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Article

Cytotoxic Activity of Polymethoxyflavone Compounds Ethyl Acetate Extract of Sungkai Leaves (*Peronema canescens* Jack) as Anticancer Candidates

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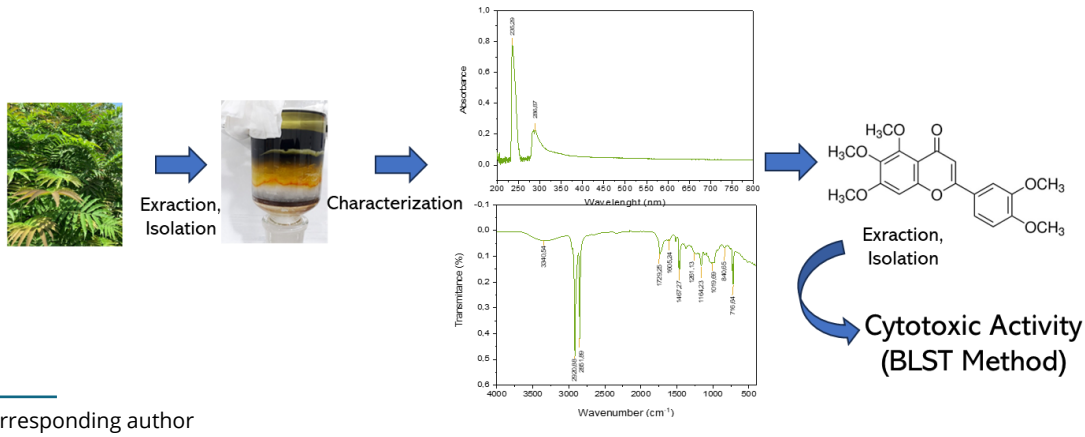
Abstract

This study presents the findings of a phytochemical screening of the ethyl acetate fraction of Sungkai leaves, revealing the presence of various secondary metabolites, including flavonoids, steroids, phenolics, and tannins. Liquid vacuum chromatography resulted in the separation of four distinct fractions: Fraction 1 (F1), Fraction 2 (F2), Fraction 3 (F3), and Fraction 4 (F4). An isolate obtained from Fraction 2 was designated as F2.1. Characterization of this isolate was conducted using UV-Vis and FT-IR spectrophotometry. The UV-Vis analysis revealed two absorption peaks at 235.29 nm (band 1) and 286.87 nm (band 2), indicating the presence of a conjugated diene system and an aromatic system with specific substituents, respectively. FT-IR spectroscopy of the F2.1 isolate from the ethyl acetate fraction of Sungkai leaves exhibited absorption bands at 2851.89 cm^{-1}, suggesting the presence of methoxy (-OCH_3) functional groups, and at 1261.13 cm^{-1}, corresponding to C-O-C stretching vibrations. Additional absorption peaks at 1467.27 cm^{-1} and 840.65 cm^{-1} indicated the presence of aromatic C=C and C-H bonds, respectively, while the absorption at 1729.25 cm^{-1} was characteristic of carbonyl functional groups. These spectral characteristics suggest that the isolate belongs to the flavonoid class, specifically the flavone subclass, with structural similarities to polymethoxyflavones such as sinensetin. The cytotoxic evaluation, performed using the Brine Shrimp Lethality Test (BSLT) and analyzed via the probit method in SPSS version 25 (SPSS Inc., Chicago, IL, USA), determined the LC_{50} value of the ethyl acetate extract to be 408.32 ppm. The LC_{50} values for the VLC fractions (F1, F2, F3, and F4) were 168.66 ppm, 242.66 ppm, 1936.42 ppm, and 857.03 ppm, respectively. Meanwhile, the isolate exhibited an LC_{50} value of 191.43 ppm, indicating potential anticancer properties.

Keywords: *Biactive compound, cytotoxic; Peronema canescens.*

Graphical Abstract



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Introduction

Cancer is a disease characterized by the uncontrolled growth and spread of abnormal cells [1]. Four main factors cause cancer such as environment, food, biological and psychological. Cancer is a disease with no known exact cause but is influenced by many factors, such as smoking/exposure to cigarette smoke, consuming alcohol, excessive exposure to ultraviolet light on the skin, obesity, an unhealthy diet, lack of physical activity, and infections related to cancer. Cancer can be prevented by reducing the risk factors for cancer. In developments in the health sector, anticancer drugs have been discovered, and chemotherapy has been carried out, but the high-cost factor is an obstacle [2]. Cancer is a deadly disease for humans. The World Health Organization (WHO) states that cancer is the main cause of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 million deaths from cancer in 2012. In Indonesia, the prevalence of cancer is 1.4 out of 1000 population or around 330 thousand Persons. The number of cancer patients in Indonesia is very high. This was published based on data on people affected by cancer from the government and cancer institutions [3]. Deaths caused by cancer continue to grow. From the World Health Organization (WHO), 9.6 million people died from cancer in 2018 [4].

Most patients with cancer receive chemotherapy, which involves using anticancer drugs and supporting drugs to reduce the side effects of using anticancer drugs. One example of an anticancer drug is doxorubicin, but continuous use of doxorubicin can be toxic to organs [5]. Chemotherapy drugs kill not only cancer cells but also normal cells [6]. Early prevention against the risk of cancer is to be able to consume fruits and vegetables that contain phytochemicals that are beneficial to the body [7]. Thus, it is necessary to develop therapies using natural medicines as alternative treatment materials [6]. Traditional medicine has been widely used and developed in several countries [8].

Traditional medicine is a treatment derived from herbal plants and has been known since ancient times. Traditional medicine has been relied on for generations because it is inexpensive and often has fewer side effects than modern medicine. Additional research on plants used as traditional medicines is still needed through preclinical trials and clinical trials to be used safely in healthcare facilities. Many herbal plants have no known degree of cytotoxicity. One of the natural ingredients that is often used in traditional medicine is sungkai leaves [9].

The sungkai leaf plant is one of the plants used as a source of traditional medicine for the community and is unique (endemic) to Indonesia. There are bioactive compounds from Sungkai leaf extract, including flavonoids, alkaloids, steroids, phenolics, tannins, and saponins [10–12]. Several studies reported that Sungkai leaves have bioactivities such as antimalarial, antibacterial, analgesic, immunomodulatory, antiplasmodial, antidiabetic, anthyperusemia, and immunostimulant [13].

Secondary metabolite compounds (such as alkaloid compounds, terpenoids, flavonoids, phenolics, and organic acids) indicate that plants have secondary metabolite compounds that inhibit the growth of cancer cells. Potential anticancer activity of chloroform subfraction from Sungkai leaves against HT-29 cancer cells in vitro for secondary metabolite compounds from chloroform extract, namely alkaloids, terpenoids, steroids, flavonoids, and phenolics. The cytotoxic activity (IC_{50}) value against HT-29 colon cancer cells ranged from 14.807 to 34.448 µg/ml. Tests showed that chloroform subfraction 3 showed potential cytotoxic activity against human HT-29 cancer cells with an IC_{50} value of 14.807 µg/ml [14].

Cytotoxic activity of *Peronema canescens* Jack sungkai leaves in human cells: HT-29 and primary colon cancer adenocarcinoma methanol extract. Cytotoxicity value (IC_{50}) to AdenoCa cells 1.897 µg/ml. The inhibitory activity of the synthesis and mitotic phases in the cell cycle showed that different concentrations of SF3 had inhibitory activity on HT-29 (29,614 µg/ml) of 26.79% and 0.16%, AdenoCa cells (14,807 µg/ml) of 10.27% and 19.29% respectively. The apoptotic activity induced in HT-29 cells (29,614 µg/ml) and AdenoCa cells (14,807 µg/ml) were 26.58% and 11.50% [15].

Material and Methods

Materials and Instrumentations

The sample used in this study was sungkai leaves (*Peronema canescens* Jack). Samples were obtained from Kademangan Village, Jaluko District, Muaro Jambi Regency, Jambi Province, Indonesia. The chemicals were used ethyl acetate, n-hexane, 2N sulfuric acid, Dragendorff reagent, Meyer reagent, Lieberman-Burchard reagent, concentrated HCl, Mg powder, 2N HCl, FeCl3, acetic acid. Anhydrous (Sigma Aldrich), fine silica gel (packing) 0.040 - 0.063 mm and coarse silica gel (imprent) 0.063 -0.200 mm (Merck), Artemia salina larvae eggs, NaCl.

The equipments and instrumentations used in this study were maceration bottles, filter paper, rotary evaporator components, funnels, measuring cups, Erlenmeyer cups, VLC and GCC columns, vacuum pumps, capillary tubes, TLC plates (Merck), drip plates, test tubes, test tube rack, dropping pipette, 1 ml micropipette, 10 ml micropipette, KBR pellet, volumetric flask, vial, stir bar, watch glass, label paper, aerator, Artemia salina larvae breeding vessel, incandescent lamp, loop (magnifying glass), UV-Vis spectrophotometer (Shimadzu, Japan), FTIR spectrophotometer (Bruker, USA)

Methods

Extraction and Screening. Sungkai leaves as much as 1.5 kg were macerated in stages starting in 1.5 L n-hexane solvent for 2 x 24 hours with two repetitions. The simplicia was macerated again using ethyl acetate solvent in the same way. Both maserates were evaporated at 60 °C. The yield obtained was calculated as a weight percentage (w/w). Sungkai leaf phytochemical screening, including tests for alkaloids, saponins, phenolics, tannins, steroids, and flavonoids [10].

Separation and Purification. Thin Layer Chromatography (TLC) was prepared 1 x 5 cm with a lower and upper limit of 1 cm and 0.5 cm, respectively, so the eluent travelled 3.5 cm. Eluents are prepared by comparing organic solvents based on their polarity. The extract was spotted using a capillary tube at the bottom of the plate, and elution was carried out using the mobile phase. After the mobile phase reaches the upper limit on the plate, the elution process is stopped. Then the stain was examined directly under a UV lamp with a wavelength of 254 or 395 nm. All fractions were subjected to the TLC test, and the fractions with the same stain spots were put together and analyzed by TLC.

Furthermore, Isolation of compounds was carried out using vacuum liquid chromatography (VLC). VLC was performed using silica gel as the stationary phase with a sample:silka gel ratio (1:20). The sample extract was impregnated using silica gel and added to the column containing the stationary phase. At the same time, the mobile phase used is a mobile phase with gradient polarity. 10-15 grams of sample was impregnated with 15 grams of coarse silica gel (0.063 – 0,200 mm silica imprent). VLC was carried out using a column with a diameter of 5 cm with the stationary phase in the form of fine silica gel (silica packing 0.040-0.063mm) and the mobile phase in the form of a solvent with gradient polarity. Gradient elution increases the resolution of complex mixtures, especially when the sample has a broad polarity range. VLC starts from 100% n-hexane, n-hexane: ethyl acetate, ethyl acetate: methanol, to 100% methanol. The resulting fraction is then accommodated in a vial. The eluate is accommodated based on each band obtained and then evaporated. Column chromatography results were carried out by TLC again. Rf values that are close to and have identical spots are combined into one fraction.

BSLT Method Cytotoxic Test. The cytotoxicity test treatment was carried out on four repetitions. Artemia salina larvae were prepared by incubating the eggs 48 hours before testing and prepared mother liquor and serial concentrations of 1000 ppm, 100 ppm, and 10 ppm solvent used according to the fraction. Sea water was used as a negative control. Added 5 ml of engineered seawater and 1 ml of each test solution which had been evaporated for 24 hours into a test tube and then homogenized, then ten larvae were added. Observations were made (24 hours) on the death of shrimp larvae with the help of a magnifying glass

Characterization and Data analysis. Isolate was characterized using a UV-Vis spectrophotometer and FTIR spectrophotometer. Cytotoxicity data analysis was carried out by knowing the mortality of Artemia salina larvae, then looking for the probit number using the probit analysis program SPSS version 25 (SPSS Inc., Chicago, IL, 250 USA).

Results and Discussions

A dry sample of 1500 g was macerated in stages. The initial solvent used for maceration was n-hexane, as much as 1.5 L. From the maceration results, a yellow extract was obtained. Maceration with hexane was repeated two times until the yellow extract faded. Then, maceration was continued with 1.5 litres of ethyl acetate and two repetitions. The EtOAc extract fraction obtained was dark green. The extracts from the two solvents were then concentrated using a rotary evaporator. The dry hexane extract fraction from Sungkai leaves has a sticky character and a yellowish-green colour. Meanwhile, the dry EtOAc extract fraction also has a sticky character and is green-black. The mass and yield of the extract are presented in Table 1.

Table 1. Mass and yield of dried crude extracts

Fraction extracts	Mass (g)	Yield (%)
Hexane	14.2	0.94
Ethyl acetate	18.3	1.22

Secondary Metabolites

Phytochemical screening was carried out qualitatively using specific reagent solvents for each secondary metabolite, with the colour test method to determine the content of chemical compounds (Table 2 dan Table 3). There is a difference in the phytochemical screening using hexane and ethyl acetate solvents. Ethyl acetate solvents contain more diverse secondary metabolite compounds than hexane solvents because hexane solvents tend to be non-polar, which can extract non-polar compounds such as steroid compounds. The existence of various metabolite compounds in phenolic ethyl acetate solvents, tannins, steroids and flavonoids are a class of compounds that have cytotoxic activity.

Table 2. Phytochemical screening of extracts

Secondary Metabolites	Fractions	
	Hexane	EtAc
Alkaloids	-	-
- Dragendorf	-	-
- Meyer	-	-
Saponins	-	-
Phenolics/Tanins	-	+
Steroids	+	+
Flavonoids	-	+

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Table 3. Phytochemical screening of VLC EtOAc fraction

Secondary Metabolites	F1	F2	F3	F4
Alkaloids	-	-	-	-
Saponins	-	-	-	-
Phenolics/tanins	+	+	-	-
Steroids	-	-	+	+
Flavonoids	+	+	+	+

In the separation process using VLC, 21 vials were obtained and then evaporated at room temperature to approximately ½ vial. After that, TLC was performed on each eluate in the vials to identify the fractions in the 21 vials. The eluate in the vial, which has the same stain pattern as the TLC, is combined into 1 fraction. The results of this VLC are 4 fractions, namely F1, F2, F3, and F4. The four fractions were then subjected to a phytochemical screening test. The results of the phytochemical screening showed that all the VLC fractions of the EtOAC extract of Sungkai leaves were still positive for several classes of secondary metabolite compounds in Table 3. This phytochemistry aims to classify secondary metabolite compounds that can describe isolate from the isolate as belonging to a specific class of secondary metabolite.

In the F2 fraction, there is a precipitate, and then the precipitate is separated from the solution to be recrystallized. Recrystallization is carried out for the purification stage of impurities that come down during the elution process. The isolate is recrystallized with n-hexane solvent then ethyl acetate is called F2.1. After recrystallization was complete, TLC and phytochemical screening were carried out. In isolate, the phytochemical screening usually shows 1 positive test on the results of the phytochemical screening.

Based on the TLC test for isolate F2.1, ethyl acetate: methanol (3:7) was used with an Rf value of 0.51 and acetone: methanol (6:4) with an Rf value of 0.77. In this study, the isolate was positive for secondary metabolite compounds, namely flavonoids, on the results of the phytochemical screening

Characterization of the Isolate

The UV-Vis spectrum showed that the isolate gave two absorption peaks: band 1 λ = 235.29 nm and band 2 λ = 286.87 nm (Figure 1). The absorption at 235 nm indicates the presence of a conjugated diene/double system in the structure. Meanwhile, the absorption at 286 indicates the presence of an aromatic system with specific substituents.

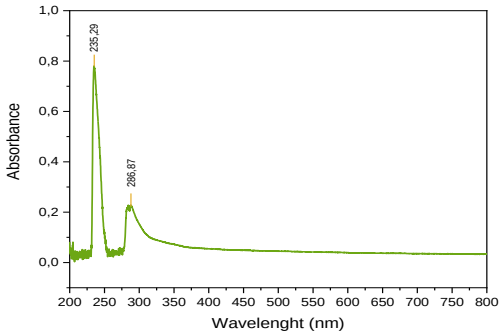

Figure 1. UV-Vis spectrum of the isolate

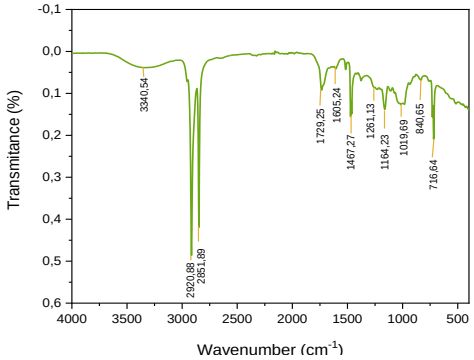

Figure 2. FTIR spectrum of the isolate

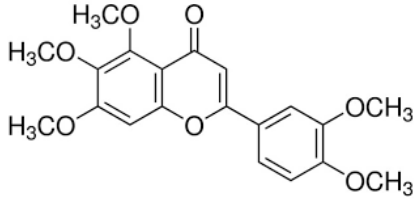

Figure 3. Chemical structure of Sinensetin [16]

Table 4. IR spectrum comparison and interpretation

Adsorption (cm^{-1})			
Isolate	PMF	Sinensetin [16]	**Interpretation**
2851.89 cm^{-1}	2854 cm^{-1}	2838 cm^{-1}	-OCH_3
1261.13 cm^{-1}	1266 cm^{-1}	1264 cm^{-1}	-C-O-C streching
1467.27 cm^{-1}	1463 cm^{-1}	1486 cm^{-1}	-C=C stretching aromatic
840.65 cm^{-1}	-	-	-C-H bending aromatic
1729.25 cm^{-1}	1733 cm^{-1}	1639 cm^{-1}	-C=O streching

IR spectrum on isolate F2.1 ethyl acetate fraction of Sungkai leaves showed absorption in the area of 2851.89 cm^{-1}, indicating the presence of –OCH_3 from methoxy, stretching vibration of C-O-C in the absorption area of 1261.13 cm^{-1}, stretching vibration in the absorption area of 1467 .27 cm^{-1} which indicates an aromatic C=C characteristic, bending vibrations in the absorption area of 840.65 cm^{-1} indicate the presence of aromatic C-H, and stretching vibrations in the absorption area of 1729.25 cm^{-1} which indicates the characteristics of carbonyl compounds (Figure 2). This IR absorption is significant in indicating the characteristics of the secondary metabolite class of flavonoids from sub-flavonoids, namely flavones. Based on the comparison of the IR spectra in Table 4, shows that these compounds, including polymethoxyflavone (PMF), are particularly close to the IR spectrum of sinensetin (5,6,7,3',4'-pentamethoxyflavone) (Figure 3).

Cytotixic Activity

The cytotoxicity test of a compound can be carried out using the Brine Shrimp Lethality Test (BSLT) method. The BSLT method was carried out to see the effect of cytotoxicity on cells and is often used in preliminary tests for screening or screening of pharmacological activity in medicinal plants and is also widely used for screening new anticancer compounds derived from plants. Toxicity test results with this method have been shown to correlate positively with the cytotoxicity of anticancer compounds [17].

The use of *Artemia salina* larvae eggs is used after 48 hr. It was the first instar phase when *Artemia salina* was 24 hr old. The larvae did not have a complete mouth and digestive system in this phase. While at the age of 36-48 hr after breeding the larvae, the larvae will metamorphose into the second instar stage. In this second stage, the mouth and digestive system are complete [18]. *Artemia salina* larvae are known to have skin sensitive to the environment, for example, foreign compounds such as bioactive compounds that can diffuse into cells. If the bioactive compound has cytotoxic activity, *Artemia salina* larvae will die [19].

The testing time for *Artemia Salina* larvae significantly affects the mortality rate of the larvae. In previous studies, the 12-hour test time was classified as an acute time for larvae, for 24 hr was a chronic time.

From Table 5, the results of the cytotoxic test show successively increased toxicity of F4, EtOAc Extract Fraction, F2, F2.1 and F1 with LC_{50} values of 857.03 ppm, 408.32 ppm, 242.66 ppm, 191.43 ppm and 168 .66 ppm is classified as toxic, for F3 it is classified as non-toxic with an LC_{50} value of 1936.42 ppm. While the hexane fraction, the LC_{50} value of 1066.66 ppm, is classified as non-toxic, this is the basis for isolating the ethyl acetate fraction. LC_{50} values have a toxic range, LC_{50} values < 30 ppm are very toxic, LC_{50} values are 30-1000 ppm toxic and LC_{50} values > 1000 ppm are not toxic [20]. When compared to the LC_{50} values of the EtOAc, F2, and F2.1 (Isolate) extract fractions indicates the presence of bioactive compounds, with a decrease in the LC_{50} value on the mortality of *Artemia salina* larvae.

The LC_{50} value of F1 is more toxic than F2.1. This is possible because of the synergistic effect of other compounds in Sungkai leaves, which can increase cytotoxicity, whereas F3, classified as non-toxic, can be influenced by other compounds in Sungkai leaves with antagonistic effects. The synergistic (supporting) and antagonistic (inhibiting) effects affect the

bioactivity of the compounds. Based on the results of the characterization of the isolated compounds, it is possible that they belong to the flavonoid class, which has a methoxy functional group. In previous studies, flavonoid compounds that have a methoxy functional group have the potential to inhibit leukaemic cancer cells HL60 and prostate cancer cells (PC-3 and DU145) [21,22].

Table 5. BSLT method cytotoxic test result

Samples	Conc (ppm)	Total deaths	Probit values	LC_{50} (ppm)
n-hexane extract fraction	10	8	3.028	1066.6
	100	11		
	1000	23		
EtOAc extract fraction	10	7	2.611	408.32
	100	14		
	1000	27		
F1	10	6	2.227	168.66
	100	7		
	1000	37		
F2	10	3	2.385	242.66
	100	6		
	1000	35		
F3	10	3	3.287	1936.42
	100	5		
	1000	18		
F4	10	4	2.933	857.03
	100	10		
	1000	21		
F2.1 (Isolate)	10	4	2.282	191.43
	100	8		
	1000	36		

Conclusion

The profile of bioactive compounds from the ethyl acetate fraction of Sungkai leaves refers to the flavonoid group, especially flavone, namely polymethoxyflavone. Based on the results of the cytotoxic test, the bioactive compound from the ethyl acetate fraction had an LC50 value of 191.43 ppm against Artemia salina.

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Author Contributions

Conceptualization, ZA and MF.; Methodology, MF and S; Software, ZA and ILT.; Validation: ILT and S; Formal Analysis, ZA and MF.; Investigation, MF and ZA.; Resources, ILT and S.; Data Curation, S and MF; Writing – Original Draft Preparation, ZA, MF, ; Writing – Review & Editing, S and ILT; Visualization: ZA and MF.; Supervision, S and ILT; Project Administration, S.

Conflic of Interest

The authors declare no conflict of interest

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