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Quantitative Analysis of Flavonoid Content, Total Phenolic and Toxicity Test of Extract *Smilax rotundifolia* Leaves From Papua

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ABSTRACT

This study investigates Bungkus leaf (*Smilax rotundifolia*), a well-known plant in Papua commonly referred to as the three-finger leaf. Indigenous Papuans traditionally use this plant to enlarge genitals, buttocks, and breasts, as well as to treat syphilis. The plant is known to contain flavonoid and phenolic compounds with pharmacological activity. This study aimed to determine the total flavonoid and total phenolic content of the 70% ethanol extract of Bungkus leaf. The research began with simplicia preparation, followed by extraction using 70% ethanol. Qualitative tests, including phytochemical screening and thin-layer chromatography (TLC), were conducted, followed by quantitative analysis using UV-Vis spectrophotometry. The results showed total flavonoid content of 54.696 mg QE/g ± 0.565 and total phenolic content of 32.908 mg GAE/g ± 0.263. The study concluded that the total flavonoid content was higher than the total phenolic content. Toxicity testing categorized the extract as toxic, with an LC_{50} value of 442.92 ppm.

Key words: BSLT; S*milax rotundifolia;* spectrophotometry; total flavonoids; total phenolic

INTRODUCTION

Papua is one of the provinces located in Indonesia. This region has rich natural biodiversity, including plants and vegetation. One of the well-known plants in Papua is the leaf wrapping plant (*Smilax rotundifolia*). Ethnopharmacologically, *S. rotundifolia* is widely used as an aphrodisiac, to enlarge genitalia, buttocks, and breasts, as well as to treat syphilis (Jonatan, 2016). The aphrodisiac activity test of *S. rotundifolia* extract on *Rattus norvegicus* showed differences in pregnancy index percentages and significant effects on fertility index values (Wulandari *et al*., 2022).

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The aphrodisiac activity is closely related to the secondary metabolite compounds found in the leaf wrapping plant. Extracts of *S. rotundifolia* are known to contain secondary metabolites such as flavonoids, saponins, alkaloids, tannins, and steroids. Phenolic compounds like trans-resveratrol and trans-3,3′,5,5′-tetrahydroxy-4′-methoxy-stilbene have been successfully isolated from this plant and are believed to possess aphrodisiac activity (Akma *et al.*, 2022).

Polyphenol compounds such as flavonoids and phenolics not only contribute to aphrodisiac activity but also act as antioxidants, anti-inflammatory agents, anticancer agents, cardiovascular protectors, and neuroprotectors (Sunarti, 2021). Flavonoids and phenolics are chemical compounds with conjugated double bonds and chromophore groups, allowing for quantitative analysis of their content using UV-Vis spectrophotometry (Hariana, 2013).

Quantitative analysis of flavonoid and phenolic contents in the leaf wrapping plant extract can assist in a broader pharmacological assessment of the plant. The choice of solvent plays an important role in the extraction process of secondary metabolites. A 70% ethanol solvent is one of the polar solvents commonly used to extract flavonoid and phenolic compounds (Mahfirohtun, 2020).

One of the preclinical tests that can be performed on a plant extract before it is used as a traditional medicine is toxicity testing. The Brine Shrimp Lethality Test (BSLT) is a simple method used to observe the toxicity of extracts or compounds and serves as a screening method for anticancer activity. This method measures the mortality rate of *Artemia salina* Leach larvae caused by the test extract, with results calculated as LC_{50} values (Agustina *et al*., 2017). Therefore, this study aims to determine the total flavonoid and phenolic content of 70% ethanol extracts of *S. rotundifolia* as well as to conduct toxicity tests on the extract.

MATERIALS AND METHODS

Time and location of research

This research was conducted from May to August 2024 at the Pharmaceutical Research Laboratory, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Cenderawasih University, and the Pharmacy Laboratory of the University of Science and Technology Jayapura.

Tools and materials

The equipment used in this study included standard laboratory glassware, sieve no. 80, separating funnel, rotary evaporator, analytical balance, aluminum foil, spot plate, micropipette, filter paper, aerator, 25-watt lamp, magnifying glass, flashlight, maceration container, hatching container for *A. salina* larvae eggs, universal pH indicator, porcelain dish, water bath and UV-Vis spectrophotometer.

Materials used were *bungkus leaves* (*S. rotundifolia*), 70% ethanol, distilled water, Mg powder, 10% $FeCl_3$, gelatin, quercetin, gallic acid, 7.5% Na_2CO_3, 2 N HCl, 1 N NaOH, 1 M CH_3COONa, distilled water, Folin-Ciocalteu reagent, 10% $AlCl_3$, $K_2Cr_2O_7$, H_2SO_4, $CHCl_3$, DMSO, glacial acetic acid, Mayer's reagent, Wagner's reagent, Dragendorff's reagent, and Lieberman-Burchard reagent.

Working procedure

The extraction was carried out using the maceration method, which is advantageous due to its simple procedure and equipment, and its ability to extract thermolabile compounds (Harborne, 1987). After the extract was free of solvent, a phytochemical screening was conducted to identify the secondary metabolite content (Vifta & Advistasari, 2018). Phytochemical screening supports the phytopharmacological evaluation process by providing preliminary information about plants used in traditional medicine.

Preparation of simplisia and extract

Bungkus leaves collected from Skouw Yambe Village, Muara Tami District, Jayapura City, was washed, air-dried and sorted. The sample was blended and sieved using mesh no. 80 to obtain powdered simplicia. A total of 500 g of simplicia was soaked in 2500 mL of 70% ethanol (1:5 ratio) in a closed container for 24 hours with occasional stirring to obtain the first macerate. Remaceration was carried out, and the combined macerates were concentrated using a water bath at 50°C to yield a thick extract. The percentage yield was then calculated.

Ethanol-free test and phytochemical screening

The ethanol-free test was performed by adding 1 mL of $K_2Cr_2O_7$ and 6 drops of H_2SO_4 to the extract, then mixing. The extract was considered ethanol-free if no color change occurred. The ethanol-free extract was then screened for secondary metabolites (phyto-chemical screening).

Qualitative analysis of flavanoids and phenolics

Further identification of flavonoids and phenolics was performed using Thin Layer Chromatography (TLC), with EtOH : EtOAc (5:5) as the mobile phase. $AlCl_3$ was used as the spray reagent for flavonoids, and $FeCl_3$ for phenolics.

Quantitative analysis of total flavanoid content

Steps included the preparation of a quercetin stock solution (1000 ppm), preparation of a standard series (20, 40, 60, 80, and 100 ppm), and a blank solution (3 mL ethanol + 0.2 mL 1 M CH_3COONa + 0.2 mL 10% $AlCl_3$ + aquades). The maximum wavelength was determined in the 350–500 nm range.

Operating time at maximum wavelength was recorded every 5 minutes for 30 minutes. Flavonoid content was determined using UV-Vis spectrophotometry and measured in triplicate using the 1000 ppm extract of *bungkus leaves*.

Quantitative analysis of total phenolic content

Steps included the preparation of a gallic acid stock solution (1000 ppm), preparation of a standard series (20, 40, 60, 80, and 100 ppm), and a blank solution (1.5 mL 10% Folin-Ciocalteu + 4 mL 7.5% Na_2CO_3 + aquadest).

The maximum wavelength was determined in the 600–800 nm range. Operating time at maximum wavelength was recorded every 5 minutes for 30 minutes. Phenolic content was determined using UV-Vis spectrophotometry and measured in triplicate using the 1000 ppm extract of *bungkus leaves*.

Toxicity test

The stages of the toxicity test included: hatching of *A. salina* larvae; preparation of stock solutions (10, 50, 150, 300, 450, 550, 750 and 1000 ppm); observation of larval mortality after 48 hours; LC_{50} analysis by calculating the percentage of larval mortality; and data analysis using linear regression by plotting probit values against the logarithm of the concentration.

RESULTS AND DISCUSSION

Extraction results of *S. Rotundifolia* leaves

Bungkus leaves Leaves from Skow Yambe, Keerom Regency, Papua Province were sorted, dried, and ground into simplicia, then extracted using the cold maceration method. The final result of the extraction process was a thick extract, and the yield percentage was calculated. Using 550 g of simplicia, the yield percentage obtained was 23.81%. This result meets the requirements of the Indonesian Herbal Pharmacopoeia, which states a minimum of 10% (Ministry of Health, 2017).

A study conducted by Vonna *et al.* (2021) showed that the extract yield percentage obtained was 20.1%. One of the key factors influencing yield percentage is the remaceration process,

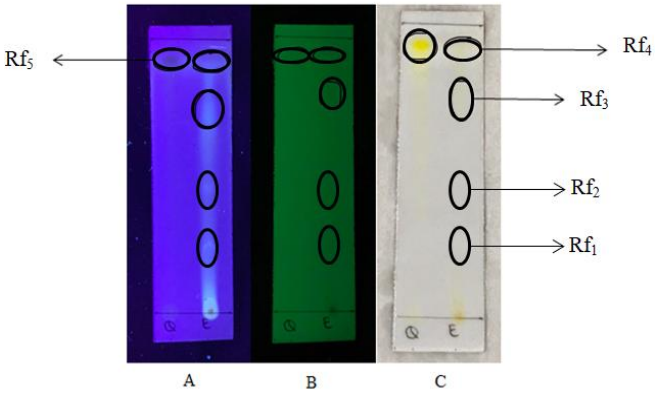

Figure 1. Chromatogram of flavonoid compounds in *S. rotundifolia* ethanolic extract.

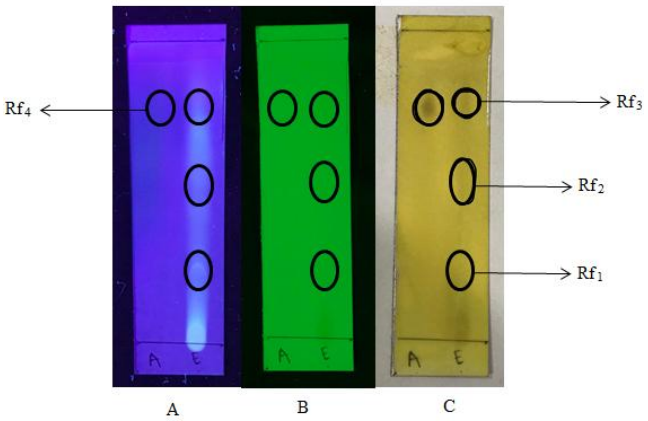

Figure 2. Chromatogram of phenolic compounds in the ethanolic exctact *S. rotundifolia*.

which allows for maximum extraction of secondary metabolite compounds.

Results of qualitative analysis

Qualitative analysis using ethanol: ethyl acetate (5:5) eluent for identification of flavonoid and phenolic compounds. Qualitative analysis was carried out using the eluent Ethanol:Ethyl Acetate (5:5) to identify flavonoid and phenolic compounds. A positive result for flavonoids was indicated by the appearance of yellow spots on the extract sample after being sprayed with $AlCl_3$ reagent, with quercetin used as a reference compound. The yellow color change is due to the reaction of $AlCl_3$ with the hydroxyl and ketone groups in the flavonoid structure, forming a yellow-colored complex (Harborne, 1987).

Based on the chromatogram, the Rf value of quercetin was found to be 0.94, while the Rf values of the ethanolic extract of *S. rotundifolia* leaves were Rf1 = 0.21, Rf2 = 0.42, Rf3 = 0.80, and Rf4 = 0.94. The Rf4 value matched the Rf value of the quercetin reference compound. The extract's Rf values fall within the typical quercetin Rf range of 0.81–0.96, indicating that the leaf extract contains flavonoid compounds, specifically quercetin (Putri, 2023). These results are consistent with those of Putri (2020), who reported an Rf value of 0.94 for quercetin. According to the Indonesian Ministry of Health (2017), the standard Rf value for quercetin is 0.86.

Flavonoid compounds have antioxidant activity, making them beneficial for cancer prevention, protecting cellular structures, anti-inflammatory effects, preventing osteoporosis, and acting as antibiotics (Manopo *et al*., 2023).

A positive result for phenolic compounds was indicated by the appearance of black spots on the extract sample after being sprayed with $FeCl_3$ reagent, with gallic acid used as the reference compound. This result is consistent with the theory that a sample is considered positive for phenolic content if the spot turns black after being sprayed with $FeCl_3$ reagent. The appearance of the black color is caused by the interaction between iron ions (Fe^{3+}) and hydroxyl (-OH) groups present in phenolic compounds, forming a complex compound (Harborne, 1987).

Based on the chromatogram, gallic acid was identified with an Rf value of 0.78, while the

Table 1. Phytochemical screening results of *S. rotundifolia.*

No	Compound group	Test results	Description
1.	Alkaloids	+	Formation of reddish-brown precipitate (Wegner)
		+	Formation of white precipitate (Meyer)
		+	Formation of orange-red precipitate (Dragendorff)
2.	Flavonoids	+	Orange-colored solution
3.	Saponins	+	Formation of stable foam
4.	Tannins	+	Greenish-black colored solution
5.	Steroids	-	No change to greenish-blue color
6.	Triterpenoids	+	Formation of brownish ring
7.	Quinones	+	Formation of yellow color

Table 2. Flavanoid content calculation results of *S. rotundifolia*

Replication	Abs measurement	Abs sample	X (mg/mL)	X (mgQE/g)	KTFe (mgQE/g) $\pm$ SD
1	0.375	0.293	0.055	55.342	
2	0.368	0.286	0.054	54.298	54.696 $\pm$ 0.565
3	0.396	0.287	0.058	54.477	

ethanol extract of the bungkus leaves leaves showed Rf1 = 0.20, Rf2 = 0.50, and Rf3= 0.78. The Rf3 value of 0.78 aligns with the Rf value of the gallic acid reference standard. Since the difference between the sample's Rf value and the reference is less than 0.05, and the Rf value of the extract falls within the gallic acid Rf range of 0.75–0.78, the analysis results indicate that the bungkus leaves extract contains phenolic compounds, specifically gallic acid (Putri, 2023). These results are consistent with the study conducted by Saputri (2021), which reported a gallic acid Rf value of 0.76, and with the Ministry of Health (2017) reference Rf value of 0.76.

Phenolic compounds have antioxidant activity, making them potentially useful in the prevention or treatment of degenerative diseases, cancer, premature aging, and immune system disorders (Padamani *et al*., 2020).

Other compounds were also identified in the ethanol extract of bungkus leaves, as evidenced by the results of phytochemical screening. Phytochemical screening is one method used to qualitatively identify the presence of secondary metabolite compounds in natural materials. The 70% ethanol extract of *S. rotundifolia* leaves revealed the presence of secondary metabolite groups including alkaloids, flavonoids, saponins, tannins, triterpenoids and quinones (Table 1).

According to research by Nur *et al*. (2019), phenolic and flavonoid compounds are secondary metabolites found in various plant species. These two types of compounds play important roles in antioxidant activity; the higher the phenolic content, the stronger the antioxidant activity. The presence of flavonoids and phenolics provides many advantages in the development of natural-based medicinal agents, particularly as antioxidant agents. Based on this explanation, the total content

Tabel 3. Results of phenolic from *S. rotundifolia*. content by ethanol

Replikasi	Abs Int.	Abs sample	X (mg/mL)	X (mgGAE/g)	KTFe (mgGAE/g) ± SD
1.	0.338	0.295	0.032	32.620	
2.	0.340	0.297	0.032	32.965	32.908 ± 0.263
3.	0.341	0.298	0.033	33.137	

Tabel 4. Test toxicity of *S. rotundifolia*.

Concentration (ppm)	Log concentration (ppm)	Avg. mortality	Mortality (%)	Probit
0	0.00	0.0	0.0	0.0
50	1.69	0.3	3.0	3.12
100	2.0	1.4	14.0	3.92
150	2.18	2.2	22.0	4.23
200	2.30	2.3	23.0	4.26
250	2.40	3.4	34.0	4.59
300	2.48	4.0	40.0	4.75
500	2.70	5.5	55.0	5.13
750	2.88	6.2	62.0	5.31
1000	3.0	10.0	100.0	8.09

of flavonoid and phenolic compounds in the bungkus leaves extract was determined using UV-Vis spectrophotometry.

Results of quantitative analysis

Total flavonoid content

The initial stage of testing involved determining the maximum wavelength (λ_{max}). This step aimed to identify the wavelength at which a complex is formed between quercetin and $AlCl_3$, resulting in optimal absorbance. This process is essential in chemical analysis using the UV-Vis spectrophotometric method. The obtained λmax was 441 nm.

The operating time (incubation period) was established using a standard series solution of 40 ppm, where stable absorbance readings were observed at the 20th minute. The standard curve was prepared using standard series solutions of 20, 40, 60, 80 and 100 ppm, left for the determined incubation time, and measured at 441 nm.

The total flavonoid content was determined using the spectrophotometric method described by Chang *et al*., (2002). In this method, the bungkus leaves extract was mixed with $AlCl_3$ and potassium acetate, resulting in a yellow-colored solution. This yellow color appears due to the formation of a stable complex between the acid and the ketone group at C-4 or the hydroxyl group at C-3 or C-5 of flavones and flavonols, as illustrated in Figure 1.

Table 2 shows that the average total flavonoid content in the bungkus leaves extract was 54.696 mgQE/g. In a previous study, the average flavonoid content was found to be 59.37 mgQE/g (Rahayu, 2021). Flavonoids are derived from a C6-C3 (phenylpropane) unit originating from shikimic acid and a C6 unit derived from the polyketide pathway. Flavonoids are present in all parts of plants, including leaves, roots, wood, bark, flowers, fruits, and seeds. The main groups of flavonoids include anthocyanins, flavonols, and flavones, which are widely distributed in plants, whereas chalcones, aurones, flavonols, dihydrochalcones, and isoflavones are less commonly found (Haslindha, 2022). Various medicinal plants containing flavonoids have been proven to exhibit multiple bioactivities, including antioxidant, antibacterial, antiviral, anti-inflammatory, antiallergic, and anticancer properties.

Total phenolic

The determination of the maximum wavelength was carried out using a 100 ppm solution, to which 7.5% Folin-Ciocalteu reagent and 1M Na_2CO_3 were added, followed by incubation for 20 minutes (incubation time was determined based on the operating time). The obtained λmax was 733 nm. This result is close to the findings of Susanti and Dewantara *et al*. (2021), who reported the λmax of gallic acid at 760 nm, and the results of Kumalasari *et al*., (2021), who reported it at 766 nm.

The standard curve was prepared using gallic acid at concentrations of 20, 40, 60, 80, and 100 ppm, incubated for the determined operating time. The total phenolic content was determined using the Folin-Ciocalteu reagent. This reagent was chosen because phenolic compounds can react with it by oxidizing phenolate (alkaline salts) or phenolic hydroxyl groups, which in turn reduce the heteropoly acids (phosphomolybdic-phosphotungstic acid) contained in the Folin-Ciocalteu reagent to form a molybdenum-tungsten complex. This complex can then be measured using UV-Vis spectrophotometry.

Table 3 shows that the average total phenolic content in the *bungkus leaves* extract was 32.908 mgGAE/g. A previous study reported an average phenolic content of 35.80 mgGAE/g (Defri, 2022). Quantitative analysis of total flavonoids and phenolics using the UV-Vis spectrophotometry method revealed that the *bungkus leaves* extract contained a higher total flavonoid content compared to its total phenolic content.

According to Ramadhani *et al*., (2019), the flavonoid content was found to be 90.58 mgQE/g, while the phenolic content was 67.41 mgGAE/g, indicating a higher flavonoid level than phenolic content. However, in theory, the phenolic content should be higher than the flavonoid content, since flavonoids are a subclass of phenolic compounds. This discrepancy may be due to the instability of

gallic acid, which can also be influenced by factors such as operating time and the determined wavelength, potentially affecting the accuracy of the results.

Phenolic compounds are characterized by having one or more hydroxyl groups directly attached to a carbon ring. The aromatic ring affects the hydroxyl groups of phenols, making the hydrogen and hydroxyl groups more labile and classifying phenolics as weak acids (Ifadah, 2022).

Toxicity test results

The cytotoxicity test results were processed using LC_{50} (*Lethal Concentration*), which refers to the concentration causing 50% mortality, based on the percentage mortality of *A. salina* larvae at each sample concentration. The LC_{50} value was derived from the average mortality percentage obtained from each replication and then analyzed using probit analysis.

Based on the toxicity test results (Table 4), it was found that the 1000 ppm concentration of the ethanol extract from *S. rotundifolia* leaves caused the highest average mortality of 100%, with a probit value of 8.09. The 70% ethanol extract is considered to have a toxic effect on *A. salina* larvae. According to the study, the cytotoxic activity test on *A. salina* larvae using the Brine Shrimp Lethality Test (BSLT) showed that the extract is highly toxic, with an LC_{50} value of 442.92 ppm. The toxic effect of *S. rotundifolia* leaves is influenced by the secondary metabolite content in the sample.

Furthermore, toxicity testing has also been conducted on *Smilax zeylanica* extract, which showed a 50% mortality rate of *A. salina* larvae at 84 ppm. This level is categorized as toxic and has potential for further anticancer testing (Malge *et al*., 2021).

One of the secondary metabolites involved is flavonoids, which act as respiratory and metabolic toxins, causing rapid death of *A. salina* larvae. Additionally, flavonoids can inhibit taste receptors in the oral region of *A. salina*, causing the larvae to fail to detect food stimuli, leading to starvation and death (Kurniawan & Ropiqa, 2021).

Regarding the mechanism of inhibition on cancer or tumor cells, flavonoids have the potential to inhibit tyrosine kinase receptor activity, which plays a role in cancer cell growth. Moreover, flavonoids can reduce and control cancer pathogenic factors through their antioxidant capabilities (Swasana, 2019).

CONCLUSION

The 70% ethanol extract of *S. rotundifolia* leaves (bungkus leaves) contains secondary metabolites such as alkaloids, flavonoids, saponins, tannins, triterpenoids, and quinones. The total flavonoid content in the leaf extract is 54.696 mg QE/g, while the total phenolic content is 32.908 mg QE/g. The 70% ethanol extract of *S. rotundifolia* leaves has an LC_{50} value of 442.92 ppm, which falls into the toxic category.

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