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Analysis of The Essential Oil from *Lantana camara* Leaves and Its Cytotoxic Potential Against T-47D Breast Cancer Cells

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Abstract

Lantana camara Linn is a family of Verbenaceae which grows wild and is widespread in various both tropical and sub-tropical countries. Isolation essential oil of the *L. camara* leaves extracted by hydrodistillation and were analyzed by Gas Chromatography-Mass Spectrometry (GC-MS). The results of GC-MS analysis show isocaryophyllene (14.39%), *ρ*-cymene (8.17 %), β-cubebene (7.8 %), *α*-pinene (7.64 %), and β-elemene (5.51 %) as the main compound. The cytotoxic activity of the isolated essential oil was highly toxic both to *Artemia salina* shrimp larvae (LC_{50} 15.92 μg/mL) and to breast cancer cells T-47D (IC_{50} 10.67 μM).

Keywords: *Lantana camara, cytotoxic, BSLT assay, MTT assay*

Introduction

Lantana camara Linn is one of Indonesia's traditional medicinal plants, including the Verbenaceae family, which is widespread in tropical and sub-tropical regions. This plant can thrive up to an altitude of 2000 meters above sea level. Traditionally, *L. camara* has been used to cure various diseases such as skin diseases, digestive disorders, inhalation, and also as an antiseptic[1].

From previous studies, it has been reported that the cytotoxic activity of the hexane extract of *L. camara* leaves showed strong activity with LC_{50} value 34.2972 μg/mL[2]. Several cytotoxic compounds have also been isolated from this plant, such as lantanilic acid which shows strong activity against shrimp larvae of Artemia salina L with LC_{50} value 27.99 μg/mL[2], lantadene A with LC_{50} value 48.97 μg/mL[3], and lantadene B had even been tested for its cytotoxic potential against MCF-7 breast cancer cells, and showed strong activity with IC_{50} value 1.134 μM[4]. Several studies have reported on the use of essential oils in herbal medicine, including for treating various human diseases such as skin itching, leprosy, cancer, chickenpox, measles, asthma, ulcers, tumors, high blood pressure, tetanus, and rheumatism[5]. Moreover, essential oils of *L. camara* leaves have also been used as insecticides, repellents for bees, mosquitoes, and flies. This is caused by chemical components such as those that give essential oils their distinctive odor[6].

Essential oils isolated from *L. camara* leaves have been reported and shown various biological

activities such as antibacterial, cytotoxic[7], antimicrobial[8], insecticidal[9], allelopathic[10], fumigant[11] and larvicidal[12]. Though, the essential oil composition and bioactivity of *L. camara* leaves has been previously reported widely, but the cytotoxicity activity of the oil against T-47D breast cancer cells have not been investigated. In this paper, will report the cytotoxicity activity against T-47D breast cancer cells. Isolation of essential oils was carried out using the hydrodistillation method, the analysis was carried out by Gas Chromatography-Mass Spectrometry (GC-MS). Furthermore, for the preliminary cytotoxic test was carried out using the Brine Shrimp Lethality Test (BSLT) method and continued with the MTT test against T-47D breast cancer cells. The results of this study will complement the information about the chemical content of *L. camara* and its cytotoxic activity.

Experimental

Materials

The materials used for the isolation of essential oils were the dried powder of the leaves of the *L. camara* plant. Distilled water, and anhydrous sodium sulphate (Sigma-Aldrich) were obtained from Natural Product Organic Chemistry Laboratory, Department of Chemistry, Universitas Andalas. Materials for the cytotoxic activity test were used larvae of *Artemia salina* shrimp, seawater, T-47D breast cancer cells, Roswell Park Memorial Institute (RPMI) 1640 medium, MTT reagent (3- (4, 5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide), Fetal Bovine Serum (FBS), antibiotics (mixture of penicillin and streptomycin) and dimethyl sulfoxide (DMSO) were preserved in Cell Culture Laboratory, Faculty of Pharmacy, Universitas Andalas. Helium gas were purchased from PT. Air Liquide Indonesia.

Instruments

Clevenger-type apparatus, Eppendorf tube, Eppendorf serological pipets, oven, aerator, micropipette, 96-well plates, syringe pumps, syringe filters, automated hemocytometer plates, and laminar airflow safety cabinets. The instruments were used for essential oil analysis is GC-MS (GC-MS-QP-2010, Shimadzu, Tokyo, Japan) using the capillary column Rxi-5MS (60 m × 0.25 mm × 0.25 μm).

Essential oil hydrodistillation

The leaves of *L. camara* were obtained from Batu Busuk, Lambuk Bukik, Pauh, Padang (0°54'06.7 "S 100°27'35.0"E). Fresh leaves of *L. camara* (520 g) were cleaned, coarsely chopped, then dried, and dried leaves (145 g) were obtained. The essential oil was isolated using the steam distillation method for 7 hours using a Clevenger apparatus[13]. The essential oil obtained is then added with sodium sulfate anhydrous and stored in the refrigerator until further use. The percentage of oil and its specific gravity were analyzed for essential oils.

GC-MS and data analysis

The essential oil of *L. camara* leaves was analyzed using GC-MS-QP-2010 (Shimadzu, Tokyo, Japan) equipped with AOC-20i auto-sampler and single quadrupole MS as a detector. The column used is the capillary column Rxi-5MS (60 m × 0.25 mm × 0.25 μm). Samples were injected as much as 0.1 μL with a split ratio of 1:250 without using solvents and the temperature of the injectors and detectors was 200-230 °C. Helium as the carrier gas flowing at a rate of 45.5 mL/min. The column temperature is set at 60 °C for 1 minute, after which the temperature will increase by 10 °C/ minute to 210 °C and then left constant for 1 minute. The m/z value read from MS is 45-500 AMU with ionization energy of 70 eV with a scanning time of 3 seconds. Parameter settings for GC-MS as well as data recording and processing were carried out using the GC-MS Shimadzu version 4 software. Isolated essential oils were identified using comparisons with data from the National Institute of Standards and Technologies (NIST) 14.

Cytotoxic activity test

The preliminary cytotoxic activity test of the isolated essential oil was carried out on *Artemia salina* shrimp larvae using the BSLT method referring to the work procedure performed by Meyer, et al. (1982) with slight modifications[14]. The cytotoxic activity test using the MTT method refers to the protocol that has been carried out by Mosmann (1983) with slight

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modifications[15]. The essential oil of *L. camara* leaves was dissolved with DMSO to obtain a 100 mM stock solution. Variation's concentration of test solutions of 100 µM, 10 µM, 1 µM, and 0.1 µM were prepared by diluting stock solutions with RPMI medium.

T-47D breast cancer cells are taken from storage and grown on RPMI medium that containing 10% serum fetal bovine (FBS) and antibiotics (1% penicillin-streptomycin), incubated for 24 hours in an incubator at 37 °C, humidity 95% and 5% CO_2 concentration. Cell growth is observed every day using an inverted microscope. Cells are harvested when confluent cells reach 80%. Harvested cells are suspended in 96-well plates containing complete medium and the cell density is determined to be 2×10^3 cells / mL T-47D breast cancer cells using a hemocytometer.

MTT assay is a common method used in determining the toxicity of a sample against a target cancer cells. Harvested T-47D breast cancer cells (2×10^3 cells / mL) were grown on 96-well plates and added 20 µl each test solution. Cells were incubated in an incubator for 24 hours at 37 °C, 95% humidity, and 5% CO_2 concentration. After that, the cell media was removed and washed with 100 µl FBS. MTT reagent (0.5 mg/mL) was added to the plate and re-incubated for 4 hours. The MTT solution was removed, the formazan salt crystals formed were then dissolved with 100 µL DMSO and absorbance is measured at 550 nm using an ELISA plate reader[16]. The absorbance data obtained is converted to percent cell viability using the formula:

$$\% \text{ Cell viability} = \frac{(\text{sample absorbance - negative control absorbance})}{(\text{positive control absorbance - negative control absorbance})} X\ 100\%$$

The level toxicity of the sample is determined based on IC_{50} values. The variation of the concentration of the test solution with the percentage of cell viability is displayed in graphical form and the IC_{50} value is determined using Graph Pad Prism 9.0 software.

Results and Discussion

Essential oil of *L. camara* leaves

The *L. camara* leaves sample contained 72.2% moisture content, from 520 grams of fresh samples obtained 145 grams of dry samples. Isolation of the essential oil from 145 grams of dry leaves using the hydrodistillation method obtained 0.125 mL of essential oil (yield 0.0781% w/w) and a specific gravity of 0.906 g/mL.

The results of the analysis of the chemical content of essential oils using GC-MS showed that there were 38 peaks identified as 38 chemical compounds with different levels, the results were listed in Table 1.

Tabel 1. The composition of the essential oil of *L. camara* leaves

No	RT [a] (Minutes)	Compound	Molecular formula	Area (%)	SI [b] (%)
1	5.938	5,5-Dimethyl-2-ethyl-1,3-cyclopentadiene	C_9H_{14}	0.04	92
2	6.071	Cis-3-hexenol	$C_6H_{12}O$	0.19	90
3	6.248	Hexyl formate	$C_7H_{14}O_2$	0.07	92
4	7.330	α-Phellandrene	$C_{10}H_{16}$	2.72	95
5	7.511	α-Pinene	$C_{10}H_{16}$	7.65	95
6	7.776	Camphene	$C_{10}H_{16}$	0.71	93
7	8.159	2-ρ-Menthadiene	$C_{10}H_{16}$	4.16	95
8	8.278	β-Pinene	$C_{10}H_{16}$	4.72	95

9	8.343	β-Myrcene	$C_{10}H_{16}$	0.95	91
10	8.668	α-Phellandrene	$C_{10}H_{16}$	1.42	96
11	8.871	α-Terpinene	$C_{10}H_{16}$	1.20	96
12	9.062	ρ-Cymene	$C_{10}H_{14}$	8.18	93
13	9.119	Sylvestrene	$C_{10}H_{16}$	1.04	93
14	9.300	β-Ocimene	$C_{10}H_{16}$	2.34	94
15	9.563	γ-Terpinene	$C_{10}H_{16}$	3.24	96
16	10.054	α-Terpinolene	$C_{10}H_{16}$	0.54	82
17	10.140	β-Linalool	$C_{10}H_{18}O$	0.71	93
18	11.326	Pinocarvone	$C_{10}H_{14}O$	0.29	88
19	11.379	Borneol	$C_{10}H_{18}O$	0.14	91
20	11.514	Terpinen 4-ol	$C_{10}H_{18}O$	2.24	95
21	11.688	Linalyl propionate	$C_{13}H_{22}O_2$	0.51	90
22	13.050	ρ-Thymol	$C_{10}H_{14}O$	1.78	93
23	13.208	ρ-Thymol	$C_{10}H_{14}O$	0.53	94
24	13.808	δ-Elemene	$C_{15}H_{24}$	1.74	90
25	13.982	α-Cubebene	$C_{15}H_{24}$	0.91	89
26	14.600	β-Elemene	$C_{15}H_{24}$	5.52	91
27	15.194	Isocaryophyllene	$C_{15}H_{24}$	14.42	92
28	15.242	γ-Muurolene	$C_{14}H_{20}O$	0.31	82
29	15.558	α-Humulene	$C_{15}H_{24}$	4.31	93
30	15.632	β-Panasinsene	$C_{15}H_{24}$	0.77	89
31	15.933	β-Cubebene	$C_{15}H_{24}$	7.81	92
32	16.298	δ-Cadinene	$C_{15}H_{24}$	4.26	90
33	17.250	Epoxycaryophyllene	$C_{15}H_{24}O$	4.15	92
34	17.508	Ginsenol	$C_{15}H_{26}O$	1.42	74
35	17.644	Isospathulenol	$C_{15}H_{24}O$	3.28	81
36	17.817	τ-Muurolol	$C_{15}H_{26}O$	2.49	82
37	18.121	Solium	$C_{15}H_{24}O$	1.27	78
38	18.329	Eudesma-4,11-dien-2-ol	$C_{15}H_{24}O$	1.97	77
Class composition					
Hydrocarbon monoterpenes					34.2
Hydrocarbon sesquiterpenes					21.1
Oxygenated monoterpenes					15.8
Oxygenated sesquiterpenes					15.8
Other					10.8
Total compounds identified					97.8

[a] Retention time (Minutes)
[b] Similarity index (%)

Thirty-eight compounds were identified, there were 14 compounds that had levels smaller than 1%, 19 compounds that have levels between 1-5% and 5 compounds that have levels greater than 5%. Based on these data, there are 5 main compounds with percent area content >5%, namely isocaryophyllene (14.39% SI 92%), ρ-cymene (8.17% SI 93%), β-cubebene (7.8% SI 92%), α-pinene (7, 64% SI 95%) and β-elements (5.51% SI 91%) (Figure 1).

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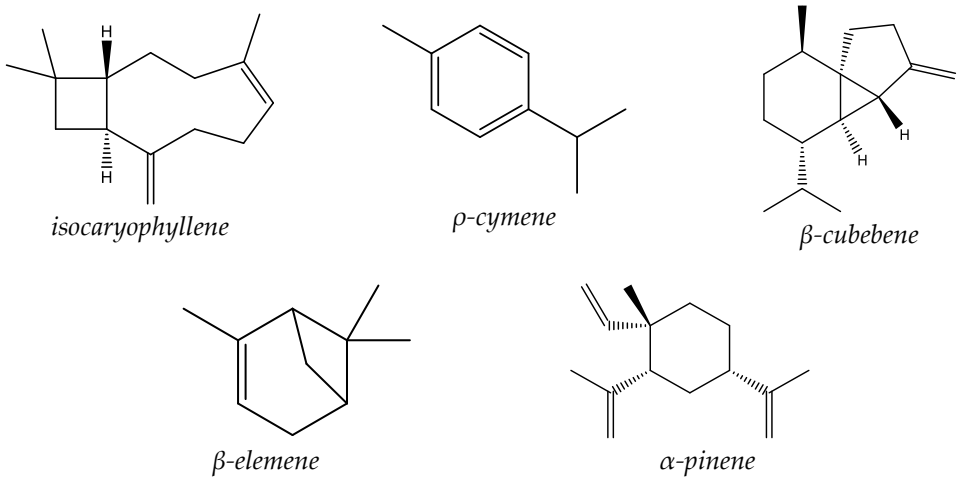

Figure 1. The main compound structure of the isolated essential oil.

The group of caryophyllene compounds has been reported as the main constituent of the essential oil of *L. camara* leaves in various regions with different percentages such as (E) - caryophyllene (14.31%) in Brazil[17], β-caryophylene (8.9%) in Nigeria[5], β-caryophyllene (27.1%) in Cote d'Ivoire[18], β-caryophylene (9.4%) in India[13], caryophyllene oxide (7.5%) in Saudi Arabia[19], β-caryophylene (35.70%) caryophyllene oxide (10.04%) in Algeria[11] and cis-caryophyllene (16.2%) in Iran[20]. Besides, caryophyllene derivatives (10.87%) are also reported to be the main component of *L. camara* flower essential oil which is obtained from the North Sumatra region[21].

The difference in the percentage and constituent components contained in each essential oil varies widely. This is influenced by the genetic diversity in a different plant population. Several extrinsic factors such as geographic and climatic conditions, stress caused by drought, insects or microorganisms, collection time, drying and storage conditions, and extraction methods can also affect the composition of these essential oils[17],[22].

Cytotoxic Activity Test

As a preliminary test, the cytotoxic activity of the essential oil isolated from *L. camara* leaves was evaluated using the BSLT method, then tested for its cytotoxic potential using the MTT method against T-47D breast cancer cells. The results of the cytotoxic activity test using the BSLT method showed the effect of mortality on *A. salina* shrimp larvae as test animals. The concentration of the test solution greatly influences this mortality effect. At high concentrations (100 µg / mL or log 2 concentration) the test compound showed a mortality rate of 87% (probit value 6.13) (Figure 2).

Bioactivity is one of the most studied subjects in essential oil research both in vivo and in vitro. Generally, these major components of essential oils such as terpenes/terpenoids and aromatic and aliphatic compounds determine their biological properties[23]. Caryophyllene is the main constituent of essential oil, which is known to show a rich in bioactivities[24],[25]. Besides, components containing only an aromatic ring with alkyl substituents as in p-cymene has been observed to be rich in bioactivities although some activities are considered weak[26].

The antagonistic and synergistic effects are also important in evaluating the biological activities because there are many components present in essential oils either as main components with high concentrations or as small components. Besides that, essential oils which generally have lipophilic properties to make it easier shrimp larvae death by impaired cell membrane permeability with allow them to cross cell membranes, alter membrane composition, and

increase membrane fluidity, leading to leakage of ions and cytoplasmic molecules[25],[27]. This is explained by Raineri (1981) where the toxic compounds can be absorbed through cell membranes. A drastic change in concentration between inside and outside the cell will cause toxic compounds to be well distributed and cause metabolic reactions to damage in larvae[28]. On cells, the main mechanisms the cytotoxic effects of essential oils include the induction of cell death by activation of apoptosis or necrosis processes, cell cycle arrest, and loss of function essential organelles[25].

Cytotoxic activity tests were carried out using the MTT assay This method is based on the formation of formazan salt crystals from the reduction of MTT solution by the enzyme dehydrogenase produced by mitochondria in cells. Formazan salt crystals that are formed are purple and this can indicate the viability of the target cell being tested. In living cells, the dehydrogenase enzyme will reduce MTT to produce formazan salt crystals and will be characterized by the formation of an increasingly deep purple color. In dead cells, the dehydrogenase enzyme can no longer be produced, so the MTT reduction reaction cannot take place and formazan salt crystals are not produced[15].

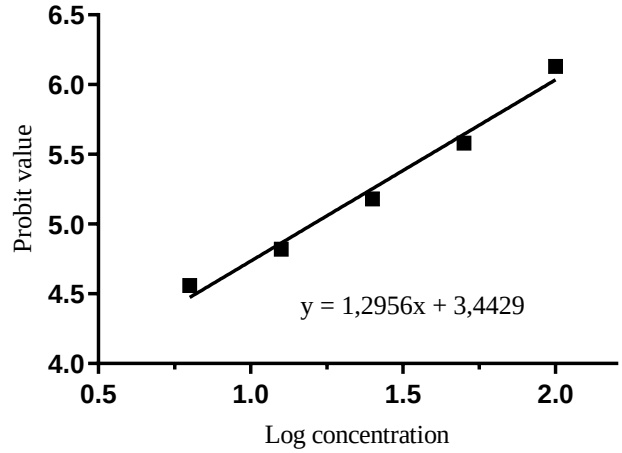

Figure 2. Cytotoxic activity of *L. camara* leaf essential oil using the BSLT method.

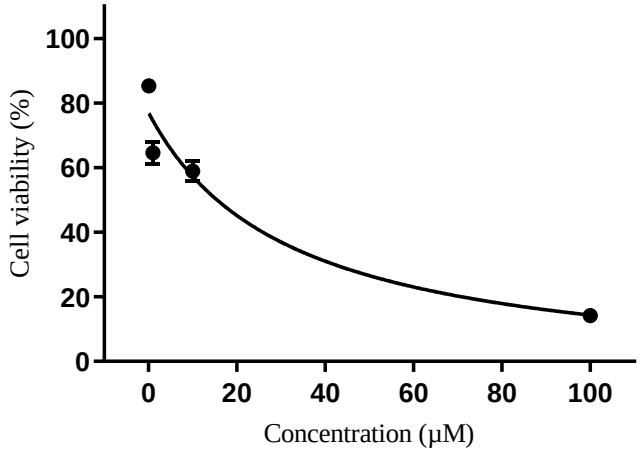

Figure 3. Cytotoxic activity of *L. camara* leaf essential oil using the MTT method.

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Cell viability provides cell information that can survive after being exposed to a substance. The results of the cytotoxic activity of the test compounds on T-47D breast cancer cells (Figure 3) showed a correlation between cell viability and variations in the concentration of the test solution. The percentage of cell viability will decrease with an increase in the concentration of the test solution used. So that the high concentration of the test solution will cause inhibition of more cell growth.

The Figure 3 shows that the test solution with a concentration of 0.1 μM has almost no inhibition for the growth of cancer cells (cell viability 85%). Likewise, with the test solution with a concentration of 10 μM where the test solution was not able to inhibit 50% of cancer cell growth. However, at high concentrations, the 100 μM test solution provided high inhibition with 14% cell viability. This shows that the test solution can inhibit the growth of cancer cells as a whole. The calculation of the IC_{50} value of the test solution was analyzed using GraphPad Prism 9 software and obtained an IC_{50} value of 10.67 μM.

Hamidi et al. (2014) explained that the level of toxicity of a compound is said to be non-toxic if the LC_{50} value is> 1000 μg / mL, low toxic if the LC_{50} is 500-1000 μg/mL, medium toxic if LC_{50} 100-500 μg/mL and high toxic if LC_{50} 0-100 μg/mL[29]. Prasetyaningrum et al (2018) also explained that if the toxic level of a compound with an IC_{50} value of <12 μg/mL or <30 μM) the compound is considered to have high cytotoxic activity (high toxic)[30]. Based on these data, it is known that the activity of the essential oil isolated from *L. camara* leaves is highly toxic, both tested against *A. salina* shrimp larvae (LC_{50} 15.92 μg/mL) and T-47D breast cancer cells (IC_{50} 10.67 μM). This also indicates that the essential oil of *L. camara* leaves has potential as an anticancer[14].

Conclusions

The main chemical content of essential oil isolated from *L. camara* leaves is isocaryophyllene (14.39%), *ρ*-cymene (8.17%), β-cubebene (7.8%), *α*-pinene (7.64%). and β-elements (5.51%). The cytotoxic activity of the isolated essential oil is highly toxic both *Artemia salina* shrimp larvae (LC_{50} 15.92 μg / mL) and T-47D breast cancer cells (IC_{50} 10.67 μM).

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