate heptahydrate (FeSO<sub>4</sub>•7H<sub>2</sub>O) and ferric chloride (FeCl<sub>3</sub>) were purchased from Sigma Chemical Co (St. Louis, MO, USA).

**Germination:** About 100 g of each legume was sterilized with 70% ethanol for 2.5 min, followed by 2.5% sodium hypochlorite for 15 minutes (Huang *et al.*, 2003). Ethanol and sodium hypochlorite were removed with several rinses of sterile water. After disinfection, legumes were allowed to imbibe water at 20°C for 17 hours. Then water was removed and seeds were dark-germinated in basin with humidified Whatman no.4 filter papers at 20°C for 7 days. The germinated seeds were freeze-dried and grinded to pass a sieve to ensure comparable particle size. For both of germinated and non-germinated samples of legume, the process of defatting was necessary to remove its high fat content by soaking the freeze-dried legumes with hexane overnight before grinding. The fine flours of legumes obtained were stored in darkness at 4°C until ready for extraction.

**Extraction:** The legumes samples were extracted as described by Kim *et al.* (2006) with slight modifications. The fine flour of legumes was transferred to an Erlenmeyer flask, defatted twice with hexane at a 4:1 ratio (v/w) and kept on a orbital shaker (Unimax 1010, Heidolph Instruments GmbH and Co. KG, Germany) for 1 hour at room temperature. Each time, the mixture was filtered through a Whatman No.1 filter paper and the final defatted legumes were dried at room temperature. The defatted legumes were then extracted twice with 80% methanol at a 5:1 ratio (v/w) for 1 hour at room temperature. The mixture was filtered through a Whatman No.1 filter paper and the combined supernatant (methanolic extract) was stored in a sealed container at -80°C prior to being analyzed. Aliquots of the methanolic extracts were subjected to the determination of total phenolic contents, DPPH and FRAP assay.

**Hydrolysis:** The residue, after methanol extraction, was hydrolyzed by alkaline followed by acid to extract bound phenolic compound in the legume that were not extractable by 80% methanol, following the method from Ross *et al.* (2009) and Kim *et al.* (2006) with slight modifications. Firstly, this method started with alkaline

hydrolysis (1 g residue in 40 ml 2 M sodium hydrochloride for 4 hours, at room temperature) and was subsequently followed by acid hydrolysis of sample residue (40 ml 6 M hydrochloric acid for 1 hour at 95°C, resulting in two fractions which are P and Q, respectively. All the fractions (P and Q) were extracted with ethyl ether (40 ml x 3). Then, ethyl ether was evaporated to dryness using rotary evaporator (Rotavapor R-210, Buchi, Switzerland). Total phenolic determination, DPPH assay and FRAP were done for all these fractions P and Q.

**Determination of total phenolic content:** The total phenolic content was determined as described by Velioglu *et al.* (1998) with slight modifications. The sample extract (200 µl) was mixed with 1.5 ml of Folin-Ciocalteu reagent and allowed to stand at room temperature for 5 minutes. Later, 1.5 ml sodium bicarbonate solution was added to the mixture. After incubation for 90 minutes at room temperature, the absorbance was measured at 750 nm. Total phenolic was quantified by calibration curve obtained from measuring the absorbance of the known concentrations of gallic acid standard solutions (25-150 µg millilitre<sup>-1</sup> in 80% methanol solution). The results were calculated as milligram gallic acid equivalent (mg GAE) 100g<sup>-1</sup> dry weight of legumes and reported as mean value±standard deviation.

**Determination of antioxidant activity:** DPPH Radical Scavenging Assay: DPPH-free radical scavenging capacity of legume extracts was evaluated according to method of Chen and Ho (1995) with slight modifications. Briefly, 0.2 ml of sample was added to 3.8 ml 80% methanol solution of DPPH radical. The mixture was shaken vigorously for 1 minute by vortex and left to stand at room temperature in the dark for 30 minutes. Later, the absorbance for the sample ( $A_{sample}$ ) was measured using the spectrophotometer (Prim, Secomam, France) at 517 nm. A negative control ( $A_{control}$ ) was taken after adding DPPH solution to 0.2 ml of the 80% methanol extraction solvent. The percent of DPPH discoloration of the sample was calculated according to the following equation of discoloration percent.

$$\text{Discolouration (\%)} = [1 - (A_{sample}/A_{control})] \times 100$$

The free radical scavenging activity of legume extracts was expressed as an equivalent of that of Trolox. The results were calculated and expressed as micromoles of Trolox Equivalents (TE) per gram of legume using the calibration curve of the Trolox. Linearity range of the calibration curve was 10-900 µM (r = 0.99).

**Ferric reducing/antioxidant power assay:** The ability to reduce ferric ions was measured using the method described by Benzie and Strain (1996) and Katalinic *et*

al. (2005). Freshly prepared FRAP reagent was warmed at 37°C in a water bath which gives the initial reading ( $A_{\text{initial}}$ ; t = 0 minute). The reagent was prepared by mixing 10 m mole of 2, 4, 6-tripyridyl-s-triazine (TPTPZ) in 40 mM HCl, 20 mM  $\text{FeCl}_3$  and 0.3 M acetate buffer (pH 3.6) in the ratio of 1:1:10. Sample extract (100  $\mu\text{l}$ ) were added to 100  $\mu\text{l}$  of distilled water and 1.8 ml of FRAP reagent. The mixture was incubated at 37°C for 4 min. Absorbance was read at 593 nm using spectrophotometer. FRAP value was calculated based on the following equation.

$$\text{FRAP value} = A_{\text{final}} - A_{\text{initial}}$$

$A_{\text{final}}$ -Final absorbance at 593 nm (4 minutes)

$A_{\text{initial}}$ -Initial absorbance at 593 nm (0 minutes)

Antioxidants reducing ability in FRAP assay was calculated with reference to the reaction given by  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  at concentrations ranging from 50-1000  $\mu\text{M}$ . The values were expressed as  $\mu\text{mol}$  of  $\text{Fe}^{2+}$  equivalents per 100 gram dry weight of legumes.

**Statistical analyses:** Mean values and standard deviation of total phenolic content and antioxidant activity from legumes extracted by 80% methanol were calculated. Data analyses were performed using Minitab software version 15.0. Comparisons of means were carried out using 2-sample t-test and 1-way analysis of variance (significant level was considered at  $p < 0.05$ ). Pearson's correlation was used to determine the relationship between total antioxidant activities and total phenolic content.

## RESULTS AND DISCUSSION

**Total phenolic content:** The total phenolic content in non-germinated peanut and non-germinated soybean were  $1090.58 \pm 60.42$  and  $888.08 \pm 23.51$  mg GAE/100g dry weight, respectively. After germination, total phenolic content in peanut increased 5.57% to  $1151.33 \pm 80.99$  mg GAE/100g dry weight; while in soybean decreased

significantly 25.96% to  $657.50 \pm 49.36$  mg GAE/100g dry weight (Table 1).

Different trends of phenolic content in the portion of M (free phenolics), P (alkaline hydrolyzable phenolic acids) and Q (acid hydrolyzable phenolic acids) can be observed in the legumes samples due to germination process. In the case of peanut, total phenolic content in M increased 12.7% from  $255.25 \pm 23.21$  (non-germinated peanut) to  $287.75 \pm 22.75$  (germinated peanut) mg GAE/100g dry weight. On the other hand, total phenolic content in P decreased 11.37% after germination while Q have an increased of 17.18%. The total increased of total phenolic content in germinated peanut was solely contributed by the M and Q portions. All M, P and Q portions in soybean sample decreased 4.64, 23.22 and 51.79%, respectively after germination (relative to non-germinated peanut). Consequently, soybean decreased in total phenolic content after germination.

From the results obtained in the evaluation of the total phenolic content in M, P and Q of both samples, it can be deduced that germination caused an increased of total phenolic content in peanut but not in soybean. The increased of phenolic content in peanut is in agreement with Dueñas *et al.* (2009) who germinated Lupin seeds (*Lupinus angustifolius* L.). Another study that supported this finding was Fernandez-Orozco *et al.* (2006) who reported an increment of 53% of total phenols in lupin sprouts. Furthermore, Khattak *et al.* (2007) also observed a rise of total phenols after five days germination of chickpea (*Cicer arietinum* L.).

### Antioxidant activities

**DPPH scavenging effects of antioxidant:** After the germination of peanut, scavenging activities of germinated peanut in M portion increased 16.71% while its bound phenolics act in a contrary way as Q portion decreased 66.94%, relative to non-germinated peanut. As for soybean, M and P showed increments of 107.26 and 79.74%, respectively after germination but Q decreased 53.68% after germination. In terms of scavenging activity of the antioxidant compounds as a whole, peanut showed significant decreased of 21.34%

Table 1: Total phenolic content (TPC), DPPH and FRAP in germinated and non-germinated legume extracts

Assay	Sample		Mean $\pm$ SD (n = 3)	CV (%)
TPC (mg GAE/100g dry weight)	Peanut	N	1090.58 $\pm$ 60.42	6
		G	1151.33 $\pm$ 80.99	7
	Soybean	N	888.08 $\pm$ 23.51	3
		G	657.50 $\pm$ 49.36	7
DPPH ( $\mu\text{M}$ TE/100g dry weight)	Peanut	N	1084.99 $\pm$ 113.28	10
		G	853.40 $\pm$ 25.57	3
	Soybean	N	507.59 $\pm$ 37.03	8
		G	650.86 $\pm$ 21.74	3
FRAP ( $\mu\text{M}$ $\text{Fe}^{2+}$ equivalent/100g dry weight)	Peanut	N	47966.67 $\pm$ 5278.32	10
		G	32810.00 $\pm$ 2055.89	6
	Soybean	N	23010.00 $\pm$ 475.71	6
		G	27513.33 $\pm$ 2242.68	8

Each value represents the mean  $\pm$  SD of triplicates. G: Germinated, N: Non-Germinated

from 1084.99 to 854.40  $\mu\text{M TE}/100\text{g}$  dry weight; soybean showed significant increase of 28.20% from 507.59 to 650.86  $\mu\text{M TE}/100\text{g}$  dry weight (Table 1).

The decreasing trend of antioxidant activity in peanut is similar to trend observed in lentils (*Lens culinaris*) while the increasing trend of antioxidant activity in soybean is similar to beans (*Phaseolus vulgaris*) and peas (*Pisum sativum*) (López-Amorós *et al.*, 2006). Wong *et al.* (2006) stated that one of the possible reasons for the lower value obtained from DPPH assay for plant samples could be due to the presence of compound not reactive towards DPPH free radicals. Polyphenols may be more efficient as reducing agents in reducing ferric iron but some may not scavenge DPPH free radicals as efficiently due to steric hindrance. In this present study, the reactive compound in peanut after alkaline hydrolysis may be too low to present its antioxidant activity through scavenging of DPPH radicals.

**Ferric ion reducing power of antioxidant:** In terms of activity that reduce ferric ion to ferrous ion, antioxidant activity of peanut decreased significantly 31.60% from 47966.67 to 32810.00  $\mu\text{M Fe}^{2+}$  equivalent/100g dry weight; soybean increased significantly 19.60% from 23010.00 to 27513.33  $\mu\text{M Fe}^{2+}$  equivalent/100g dry weight (Table 1).

Specifically, the ferric acid reducing ability of the portions of M, P and Q in peanut decreased 17.11, 5.38 and 74.43%, respectively in relative to non-germinated peanut. In the case of soybean, only Q showed a decreased of 1.93% while M and P increased 41.88 and 48.90%, respectively after germination.

**Correlation test:** Correlation tests were carried out to link antioxidant activities assayed by DPPH and FRAP with the total phenolic content of the legume extracts. There was no correlation ( $p > 0.05$ ) observed between antioxidant activities and total phenolic content in both peanut and soybean extracts. In other words, the results do not demonstrated the contribution of phenolic compounds to the antioxidant activity of the germinated and non-germinated legumes.

These results are in agreement with the study of Bajpai *et al.* (2005) using medicinal plant sample. This indicated that the amount of total phenolic content did not reflect in their antioxidant activity or antioxidant capacity. Furthermore, Sun and Ho (2005) also reported that the antioxidant activity of buckwheat seed was inversely correlated with total phenolic content. While Tsuda *et al.* (1993) who used pecan and cashew nut as samples reported similar results with this study. In addition, Amarowicz *et al.* (1993) also showed that the lowest total phenolic content exhibited the highest antioxidant activity in flaxseed. Azizah *et al.* (2007) proved that there was no significant correlation between antioxidant activity and total phenolic content in cocoa bean extract.

However, there are some studies using legume as sample reported findings that in contrast to the present study. They found that there were close relationship between antioxidant activities and total phenolic content in legume such as petai (*Parkia speciosa*) (Wong *et al.*, 2006) and lupin (*Lupinus angustifolius* L.) (Dueñas *et al.*, 2009). The results of the present study also were in contrast with findings from Shukla *et al.* (2009) who indicated that the phenolic compounds could be the major contributors to antioxidant activity.

In the case of soybean, their antioxidant activities increase with a decrease of total phenolic content after germination. This might due to the presence of compounds with antioxidant activities that is not phenolic (Bajpai *et al.*, 2005).

While for peanut, the results observed in peanut is contradicting with the results obtained from soybean. The increased in total phenolic content followed by the decrease of antioxidant activities after germination of peanut indicating that not all phenolics in peanut contribute to antioxidant activity of this legume.

According to Sengul *et al.* (2009), the no correlation between total phenolic content and antioxidant capacity in plant samples is possibly due to the following reasons: the antioxidant capacity observed was not solely from the phenolic contents but could be due to the presence of some other phytochemicals such as ascorbic acid, tocopherol and pigments and also the synergistic effects among them, that may also contribute to total antioxidant capacity. The same factors might be contributing this present study since peanut and soybean are rich in phytochemicals, vitamin and protein as well.

**The bound phenolics:** Total phenolic content of legume consist of free phenolics and bound phenolics. The bound phenolics were extracted by alkaline hydrolysis and subsequently by acid hydrolysis. The phenolics extracted by alkaline hydrolysis are called alkaline-hydrolyzable phenolic acids (P) while the phenolics extracted by acid hydrolysis are called acid-hydrolyzable phenolic acids (Q).

In this study, alkaline hydrolysis contributed 31-49% of the total phenolic content; acid hydrolysis contributed 22-44% of the total phenolic content (Table 2). This reflects that both alkaline and acid hydrolysis must be carried out in order to fully extract the phenolics in the legumes since combination of both hydrolysis conditions had contributed to a considerable amount (71-77%) of phenolic acids to total phenolic content. These results indicate that the majority of the phenolic acids existed in bound form in peanut and soybean. Hence, this study strongly suggest that total phenolic content of legumes could be underestimated without including the bound phenolic compounds.

The content of insoluble bound phenolics was significantly higher than those of free phenolics among

Table 2: Percentage of free and bound phenolic acids in legume extracts

Sample	M	Percentage (%)			
		Total bound phenolic acids	P	Q	
Peanut	Non-germinated*	23	76	37	39
	Germinated*	25	75	31	44
Soybean	Non-germinated*	23	77	40	37
	Germinated*	29	71	49	22

\*Means values significantly different between free and bound phenolic acids in legume extracts at the level of  $p < 0.05$ .

M: Free phenolic acids, P: Alkaline-hydrolyzable phenolic acids, Q: Acid-hydrolyzable phenolic acids

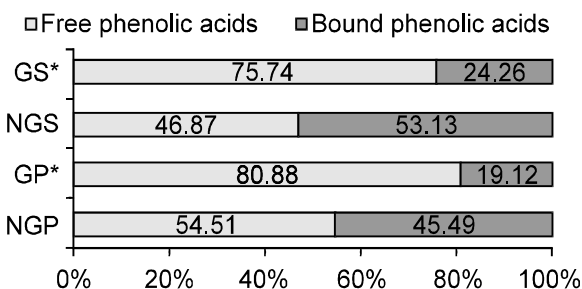


Fig. 1: DPPH of free and bound phenolic acids in legume extracts.

\*Means values significantly different between free and bound phenolic acids in legume extracts at the level of  $p < 0.05$ . Each value represents the mean  $\pm$  SD of triplicates

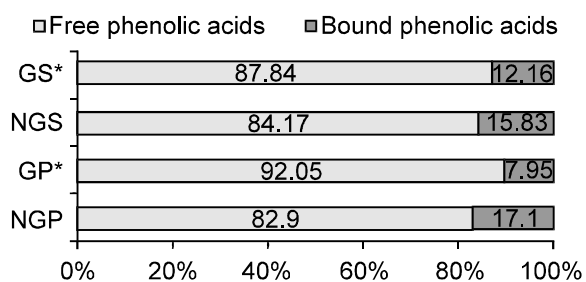


Fig. 2: FRAP of free and bound phenolic acids in legume extracts.

\*Means values significantly different between free and bound phenolic acids in legume extracts at the level of  $p < 0.05$ . Each value represents the mean  $\pm$  SD of triplicates

all legume extracts regardless of being germinated or non-germinated. Similar results were found by Nacz and Shahidi (1989) which using canola seeds as sample. According to Madhujith and Shahidi (2009), insoluble bound phenolics are associated with cell wall materials, for instance arabinoxylan which is a complex carbohydrate.

According to John and Shahidi (2010), some phenolics are present only in the bound form. Unfortunately, in this study, the higher bound phenolic acids content do not resulted to higher percentage of antioxidant activity in DPPH and FRAP assays. Most probably, the antioxidants were partially destroyed by multiple hydrolysis as shown in Fig. 1 and 2. Obviously, the free phenolics had performed a higher antioxidant activity in scavenging the DPPH radical and reducing the ferric ion to ferrous ion when compared to the bound phenolic acids. This could be due to the extreme condition of concentrated alkaline followed by hot acid that lead to the degradation of antioxidant compounds that are very sensitive to heat or hot condition. A recent research (Inglett *et al.*, 2011) suggested acid hydrolysis that followed the alkaline hydrolysis may also results in proteins precipitation. Therefore, protein-bound antioxidants could be discarded with the precipitate. Besides that, severe chemical hydrolysis also may change structures that no longer represent the real antioxidant activity.

Alkaline acid hydrolysis had increased the total phenolic content by maximizing the extraction yield of bound phenolics but not much increase in their antioxidant activities. Therefore, it is suggested that ascorbic acid

and Ethylene Diaminetetraacetic Acid (EDTA) should be added to the hydrolysis steps, since both chemicals have a protective effect toward these phenolic acids under aggressive hydrolysis condition (Ross *et al.*, 2009).

**Conclusion:** Germination has resulted to an increment of antioxidant activities in soybean and total phenolic content in peanut which reflected the enhanced of the nutritional level of the studied legumes. In conclusion, germination changed the total phenolic content and antioxidant activities of peanut and soybean. However, based on this present study, total phenolic content and antioxidant activities of legumes cannot be concluded as enhanced after germination since the antioxidant activities of peanut and total phenolic content of soybean showed a decrease after seven days of germination. But germination is still used as one of the several processing technique to decrease or eliminate anti-nutritional factors and toxicant in the legumes.

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