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Effect of Different Solvents on Toxicity and Secondary Metabolites of Mangkokan Leaves (*Polyscias scutellaria* (Burm.f.) Fosberg) by Brine Shrimp Lethality Test (BSLT) Method

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ABSTRACT

Mangkokan (*Polyscias scutellaria* (Burm.f.) Fosberg) is one of the plants belonging to the Araliaceae family. Empirically, mangkokan plants, especially the leaves, are used by the community as traditional medicine. Mangkokan leaves need to be developed into a source of natural materials such as biopesticides, biolarvicide, and anticancer agents because they have toxic compounds. Secondary metabolite compounds of mangkokan leaves consist of flavonoids, saponins, monoterpenes-sesquiterpenes, and steroids. This study aims to determine the toxicity of n-hexane, ethyl acetate, and ethanol extracts of mangkokan leaves using the Brine Shrimp Lethality Test (BSLT) method. Mangkokan leaves were extracted by graded maceration method using n-hexane, ethyl acetate, and 96% ethanol solvents. All three extracts were tested BSLT on Artemia salina larva test animals. The toxicity level is expressed by the LC50 value obtained from the probit analysis. The LC50 value of n-hexane, ethyl acetate, and ethanol extracts was 146.724 µg/mL, respectively; 66.029 µg/mL; and 92.007 µg/mL. The results showed that all three extracts were toxic.

INTRODUCTION

One of the diseases that can cause the most deaths worldwide and is also non-communicable is cancer (Rosita, Binakada and Kusnan, 2021). The number of cancer cases continues to increase along with the high prevalence of cancer risk factors such as smoking, obesity, physical inactivity, and changes in reproductive patterns related to urbanization and economic growth. (Sampoerna and Pandapotan Nasution, 2022). Based on data from the Global Burden of Cancer Study (Globocan) issued by the World Health Organization (WHO), Indonesia's total number of cancer cases reached 396.914 cases in 2020 and total deaths of 234.511 cases. In the next 20 years, it is estimated that cancer cases will increase to 22 million from 14 million in 2012 (Pusat Data dan Informasi Kementrian Kesehatan RI, 2015). Likewise, the cancer death rate is expected to continue to increase to reach 13.1 million in the next 7 years. Chemotherapy, radiation, and surgery are the main treatments for cancer patients in Indonesia. However, the side effects that occur and the relatively expensive treatment make people start to choose alternative anticancers using herbal plants whose availability in Indonesia is abundant.

Indonesia is second only to Brazil for its abundant biodiversity. Its strategic geographical position means that many plants can grow well. This biodiversity creates the potential for

developing plants in Indonesia into herbal medicines that can be used as economical alternative medicines. However, there is still a lack of public knowledge so plants in Indonesia are not optimally utilized.

One of the plants whose utilization is still lacking and has the potential as an anticancer is the mangkokan plant. The mangkokan plant (*Polyscias scutellaria* (Burm.f.) Fosberg) is a wild plant widely used as an ornamental plant or hedge plant in the yard of a house. (Novitasari and Adawiyah, 2018). Some people use it as a plant that is mixed into medicine and then believed to have properties for various diseases commonly suffered by the community, especially in the leaves. (Primadiamanti et al., 2020).

Mangkokan leaves have a variety of benefits for the digestive system, including preventing hair loss, treating wounds, diuretic effects, improving blood circulation, and antioxidizing the body. (Hanum and Ardiansyah, 2017). The pharmacological effects produced by mangkokan leaves are due to the presence of secondary metabolite compounds. Secondary metabolite compounds can treat various diseases due to their toxic properties. (Baud, Sangi and Koleangan, 2014). Mangkokan leaves contain secondary metabolites such as alkaloids, flavonoids, triterpenoids, saponins, tannins, and phenols. (Helmin et al., 2021).

Based on antioxidant activity testing using the DPPH method, it is known that ethanol extract from mangkokan leaves has the potential to treat diseases caused by free radicals with an IC50 value of 161.39 ppm. (Sari and Hidayati, 2021). Cancer is a deadly degenerative disease caused by free radicals. (Sa'adah, 2016). Based on research (Ramadan, Wardatun and Wiendarlina, 2015) ethanol extract from mangkokan leaves has a toxic ability with an LC50 value of 104.14 ppm against Artemia salina Leach so it has a positive correlation as an anti-cancer.

To determine the effectiveness of active components such as herbal plants containing anticancer, it is necessary to have an initial analysis, namely toxicity testing using the Brine Shrimp Lethality Test (BSLT) method. BSLT is one method that is often used as a search for new compounds for cancer derived from plants. (Tianandari and Rasidah, 2017). In addition, BSLT testing is also easy to perform, cost-effective, does not require a long time, and is quite accurate. (Meyer et al., 1982 dalam Kurniawan and Ropiqa, 2021). The presence of biological activity of a compound against Artemia salina L. is characterized by its death (Sukmawati, Hayati and Muti'ah, 2014). Information on the toxicity of mangkokan leaves is still limited so it is necessary to test the toxicity activity of n-hexane, ethyl acetate, and ethanol extracts of mangkokan leaves (*Polyscias scutellaria* (Burm.f.) Fosberg) against Artemia salina L. using the BSLT method.

So that the results of this study are expected to obtain data related to the toxic activity of mangkokan leaves to provide scientific information to the public regarding the safety of mangkokan leaves through the BSLT method.

METHODS

Tools and Materials

The tools used in this research are blender, oven, analytical balance, microscope, object glass, porcelain crucible, weighing bottle, author's stove, furnace, macerator, rotary evaporator (Heidolph®), desiccator, round bottom flask, water bath, chromatography column, cuvette, chromatography vessel, vial, porcelain cup, ash-free filter paper, micropipette, set of glassware, set of BSLT equipment.

The materials used in this study were mangkokan leaves Fosberg, *Artemia salina* larvae, phytochemical screening reagent, spotting reagent, ethanol 96%, ethyl acetate, n-hexanes, seawater, filter paper, tween 80.

Mangkokan Leaf Material Collection and Preparation of Shrimp Larvae

Mangkokan leaves were obtained from Sikanco Village, Nusawungu, Cilacap, Central Java. Randomly picked which was done in November 2022 and *Artemia salina* shrimp eggs were obtained from Laksana Aquarium Marine Animal Shop on Jalan Karapitan, Bandung.

Crude Drugs Characterization

Crude drugs of mangkokan leaves were characterized in the form of determination of ash content, extractable content in certain solvents, water content, loss on drying, and specific gravity.

Phytochemical Screening and Extracts

Crude drugs and extracts of mangkokan leaves were determined for secondary metabolites contained therein using the phytochemical

screening method for flavonoids, polyphenols, alkaloids, tannins, saponins, quinones, zztriterpenoid -steroids.

Extraction

Extraction was carried out coldly with a multistage maceration method using solvents with different levels of polarity, namely n-hexane, ethyl acetate, and 96% ethanol. Put a certain amount of weighted simplisia powder into the macerator, and added a certain amount of n-hexane solvent. Extraction was carried out for 3 x 24 hours with occasional stirring, repeating soaking every 24 hours, and then separating the filtrate and residue. The collected n-hexane filtrate was stored in a container. Furthermore, the filtered residue was added with several ethyl acetate solvents and then macerated again following the extraction procedure in n-hexane solvent. The above procedure applies equally to 96% ethanol solvent. Each extract (n-hexane, ethyl acetate, and 96% ethanol) collected was evaporated using a rotary evaporator to obtain a thick extract.

Thin Layer Chromatography of Extracts

Mangkokan leaf extract was observed using thin-layer chromatography. Silica gel GF 254 or a suitable stationary phase and suitable mobile phase were used.

Brine Shrimp Lethality Test

Preparation of Shrimp Larvae

Preparation of shrimp larvae is done by weighing 50-100 mg of *Artemia salina* eggs per liter of water. Prepared a container containing seawater or salt water as much as 500 mL. The container is partitioned into two spaces connected by small holes as a medium for hatching. The hatching room is given dark conditions covered with aluminum foil while the other room is given lighting and aerated to supply oxygen. Sow the eggs in the dark area. Eggs will hatch into larvae and swim to the light area after 48 hours. The 48-hour-old shrimp larvae were used as test animals.

Preparation of Sample and Control Solutions

The n-hexane, ethyl acetate, and 96% ethanol extracts of mangkokan leaves were weighed as much as 100 mg. Dissolved the samples in their respective solvents as much as 100 mL to obtain a solution of 1000 µg/mL. Optimization was carried out by making 3 concentration variation points, namely 10 µg/mL, 100 µg/mL, and 1000 µg/mL. Put each sample into a vial. Put 10 Artemia salina and then 10 mL seawater. Let stand for 24 hours and calculate the percent value of larval mortality. Each concentration variation is done 3 times repetition, the control solution is done without the addition of extracts.

Toxicity Testing Using BSLT Method

Tests are carried out by taking 6 points of concentration variations that have a percent mortality value exceeding 50% of each extract, namely n-hexane, ethyl acetate, and ethanol extracts, and then putting each sample into a vial. Selected *Artemia salina* 48 hours old health. Put 10 Artemia salina into the vial and seawater and 10 mL. Then allowed to stand for 24 hours under lighting. After 24 hours, the number of living and dead larvae was counted with the help of a magnifying glass. There were 3 repetitions for each concentration variation. Percent larval mortality was calculated using the formula:

$$\%Larval\ mortality = \frac{number\ of\ deaths}{number\ of\ test\ larvae} x\ 100 \quad \text{.....(1)}$$

Data Analysis

The data obtained as the percent mortality value of the test larvae produced by the samples of mangkokan leaf extracts were calculated using probit analysis to obtain the LC50 value using the SPSS Statistics program.

RESULT AND DISCUSSION

The mangkokan plants used in the study were obtained from Sikanco Village, Nusawungu, Cilacap, Central Java. The plants were determined at the Central Laboratory of Padjajaran University Jl. Bandung Sumedang, Jatinangor, Sumedang Regency, West Java 45363 to find out and confirm the correct identity of the mangkokan plant. The determination results showed that the plant was a mangkokan leaf (*Polyscias scutellaria* (Burm.f.) Fosberg). The results of the characterization of mangkokan leaf simplisia in testing water content, ash content, extraction content in certain solvents, loss on drying, and specific gravity of extracts are presented in **Table 1**.

Table 1. Mangkokan Leaf Characterization

Examination	Result
Water content (% v/w)	6.2 ±1.31%
Total ash content (% w/w)	12.92±0.46%
Water soluble ash content (% w/w)	5.83±0.23%
Acid insoluble ash content (% w/w)	0.45±0.12%
Water soluble extraction content (% w/w)	32.12±0.75%
Ethanolic soluble extraction content (% w/w)	20.04±0.59%
Loss on drying (% w/w)	7.5±0.14%
Specific gravity of n-hexane extract	0.999
Specific gravity of ethyl acetate extract	1.000
The specific gravity of ethanol extract	1.004

Table 2. Yield of n-Hexane, Ethyl Acetate, and Ethanol Extracts of Mangkokan Leaf

Extracts	Yields (%w/w)
n-Heksana	2.57
Ethyl acetate	3.22
Ethanol	12.6

Extraction was carried out by cold extraction method, namely multistage maceration. Maceration is done at room temperature by soaking the simplisia powder using the appropriate solvent. This method was chosen because it is simple, low cost, easy to work with, and does not use heating so it is safe for thermolabile compounds. Multistage maceration is an extraction using solvents of different polarity. The purpose of multistage maceration is to separate or withdraw compounds according to their polarity distribution. The process of separating these compounds is based on the principle of like dissolved like, namely non-polar solvents will attract non-polar compounds, semi-polar solvents will attract semi-polar compounds and so will polar solvents attract polar compounds. The extract yield results can be seen in **Table 2**.

Phytochemical screening is carried out qualitatively on crude drugs and extracts to identify secondary metabolite compounds. Qualitative phytochemical screening will produce different color reactions in each treatment by using a reagent to bring out a metabolite (Vifta & Advistasari, 2018).

Ethyl acetate extract positively contains flavonoids, monoterpenes-sesquiterpenes, and steroids. Ethanol extract of mangkokan leaves positively contains flavonoids, saponins, and quinones. Phytochemical screening of all extracts showed multistage solvent extraction process successfully distributed compounds of mangkokan leaves to n-hexane to non-polar compounds, ethyl acetate to semi-polar compounds, and ethanol to polar compounds. Phytochemical results can be used to predict which compounds that responsible for toxicity activity in any extract from Mangkokan leaves.

The mangkokan leaf extract was then observed by thin-layer chromatography to qualitatively analyze the compounds contained in the extract. In the chromatogram profile of the n-hexane extract (**Figure 1**) using silica GF_{254} as stationary phase and n-hexane: ethyl acetate (8: 2) as mobile phase is suspected positive of secondary metabolite compounds of steroid group with Rf 0.636 and 0.690 as presence of greenish spots on visual appearance after spraying Lieberman-bouchard reagent and monoterpene-sesquiterpene group with Rf 0.673 and 0.727 as presence of brownish spots after spraying vanillin sulfate reagent.

Table 3. Phytochemical Screening of Mangkokan Leaf Simplisia and Extracts

Secondary Metabolite Compounds	Results			
	Crude drugs	n-hex	EtoAc	EtOH
Alkaloids	-	-	-	-
Flavonoids	+	-	+	+
Tannins	-	-	-	-
Polyphenols	+	-	-	-
Saponins	+	-	-	+
Quinones	+	-	-	+
Monoterpenes-sesquiterpenes	+	+	+	-
Triterpenoid-steroids	+	+	+	-

The chromatogram profile of ethyl acetate extract (**Figure 2**) using the mobile phase toluene: ethyl acetate: formic acid (5:4:1) are suspected secondary metabolite compounds of steroid groups with Rf 0.636; 0.690 and monoterpene-sesquiterpene groups with Rf 0.654; 0.709 characterized by a greenish spot on

visual appearance after spraying Lieberman-Bouchard for steroid groups and spraying vanillin sulfate showed a brownish spot for monoterpene-sesquiterpene groups. The flavonoid group is characterized by a change in color to a weak purple with an Rf of 0.945; after spraying the cyroborate spot.

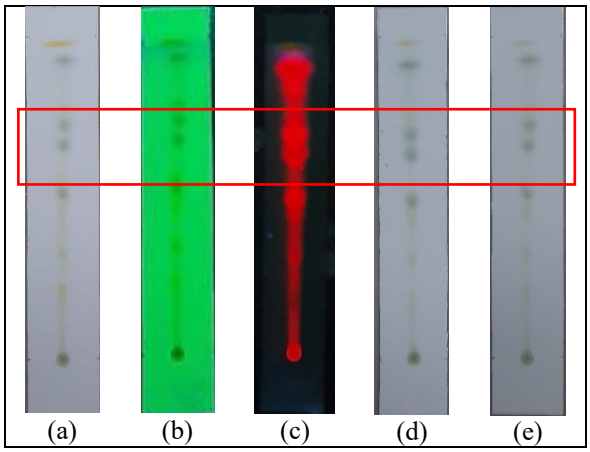

Figure 1. Chromatogram Profile of n-Hexane Extract. (a) Visual observation (b) UV 254 nm (c) UV 366 nm (d) Visual observation with Lieberman-Bouchard spray reagent (e) Visual observation with vanillin sulfate spray reagent

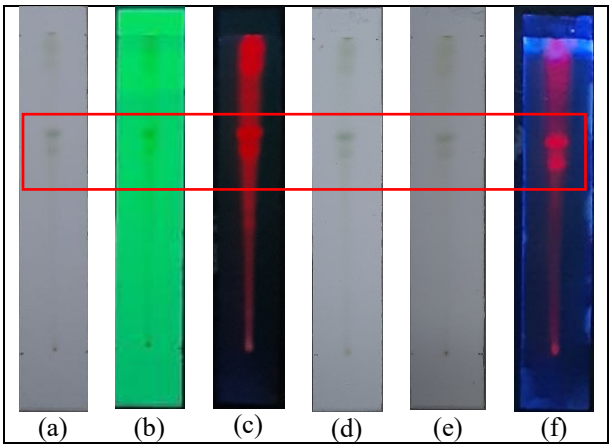

Figure 2. Chromatogram Profile of Ethyl Acetate Extract of Mangkokan Leaf, (a) Visual observation (b) UV 254 nm (c) UV 366 nm (d) Visual observation with Lieberman-Bouchard spray reagent (e) Visual observation with vanillin spray reagent

The chromatogram profile of the ethanol extract (**Figure 3**) using the mobile phase chloroform: methanol: formic acid (4:0.5:0.5) is suspected flavonoid secondary metabolite compounds with Rf 0.290 and 0.381 marked by a change in spot color to bluish green under UV 366 nm after being sprayed with cyroborate spotting agent.

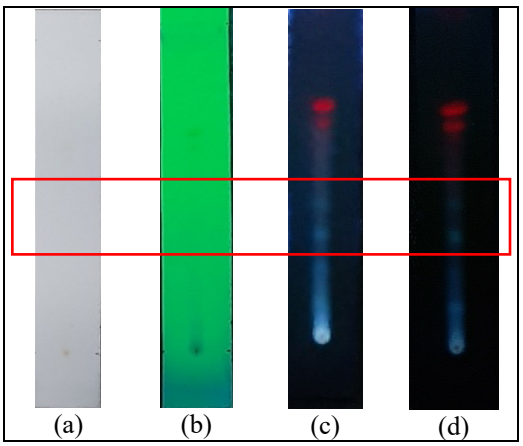

Figure 3. Chromatogram Profile of 96% Ethanol Extract of Mangkokan Leaf, (a) Visual observation, (b) UV 254 nm, (c) UV 366 nm (d) UV 366 nm with cytoborate spotting agent.

Toxicity testing was conducted using the BSLT method on n-hexane, ethyl acetate, and 96% ethanol extracts of mangkokan leaves. The BSLT method is a preliminary test that is widely carried out using test animals in the form of *Artemia shrimp* larvae to determine the presence of toxic compounds in an extract. The presence of compounds in the extract contains biological activity that is toxic to cause death to *Artemia* larvae.

The test was conducted 3 times on each test sample. Each extract was dissolved in seawater until homogeneous with the help of sonification. BSLT testing of n-hexane, ethyl acetate, and ethanol extracts provides a percent mortality value and produces an LC50 value, which is the result of probit analysis using SPSS Statistics (**Table 4**).

Table 4. LC50 Value of Each Mangkokan Leaf Extract

Sample	LC50 Value (µg/mL)
n-Heksane	146.724±6.1
Ethyl acetate	66.029±9.2
Ethanol 96%	92.007±5.9

An extract can be categorized as toxic if it has an LC50 value below 1000 µg/mL, it is called highly toxic if it has an LC50 value <30 µg/mL, toxic 31-1000 µg/mL, and non-toxic >1000 µg/mL (Meyer et al., 1982). The test results showed that n-hexane, ethyl acetate, and ethanol extracts were toxic, with LC50 values of 146.724 ± 6.1 µg/mL, 66.029 ± 9.2 µg/mL, and 92.007 ± 5.9 µg/mL respectively. The n-hexane, ethyl acetate, and ethanol extracts were categorized as toxic because the LC50 values were in the range of 31-1000 µg/mL.

Ethyl acetate extract showed the lowest LC50 with 66,029 µg/mL as the highest toxic activity with suggested responsibility compounds are flavonoids and terpenoids. Ethanol extract is categorized as a toxic extract with suggested responsible compounds such as flavonoids, quinones, and saponins. N-hexane extracts showed toxic activity with suggested responsibility compounds are terpenoid groups.

Larval mortality in various variations of extract concentrations is due to direct contact of active compounds with larvae. Mangkokan leaves have active compounds that are toxic such as flavonoids, saponins, monoterpenes-sesquiterpenes, and steroids. These compounds work to attack the larval digestive apparatus as a stomach poison so that the lack of larval food intake is reduced (Setyowati & Cahyanto, 2016). These compounds cause the larvae to not get a taste stimulus in the mouth so that the larvae are unable to recognize their food, resulting in starvation (Yulistyana et al., 2020).

The mechanism of action of shrimp larvae death due to the presence of flavonoid compounds in the cell environment causes the OH- group on flavonoids to bind to integral proteins of the cell membrane. This causes the active transport of Na^+ and K^+ to be blocked. Active transport that stops causes uncontrolled entry of Na^+ ions into the cell, this causes rupture of the cell membrane, resulting in cell death or shrimp larvae (Sanjayasari & Pliliang, 2011) . In addition, flavonoids work by activating the apoptotic pathway of cancer cells. This apoptotic mechanism occurs due to the breakdown of DNA, which is characterized by the removal of the DNA proximal chain by relative oxygen compounds such as hydroxyl radicals. (Woo & Kim, 2013)

Saponins work by lowering the surface tension of the larval digestive membrane so that the digestive tract exposed to the compound is damaged (Abriyani et al., 2022). The content of saponin compounds has the potential as an anticancer with the mechanism of cell apoptosis and inducing cell cycle arrest. (Supriningrum et al., 2017).

Monoterpenes-sesquiterpenes belong to the class of essential oils that cause damage to cell walls and enzymes due to disruption of larval cell metabolism so that larvae die of starvation. (Puspa et al., 2017). Steroids can cause cancer cells to undergo necrosis and cell death due to damage to mitochondrial membrane permeability in cancer cells. (Putri & Winata, 2019).

CONCLUSIONS

The content of secondary metabolites in mangkokan leaves are flavonoids, saponins, monoterpenes-sesquiterpenes, and steroids. The results of BSLT testing on n-hexane, ethyl acetate, and 96% ethanol extracts of mangkokan leaves respectively are 146.724 ± 6.1 µg/mL; 66.029 ± 9.2 µg/mL; and 92.007 ± 5.9 µg/mL. The results showed that the three extracts were toxic. Ethyl acetate extract showed the lowest LC50 with 66.029 µg/mL as the highest toxic activity with suggested responsibility compund are flavonoids and terpenoids. Ethanol extract categorized as toxic extract with suggested responsibility compound are flavonoids, quinones, and saponins. N-hexane extracts showed toxic activity with suggested responsibility compound are terpenoids groups.

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AUTHORS' CONTRIBUTIONS

Conception and study design were done by Soraya and Akhirul. All authors provided administrative technical/logistic support, conducted data collection, and data analysis. Critical revision of the article for important intellectual content was done by Akhirul and Soraya; all authors contributed to article drafting and approved final version.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this article.

ETHICAL CONSIDERATION

Ethical issues (including plagiarism, data fabrication, double publication, etc) have been completely observed by the author.

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