Isolation and Characterization of Stigmasterol and β-sitosterol from Plectranthus scutellarioides var. color blaze dark star and Cytotoxicity of its Fraction

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Isolation and Characterization of Stigmasterol and β-sitosterol from *Plectranthus scutellarioides* var. *color blaze dark star* and Cytotoxicity of its Fraction

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Abstract

Plectranthus scutellarioides is one of medicinal plants in Indonesia, traditionally known as iler/miana/jawer kotok. Due to a large number of varieties, certain varieties of *P. scutellarioides* have not been extensively studied. This study focused on the isolations of two phytosterols, stigmasterol and *β*-sitosterol, which were characterized by various 1D and 2D NMR analyses, from the ethyl acetate extract of *P. scutellarioides* var. *color blaze dark star*. Cytotoxicity of the ethyl acetate extract and some selected fractions were evaluated against A549, MDA-MB-231, MCF-7, KB, and KB-Vin cell lines using SRB (Sulforhodamine B) method. Among all tested sample, fractions 15 and 17 have significantly inhibited cell growth with IC_{50} of 14.3-24.3 *μ*g/mL and 7.3-14.1 *μ*g/mL, respectively.

Keywords: Plectranthus scutellarioides, stigmasterol, β-sitosterol, cytotoxicity

1. Introduction

Plectranthus scutellarioides (syn. *Coleus blumei*, *Coleus scutellarioides*, *Plectranthus blumei*), belonging to Lamiaceae, is a perennial plant [1] that is widely distributed in the continents of Southeast Asia, Africa, Australia [2], America, and Europe [3] and has hundreds of varieties [4]. Most of them are cultivated as ornamental plants due to their beautiful colors and leaf patterns [5]. One of the varieties of *P. scutellarioides* is color blaze dark star, which has brown to black purple leaves, known as “miana” or “jawer kotok” or “iler” in Indonesia has been approved as a medicinal plant from the Minister of Agriculture. The leaves are used empirically to treat ulcers, swelling of the eyes and ears [6], constipation, dysmenorrhea, fever, alleviating symptoms of diabetes mellitus [7], and pulmonary tuberculosis [8]. Several compounds have been isolated from *P. scutellarioides*, such as p-coumaric acid, caffeic acid, p-hydrophenyllactic acid, dihydrophenyllactic acid, and trans-rosmarinic acid [5] from the *henna* variety, sincoetsin C, 3-hydroxyspirocoleon 7-O-*β*-D-glucoside, scutellarioidone A, spirocoleon 7-O-*β*-D-glucoside [1] from the *inky finger* variety, scutellarioidones A, B, C, and D, 6-acetyl derivative of fredericone B, scutellarioidolide A, coleon O, coleon G, lanugone K, fredericone B, 2,16-diacetyl-6,11,12,14,17-heptahydroxy-5,8,11,13-abietatetraen-7-one [9] from the *watermelon* variety, spiroscutelone A-C and abietane-type diterpenoid [10] from the *color blaze dark star* variety. (2,4,4,4,16,16-D6)-3α,17β-Dihydroxy-5*β*-

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androstane, (E,E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-ol acetate, 1,8-bis(3,4-dicyanophenyl)anthracene, (23R)-methylcholesterol, stigmasterol, stigmast-8(14)-en-3β-ol and α-amyrin acetate were also detected by GCMS in *wizard mix* variety [11].

Among all *P. scutellarioides*, the *color blaze dark star* variety has not been widely studied. This study presents the first report for the isolations of stigmasterol and β-sitosterol from *P. scutellarioides* var. *color blaze dark star* and cytotoxicity of its fractions against A549, MDA-MB-231, MCF-7, KB, and KB-Vin tumor cell line.

2. Methods

2.1 General experimental procedures

NMR spectra were measured on JEOL JMN-ECA600 and JMN-ECS400 spectrometers with tetramethylsilane as an internal standard and $CDCl_3$ as solvent for both ^1H and ^{13}C NMR, and chemical shifts are recorded as δ values. Analytical and preparative thin-layer chromatography (TLC) were performed using precoated silica gel 60 F_{254} plates (0.25 mm, Merck) for normal-phase and silica gel 60 RP-18 $F_{254}S$ plates (0.25 mm, Merck) for reversed-phase. Column chromatography was carried out with silica gel 60 N (Spherical, 63-210 μm, neutral, Kanto Chemical).

2.2 Plant material

The leaves of *P. scutellarioides* var. *color blaze dark star* were collected from Rantebelu village, East Luwu, South Sulawesi, Indonesia and identified by the Faculty of Biology, State of Makassar University. A voucher specimen (ADA-FFUH-01) has been deposited in the Pharmacognosy-Phytochemistry Laboratory, Hasanuddin University.

2.3 Extraction, fractionation, and isolation

Fresh leaves (400 g) were oven-dried at 50 ^{0}C for three days (200 g), extracted with ethanol by maceration for five days, and the volatile solvent was evaporated (35.1 g). The residue was partitioned with H_2O and ethyl acetate (EtOAc) (1:2). The EtOAc layer (12.61 g) was subjected to normal phase open column chromatography with *n*-Hexane:EtOAc (7:1, 6:1, 5:1, 4:1, 3:1, 2:1, 1:1, 1:2 v/v) to get 60 fractions. All fractions were analyzed using normal-phase thin layer chromatography (TLC), respectively. Fractions which have similar TLC profile were combined to make 21 fractions. Fraction 6 (58.2 mg) was selected for further purification using preparative TLC developed with *n*-Hexane:EtOAc (3:1). The single spot was confirmed for white crystalline powder (19.5 mg) by TLC analysis using different solvent systems and characterized using 1D and 2D NMRs.

2.4 Assay for cytotoxicity against cancer cell lines

The EtOAc extract, fractions 1, 8, 10, 12, 15, 17, 18, and 21 were selected for *in vitro* cytotoxicity against A549 (lung carcinoma), MDA-MB-231 (triple-negative breast cancer), MCF-7 (estrogen receptor-positive and HER2 negative breast cancer), KB (cervical cancer cell line HeLa derivative), and KB-Vin [multidrug-resistant (MDR) subline of KB] cells using sulforhodamine B (SRB) assay as previously described [12]. Briefly, all cell lines were sub-cultured at 37 ^{0}C and 5% of CO_2 in T-75 flasks. The fresh trypsinized cell suspensions were introduced into 96-well plates at a density of 4000–11000 cells per well with the selected fraction. After incubation for 72 hours, cells were fixed in 10% trichloroacetic acid and stained with 0.04% sulforhodamine B. The cells were then washed with 10 mM Tris base to help dissolve the protein-bound dye, and then the optical density (OD) was measured at 515 nm. The results were expressed as % cell growth inhibition (Table 2).

3. Result and Discussion

A crude ethanol extract of *P. scutellarioides* was partitioned between H_2O and EtOAc. The EtOAc layers were fractionated by column chromatography using various solvent systems and led to the isolation of white crystalline powder. The ^{13}C and ^1H NMR along with HMBC and HMQC spectra of the powder supported the presence of sterol skeleton with two double bonds at δ_C 140.9 and 121.8 (C5/C6) as well as at δ_C 138.4 and 129.3 (C22/C23), and a hydroxyl group at δ_C 71.9 (C3) and δ_H 3.51 (1H, m). Some signals also appeared at position δ_C 34.0 and 26.1 (C22/C23), supported the presence of single bond.

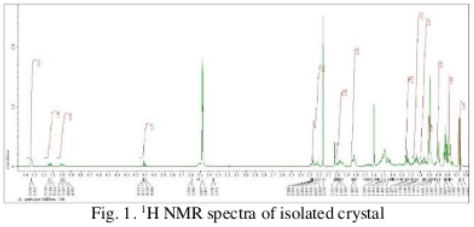

Fig. 1. ^1H NMR spectra of isolated crystal

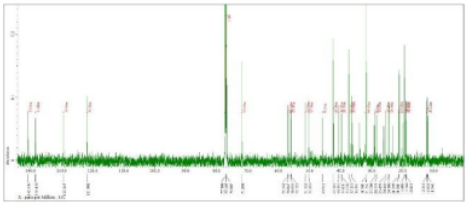

Fig. 2. ^{13}C NMR spectra of isolated crystal

The spectral data were identical with those of the reported stigmasterol and *β*-sitosterol [13]. Both stigmasterol and *β*-sitosterol have six methyls at C18, C19, C21, C26, C27, and C29; three quaternary carbons at C5, C10, and C13; nine methylenes at C1, C2, C4, C7, C11, C12, C15, C16, and C28; eleven methines at C3, C6, C8, C9, C14, C17, C20, C22, C23, C24, and C25 for stigmasterol, while *β*-sitosterol have eleven methylenes at C1, C2, C4, C7, C11, C12, C15, C16, C22, C23, and C28; nine methines at C3, C6, C8, C9, C14, C17, C20, C24, and C25. The structural difference between stigmasterol (**1**) and *β*-sitosterol (**2**) is a C-C bond formation between C22 and C23. The double bond for stigmasterol (**1**) appeared at δ_H 0.99 (s, 1H) and 5.11 (dd, 1H) in the ^{1}H NMR as well as δ_C 138.4 and 129.3 in the ^{13}C NMR, respectively, while all other chemical shifts were overlapped due to structural similarities.

The determination of carbon resonances was carried out using two dimensional NMR data. The correlation observed between the methine proton at δH 1.83 (m, 2H) with carbons at δC 37.3 (C1) and δC 140.9 (C5), methyl proton at δH 1.14 (s, 3H) with carbon at δC 140.9 (C5), methyl proton at δH 1.09 (s, 3H) with carbon at δC 56.0 (C17), methylenes proton at δH 0.90 (d, 2H) with carbons at δC 19.1 (C21) and 51.3 (C24), δH 0.79 (t, 2H) with carbons at δC 32.0 (C25) and 25.5 (C28).

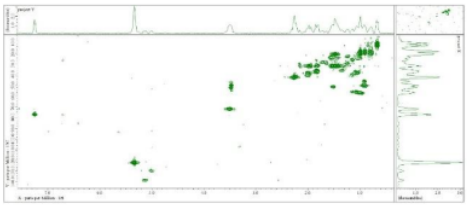

Fig. 3. HMQC spectra of isolated crystal

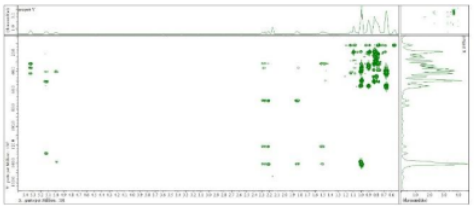

Fig. 4. HMBC spectra of isolated crystal

Based on spectral data, those white crystalline powder consists of a mixture of stigmasterol and β-sitosterol in a ratio of about 1:1. The small structural differences made them difficult to separate and often obtained in mixed form. The challenge of attaining pure sitosterol has also been confirmed by other studies [14,15,16,17,18].

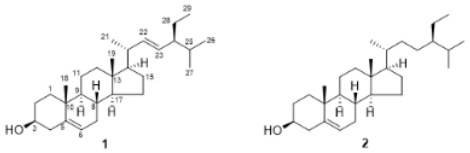

Fig. 5. Structures of Stigmasterol (**1**) and *β*-Sitosterol (**2**)

EtOAc extract, fractions 1, 8, 10, 12, 15, 17, 18, and 21 were evaluated for cytotoxicity against five human tumor cell lines, A549, MDA-MB-231, MCF-7, KB, and KB-Vin (Table 2). EtOAc extract, fractions 1 and 8 showed the lowest cell growth inhibition among all tested samples, while fractions 15 and 17 showed promising inhibitory effects. Low growth inhibition effect of EtOAc extract may be due to the antagonistic effect of the compounds in the extract. Fractions 1 and 8 were the initial fractions which may be residual wax or compounds that have very low polarity. Fraction 15 has low toxicity and sensitivity with IC50 ranging from 14.3 – 24.3 *μ*g/mL, whereas fraction 17 has moderate toxicity and sensitivity against A549 and MCF-7 cell lines with IC50 7.3 and 9.9 *μ*g/mL, respectively. The toxicity was classified into four category, IC50 < 1.0 *μ*g/mL: high toxicity and sensitivity, 1.0 < IC50 < 10.0 *μ*g/mL: moderate toxicity and sensitivity, 10.0 < IC50 < 50.0 *μ*g/mL: low toxicity and sensitivity, and IC50 > 50.0 *μ*g/mL: non toxic and insensitive (19). Moderate toxicity against KB-Vin cell line, a p-glycoprotein (p-gp) over-expressing MDR cell, owned by fractions 15 and 17 (IC50 of 18.1 and 11.3

μg/mL, respectively), indicated that the active components of fractions 15 and 17 might not be p-gp substrates and could be effective against MDR tumors.

Table 1. ^{1}H and ^{13}C NMR chemical shift of isolated crystal, a mixture of stigmasterol (**1**) and β-sitosterol (**2**)

Position	Observed Isolated Crystal		Reported Values [13] Stigmasterol	Reported Values [13] β-Sitosterol
	^{1}H[c]	^{13}C[d]	^{13}C	^{13}C
1		37.3	37.3	37.3
2		32.0	31.8	32.0
3	1.83 (m, 2H)	71.8	71.9	71.9
4		42.4	42.4	42.4
5		140.9	140.8	140.8
6		121.7	121.8	121.8
7		31.8	31.8	31.8
8		32.0	32.0	32.0
9		50.2	50.2	50.2
10		36.6	36.6	36.6
11		21.2	21.2	21.2
12		39.8	39.8	39.9
13		42.3	42.3	42.3
14		56.9	57.0	56.8
15		24.4	24.5	24.4
16		29.0	29.0	28.3
17		56.0	56.0	56.1
18	1.14 (s, 3H)	12.3	12.4	11.9
19	1.09 (s, 3H)	19.5	19.5	18.9
20	1.01 (s, 1H)	40.6	40.6	36.2
21		19.1	21.2	19.1
22 for **1**	5.00 (dd, J=15.3, 8.8 Hz, 1H)	138.4	138.4	
22 for **2**	0.90 (d, J=6.5 Hz, 1H)	34.0		34.0
23 for **1**	5.13 (dd, J=15.1, 8.6 Hz, 1H)	129.3	129.3	
23 for **2**	0.79 (t, J=1.9 Hz, 2H)	26.1		26.1
24		51.3	51.3	45.9
25		32.0	32.0	29.2
26		19.1	19.1	19.9
27		19.5	21.3	19.5
28		25.5	25.5	23.1
29		12.1	12.1	12.1

[a] assignments made on the basis of HMBC and HMQC correlation; [b] Chemical shift values are in δ (ppm); [c] $CDCl_3$ at 600 MHz; [d] $CDCl_3$ at 150 MHz.

Table 2. Cytotoxicity data of selected fractions of EtOAc extract

Sample	Concentration (μg/mL)	% Cell growth inhibition [a] A549	MDA-MB-231	MCF-7	KB	KB-Vin
EtOAc extract	20	0.4	5.0	1.4	9.7	10.0
Fr 1	20	0	0	0	0	0
Fr 8	20	0	0.8	8.5	2.7	0
Fr 10	20	40.9	47.0	37.3	34.8	29.3
Fr 12	20	14.0	19.1	29.1	21.6	9.9
Fr 15	20	73.0	66.0	67.0	52.4	61.3
Fr 17	20	100	100	100	100	100
Fr 18	20	67.2	61.7	49.2	56.8	37.9
Fr 21	20	11.9	17.0	22.2	22.4	17.6
DMSO	0.2%	0	0	0	0	0

[a] Cytotoxicity stated as % cell growth inhibition for each cell line.

Table 3. Cytotoxicity data of fraction 15 and 17

Sample	IC_{50} [a]	A549	MDA-MB-231	MCF-7	KB	KB-Vin
Fr 15	μg/mL	14.3	24.3	15.8	19.6	18.1
Fr 17	μg/mL	7.3	14.1	9.9	11.0	11.3

[a] Cytotoxicity stated as IC_{50} (μg/mL), the concentration of fraction required for 50% inhibition.

4. Conclusions

Stigmasterol and β-sitosterol were isolated as a mixture from the leaves of *P. scutellarioides* var. *color blaze dark star*. The EtOAc extract was roughly fractionated by silica gel column chromatography. Fractions 15 and 17 showed promising inhibitory effects on all tested tumor cell growth, including the MDR tumor cell, with IC_{50} of 14.3-24.3 μg/mL and 7.3-14.1 μg/mL, respectively. Further research is needed to isolate these bioactive compounds.

Conflicts of interest

Authors declare there is no conflicts of interest.

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