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Cytotoxic Activity of Citronellyl Caproate on Murine Leukemia (P388) Cells

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Abstract

Citronellol is a monoterpene compound and has been known having antibacterial, antioxidant, antiinflamation, and anticancer activity. To increased activity, ester compound was synthesized from citronellol and caproic acid by using sodium hydroxyde as catalyst. Analysis of ester compound was carried out by TLC, IR, LC-MS, and NMR. Whilst analysis of in-vitro cytotoxic activity was carried out by BSLT and MTT methods against Artemia salina and murine leukemia (P388) cells, respectively. The result showed that citronellyl caproate was very active against Artemia salina with LC_{50} 1.21 μg/mL and has potency as anti-leukemia cancer, indicated by IC_{50} 10.63 μg/mL against murine leukemia (P388) cells.

Keywords: citronellol, citronellyl caproate, leukemia.

INTRODUCTION

Cancer is a top ten killer diseases in the world. Cancer also has become one of the major causes of death in Indonesia. Every year, the number of cancer patients is keep increasing in Indonesia, and now cancer is a second largest killer after heart disease [1-3]. Cancer treatment can be done by surgery, radiation, chemotherapy, and cancer medicine usage. The development of cancer medicine either natural or synthetic medicine is expected to result the medicine with higher activity, lower toxicity, and more selectively.

During the last few decades, chemopreventive and chemotherapeutic compounds against various types of cancer have been isolated from a number of medicinal plants [4-7]. Indonesian biodiversity has potential as a source of bioaktive compounds as chemopreventive and chemotherapeutic agent for cancer treatment. Citronella oil is a

bioactive compound derived from *Lemongrass* plant, including in genus of *Cymbapogon* and family of Poaceae [8]. Citronella oil is one of the main commodities of various essential oils in world trade and widely used in the perfume, cosmetics, food, and pharmaceuticals industry. Indonesia is the third citronella oil producer in the world, after China and Vietnam [9]. The main component of citronella oil are citronellal, citronellol, and geraniol [10]. Citronellol is a monoterpene compound in the form colorless asyclic oil that have been known as antimicrobial, antifungal, antioxidant, anti-inflammatory, and potencial as a chemopreventive agent [11-15]. The monoterpene compound is a new class compound of cancer chemopreventive**.**

Citronellol can be used as natural medicine for cancer treatment. In addition, citronellol can also used as a starting material for synthesize of derivatives or analogs of the bioactive compound for cancer treatment. Citronellol contained in lemongrass oil and it's derivative, citronellyl 5-aminolevulinate were reported effective in inhibition of cells growth of skin tumor cells. The cytotoxic activity of citronellyl 5-aminolevulinate reported increase significantly in the deadly skin tumor cells. The preclinical assay result of effectiveness of these compounds by photodynamic therapy on mice with a dose of 50 mg / kg body weight showed that a decline in tumor volume significantly, about 10 times smaller than citronellol. The volume of tumor formed of citronellol and citronellyl 5-aminolevulinate were 3.06 and 0.30 mL, respectively [14].

In Indonesia, almost all the raw materials for synthesis of synthetic medicine are still imported until now. Therefore, it is necessary to utilize the potential of local natural compounds as raw materials for synthesis of medicine for cancer treatment. In this study, we synthesis of ester compound from citronellol and caproic acid that derived from Indonesian native plants. Caproic acid (C_6) that can be isolated from palm kernel oil (PKO) was also reported to have bioactivity as antimicrobial, antifungal, antiviral, and potentially as anti-neoplastic agents [16,17]. Indonesia is the second palm oil producer in the world, after Malaysia [18].

The aim of this study is synthesis of citronellyl caproate by esterification process by using sodium hydroxide (NaOH) as catalyst to increase activity of this derivative. Identification of ester compound was carried out by TLC, FT-IR, LC-MS, and spectrometer NMR. The *in-vitro* cytotoxic activity of citronellyl caproate against *Artemia salina* and murine leukemia (P388) cells by using BSLT and MTT methods, respevtively.

EXPERIMENTAL SECTION

Materials

Materials used were citronellol (Sigma Aldrich C 5904) and caproic acid (Sumi Asih with purity 99%) as starting material, NaOH (E.Merck 106469), 1% of HCl solution, *n*-hexane, ethyl acetate (EtOAc), distilled water, and analytical grade chemicals were used for molecular weight, structure, and *in-vitro* cytotoxic activity analysis.

Equipments used in this study were esterification process unit, evaporation unit, and one set of ester compound identification unit. Thin Layer Chromatography (TLC) was carried out using precoated silica gel plates (Merck Kieselgel 60F 254, 0.25 mm),

spots were visualized under UV light (254 and 365 nm) irradiation and by spraying with 10% sulphuric acid solution followed by heating at 110 °C. Silica gel column chromatography was carried out on Merck (70-230 mesh and 230-400 mesh). IR spectra was measured by Fourier Transform Infra Red (FT-IR) Spectrophotometer Shimadzu prestige 21 using KBr pellets. Mass spectra (MS) or molecular weighy (MW) was obtained by using *Liquid Chromatography-Mass Spectroscopy* (LC-MS) Mariner Biospectrometry spectrometer using Electrospray Ionization (ESI) System and positive ion mode. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were recorded on a Nuclear Magnetic Resonance (NMR) Jeol spectrophotometer *using $CDCl_3$ as solvent and TMS as internal standard.*

METHODS

Synthesis of citronellyl caproate ester compound

A total of 1.56 g of citronellol was reacted with 1.17 g of caproic acid, and 58.3 mg of NaOH was added and stirred for 8 hours at 80°C. NaOH catalyst was separated from synthesis product by using 1 % of HCl solution. The synthesis product was extracted with EtOAC, washed with water (distilled water), and separated between water and EtOAC. The flitrate was concentrated by evaporating of EtOAc at 45°C under vacuum to obtain product containing of a mixture esterification product. The crude ester was analyzed by TLC with eluent *n*-hexane : EtOAc = 95 : 5. In addition, crude ester was analyzed by GC-MS to ensure that the desired ester products have been formed.

Purification of ester products

Ester product was purified by column chromatography (silica gel Merck 64271) eluting with *n*-hexane, a gradient of EtOAc to 100 %. The pure ester product was identified by spectroscopic methods (FT-IR, LC-MS, NMR).

in-vitro Cytotoxic Activity Analysis

Brine Shrimp Lethality Test (BSLT)

BSLT (Meyer's method) was conducted using nauplii of *Artemia salina* Leach that are generally considered as a bench top assay aiming at the discovery of cytotoxic compounds. About 50-100 mg brine shrimps eggs were placed in seawater in the vessel, and allowed the shrimps to mature as shrimp larvae for 48 hours. An amount of 10, 100, 500, and 1000 µg/mL of sample concentration were prepared. About 100 mL of seawater containing 10-11 shrimp larvaes of *Artemia salina* were transfered into the test container. About 100 µL of each sample concentration of 10, 100, 500, and 1000 µg/mL was added into sample solution. Each concentration was carried out three replicates. Solutions were incubated for 24 hours at room temperature under illumination. The number of dead and live of shrimp larvae in every hole were counted, and LC_{50} value was determined after 24 hours exposure. LC_{50} value is the concentration of a substance that caused 50% of the death of shrimp larvae [19].

MTT Method

The inhibitory effect of ester product on murine leukemia (P388) cells assessed using MTT method (Mosmann's method) with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colouring. The devolepment of cells culture performed by growing of cells culture in RPMI 1640-serum (phosphat bovine serum (PBS)) medium and incubated in a humidified atmosphere of 37^0C and 5% of CO_2 for 24 hours. Serum medium was replaced new serum medium and incubated in a humidified atmosphere of 37^0C and 5% of CO_2 again so on to obtain sufficient cells number for testing. After a sufficient number of cells, cell medium removed, and washed with PBS, and then RPMI medium added. The cells were transferred into tubes and centrifuge at 1200 rpm for 5 minutes. Supernatant was discarded and the precipitate was added RPMI 1640 medium containing 10% PBS. The cells density was calculated by hemocymeter and cells number was counted. The number of cells that had been known then made dilution by adding with RPMI-serum medium to obtain cell number of 2 X 10^4 cells/mL of cell suspence. Cells with densities 1-2 X 10^4 cells/well in 96 well-plates cultivated in a humidified atmosphere of 37^0C and 5% of CO_2 for 24 hours. Afterwards the cell cultures were replaced, washed with PBS, and added with 100 µL fresh culture medium containing sample at concentration of 100, 50, 25, 10, 5, 2.5, and 1 µg/mL. Furthermore, the plates were incubated at 37^0C in CO_2 5% for 48 hours. At the end of the treatment, medium was replaced and the cells were washed with PBS and added with 100 µL of a new fresh medium containing MTT 5 mg/mL. Plates were incubated in a humidified atmosphere at 37^0C in 5% CO_2 for 4 hours to bioreduction of the MTT dye into purple formazan crystals. After 4 hours, the medium containing MTT was discarded, washed with PBS, added 200 µL isopropanoat solution, and incubated at room temperature for 12 hours to completely solubilize the formazan crystals. The bioreduction of MTT was assessed by measuring the absorbance of each well at 550 nm by ELISA reader. Viable cells were expressed as a percentage of the absorbance treatment cells divided by abcorbance control cells multiplied viable cell versus contentration. The IC_{50} express 50% death induced by the concentration and is a measure of the effectiveness of a compound in inhibiting biological function [20,21].

RESULTS AND DISCUSSION

Synthesis of ester compound from citronellol and caproic acid has been done by using sodium hydroxide (NaOH) as catalyst at 80^0C for 8 hours. The crude ester product that obtained was oily liquid. The TLC analysis of synthesis product showed that synthesis product contained ester compound which was indicated by a spot with Rf value of 0.42 of ester compound, lower than Rf of citronellol (0.45) and caproic acid (0.82) as starting material.

Crude ester was then analyzed by GC-MS to ensure that desired product of citronellyl caproate have been formed. The GC-MS analysis result of crude ester product showed that, citronellyl caproate has been formed with indicated a dominant peak at retention time of 17.03 minutes (peak of hexanoic acid 3,7-dimethyl-6-octenyl ester, $C_{16}H_{30}O_2$, MW of 254). The GC-MS chromatograms also showed another peak with higher

intencity of residual unreacted reactant of citronellol at retention time of 10.92 minutes (Figure 1).

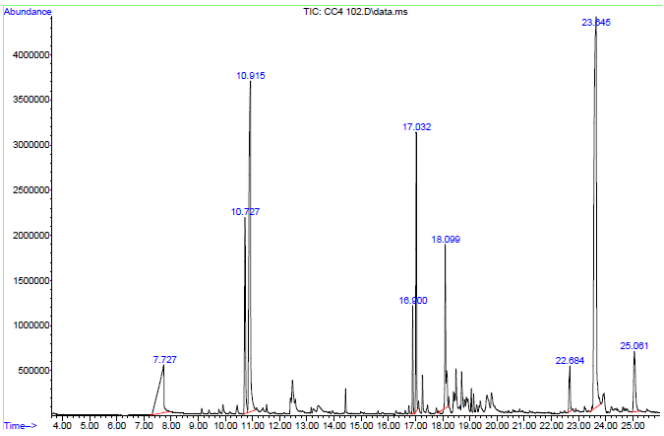

Figure 1. GCMS chromatogram of crude ester of citronellyl caproate

Crude ester further purified by using chromatography column using silica gel as the stationary phase, and a mixture solvent of *n-hexane* : EtOAc 100 : 0, 99 : 1, 98 : 2, and so on as the mobile phase. Pure citronellyl caproate compound was obtained by using the mixture solvent of *n*-hexane : EtOAc = 98 : 2. The weight and yield of pure citronellyl caproate was 1.29 g and 50.48%, respectively.

The IR spectra of citronellyl caproate compound displayed the formation of a new group at wave number, v_{max} = 1728.22 cm^{-1} which is stretching vibration carbonyl (C=O) of citronellyl caproate of ester formed, different from the absorption of carbonyl (C=O) of caproic acid at wave number, v_{max} = 1705.07 cm^{-1} as shown at the Figure 2.

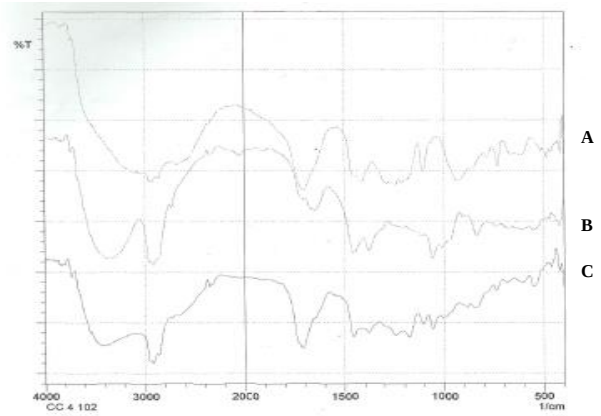

Figure 2: IR spectra of caproic acid (A), citronellol (B) and crude ester of citronellyl caproate (C)

The mass spectrum showed a $(M+H)^{+1}$ at m/z 255.08 corresponding to molecular formula of $C_{16}H_{30}O_2$ and molecular weight (MW) of citronellyl caproate was 254.08 g/mol. The chromatograms of citronellyl caproate illustrated 2 peaks, which the retention time of the dominant peak was about 3.8 minutes, area under curve (AUC) of 31564.1, and MW of 254.08 was recognized as citronellyl caproate compound cause the MW of citronellyl caproate compound according to literature was 254.41. The second peak at retention time of 6.8 minutes and AUC of 1951.3 most likely was impurity. Citronellyl caproate compound was obtained quite purity of about 94.18% as shown at the Figure 3.

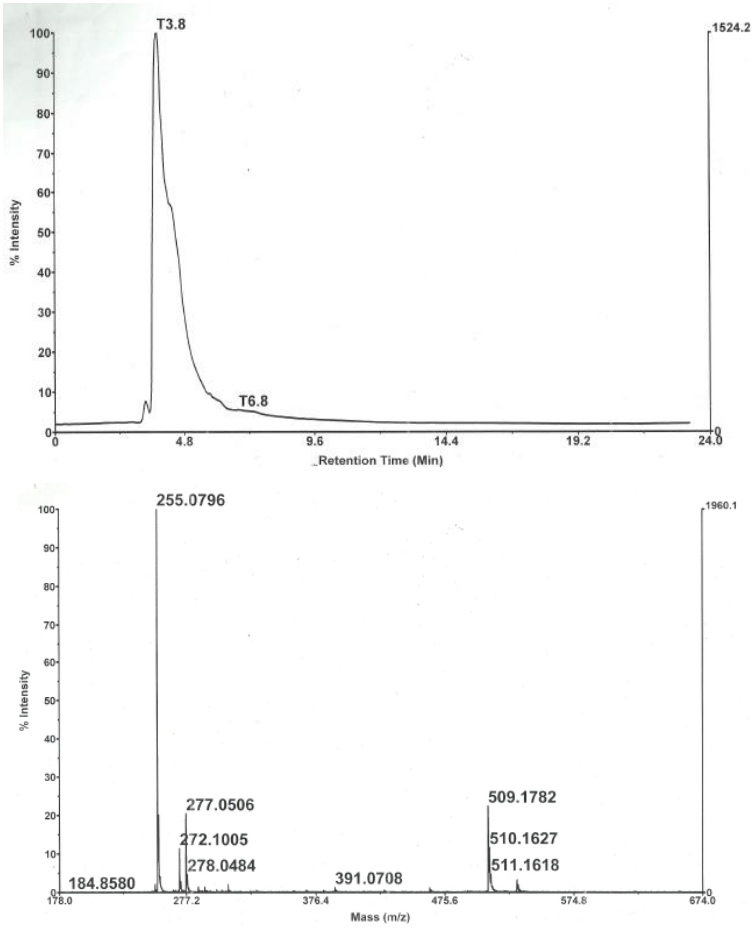

Figure 3. Mass spectra of citronellyl caproate compound that analysis by using LCMS

The ^{1}H-NMR spectra data can be explained as follows : at δ_H = 4.08 ppm (2H, *t,* 7.4 Hz) is a chemical shift of 2 protons (H) of the methylene (CH_2) group that formed triplet as adjacent to CH_2 group and downfiled due to interacting with electronegative of oxygen, δ_H = 1.60 ppm (2H, *q,* 7.4 Hz) is a chemical shifts of 2 protons of CH_2

group, and formed quartet as adjacent to CH_2 and methine (CH) groups. The proton signal δ_H = 1.65 ppm (1H, *m,* 7.4 Hz) is a chemical shifts of one proton of the CH group that formed multiplet as adjacent to CH_3 and CH_2 groups, δ_H = 1.58 ppm (2H, *q, 7*.8 Hz) is a chemical shift of 2 proton of CH_2 group that formed quartet cause interacting with 2 protons of the CH_2 group and one proton of the CH group. At δ_H = 1.95 ppm (2H, *t,* 6.7 Hz) is a chemical shift of 2 protons of the CH_2 group that interact with CH_2 group and δ_H = 5.06 ppm (1H, *t,* 6.7 Hz) is a chemical shifts of one proton of the CH group that interact with 2 protons from the CH_2 group, and downfield due to its double bond. At δ_H = 1.79 ppm (3H, s) is a chemical shifts of methyl (CH_3) group that interact with quaternary carbon atoms (C). At δ_H = 0.90 ppm (3H, *d,* 7.4 Hz) is a chemical shifts of 3 protons of CH_3 group that interacts with CH_2 group, δ_H = 2.26 ppm (2H, *t,* 7.8 Hz) is a chemical shifts of 2 protons of CH_2 group that interacts with CH_2 group. Furthermore the proton signals at δ_H = 1.28-1.66 ppm (2H, *q, 7.8*) is a chemical shifts of 2 protons for three CH_2 groups that interacts with 2 CH_2 groups, and signal at δ_H = 0.88 (3H, *t,* 6.9 Hz) is a chemical shifts of 3 proton of CH_3 group that interacts with CH_2 group.

The ^{13}C-NMR spectra data of citronellyl caproate compound according to Figure 4 showed 16 peaks, 4 methyls (4 x CH_3), 9 methylenes (9 x CH_2), 2 methines (2 x CH), and one quaternary carbons of the chemical shift as follows : δc = 62.8 ppm showed methylene (CH_2) carbon (C1') and interacting with the ester carbonyl. At 124.7 ppm showed chemical shifts of carbon (C6') of methine (CH) that double bonded with C7 (131.4 ppm). The most downfield shift is the ester carbonyl group of C1 (174.1 ppm). At 14.1 ppm (C6'), 17.8 ppm (C9), 19.7 ppm (C10) and 25.5 ppm (C8) showed the chemical shifts of methyl carbon (CH_3). At 22.5 ppm (C5), 24.8 (C5'), 29.6 ppm (C3'), 31.5 ppm (C3 and C4), 34.5ppm (C2), 35.6 ppm (C2'), and 37.1 ppm (C4') showed the chemical shiffs of carbon of methylene (CH_2). The ^{1}H and ^{13}C-NMR spectra data of citronellyl caproate compound according to Figure 4 as shown at Figure 5 and Table 1.

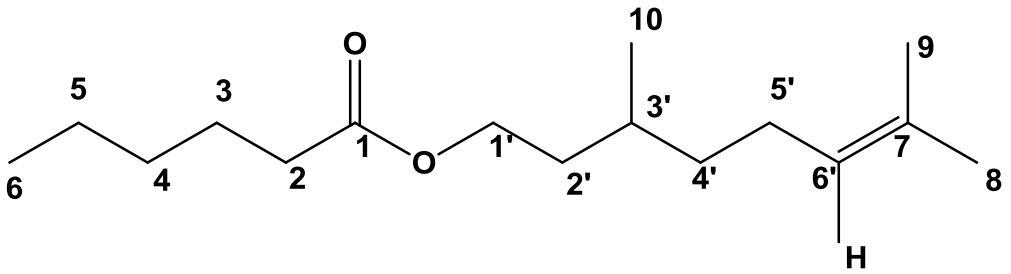

Figure 4: Molecular structure of citronellyl caproate

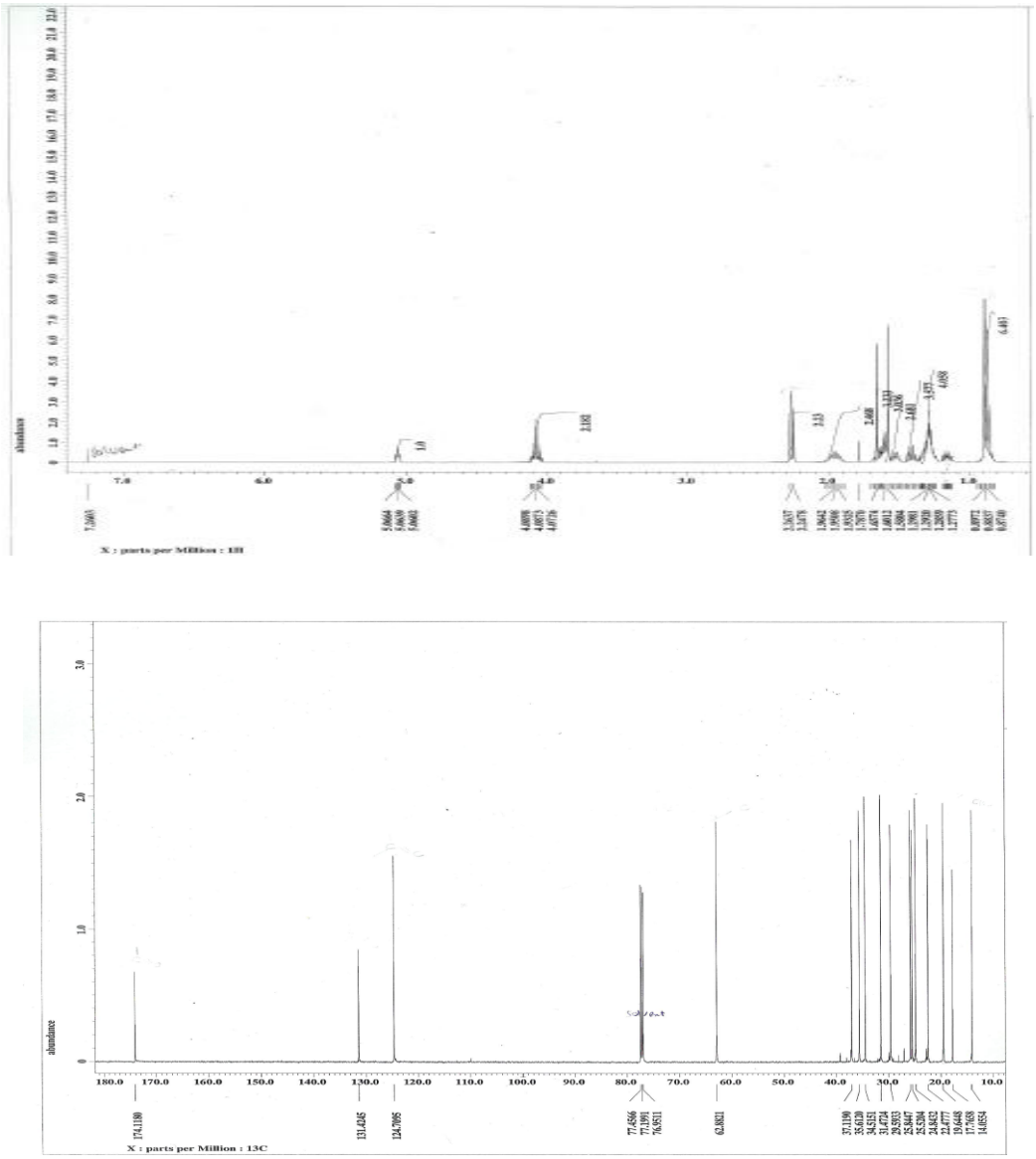

Figure 5: ^{1}H and ^{13}C-NMR spectra of citronellyl caproate

Table 1: ^{1}H-NMR ($CDCl_3$, 500 MHz) and ^{13}C-NMR ($CDCl_3$, 125 MHz) spectra of citronellyl caproate

^{1}H/^{13}C	**Chemical shift (δ, ppm)**	
	^{1}H (ΣH, *m*, *J* Hz)	**^{13}C**
1	-	174.1
2	2.26 (2, *t*, 7.8)	34.5
3	1.66 (2,*q*, 7.8)	31.5
4	1.28 (2, *q*, 7.8)	31.5

5	1.30 (2, *q*, 7.8)	22.5
6	0.88 (3, *t*, 6.9 Hz)	14.1
7	-	131.4
8	1,79 (3, *s*)	25.5
9	1,78 (3, *s*)	17.8
10	0,90 (3, *d*, 7.4)	19.7
1’	4.08 (2, *t*, 7,4)	62.8
2’	1.60 (2, *q*, 7.4)	35.6
3’	1.65 (1, *m*, 7.4)	29.6
4’	1.58 (2, *q*, 7.8)	37.1
5’	1.95 (2, *t*, 6,7)	24.8
6’	5.06 (1H, *t*, 6.7)	124.7

Note : s = singlet, d= doublet, t = triplet, q = quartet, q = quintet, m = multiplet.

The preliminary assay of cytotoxicity of citronellyl caproate, the derivative ester compound of citronellol has been done by using Brine Shrimps Lethality Test (BSLT) method. BSLT is an easy, cheap, and reliable to be used as preliminary assay of an anticancer activity. The compound is active against *Artemia Salina* if the value of $LC_{50} \leq 30$ μg/mL [19,22]. The result of BSLT assay (Figure 6) showed that citronellol and citronellyl caproate were very active against *Artemia Salina* with LC_{50} 1.15 and 1.21 μg/mL, respectively, so the compounds were a potential source of cytotoxic agents. Based on the LC_{50}, the citronellyl caproate compound was lower toxicity than citronellol, so it is expected to be used as active compound for medicine candidate.

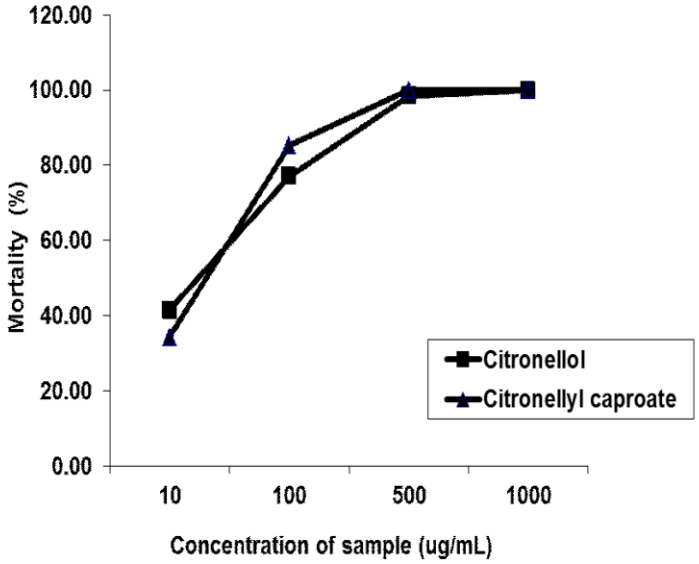

Figure 6: The Preliminary cytotoxic activity test of sample by using BSLT method

The cytotoxicity of sample was expressed with IC_{50} value. The IC_{50} express 50% death induced by the concentration and is a measure of the effectiveness of a compound in inhibiting the growth of cancer cell. The *in vitro* cytotoxic test of citronellol and citronellyl caproate compounds against murine leukemia P388 cells has been done by using MTT method. The test results (Figure 7) showed that citronellol and citronellyl caproate compounds have leukemia anticancer activity, indicated by IC_{50} values of the compounds against murine leukemia P388 cells lower than 100 μg/mL (IC_{50} < 100 μg/mL) [21]. The concentration of citronellol and citronellyl caproate that inhibit 50% of growth of murine leukemia (P388) cells (IC_{50}) was 38.49 and 10.63 μg/mL, respectively, so it can be promising anticancer therapeutic agent for leukemia.

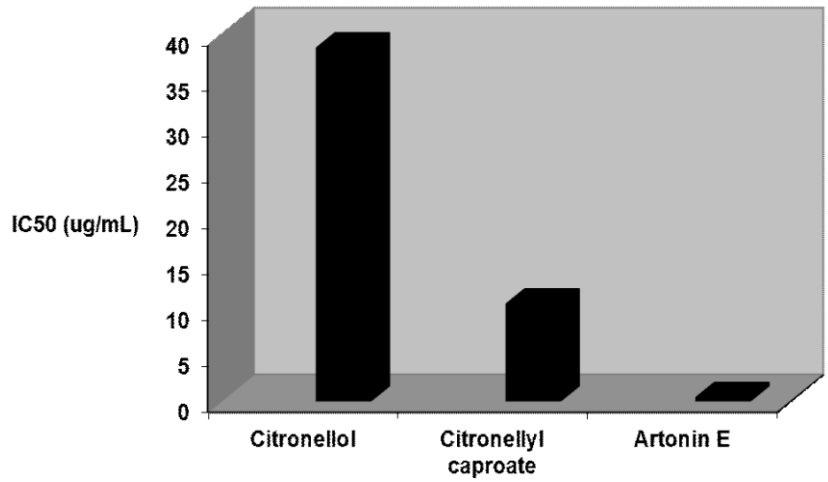

Figure 7: The result of *in-vitro* cytotoxic activity test of sample against murine leukemia cancer P388 cells.

There was a significant decrease of IC_{50} values of citronellyl caproate compared to citronellol, which the IC_{50} value of citronellyl caproate lower than citronellol. It means that leukemia anticancer activity of citronellyl caproate was higher than citronellol, so the esterification of citronellol can increase leukemia anticancer activity. The activity increasing of leukemia anticancer was possible due to the increasing lipophilicity of citronellyl caproate than citronellol. The increased lipophilicity of the citronellyl caproate ester compound (Log P 4.96, BM 254.41), while the log P of citronellol was 2.86. The increasing of lipophilicity will be changing of physical properties such as rising of boiling point, reducing of solubility in water, increasing of the partition coefficient of fat/water, surface tension and viscosity. The increasing of lipophilicity of citronellyl caproate will be increasing the solubility of the compound in fat, thus increasing the ability of the compound to penetrate the walls of the cell membrane. It also means the solubility of the fluid of

the cells outside was also increaced that associated with the transport of medicines to the working side or the receptor. This is in accordance with the Lipinski's rules (Log P value of < 5 and the molecular weight < 500 [23-25].

On the *in-vitro* cytotoxic activity test, we used artonin E that commonly used as leukemia drug as standard. The concentration of artonin E that inhibit 50% of growth of murine leukemia (P388) cancer cells (IC_{50}) was 0.4 μg/mL (Figure 7). The IC_{50} of citronellyl caproate (IC_{50} = 10.63 μg/mL) was still high and lower than artonin E maybe caused the the purity of the product was still low (only 94% lower than 99%).

CONCLUSION

Citronellol, one of the main component of citronella oil has potential as a source of cytotoxic agents. Esterification of citronellol with caproic acid in the presence base catalyst of sodium hydroxide, gave citronellyl caproate, in medium yield (50.48%). Citronellyl caproate colud be used as anticancer medicine candidate cause has lower toxicity and higher activity than citronellol. The cytotoxic activity of citronellyl caproate againts murine leukemia (P388) cells is more active than citronellol about 4 times, so it has potency as anticancer therapeutic agent for human leukemia cancer.

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