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Original Article

Leucaena leucocephala (Lam.) de Wit Seeds: A New Potential Source of Sulfhydryl Compounds

Sri Wardatun[1], Yahdiana Harahap[1,*], Abdul Mun'im[2], Fadlina Chany Saputri[3], Noorwati Sutandyo[4]

ABSTRACT

Background: *Leucaena leucocephala* (Lam.) de Wit seeds are considered the most widely consumed legumes by ruminants. The seeds contain around 1.5 mmol of thiol/sulfhydryl compounds per 100 g of dried seeds. The contents of the sulfhydryl compounds can act like glutathione. On the other hand, the intake of a high amount of *Leucaena leucocephala* (Lam.) de Wit seeds is limited by mimosine because it can induce toxicity and death in ruminants. **Objective**: The aim of this study was to determine sulfhydryl compound levels in *Leucaena leucocephala* (Lam.) de Wit seeds after the mimosine removal process. **Materials and methods:** *Leucaena leucocephala* (Lam.) de Wit seeds were soaked in aquadest for 24 hours and then dried at 40°C for 86 hours. The dried seeds were macerated at room temperature using 30%, 50%, 70%, and 96% (v/v) of ethanol as the solvent with a 1:10 solvent-to-solid ratio. Levels of dried extract yield were determined for sulfhydryl compounds and mimosine using a spectrophotometer. **Results:** The 30% ethanol extract without soaking produced the highest levels of extract yield, but 70% ethanol was the most effective solvent for extracting the maximum sulfhydryl and minimum mimosine levels. **Conclusion:** Ethanol solvent (70%) can be used to extract maximum levels of sulfhydryl compound and minimum levels of mimosine from *Leucaena leucocephala* (Lam.) de Wit soaked seeds.

Key words: Ethanol; *Leucaena leucocephala* (Lam.) de Wit; Mimosine; Sulfhydryl compounds.

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INTRODUCTION

Leucaena leucocephala (Lam.) de Wit is a tropical leguminous tree in the family *Leguminosae* and is found in Indonesia and other tropical areas.[1,2] Seeds produced from trees in this family are often eaten as food and have several health benefits. *Leucaena leucocephala* (Lam.) de Wit is used as an antihelmintic, antidiabetic,[3,4] anticancer agent, and antimetastatic agent.[4] *Leucaena leucocephala* (Lam.) de Wit seeds contain around 1.5 mmol of thiol compounds per 100 g of dried seeds.[5] Thiol compounds are compounds that contain functional groups consisting of sulfur and hydrogen atoms (-SH), often referred to as sulfhydryl compounds.[6] Sulfhydryl compounds in the body can act like glutathione (ϒ-glutamyl-cysteinyl-glycine; GSH) and function as antioxidants to reduce reactive oxygen or nitrogen compounds and electrophiles, which are cofactors in several types of enzymes.[7,8] *Leucaena leucocephala* (Lam.) de Wit trees are easily found in abundant quantities and widely used as animal feed currently.[9] The utilization of *Leucaena leucocephala* (Lam.) de Wit as a source of nutrients, producing sulfhydryl compounds, has increased the value of the tree.[5]

The utilization of *Leucaena leucocephala* (Lam.) de Wit seeds as a source of sulfhydryl compounds is limited by the presence of mimosine,[10] another compound contained in the seeds. *Leucaena leucocephala* (Lam.) de Wit seeds contain 6.58% dry weight mimosine.[11] A side effect that arises from mimosine is the onset of hair loss.[12] The concentration of mimosine in the body must be considered,[9,13] and mimosine levels causing undesirable effects in goats was achieved at 800 mg/kg/day[14] and 1000 mg/kg/day[15] in rabbits or the equivalent of 32 mg/kg/day in humans.

Extracting compounds from plants can be done through plant parts. The extraction process is an important procedure when developing active plant compounds in dosage forms.[16] The extraction process isolates a number of active compounds contained in plants. The possibility of mimosine being extracted must be considered when isolating sulfhydryl compounds from *Leucaena leucocephala* (Lam.) de Wit seeds, and the sample preparation process is an important step.[6] The solvent choice determines the type of compound to be extracted from the sample.[17] The extraction of sulfhydryl compounds from *Leucaena leucocephala* (Lam.) de Wit seeds has been done using water.[5] Water has no impact on the environment but can dissolve undesired proteins and polysaccharides.[18,19] The presence of proteins and polysaccharides can make purification difficult when a filter membrane is used.[19] A high energy level is also needed to remove the water solvent by evaporation.[18] Ethanol is an organic and nontoxic solvent that can be used for extraction.[20] High concentrations of ethanol increase the cost of extraction.[19] The solvent concentration plays an important role in efficiency and will affect the quantity of the secondary metabolite.[21,22]

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This study aims to extract sulfhydryl compounds from *Leucaena leucocephala* (Lam.) de Wit seeds using different ethanol concentrations. Mimosine contained in the seeds can be removed by soaking.[11] The effect of soaking on the levels of mimosine and sulfhydryl compounds was determined.

MATERIALS AND METHODS

Chemicals and samples: The chemicals used in this study include reduced L-glutathione and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB; Ellman's reagent) from Sigma-Aldrich, ethanol, potassium phosphate monobasic, and sodium hydroxide (Merck). All chemicals used were analytical grades purchased from Merck.

Fresh *Leucaena leucocephala* (Lam.) de Wit seeds were collected from Ciomas, Bogor, Indonesia, in December 2018. All samples used were mature seeds. The seeds were characterized at the Bogor Botanic Gardens (Center for Plant Conservation), Indonesia.

Preparation of the dry powder: A number of samples were cleaned and then divided into three parts. Part A seeds were directly dried in an oven at 40°C for 86 hours. The seeds were ground to make powdered samples. Part B seeds were soaked for 24 hours with aquadest (1:10) and dried in an oven at 40°C for 86 hours. Part C seeds were split into two parts, soaked for 24 hours with aquadest (1:10), and then dried in an oven at 40°C for 86 hours.

Preparation of the extract: Dry powdered seeds amounting to 50 grams of dried powdered seeds were macerated with 250 ml of different ethanol solvent concentrations (30%, 50%, 70%, and 96%) for 24 hours. Maceration was repeated twice for 24 hours using 150 ml and 100 ml of solvent, respectively. The liquid extract was separated from the powdered seeds using a rotary evaporator. Furthermore, the extract yield was calculated.

Analysis of the sulfhydryl group in the extracts: The determination of the sulfhydryl compounds was carried out according to Ellman's method.[23-25] A number of extracts were dissolved with aquadest in a conical flask and then filtered. In brief, 2400 µl of potassium phosphate buffer, pH 7.4 (K_2HPO_4/NaOH, 200 mM), and 600 µl of extract filtrate were added to 300 µl of DTNB reagent in 1 mM potassium phosphate buffer, pH 7.4. The liquid was measured using Jasco V-730 UV-VIS double beam spectrophotometer at 411 nm after two minutes. The sulfhydryl content was calculated using a standard calibration curve for GSH solution. The results of the determination were expressed in gram GSH/100 g of powdered seeds.

Analysis of mimosine in the extracts: The extracts were carefully weighed (amounting to as much as 500 mg) and put in a beaker. Distilled water (2.0 ml) was added. Then 30 mg of activated carbon was added, and the solution was boiled for 15 minutes. The solution was filtered with a syringe filter (Axiva, 0.45 µm) into a 5 ml conical flask. HCl 0.1N (2.5 ml) was then added and diluted with distilled water to the limit. About 200 µl of this solution was pipetted and then put into a 5 ml conical flask. Subsequently, 400 µl HCl 0.1N and 200 µl 0.5% $FeCl_3$ in 0.1N HCl was added and then diluted to the limit with aquadest. The liquid was measured after 10 minutes at 534 nm, as maximum wavelength, using a Jasco V-730 UV-VIS double beam spectrophotometer.[10] The mimosine content was calculated using a standard calibration curve for mimosine solution. The results of the determination were expressed in mg/100 g of powdered seeds.

Standard curve for reduced glutathione levels: Phosphate buffer solution (2400 µl) of pH 7.4 was added to 600 µl of 15, 30, 45, 60, and 75 µg/ml solutions of GSH, followed by the addition of 300 µl of 1 mM DTNB stock solution, respectively. The mixture was shaken for 10 seconds using vortex and kept at room temperature (20°C) for 2 minutes. After two minutes, the absorbance was measured in λ_{max} as 411 nm. A standard curve was constructed between concentration and absorbance, and a straight line was obtained. The coefficient regression of the plot was 0.9996.

Standard curve for mimosine levels: Mimosine stock solution (1000 µg/ml) was pipetted (75, 150, 225, 300, and 375 µl) and then put into a 5 ml conical flask. Subsequently, 400 µl HCl 0.1N and 200 µL 0.5% $FeCl_3$ in 0.1N HCl was added and then diluted to the limit with aquadest. The liquid was measured after 10 minutes at 534 nm, as maximum wavelength, using a Jasco V-730 UV/VIS double beam spectrophotometer.[10] A standard curve was constructed between concentration and absorbance, and a straight line was obtained. The coefficient regression of the plot was 0.9997.

Statistical analysis: All the experiments were carried out in triplicates. The results are presented as average ± standard deviation. ANOVA was used to test the statistically significant difference between each treatment. $P < 0.05$ was considered as the level of significance.

RESULTS

The results show the extract yield produced from soaked *Leucaena leucocephala* (Lam.) de Wit seeds was lower than the extract yield produced by seeds that were not soaked and the seeds that were soaked and split (Figure 1). The analysis indicates that the 30% ethanol solvent produced a higher extract yield than other ethanol solvents. The 96% ethanol solvent yielded the lowest both in the soaked and unsoaked seeds. The 50% and 70% ethanol solvents produced some levels of extract yield ($P<0.05$).

The sulfhydryl levels in *Leucaena leucocephala* (Lam.) de Wit seeds can be seen in Figure 2. The levels of sulfhydryl compounds varied depending on the treatment of the seed samples. The sulfhydryl compounds levels from soaked seeds were lower than those of the unsoaked seeds. Furthermore, the concentration of the extracting solvent used affected the sulfhydryl compounds levels. The analysis show that the lower ethanol concentration produced higher levels of extracted sulfhydryl compounds, while the higher concentration of ethanol produced lower levels of extracted sulfhydryl compounds. The concentration of ethanol significantly affected the sulfhydryl compound levels ($P < 0.05$).

Soaking and splitting the *Leucaena leucocephala* (Lam.) de Wit seeds before extraction had a significantly different effect on the levels of extracted mimosine (Figure 3). The seeds split in half before extracting produced lower levels of mimosine than the soaked and unsoaked seeds. The difference in ethanol concentration used had a significant effect on the levels of mimosine extracted ($P < 0.05$). The lower ethanol concentrations produced higher levels of mimosine. The 96% ethanol solvent produced the lowest amount of mimosine compounds, compared to other ethanol concentrations.

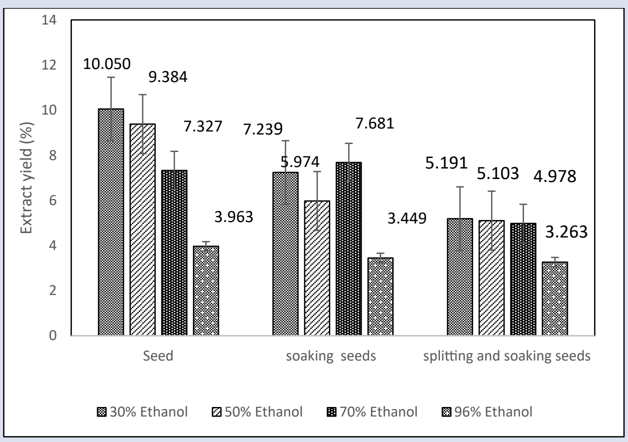

Figure 1: Effect of ethanol concentration and soaking treatment on the extract yield of *Leucaena leucocephala* (Lam.) de Wit seeds.

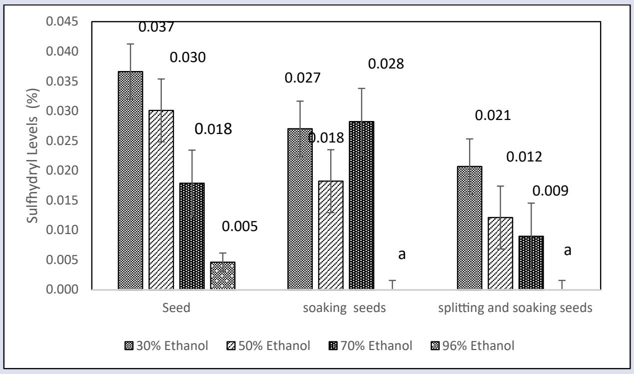

Figure 2: Effect of ethanol concentration and soaking treatment on the levels of sulfhydryl compounds in *Leucaena leucocephala* (Lam.) de Wit seeds.

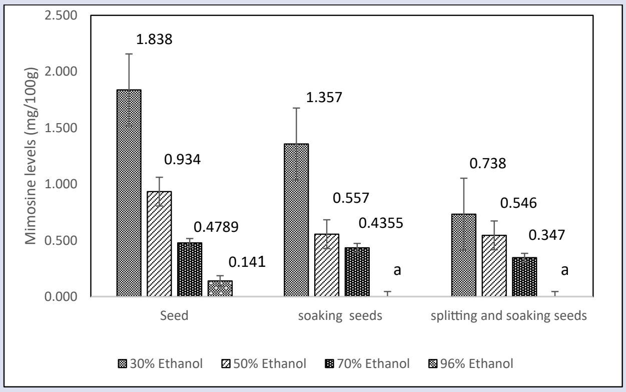

Figure 3: Effect of ethanol concentration and soaking treatment on the levels of mimosine in *Leucaena leucocephala* (Lam.) de Wit seeds.

DISCUSSION

Sulfhydryl compounds in the body can act like glutathione (GSH). In the body, glutathione can act as an antioxidant to reduce free radicals, reactive oxygen or nitrogen compounds, and electrophiles, which is a cofactor in several types of enzymes.[7,8] *Leucaena leucocephala* (Lam.) de Wit seeds contain around 1.5 mmol/100 g of dried thiol compounds using water as the solvent.[5] Solvents have an important role in the amount of extracted compounds.[21] The use of various concentrations of ethanol is expected to be more effective in extracting sulfhydryl compounds. The use of ethanol as a solvent affected the extracted levels of sulfhydryl compounds ($P < 0.05$). The lower ethanol concentrations produced higher levels of extracted sulfhydryl compounds (Figure 2). Sulfhydryl compounds are water-soluble and oil-soluble.[26] Water-soluble sulfhydryl compounds can be extracted by ethanol because ethanol is a polar solvent.[27] The levels of sulfhydryl compounds obtained in this research were different from those from Suvachittanont et al.'s study[5]—where fresh seeds were used, water was used for extraction, and the extract was measured without evaporation. The drying process may be affected by the available content of the sulfhydryl compounds.[5] Moreover, the evaporation of the extract using a rotary evaporator at 40°C may affect the total sulfhydryl compound content.

The presence of mimosine is a limitation for the use of *Leucaena leucocephala* (Lam.) de Wit seeds because mimosine is a toxic compound.[10] Mimosine can cause hair loss,[11] damage male reproductive organs in mice,[28] and impair growth performance.[29] The seeds contain 6.58% dry weight mimosine, which can be removed by soaking in aquadest for 24 hours.[11] The results of this study indicate seeds split in half and then soaked will lose more mimosine, compared to seeds that are not soaked.[11] Lower mimosine levels were extracted by the 96% ethanol solvent than by the 30% ethanol solvent.

The 30% ethanol solvent produced sulfhydryl compounds in higher amounts than the other ethanol concentrations. But the use of 30% ethanol can also cause the extraction of higher levels of mimosine. This is possible because the solvent with a lot of water was able to extract more mimosine.[10] Furthermore, the soaking process affected the levels of sulfhydryl compounds and mimosine extracted. Splitting the seeds in half before soaking eliminated a higher level of mimosine and produced less sulfhydryl compounds, compared to seeds soaked without splitting. Therefore, to develop the potential of *Leucaena leucocephala* (Lam.) de Wit seeds as a source of sulfhydryl compounds, it is necessary to prepare seeds before extracting. The use of 70% ethanol solvent in soaking seeds produced a higher level of sulfhydryl compounds and a lower level of mimosine (0.000435%), compared to mimosine in fresh seeds (6.58%).[11] The 70% ethanol for extracting soaked seeds is the best solvent to extract sulfhydryl levels with lower mimosine levels. Using 70% ethanol solvent for extracting *Leucaena leucocephala* (Lam.) de Wit seeds without soaking, soaked seeds, and soaked and split seeds can reduce levels of mimosine up to 99.99%, but the use of 70% ethanol with soaked seeds can extract higher sulfhydryl levels.

CONCLUSION

The use of 70% ethanol solvent can be an alternative in the preparation process of *Leucaena leucocephala* (Lam.) de Wit soaked seeds to extract maximum sulfhydryl compounds and minimum mimosine levels.

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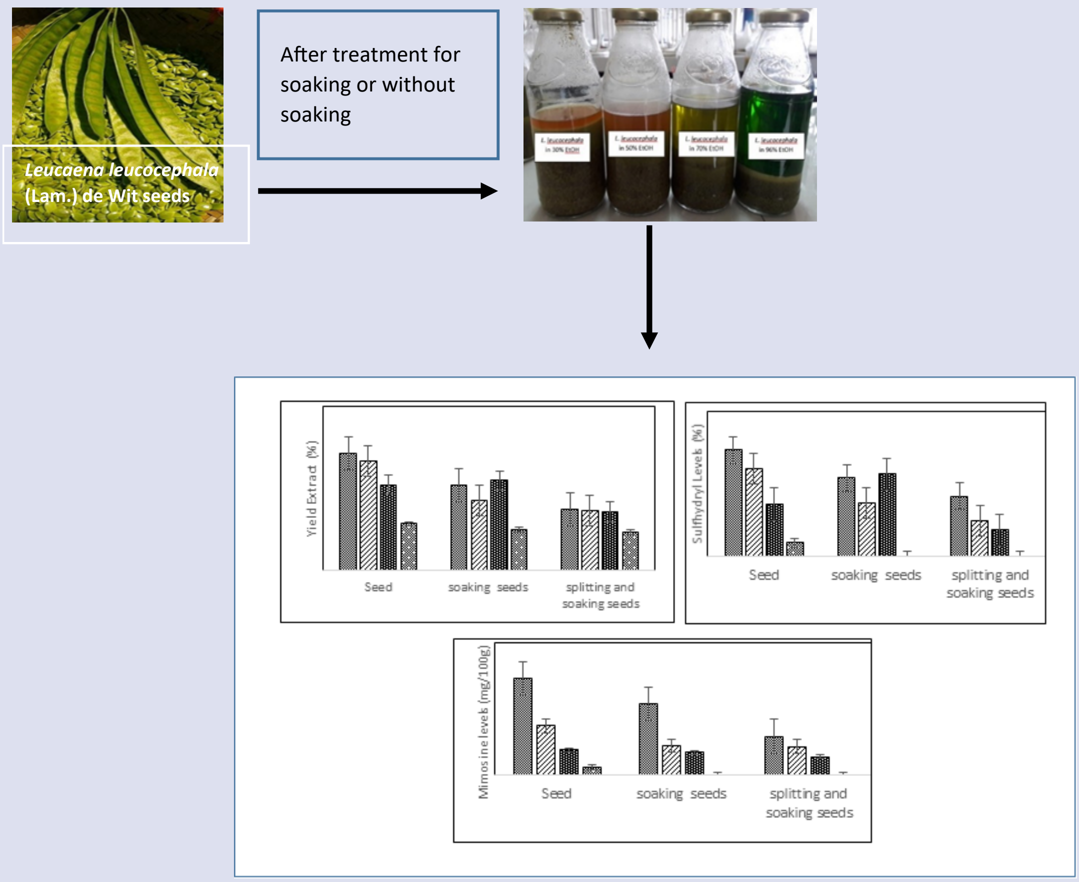
GRAPHICAL ABSTRACT



SUMMARY

Leucaena leucocephala (Lam.) de Wit seeds contain thiol/sulfhydryl compounds which can act like glutathione. The intake of a high amount of *Leucaena leucocephala* (Lam.) de Wit seeds is limited by mimosine. The 30% ethanol extract without soaking produced the highest levels of extract yield, sulfhydryl compounds and mimosine levels, but 70% ethanol was the most effective solvent for extracting the maximum sulfhydryl and minimum mimosine level. The *Leucaena leucocephala* (Lam.) de Wit seeds can be used as potential source of sulfhydryl compounds.

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