

PHYTOSTEROL CONTENT IN BENGKOANG (*Pachyrhizus erosus*)

KANDUNGAN PHYTOSTEROL DALAM BENGKOANG (*Pachyrhizus erosus*)

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ABSTRACT

Bengkoang has long been used by ancestors as a raw material for cosmetics. In Indonesia, it is usually eaten raw, sometimes with salt, lemon juice and powdered chili. However, scientific evidences that support the use of bengkoang have not been widely published. Phytosterols from the bengkoang root have been isolated and identified based on their NMR spectrum data and mass spectrum. The result of the structure elucidation informed that β -sitosterol and stigmasterol are major components of phytosterol in bengkoang root. Phytosterol content was about 0.02% of dry weight bengkoang or 2.76 % in petroleum ether extract of bengkoang, with a ratio of β -sitosterol and stigmasterol (65:35). Therefore, bengkoang can be further exploited as a raw material of phytosterol supplement.

ABSTRAK

Bengkoang telah lama digunakan oleh nenek moyang sebagai bahan baku kosmetika. Di Indonesia, bengkoang biasa dimakan secara langsung bersama dengan garam, jus jeruk atau cabe. Namun hingga saat ini bukti ilmiah yang mendukung pemanfaatan bengkoang tersebut belum banyak dipublikasikan. Phytosterol dari umbi bengkoang telah berhasil diisolasi dan diidentifikasi berdasarkan data spektra NMR dan spektra massa. Hasil elusidasi struktur menginformasikan bahwa β -sitosterol dan stigmasterol merupakan komponen utama phytosterol dalam umbi bengkoang. Kandungan phytosterol berkisar 0,02% per berat kering bengkoang atau 2,76% dalam ekstrak petroleum eter, dengan perbandingan β -sitosterol dan stigmasterol (65:35). Dengan demikian bengkoang dapat dimanfaatkan lebih lanjut sebagai bahan baku suplemen phytosterol.

INTRODUCTION

Phytosterol is a class of sterol compounds contained in plants. It is an integral component of the membrane lipid bilayer (Demel and DeKruyff, 1976; Schuleretal., 1991). Phytosterols are different from the cholesterol on their side chain substitution and the position of double bonds in their cyclic chain. Because they have a double bond in their cyclic group, then either the phytosterol or cholesterol are susceptible to oxidation (Zhang, *et al.*, 2005). Each plant species has its own characteristic distribution of phytosterols. There are three phytosterol constituents are most commonly found, namely beta-sitosterol, campesterol and stigmasterol (Benveniste, 1986; Benveniste, 2004).

Now, phytosterol is not only used as ingredients such as anti-inflammatory drugs (Parra-Delgado *et al.* 2004; Dickson *et al.* 2007), hormones and vitamins (Dewick, 2002), but also as a nutritional supplement (anti-cholesterol additives) and cosmetics (as a cream and lipsticks) (Berezin *et al.*, 2001). Gomes *et al.* (2007) reported that beta-sitosterol and stigmasterol isolated from *Pluchea indica* Less plant was able to against the snake venom.

In addition, phytosterols are also able to reduce cholesterol absorption, so the prevalence of cardiovascular disease and

cancer can be reduced by dietary phytosterol. Sitosterol was reported to inhibit the growth of colon cancer cells HT-29 (Awad, *et al.*, 1996) and LNCaP prostate cancer cells (Holtz, *et al.*, 1998). The effect of sitosterol and campesterol on breast cancer cell apoptosis MDAMB-231 has also been reported by Downie *et al.* (1999). However, until now the mechanism of inhibition of cell growth and stimulate apoptosis of the sitosterol is not well known (Moon, *et al.*, 2007).

METHODOLOGY

Plant materials

The bengkoang belongs to the taxonomic class of Magnoliopsida; order Fabales; family Fabaceae; subfamily Faboideae; genus *Pachyrhizus*; species *Pachyrhizus erosus*. The bengkoang *Pachyrhizus erosus* (L) Urb roots were collected from Purworejo, Central Java, Indonesia in dry season.

Chemicals and solvents

The chemicals used in the detection and isolation methods were anisaldehyde (4-methoxybenzaldehyde), glacial acetic acid, aluminium chloride, hydrochloric acid and concentrated sulphuric acid (all purchased from Merck, Darmstadt, Germany), Sephadex LH20 (Aldrich, Steinheim, Germany), Silica gel 60 (particle sizes 0.063-0.200mm, Merck, Darmstadt, Germany), TLC Aluminium sheets,

silica gel 60 F254 (layer thickness 0.2 mm, Merck, Darmstadt, Germany).

Solvents for separation techniques were petroleum ether, ethyl acetate (Fisher Scientific, Leicestershire, UK), methanol (Merck, Darmstadt, Germany), chloroform, dichloromethane, from the Fluka, Seelze, Germany.

Instruments

Melting point SMP3 Stuart[®] apparatus (Staffordshire, UK), Cary 50 Bio UV-Visible spectrophotometer (Varian, California, USA), JASCO FT/IR-6100 Spectrophotometer (Gross-Umstadt, Germany), ALPHA II-12 Freeze dryer (Osterode, Germany), Bruker Avance 400 NMR spectrometer (Rheinstetten, Germany), Shimadzu GC/MS-QP 20105 gas chromatography (Kyoto, Japan).

Extraction and isolation of phytosterol from bengkoang

The bengkoang roots (45 kg) were peeled and washed with water, subsequently dried at 60°C and milled into fine powder. The fine powder (4.75 kg) was extracted by soxhlet using petroleum ether.

The petroleum ether extract (26.3 gram) was further subjected to silica gel chromatography and eluted using gradient mixture of PE-EtOAc (from 100% of PE to 100% of EtOAc) and followed by gradient mixture of EtOAc-MeOH (from 100% of EtOAc to 100% of MeOH). 29 fractions of 100 ml were collected according to the TLC results. TLC was conducted to each fraction respectively using silica gel as a stationary phase, chloroform-ethyl acetate (6:4) as a mobile phase. Detection was performed with UV light at 254 and 366 nm and followed by spraying the TLC plates with anisaldehyde-H₂SO₄ reagent and subsequent heating at 110°C. Phytosterol will react positive with anisaldehyde and give blue-purple colour.

Fractions 8, 9, 10, 11 have the same spot on TLC with an R_f value of 0.48. Therefore, they were combined and then subjected to another silica gel column chromatography using dichloromethane-ethyl acetate (60:40) as a mobile phase. Ten millilitre fractions were collected and evaluated by TLC using dichloromethane-ethyl acetate (60:40) as mobile phase. Fractions 9-16 have the same retention factor (R_f = 0.43) on TLC. They were combined and evaporated. The obtained solid was washed with methanol and recrystallized from petroleum ether and ethyl acetate to get pure compound (727 mg). The compound was further analysed by NMR spectrophotometer and gas chromatography equipped by mass spectrophotometer.

RESULT AND DISCUSSION

Structure elucidation of isolated phytosterol compound

Compound P-1 (Stigmasterol)

White needle crystals; m.p. 136-139 °C; MW 412; IR (cm⁻¹): 3364, 2925, 2834, 1463, 1375, 1051, 1022, 957, 801. EIMS m/z (% intensity): 412 ([M]⁺, 30.72), 394 ([M-H₂O]⁺, 5.48), 379 ([M-H₂O-CH₃]⁺, 5.70), 351 (17.77), 273 (10.94), 271 (26.90), 255 (30.94), 213 (16.51), 159 (35.91), 133 (40.83), 123 (22.21), 105 (15.01), 97 (33.98), 83 (70.32), 69 (55.71), 55 (100), 41 (47.21). ¹H NMR (500 MHz, CDCl₃): δ 0.68 (s, 3H, 3H¹⁸), 0.83 (d, 9H, 3H²⁶, 3H²⁸, 3H²⁹), 0.92 (s, 3H, 3H²¹), 0.95 (m, 1H, H⁹), 1.01 (s, 3H, 3H¹⁸), 1.09 (m, 1H, H^{15a}), 1.10 (m, 1H, H¹⁴), 1.20 (m, 3H, 2H²⁵, H¹⁷), 1.28 (m, 1H, H²⁰), 1.30 (m, 1H, H^{1b}), 1.50 (m, 6H, 2H², 2H¹¹, 2H²⁴), 1.70 (m, 6H, H¹⁶, H^{15b}, H⁸), 1.84 (m, 1H, H^{1a}), 1.98 (m, 2H, 2H⁷), 2.01 (m, 2H, 2H¹²), 3.50 (m, 3H, 3H³), 2.27 (m, 2H, 2H⁴), 5.01 (dd, 1H, H²²), 5.16 (dd, 1H, H²³), 5.34 (dd, 1H, H⁶). ¹³C NMR (125 MHz, CDCl₃): δ 37.4 (C¹), 28.4 (C²), 71.9 (C³), 42.5 (C⁴), 140.9 (C⁵), 121.9 (C⁶), 32.0 (C⁷), 32.1 (C⁸), 50.3 (C⁹), 36.7 (C¹⁰), 20.0 (C¹¹), 39.4 (C¹²), 46.0 (C¹³), 56.9 (C¹⁴), 23.2 (C¹⁵), 26.3 (C¹⁶), 56.2 (C¹⁷), 12.0 (C¹⁸), 19.2 (C¹⁹), 40.6 (C²⁰), 19.6 (C²¹), 138.5 (C²²), 129.4 (C²³), 51.4 (C²⁴), 24.5 (C²⁵), 12.3 (C²⁶), 29.3 (C²⁷), 18.9 (C²⁸), 21.2 (C²⁹).

Compound P-2 (β-Sitosterol)

White needle crystals; m.p. 136-139 °C; MW 414; IR (cm⁻¹): 3364, 2925, 2834, 1463, 1375, 1051, 1022, 957, 801; EIMS m/z (% intensity): 414 ([M]⁺, 40.18), 396 ([M-H₂O]⁺, 24.76), 381 ([M-H₂O-CH₃]⁺, 18.14), 329 (29.71), 303 (20.05), 273 (11.61), 255 (22.11), 231 (14.86), 213 (20.06), 185 (5.63), 173 (15.89), 159 (25.87), 145 (38.42), 133 (26.78), 119 (31.11), 107 (48.03), 105 (46.22), 95 (45.18), 93 (35.74), 81 (52.19), 57 (60.27), 43 (100), 41 (40.2); ¹H NMR (500 MHz, CDCl₃): δ 0.68 (s, 3H, 3H¹⁸), 0.83 (d, 9H, 3H²⁶, 3H²⁸, 3H²⁹), 0.92 (s, 3H, 3H²¹), 0.95 (m, 1H, H⁹), 1.01 (s, 3H, 3H¹⁸), 1.09 (m, 1H, H^{15a}), 1.10 (m, 1H, H¹⁴), 1.20 (m, 3H, 2H²⁵, H¹⁷), 1.28 (m, 1H, H²⁰), 1.30 (m, 1H, H^{1b}), 1.50 (m, 6H, 2H², 2H¹¹, 2H²⁴), 1.70 (m, 6H, H¹⁶, H^{15b}, H⁸), 1.84 (m, 1H, H^{1a}), 1.98 (m, 2H, 2H⁷), 2.01 (m, 2H, 2H¹²), 3.50 (m, 3H, 3H³), 2.27 (m, 2H, 2H⁴), 1.20 (m, 2H, 2H²²), 1.70 (m, 2H, 2H²³), 5.34 (dd, 1H, H⁶); ¹³C NMR (125 MHz, CDCl₃): δ 37.4 (C¹), 28.4 (C²), 71.9 (C³), 42.5 (C⁴), 140.9 (C⁵), 121.9 (C⁶), 32.0 (C⁷), 32.1 (C⁸), 50.3 (C⁹), 36.7 (C¹⁰), 20.0 (C¹¹), 39.4 (C¹²), 46.0 (C¹³), 56.9 (C¹⁴), 23.2 (C¹⁵), 26.3 (C¹⁶), 56.2 (C¹⁷), 12.0 (C¹⁸), 19.2 (C¹⁹), 40.6 (C²⁰), 19.6 (C²¹), 33.9 (C²²), 28.3 (C²³), 51.4 (C²⁴), 24.5 (C²⁵), 12.3 (C²⁶), 29.3 (C²⁷), 18.9 (C²⁸), 21.2 (C²⁹).

Tabel 1- ^1H NMR and ^{13}C NMR spectroscopic data of the isolated compound

C	Compound 109a (Stigmasterol)			Compound 109b (β -sitosterol)		
	δH	δC	δC^*	δH	δC	δC^*
1	1.84 (1H, m); 1.30 (1H, m)	37.4	37.2	1.84 (1H, m); 1.30 (1H, m)	37.4	37.3
2	1.50 (2H, m)	28.4	31.3	1.50 (2H, m)	28.4	31.6
3	3.50 (3H, m)	71.9	70.3	3.50 (3H, m)	71.9	71.7
4	2.27 (2H, m)	42.5	42.0	2.27 (2H, m)	42.5	42.3
5	-	140.9	141.6	-	140.9	140.8
6	5.34 (1H, dd)	121.9	120.2	5.34 (1H, dd)	121.9	121.6
7	1.98 (2H, m)	32.0	31.6	1.98 (2H, m)	32.0	31.9
8	1.70 (1H, m)	32.1	31.8	1.70 (1H, m)	32.1	31.9
9	0.92 (1H, m)	50.3	50.1	0.92 (1H, m)	50.3	50.2
10	-	36.7	36.3	-	36.7	36.5
11	1.50 (2H, m)	20.0	20.8	1.50 (2H, m)	20.0	21.1
12	2.01 (2H, m)	39.9	39.4	2.01 (2H, m)	39.9	39.8
13	-	46.0	42.4	-	46.0	42.3
14	1.10 (1H, m)	56.9	56.5	1.10 (1H, m)	56.9	56.8
15	1.09 (1H, m), 1.70 (1H, m)	23.2	23.9	1.09 (1H, m), 1.70 (1H, m)	23.2	24.3
16	1.70 (2H, m)	26.3	28.1	1.70 (2H, m)	26.3	28.3
17	1.20 (1H, m)	56.2	55.9	1.20 (1H, m)	56.2	56.1
18	0.68 (3H, s)	12.0	11.8	0.68 (3H, s)	12.0	11.9
19	1.01 (3H, s)	19.2	19.1	1.01 (3H, s)	19.2	19.4
20	1.28 (1H, m)	40.6	40.3	1.28 (1H, m)	40.6	36.2
21	0.92 (3H, s)	19.6	20.6	0.92 (3H, s)	19.6	18.8
22	5.01 (1H, dd)	138.5	137.7	1.20 (2H, m)	33.90	33.9
23	5.16 (1H, dd)	129.4	129.3	1.7 (2H, m)	28.30	26.1
24	1.50 (2H, m)	51.4	50.6	1.50 (2H, m)	51.4	45.9
25	1.20 (2H, m)	24.5	24.7	1.2 (2H, m)	24.5	23.1
26	0.83 (3H, d)	12.3	11.9	0.83 (3H, d)	12.3	12.3
27	1.90 (1H, m)	29.3	31.50	1.90 (1H, m)	29.3	29.2
28	0.83 (3H, d)	18.9	18.90	0.83 (3H, d)	18.9	19.1
29	0.83 (3H, d)	21.2	21.04	0.83 (3H, d)	21.2	19.8

Note : δC^* is data base of carbon chemical shift from SDBS AIST Japan

The TLC analysis of petroleum ether extract of bengkoang has been carried out to find out how many compounds are in the extract and their distribution. This step was

necessary before doing isolation and determination. Figure 1 displayed the TLC chromatogram of bengkoang extract and fraction containing phytosterol.

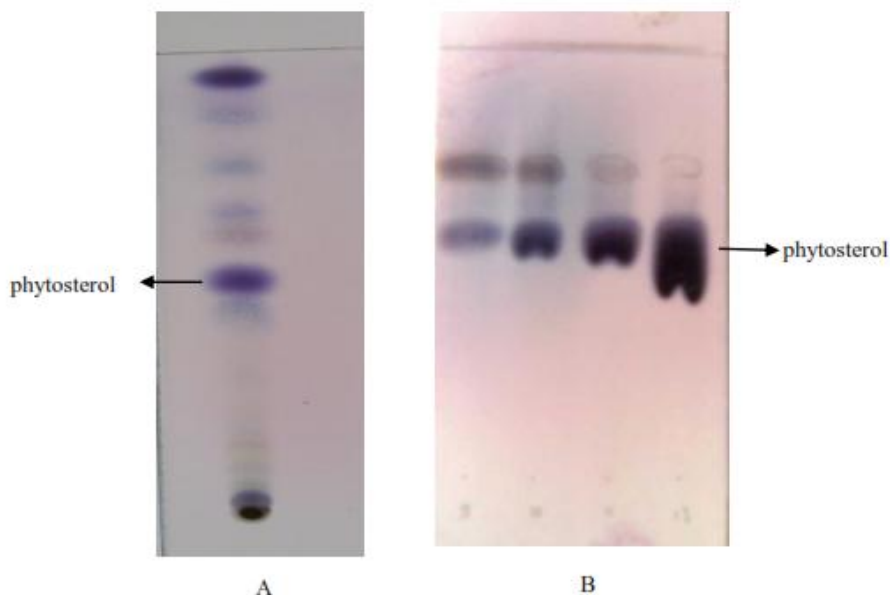


Fig.1- The TLC chromatogram of petroleum extract (A) and fraction 8, 9, 10, 11 containing phytosterols (B). The TLC system consists of silica as stationary phase and a mixture of chloroform-methanol (60:40) as mobile phase and detected by anisaldehyde

The isolated compound from fractions 8, 9, 10, 11 of the petroleum ether extract as white needle crystals having a melting point of 136-139 °C. The IR spectrum showed bands at

1699 cm^{-1} indicated the presence of C=C or C=O. Additionally the IR spectrum showed 3364 cm^{-1} (-OH group), 2925 cm^{-1} (-C-H aliphatic group), 1463 cm^{-1} (-CH group), 1375

cm^{-1} (-CH group) and 1051 cm^{-1} (-C-O-C group).

An analysis using gas chromatogram has been conducted and the chromatogram can be seen in Figure 2. The chromatogram showed two peaks with retention times of 31.2 and 32.4

min, corresponding to the molecular ion peaks at m/z 412 and 414, respectively. They are consistent with the molecular formula $\text{C}_{29}\text{H}_{48}\text{O}$ (e.g. stigmasterol) and $\text{C}_{29}\text{H}_{50}\text{O}$ (e.g. β -sitosterol).

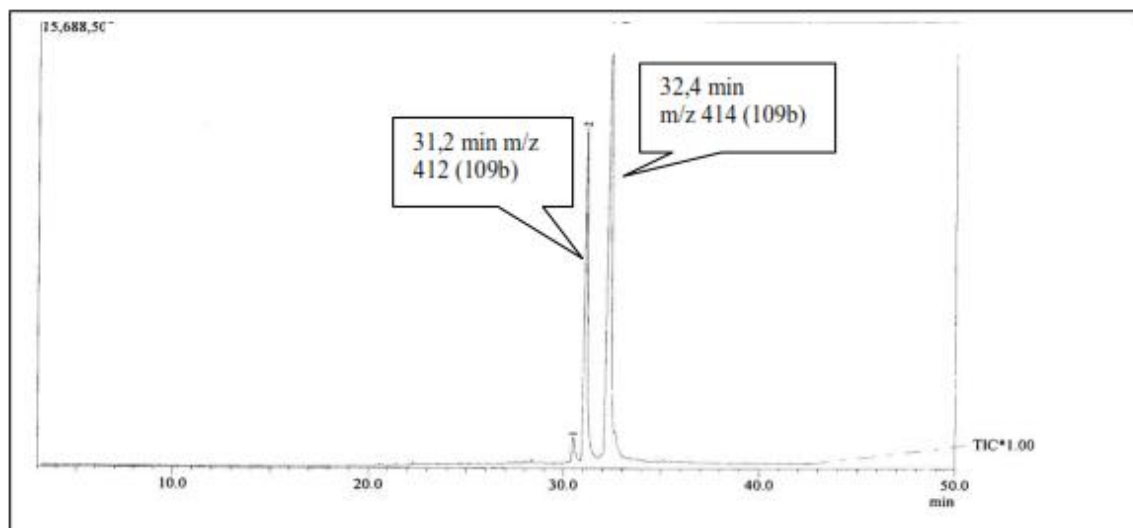


Figure 2. Gas chromatogram Mass Spectroscopy of the sample, the chromatogram showed two peaks with retention times of 31.2 and 32.4 min, corresponding to the molecular ion peaks at m/z 412 and 414, respectively.

The hypothesis that the isolated compound was a mixture of a β -sitosterol and stigmasterol supported by the results of NMR spectrum and the fragmentation pattern in MS spectrum. The EI-MS spectrum showed the presence of the hydroxyl group was supported by the strong loss of 18 mass units in both EI mass spectrums. The existence of a double bond of C-C was represented in the ^{13}C -NMR by four downfield signals at δ 140.9 ppm (quarternary carbon), 121.9 ppm (CH), 129.4 ppm (CH) and 138.5 ppm (CH). The carbon chemical shift data of **P-1** was similar to **P-2**, except δ C^{22} and C^{23} . The carbon chemical shifts of C^{22} from P-1 and P-2 were 33.9 ppm and 138.5 ppm, respectively. Meanwhile the carbon chemical shifts of C^{23} of **P-1** and **P-2** were 28.3 ppm and 129.4 ppm, respectively. All the carbon chemical shift data of the ^{13}C NMR spectrum (Table I) were in a close agreement with those of stigmasterol and β -sitosterol in literature reported (Kovganko et al. 2000) and also with the database in SDBS AIST Japan.

The downfield signal in the ^1H -NMR spectrum at δ 5.34 (1H, dd) was due to an

olefinic proton at C^6 and a methine proton at C^3 was represented by multiplet signal at 3.50 (1H, m). The presence of a pair of doublets at δ 5.01 and δ 5.16 was due to the sp^2 methine protons at C^{22} and C^{23} in molecule **P-1**. The existence of six methyl signals was also noted at δ 0.68 ($\text{H}^3\text{-C}^{18}$), 1.01 ($\text{H}^3\text{-C}^{19}$), 0.92 ($\text{H}^3\text{-C}^{21}$), 0.83 ($\text{H}^3\text{-C}^{28}$), 0.83 ($\text{H}^3\text{-C}^{29}$).

The COSY correlations between H^6 and H^7 ; H^{22} and H^{23} ; H^3 and H^4 ; H^{11} and H^{12} ; H^1 and H^2 ; H^2 and H^3 ; H^{20} and H^{22} supported the stigmasterol and β -sitosterol (Fig. 3). Some of the ^1H - ^{13}C -long range correlations (Fig. 4) observed in the HMBC diagram confirmed that the structures of **P-1** and **P-2** were stigmasterol and β -sitosterol.

The mass fragmentation pattern of the **P-1** is displayed in Figure 5. The molecular ion of **P-1** was observed at m/z 412. The loss of water from **P-1** was indicated by the presence of a fragment ion at m/z 394. The subsequent fragment at m/z 379 might be due to the loss of a methyl group. The signal at m/z 271 was the characteristic of the stigmasterol fragmentation due to the loss of side chain followed by the loss of two hydrogen atoms.

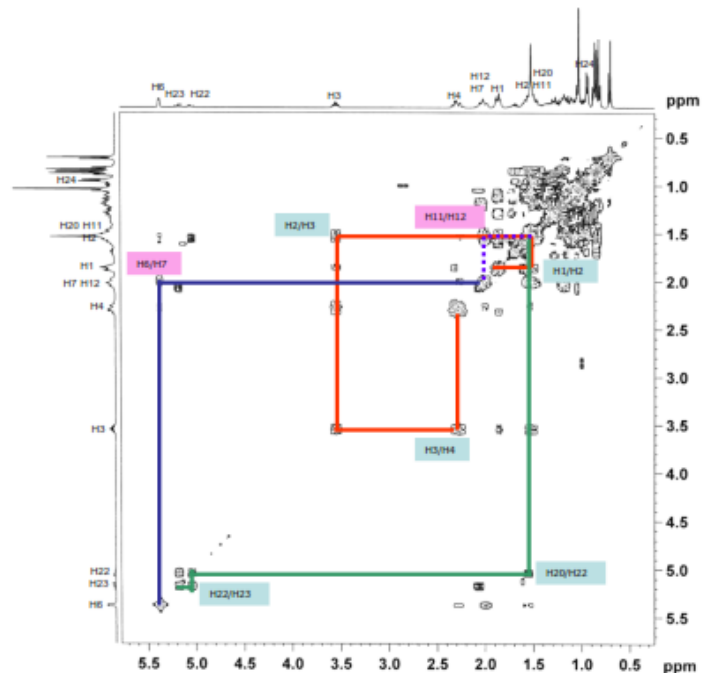


Fig. 3-COSY diagram of the mixture of the isolated compound

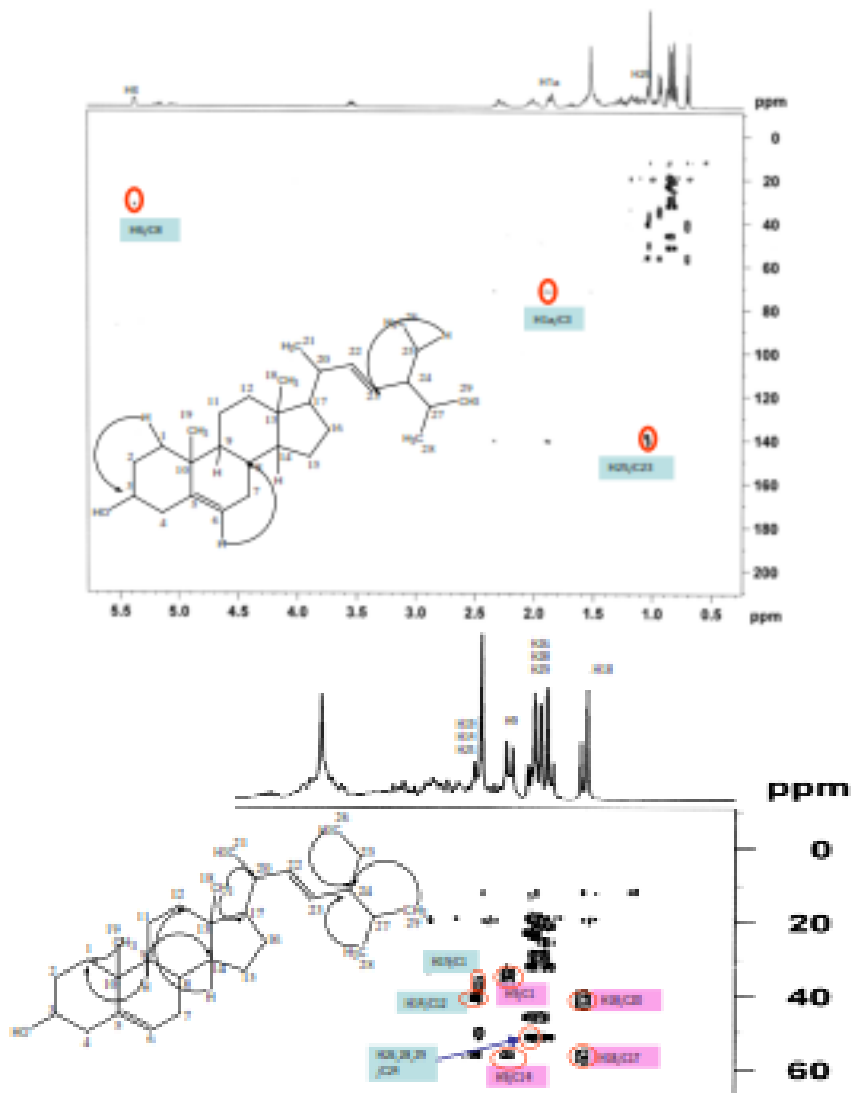


Fig. 4-MBC diagram of the mixture of compounds 109a and 109b

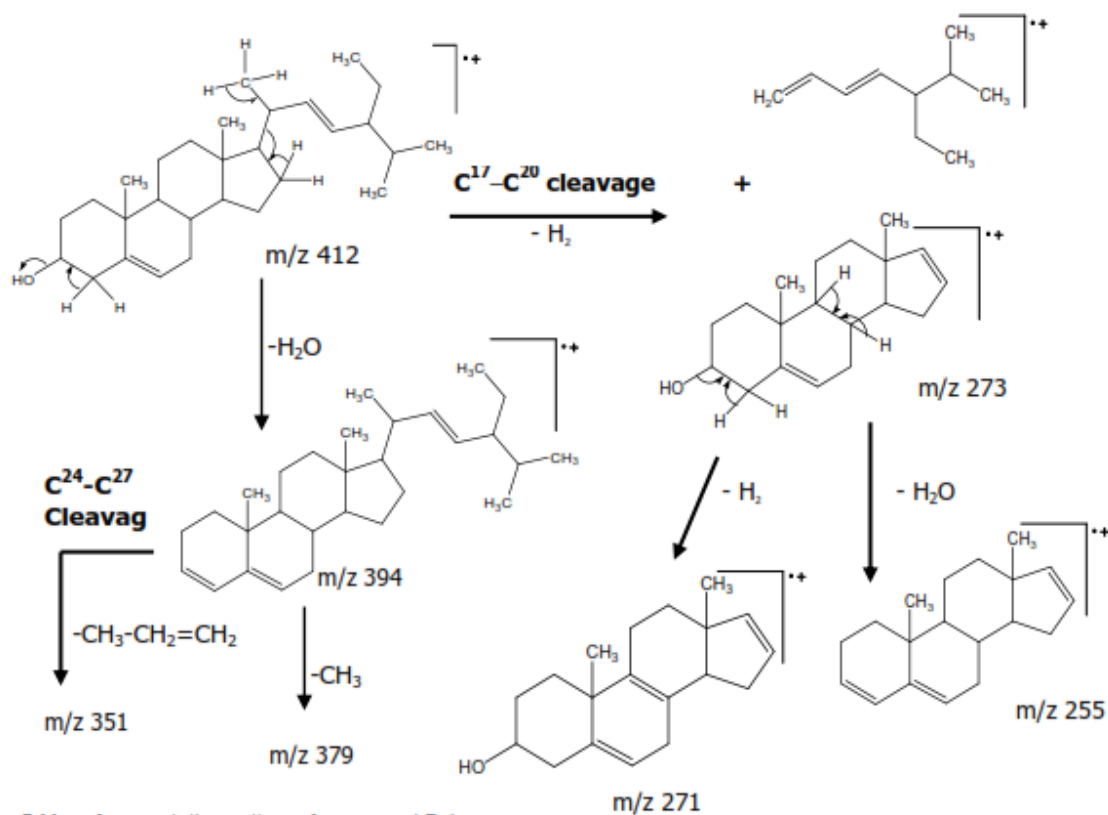


Fig. 5-Mass fragmentation pattern of compound P-1

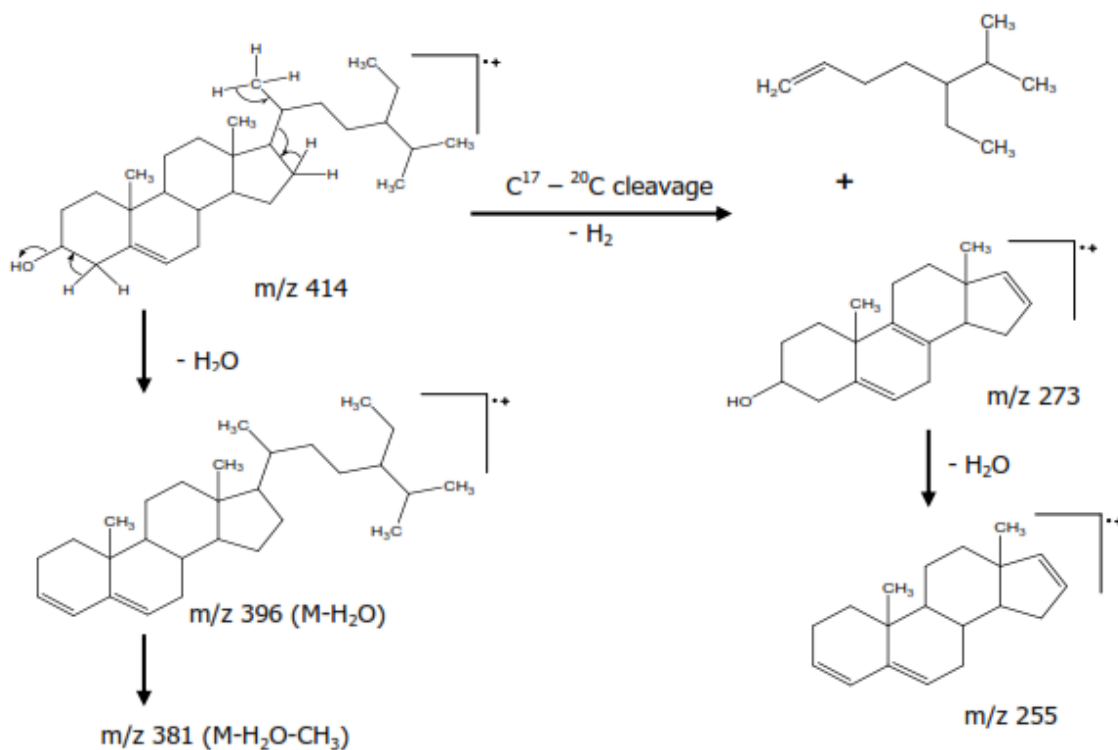


Fig. 6- Mass fragmentation pattern of compound P-2

The mass fragmentation pattern above was in accordance with previously paper. Chaves et al. (2004) found in GC/MS the fragments with m/z value of 412 (M^+), 271 and 273. Based on the above fragmentation, this substance **P-1** was identified as stigmasterol.

The Figure 6 displayed a mass fragmentation pattern of **P-2** having a molecular ion m/z 414. The fragment ions at m/z 396 and 381 were due to the loss of water and followed by a methyl group from the molecular ion. The characteristic observed in the mass spectrum of **P-2** was the presence of a fragment ion peak at m/z 273. This signal was in a close agreement with the loss of side chain caused by the fission of $C^{17}-C^{20}$ bond. This was further fragmented producing water. From this process, the signal at m/z 255 was observed.

The fragmentation pattern above is in accordance to Zhang et. al. (2005): β -sitosterol in acid solution produces positive signals at m/z 382 and 397 with intensities 52 and 100, respectively. The signal at m/z 397 was assigned to $(M+H^+ - H_2O)$ and the signal at m/z 382 to $(M+H^+ - H_2O - CH_3)$. In addition, Berezin et al. (2004) and Huang et al. (2007) have investigated the fragmentation of β -sitosterol and stigmasterol using HPLC-MS equipped with APCI. The result showed that β -sitosterol and stigmasterol were protonated by a reactive species in the plasma of

ion source. β -Sitosterol had an exact mass of 414.39, which became 397.38 after protonation and loss of water. While stigmasterol had an exact mass of 412.41 which became 395.4 after protonation and loss of water.

All these spectroscopy data proved the structure of the substance **P-1** as stigmasterol and **P-2** as β -sitosterol. Based on the gas chromatogram at Figure 2, the concentration ratio of stigmasterol and β -sitosterol from this fraction was 35 : 65.

This research proved that the total phytosterol content in petroleum ether extract of bengkoang was relatively high (2,76%) or about 0.02 % of dry weight bengkoang. Therefore, bengkoang can be used as a good source of phytosterol and potentially used as nutritional supplement of cholesterol replacement.

CONCLUSION

Bengkoang contained a relatively high of phytosterols consisted of two major compounds, namely β -sitosterol and stigmasterol. Therefore, bengkoang can be further exploited not only as cosmetics material, but also as nutritional supplement especially for cholesterol replacement.

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