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Phytochemical Screening, Total Flavonoid Content, and Total Phenolic Content of Ethanol Extract of the Indonesian Fern Selaginella Plana

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ABSTRACT
Selaginella plana (*Cakar ayam*) is one of the ferns that grow widely in Indonesia and has been used by the community as food, ornamental plants and traditional medicines to treat wounds, respiratory infections, liver disorders, urinary tract infections, fractures, rheumatism, cancer and increase the body immunity. Research on the chemical constituents of this plant has not been widely reported. This study aimed to describe secondary metabolites from ethanol extract of *S. plana* and determine total flavonoid and total phenolic content. Secondary metabolites content was determined using phytochemical tests, total phenolic and total flavonoid content were determined using UV-Vis spectroscopy. The results showed that the ethanol extract of *S. plana* contained secondary metabolites of steroids, saponins, phenolics, flavonoids, alkaloids, and tannins. Based on the results of determination using the UV-Vis spectroscopy, it was known that the ethanol extract of *S. plana* has total flavonoid and a total phenolic content of 35.725 ± 0.110 mg QE/ g sample mass and 36.363 ± 0.143 mg GAE/ g dried extract, respectively.

Keywords*: Selaginella plana, Phytochemical screening, Total phenolic content, Total flavonoid content.*

1. INTRODUCTION

Selaginella plana (cakar ayam) is one of the ferns which becomes Indonesia's biological wealth [1]. This plant is spread in Southeast Asia, in Java it grows to a height of approximately 750 above sea level, generally on the moist and protected riverbank and on the slopes of ravines. S. plana has been known and used by society as food (vegetables), ornamental plants, and traditional medicines. It has been used for postpartum care, treating wounds, treating bleeding, menstrual disorders, increasing the body immunity, as well as a postnatal tonic [2-4]. Traditionally, *S. plana* has been used in the treatment of several diseases such as cancer, respiratory infections, liver disorders, urinary tract infections, fractures and rheumatism [5].

Chikmawati, et al. reported that *Selaginella* species on the island of Java was found to have secondary metabolites of the flavonoid group, with the highest content of biflavonoid compounds [6]. Biflavonoid compounds from *Selaginella* genus have various bioactive properties, including antioxidants, anticancer, antibacterial, antiviral, antifungal, antimalarial, and anti-inflammatory properties [7-10].

As a phenolic compound, biflavonoid had been extracted from several fern species in Selaginellaceae family. Amentoflavone was extracted from *S. tamariscna* and *S. bryopteris*, [11,12]. From *S. lepydophylla*, robustataflavone, ginkgetin, kayaflavone and podocarpus flavone A had been extracted [13]. Meanwhile, hinokiflavone had been reported from *S. crysocaulos* and *S. bryopteris* [12][14]. Research on the content of phenolic compounds and flavonoid content in the fern *S. plana* has never been reported. This paper will report on results of the phytochemical screening

of ethanol extract from *S. plana* and determining total flavonoid and total phenolic content.

2. METHODS

2.1. Material

The dried powder of *S. plana* aerial parts, ethanol (80%, p.a.), methanol, acetic anhydride, concentrated sulfuric acid, hydrochloric acid (1 M, 2 M, concentrated), magnesium ribbon, $FeCl_3$ (1%, 5%), Mayer reagent, Dragendorff reagent, Wagner reagent, gallic acid (Merck), Folin-Ciocalteu reagent (Merck), Na_2CO_3 7.5%, quercetin (Sigma), $AlCl_3$ 10%, amonia, sodium acetate 1 M.

2.2. Instruments

Spectrophotometer UV-Vis (Shimadzu UV-1800), magnetic stirrer (Heidolph), rotary vacuum evaporator (Buchi R-300), oven (Heraeus ST-5042), analytical balance (Advanturer Ohaus), vacuum pump (Gast DOA-P-504-BN), water bath (Memmert), freeze dryer (Martin Christ Alpha 1-2 LDplus), vortex mixer, volumetric flask, beaker glass, Buchner funnel, micropipette, test tube, drip plate, spatula, maceration vessel.

2.3. Research procedures

2.3.1. Collection and preparation of sample

Samples of *S. plana* were collected from the Kletak forest, Nongkojajar, Pasuruan, East Java. Before the further investigation, the sample was identified at LIPI Botanical Garden, Purwodadi, East Java. Sample was cleaned of attached dirt, then dried at room temperature for 12 days. The dried sample was grinded into a fine powder ready for extraction [15].

2.3.2. Extraction

Dried powder of *S. plana* aerial part (1000 g) was macerated with ethanol 80% for 3 x 24 hours. Then filtered using a Buchner funnel. The ethanol extract obtained was evaporated in vacuum using rotary vacuum evaporator, resulted the concentrated extract. It was dried in freeze dryer to produce dried extract [15].

2.3.3. Phytochemical screening

2.3.3.1. Triterpenoid and steroid test (Liebermann-Burchad test)

About 1 mg of ethanolic extract was placed on a drip plate, added 3 drops of acetic anhydride and stirred with a spatula until dissolved. Then one drop of concentrated sulfuric acid was added to the mixture [16].

2.3.3.2. Phenolic test

About 1 mg ethanolic extract was placed on a drip plate, added 5 drops of methanol, then stirred with spatula until dissolved. Then 3 drops of 5% $FeCl_3$ solution in ethanol was added into mixture [16].

2.3.3.3. Flavonoid test (Shinoda test)

About 1 mg of ethanolic extract was placed on a drip plate, added 5 drops of methanol, then 3-4 small pieces of Mg ribbon and 2 drops of concentrated HCl were added to the mixture [17].

2.3.3.4. Alkaloid test

A total of 4 mg of ethanolic extract was dissolved with 3 mL of methanol and 5 mL of ammonia to pH 8-9, then the mixture was filtered. The filtrate was added with 2 mL of 2 M HCl solution. After the mixture was shaken, 5 drops of the top layer were added into 4 test tubes. The solution in tube 1 was used as a blank, solution in tube 2, 3, and 4 was added one drop of Mayer, Wagner, and Dragendorff reagents, respectively [16].

2.3.3.5. Saponin test

About 1 mg ethanolic extract was put into a test tube, then added 5 mL of distilled water, shaken for 1 minute. If foam appeared, 2 drops of 1 M HCl solution was added. If foam with height of 1-3 cm did not disappear or lasted for 10 minutes, sample contained saponins [16].

2.3.3.6. Tannin test

About 1 mg of ethanolic extract was placed on a drip plate, added 5 drops of methanol, stirred with a spatula until dissolved. Then 2-3 drops of 1% $FeCl_3$ solution were added. Appearance of blue, green, or brownish green color indicated the presence of tannins [17].

2.3.4. Determination of total phenolic contents

A total of 1.0 mL of the extract solution was pipetted from the stock solution (1000 ppm) was put in a test tube, then added 1.5 mL of Folin Ciocalteau reagent (10% = 1:10). After being allowed to stand for 3 minutes, 1.2 mL of 7.5% Na_2CO_3 was added, mixed for 3 seconds using a vortex mixer. The mixture was allowed to stand for 30 minutes, then its absorbance was measured by UV-Vis spectroscopy

at a maximum absorption wavelength of 763 nm. The concentration of phenolic compounds in the extract was determined using a standard gallic acid curve with various concentrations of 10, 12, 14, 16, 18, and 20 ppm. The content of phenolic compounds was expressed as milligrams of gallic acid equivalent (GAE)/gram dried extract [18].

2.3.5. Determination of total flavonoid contents

A total of 0.5 mL of 2000 ppm extract solution was put in a test tube, then 0.1 mL of 10% aluminum (III) chloride, 0.1 mL of 1 M sodium acetate and 2.8 mL of distilled water were added. After being incubated for 30 minutes. The absorbance was measured using a UV-Vis spectrophotometer at a maximum wavelength of 433 nm. The total flavonoid content of the ethanol extract of the fern *S. plana* was determined based on the standard curve of quercetin with various concentrations of 20, 30, 40, 50, and 60 ppm. The total flavonoid content was expressed as milligrams of quercetin equivalent (QE)/gram of dried extract [19].

3. RESULTS AND DISCUSSION

3.1. Extraction of S. plana aerial part

The dried powder of *S. plana* aerial part (1000 g) was macerated using ethanol 80% as a solvent for 24 hours and repeated three times. Evaporation of the solvent in vacuo, followed by drying at freeze drier for 16 hours, yielded the dark green solid (49.14 g).

3.2. Phytochemical screening

Phytochemical screening was carried out to determine the group of secondary metabolites contained in the ethanol extract of the fern *S. plana*. The tests carried out included steroids-triterpenoids test, phenolic test, flavonoids test, alkaloid test, saponin test, and tannin test.

Steroid-triterpenoid test was carried out using Liebermann-Burchard reagent. Ethanol extract of *S. plana* after being dissolved with acetic anhydride and dripped with concentrated sulfuric acid produced a bluish-green solution. The appearance of blue color in the test with the Liebermann-Burchard reaction indicated that the sample contained steroid compounds [16].

Phenolic test was carried out using ferric chloride reagent with a concentration of 5% in ethanol. The ethanol extract of *S. plana* after being dissolved in methanol, then dripped with 5% ferric chloride solution turned out to be a purple solution. The appearance of green, blue, or purple color indicates that the sample contains phenolic compounds. These colors occur as result of formation of colored complex ions between ferric ions (Fe^{3+}) and phenolic ions in phenolic compounds [16,20].

In this study, flavonoid test was conducted using Shinoda test. After being dissolved in methanol, 3-4 pieces of magnesium ribbon were added to the ethanol extract of *S. plana*. After adding 1 M hydrochloric acid solution, a yellow, orange, blue, or red colored solution was formed indicating the presence of flavonoid compounds in the ethanol extract of *S. plana*. The color occurred was result of the formation of colored complex ions between magnesium ions (Mg^{2+}) and phenolic ions in phenolic compounds [17].

The alkaloid test was carried out using Mayer, Wagner and Dragendorff reagents. The ethanol extract of *S. plana* after being tested using the three reagents showed positive results because it showed white, brown and orange precipitates after being added with Mayer, Wagner and Dragendorff reagents, respectively [16,20].

The test for the presence of saponin compounds in the ethanol extract was conducted using Forth test. A number of *S. plana* ethanolic extract was dissolved in water and then heated. It turns out that a stable foam was formed. These results indicated that the extract contained secondary metabolites of the saponin group [16,20].

In this study, the tannin test was carried out with 1% ferric chloride reagent in ethanol. Ethanol extract solution of *S. plana* after being reacted with 1% ferric chloride solution formed a greenish-blue solution. According to Edeoga, *et al* the formation of blue, green, or brownish green color indicated the presence of tannin compounds in the sample. Thus, the ethanol extract of *S. plana* contained compounds belonging to the tannin group. The color was produced due to the formation of a complex between ferric ions and phenolic ions from tannin compounds [17]. Results of the phytochemical tests above are briefly presented in the Table 1.

Table 1. Phytochemical test of ethanol extract of *S. plana*

No.	Phytochemicals	Test methods	Results (Indicating positive test)	
1	Steroids and triterpenoids	Liebermann-Burchard test	A bluish green color	+ (Steroids)
2	Phenolic	Ferric chloride test	A purple color	+
3	Flavonoids	Shinoda test	A orange color	+
4	Alkaloid	- Mayer test - Dragendorff test - Wagner test	- A white precipitate - A orange precipitate - A chocolate precipitate	+
5	Saponins	Forth test	Persistent foam	+
6	Tannins	Ferric chloride test	A greenish blue color	+

Phytochemical test showed that the ethanol extract of *S. plana* contained secondary metabolites of steroids, phenolics, flavonoids, alkaloids, saponins, and taninis. The presence of phenolics and flavonoids compounds supports the results of previous studies which stated that the ferns of the genus *Selaginella* from Java contain flavonoid compounds, especially biflavonoids [6]. Beside that it also supports the potential use of *S. plana* as an Indonesian medicinal plant.

3.3. Total phenolic content

The total phenolic content in the ethanol extract of the fern *S. plana* was determined by the spectroscopic method using Folin-Ciocalteu reagent. The reagent will be reduced by phenolic compounds in alkaline condition to produce a blue phosphotungstate-phosphomolybdate complexes. Alkaline condition is needed to convert the phenolic hydoxyl group into phenolic ions due to proton dissociation [18]. Based on measurement of the absorbance of the standard solution of gallic acid, it was obtained the standard curve presented in Figure 1.

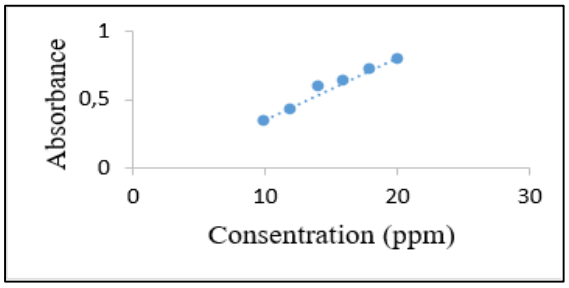

Figure 1 Standard curve of gallic acid solution.

The total phenolic content was determined based on the standard gallic acid curve determined by UV-Vis spectroscopy at a wavelength of 763 nm. From the standard curve (**Fig 1**), it was obtained the regression equation Y = 0.462X – 0.113 with R^2 = 0.9768. The results of determining the total phenolic content of the ethanol extract of the fern *S. plana* 1000 ppm using the spectroscopic method from three measurements obtained an absorbance value of 0.558; 0.562 and 0.557. Based on the gallic acid standard curve and taking into account the dilution factor, the total phenolic content in the ethanol extract of the fern *S. plana* was 36.310 + 36.526 + 36.255, respectively, with an average value of 36.364 ± 0.143 mg gallic acid equivalent (GAE)/gram dried extract.

3.4. Total flavonoid content

Total phenolic content in ethanol extract of fern *S. plana* was determined by an aluminium chloride reagent. This reagent will form yellow complexes with the carbonyl group of flavonoids at the C-4 with an adjacent hydroxyl group at the C-3 (flavonols) or C-5 (flavonols and flavone) and provide specific absorption in UV-Vis spectroscopy [19,21]. The total flavonoid content was determined based on the standard curve of quercetin as measured by UV-Vis spectroscopy at a wavelength of 433 nm. Based on the measurement results of the absorbance of the standard solution of quercetin, the standard curve was obtained which was presented in Figure 2.

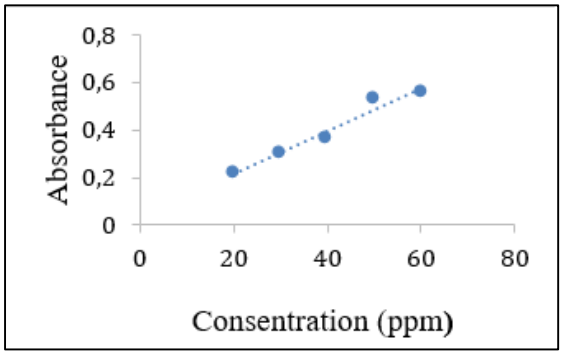

Figure 2 Standard curve of quercetin solution.

The linear regression equation obtained from the standard curve was Y = 0.0091X + 0.0298 with R^2 = 0.9649. The results of the determination of the total flavonoid content of the ethanol extract of the fern *S. plana* 1000 ppm using the spectroscopic method from three measurements obtained an absorbance value of 0.678; 0.680 and 0.682. Based on the standard curve of quercetin, the total phenolic content in the ethanol extract of the fern *S. plana* were 35.615; 35.726; 35.835, respectively, with an average value of 35.725 ± 0.110 mg of quercetin equivalent (QE)/gram dried extract. The total phenolic content and total flavonoid content of *S. plana* correspond with the results of phytochemical tests that it contains phenolics, flavonoids and tannins. The plant containing phenolics and flavonoids compounds such as *S. plana* has the potential to be used as antioxidants [18-20].

4. CONCLUSION

The study concludes that the ethanol extract of S. plana contains secondary metabolites of steroids, saponins, phenolics, flavonoids, alkaloids, and tannins. It was found to have total flavonoid and total phenolic content of 35.725 ± 0.110 mg QE/ g dried extract and 36.363 ± 0.143 mg GAE/ g dried extract, respectively.

AUTHORS' CONTRIBUTION

Suyatno Sutoyo: conceptualizing the research and writing the manuscript. Amaria, I Gede Made Sanjaya and Nurrulhidayah Ahmad Fadzlillah: developing research methods and analyzing data. Rusly Hidayah and Devy Puspita Sari: presenting data and doing data analysis.

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