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Original Research Article

Cytotoxicity of Butyrospermol and Sitosterone from the Stem Bark of *Syzygium aqueum*

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ABSTRACT

Syzygium aqueum is a public large species of *Syzygium* genus in Myrtaceae family and it is native to Indonesia and Malaysia. Various parts of *S. aqueum* have been widely known to be used for traditional medicine, therefore this plant is documented as a medicinal plant. The isolation of some phytochemical compounds from the stem bark of *Syzygium aqueum* and their cytotoxicity were determined. The extraction and separation of phytochemical compounds in the stem bark of *S. aqueum* was carried out using standard methods. The structures of isolated compounds were determined by UV, FTIR and NMR. The analysis of all spectra obtained and comparison with literature data led to the conclusion that the isolated compounds were steroids, namely butyrospermol and sitosterone. The cytotoxicity of the isolated compounds was then evaluated against HeLa and T47D cell lines by the MTT method and A549 cell lines by the XTT method.

Keyword: *Syzygium aqueum*, Butyrospermol, Sitosterone, HeLa, MTT assay, XTT assay.

Introduction

Cancer is a worldwide health problem leading to high mortality and morbidity rates in children and adults.[1] Mortality cases are increasing all over the world and Weasam Kooti *et al*. reported that the deaths due to cancer will rise up to 13.1 million by 2030.[2] In the aim of numerous studies, multiple researchers have described plant-derived known and unknown bioactive compounds from several plants for the treatment of cancer.[3-5] The bioactive compounds refer to phytochemical constituents from various parts of medicinal plants such as flowers, pulps, seeds, leaves, barks, etc.[6] The phytochemicals from medicinal plants have low side effects and high effectiveness.[7]
Syzygium in Myrtaceae family is a genus of flowering plants which comprises about 1100-1200 species and it has high levels of diversity.[8,9] Several public large species in this genus are cultivated and they are used as traditional medicine.[10-11] Phytochemicals and bioactivities have been widely studied from many species in this genus.[12] One of public large species, *Syzygium aqueum*, is also known as *Eugenia aquea* and it is native to Malaysia and Indonesia.[11,13] The various parts of the plant are applied for traditional medicine and this plant is well documented as a medicinal plant.[14] The previous researchers reported tannin and flavonoid compounds from the leaf of *S. aqueum*.[15-16] Moreover, antidiabetic[15] and cytotoxicity on MCF-cell line[17] were evaluated from some of these flavonoids. Cytotoxicity against MCF-7, MDAMB-231 and HS-27 cancer cell lines was tested on aqueous and methanol extracts of *S. aqueum* fruits.[18]

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Cervical cancer (HeLa) and breast cancer (T47D) are common in women. Breast cancer is the second leading mortality after cervical cancer.[19-20] Lung cancer (A549) also has a high incidence a leading cause cancer death worldwide both in men and women.[21-23] Cytotoxicity test on HeLa, T47D and A549 cancer cell lines of isolated compounds from *S. aqueum* (various parts) have not been reported by any researchers. Therefore, the focus of this study is to isolate phytochemical constituents from stem bark of *S.aqueum*, and then followed by cytotoxicity test against on the important cancer cell lines (HeLa, T47D and A549) using MTT and XTT assay. From this study, two steroids, namely Butyrospermol and Sitosterone, were isolated successfully. The structure of these compounds were presented in Figure-1. These two compounds were then subject to cytotoxicity evaluation.

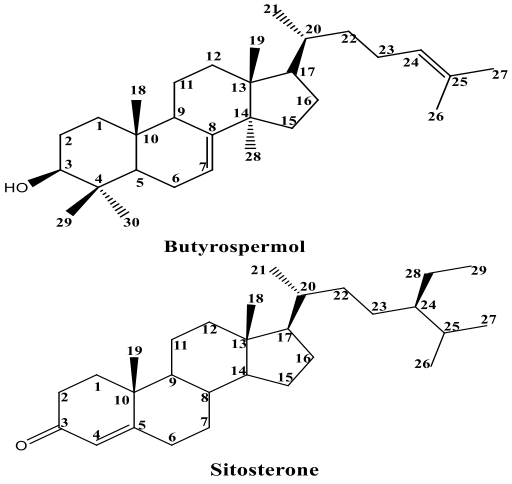

Figure 1: Chemical structures of butyrospermol and sitosterone of *S. aqueum*

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Materials and Methods

General experimental procedures

For Analytical TLC (Thin Layer Chromatography), a pre-coated Silica gel 60 F_{254} (25 Aluminum sheets 20×20 cm, Merck) was used. Silica gel 60 (700-200 mesh ASTM) was used for Column chromatography. UV-Vis spectra were measured on UV-Vis spectrophotometer (Shimadzu). Tracer-100 FT-IR spectrophotometer (Shimadzu) was applied for obtaining FT-TR (Fourier Transform Infrared spectroscopy) spectra which was measured in cm^{-1}. ^{1}H- and ^{13}C-NMR (Nuclear Magnetic Resonance) were recorded on BRUKER 600 Hz with Tetramethylsilane (TMS). Chemical shift was given in δ (ppm) and coupling constants (*J*) were expressed in hertz (Hz). HR-DART-MS was accomplished with a JEOL JMS HX-110 mass spectrometer. ELISA (Enzyme-Linked Immunosorbent Assay) reader was used for measuring cytotoxicity.

Plant material

The stem bark of *S. aqueum* was obtained from Wage, Taman, Sidarjo, East Java, Indonesia on September 5th, 2018 and the voucher specimen (UA-MSa050918) was deposited at the Herbarium of Universitas Airlangga. Laboratory of Biosystematic, Department of Biology, Faculty of Science and Technology, Universitas Airlangga.

Extraction and Separation

The stem bark powder (2 kg) was macerated in methanol (40 L) at room temperature for 3 days. After that, the methanol extract was evaporated using evaporator (Buchi Rotary). The extract (450 g) was partitioned with n-hexane (6 L) in triplicate. N-hexane extract (50 g) was separated utilizing silica gel chromatography with n-hexane:ethyl acetate (6:4, 3 L) as eluent. The similar fractions were combined yielding 19 fractions (SA-1 to SA-19). Fraction SA-6 (800 mg) was re-chromatographed with eluent n-hexane:dichloromethane (6:4, 1 L) which yielded 3 fractions (SA-6-1 to SA-6-3). Butyrospermol (58 mg) was obtained from fraction-1 (SA-6-1) and sitosterone (51 mg) was gotten form fraction-3 (SA-6-3).

Cytotoxicity of isolated compounds
Cell culture

Three cancer cell lines, HeLa, T47D and A459 were obtained from American Type Culture Collection (ATCC). Cells were cultured at 37ºC and 5% CO_2 for 24 h and 100% humidity in medium supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin.

MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay method was used. HeLa and T47D cells were cultured in a 96-well plate at 213 x 10^4 cell/well density. The cells were then treated with 100 µL of the prepared compounds with various concentrations (1.5625, 3.125, 6.25, 12.5, 25, 50 and 100 µg/mL) for 24 h. A 100 µL of MTT solution (50 mg in 10 mL of PBS) was added, followed by incubation in CO_2 incubator for 4 h. The amount of viable cells was visualized by the formation of purple colour due to formation of formazan crystals. The formed formazan that was proportional to the total number of viable cells was calculated using spectrophotometer at 560 nm. The number of viable cells was calculated using the following formula;

$$\%\ viable\ cells = \frac{\text{treatment absorbance} - \text{control medium absorbance}}{\text{negative control absorbance} - \text{control medium absorbance}}\ x\ 100\%$$

IC_{50} was obtained after statistical analysis using SPSS program. Doxorubicin was used as a positive control in this assay. The test was conducted in triplicate.

XTT assay

XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) assay method was carried out. The XTT method is basically the same as MTT. The only difference lies in the tetrazolium salt used. The XTT method used 1 mg/mL XTT reagent (4 mg in 4 mL DMEM/MEM/RPMI-1640 culture medium) added with 10 µL of PMS. The absorbance was determined at 450 nm.

Results and Discussion

Butyrospermol was collected as pink oil. UV spectrum in MeOH appeared at 239 nm. DART-MS: m/z 427.3934 $[M+H]^+$ suitable for molecular formula $C_{30}H_{50}O$, FTIR spectra showed the bands at 3385 cm^{-1} (-OH), 2927-2929 cm^{-1} (sp^3, C-H stretching), 1668 cm^{-1} (C=C), 1452 cm^{-1} and 1064 (C-O). ^{1}H-NMR δ_H (ppm): 0.75 (3H, s), 0.81 (3H, s), 0.86 (3H, s), 0.89 (3H, d, *J* = 6.5 Hz), 0.97 (6H, s), 1.04 (2H, m), 1.14 (1H, m), 1.31 (1H, m), 1.40 (1H, m), 1.44 (1H, m), 1.50 (4H, m), 1.61 (3H, s), 1.64 (3H, m), 1.67 (1H,m), 1.69 (3H, s), 1.79 (1H, m), 1.87 (1H, m), 1.94 (2H, m), 1.97 (1H, m), 2.04 (1H, m), 2.15 (1H, m), 2.21 (1H, m), 3.25 (1H, dd, *J* = 3.9, 11.5 Hz), 5.10 (1H, m), 5.26 (1H, m). The ^{13}C-NMR δ_C (ppm): 37.2 (C-1), 27.7 (C-2), 79.3 (C-3), 38.9 (C-4), 50.6 (C-5), 23.9 (C-6), 117.8 (C-7), 145.9 (C-8), 48.9 (C-9), 35.0 (C-10), 18.1 (C-11), 33.8 (C-12), 43.5 (C-13), 51.2 (C-14), 34.0 (C-15), 28.2 (C-16), 52.9 (C-17), 22.0 (C-18), 13.1 (C-19), 35.9 (C-20), 18.3 (C-21), 36.1 (C-22), 25.0 (C-23), 125.1 (C-24), 130.9 (C-25), 17.6 (C-26), 25.7 (C-27), 27.6 (C-28), 14.7 (C-29), 27.3 (C-30). ^{1}H-NMR δ_H : the signals at 5.26 ppm and 5.10 ppm belonged to protons of olefinic (C-7 & C-24) and confirmed by HSQC. And then, the carbon and proton (δ_C 79.29 ppm & δ_H 3.35 ppm) at position C-3 appeared in downfield region because it was connected to electronegative group (-OH). COSY and HMBC spectra were linked to confirm the structure (Figure 2). The analysis of NMR spectra is presented in Table 1. This NMR data was also compared with literture.

Sitosterone was obtained as pink oil. DART-MS: m/z 411.3632 $[M-H]^-$ suitable for molecular formula of $C_{29}H_{48}O$, FTIR spectra showed some bands at 2926-2848 cm^{-1} (sp^3 C-H stretching), 1678 cm^{-1} (C=O), 1618 cm^{-1} (C=C). ^{1}H-NMR δ_H (ppm): 0.72 (3H, m), 0.82 (3H, d, *J* = 6.8 Hz), 0.83 (3H, d, *J* = 6.0 Hz), 0.84 (3H, t, *J* = 6.0 Hz), 0.92 (5H, m), 1.02 (3H, m), 1.11 (1H, m), 1.16 (3H, m), 1.18 (3H, s), 1.26 (2H, m), 1.32 (1H, m), 1.36 (1H, m), 1.44 (1H, m), 1.52 (1H, m), 1.53 (1H, m), 1.61 (2H, m), 1.67 (1H, m), 1.71 (1H, m), 1.85 (3H, m), 2.02 (2H, m), 2.27 (2H, m), 2.35 (1H, m), 2.41 (1H, m), 5.73 (1H, s). ^{13}C-NMR δ_C (ppm): 35.7 (C-1), 34.0 (C-2), 199.6 (C-3), 123.7 (C-4), 171.6 (C-5), 33.9 (C-6), 32.9 (C-7), 36.1 (C-8), 53.8 (C-9), 38.6 (C-10), 21.0 (C-11), 39.6 (C-12), 42.2 (C-13), 56.0 (C-14), 24.2 (C-15), 28.2 (C-16), 55.9 (C-17), 12.0 (C-18), 17.4 (C-19), 35.7 (C-20), 18.7 (C-21), 32.1 (C-22), 26.1 (C-23), 45.9 (C-24), 29.2 (C-25), 19.8 (C-26), 19.0 (C-27), 23.1 (C-28), 11.8 (C-29). The signal at chemical shift δ_C 199.6 ppm was a signal belonging to C_{sp2}. COSY and HMBC correlations were joined for structure confirmation (Figure 2). The analysis of NMR spectra was presented in Table 2. This NMR data was also compared with literture.

The cytotoxicity evaluation of isolated compounds was determined against HeLa, T47D and A549 cancer cell lines (Table 3). Butyrospermol (43.59 ± 0.393 µg/mL) was moderately active against HeLa cell line and it was less cytotoxic on T45D and A549 cell lines. Moreover, sitosterone (29.96 ± 0.0422 µg/mL) also had moderate toxicity against A549 cell line using XTT assay. In literature, pure compound is considered as highly toxic (≤ 4 µg/mL), toxic (≤ 20 µg/mL), moderately toxic (20-100 µg/mL) and non-toxic (> 100 µg/mL).[27,28] In XTT assay, orange-coloured formazan is water soluble and its intensity can be measured with a spectrophotometer. However, the purple needle-shaped crystals or formazan needles in the cells are insoluble in water and the formazan has to be dissolved in DMSO or isopropanol for MTT assay before measuring the absorbance.[29]

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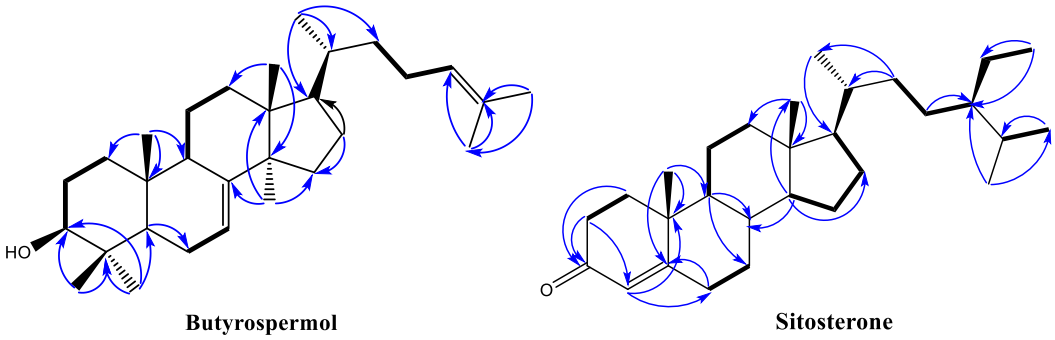

Butyrospermol **Sitosterone**

Figure 2: ( ) HMBC and ( ) COSY correlations of **Butyrospermol** and **Sitosterone**

Table 1: NMR Spectra Data of Butyrospermol Recorded in $CDCl_3$ / 600 Hz

Position	DEPT	Butyrospermol		Literature [24-25]	
		δ_C (ppm)	δ_H (ppm) (mult, J in Hz)	δ_C (ppm)	δ_H (ppm) (mult, J in Hz)
1	CH_2	37.2	1.67 (*m*), 1.14 (*m*)	37.5	-
2	CH_2	27.7	1.64 (*m*)	27.7	-
3	CH	79.3	3.25 (*dd*, *J*= 3.9, 11.5 Hz)	79.3	3.24 (*m*)
4	C	38.9	-	38.9	-
5	CH	50.6	1.31 (*m*)	50.6	-
6	CH_2	23.9	1.97 (*m*), 2.15 (*m*)	23.9	-
7	CH	117.8	5.26 (*m*)	117.8	5.30 (*dd*, *J* = 3.5 Hz)
8	C	145.9	-	145.9	-
9	CH	48.9	2.21 (*m*)	48.9	-
10	C	35.0	-	35.0	-
11	CH_2	18.1	1.50 (*m*)	18.2	-
12	CH_2	33.8	1.79 (*m*), 1.64 (*m*)	33.8	-
13	C	43.5	-	43.5	-
14	C	51.2	-	51.3	-
15	CH_2	34.0	1.44 (*m*), 1.50 (*m*)	33.9	-
16	CH_2	28.2	1.94 (*m*)	28.5	-
17	CH	52.9	1.50 (*m*)	52.2	-
18	CH_3	22.0	0.81 (*s*)	22.1	0.81 (*s*)
19	CH_3	13.1	0.75 (*s*)	13.1	0.74 (*s*)
20	CH	35.9	1.40 (*m*)	35.8	-
21	CH_3	18.3	0.89 (*d*, *J* = 6.5 Hz)	18.6	0.84 (*brs*)
22	CH_2	36.1	1.04 (*m*)	35.2	-
23	CH_2	25.0	2.04 (*m*) 1.87 (*m*)	25.4	-
24	CH	125.1	5.10 (*m*)	125.1	5.10 (*dd*, *J* = 6.8, 7.2 Hz)
25	C	130.9	-	130.9	-
26	CH_3	17.6	1.61 (*s*)	17.7	1.61 (*s*)
27	CH_3	25.7	1.69 (*s*)	25.7	1.68 (*s*)
28	CH_3	27.6	0.97 (*s*)	27.6	0.97 (*s*)
29	CH_3	14.7	0.86 (*s*)	14.7	0.86 (*s*)
30	CH_3	27.3	0.97 (*s*)	27.3	0.97 (*s*)

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Table 2: NMR Spectra Data of Sitosterone Recorded in $CDCl_3$ / 600 Hz

Position	DEPT	Sitosterone		Literature [26]	
		δ_C (ppm)	δ_H (ppm) (mult, *J* in Hz)	δ_C (ppm)	δ_H (ppm) (mult, *J* in Hz)
1	CH_2	35.7	2.02 (*m*), 1.71 (*m*)	35.8	-
2	CH_2	34.0	2.41 (*m*), 1.32 (*m*)	34.1	-
3	C	199.6	-	199.8	-
4	CH	123.7	5.73 (*s*)	123.8	5.72 (*s*)
5	C	171.6	-	171.8	-
6	CH_2	33.9	2.35 (*m*), 1.02 (*m*)	33.1	-
7	CH_2	32.9	2.27 (*m*)	32.2	-
8	CH	36.1	1.36 (*m*)	35.7	-
9	CH	53.8	0.92 (*m*)	53.9	-
10	C	38.6	-	38.7	-
11	CH_2	21.0	1.52 (*m*), 1.44 (*m*)	19.9	-
12	CH_2	39.6	2.02 (*m*), 1.16 (*m*)	39.7	-
13	C	42.2	-	42.5	-
14	CH	56.0	1.11 (*m*)	56.0	-
15	CH_2	24.2	1.61 (*m*)	24.3	-
16	CH_2	28.2	1.85 (*m*)	28.3	-
17	CH	55.9	1.02 (*m*)	56.1	-
18	CH_3	12.0	0.72 (*s*)	12.1	0.74 (*s*)
19	CH_3	17.4	1.18 (*s*)	17.5	1.18 (*s*)
20	CH	35.7	1.53 (*m*)	36.2	-
21	CH_3	18.7	0.92 (*m*)	19.9	0.91 (*d, J*= 6.5 Hz)
22	CH_2	32.1	1.85(*m*), 1.02 (*m*)	34.0	-
23	CH_2	26.1	1.16 (*m*)	26.2	-
24	CH	45.9	0.92 (*m*)	45.9	-
25	CH	29.2	1.67 (*m*)	29.3	-
CH_2	CH_3	19.8	0.83 (*d, J* = 6.0 Hz)	21.1	0.83 (*d, J* = 6.8 Hz)
27	CH_3	19.0	0.82 (*d, J* = 6.8 Hz)	19.1	0.80 (*d, J* = 6.4 Hz)
28	CH_2	23.1	1.26 (*m*)	23.1	-
29	CH_3	11.8	0.84 (*t, J* = 6.0 Hz)	12.1	0.84 (*t, J* = 7.2 Hz)

Table 3: Cytotoxicity (IC_{50}) data of Butyrospermol and Sitosterone against HeLa, T47D and A549

	IC_{50} (µg/mL)		
	MTT assay		XTT assay
	HeLa	**T47D**	**A549**
Butyrospermol	43.59 ± 0.393	419.05 ± 0.246	354.85 ± 0.017
Sitosterone	ND*	ND*	29.96 ± 0.0422
Doxorubicin	2.67± 0.247	0.035 ± 0.012	ND*

*ND = not determined

Conclusion

In the current study, two steroids, butyrospermol and sitosterone, were isolated from the stem bark of *S. aqueum*. These two steroids were reported to have been isolated from this species for the first time. They were evaluated for their cytotoxicity on different cell lines with MTT assay and XTT assay. Both compounds have moderate cytotoxicity on different cancer cell lines.

Conflict of interest

The Authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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