# Cytotoxic Steroids From The Stembak of Chisocheton celebicus KOORD

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**Submission date:** 08-Jan-2020 02:01PM (UTC+0700)
**Submission ID:** 1239983118
**File name:** komplikasi_artikel_ilmiah_jurnal_nasional_terakreditasi_5.pdf (607.6K)
**Word count:** 3976
**Character count:** 18730

# Cytotoxic Steroids From The Stembak of *Chisocheton celebicus* KOORD

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Received: August 2019; Revision: October 2019; Accepted: November 2019; Available online: November 2019

**Abstract**

In the course of our continuing search for anticancer compounds from *Chisocheton* species, three steroids, stigmast-5-en-3β-ol (**1**), stigmast-5-en-3β-ol-3-*O*-β-D-glucopyranoside (**2**) and stigmast-5,22-dien-3β-ol-3-*O*-β-D-glucopyranoside (**3**), were obtained from the stembark of *Chisocheton celebicus*. The structures of compound **1-3** were identified with spectroscopic data including IR, 1D-NMR, 2D-NMR and TOF-MS, as well as by comparing with those spectral data previously. Compounds **1-3**, were evaluated for their cytotoxic effects against P-388 murine leukemia cells and displayed the cytotoxicity activity with $IC_{50}$ values of 12.45 ± 0.050, 52.27 ± [503] 031 and 62.52 ± 0.076 µg/mL, respectively.

***Keyword:*** *Chisocheton celebicus* koord, cytotoxic activity, Meliaceae, P-388 murine leukemia cells, steroid.

**Abstrak**

Sebagai bagian dari studi kami tentang senyawa kandidat antikanker dari tumbuhan *Chisocheton* Indonesia, tiga senyawa steroid, stigmast-5-en-3β-ol (**1**), stigmast-5-en-3β-ol-3-*O*-β-D-glucopyranoside (**2**) dan stigmast-5,22-dien-3β-ol-3-*O*-β-D-glucopyranoside (**3**), telah diperoleh dari kulit batang *Chisocheton celebicus*. Struktur kimia senyawa **1-3** diidentifikasikan berdasarkan data spektroskopi meliputi, IR, NMR-1D, NMR-2D dan TOF-MS serta perbandingan dengan data spektra sebelumnya. Senyawa **1-3** diuji efek sitotoksiknya terhadap sel murin leukemia P-388 dan memperlihatkan aktivitas sitotoksik dengan nilai $IC_{50}$ berturut-turut, 5.14 ± 0.015, 30.12 ± 0.0025 dan 35.86 ± 0.053 µM.

***Kata kunci:*** Aktivitas sitotoksik, *Chisocheton celebicus* koord, Meliaceae, sel murin leukimia P-388, steroid.

***DOI:*** *10.15408/jkv.v5i2.12132*

## 1. INTRODUCTION

The genus *Chisocheton*, belong to family Meliaceae, consisting more than 50 plants and distributes in Nepal, India, Myanmar, China, Thailand, Indonesia, Malaysia, and Papua New Guinea (Vossen and Umali, 2002). Previous investigation of chemical constituents on *Chisocheton* plants had reported contain sesquiterpenoids (Phongmaykin *et al.*, 2008), mexmarane-type triterpenoids (Phongmaykin *et al.*, 2008; Inada *et al.*, 1993), lanostane-type triterpenoid (Katja *et al.*, 2017a), tirucallane-type triterpenoids (Zhang *et al.*, 2012) apo-tirucallane-type triterpenoids (Zhang *et al.*, 2012; Yang *et al.*, 2011), euphane-type triterpenoids (Supratman *et al.*, 2019); limonoids (Maneerat *et al.*, 2008; Laphookhieo *et al.*, 2008; Mohamad *et al.*, 2009; Yang et al., 2009; Najmuldeen et al., 2011; Wong *et al.*, 2011; Nurlelasari *et al.*, 2017; Supriatno et al., 2018), steroids


P-ISSN: 2460-6065, E-ISSN: 2548-3013

(Najmuldeen *et al.*, 2011) and phenolics (Inada *et al.*, 1993).

As part of our investigation on cytotoxic compounds from *Chisocheton* plants, we reported a mexicanolide-type limonoid from *C. macrophyllus* (Nurlelasari *et al.*, 2017), a trijugin-type limonoid and lanostane-type triterpenoid from *C. cumingianus* (Katja *et al.*, 2017a; Katja *et al.*, 2017b), vilacinine-type limonoid from *C. pentandrus* (Supriatno *et al.*, 2018) and euphane type-triterpenoid from *C. patens* Blume (Supratman *et al.*, 2019). In the our continuing search for cytotoxic compounds from Indonesia *Chisocheton* plants, we found that the *n*-hexane and ethyl acetate extracts of the stembark of *C. celebicus* exhibited a cytotoxic activity against P-388 murine leukemia cells with $IC_{50}$ of 20.72 ± 0.02 and 18.48 ± 0.03 µg/mL, respectively. In this paper, the isolation and structural identification of three steroids along with their cytotoxic activity against P-388 murine leukimia cells are discribed.

## 2 MATERIALS AND METHODS

### Experimental Procedure

Melting points were obtained on an electrothermal melting point instrument. The infrared spectra and mass spectra were obtained on a SHIMADZU IR Prestige-21 in KBr and Waters Xevo QTOF-MS, respectively. The NMR data was recorded using a JEOL ECZ-500 at 500 MHz for $^{1}H$ and [125] MHz for $^{13}C$, using tetramethylsilane as internal standard. Column chromatography was carried out on the silica gel 60 (70–230 and 230–400 mesh), after which TLC analysis was carried out on 60 $GF_{254}$ (0.25 mm) using various solvent systems, spots was detected by spraying with 10% sulfuric acid in ethanol followed by heating.

### Plant Material

The stembarks of *C. celebicus* were obtained in Bogor Botanical Garden, West Java Province, Indonesia in April 2012. The plant was determined by Mr. Ismail at the Bogoriense Herbarium, Bogor, Indonesia and deposited at the herbarium with the number of Bo-1305316.

### Determination of Cytotoxic Activities

The P388 cells were growth into 96-well plates at an initial cell density of approximately 3 x $10^4$ cells $cm^{-3}$. After 24 hours of incubation for cell attachment and growth, several concentrations of samples were added. The samples added were first dissolved in dimethyl sulfoxide at the required concentration as a negative control. Subsequent six desirable concentrations were prepared using phosphoric buffer solution, pH [27] 7.30-7.65). Control wells received only dimethyl sulfoxide. The assay was terminated after a 48 hours incubation period by adding MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; also named as thiazol blue] and the incubation was continued for another four hours, in which the MTT-stop solution containing sodium dodecyl sulphate was added and another 24 hours incubation was conducted. Optical density was read by using a micro plate reader at 550 nm. $IC_{50}$ values were taken from the plotted graph of percentage live cells compared to control (%), receiving only phosphoric buffer solution, pH = 7.30 - 7.65 and dimethyl sulfoxide, versus the tested concentration of compounds (µg/mL). The $IC_{50}$ value is the concentration required for 50% growth inhibition. Each assay and analysis was run in triplicate and averaged.

### Extraction and Isolation

The dried stembark (1.5 kg) was soaked in methanol (12 L) for 3 days. After evaporate of the methanol on the rotary evaporator, the concentrated of MeOH extract (120.5 g) was dissolved in $H_2O$ and then partitioned successively with *n*-hexane, EtOAc, and *n*-BuOH. Evaporation on the rotary evaporator produced the crude extracts of *n*-hexane (20.3 g), EtOAc (10.4 g), and *n*-BuOH (11.6 g), respectively. The *n*-hexane extract (20.3 g) was separated by vacuum liquid chromatography on silica gel 60 by using *n*-hexane and ethyl acetate as a gradien eluent to give nine fractions (A–I). Fraction A (6 g) was separated by column chromatographed on silica gel with a *n*-hexane–$CH_2Cl_2$ as a gradient eluen (10:0–1:1) to give ten subfractions (A01–A10). Subfraction A03 was further separated by column chromatographed on silica gel with *n*-hexane:$CHCl_3$ (9:1) as an eleunt to give **1** (14.5 mg). The EtOAc extract (12.4 g) was separated by column chromatography on silica gel using a *n*-hexane and ethyl acetate as an eleunt to give eight fractions (J–Q). Fraction K (927.6 mg) was column chromatographed on silica gel, eluted with a *n*-hexane–EtOAc (10:0–0:10) as eluent

to give **2** (16.3 mg). Fractions P (3.86 g) was column chromatographed on silica gel, eluted with a $CHCl_3$-$Me_2CO$ as a eleunt (10:0–4:1) to give **3** (5.5 mg).

## 3. RESULTS AND DISCUSSION

The *n*-hexane and EtOAc fraction were separated by several column chromatography followed by cytotoxic test to produce three cytotoxic steroids 1–3 (Figure 1).

**Stigmast-5-en-3β-ol (1)**, white crystals, m.p. 134-136 $^{o}$C; IR (KBr) $\nu_{max}$ 34[illegible], 2950, 2860, 1460, 1360, 1240, 1060 cm$^{-1}$; $^{1}$H-NMR ($CDCl_3$, 500 MHz) and $^{13}$C-NMR ($CDCl_3$, 125 MHz), see Table 1; TOFMS (negative ion mode) *m/z* 413.0811 $[M-H]^{-}$, (calcd. $C_{29}H_{49}O^{-}$, *m/z* 413.3789).

**Stigmast-5-en-3β-ol-3-*O*-β-D-glucopyranoside (4)**, white amorphous powder; m.p. (decomposed); IR (KBr) $\nu_{max}$ 3433, 1639, 1461, 1380, 1053 cm$^{-1}$; $^{1}$H-NMR (pyridine-$d_5$, 500 MHz) and $^{13}$C-NMR (pyridine-$d_5$, 125 MHz), see Table 1.

**Stigmast-5,22-dien-3β-ol-3-*O*-β-D-glucopyranoside (5),** white amorphous powder; m.p. (decomposed); IR (KBr) $\nu_{max}$ 3450, 1630, 1445, 1370, 1050 cm$^{-1}$; $^{1}$H-NMR (pyridine-$d_5$, 500 MHz) and $^{13}$C-NMR ($CDCl_3$, 125 MHz), see Table 1.

Compound 1. The HR-TOFMS spectrum of compound 1 showed molecular ion at *m/z* 413.0811 (calcd. *m/z* 413.3789), which consistent to the molecular formula of $C_{29}H_{50}O$ and thus requiring hydrogen deficiency index of five, consisting of one pairs of C sp$^2$ and tetracyclic stigmastane-type steroid. The infra red spectra displayed the presence of hydroxyl (3430 cm$^{-1}$) alipahtics (2950 and 2860 cm$^{-1}$), olefinic (1460 cm-1), *gem*-dimethyl (1360 and 1240 cm$^{-1}$) and ether group (1060 cm$^{-1}$).

The $^{1}$H-NMR spectrum displayed two tertiary methyl at $\delta_H$ 1.00 (Me-18) and 0.68 (Me-19), three secondary methyl at $\delta_H$ 0.92 (3H, d, $J$ = 6.2 Hz, Me-21), 0.83 (3H, d, $J$ = 6.5 Hz, Me-26), and 0.81 (d, $J$ = 5.2 Hz, Me-27), one primary methyl group at $\delta_H$ 0.84 (t, $J$ = 5.2 Hz, Me-29), corresponding to stigmastane-type steroid (Cayme and Ragasa, 2004; Farabi et al., 2017). An oxygenated sp$^2$ methine at $\delta_H$ 5.35 (d, $J$ = 5.2 Hz, H-6) and oxygenated sp$^3$ methine at $\delta_H$ 3.52 (1H, m, H-3), were identified at $^{1}$H NMR spectra. The vicinal proton was also confirmed by the $^{1}$H-$^{1}$H Correlated Spectroscopy (COSY) spectrum (Figure 2). $^{1}$H-$^{1}$H COSY countour was identified at $C_2$-$C_3$-$C_4$ suggested that position of a secondary alcohol at C-3. The countour was also observed at $C_6$-$C_7$-$C_8$, indicated that the position of double bond at $C_5$-$C_6$ ($\Delta^{5,6}$). The $^{13}$C-NMR ($CDCl_3$ 125 MHz) and heteronuclear single quantum coherence (HSQC) and Distortionless enhancement by polarization transfer (DEPT) spectra displayed the presence of six methyl, an olefinic methine, an olefinic quartenary carbon, and a oxygenated methine at $\delta_C$ 72.0 (C-3), suggested the presence of stigmastane-type steroid (Cayme and Ragasa, 2004; Farabi *et al.*, 2017). These unsaturations were determined for one of total hydrogen deficiency index of five. The remaining four degrees of hydrogen deficiency index were corresponding to stigmastane-type steroid. A detail analysis cof the NMR data of **1** with to those of β-sitosterol (Chaturvedula and Prakash, 2012; Farabi *et al.*, 2017), indicated that the structure of both compounds displayed highly similarity, therefore compound **1** was identified as a stigmast-5-en-3β-ol (β-sitosterol).

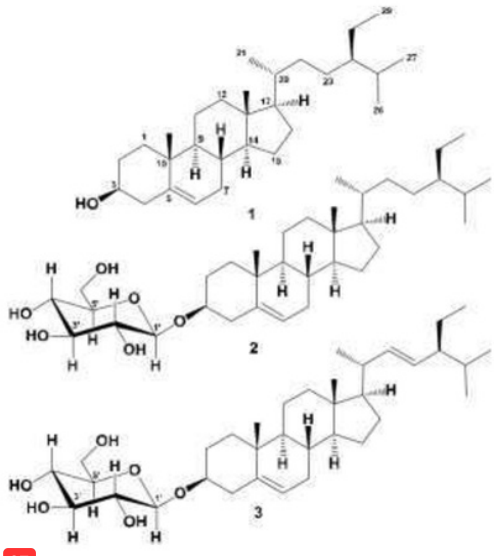

**Figure 1.** Chemical structure of compounds **1-3**.

Compound **2,** the molecular formula of **2** was identified as C $_{35}H_{60}O_6$, from NMR data (Table 1). The infra red spectrum showed absorption band at 3460, 2890, 1650, 1475

and 1380, and 1070 $cm^{-1}$, respectively, corresponding to hydroxyl, aliphatics, *m*-dimethyl, olefinic and ether groups. A NMR spectra of **2** similar to those of **1**, except the presence of sugar moiety in **2**. The signals of oxygenated methylene at $\delta_H$ 4.27 (2H, m, H-6′) and an anomeric signal proton at $\delta_H$ 4.53 (1H, d, $J$ = 7.5 Hz, H-1′), as well as of four oxygenated methines at $\delta_H$ 4.25 (1H, dd, $J$ = 5.5, 7.5 Hz, H-2′), 3.95 (1H, dd, $J$ = 5.5, 7.2 Hz, H-3′), 4.03 (1H, dd, $J$ = 74.5, 7.2 Hz, H-4′), and 4.39 (1H, d, $J$ = 4.5 Hz, H-5′), supporting for a glucose moiety. The $^{13}C$ NMR signal of anomeric carbon located at $\delta_C$ 102.4 (C-1′), suggesting the β-glucose. In comparison of **2** with literature data (Harneti *et al.*, 2014; Farabi *et al.*, 2017), showed good agreement, therefore compound **2** was identified as stigmast-5-en-3β-ol-3-*O*-β-D-glucopyranoside (β-sitosterol glucoside).

**Table 1.** NMR data for compounds **1-3**.

| C | **1** | | **2** | | **3** | |
|---|---|---|---|---|---|---|
| | $\delta_C$ (mult.) | $\delta_H$ (Σ H, mult., $J$ (Hz) | $\delta_C$ (mult.) | $\delta_H$ (Σ H, mult., $J$ (Hz) | $\delta_C$ (mult.) | $\delta_H$ (Σ H, mult., $J$ (Hz) |
| 1 | 37.4 (t) | 1.68 (1H, m)<br>1.70 (1H, dd, 2.4, 4.5) | 37.3 (t) | 1.83 (1H, m)<br>1.73 (1H, dd, 1.2, 8.5) | 37.3 (t) | 1.83 (1H, m)<br>1.90 (1H, dd, 3.4, 7.8) |
| 2 | 31.8 (t) | 1.50 (1H, m)<br>1.60 (1H, m) | 31.9 (t) | 1.90 (1H, dd, 3.0, 9.0)<br>2.42 (1H, t, 10.8) | 31.9 (t) | 1.90 (1H, dt, 1.2, 9.4)<br>2.42 (1H, dd, 2.3, 9.4) |
| 3 | 72.0 (d) | 3.52 (1H, m) | 78.5 (d) | 3.90 (1H, m) | 78.5 (d) | 3.90 (1H, m) |
| 4 | 42.3 (t) | 2.23 (1H, dd, 3.4, 8.5)<br>2.45 (1H, m) | 39.2 (t) | 2.06 (1H, dd, 1.8, 12.8)<br>2.68 (1H, dd, 1.8, 9.4) | 39.2 (t) | 1.66 (1H, dd, 1.2, 9.7)<br>2.67 (1H, m) |
| 5 | 140.9 (s) | - | 140.7 (s) | - | 140.7 (s) | - |
| 6 | 121.9 (d) | 5.35 (1H, t, 5.2) | 121.8 (d) | 5.28 (1H, d, 4.8) | 121.8 (d) | 5.28 (1H, d, 3.6) |
| 7 | 32.1 (t) | 1.99 (1H, dd, 5.2, 8.5)<br>2.01 (1H, m) | 29.2 (t) | 1.76 (1H, m)<br>1.80 (1H, dd, 1.2, 4.8) | 29.3 (t) | 1.80 (1H, m)<br>1.84 (1H, dd, 4.2, 8.2) |
| 8 | 32.1 (d) | 0.85 (1H, dd, 4.5, 8.5) | 32.0 (d) | 1.69 (1H, m) | 32.0 (d) | 1.69 (1H, m) |
| 9 | 50.3 (d) | 1.43 (1H, dd, 8.5, 9.8) | 50.1 (d) | 0.96 (1H, t, 7.1) | 50.2 (d) | 1.00 (1H, d, 9.6) |
| 10 | 36.7 (s) | - | 36.7 (s) | | 36.7 (s) | - |
| 11 | 21.3 (t) | 1.46 (1H, m)<br>1.55 (1H, dd, 4.8, 9.2) | 21.1 (t) | 1.47 (1H, m)<br>1.68 (1H, m) | 21.1 (t) | 1.47 (1H, m)<br>1.52 (1H, m) |
| 12 | 39.9 (t) | 1.23 (1H, m)<br>1.35(1H, dd, 2.5, 9.2) | 39.7 (t) | 1.30 (1H, m)<br>1.50 (1H, dd, 4.5, 9.8) | 39.8 (t) | 1.43 (1H, dd, 3.0, 9.5)<br>1.32 (1H, m) |
| 13 | 42.4 (s) | - | 42.3 (s) | - | 42.3 (s) | - |
| 14 | 56.9 (d) | 0.95 (1H, m) | 56.6 (d) | 1.04 (1H, dd, 4.5, 7.6) | 56.7 (d) | 1.05 (1H, m) |
| 15 | 26.2 (t) | 1.58 (1H, dd, 4.5, 9.8)<br>1.66 (1H, m) | 24.3 (t) | 1.49 (1H, m)<br>1.54 (1H, m) | 24.4 (t) | 1.48 (1H, m)<br>1.50 (1H, dd, 6.8, 9.6) |
| 16 | 28.4 (t) | 1.16 (1H, m)<br>1.30 (1H, m) | 28.4 (t) | 1.53 (1H, m)<br>1.62 (1H, m) | 28.4 (t) | 1.66 (1H, m)<br>1.72 (1H, m) |
| 17 | 56.2 (d) | 1.10 (1H, m) | 56.0 (d) | 1.20 (1H, dd, 9.5, 11.4) | 56.1 (d) | 1.19 (1H, m) |
| 18 | 12.0 (q) | 1.00 (3H, s) | 11.8 (q) | 0.58 (3H, s) | 11.8 (q) | 0.59 (3H, s) |
| 19 | 19.0 (q) | 0.68 (3H, s) | 19.8 (q) | 0.86 (3H, s) | 19.8 (q) | 0.86 (3H, s) |
| 20 | 36.3 (d) | 1.86 (1H, dd, 2.5, 6.2) | 36.2 (d) | 1.36 (1H, m) | 36.2 (d) | 1.36 (1H, m) |
| 21 | 10.2 (q) | 0.92 (3H, d, 6.2) | 19.0 (q) | 0.91 (3H, d, 6.0) | 19.0 (q) | 0.92 (3H, d, 4.2) |
| 22 | 34.1 (t) | 1.15 (2H, m) | 34.0 (t) | 1.17 (2H, m) | 138.7 (d) | 4.98 (1H, dd, 7.8, 8.4) |
| 23 | 26.2 (t) | 1.23 (2H, m) | 26.1 (t) | 1.29 (2H, m) | 129.3 (d) | 5.13 (1H, dd, 7.8, 8.4) |
| 24 | 45.9 (d) | 1.52 (1H, dd, 1.6, 5.6) | 45.8 (d) | 1.01 (1H, dd, 3.4, 7.8) | 45.8 (d) | 1.01 (1H, dd, 4.6, 8.3) |
| 25 | 29.3 (d) | 1.60 (1H, dd, 1.2, 7.8) | 30.1 (d) | 1.59 (1H, dd, 2.1, 6.5) | 30.1 (d) | 1.65 (1H, m) |
| 26 | 19.6 (q) | 0.83 (3H, d, 6.5) | 19.3 (q) | 0.80 (3H, d, 5.0) | 19.3 (q) | 0.81 (3H, d, 4.9) |
| 27 | 20.0 (q) | 0.79 (3H, d, 5.2) | 18.8 (q) | 0.78 (3H, d, 5.0) | 18.8 (q) | 0.79 (3H, d, 4.9) |
| 28 | 23.2 (t) | 1.30 (2H, m) | 23.2 (t) | 1.34 (2H, m) | 23.2 (t) | 1.25 (1H, m)<br>1.32 (1H, dd, 2.4, 7.4) |
| 29 | 12.2 (q) | 0.83 (3H, t, 5.2) | 12.0 (q) | 0.83 (3H, t, 2.5) | 12.0 (t) | 0.83 (3H, t, 2.4) |
| 1′ | | | 102.4 (d) | 4.53 (1H, d, 7.5) | 102.4 (d) | 4.52 (1H, dd, 2.4, 8.5) |
| 2′ | | | 75.2 (d) | 4.25 (1H, dd, 5.5, 7.5) | 75.2 (d) | 4.22 (1H, dd, 8.5, 9.3) |
| 3′ | | | 77.7 (d) | 3.95 (1H, dd, 5.5, 7.2) | 77.7 (d) | 3.94 (1H, dd, 7.3, 9.3) |
| 4′ | | | 78.4 (d) | 4.07 (1H, dd, 4.5, 7.2) | 78.4 (d) | 4.02 (1H, dd, 7.3, 7.5) |
| 5′ | | | 71.5 (d) | 4.39 (1H, d, 4.5) | 71.5 (d) | 4.37 (1H, dd, 7.5, 7.7) |
| 6′ | | | 62.6 (t) | 4.27 (2H, m) | 62.6 (t) | 4.25 (2H, m) |

*Measured in $CDCl_3$ (500 MHz for $^1H$ and 125 MHz for $^{13}C$)

Compound **3,** the molecular formula was determined to be $C_{35}H_{58}O_6$ from NMR data (Table 1). The IR spectrum showed absorption peak at 3475, 2890, 1630, 1445 and 1370 and 1050 $cm^{-1}$, respectively, corresponding to hydroxyl, aliphatic, olefinic, *gem*-dimethyl and ether groups. NMR spectra of **3** very similar with **2**, except the presence of an additional double bond at $\delta_H$ 4.98 and 5.13 (each 1H, dd, $J$ = 7.8, 8.4 Hz, H-22, H-23) and $\delta_C$ 138.7 (C-22) and 129.3 (C-23) in **3**. In comparison of **3** with literature data (Harneti et al., 2014; Farabi et al., 2017), showed good agreement, therefore compound **3** was identified as stigmast-5,22-dien-3β-ol-3-*O*-β-D-glucopyranoside (stigmasterol glucoside).

The cytotoxic assay was conducted as mentioned in the previous papers (Alley *et al.*, 1988; Hakim *et al.*, 2007; Supratman *et al.*, 2019) and was used an artonin E ($IC_{50}$ 0.75 µg/mL) as a positive control (Hakim et al., 2007). The cytotoxic activity of stigmast-5-en-3β-ol (**1**) stronger than stigmast-5-en-3β-ol-3-*O*-β-D-glucopyranoside (**2**) and stigmast-5,22-dien-3β-ol-3-*O*-β-D-glucopyranoside (**3**), indicated that the presence of sugar group can decrease cytotoxic activity in steroid structure.

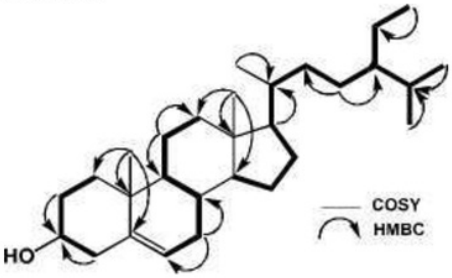


**Figure 2.** Selected COSY and HMBC correlation for compound **1**

## 4. CONCLUSION

Three cytotoxic steroids were investigated from the bark of *C. celebicus* and identified as stigmast-5-en-3β-ol (**1**), stigmast-5-en-3β-ol-3-*O*-β-D-glucopyranoside (**2**), and stigmast-5,22-dien-3β-ol-3-*O*-β-D-glucopyranoside (**3**). The presence of sugar moities can decrease cytotoxic activity. The investigation of these steroids were shown in this species for the first time.

## ACKNOWLEDGMENTS

This research was supported by Kemristekdikti, Indonesia (Hibah Penelitian Dasar Perguruan Tinggi, 2018-2019, by Dewa Gede Katja). We thank Dr. Ahmad Darmawan, M.Si and Mrs. Sofa Fajriah, M.Si in the Research Center for Chemistry, Indonesian Science Institute, for NMR measurements.

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