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Effects of Leaf Maturity and Extraction Method on Phytochemical Fingerprints and HeLa Cell Viability of *Peronema canescens* Jack Leaf Extracts

Merin[1], Rini Muharini[1]*, Firman Shantya Budi[2]
[1]Chemistry Education Study Program, Faculty of Teacher Training and Education, Universitas Tanjungpura.
[2]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Tanjungpura.
Jl. Prof. Dr. H. Hadari Nawawi / Jenderal Ahmad Yani, Pontianak - Kalimantan Barat (78124), Tel. (0561) 739630, Fax. (0561) 739637, Indonesia.

Corresponding author*
rini.muharini@fkip.untan.ac.id

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Abstract

Peronema canescens Jack is an Indonesian endemic plant traditionally used as medicine to treat flu, coughs, colds, fever, and malaria, as well as to support the immune system. This study investigated the influence of extraction technique and leaf age on the chemical profile of *P. canescens* leaf extracts and their cytotoxicity against HeLa cells. Chemical profiles were characterized using FTIR-ATR spectroscopy, phytochemical screening was conducted to identify secondary metabolite groups, and cytotoxicity was evaluated using the MTT assay. Phytochemical screening revealed differences in metabolite composition between young and mature leaves, with some compounds detected only in young leaves. FTIR-ATR analysis also showed distinct functional group patterns according to leaf age. Young leaves exhibited characteristic bands associated with peronemin A2, A3, B1, B2, and B3, whereas mature leaves displayed bands characteristic of peronemin C1 and D1. The C–N functional group showed moderate intensity in young leaves but weak intensity in mature leaves. Cytotoxicity results indicated that all extracts were non-toxic toward HeLa cells, with IC50 values above 500 µg/mL. However, the mature-leaf Soxhlet extract showed the lowest IC50 tendency, with a mean value of 525.91 ± 22.46 µg/mL. Overall, leaf age influenced the chemical profile and cytotoxicity more than extraction method.

Keywords: Cytotoxicity; FTIR-ATR; MTT assay; *P. canescens* Jack; Phytochemistry.

INTRODUCTION

Cancer is one of the leading causes of death worldwide. According to the Global Cancer Statistics (GLOBOCAN) data for 2022, cervical cancer ranks eighth among the types of cancer with the highest number of cases, accounting for 3.3% of all cases, with an estimated 348,189 deaths (3.6%), and ranks ninth in cancer-related deaths (Bray et al., 2024). According to data from the Ministry of Health (2025), cervical cancer is the second most common type of cancer in Indonesia, with an estimated more than 36,000 new cases detected annually. Cervical cancer is generally treated with chemotherapy, surgery, and radiation therapy. These treatments have side effects for patients, such as fatigue, nausea, vomiting, hair loss, skin peeling due to radiation, as well as vaginal pain, changes in the menstrual cycle, anemia, and others (Ansharina et al., 2025). To minimize these side effects, the search for new anticancer agents from natural sources remains an important area of research. One promising medicinal plant that has attracted attention as a potential source of anticancer compounds is the *Sungkai* plant (Fikriansyah et al., 2023).

The *Sungkai* plant (*P. canescens* Jack) is classified in the *Lamiaceae* family. *Sungkai* is an ethnobotanical plant used by local communities as a traditional medicinal plant endemic to Indonesia. The *Sungkai* plant is found in Sumatra, Kalimantan, Jambi, Bengkulu, Lampung, Sulawesi, and West Java. According to local beliefs, an infusion of *Sungkai* leaves is believed to lower blood pressure and uric acid levels, alleviate the flu, cough, the common cold, fever, and malaria; boost the immune system; and may be used to prevent cancer (Vidi et al., 2024). The pharmacological potential of *Sungkai* leaves has been demonstrated through their anti-inflammatory, antibacterial, antioxidant, and cytotoxic activities against various cancer cells (Ningsih & Ibrahim, 2023; Aztur et al., 2023; Vidi et al., 2024).

A literature review revealed that the maceration and Soxhlet extraction methods have been used to analyze the total phenolic content of *Sungkai* leaves (Jaya & Hasnah, 2024b). The results showed that maceration yielded a higher extraction yield than Soxhlet, while Soxhlet yielded higher total phenolic content than

maceration. The chemical profiles of Sungkai leaf extracts obtained from both methods were analyzed using GCMS. The analysis results showed differences in the compounds obtained from the maceration and Soxhlet methods. However, these studies have not examined biological activity nor considered the age of the *Sungkai* leaves used. Meanwhile, a study by Rahmi et al. (2023) (Rahmi Z J et al., 2023) evaluated the antioxidant activity in young, semi-mature, and mature *Sungkai* leaves. Therefore, this paper compares the phytochemical profiles of extracts from young and mature *Sungkai* leaves obtained using different extraction techniques through phytochemical screening analysis, FTIR-ATR, and cytotoxic biological activity assays to determine their potential as safe and effective anticancer agents for cervical cancer.

MATERIALS AND METHODS

Materials

The *Sungkai* leaves used in this study were young and mature leaves collected on January 24, 2025, in Sanggau Regency, West Kalimantan. The solvent used for the extraction and phytochemical screening was 96% distilled ethanol. Phytochemical screening was carried out using freshly prepared Mayer's, Dragendorff's, Wagner's, Liebermann–Burchard's, 5% $FeCl_3$, and Shinoda's reagents prepared from analytical-grade chemicals (Merck, Darmstadt, Germany). Thin-layer chromatography (TLC) analysis was performed using silica gel 60 F254 plates (Merck, Darmstadt, Germany), while the spot-developing reagent used was 0.15% $CeSO_4$ (Merck, Darmstadt, Germany). Cytotoxicity testing was performed using HeLa cell lines (ATCC, Manassas, VA, USA), RPMI-1640 culture medium, Fetal Bovine Serum (FBS), and Phosphate-Buffered Saline (PBS) (Gibco, Grand Island, NY, USA), dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany), MTT reagent (Sigma-Aldrich, St. Louis, MO, USA), and a 10% sodium dodecyl sulfate (SDS) (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The Parasitology Laboratory, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia, supplied all reagents and cell culture materials.

Procedures

Sample Determination

Experts at the Bogor Herbarium, BRIN, in Cibinong, West Java, determined the Sungkai plant. The identification results (reference number B-641/II.6.2/IR.01.02/2/2025) indicate that the sample is P. canescens Jack and belongs to the Lamiaceae family.

Sample Preparation

The mature leaves collected were green and fresh, while the young leaves collected were purple (5–7 terminal leaves). The collected Sungkai leaf samples were washed with running water, then air-dried in a closed room. The dried samples were finely chopped and subsequently pulverized using a grinder to obtain a powdered crude drug material, with the following weights: 28 grams of young Sungkai leaves and 1,530 grams of mature Sungkai leaves.

Extraction

The extraction process in this study utilized two techniques: maceration and Soxhlet extraction. The maceration of young and mature Sungkai leaves was performed using distilled ethanol. Powdered crude material from mature *Sungkai* leaves (750 grams) and powdered crude material from young Sungkai leaves (14 grams) were macerated in ethanol for 3 × 24 hours. The liquid extract obtained from maceration was concentrated using a rotary vacuum evaporator, yielding concentrated macerated extracts of young Sungkai leaves (SMM) and mature *Sungkai* leaves (STM). Meanwhile, for the Soxhlet extraction method, powdered crude material from mature Sungkai leaves (750 grams) and powdered crude material from young Sungkai leaves (14 grams) were placed into a filter paper sleeve. The prepared sample was subsequently loaded into the Soxhlet extractor, while 250 mL of ethanol was placed in the round-bottom flask. The extraction process was carried out until the extract solvent in the siphon tube became clear (Jaya et al., 2024a). In this study, young Sungkai leaves required 11 cycles, while mature Sungkai leaves required 14 cycles until the extract solvent in the siphon tube became clear. The extracts obtained through Soxhlet extraction were subsequently concentrated using a rotary vacuum evaporator, yielding concentrated extracts from young Sungkai leaves (SMS) and mature Sungkai leaves (STS).

Phytochemical screening

Phytochemical screening was conducted using colour reaction tests with specific reagents, as previously described (Harborne, 1987). Alkaloid testing was performed by adding Dragendorff, Mayer, and Wagner reagents, respectively, to approximately 1 mL of the dissolved sample; the presence of alkaloids was confirmed by the formation of characteristic precipitates: a reddish-brown precipitate upon reaction with Dragendorff's reagent, a yellowish-white precipitate upon reaction with Mayer's reagent, and a brown precipitate after treatment with Wagner's reagent. (Sangi et al., 2012). The flavonoid test was performed by adding a magnesium strip, and A few drops of concentrated hydrochloric acid (HCl) were added to the sample. The presence of flavonoids was confirmed by the development of a red colouration accompanied by foam formation (Mukhriani et al., 2019). The phenolic test was performed by adding 5% $FeCl_3$ to the sample. The development of a dark green or bluish-black colouration confirmed the presence of phenolics. (Yanti et al., 2019).

Meanwhile, the terpenoid and steroid tests were performed using the Liebermann–Burchard reagent. The presence of terpenoids and steroids was confirmed by the formation of a red colour for terpenoids and a blue colour for steroids (Habibi et al., 2018).

Thin-Layer Chromatography (TLC)
A 5 × 6 cm TLC plate was prepared by marking an upper boundary at 0.5 cm and a lower boundary at 1 cm. The eluent was prepared using a gradient system of n-hexane: ethyl acetate (7:3, 9:1) and chloroform: methanol (9:1). The extract was spotted at the lower boundary of the plate using a capillary tube, then allowed to dry. Next, the plate was placed in a chamber saturated with the mobile phase to allow the elution process to proceed. The process was terminated once the solvent front reached the upper boundary of the plate. The resulting spots were observed with the naked eye and under a UV lamp, and sprayed with the spot-revealing reagent cerium sulfate ($CeSO_4$).

Fourier Transform Infrared Spectroscopy (FTIR-ATR)
Sample preparation was carried out as described previously (Suryelita et al., 2017). A 10-mg sample was taken using a spatula and placed into the sample holder of the FTIR-ATR instrument, which had been previously cleaned with alcohol. The sample was then measured using the FTIR-ATR spectrometer to obtain an IR spectrum.

Cytotoxicity Assay
The cytotoxicity of the samples was assessed on HeLa cells using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method, as previously described (Arifianti et al., 2014). A total of 1 × 10^4 HeLa cells were transferred into complete culture medium (consisting of FBS, penicillin-streptomycin, amphotericin B, and RPMI 1640). On the first day, 100 µl of a cell suspension containing 5 × 10^3 cells was pipetted into each well, then the plate was placed in a 96-well plate and incubated for 24 hours in a 5% CO_2 incubator at 37°C. On the second day, test samples with a series of concentrations (500, 250, 125, 62.5, 31.25 µg/mL) were added in triplicate (three times), then incubated again for 24 hours. On the third day, 100 µL of MTT reagent was added to the cells, followed by a further 4-hour incubation period. The assay results indicated the formation of formazan crystals in the viable cells, reflecting the reduction of MTT by metabolically active cells. The reaction was subsequently terminated by adding a 10% SDS stopping solution. Finally, the absorbance, expressed as optical density (OD), of the diarylheptanoid-treated samples was determined using an ELISA reader at 595 nm. Cytotoxicity was determined as the cytotoxic concentration causing 50% inhibition of cell growth (IC_{50} value).

Data analysis
Phytochemical screening data, TLC chromatograms, and FTIR-ATR spectra for each extract were analysed qualitatively. Cytotoxicity and selectivity data for each extract were analysed quantitatively by calculating the IC_{50}, expressed as the mean ± standard deviation. Cell viability percentage calculations were as described previously by Arifianti et al. (2014), using the formula:

$$\%Viability = \frac{(absorbance\ of\ the\ treatment - absorbance\ of\ the\ control\ medium)}{(absorbance\ of\ the\ control\ cell - absorbance\ of\ the\ control\ medium)} \times 100\% \quad (1)$$

The viability percentage was analysed using linear regression in Microsoft Excel 2021 to obtain the final IC_{50} value. A t-test was then performed using IBM SPSS 21 to compare the sample means of two groups to determine whether they are different or the same. If the significance value (2-tailed) > 0.05, then there is no significant difference between the groups. Conversely, if the significance value (2-tailed) < 0.05, then there is a significant difference between the groups. (Arif et al., 2023).

RESULTS AND DISCUSSION

Extraction and Yield
Powders of young and mature *Sungkai* leaves were extracted using two methods: maceration and Soxhlet extraction with ethanol as the solvent. These methods were selected to compare yields, phytochemical profiles, and cytotoxic activity obtained from extractions performed at different temperatures (Jaya et al., 2024a). The use of ethanol as a solvent is based on its ability to dissolve both polar and nonpolar compounds, thereby enabling the extraction of active compounds in a more optimal quantity compared to other solvents (Atun, 2014). The extraction results using the maceration and Soxhlet methods yielded a thick, blackish-green extract. The yields obtained from extraction using the maceration and Soxhlet methods are shown in **Table 1**.

Table 1. Yield data for the maceration and Soxhlet extraction of young and mature Sungkai.

Sample	Sample weight (g)	Extract weight (g)	Yield (%)
STM	750	49.5	6.6
SMM	14	2.3	16.4
STS	750	57.9	7.7
SMS	14	2.9	20.7

Variations in the yield of *Sungkai* leaf extract are associated with differences in leaf age and the extraction method applied. Based on leaf age, the yield obtained from young leaves was 16.4% and 20.7%, respectively, compared to that from mature leaves. Meanwhile, based on the extraction method, Soxhlet extraction yielded higher yields than maceration, at 7.7% and 20.7%, respectively (**Table 1**). These results indicate that the age of the leaves used can influence the yield (Wijaya et al., 2022). Additionally, the extraction method used is also expected to affect the magnitude of the yield produced. However, based on this study, the results are descriptive in nature and therefore cannot be used to conclude that either leaf age or the extraction method has a causal effect on the yield.

Chemical Composition Profile Using Phytochemical Screening Methods

One of the tests in the field of chemistry used to identify secondary metabolites in natural materials is called phytochemical screening. This phytochemical screening is an initial step conducted to provide an overview of the chemical compounds present in the natural materials under study (Harborne, 1987). Results of the phytochemical screening of young and mature Sungkai leaves using maceration and Soxhlet extraction methods (**Table 2**).

Table 2. Data from phytochemical tests using specific reagents.

Compound Identification	Observation Results	Sample			
		SMM	**STM**	**STS**	**SMS**
Alkaloids					
*** Dragendorff**	↓ Reddish brown	+	+	+	+
***Mayer**	↓ White	+	+	+	+
***Wagner**	↓ Brown	+	+	+	+
Flavonoids	Red,	+	+	+	+
Fenoliks	Dark green	+	+	+	+
Terpenoids	Red	+	-	+	-
Steroids	Blue	-	+	-	+

Note: (+) detected, (-) not detected

Sungkai leaf extract contains secondary metabolites such as alkaloids, flavonoids, and phenolics (Table 2). Differences in leaf age result in distinct chemical profiles in *Sungkai* leaf extracts, particularly in terms of terpenoid and steroid content. Conversely, differences in extraction methods did not result in differences in the chemical composition of the Sungkai leaf extract. The biosynthesis of terpenoids and steroids causes the differences in chemical composition between young and mature Sungkai leaves. Biochemically, steroids are derivatives of terpenoids via the mevalonate pathway, beginning with the formation of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which subsequently produce farnesyl pyrophosphate (FPP), squalene, and lanosterol as steroid precursors (Duan et al., 2022) As leaves age, squalene undergoes further epoxidation and cyclization to form steroid structures, resulting in changes in the composition of secondary metabolites due to the physiological and metabolic development of the tissue. Therefore, terpenoid compounds tend to be dominant in young leaves, while steroid compounds are more dominant in mature leaves.

Chemical composition profile using thin-layer chromatography

TLC tests were conducted to compare the chemical composition of young and mature *Sungkai* leaves using different extraction methods, to determine whether there are differences in the chemical composition of Sungkai leaves based on age and extraction method. The results of the TLC tests are presented in **Figure 1.**

There are differences in the composition of compounds contained in young and mature *Sungkai* leaves, as evidenced by the colour of the spots and the Rf values (**Figure 1**). Some spots with the same Rf value exhibit different colours in the extracts of young and mature Sungkai leaves after being sprayed with cerium sulfate ($CeSO_4$ 0.15%). Furthermore, not all *Sungkai* leaf extracts produced spots with the same Rf values. This indicates differences in the chemical composition of

Sungkai leaves, influenced by the extraction technique and leaf age. These findings are consistent with the phytochemical analysis (**Table 2**), which shows differences in the chemical components present in young and mature Sungkai leaves.

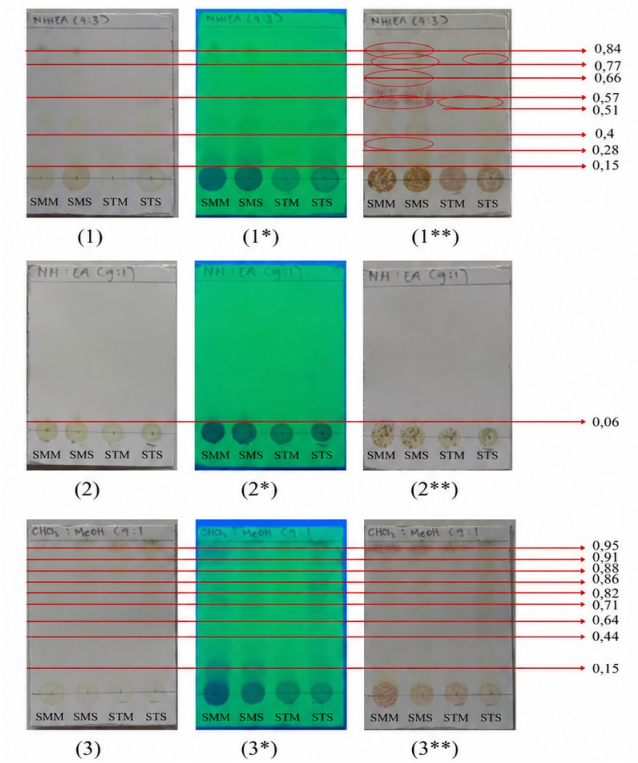


Figure 1. TLC results using various eluent systems. n-hexane:ethyl acetate (7:3) (1); n-hexane:ethyl acetate (9:1) (2); chloroform:methanol (9:1) (3), *viewed under UV at 254 nm, **+ $CeSO_4$ spot

FTIR-ATR Characterization

Characterization of compounds using an FTIR-ATR spectrometer was performed to identify the functional groups of the compounds contained in extracts of young and mature Sungkai leaves obtained via maceration and Soxhlet extraction. Functional group identification was based on the specific wavenumber absorptions detected by the infrared spectrometer. The FTIR-ATR spectra are shown in **Figure 2.**

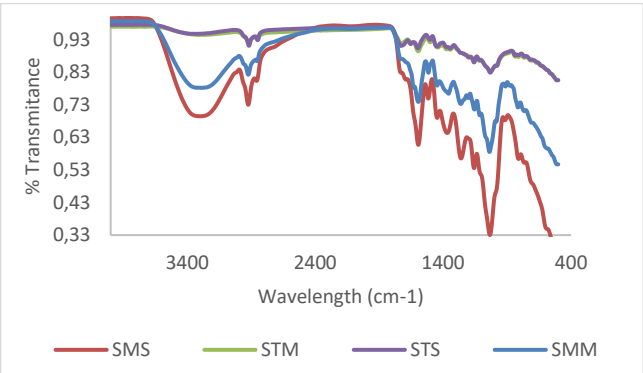


Figure 2. FTIR-ATR spectrum of *P. canescens* Jack

The FTIR-ATR spectra show that each *Sungkai* leaf extract exhibits a relatively similar spectral pattern, although there are some differences in the intensity of the absorption peaks (**Figure 2**). These differences indicate that the composition of the compounds contained in each extract is not entirely the same. This suggests differences in the compounds produced by the maceration and Soxhlet extraction processes applied to young and mature *Sungkai* leaves. The results of the functional group interpretation based on the FTIR-ATR spectra are presented in **Table 3**.

There was a difference in the intensity of the carbonyl (C=O) functional group band between young and mature *Sungkai* leaves (**Table 3**). The absorption band at a wavenumber of 1729–1730 cm^{-1}, which is indicative of carbonyl (C=O) stretching vibration, was detected with low intensity in young Sungkai leaves and moderate intensity in mature Sungkai leaves. This difference in band intensity is thought to be related to differences in the structure and number of carbonyl groups in the peronemin compounds contained in the Sungkai leaf extract. Based on previously reported structural analyses of compounds in the Sungkai leaf extract (Kitagawa et al., 1994), peronemin compounds have different numbers of carbonyl groups. Specifically, the compounds peronemin A2 **(1)**, peronemin A3 **(2)**, peronemin B1 **(3)**, peronemin B2 **(4)**, and peronemin B3 **(5)** contain one carbonyl group (C=O), whereas the compounds peronemin C1 **(6)** and peronemin D1 **(7)** contain two carbonyl groups (C=O). Therefore, the moderate band intensity in mature leaves is thought to indicate the presence of compounds with a greater number of carbonyl groups compared to young leaves. Thus, the compounds present in young Sungkai leaves are presumed to be peronemin A3, peronemin B1, peronemin B2, and peronemin B3. In contrast, the compounds present in mature leaves are presumed to be peronemin C1 and peronemin D1.

There was a difference in the intensity of the C–N functional group bands between young and mature *Sungkai* leaves (**Table 3**). Absorption bands at wavelengths of 1180–1360 indicated the presence of C–N stretching vibrations, with moderate intensity in young *Sungkai* leaves and weak intensity in mature leaves. The C–N functional group is thought to indicate the presence of alkaloid compounds, which is consistent with the phytochemical analysis (**Table 2**), which showed that both young and mature Sungkai leaves tested positive for alkaloids. The difference in the intensity of the C–N absorption band between young and mature leaves is likely due to lower alkaloid concentrations in mature leaves, resulting in absorption intensity that is not strong enough to be detected. Plant physiological factors can also influence secondary metabolite composition, with young leaves tending to have higher levels of protective compounds such as alkaloids than mature leaves. Alkaloids function to regulate plant growth and development and to protect against pests and diseases

(Sianipar & Siahaan, 2017). Therefore, alkaloids are more prevalent in young leaves.

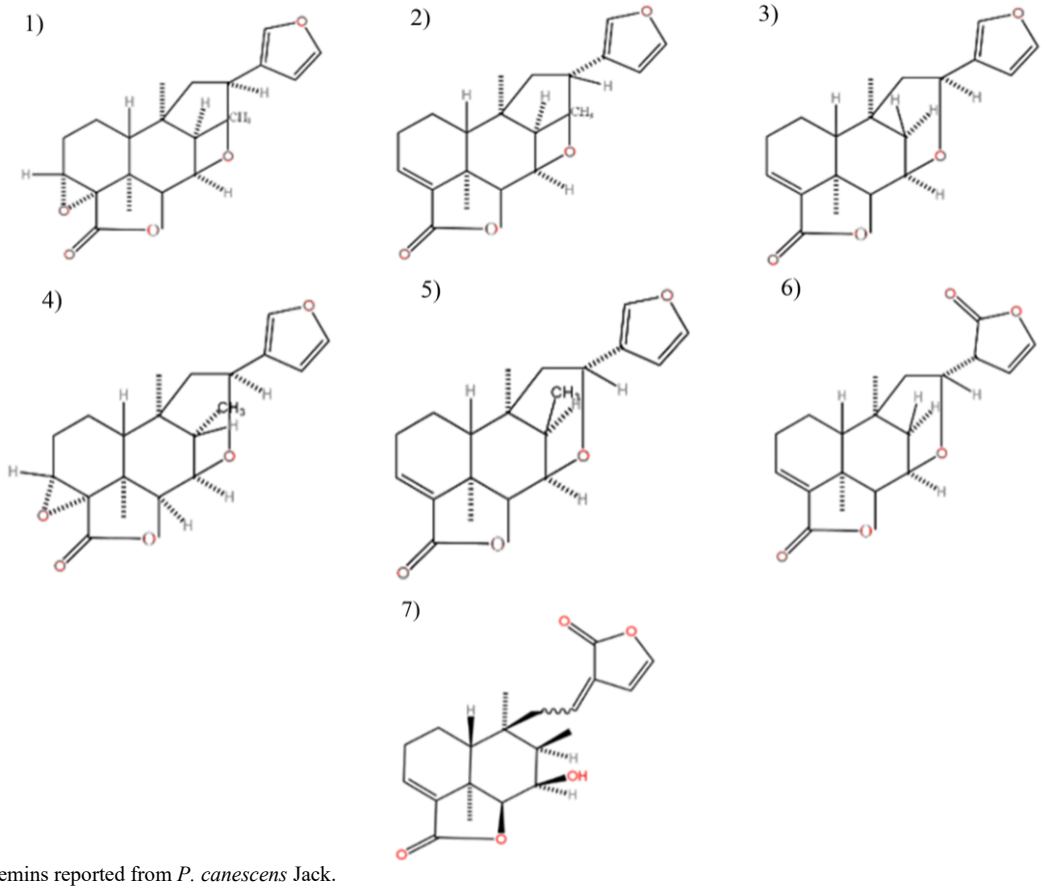


Figure 3. Peronemins reported from *P. canescens* Jack.

Table 3. Interpretation of functional groups based on FTIR-ATR spectra of young and mature *P.canescens* Jack leaf extracts using the maceration and Soxhlet method.

Range Literature (cm^{-1})	Group Function	SMM (cm^{-1})	Intensity band	STM (cm^{-1})	Intensity band	SMS (cm^{-1})	Intensity band	STS (cm^{-1})	Intensity band	Type Vibration
3200-3600	O-H	3297.90	Strong	3307.13	Moderate	3290.24	Strong	3341.12	Moderate	Stretching
2850-2970	C-H,	2923.98	Strong	2920.88	Strong	2923.72	Strong	2918.45	Strong	Stretching
	Alkana	2858.61	Weak	2851.42	Moderate	2854.27	Weak	2850.20	Moderate	Stretching
1690-1760	C=O	-	Weak	1730.76	Moderate	-	Weak	1729.61	Moderate	Stretching
1610-1680	C=C	-	Weak	1651.85	Weak	1629.40	Weak	1654.01	Weak	Stretching
1500-1600	C=C,	1594.11	Strong	1595.42	Strong	1594. 05	Moderate	1597.79	Moderate	Stretching
		1516.06	Moderate	1501.16	Moderate	1515.10	Moderate	1502.17	Moderate	Stretching
1340-1470	C-H,	1446.64	Moderate	1439.75	Weak	1446.82	Weak	1446.28	Moderate	Bending
	Alkana	1368.22	Moderate	1373.11	Weak	1357.38	Moderate	1371.33	Weak	Bending
		-	Weak	1344.74	Weak	-	Weak	1345.62	Weak	Bending
1180-1360	C-N	1259.70	Moderate	-	Weak	1261.66	Moderate	-	Weak	Stretching
		-		-	Weak	1203.17	Weak	-	Weak	Stretching
1050-1300	C-O	1157.94	Moderate	1198.59	Moderate	1156.13	Moderate	1198.98	Moderate	Stretching
		1034.19	Strong	1157.49	Moderate	1113.57	Weak	1158.43	Weak	Stretching
		-		1032.02	Strong	1037.55	Strong	1031.54	Strong	Stretching
675-995	C-H,	915.84	Weak	836.08	Weak	983.94	Weak	981.11	Weak	Bending
	Alkena	810.43	Weak	811.47	Weak	912.47	Weak	838.06	Weak	Bending
				765.59	Weak	814.53	Weak	812.62	Weak	Bending
								764.47	Weak	Bending
								715.53	Weak	Bending

The cytotoxicity of young and mature P. canescens* Jack *leaf extracts using the maceration and Soxhlet method against HeLa Cells

Anticancer activity testing was conducted to determine the ability of ethanol extracts obtained via maceration and Soxhlet extraction from young Sungkai leaves to inhibit the growth of cervical cancer cells. Cytotoxicity testing was performed in vitro on HeLa cervical cancer cells using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The cytotoxicity test results showed that Sungkai leaf extracts obtained via maceration and Soxhlet extraction did not inhibit HeLa cancer cells (**Table 4**).

Table 4. IC_{50} results of young and mature Sungkai leaf extracts using the maceration and Soxhlet method

No	Sample	IC_{50} value (µg/ml)			Mean IC_{50} ((µg/ml) ± standard deviation)
		I	II	II	
1.	SMM	837.54	856.38	669.10	787.67 ± 103.12
2.	STM	736.80	585.79	460.37	594.32 ± 138.41
3.	SMS	659.07	554.35	561.06	591.49 ± 58.62
4.	STS	546.73	502.10	528.90	525.91 ± 22.46

Note: IC_{50} values are represented as the mean and standard deviation; n = 3.

A substance is considered to have very strong cytotoxicity if the IC_{50} value is ≤ 20 µg/mL, moderate if the IC_{50} value is 21–200 µg/mL, and weak if the IC_{50} value is 201–500 µg/mL, and non-toxic if the IC_{50} value is ≥ 500 µg/mL (22). Young and mature Sungkai leaf extracts using the maceration and Soxhlet method have IC_{50} values > 500 µg/mL, indicating that all four samples are non-toxic to HeLa cells (Table 4). However, based on the IC_{50} values for HeLa cells obtained, it is evident that mature Sungkai leaves have a lower IC_{50} value compared to young leaves. The age of these leaves influences the IC_{50} values of HeLa cells from Sungkai leaf extracts, suggesting differences in the chemical composition between young and mature Sungkai leaves.

The peak intensity of the carbonyl group (C=O) in mature Sungkai leaves was higher than in young leaves, indicating a higher carbonyl group content (Table 3). Carbonyl groups are known to be highly polar and play a role in forming noncovalent interactions with biomolecules, thereby enhancing the biological activity of compounds (Newberry & Raines, 2017). The high intensity of the C=O band in mature leaves is suggested to be associated with the predominance of peronemin compounds, which contain more carbonyl groups. This condition is believed to contribute to the higher cytotoxic activity of the mature leaf extract, as indicated by the lower IC_{50} value.

T-Test

The t-test was employed to determine whether there were significant differences between the means of two sample groups. A significance level (two-tailed) > 0.05 indicates no significant difference, while < 0.05 indicates a significant difference. In this study, the t-test was performed to compare the mean values obtained from maceration and Soxhlet extraction of young and mature leaves.

Table 5. The t-test data for each Sungkai leaf extract

Sample groups	F	t	Sig. (p)	Note
SMM & STM	0.111	1.904	0.124	Not significant
SMS & STS	4.926	1.809	0.145	Not significant
SMM & SMS	2.099	2.865	0.046	Significant
STM & STS	3.265	0.845	0.446	Not significant

A statistically significant difference was identified between the SMM and SMS extracts, with a significance value (2-tailed) of 0.046 (sig < 0.05). In contrast, other tests showed no significant difference in IC_{50} values among these groups (sig (2-tailed) > 0.05) (Table 5). The significant difference in IC_{50} values between the SMM and SMS extracts is likely attributable to differences in the extraction efficiency of the two methods. Maceration is a cold extraction method performed at room temperature, whereas Soxhlet extraction uses heating and continuous solvent circulation, thereby increasing the amount of bioactive compounds extracted compared to maceration. The results of the phytochemical analysis indicate the presence of secondary metabolites in young Sungkai leaves, which are believed to be extracted more effectively via Soxhlet extraction, resulting in significantly different cytotoxic activities. However, this increase in the amount of bioactive compounds extracted has not yet been sufficient to produce strong cytotoxic activity against HeLa cells.

CONCLUSIONS

Phytochemical screening and FTIR-ATR characterization revealed differences in chemical composition between extracts of young and mature leaves. All extracts exhibited non-toxic activity against HeLa cancer cells, with IC_{50} > 500 µg/mL. However, the mature leaf extract exhibited a lower IC_{50} value compared to the young leaf extract, which may be related to differences in their chemical composition. Differences in the intensity of C–N functional groups between young and mature leaves suggest that total alkaloid content could be considered for further chemical screening. These findings indicate that leaf age influences the chemical profile and cytotoxic activity of *Sungkai l*eaf extracts.

Authors' Contributions: Rini Muharini designed the study. Merin carried out the laboratory work. Merin & Rini Muharini analyzed the data. Merin wrote the manuscript. Firman Shantya Budi reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

Competing Interests: The authors declare that there are no competing interests.

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