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STEROIDS FROM *Chisocheton tomentosus*

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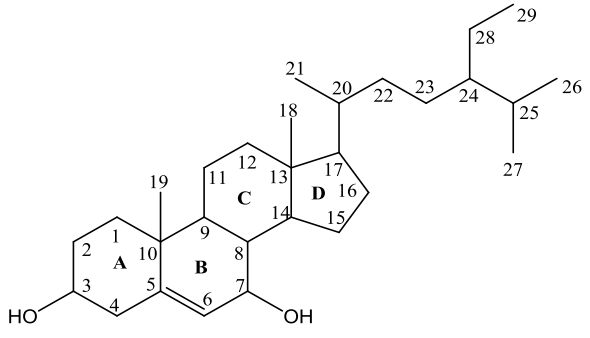
ABSTRACT Four steroids have been isolated from dichloromethane extract of *Chisocheton tomentosus* (Meliaceace), they were; 7α-hydroxy-β-sitosterol **1**, stigmasta-4,6-diene-3-one **2**, stigmasterol **3** and sitosterol **4**. 7α-Hydroxy-β-sitosterol **1** was isolated as a colourless crystal and this compound was a new compound in crystal structure. The isolation and purification of the compounds were achieved by using column and PTL chromatographic techniques. The compounds were identified by spectroscopic techniques such as UV, IR, MS, 1D, and 2D NMR. In addition; 7 α -hydroxy -β-sitosterol was also identified by X-Ray diffraction technique.

(Key word: Meliaceace, *Chisocheton tomentosus, steroids)*

INTRODUCTION

In continuing of our researches on the Meliaceae genera [1-4], we have studied the phytochemical constituents of the dichloromethane extract of *Chisocheton tomentosus* (bark). *Chisocheton tomentosus* is a 14 m tall and 15 cm diameter tree with a dark brown bark. There is no phytochemical investigation reported for this plant.

Previous phytochemical studies on *Chisocheton* species have yielded a number of interesting bioactive compounds including limonoids, antifungal meliacine-type compounds, spermidine alkaloids, sesquiterpenes, and dammarane triterpenoids with an inhibibitory effect on Epstein-Barr virus activation [5-10]. In this work, we report the isolation and characterization of four steroids isolated from *Chisocheton tomentosus*.



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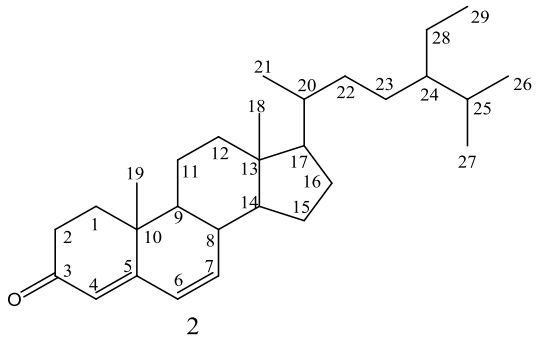

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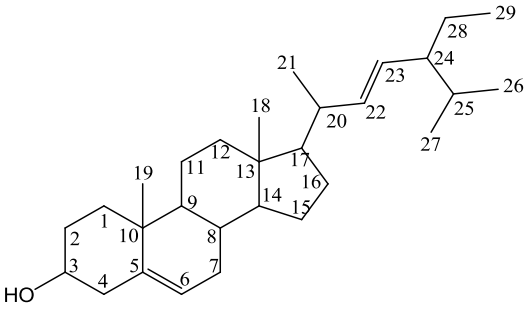

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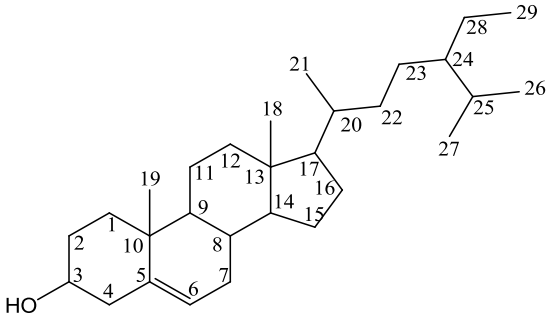


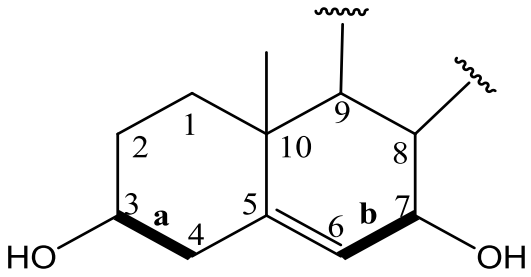
4

7*α*-Hydroxy-*β*-sitosterol **1** was isolated as a colorless crystal mp 138-140 °C, the UV spectrum showed absorption at λ_{max} 302nm and 254nm. The IR spectrum indicated the presence of hydroxyl group by the absorption band at ν_{max} 3430 cm^{-1}; the GCMS spectrum revealed a molecular ion peaks $[M]^+$ at m/z 430, corresponding for molecular formula $C_{29}H_{50}O_2$.

The ^{1}H-NMR spectrum **(Table 1)** of **1** showed six methyl groups resonated as singlet, doublet and triplet in the region of δ_H 0.65-0.95. A characteristic doublet for H-6 methine proton appeared at δ_H 5.55 indicating the presence of double bond functionality between C-5 and C-6, two downfield signals at δ 3.81 and 3.54 were assigned to the H-7 and H-3 respectively, indicating to the presence of oxygen functionality at their carbons.

The ^{13}C- NMR spectrum **(Table 2)** of **1** displayed twenty-nine carbon atoms in the molecule. The DEPT spectrum exhibited six methyls, ten methylenes and ten methines, while the remaining three carbons were quaternary. No signals was observed beyond δ_C 146.3 and, therefore, it was concluded that no ketonic function in this molecule. The downfield signals at δ_C 146.3 was attributed to olefinic quaternary carbon, the C-6 olefinic methine carbon appeared at δ_C 123.8, two more signals for oxygen-bearing carbons at δ_C 71.3 and 65.4 were ascribed to C-3 and C-7 respectively.

The COSY spectrum indicated the presence of two major spin systems, spin system "**a**" and spin system "**b**". Spin system "**a**" started with the couplings of C-3 proton with the C-4 methylene protons, meanwhile, spin system "**b**" the olefinic proton of C-6 showed vicinal connectivity with C-7methineproton.



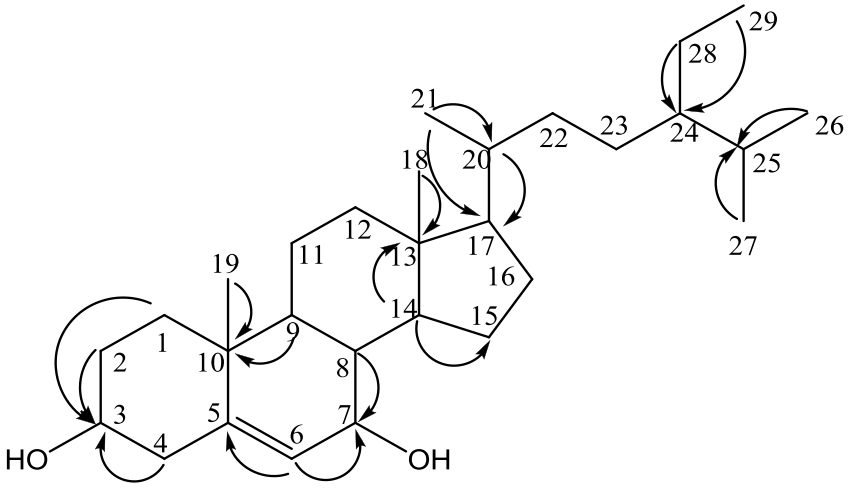
selected ^{1}H-^{1}H COSY of Compound **1**

Table 1. ^{1}H-NMR (400 MHz, in $CDCl_3$) spectral data for compounds **1**, **2**, **3**, and **4**

	δ_H (int.; mult.; *J*(Hz))			
Positions	**1**	**2**	**3**	**4**
1a	1.80 (1H, m)	1.96 (1H, m)	1.81 (1H, m)	1.81 (1H, m)
1b	1.01 (1H,m)	1.70 (1H, m)	1.04 (1H, m)	1.04 (1H, m)
2a	1.80 (1H, m)	2.55 (1H, m)	1.79 (1H, m)	1.79 (1H, m)
2b	1.47 (1H, m)	2.42 (1H, m)	1.50 (1H, m)	1.50 (1H, m)
3	3.54 (1H, m)		3.51 (1H, m)	3.46 (1H, m)
4	2.29 (2H, d, 5)	5.64 (1H, s)	2.27 (2H, m)	2.27 (2H, m)
6	5.55 (1H, d, 5.04)	6.06 (1H, dd, 10, 2.5)	5.32 (1H, m)	5.32 (1H, m)
7	3.81 (1H, brs)	6,09 (1H, t, 10)	1.93 (2H, m)	1.93 (2H, m)
8	1.43 (1H, m)	2.18 (1H, m)	1.45 (1H, m)	1.45 (1H, m)
9	1.15 (1H, m)	1.18 (1H, m)	0.92 (1H, m)	0.92 (1H, m)
11	1.49 (2H, m)	1.57 (2H, m)	1.50 (2H, m)	1.50 (2H, m)
12a	1.97 (1H, m)	2.05 (1H, m)	1.95 (1H, m)	1.95 (1H, m)
12b	1.12 (1H, m)	1.24 (1H, m)	1.17(1H, m)	1.17 (1H, m)
14	1.41 (1H, m)	1.25 (1H, m)	1.00 (1H, m)	1.00 (1H, m)
15a	1.66 (1H, m)	1.77 (1H, m)	1.54 (1H, m)	1.54 (1H, m)
15b	1.08 (1H, m)	1.25 (1H, m)	1.04 (1H, m)	1.04 (1H, m)
16	1.83 (2H, m)	1.54 (2H, m)	1.65 (2H, m)	1.65 (2H, m)
17	1.14 (1H, m)	1.14 (1H, m)	1.12 (1H, m)	1.12 (1H, m)
18	0.65 (3H, s)	0.74 (3H, s)	0.67 (3H, s)	0.65 (3H,s)
19	0.95 (3H, s)	1.09 (3H, s)	1.00 (3H, s)	1.00 (3H, s)
20	1.33 (1H, m)	1.36 (1H, m)	2.00 (1H, m)	1.41 (1H, m)
21	0.89 (3H, d, 6.4)	0.90 (3H, m)	0.98 (3H, m)	0.98 (3H, m)
22	1.24 (2H, m)	1.36 (2H, m)	5.09 (1H, *d*, 15.1)	1.32 (2H, m)
23	1.22 (2H, m)	1.30 (2H, m)	4.96 (1H, *d*, 15.1)	1.33 (2H, m)
24	0.93 (1H, m)	0.90 (1H, m)	1.52 (1H, m)	1.47 (1H, m)
25	1.64 (1H, m)	1.66 (1H, m)	1.53 (1H, m)	1.47 (1H, m)
26	0.81 (3H, m)	0.81 (3H, m)	0.83 (3H,m)	0.80 (3H, m)
27	0.77 (3H, m)	0.77 (3H, m)	0.80 (3H, m)	0.78 (3H, m)
28	1.22 (2H, m)	1.22 (2H, m)	1.43 (2H, m)	1.21 (2H, m)
29	0.83 (3H, m)	0.83 (3H, m)	0.81 (3H, t)	0.89 (3H, m)

Table 2. ^{13}C-NMR (100 MHz, in $CDCl_3$) spectral data for compounds **1**, **2**, **3**, and **4**

	δ ^{13}C			
Positions	**1**	**2**	**3**	**4**
1	37.08	39.9	37.2	37.2
2	31.3	34.0	29.7	31.6
3	71.3	199.8	71.8	71.8
4	42.0	123.5	42.3	39.8
5	146.3	164.2	140.7	140.7
6	123.8	127.8	121.7	121.7
7	65.4	141.8	31.9	31.9
8	37.5	37.8	31.9	31.9
9	42.3	50.7	50.1	50.1
10	37.4	36.1	36.5	36.5
11	20.7	20.7	21.1	21.1
12	39.2	39.6	39.7	42.3
13	42.2	43.5	42.2	42.3
14	49.4	53.4	56.8	56.7
15	24.3	23.8	24.3	24.3
16	28.3	26.1	28.9	28.2
17	55.7	56.0	55.9	56.0
18	11.7	11.9	12.0	11.8
19	19.2	16.4	19.4	19.4
20	36.1	36.2	40.5	36.1
21	18.3	18.7	21.2	18.8
22	33.8	34.9	138.3	33.9
23	29.8	28.3	129.2	26.0
24	49.4	45.9	51.2	45.8
25	29.0	29.1	31.9	29.1
26	19.9	19.9	21.1	19.8
27	18.9	19.0	19.9	19.0
28	23.1	23.1	25.4	23.0
29	12.1	12.0	12.2	12.0



Selected HMBC Correlations of Compound **1**

HMBC spectrum of **1** showed correlation of H_3-19 to C-10 , similarly H_3-18 to C-13. It means that the methyl group at C-19 and C-18 should be attached directly to C-10 and C-13 respectively. Other protons to carbon connectivity were shown in the following structure.

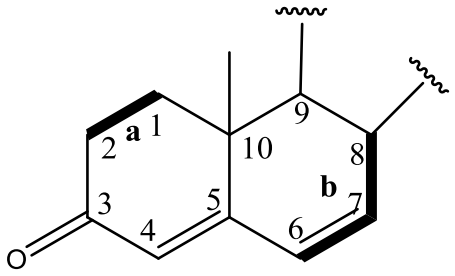
Based on the spectral data and comparison with literature review [11-14], it was concluded that the steroid **1** was 7*α*-hydroxy-*β*-sitosterol. This was further supported by X-Ray reflection technique.

4,6-Stigmastadiene-3-one **2**, was isolated as white amorphous, UV spectrum showed an absorption at λ_{max} 282 nm. The IR spectrum indicated the presence of conjugated ketone at ν_{max} 1670, 1620, 1588, and 875 cm^{-1} indicating the presence of conjugated diene. The LCMS spectrum revealed a molecular ion peaks $[M]^+$ at m/z 410 corresponding for molecular formula $C_{29}H_{46}O$.

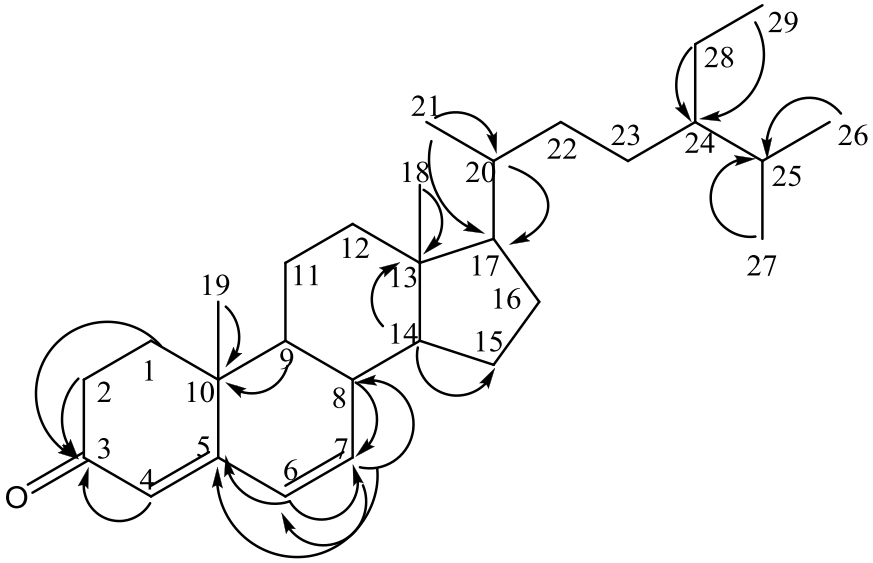
The ^{1}H-NMR spectrum **(Table 1)** of **2** closely resembled compound **1** (7*α* -hydroxy *β*-sitosterol). It showed six methyl groups resonated as singlet, doublet and triplet in the region of δ_H 0.74-1.09. A diagnostic downfield signals at δ_H 5.64 (singlet), 6.06 (dd, $J_{6,7}$=10 Hz, $J_{6,4}$= 2.5 Hz), and 6.09 (triplet, $J_{7,6}$ and $J_{7,8}$= 10 Hz) were ascribed to C-4, C-6, C-7 olefinic protons, respectively. Downfield multiplet signals at δ_H 2.55 and 2.42 were attributed to two geminal protons of C-2 vicinal to ketone, the multiplicity signals of C-2 protons again indicated the presence of carbonyl at vicinal C-3. This indicated the presence of 4,6-diene-3-oxo system in the ring **A** and **B**.

The ^{13}C-NMR spectrum (**Table 2**) of **2** displayed twenty-nine carbon signals; the DEPT spectra indicated the presence of six methyl, nine methylene and ten methine carbons, while the remaining four carbons were quaternary as deduced from broadband spectrum. The downfield signals at δ_C 199.8 were assigned to the 3-oxo-carbon, the signals at δ_C 123.5, 164.2, 127.8, and 141.8 were ascribed to the olefinic C-4, C-5, C-6, and C-7 respectively. By comparison with the reported data, compound **2** was identified as a known stigmasta-4,6-diene-3-one.

The COSY spectrum indicated the presence of two major selected fragments, spin system in the fragment "**a**" and spin system in the fragment "**b**". Spin system "**a**" started with the couplings of C-1 methylene protons with the C-2 methylene protons, meanwhile, spin system "**b**" started with the couplings of olefinic proton of C-6 with C-7 methine proton which in turn showed couplings with C-8 methine proton and so on. These homonuclear couplings helped in the construction of the double bond in the fragment "**b**" which conjugated with the double bond of C-4 and C-5.



selected ^{1}H-^{1}H COSY of Compound **2**



Selected HMBC Correlations of Compound **2**

The H_3-19 at junction of rings **A** and **B** showed HMBC connectivity with sp^3 quaternary carbon of C-10, sp^2 quaternary carbon of C-5, methylene of C-1 and methine carbon of C-9. Similarly, C-2 methylene protons showed HMBC correlations with ketonic carbon at C-3 and methylene carbon of C-1, indicating that the ketone should be at the position C-3. By comparing the spectral data and LCMS of **2** with reported data [15-16], the compound **2** was identified as stigmasta-4,6-diene-3-one.

Compound **3** and **4** were isolated as a mixture of a white solid with the same R_f values. The UV spectrum showed absorption bands at λ_{max} 302 and 254 nm. The infrared spectrum indicated the presence of hydroxyl group by the absorption bands at ν_{max} 3430 cm^{-1}. The presence of stigmasterol **3** and 4 was confirmed by the LCMS showing a molecular peak $[M+H]^+$ at *m/z* 413 and 415, corresponding to the molecular formula $C_{29}H_{49}O$ and $C_{29}H_{51}O$ respectively.

Sterols with an ethyl group at C-24, such as stigmasterol and *β*-sitosterol are by far the most abundant compounds in most plants. The mixture of these two compounds was analyzed by ^{1}H and ^{13}C-NMR spectroscopy. The ^{1}H-NMR spectrum (Table 1) of this mixture showed twelve methyl groups (six methyl groups for each compound) resonated as singlet, doublet and triplet in the region of δ_H 0.65-1.00. A methine proton attached to C-6 of both compounds resonated further downfield as a doublet at δ_H 5.32, the most significant differences shown by the ^{1}H-NMR chemical shift of these two molecules were the proton signals of C-22 and C-23. In compound **3,** the presence of a double bond at position C-22 gave rise to two-doublet of a doublet signals at δ_H 4.96 and 5.09 which belong to H-23,

and H-22, respectively. In compound **4**, the protons of two methylene groups at C-22 and C-23, gave rise

as multiplets in the region of δ_H 0.90- 2.00. The rest of the protons resonated as multiplets in the region of δ_H 0.7-3.5.

The integration of H-6, H-22 and H-23 appeared to be in the ratio of 1:0.25:0.25. Therefore, it could be deduced that the mixture of isolated stigmasterol and *β*-sitosterol was in the ratio of approximately 1:2.

Since compound **3** and **4** have an identical sterol skeleton, the ^{13}C/DEPT spectra **(Table 2)** of this mixture showed quite similar chemical shifts. The most significant differences on the chemical shift of these two molecules were the signals of C-22 and C-23For compound **3**, the sp^2 carbons; C-22 and C-23 resonated at δ_C 138.3 and 129.2, respectively.

The presence of the double bond also moved C-20, C-21, C-24, C-25 and C-28 further downfield at δ_C 40.5, 21.2, 51.2, 31.9 and 25.4 respectively, as compared to that of compound **4**, which showed the signals at δ_C 36.1, 18.8, 45.8, 29.1 and 23.1 for C-20, C-21, C-24, C-25 and C-28, respectively.

By comparing the NMR spectra data with the literature value [17-23], it was confirmed that compound **3** was stigmasterol and compound **4** was *β*-sitosterol.

Experimental Part

General Methods

All solvents used in this experiment are distilled industrial grade. Silica gel 60, 230-400 mesh ASTM (Merck 9385) was used for column chromatography. A slurry of silica gel 60 (approximately 30:1 silica gel to sample ratio). The NMR spectra were obtained using JEOL LA400 FT NMR and JEOL ECA400 FT NMR Spectrometer System using deuterated chloroform as solvent. Chemical shifts were reported in ppm and coupling constants were given in Hertz (Hz). Mass spectra were carried out on Agilent Technologies 6530 Accurate-Mass Q-TOF LC/MS, with ZORBAX Eclipse XDB-C18 Rapid Resolution HT 4.6 mmi.d. x 50 mm x 1.8 µm column, the EI MS spectra were obtained on Shimadzu GC-MS QP2000A spectrometer 70 eV , the high-resolution ESI MS were measured on a LTQ Orbitrap XL (Thermo Scientific). UV spectra were recorded on a Shimadzu UV-Visible Recording Spectrophotometer using HPLC grade ethanol as solvent with mirror UV cell.The infrared (IR) spectra were obtained through Perkin Elmer FT-IR Spectrometer Spectrum RX1 using chloroform as solvent. Melting points were taken on hot stage Gallen Kamp melting point apparatus and were uncorrected.

Plant Materials

Bark of *Chisocheton tomentosus* was collected and identified from Mersing Johor, Malaysia in 1993 by the team of Herbarium of Chemistry Department, University of Malaya, Kuala Lumpur, Malaysia. It has been deposited in the above herbarium under voucher specimens KL 4251.

Extraction and Isolation

The ground and dried bark of *Chisocheton tomentosus* (1.0 kg) was first extracted using hexane for 3 to 5 days followed by CH_2Cl_2 and MeOH. Then, the CH_2Cl_2 extract underwent the fractionation using silica gel column chromatography. The fractions from the column were subjected to thin layer chromatography (TLC) and further purified by preparative TLC and micro-column. The structures of isolated compounds were determined by spectroscopic methods such as UV, Infrared, 1D and 2D Nuclear Magnetic Resonance, Mass Spectroscopy as well as X-Ray reflectometer. **Figure 1** showed the extraction and isolation procedures of steroids from *Chisocheton tomentosus*.

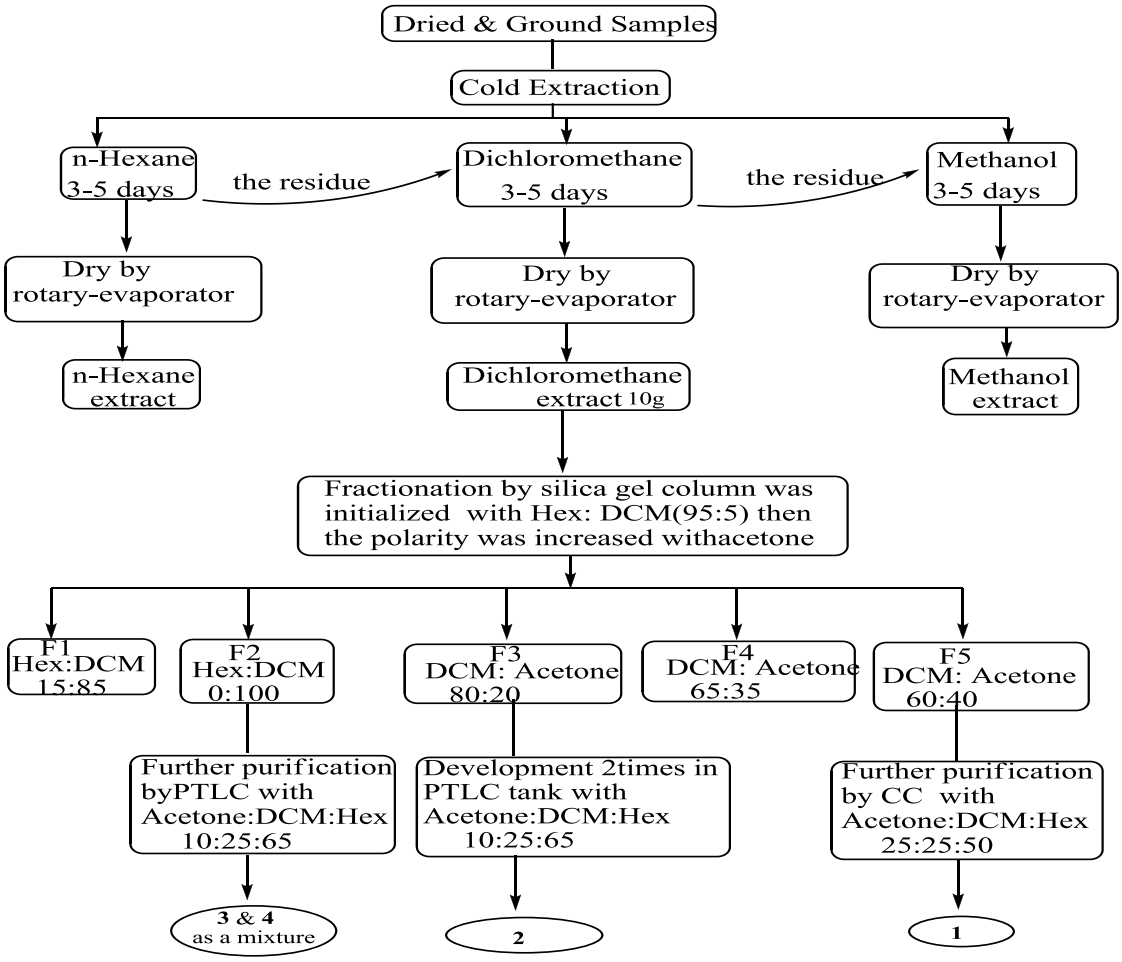


Figure 1**.** Extraction and isolation of chemical constituents from *Chisocheton tomentosus*

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