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Steroids from N-Hexane Fraction of the Stem Bark of *Shorea singkawang* Mig and Anticancer Activity as Tested with Murin Leukemia P-388 Cells.

Yusnelti, Yunazar Manjang*, Abdi Dharma, Djaswir Darwis and Edison Munaf.

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

ABSTRACT

The isolation of steroid compounds from n-hexane fraction of bark of *shorea singkawang* has been identified as campesterol or *5-ergost-en-3-ol (3beta)* based on the data of UV spectroscopy, IR, GC-MS and ^{1}H-NMR and ^{13}C N-MR, HMBC and HSQC. This compound is derived from steroid which was first discovered in the current plant and bioassay cytotoxic activity by using Murin leukemia of P-388 cells, with IC_{50}> 100 µg/ mL

Keywords: *Shorea Singkawang*, Steroids, Murin leukemia P-388 cells, IC_{50} µg/ mL

Corresponding author

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INTRODUCTION

Shorea is one of the family of *dipterocarpaceae* which spread throughout tropical forests of Indonesia. Shorea genus consists of 150 species. One of them is *Shorea singkawang* (Miq). Which known as red meranti *Singkawang* nut. This plant is Sumantera's endemic plant, especially in Jambi Province. It has high economic value of timber and the fruit produces oil which is known as tengkawang oil. The oil is bitter and can degrade spicy flavor. The timber of singkawang is important export commodities as lumber or plywood. This plant also produces various types of chemical compounds such as flavonoids, phenolics, steroids, coumarins and triterpenoids, polifenol. It has been reported that reported that *Dipterocarpaceae* is the source of polyphenolic chemical compounds that show various bioactivities, such as chemo preventive for cancer, cytotoxicity against human tumor cells, hepatoprotective, anti-inflammatory, inhibiting the spread histamine STP-ase of gastric and topoisomerase II, antibacterial, antifungal, and anti-HIV[3]. Concerning to several phytochemical studies toward genus *Shorea,* its have been reported that its contains flavonoid compounds, such as fenil praponoid, phenolic acids, and steroids triterpenoids, and coumarine.

One of the species is *Shorea singkawang*. From the bark of *Shorea singkawang*, we isolated three compounds of the steroid, including *ergost-5-en-3-ol* (3βeta) or campesterol. Molecular structure of these compounds is determined based on the data of UV spectroscopy, IR and ^{1}H-NMR, and ^{13}C-NMR. Toxicity test is conducting by using shrimp larvae *Artemia Salina* and bioactivity as anticancer against murine leukemia cells P-388.

Research on the content of chemical compounds from the stem bark of *Shorea singkawang* and its bioactivity as anticancer against murine leukemia cells P-388 has never been conducted before. From preliminary research of phytochemical test, it has been found that the bark of this plant contains flavonoids, phenolics, coumarins, steroids and triterpenoids. From the research findings, it has been obtained campesterol steroid compounds campesterol test cell activity against murine leukemia P-388 indicated very weak with IC_{50} value > 100 µg/ml, test sytocity test toward, contains flavonoid compounds, fenilpraponoid, phenolic acids, and steroids and triterpenoids, coumarins.

One of the species of is *Shorea singkawang*. From the bark of *Shorea singkawang* extract, we have isolated three compounds of the steroid, including *ergost-5-en-3-ol* (3βeta) or campesterol, In the current paper, it will be presented the determination of the structure of compound campesterol, ergost-en-3-ol (2βeta) which well known as campesterol (Figure 1) that can be isolated from the extract of n-hexane in bark of *Shorea singkawang* (Miq).Miq). Molecular structure of these compounds is determined based on the data of UV spectroscopy, IR and ^{1}HNMR, and ^{13}C NMR, and supported by data comparison that have been reported before. Toxicity test is conducting by using shrimp larvae *Artemia Salina* and biokactivity as anticancer against murine leukemia P-388 cells.Research on the content of chemical compounds from the stem bark of *Shorea singkawang* and its bioactivity as anticancer against murine leukemia P-388 cells has never been conducted since then. From the preliminary research of phytochemical test, it has been found that the bark of this plant contains flavonoids, phenolics, coumarins, steroids and triterpenoids. From the research findings, it has been obtained that campesterol steroid compounds (1) test cell activity against murine leukemia P-388 indicated very weak with LC_{50} value > 100 µg/mL test sytocity test toward shrimp larvae *artemia salina* with LC_{50} value 2,39 µg/mL.

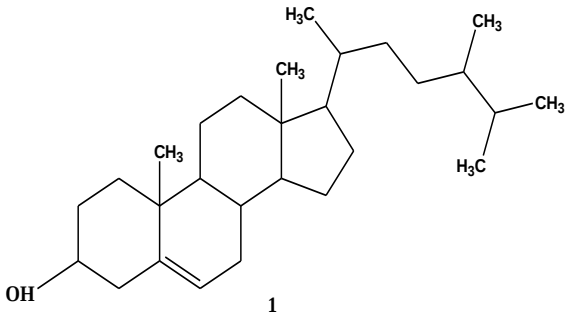

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Figure 1: Campesterol

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EXPERIMENTAL

General

The melting point is determined by using Melting Point Asparatus Jhons Fischer, UV-VIS spectrophotometer, Pharmaspec 1700 (Shimadzu), FT-IR spectrophotometer (Spectrum One FT-IR). Spectrum of ^{1}H-NMR and ^{13}C-NMR are determined with a spectrophotometer JEOL JNM ECA-500 operating at 500 MHz (^{1}H) and 125 MHz (^{13}C) using TMS as a standard. Vacuum Liquid Chromatography (VLC) was performed by using Si gel Merck 60 GF_{254} (Merck), analysis toward Thin Layer Chromatography (TLC) on aluminium plates coated by Si gel Merck Kieselgel 60 $_{F254}$, 0.25 mm Merck). The solvent used is technically distillation qualified, and solvent pa, toxicity testing using *Artemia salina* shrimp larvae, and for sytotoxicity activity is performed by using cells murine leukemia P-388.

Plant Material

Plant materials such as bark of *Shorea singkawang*, plant material derived from the community garden village Rantau Panjang subdistrict Seling Marangin Jambi district. Specimens of this plant has been inspected and stored in the Herbarium of Biological Science, University of Andalas.

Extraction an Isolation

The stem bark of *Shorea singkawang* that had been (3,8 kg) was macerated with methanol (20 l) 3 x 24 hours. After the solvent was evaporated at low pressure, the thick extracts was obtained 380 gr with red-brown colored residue. Then 250 g of condensed methanol extract was fractionated by using an organic solvent n-hexane, dichloromethane and ethyl acetate, then the solvent was evaporated and produce n-hexane fraction 28,6456 g, dichloromethane fraction (DCM) 8,8792 g and 18, 0374 g of ethyl acetate fraction, residual faction 216.8 g.

Vacuum liquid chromatography toward 10 g n-hexane fraction was performed gradually by using silica gel G 60 GF 254 as stative phase and the eluent of n-hexane compound, ethyl acetate and methanol. Based on the results of monitoring by using thin layer chromatography (TLC), 14 fractions could be combined into 5 combined fractions A = 0.9639 g, B =0.3110, C = 0,9240 g,D = 0.1031 g, E = 0, 0652 g.

Separation fraction C was conducted thin layer chromatography in order to determine the levels of the chemical composition. The spots on TLC plate was monitorized under UV light 245 nm, then the active spots unde UV was marked. Some eluent mixtures with different polarity had been tested in TLC, including: n-hexane, n-hexane-ethyl setat, with comparisons between the other 9:1; 8:2; 7:3; 6:4 and 1:1 ethyl acetate. The 17 -19 vial contained stain spots 1, then it been recrystalized by using the ethyl asetat- n-hexane. The result obtained pure compounds with R_f 0.73 toward n-hexane-etil eleuen asetat 7:3.

RESULTS AND DISCUSSION

Compound of campesterol obtained from the process was steroid which seem like white needles. Under UV light, the stain was not visible with wavelength of 234 nm, but with wavelength 354 there was purple stain. In order to clarified that the compounds including steroids, the process was continued by using vanillin and 10% H_2SO_4. At spot, the vanillin was smeared and then bing eluted with the same eluent. It is heated approximately 5 minutes to form redish purple stain. Crystals produced in the process was 103,3 mg, melting point 138-140 and UV-vis spectrum $(MeOH)_{\lambda maks}$ 203 nm, 280 nm and 397 nm, 413 nm, 414 nm, 470 nm, 481 nm. Data of IR spectrum (KBr) ν_{maks}: 3474 cm^{-1}, 2920 cm^{-1}, 2850 cm^{1}, 1685 cm^{-1} 1463 cm^{-1}, 1376 cm^{-1}, 1238 cm^{-1} examination of ^{13}C-NMR of compound campesterol in chloroform showed 28 singal with chemical shifts (ppm): δ_C 140,7, δ121,7, δ71,8, δ56,8, δ56,1, δ50,1, δ45,8, δ42,3, δ39,8, δ37,3, δ36,5, δ36,2, δ34,0, δ31,9, δ31,6, δ29,7, δ29 , 2, δ28,3, δ26,1, δ24,1, δ23,1, δ21,1, δ19,8, δ19,4, δ19,0, δ18,8, δ12,0, δ11,9 : ^{1}H NMR : δ5,35 (1H, H-6), δ3,55 (tdd, OH, H-3) δ 2.31 (s, 3H), δ2,02 (s, 3H), δ1,86 (s, 3H), δ 1.08 (s, 3H). δ 1.00 (s, 3H), δ 0.92 (s, 3H), δ0, 86 (s, 3H), GC-MS m/z: 400, 362,367,315, 289, 273, 231, 213, 173, 159 , 145, 133, 107, 95, 81, 57, 43, 41, Data ^{1}H-NMR and ^{13}C-NMR listed in Table 1 (4,11-12)

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Bioassay Cytotoxic Activity Using cell P 388

Cytotoxic test was using cultured protocol murine leukemia cell P-388 over the plate, compound 1 showed IC_{50}> 100 µg/mL, whereas sitoxicity test was conducted by using shrimp fry *Artemia salina* with bioassay method brine shrimp lethality (Anderson, Guetz, and Mc Laughlin, 1991) compound compesterol showed LC_{50} ,2,39 µg/mL from n-hexane and compound fraction showed LC_{50} ,1,81 µg /mL.

Research using samples of bark extraction *Shorea Singkawang* with methanol, then been partition by using n-hexane has been found a compound campesterol derivate from steroid, These compounds were obtained through several stages of fractionation followed by testing on thin layer chromatography (TLC) and chromatographic separation column. These compounds were obtained in several stages of fractionation, and was followed by thin-layer chromatography analysis and separation by chromatography, followed by melting point test, UV spectrum, IR, GC-MS, ^{1}H-NMR and ^{13}C- NMR. The data obtained has been shown in Table 1. On the other hand the data from Gas Chromatography-Mass Spectrometry has been shown in Table 2.

Table 1: Comparison of IR Compound of Isolation with Campesterol

Product	Isolation	Campesterol
(OH)	3474.91	3425.89
(C-H)	2920.27	2935.92
(C-H)	2850.15	2868.41
(C=H)	1685.93	1639.64
(C-H)	1463.98	1466.03
(C-H)	1376.11	1379.23
(C-H)	1238.49	1063.41
(C-H)	1022.27	1022.36

Tabel 2. Comparison of GC-MS Compound Isolation with Compared Compound (campesterol) (1,3,11)

Compound of Isolation Product
400 (M^+ $C_{28}H_{48}$ O); 43 (100) 382 ; 367; 289 ; 273; 255;231; 213; 173; 161; 145 ;133, 119; 107 ; 95 ; 81;57;41
Compound of Isolation Campesterol
400 (M^+, $C_{28}H_{48}O$), 43 (100); 382 (6); 367 (4), 261 (14), 213 (15); 161 (16) ;159 (19) 147 (28); 145 (37) ; 105 (38); 91 (37) ; 81 (50) ; 67 (32) ; 57 (41); 55 (62); 29(16) (15)

The mass spectrum of the mixture is characterized by the protonated molecular ion (m/z) of the compound and abundant signal corresponding to the fragment ions due to loss of water molekule. A signal at m/z 383 indicates the loss of water $[M + Z\text{-}H_2O]^+$ seems to be characteristic of sterols. The peaks at m/z 400, and 382, 367, 315, 289, 273, 231, 213, 173, 159, 145, 133, 119, 107, 95.81, 57 and 43 (100) were found to be identical with the mass spectrum of appropriate campesterol[11]. IR spectrum showed the presence of hydroxyl groups (3474.91 cm^{-1}) and C=C (1685,93 cm^{1}) showed saturation in molecule A at 2920,27 cm^{1} indicated the presence of a methyl group. Maximum UV spectrum was observed at 203 nm. Positive identification test for the Liebermann-Burchard sterols in the ^{1}H-NMR spectrum of the mixture, one-proton signal appeared at d 5.35 which indicated the presence of the olefin proton at C-6 because the double bond between C-5 and C-6. singlets at 0.809 and 1.009 for the methyl group angle C-18 and C-19 and the doublet at 0.929, 0.916 and triplet at 0.84, the presence of the C-21, C-25 methyl group and C-28-27. From this analysis by comparing the FTIR spectrum, GC-MS and ^{1}H-NMR, and ^{13}C-NMR compounds reported in the literature [1,3,10,11] This compound is called *ergost-5-en-3-ol (3ẞeta)* or campesterol with molecular formula $C_{28}H_{48}O$.

Furthermore, distribution pattern of compound 1 from is obtained from measurements of ^{13}C-NMR and ^{1}H-NMR, 28 carbons and 48 protons which each of them bonded to carbon seen in the ^{13} CNMR spectrum and ^{1}H-NMR. A total of 28 signals appear on the ^{13} C-NMR spectrum (Cx 3, CHx9, x10 CH2, CH3 x 6) ^{1} H spectrum showed a singlet methyl peaks [(δH 0, 80 (H, s, H-18): δH 1.08 (3H, H19) and the double methyl peaks δH 0.92 (3H, d, Hz, H-21); δH 0.86 (3H, d, J = 5.35 Hz, H-25); δH 0.86 (3H, d, J = 7.26 Hz H-28)] (in table 3). Compound compesterol was obtained as white crystals t.l 138-140 ^{0}C. IR spectrum (KBR) of compound showed the presence of hydroxyl group (OH) at 3474 v_{max} (cm^{-1}) at v_{max} 2920 C-H, 2850 (cm^{-1}), group C=C on v_{max} 1685 (cm^{-1}), maximum absorption at 203 nm in length λ_{max} indicated that compound 1 is nonconjugative

chromotor. Generally, compounds with non-conjugated system chromotor has maximum absorption at wavelength of around 190 nm[10].

Further evidence, from the analysis of ^{1}H-NMR and ^{13}C-NMR spectrum of compound 1 showed the presence of six methyl groups, and two methyl tertiary (C includes one primary methyl (C-28) and three secondary methyl (C-21, C-25, and C-27) and two methyl (C-18 and C-19) and the methylene (C-1, C-2, C-4, C-7, C-11, C-12, C-14, C-15, C-20, C-23 and C-24) and one hikdrosimetin (C-3) and eight metin sp^3-hibrid (C-4, C-8, C-7, C-9, C-16, C-22, C-19, C-23, C-24, and C-3 and two sp^3 quaternary carbon (C-10 and C-13) of the double bond (C-5 and C-6) 10 (table 3). This steroids compound is composed of atoms C, H, and O, and OH groups located at third C atom in the ring A. Meanwhile, atoms C are bonded to each other to form either cyclic chain or non-cyclic.

Cytotoxic Activity Using cell P 388

Cell cytotoxicity testing using murine leukemia P-388 compound 1 were cultured according to the protocol. Compound 1 shows the results of the IC $_{50}$ of 100 lm / mL (> 100 ug/mL), compared with standard compounds 4 lm/ml that compound 1 is weak against murine leukemia cells P-388. And toxicity tests using fry shrimp *Artemia salina* Leach, using Brine Shrimp Lethality Bioassay of compounds showed LC_{50} 50 mg/ml is a toxic compound, cytotoxicity testing using murine leukemia cells P-388 bred pure compounds according to the protocol. Pure compounds showed IC_{50} showed results above 100 lm / ml> 100 ug/mL), compared with standard compounds 4 lm / ml that compound 1 is weak against murine leukemia cells P-388. And toxicity tests using fry shrimp Artemia salina Leach, using Brine Shrimp Lethality Bioassay of compound 1 showed LC_{50} of 1.81 ug/mL showed LC_{50} toxic because the concentration of 50 ppm. While the pure compound expressed tokik when LC_{50} values <200 ppm14,15. Compound 1 is derived from the fraction of n-hexane, n-hexane fraction testing on larval Artemia salina leach LC_{50}nya value of 2.39 was obtained at a concentration of 250 ppm, while the cytotoxic strander of an extract of the larvae of Artemia salina Leach, can be determined based on the extreme value of these plants can be toxic if the LC_{50} values <1000 ppm. With the parameters of an extract is considered highly toxic when LC_{50} values below 30 ppm are considered toxic when 30-1000 ppm LC_{50} value and is not considered toxic when the LC $_{50}$ values above 1000 ppm14,15, the fraction of n-hexane in the research said the toxic LC_{50} <1000 ppm is obtained 250 (2.39 ug / mL) pure substances ppm and <30 ppm of the compound obtained was 1.81 ug / mL can be said to be toxic.

Compound 1 is a steroid compound suspected kampesterol, these compounds have not been reported from the genus Shorea dipterocarp family, especially the species *Shorea Singkawang*, is very interesting and may have chemotaxonomic significance.

CONCLUSION

Continuing our previous study on tropical forest vegetation in Indonesia which have high economic value, especially herbs including *Dipterocarpaceae*, the current research has succesed found for the first time campesterol compound (1) melting point t.l 138-140 ^{0}C, UV (MeOH) λ_{max} 203, and 280 nm, IR (KBr): 3474 cm^{-1}, 2920 cm^{-1}, 2850, 1685, cm^{-1} 1463 cm^{-1}, 1376 $cm^{-1,}$ 1238 cm^{-1},treatment ^{13}C-NMR of compound campesterol in chloroform showed 28 signals with chemical shifts (ppm): δ140,7, δ121 , 7, δ71,8, δ56,8, δ56,1, δ50,1, δ45,8, δ42,3, δ39,8, δ37,3, δ36,5 δ36,2, δ34,0, δ31,9, δ31,6, δ29,7, δ29,2, δ28,3, δ26,1, δ24,1, δ23,1, δ21,1, δ19,8, δ19,4, δ19,0, δ18,8, δ12, 0, δ11,9 ^{1}H-NMR: δ5,35 (1H, H-6), δ3,55 (tdd, OH, H-3) δ 2.31 (s, 3H), δ2,02 (s, 3H), δ1,86 (s, 3H), δ 1.08 (s, 3H). δ 1.00 (s, 3H), δ 0.92 (s, 3H), δ0,86 (s, 3H), GC-MS m/z 400, 362, 367, 315, 289, 273, 231, 213, 173, 159 , 145, 133, 107, 95, 81, 57, 43 (100), from the species *Shorea singkawang*. Compound campesterol showed toxicity both toward larva shrimp LC_{50} 2,39 µg/mL and low activity with murine leukemia P-388 cell IC_{50}> 100 µg/mL.

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