Phytochemical content, extract standardization and antioxidant activity of *Calliandra calothyrsus* Meissn leaf, a potential phytoestrogen source

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**Abstract.** Calliandra (*Calliandra calothyrsus* Meissn.) is a leguminous shrubs that almost spread throughout the area of Bali island, especially the area of Baturiti, Tabanan. The ethanol extract of 80% of *C. calothyrsus* leaves is a potential source of exogenous estrogen in terms of its phytoestrogen content. Potential extract of potassium which is very useful can be consumed in the form of phytopharmaca preparations. One of the requirements to prepare the formulation of phytopharmaceutical preparations should be done related to phytochemical screening and standardization of raw materials. This study aims to perform phytochemical screening and standardization of 80% ethanolic extract of *C. calothyrsus* leaves. Phytochemical screening can provide an overview of chemical compounds which are contained in *C. calothyrsus* leaf extract to their activity. The standardization of extracts will ensure the quality of raw materials extract to be used. The leaf extraction of 80% ethanol yielded as much as 21.85%. Phytochemical screening showed that 80% positive ethanol extracts contained flavonoid group compounds. The powder standardization obtained 8.35% of water content. While the extracts standardization obtained 5.26% of extract water content. The total ash content of the extract was obtained 4.54% and the acid soluble ash content was 0.5%. The ethanol extract of 80% *C. calothyrsus* leaf has met the standardization of raw material extract.

1. **Introduction**

Calliandra (*Calliandra calothyrsus* Meissn.) is a leguminous plant of shrubs or small trees belonging to the leguminous family [1]. Calliandra plants grow rapidly and can reach a height of 2.5-3.5 meters at 6 months of age, and 3-5 meters tall in the first year on adequate land [2]. In Bali area, *C. calothyrsus* was easily found in Baturiti, Tabanan. However, the actual of utilization of *C. calothyrsus* is very limited. Utilization of *C. calothyrsus* such as firewood, plant protection, soil conservation, green manure, bee feed, and high quality of forage [1]. The ethanolic extract of 80% of *C. calothyrsus* leaf contained phytoestrogens [3].

Pharmaceutical extraction methods involves the separation of medicinally active portions of plant tissues from the inactive (inert) components by using selective solvents. During extraction, solvents diffuse into the solid plant material and solubilize compounds with similar polarity [4]. Phytoestrogens contain three main compounds i.e. isoflavones, lignins, and coumestants. Phytoestrogens are compounds that have structures and functions similar to estrogen hormone. Therefore phytoestrogens are able to support the presence of estrogen in the body [5]. Estrogen have
an important role in the early functioning of the reproductive organs at the age of puberty characterized by estrus and ovulation [6]. Estrogen hormone deficiency can cause reproductive disorders in women. Recently there are still many cases of women who lack of estrogen hormone, however, there are phytoestrogens which are source of estrogen from outside the body (exogenous estrogen) [7]. Therefore, a formulation of phytopharmaceutical is needed.

Requirements should be done to prepare the phytopharmaceutical formulation are phytochemical screening and standardization of raw materials. The purpose of standardized extraction procedures for crude drugs (medicinal plant parts) is to attain the therapeutically desired portions and to eliminate unwanted material by treatment with a selective solvent known as menstrum. The extract obtained after standardization, may be used as medicinal agent in the form of tinctures or fluid extracts or further processed to be incorporated in any dosage form such as tablets and capsules. These products contains complex mixture of many medicinal plant metabolites i.e. alkaloids, glycosides, terpenoids, flavonoids and lignans [4]. Moreover, phytochemical screening can provide an overview of chemical compounds of the class of compounds contained in C. calothyrsus leaf extracts to their activity.

2. Materials and methods

2.1. Procedures
The research was conducted at Udayana University by using digestion method to extract, standardize and undergo the phytochemical screening.

2.2. Preparation of Sample
C. calothyrsus leaves were washed with water, then dried naturally (6 to 7 days). The leaves are separated from the stems and twigs, we used only the fresh dark-green leaves. C. calothyrsus leaves that have been separated were blend to become powder and sifted using mesh sieve number 60. The powder was oven-dried at 40-50°C overnight. Dry powder was measured until the water content reached less than 10%. The dry powder then stored in a shielded light container [8].

2.3. Extraction of C. calothyrsus Leaves
C. calothyrsus leaves extraction were done through two main processes i.e. defatting process and extraction process. Defatting process by immersing 500 g of powder into solvent of 2500 mL N-Hexane. Defatting was done at room temperature for one day. The maceration of defatting then immersed with 2500 ml of N-Hexane 2 times repetition. Maceration of defatting continues to the extraction process used digestion method with 80% ethanol solvent as much as 2500 ml for 2 hours, then the resultant pulp was filtered. The dregs were digested again with 2 repetitions for 2 hours each. The extracted results and the resulting maceration were collected, then evaporated and continued drying with oven at 40°C.

2.4. Phytochemical Screening of C. calothyrsus Extract

2.4.1. Detection of Flavonoids. Wilstatter Test: The sample (100 mg) was dissolved in a hot methanol and added 0.1 grams of Mg powder and 5 drops of concentrated HCl [9].

2.4.2. Detection of Alkaloids. Mayer Test: The sample (100 mg) was dissolved in 10 mL of solvent. The sample was filtered, filtrate (2 mL) plus 1 mL of Meyer reagent. The formation of yellow deposits indicated the presence of alkaloids [4].

2.4.3. Detection of Tannins. Iron (III) Chloride Test: The sample (100 mg) was dissolved in 10 mL of solvent. The sample was filtered, the filtrate (2 mL) was added with 1 mL FeCl3 3%. The presence of blackish sediment indicated the presence of tannins [9].
2.4.4. Detection of Saponins. Froth Method: The sample (100 mg) was dissolved in 10 mL water and filtered. The filtrate is inserted into the test tube, then shaken. The presence of a stable foam indicated the presence of saponins [4].

2.4.5. Detection of Phytosterol. Libermann Burchard's Test: 50 mg of sample was dissolved in chloroform then filtered. The filtrate plus anhydrous acetic acid is then heated and cooled. Concentrated sulfuric acid was added through the walls of the tube slowly, when a brown ring is formed, it indicated the presence of steroids [4].

2.5. Standardization of C. calothyrsus Powder

2.5.1. Organoleptic Powder. The organoleptic parameter was observed by using the five senses in describing the shape, color, smell and taste.

2.5.2. Determination of Shrinkage Drying of Powder. Determination of drying shrinkage of C. calothyrsus powder used a gravimetric method. The weighing bottle and the lid were heated at 105°C for 30 minutes, cooled in a desiccator, then towered. Approximately 1 gram of C. calothyrsus leaf powder were put in a weighing bottle, flattened by shaking the bottle up to a layer of thickness of approximately 5 to 10 mm. Then it dried in an oven at 105°C for 30 minutes with the bottle kept open. Further it was cooled in a desiccator in a closed bottle state and weighed. The drying and weighing were repeated until there was a constant weight, i.e. the difference between the first and the next weighing were not more than 0.25% [10]. The value of the drying shrink would be the same as the value of water content in the powder of C. calothyrsus leaves.

2.6. Standardization of C. calothyrsus Extract

2.6.1. Organoleptic Extract. The organoleptic parameter was observed by using the five senses in describing the shape, color, smell and taste.

2.6.2. Determination of Water Content of Extract. The determination of water content from 80%ethanolic extract of C. calothyrsus leaves did not contain essential oil so that the determination of water content was equal to the determination of drying shrinkage. The weighing bottle and the lid were heating at 105°C for 30 minutes and cooled in a desiccator, then towered. Weighed approximately 1 gram of ethanol extract of C. calothyrsus leaves in a weighing bottle which has been turreted. The extract was flatten by shaking the bottle up to a layer of thickness of approximately 5 mm to 10 mm. Then dried in an oven with the bottle open at 105°C for 30 minutes to constant weight, this step was repeated 2 times. The extract was cooled in a desiccator with the weighing bottle weighed further. The drying and weighing were repeated until the difference between the first and the next weighing was not more than 0.25% [10].

2.6.3. Determination of Total Ash Content. Total ash content was weighed carefully as much as 2 to 3 grams of ethanolic extract of C. calothyrsus leaves. The samples were inserted into a crucible silica which was first performed by annealing and riding. Slowly grew until the charcoal is exhausted, then it was cooled and weighed. If the char was not exhausted then we should add hot water and filtered through an ash-free filter paper. Filter paper and residual filtration were permitted in the same crucible. The filtrate was introduced into the crucible, then evaporated and permitted to a fixed weight. The total ash content was calculated on the sample weight and expressed in % w/w.

2.6.4. Determination of Ash Content Not Soluble Acid. The determination of acid soluble ash content was adapted from Herbal Pharmacopoeia Indonesia. The acid soluble ash content was determined by boiling the ash obtained from the determination of the total ash content by 25 mL of dilute chloride acid P for 5 minutes. The insoluble part of the acid was collected, filtered through an ash-free filter
paper, and washed with hot water. The results obtained were then permitted in crucibles to a fixed weight. The acid soluble ash content was calculated against the weight of the test material and expressed in % w/w.

2.7. Antioxidant Activity Analysis of DPPH Scavenging Extract in Vitro

The extract sample with a concentration of 1000 ppm was taken as much as 1 ml and then put into a test tube. DPPH 7.5765 10^-5 mol/L solution in ethanol was made. One ml of the solution was taken then added 3 ml of distilled water was added at the absorbance level with a wavelength of 516 nm will get an absorbance of 0.8. One ml of antioxidant sample was taken then 3 ml of DPPH solution (1,1-diphenyl-2-picrilhidrazil) was added. The test tube then was vortexed and left in the open air for 20 minutes. The mixture was treated with a spectrophotometer at a wavelength of 516 nm. Determination of IC50 value or the ability of antioxidants to capture DPPH free radicals was done using a curve of standard solutions with gallic acid concentrations of 0, 50, 100, 150, 200, 250, and 300 ppm respectively. When the absorbance results are obtained, it is sought by connecting the absorbance of the sample using a standard curve to obtain the concentration of the sample [11].

3. Results and Discussion

3.1. Extraction

In this study, we used Calliandra calothyrsus Meissn. leaves. The samples, C. calothyrsus leaves, were separated from the trunk and cleaned with water. After cleaning, leaves were dried with aerated to reduce water content. After that, the leaves were blended with a blender to become a powder. The purpose of making powder was to facilitate the extraction process so that the solvent can be absorbed maximally into the powder then the extraction results can be optimal. Extraction generally differentiated into maceration, infundation, percolation, steam distillation and often modification. Our study experiment used a modification method. For example in the maceration process, the rest would cause a concentration profile that was the equilibrium of mass transfer from the cell into the solvent and from the solvent into the cell. This can be prevent by stirring or heating [12].

The maceration can be modified into digestion, by using a weak heating at a temperature of 40-500°C and stirring. The digestion methods has the advantage that the fluid ability of the solvent to dissolve the desired substance will become larger and has the same effect as stirring [13]. The solvent used in this extraction process was ethanol. The polarity level of a solvent greatly determined the extraction and activity contained in the extract. Some of the criteria presumed to be good as solvents for use in plant phenolic extracts i.e. a constant dielectric (30-80) and a boiling point of 75-1000°C. Ethanol is a solvent which has a dielectric constant of 24.30 and a boiling point of 780°C [14]. From the extraction of approximately 500 grams C. calothyrsus leaf powder obtained 80% ethanol extract of C. calothyrsus leaves of 109.249 grams. Therefore, yield of 80% ethanol extract of C. calothyrsus leaves was 21.85%.

3.2. Phytochemical Screening

Phytochemical screening was performed to identify the compounds contained in the leaf extract of C. calothyrsus qualitatively. The results showed that leaf extract of C. calothyrsus positively contain flavonoids, alkaloids, tannins, saponins, and phytosterol (Table 1).

The identification of flavonoids using the wiltstater test showed an orange color which means positive for flavonoids. The magnesium and hydrochloric acid in the Wiltstater test reacted to form bubbles of H2 gas, while the concentrated Mg and HCl metals of this test served to reduce the benzopiron nuclei present in the flavonoid structure to form the color change to red or orange. If a plant contained a flavonoid compound, it would form a flavilium salt when the addition of Mg and HCl are red or orange [9].

Alkaloids may be attracted to ethanol solvents because alkaloid compounds are polar. The positive reaction occurring in the alkaloid test is the precipitate on the Mayer reagent [15]. The precipitate of the Mayer reagent occurs in the complex bonds between potassium and alkaloids, in the
manufacture of Mayer reagents, a solution of mercury (II) chloride plus potassium iodide will react to form a red sediment of mercury (II) iodide [16].

Tannins belong to the phenolic class, the framework of an aromatic ring containing hydroxyl groups (-OH) [17]. The color change occurred when the addition of FeCl3 reacted with one of the hydroxyl groups in the tannin compound, the addition of FeCl3 in the test extract yields a blackish-green color that indicated a tannin compound [18].

Saponins are generally in the form of glycosides so they are generally polar and surface active compounds that can cause foam if shaken in water. The foam on the test occurs because saponins have polar and non-polar groups that will form micelles. The micelles make the polar group face outward and the nonpolar group face inward and it looks like foam [15].

The identification of phytosterols by the Lieberman-Burchard test (concentrated acetic anhydride-H2SO4) gave the blue-green color. Identification of phytosterol of the extract showed positive results with the formation of brown ring at the limit of the solution when added with H2SO4 and looked green when the solution was dropped on the plate drops. The discoloration was due to oxidation of the phytosterol compounds by the formation of the conjugated double bond [9].

Table 1. Phytochemical screening

| Flavonoids  | +  |
|-------------|----|
| Alkaloids   | +  |
| Tannins     | +  |
| Saponins    | +  |
| Phytosterol | +  |

Phytochemical screening of the extracts was performed to give the release of classes of compounds contained in the extract. Based on the results of the study, the leaf extract of *C. calothyrsus* contains flavonoids, alkaloids, tannins, saponins and phytosterols qualitatively.

3.3. Standardization

After obtained the extract and then done standardization test of *C. calothyrsus* leaf extract. Standardization testing of this extract includes testing of specific and non specific parameters. In determining the value of standardization is required reference indicating that the extract meets the requirements that have been set. In *C. calothyrsus* leaf extract there was no official standardization reference published by the Ministry of Health nor from other sources, so we used the requirements of extracts in general which include non-specific parameters as a reference of this study.

Organoleptic examination is the first step in providing objective recognition as a basis for testing samples physically during storage that may affect quality and support the identification of further extract [19]. The results of organoleptic examination showed the characteristics that were shown in Table 2. and Table 3.

Table 2. Organoleptic of *C. calothyrsus* powder

| Form          | Solid, Powder, Dry |
|---------------|--------------------|
| Color         | Green              |
| Smell         | Aromatic           |
| Flavour       | Bitter             |

Table 3. Organoleptic of *C. calothyrsus* extract

| Form          | Solid, Dry, Thick |
|---------------|------------------|
| Color         | Dark Brown       |
| Smell         | Aromatic         |
| Flavour       | Bitter           |
The drying shrinkage parameter is the measurement of residual extract after drying at 105°C for 30 minutes or until a constant weight is expressed as proximal value (Table 4). In special cases (if the material does not contain volatile oil or volatile and the residual organic solvent evaporates) is identical to the moisture content. Allowed values or range of water content related to purity and contamination. Parameter moisture content is the measurement of water content within the material, which aims to provide a minimum or range of about the amount of water content in the material [8].

Based on the results in Table 4, shrinkage drying of *C. calothyrsus* leaf powder obtained 8.3477 ± 0.1034% w/w or less than 10% so it can it was concluded that the leaf extract of *C. calothyrsus* meets the standard requirements. Water content in the extract was less than 10%, thus can minimize the growth of mould and produce endurance storage, and quality of powder was still good. In the other hand, water content of the extract was 5.2610% ± 0.2022 w/w (Table 5). This result has been in accordance with the requirement that the water content for the viscous extract was between 5-30%. This aims to avoid the rapid growth of fungi in the extract.

**Table 4. Shrinkage Dried of Powder**

| No. | Shrinkage Dried of Powder (X) g | % Shrinkage Dried of Powder | Standard Deviation (SD) | % Relative Standard Deviation (RSD) |
|-----|--------------------------------|-----------------------------|-------------------------|-----------------------------------|
| 1.  | 0.9188                         | 8.3948                      | 0.1034                  | 1.2385                            |
| 2.  | 0.9245                         | 8.2291                      |                         |                                   |
| 3.  | 0.9170                         | 8.4191                      |                         |                                   |
| Average |                                | 8.3477                      |                         |                                   |

**Table 5. Water Content of Extract**

| No. | Water content of Extract (X) g | % Shrinkage Dried Powder | Standard Deviation (SD) | % Relative Standard Deviation (RSD) |
|-----|--------------------------------|--------------------------|-------------------------|-----------------------------------|
| 1.  | 0.9773                         | 5.0704                   | 0.2022                  | 3.8430                            |
| 2.  | 0.9531                         | 5.2396                   |                         |                                   |
| 3.  | 0.9551                         | 5.4731                   |                         |                                   |
| Average |                                | 5.2610                   |                         |                                   |

**Table 6. Total ash content of extract**

| No. | Total ash content of Extract (X) g | % Total ash content extract | Standard Deviation (SD) | % Relative Standard Deviation (RSD) |
|-----|-----------------------------------|-----------------------------|-------------------------|-----------------------------------|
| 1.  | 0.0454                            | 4.54                        | 0.0220                  | 0.4837                            |
| 2.  | 0.0457                            | 4.57                        |                         |                                   |
| 3.  | 0.0452                            | 4.52                        |                         |                                   |
| Average |                                | 4.54                        |                         |                                   |

**Table 7. Ash Content Not Soluble Acid**

| No. | Ash Content Not Soluble Acid of Extract (X) g | % Ash Content Not Soluble Acid | Standard Deviation (SD) | % Relative Standard Deviation (RSD) |
|-----|-----------------------------------------------|-------------------------------|-------------------------|-----------------------------------|
| 1.  | 0.0049                                        | 0.4                           | 0.0115                  | 2.2941                            |
| 2.  | 0.0051                                        | 0.5                           |                         |                                   |
| 3.  | 0.0051                                        | 0.5                           |                         |                                   |
| Average |                                | 0.5                           |                         |                                   |
The ash content parameter is the material heated at a temperature where the organic compound and its derivatives are destructed and evaporated. Thus, the mineral and inorganic elements, which provide an overview of internal and external mineral content derived from the initial process until the formation of the extract. These ash parameters are related to the purity and contamination of an extract [8]. The determination of ash content aims to provide an overview of internal and external mineral content. Extracts were heated at high temperatures to make organic compounds and its derivatives became destructible and evaporated until the remaining minerals and inorganic elements were present.

We obtained 4.54 ± 0.220% of ash content in the extract, while acid unsaturated ash content was 0.5 ± 0.115%. This showed that the remaining mineral and inorganic elements in the extract of 4.54 ± 0.220%, and the element is not soluble in the acid of 0.5 ± 0.115% [20]. Table 6 and Table 7 showed the ash content of extract and ash content not soluble acid. Standardization on powder and extract has met the criteria of Indonesian Herbal Pharmacopoeia in general. In the future, we need a similar study to explore other potential compounds of C. calothyrsus leaf extract.

3.4. Antioxidant Activity
The results of the measurement of DPPH free radical capture activity from C. calothyrsus leaf extract in this study showed a 50% Inhibition Concentration (IC) while the antioxidant capacity was indicated by the GAEAC (Gallic Acids Equivalent Antioxidant Capacity) value in ppm. The IC 50% value of the 29.6 mg sample of C. calothyrsus leaf extract was 0.156 mg/ mL or 156 ppm which means that the extract concentration of 0.156 mg/ mL was able to reduce DPPH 0.1 mM free radicals by 50%. The small IC50 value of C. calothyrsus leaf extract from this research showed the strength of this extract as a DPPH radical catcher, proved that this extract was potential as an antioxidant. A total of 29.63 mg of C. calothyrsus leaf extract contained flavonoids in the amount of 13120.15 mg/ 100 g QE and contained polyphenols of 5121.89 mg/ 100 g GAE. C. calothyrsus leaves, which are high in phenolic compounds, make the biggest contribution to the antioxidants found in C. calothyrsus leaf extract.

4. Conclusion
The leaf extract of C. calothyrsusin this study contained flavonoids, alkaloids, tannins, saponins and phytosterols qualitatively. C. calothyrsus flavonoid content gives the potential as phytoestrogen source. Standardization on powder and extract has met the criteria of Indonesian Herbal Pharmacopoeia in general. The antioxidant activity value of C. calothyrsus leaf extract in this research showed that C. calothyrsuswas potential as an antioxidant.

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