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STEROIDAL COMPOUNDS FROM THE ROOTS OF *HOLARRHENA CURTISII*

(Sebatian Steroid daripada Akar *Holarrhena curtisii*)

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Abstract

Two known steroidal compounds namely pregnenolone (**1**), an androstane steroid [3-hydroxypreg-5-en-20-one] and stigmasterol (**2**) [stigmasta-5,22-dien-3β-ol] have been isolated from methanol extract of the roots of *Holarrhena curtisii* by using several chromatographic means. Their structures were elucidated through a combination of NMR, IR and GC-MS techniques by comparison with the data obtained from the literature. Separation of pregnenolone and stigmasterol from the *Holarrhena curtisii* has never been reported.

Keyword: Apocynaceae, *Holarrhena curtisii*, roots, steroids, pregnenolone, stigmasterol

Abstrak

Dua sebatian steroid yang diketahui iaitu pregnenolon, sebatian steroid androstana (3-hydroxypreg-5-en-20-one) dan stigmasterol telah diasingkan daripada ekstrak methanol akar *Holarrhena curtisii* dengan menggunakan pelbagai kaedah kromatografi. Struktur sebatian berikut telah ditentukan melalui kombinasi teknik RMN, IM, dan KG-SJ secara perbandingan dengan data daripada kajian lepas. Pemisahan sebatian pregnenolon dan stigmasterol daripada *Holarrhena curtisii* belum pernah dilaporkan.

Kata kunci: Apocynaceae, *Holarrhena curtisii*, akar, steroid, pregnenolone, stigmasterol

Introduction

Numerous drugs with steroidal ring structures have neen isolated from plant sources. The limited yield of some bioactive compounds in plants however gives a significant challenge for drug development. The use of plants and their preparations to treat infectious diseases is an age-old practice. *Holarrhena curtisii* which is locally know as ”Pulai Tanah” is a native plant of Malaysia [1]. It has been used traditionally for the treatment of dysentery especially amoebic dysentry [2, 3]. This decidious tree [4] belongs to a very important alkaloid family Apocynaceae [5] and provide mainly steroidal alkaloids of the aminopregnane type [6]. Alkaloids isolated from *H. curtisii* are used as antibiotics [7] while the non-alkaloid extract exhibited significant cytotoxic activity [8].

The purpose of this study is to identify and characterize the bioactive principles from the roots of *Holarrhena curtisii*. This paper describes the isolation and structural elucidation of two unreported steroid compounds from this species which are identified as pregnenolone (1) and stigmasterol (2) on the basis of spectroscopic evidence.

Experimental

General Experimental Procedures

Solvents used are from analar grade. Thin layer chromatography (TLC) was carried out on a silica gel 60 F_{254}. Reagents used are 5% H_2SO_4, Vanillin and 5% H_2SO_4 in EtOH. ^{1}H, APT and 2D NMR spectra were recorded in $CDCl_3$ on a FT-NMR 600MHz Cryoprobe Model Bruker/AVANCE III equipped with TOPSPIN 2.1 software. Chemical shifts are given in δ (ppm) values referred to the internal standards, tetramethylsilane (TMS) while

coupling constant in Hz. IR spectra were measured on FTIR Model Perkin Elmer GC1605 spectrophotometer. GC-MS were determined on an Agilent 7890A gas chromatograph directly coupled to the mass spectrometer system of an Agilent 5975C inert MSD with triple-axis detector.

Plant Material

Roots of *Holarrhena curtisii* were collected from Perlis. A voucher specimen UKMB21593 is deposited at the herbarium of National University of Malaysia.

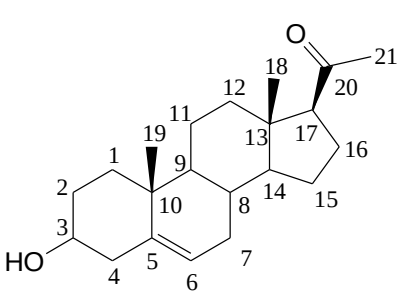
Extraction and Isolation

The roots of *Holarrhena curtisii* (573.3g) were powdered and percolated with MeOH at room temperature for 3 days x10. The concentrated MeOH extract (29.0g) was acidified with 5% HOAc. Two fractions obtained from this acidic process contain the alkaloid and non-alkaloid (4.88g) components. The alkaloidal fraction was basified with Na_2C0_3 to pH9 and partitioned with $CHCl_3$ followed by drying with Na_2SO_4 (anhydrous). The concentrated alkaloid extract (2.93g) was subjected to vacuum liquid chromatography (VLC) by using silica 7749 and eluted with $CHCl_3$: MeOH in order of increasing polarity resulting in 6 fractions. The first fraction was re-chromatograph using column chromatography (CC) (Alumina, Merck) with eluent 9Hex:1Diethylether yielded pregnenolone (1) 1.7mg. While, stigmasterol (2) was attained from the non-alkaloid part using CC that was eluted with 9Hex:1Aceton as a solvent system. Compound 2 was purified and recrystallized to yield white needle crystals (4.8mg).

Results and Discussion

Two compounds obtained from the roots of *Holarrhena curtisii* were identified as pregnenolone (1) and stigmasterol (2). These two compounds showed mass spectral data of the steroid aglycone and gave GCMS data in agreement with the proposed structures. Their IR data showed absorptions for OH and C=O functions. The NMR data of 1 and 2 can be seen in Table 1. The spectra were assigned based on the application standard of 2D NMR methods (HSQC, HMBC, COSY) and with reference to related compounds that have been previously reported [9 - 11].

Compound (1), $C_{21}H_{32}O_2$ appeared as a colourless needle-like crystal which gave a positive response with reagents vanillin followed by 5% H_2SO_4 in EtOH for a steroid. The ^{1}H NMR spectrum showed the presence of two methyl singlets (δ 0.64 and δ 0.99) , 8 complicated methylene envelope (between δ 1.02 and δ 2.31) and a vinylic proton multiplet at δ 5.36 suggesting the possibility of a steroidal moiety in 1. Additionally, one OH group at δ 3.54 and one OMe group at δ 2.13 implied that 1 is a steroidal aglycone. The APT NMR spectrum showed the presence of a total 21 carbons consistent with a C-21 pregnane skeleton. IR spectra showed absorptions for OH, C=O and C=C functions at 3383 cm^{-1} (OH), 1702 cm^{-1} (C=O) and 1460 cm^{-1} (C=C). The existance of C=C also was indicated by the presence of vinylic H-6 signal at δ 5.36 in the ^{1}H NMR spectrum and the olefinic carbon resonances at δ 140.7 and δ 121.4 in the APT NMR spectrum. Moreover, the presence of a 2^o OH group was suggested by the oxymethine resonance at δ 71.7 in the APT NMR. Determination of the OH location was also supported by HSQC and COSY data which confirmed the CH_2-CH(OH)-CH_2 fragments corresponding to the structure of C2-C3-C4 as shown in structure 1. Compound (1) was comfirmed as 3-hydroxypreg-5-en-20-one by GC-MS data at m/z 316.2.



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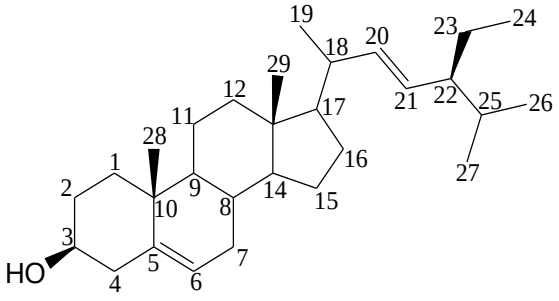

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Table 1. APT and ^{1}H NMR spectral data of pregnenolone (**1**) and stigmasterol (**2**) from roots of *H. curtisii*

	1		**2**	
Position	**APT**	**^{1}H**	**APT**	**^{1}H**
1	37.3	1.02 (4H, *s*) 1.19 (3H, *m*) 1.87 (2H, *m*)	37.3	1.04 1.17 (5H, *m*) 1.84 (1H, *m*)
2	31.8	1.58 (10H, *m*) 2.02 (2H, *m*)	31.7	1.84 (1H, *m*) 1.99 (2H, *m*)
3	71.7	1.19 (3H, *m*) 1.58 (10H, *m*) 3.54 (1H, *m*)	71.8	3.53 (1H, *m*)
4	42.3	2.21 (2H, *m*) 2.31 (1H, *m*)	42.3	2.29 (1H, *m*)
5	140.7	-	140.8	-
6	121.4	5.36 (1H, *m*)	121.7	5.36 (1H, *br-s*, *J* = 5.32 Hz)
7	31.9	1.58 (10H, *m*) 2.02 (2H, *m*) 2.13 (3H, *s*)	31.9	1.47 1.99 (2H, *m)*
8	29.7	1.87 (2H, *m*)	31.7	1.47
9	50.0	1.02 (4H, *s*)	50.2	1.01 (2H, *s*)
10	36.5	-	36.5	-
11	21.1	1.58 (10H, *m*)	21.1	1.47
12	38.8	1.58 (10H, *m*) 2.02 (2H, *m*)	39.7	1.17 (1H, *m*)
13	44.0	-	45.4	-
14	56.9	1.19 (3H, *m*)	56.9	1.01 (2H, *s*)
15	24.5	1.19 (3H, *m*) 1.58 (10H, *m*)	24.4	1.56 1.06
16	22.8	1.87 (2H, *m*) 2.21 (2H, *m*)	29.0	1.17 (5H, *m*) 1.71 (1H, *m*)
17	63.7	2.54 (1H, *t*)	55.9	1.17 (5H, *m*)
18	13.3	0.64 (3H, *s*)	40.5	1.19 (2H, *m*)
19	19.4	1.02 (4H, *s*)	21.9	1.01 (2H, *s*)
20	209.6	-	138.4	5.15 (1H, *dd*)
21	31.6	2.13 (3H, *s*)	129.3	5.01 (1H, *dd*)
22	-	-	51.3	5.15 (1H, *dd, J* = 15.2, 8.5 Hz)
23	-	-	25.4	1.17 (5H, *m*) 1.47 5.01 (1H, *dd, J* = 15.2, 8.5 Hz)
24	-	-	11.9	0.83 (4H, *m*)
25	-	-	31.3	1.47
26	-	-	19.0	0.83 (4H, *m*)
27	-	-	21.2	0.83 (4H, *m*)
28	-	-	19.4	1.01 (2H, *s*)
29	-	-	17.4	0.70 (1H, *s*)

Compound (2), $C_{29}H_{48}O$ is the major compound obtained from the non-alkaloid extract and gave positive result for a steroid when tested with reagents vanillin followed by 5% H_2SO_4 in EtOH. Compound 2 is a white crystalline needles with melting point 144-146°c [10]. The ^{1}H NMR spectrum of 2 indicated the presence of 6 methyl peaks of H-18, H-27, H-29, H-26, H-21 and H-19 that appeared at respective δ 1.19, 0.83, 0.70, 0.83, 5.01 and 1.01. The hydroxymethine proton H-3 appeared as a multiplet at δ 3.53 and revealed the existance of signals for olefinic proton at δ 5.36, 5.15, 5.01 and 3.53. Angular methyl proton at δ 0.70, 0.83 and 1.01 corresponds to C18 and C19 proton respectively [12, 13]. The APT NMR has shown recognizable signals at 145.2 and 121.7 ppm which are assigned to the C5 and C6 double bonds. The value at 21.9 ppm corresponds to angular C19. APT spectra showed 29 carbons signal including 6 methyls, 9 methylenes, 11 methane and 3 quaternary carbons. The alkene carbons appeared at 140.8, 121.4, 138.4 and 129.3 ppm [12 - 14]. Compound 2 is similar to compund 1 from C1-C17, C28-C29 except for C18-C27. The structure of compound 2 was further confirmed by its MS spectral data which showed a mass at 412.4.

Conclusion

The isolation and identification of pregnenolone (1) and stigmasterol (2) from the roots of *Holarrhena curtisii* was the first ever to be reported from this plant. The work was carried out by utilizing several kinds of chromatographic separation techniques and spectroscopy analyses.

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