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Potential Toxicity of Legundi Leaf Extract (*Vitex Trifolia* L) Using the Brine Shrimp Lethality Test (BSLT) Method

Zil Arifah[1], Mai Efdi[1]*, Syafrizayanti[1]

1 Department of Chemistry, Faculty of Mathematics and Natural Sciences, Andalas University, Padang, 25163, Indonesia.

A B S T R A C T

Many natural products can be used as starting points in developing modern medicines because of their capabilities in pharmacological activities. Vitex trifolia L is an herbal plant that has been used to treat diseases such as fever, inflammation, colds, irregular menstruation, and diseases related to the female reproductive organs. This study aims to identify the cytotoxic ability of Vitex trifolia L leaf extract using the Brine Shrimp Lethality Test (BSLT) method. Extraction was carried out by maceration and fractionation methods, followed by phytochemical assay and cytotoxic assay using BSLT method. The results showed that the n-hexane extract had a moderate cytotoxic effect (LC_{50} 241 µg/ml), the methanol extract included in the low toxic category (LC_{50} 995 µg/ml) and the other two extracts involved in the non-toxic category (ethyl acetate and butanol).

Keyword: *Vitex trifolia* L leaves, phytochemicals, BSLT, cytotoxicity

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* coresponding author: Mai Efdi (maiefdi@sci.unand.ac.id)
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INTRODUCTION

At this time the demand and use of herbal medicines among the public is constantly increasing. The use of herbal medicines is considered more secure with much less side effects than synthetic drugs. Indonesia has a variety of medicinal plants that grow well, making Indonesia one of the best herbal medicine producing countries in the world. *Vitex trifolia* L or legundi is one of the medicinal plants that has been widely used in the world of pharmacology. *Vitex trifolia* L is a tropical plant that belongs to the *Verbenaceae* family in the form of shrubs or small trees, generally *Vitex trifolia* L plants are known Three-Leaved Chaste Tree (Annamalai & Thangam, 2022)(Parkhe & Jain, 2019).

Figure 1. Vitex trivolia L plants

Vitex trifolia L has many benefits as a medicinal plant because it contains many active compounds such as secondary metabolite compounds of terpenoids, flavonoids, phenolics, lignans, and steroids. (Djimabi et al., 2022) (Kamal et al., 2022). Several research studies have investigated the benefits of the *Vitex trifolia* L plant such as anti-tumour (Gong et al., 2021), analgesics, antipyretics, anti-inflammatory (Annamalai & Thangam, 2022), antioxidants (Efdi et al., 2022), antibacterial (Ida Bagus Oka Suyasa et al., 2022), and possesses larvicidal bioactivity (Musa et al., 2020).

Various experiments have been used to determine the toxicity of herbal medicines based on several biological methods, one of which is the Brine Shrimp Lethality Test (BSLT) method. This method is one of the initial methods that is often used in testing the cytotoxic properties of an extract, fraction or compound using the *Artemia salina* shrimp larvae test. (Riskianto et al., 2022). The BSLT method has been shown to have a correlation with anticancer activity. *Artemia salina* larvae are considered representative zoological organisms for in-vivo lethality tests with the parameter used being the LC_{50} value (*lethal concentration*) (Fauziah et al., 2022). This research is to be considered as a reference in exploring the toxicity ability of *Vitex trifolia* L leaves as an effective substance in supporting the further research on anti-cancer effects.

METHODS

General Experimental Procedures

The equipment in this study is maceration container, filter paper, separatory funnel, vial, rotary evaporator, distillation instrument, Artemia salina leach egg incubator, laboratory glassware. The materials used in this study were seawater, n-hexane, ethyl acetate, butanol and methanol, chloroform, distilled water, FeCl3, mercury (II) chloride, KI, H2SO4, acetic anhydride, sodium hydroxide, Mg powder, and ammonia, Meyer reagent, KLT plates and Artemia salina Leach larval eggs.

Sample Identification

Leaf of the plant was collected from Andaleh Baruah Bukit, Batusangkar with an height of 600 - 1200 meters above sea level, which is located between 100037.22‘-100040'19’ east and 0024'36’ west. The identification of this plant was carried out at the Herbarium Laboratory of the Department of Biology, Faculty of Mathematics and Natural Sciences (MIPA), Andalas University.

Preparation and extraction

Samples of *Vitex trifolia* L leaves were collected and air-dried, grinded using a grinder and obtained a dry mass of 4 kg. Samples that have been processed were macerated using methanol solvent as much as 5 L with 8 repetitions. The extract obtained was then concentrated using a rotary evaporator to obtain methanol extract. The concentrated methanol extract was fractionated using n-hexane, ethyl acetate and butanol using a partition funnel. The methanol concentrated extract was suspended using distilled water and then 500 mL of n-hexane was added, stirred and put into a partition funnel. Then the mixture was stirred again and allowed to stand until two layers were formed. The top layer which is the n-hexane layer is separated from the methanol layer. This process was repeated until the Vitex trifolia L plant extract obtained was completely clear. The fractions obtained were concentrated using a rotary evaporator. The fractionation stage was continued for ethyl acetate solvent, with the

same working process as the fractionation process using n-hexane solvent using ethyl acetate solvent. The fractionation step was continued using buthanol solvent. Each fraction obtained was concentrated again using a rotary evaporator. After obtaining three fractions, namely n-hexane, ethyl acetate, and butanol fractions, the fractions were allowed to dry and their mass was determined.

Secondary Metabolite Assay

Vitex trifolia L leaf extract was measured at 2 mg, taken into a test tube and dissolved with solvent. Phytochemical testing of Vitex trifolia L plant extract as follows:

Flavonoid Assay

Vitex trifolia L leaf extracts that have dissolved each put into a test tube, added concentrated hydrochloric acid and a few grains of magnesium powder. Positive results are indicated by the formation of orange to red colour in the test solution.

Phenolic Assay

Vitex trifolia L leaf extracts that have dissolved each put into a test tube, then added iron (III) chloride solution and observed the colour change of the solution. If the solution is blue or purple, it indicates that it is positive for phenolic compounds

Saponin Assay

The extracts of *Vitex trifolia* L leaves that have dissolved are each shaken until foam forms, then added with concentrated hydrochloric acid. If the foam does not disappear (± 5 minutes) indicates that the sample contains saponins..

Triterpenoid dan Steroid Assay

Vitex trifolia L extract leaves that have dissolved each dripped on the hole of the drip plate is allowed to dry, then added concentrated sulfuric acid and acetic anhydride (Liebermannn Burchard). If a red to purple ring is formed, indicating the sample contains triterpenoid compounds and if a green or blue green ring is formed, indicating the presence of steroid compounds. If there are both rings, it indicates the presence of both compounds..

Alkaloid Assay

Vitex trifolia L extract leaves that have dissolved each added 5 mL chloroform ammonia then added 2N sulfuric acid to form two layers. The acid layer is separated and Mayer reagent is added. If a white precipitate occurs, it indicates that the sample contains alkaloids.

Coumarin Assay

The dissolved Vitex trifolia L leaves extracts were each spotted on a KLT plate using a pipette capillary and eluted with n-hexane:ethyl acetate eluent in the chamber. The eluted KLT plates were observed under UV light at λ 356 nm, if there was blue fluorescence and after spraying with 10% NaOH the blue colour became brighter, indicating the presence of coumarin.

Cytotoxic Bioactivity Assay of *Vitex trifolia* L Extract by BSLT Method (*Brine Shrimp Lethality Test*)

Preparation of test solution

50 mg of Vitex trifolia L extract was dissolved with each solvent to obtain a solution concentration of 1000 µg/mL. The test solution with a concentration of 1000 µg/mL was then taken back as much as 25 mL

in the same way, so as to obtain a concentration of 500 µg/mL. This was also repeated to obtain concentrations of 250; 125; 62.5; 31.25; and 15.625 µg/mL.

Culturing of Shrimp Larvae

Seawater was put into a glass box consisting of two parts, the dark section and the light section, equipped with lights and aerators. Shrimp eggs are put into the dark side of the glass box and left for 48 hours at room temperature until the larvae move to the light side. These larvae will be used as experimental animals in the cytotoxic Brine Shrimp Lethality test (BSLT).

Cytotoxic Bioactivity Assay

Each test solution with concentrations of 1000; 500; 250; 125; 62.5; 31.25; and 15.625 µg/mL was put 5 mL into a vial, and left to dry. After the fraction dried, 50 µL of DMSO was added until homogeneous. Shrimp larvae were inserted into each vial and the total volume was increased to 5 mL by adding seawater. After 24 hours, the number of shrimp larvae that died in each test solution was determined. The number of dead shrimp was used to calculate the LC50 value through probit analysis and regression equation.

RESULTS

Extracts of Vitex trifolia L Plant Leaves

Vitex trifolia L leaves samples that have been collected and plant identification carried out at the Andalas University Herbarium (letter number 488/K-ID/ANDA/XI/2022) shows the sample belongs to the family Verbenaceae with the species name Vitex trifolia L. Dry samples of Vitex trifolia L that have been macerated using methanol solvents and separated by the Fractionation method using solvents from non-polar to polar solvents starting from n hexane, ethyl acetate, and buthanol. Extraction results can be seen in Figure 1.

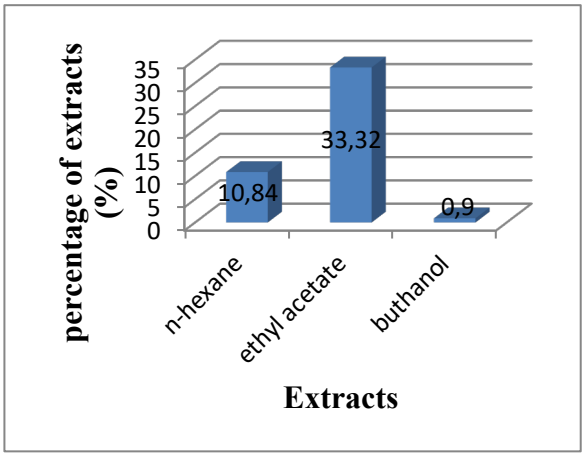

Figure 1. Extraction results of *Vitex trifolia* L leaves

Figure 1 shows the percentage of extracts obtained, which are 10.84% hexane extract, 33.32% ethyl acetate extract and 0.9% buthanol extract. Ethyl acetate extract produces the highest percentage of content, compared to other extracts. These results indicate that *Vitex trifolia* L leaf extract contains more compounds that are semi-polar compared to other classes of compounds. The results of this study are in accordance with the data obtained by (Indrayudha & Cahyani, 2020) that in *Vitex trifolia* L leaves the most compound components are in ethyl acetate extract with a yield of 24.2%.

Secondary Metabolite

Secondary metabolite content tests or phytochemical tests were carried out on the initial methanol extract from maceration and 3 types of fractionated extracts obtained. The results of the secondary metabolite content test of Vitex *trifolia* L leaves extracts can be seen in Table 1.

Table 1. Secondary metabolite test results of Vitex *trifolia* L leaves

No	Secondary Metabolite	Reagents	MeOH extract	n-hexana extract	EtOAc extract	Butanol extract
1	Phenolic	$FeCl_3$	(+)	(+)	(+)	(+)
2	Flavonoid	Sianidin test	(+)	(+)	(+)	(+)
3	Saponin	HCl	(-)	(+)	(-)	(-)
4	Triterpenoid	Liebermannn Burchard	(+)	(+)	(-)	(-)
5	Steroid	Liebermannn Burchard	(-)	(-)	(+)	(-)
6	Cumarin	NaOH	(-)	(-)	(-)	(-)
7.	Alkaloid	Mayer	(+)	(-)	(+)	(-)

Description + = contains secondary metabolites
- = does not contain secondary metabolites

Table 1 indicates that the leaves of Vitex trifolia L plant in the initial methanol fraction contain several classes of compounds such as phenolics, triterpenoids and alkaloids. Meanwhile, after the fractionation process, several different secondary metabolite compounds were obtained in the n-hexane, ethyl acetate, and butanol extracts of compounds such as saponins, steroids, and alkaloids. Research conducted by (Shukri & Hasan, 2021) which shows a slight difference with the phytochemical results on the ethyl acetate extract obtained, where (Shukri & Hasan, 2021) obtained a class of saponin compounds. The difference in the identification of secondary metabolites can be caused by environmental factors where plants grow (Sahidin, 2012).

Cytotoxic Bioactivity of *Vitex trifolia* L Extract by BSLT Method (*Brine Shrimp Lethality Test*)

The obtained *Vitex trifolia* L extracts were subjected to cytotoxic test using the Brine Shrimp Lethality Test (BSLT) method. This method is used to determine the LC_{50} value of several concentration variations of each extract. The test results are presented in Table 1.

Table 2. Toxicity Test Results of Vitex trifolia L. Leaf Extract

Sample	Concentrations (µg/ml)	shrimp larvae	Average mortality of larvae	Percentage of deaths (%)	Probit value	LC_{50} (µg/ml)
N-Hexane	1000	30	20,0	67	5,44	241
	500	30	18,3	61	5,28	
	250	30	14,7	49	4,97	
	125	30	13,0	43	4,82	
	62,5	30	10,0	33	4,53	
	31,25	30	8,0	27	4,39	
	15,625	30	5,0	17	4,05	
Ethyl Acetate	1000	30	9,0	30	4,48	21.812
	500	30	7,7	26	4,36	
	250	30	7,0	23	4,26	
	125	30	6,3	21	4,19	
	62,5	30	5,3	18	4,08	
	31,25	30	4,3	14	3,92	
	15,625	30	3,3	11	3,77	

Sample	Concentrations (µg/ml)	shrimp larvae	Average mortality of larvae	Percentage of deaths (%)	Probit value	LC_{50} (µg/ml)
Buthanol	1000	30	9,0	30	4,48	36.968
	500	30	7,0	23	4,26	
	250	30	6,3	21	4,19	
	125	30	5,7	19	4,12	
	62,5	30	5,0	17	4,05	
	31,25	30	4,0	13	3,87	
	15,625	30	3,3	11	3,77	
Methanol	1000	30	15,3	51	5,03	995
	500	30	12,3	41	4,77	
	250	30	10,0	33	4,56	
	125	30	9,0	30	4,48	
	62,5	30	6,7	22	4,27	
	31,25	30	6,0	20	4,16	
	15,625	30	3,3	11	3,77	

The data in Table 1 shows that the mortality rate of shrimp larvae varies with each concentration variation, the greater the concentration of the test solution, the greater the mortality rate of shrimp larvae, this is due to the greater and more potential amount of active compound composition contained in the solution used.

The larvae used are the larvae that have been grown for 48 hours where the organs in the larvae have been formed and are in the most sensitive state. In the calculation data (LC50) of the 4 types of extracts used, it is known that n-hexane extract has a stronger toxicity ability than the other 3 extracts and is included in the medium toxic category followed by methanol extract which is included in the low toxic category and ethyl acetate and butanol extracts are included in the non-toxic category. This toxic compound can interfere with this based on the category of toxicity level of a compound, where a compound is said to be very toxic if it has an LC_{50} value of 0-100 µg/mL, medium toxic has an LC_{50} value of 100-500 µg/mL, low toxic 500-1000 µg/mL and not toxic if it has an LC_{50} value > 1000 µg/mL (R. Hamidi et al., 2014). The mechanism of the extract obtained that can cause the death of artemia larvae is by inhibiting taste receptors in the larval mouth organs so that the larvae are unable to recognize food so that the extract is eaten by larvae and damages the digestive organs and causes larval death or also known as stomach poisoning.

Conclusion

Vitex trifolia L plant leaves are known to contain many non-polar compounds and the results of phytochemical profiles are known to contain several classes of compounds such as phenolics, flavonoids, triterpenoids, alkaloids, saponins and steroids. Based on the cytotoxicity test, it is known that the n-hexane extract has moderate toxicity with an LC_{50} value of 241 µg/ml, methanol extract is included in the low toxic category with an LC_{50} value of 995 µg/ml and ethyl acetate and butanol extracts are included in the non-toxic category.

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