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Effect of germination on antioxidant activity, total phenolics, β-carotene, ascorbic acid and α-tocopherol contents of lead tree sprouts (*Leucaena leucocephala* (lmk.) de Wit)

*Suryanti, V., Marliyana, S.D. and Putri, H.E.

Department of Chemistry, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta, Central Java 57126, Indonesia

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Abstract

This study aims to determine the effect of germination time on the antioxidant activity of *Leucaena leucocephala* (lmk.) de Wit (lead tree) sprouts. Determination of antioxidant activity was carried out on ethanol extract of germinated seeds using β-carotene bleaching assay. The results showed that the antioxidant activity of germinated seeds were affected by germination time. Germination for 4 days affected the greatest enhancement in antioxidant activity by 2.78 fold and caused a significant increment in total phenolic content by 7.29, β-carotene by 3.27, ascorbic acid by 3.81 and α-tocopherol by 4.58 fold, respect to imbibed seeds. The total phenolics content was 53.42±0.22 mg CAE/g dw, β-carotene was 530.99±71.13 10^{-3} mg/100 g, ascorbic acid was 152.37±2.06 mg/100 g, and α-tocopherol was 59.27±0.10 mg/100 g sample. The findings suggested that the *L. leucocephala* sprouts could be considered as a source of natural antioxidant.

Introduction

Since side effects have reported on synthetic antioxidants, there is a trend to substitute synthetic antioxidants with naturally occurring antioxidants for use in food, cosmetic and pharmaceutical products (Namiki, 1990). In the search for novel naturally occurring antioxidants, vegetables, fruits, leaves, cereal crops, tree barks, roots, spices and herbs have been reported to exhibit antioxidant activity (Zheng and Wang, 2001; Koleva *et al*., 2002; Marina and Noriham, 2014; Hasmida *et al*., 2014; Suryanti *et al*., 2015).

Germination is the process of seeds growing into new plants. Germination begins with imbibition of water by dry seeds and terminates with the embryonic axis extends (Cevallos-Casals and Cisneros-Zevallos, 2010). For the germination to be initiated, the seeds must be viable and the appropriate environmental conditions must be applied, such as availability of water, a proper temperature range, a supply of oxygen and sometimes, light (Jann and Amen, 1977). Sprouting is the practice of germinating seeds to be eaten either raw or cooked. Germination is a simple and economical process to improve the nutritive value of legumes by causing desirable changes in the nutrient availability, texture and organoleptic characteristics. Secondary metabolites concentrations, such as phenolics, vitamins and carotenoids, which are considered beneficial as antioxidants, increase during the germination (Prodanov *et al.*, 1998; El-Adawy, 2002; Kuo *et al*., 2004; Fernandez-Orozco *et al*., 2009).

Seeds and sprouts from legume have become increasingly popular as functional foods, because of their nutritive values including amino acid, fiber, trace elements, vitamins, flavonoids, and phenolic acids (Chon, 2013). *L. leucocephala* is a legume and belongs to Fabaceae family. It is cultivated throughout tropics and sub-tropical locations. Lead seeds have oval shape, brown hulls and yellow kernels. Lead tree seeds contain lipids, protein, carbohydrates and also a toxic and non protein substance known as mimosine. The seeds can be used as a bio energy crop since they contain more than 20% oil. The seeds are also consumed as concentrates for dairy animals, as a manure, a protein source, an oil seeds and a potential source of commercial gum (Meena Devi *et al*., 2013). Lead tree seeds have been reported to have medicinal properties (Aderibigbe *et al*., 2011). They are used to control stomachache, as contraception and abortifacient. The seeds extracts also have been reported as anthelmintic, antidiabetic and antibacterial (Meena Devi *et al*., 2013). In Central America, Indonesia, and Thailand, the leaves and seeds are consumed but are not recommended

*Corresponding author.
Email: *venty@mipa.uns.ac.id*

for extensive human consumption because of the mimosine (Rushkin, 1984; Aderibigbe *et al.*, 2011). The young seeds can be eaten as raw and cooked. In Indonesia, the mature dry seeds are germinated as sprouts and consumed as vegetables. The objective of this work was to investigate the antioxidant activity and secondary metabolites contents, such as total phenolics, β-carotene, ascorbic acid and α-tocopherol in lead tree seeds during the germination period.

Materials and Methods

Samples and chemicals

Mature and dried lead tree seeds were supplied from local market. Seeds were cleaned manually to remove broken seeds, dust and other extraneous materials and stored in darkness in polyethylene containers at 4°C. All the chemicals used were of analytical grade, purchased from Merck Chemical Company (Merck, Germany).

Germination and freeze-dried

Lead tree seeds were washed with sterile distilled water. They were placed in distilled water and allowed to imbibe water at 25°C for 2 days. Then, water was removed and the seeds were distributed into germination trays on wet filter paper and covered with the same wet paper. The seeds were incubated at 25°C in a dark chamber and filter paper was kept moist by spraying with sterile water as needed. Samples were taken at 0 (imbibed seeds as control), 1, 2, 3 and 4 germination days. The germinated seeds were freeze-dried, milled, and passed through a sieve of 0.5 mm. The germinated flour was stored in glass containers at 4°C for further analysis.

Preparation of extracts

Twenty g lead tree flour from imbibed and germinated seed samples was homogenized with 200 mL of ethanol. The mixture was kept in agitation for 24 hours at 160 rpm in an orbital shaker (IKA Labortechnik, Staufen, Germany). Then, the extract was centrifuged at 4000 rpm for 15 minutes and filtered through the filter paper (Whatman No. 41). The solvents were removed using a rotary evaporator (Buchi, Uster, Switzerland) at 40ºC under vacuum. The extracts were dried using vacuum oven at 40ºC and were kept in dry clean black glass bottle at 4ºC for further analysis.

β-Carotene bleaching method

Antioxidant activity values of the imbibed and germinated seeds were evaluated according to slightly modified version of the β-carotene bleaching method (Suja *et al*., 2005). β-Carotene (0.2 mg), linoleic acid (20 mg) and Tween 20 (200 mg) were mixed in chloroform (1 mL) and the solvent was evaporated in rotary evaporator under vacuum. The residue was diluted with 50 mL oxygenated distilled water. Sample extract (200 ppm in 0.2 mL) was added to 4.8 mL of the resulting emulsion. A solution with 0.2 mL of ethanol and 4.8 mL of the above emulsion was used as control. Absorbance readings at 469 nm in a mini UV 1240 spectrophotometer (Shimadzu, France) were carried out at 20 minutes intervals during a period of 120 minutes, keeping the cuvettes in a water bath at 50°C. In parallel, equal concentrations of a commercial antioxidant, BHT, was used as a standard antioxidant. Antioxidant activity was calculated as inhibition percentage, relative to the control, using the following equation:

$$\% \text{ inhibition of } \beta\text{-carotene oxidation} = [(AS(120) - AC(120)) / AC(0) - AC(120))] \times 100$$

where AS(120) and AC(120) is the absorbance at 120 minutes of the sample and control, respectively, and AC(0) is the absorbance of the control at zero time.

Determination of total phenolics content

Total phenolics content was determined using the Folin-Ciocalteu method (Singleton and Rossi, 1965). A calibration curve of chlorogenic acid was prepared and the results were expressed as chlorogenic acid equivalents (mg CAE/g dry matter). In this method, sample extract (0.1 g), Folin-Ciocalteu's reagent (1 mL) and saturated Na_2CO_3 solution (2 mL) were added into a test tube. A control sample was prepared at the same time using distilled water (1 mL), Folin-Ciocalteu's reagent (1 mL) and saturated Na_2CO_3 solution (2 mL). Ingredients in test tubes were mixed well using vortex and left in a dark place for 2 hours. Absorbance was measured at 640 nm using a mini UV 1240 spectrophotometer (Shimadzu, France).

Determination of β-carotene

β-Carotene was measured according to Cagampang and Rodriguez (1980) with some modification. As much as 0.1 g of extract was weighed and dissolved in 5 ml of petroleum eter - acetone (1:1), homogenized in a homogenizer at 1000 rpm for 5 minutes. The supernatant was taken and measured the absorbance at 450 nm.

Determination of ascorbic acid

Ascorbic acid concentration was measured by redox titration using iodine solution (Baily, 1974).

As much as 0.1 g of extract 0.1 g of extract was dissolved in 100 mL of distilled water. Aliquot of the sample solution (50 mL) was pipetted into a 250 mL conical flask and added 2 mL of 1% starch indicator solution (0.5 g of soluble starch and 50 mL of near boiling water were stirred to dissolve and cooled before using). The sample was titrated with 0.005 M iodine solution (2 g of potassium iodide and 1.3 g of iodine were dissolved in 1 L of distilled water). The endpoint of the titration was identified as the first permanent trace of a dark blue-black colour due to the starch-iodine complex.

Determination of α-tocopherol

α-Tocopherol was evaluated according to Emmerie-Engel reaction (AOAC, 1990). As much as 0.5 mg of extract in 1 ml of ethanol, 3.5 mL of tween 80, 3.5 ml of 0.2% 2,2'-bipyridine in ethanol were placed in a 4-ml glass test tube and were thoroughly mixed. Subsequently, 0.5 ml of 0.02% M $FeCl_3$ solution in ethanol was added, followed by mixing with a vortex mixer. After 10 minutes, 0.2 ml of 0.001 M H_2PO_4 solution in ethanol was added and the contents of the tubes were again thoroughly mixed. Standards of D-α-tocopherol were prepared at the same time. The absorbance of the solutions was determined at 520 nm using a Gilford model 240 spectrophotometer.

Statistical analysis

Experimental results were performed in triplicate and the data are presented as mean ± SD. The results were compared by one-way ANOVA. A difference was considered statistically significant if $p \leq 0.05$.

Results and Discussion

Information about of the effect of germinated time on the antioxidant capacity of lead tree seed has been found in the literature. In Indonesia, lead tree sprouts for food consumption is usually germinated for 2-4 days. In this study, the lead tree seeds were germinated for 4 days.

Antioxidant activity and total phenolics content of lead tree sprouts

The antioxidant activities of the lead tree seeds were monitored during 4 days of germination. Our pre-elementary studies show that the sprouts have grown to 5-8 cm in length after 4 days of germination time. At longer germination time, over germinated seeds were occurred and they are not suitable for consumption. They began to develop leaves, and are then known as baby greens. These studies also demonstrate that the germination percentage increases from day 1 to day 4, and after that, no significant increase in germination capacity is observed.

Table 1. Antioxidant activity and total phenolics content of the imbibed and germinated seeds

Germination time (day)	Antioxidant Activity (% inhibition)	Total Phenolics (mg CAE/g dry weight)
0	17.88±0.76	7.32±0.11
1	22.07±2.58	6.41±0.11
2	28.05±0.49	15.33±0.41
3	37.47±1.31	19.62±0.29
4	49.74±0.83	53.42±0.22

Table 1 displays the antioxidant activity and total phenolics content of imbibed and germinated lead tree seeds. The antioxidant activities at different germination times were notable significant difference from each other ($p<0.05$). Antioxidant activity increased continuously with germination time and day 4 provided the highest antioxidant activity at 49.74±0.83% inhibition. Germination resulted in a 2.78 fold increase in antioxidant activity after 4 days of germination in comparison to imbibed seeds (day 0). Antioxidant activity of standard synthetic antioxidant BHT was 67.88±1.90% inhibition and the antioxidant activity of sprouts after 4 days of germination was comparable to BHT. The results demonstrated the influence of germination process on antioxidant activity is remarkable, showing significant higher antioxidant activities during 4-days germination. Similar behavior has been reported in the literatures (Aguilera *et al.*, 2015; Duenas *et al.*, 2009).

Table 1 shows that total phenolic compounds of germinated seeds varied widely, ranging from 6.41±0.11 to 53.42±0.22 mg CAE/g dw. The phenolics content of germinated seeds in the present study was comparable to those reported by other investigators. Horax *et al.* (2005) reported phenolic contents of bitter melon *(Momordica charantia)* seed, inner tissues, and flesh ranged from 4.64 to 8.90 mg CAE/g dw which exhibited high antioxidant activities ranged from 78.5% to 88.4% inhibition. Moussaid *et al*. (2011) observed total phenolic contents of some medicinal plants in Moroccan varied between 6.7 to 33.9 mg of CAE/g (dw).

The phenolics content was insignificantly decreased ($p>0.05$) up to 1 day germination and later significantly increased ($p<0.05$) up to 4 days germination. The highest total phenolics content

Table 2. β-Carotene, ascorbic acid and α-tocopherol contents of lead tree seeds at various germination times

Germination time (day)	β-Carotene (10^{-3} mg/100 g)	Ascorbic acid (mg/100 g)	α-Tocopherol (mg/100 g)
0	162.16±4.30	39.93±0.84	12.93±0.47
1	100.93±4.26	43.52±0.91	26.43 0.89
2	116.61±8.29	110.75±1.98	34.11±0.44
3	181.30±12.67	179.73±2.57	22.79±0.72
4	530.99±71.13	152.37±2.06	59.27±0.10

was achieved at 4 days germination, in this manner a considerable increase of 729.78% in the value of total phenolics was observed, as compared to imbibed seeds. Thus, germination brought significant increases in total phenolics content. Our data agree with those established by earlier authors (Cevallos-Casals & Cisneros-Zevallos, 2010; Duenas *et al*., 2009). However, Megat Rusydi and Azrina (2012) discovered a decrease in total phenolic compounds of germinated soy bean and peanuts. It is interesting to note that the changes of phenolic compounds levels after germination depend on plant species and the germination conditions used.

The results demonstrated that the highest levels of antioxidant activity are related to germinated seeds which exhibited the highest contents of phenolic compounds. A linear correlation between the antioxidant activity of the germinated lead tree seeds and the phenolics content was observed, accordingly phenolic compounds contribute strongly to the antioxidant activity of the germinated lead tree seeds (R^2=0.883). An increase in the antioxidant activity and phenolics content after germination also has been reported by Fernandez-Orozco *et al.* (2008) for mung bean and soybean, Fernandez-Orozco *et al.* (2009) for chickpeas, Gharachorloo *et al.* (2012) for lentil *(Lens culinaris)* and Carciochi *et al*. (2014) for quinoa seed (*Chenopodium quinoa* Willd.).

β-Carotene, ascorbic acid and α-tocopherol contents

β-Carotene, ascorbic acid and α-tocopherol contents of imbibed and germinated seeds during germination process are presented in Table 2. β-Carotene, ascorbic acid and α-tocopherol contents during germination period varied widely, ranging from 100.93±4.26 to 530.99±71.13 10-3, 39.93±0.84 to 179.73±2.57 and 12.93±0.47 to 59.27±0.10/100 g, respectively. A wide variation of β-carotene, ascorbic acid and α-tocopherol of germinated seeds was observed in cereal and legume with high level of antioxidant activity, which supports our findings (Finney, 1982). In this study, the highest β-carotene and α-tocopherol contents were obtained at the end of germination time (day 4), whereas the highest ascorbic acid content was observed at day 3 of germination time. Compared to the imbibed seed (day 0), at the end of the germination (day 4) an increase of 3.27 fold for β-carotene and 4.58 fold for α-tocopherol were observed. Ascorbic acid was recorded an increase of 4.50 fold after 3 days of germination period and registered 3.81 times of increase after 4 days of germination period, compared to imbibed seeds. The vitamin contents of the samples during the germination time were significantly different from each other ($p<0.05$).

High correlations between β-carotene, ascorbic acid and α-tocopherol and antioxidant activities, (R^2 = 0.70-0.73) were obtained. Our results indicated that antioxidants as β-carotene, ascorbic acid and α-tocopherol also affect to the antioxidant activity. Such synergistic activity among β-carotene, ascorbic acid, α-tocopherol and phenolic compounds could be the main reason of the observed antioxidant activities. These results are in agreement to those reported by earlier investigators. Fernandez-Orozco *et al.* (2006) reported that germination of lupin seeds (*Lupinus angustifolius* L. var. Zapaton) increases vitamin C, vitamin E and polyphenols contents and thus, enhances antioxidant activity. Doblado *et al*. (2007) reported that germination of cowpeas (*Vigna sinensis* var. carilla) is a good procedure for improving vitamin C content and increasing antioxidant activity. Overall, these results suggested that lead tree seeds steeped for 2 days and germinated for 4 days would produce the most desirable sprouts with respect to antioxidant activity and antioxidant compounds.

Conclusions

The results demonstrated that germination for 4 days affected the greatest enhancement in antioxidant activity by 2.78 fold, compared to imbibed seeds. The germination caused a significant increment in total phenolic content by 7.29, β-carotene by 3.27, ascorbic acid by 3.81 and α-tocopherol by 4.58 fold, compared to imbibed seeds. Results suggested that germination is a good way to enhance the antioxidant properties and increase the phenolic compounds, β-carotene, ascorbic acid and α-tocopherol contents of lead tree seeds and therefore, lead tree sprouts can be used as a source of natural antioxidants in functional foods.

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