

Structure Of Stigmasterols in Bran of Red Rice from Minahasa Regency, North Sulawesi, Indonesia

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ABSTRACT

Rice bran contained important bioactive phytochemical components such as tocopherol, tocotrienol and γ -oryzanol, and one of the component of γ -oryzanol is stigmasterol. The aims of the present study was to isolate and identify stigmasterol in native red rice bran extract. The isolation of sterol from red rice bran extract was performed by using Thin Layer Chromatography (TLC) and followed by identification by Fourier Transformed Infrared Spectroscopy (FTIR) and Nuclear Magnetic Resonance (NMR). The TLC single spot originated from crude red rice hexane extract was observed at 365 nm UV and the FTIR spectra analysis result showed that OH group was indicated by 3436,9 cm^{-1} absorption. While absorption regions of 2931,6 to 2852,5 cm^{-1} indicates the presence of single bond CH. The data of ^1H -, ^{13}C - and 2D-NMR of rice bran sterol indicated that the single spot obtained in TLC analysis was stigmasterol. It can be concluded that native red rice variety of North Sulawesi contained stigmasterol and potential as native antioxidant source.

Introduction

Rice is the staple of Asia including Indonesia, where consumption's have assumed to 22.28 million tons per year [1]. In Indonesia there are several varieties of rice, among them are Cigeulis, Ciherang, IR64 and several varieties of sticky rice that are pigmented like the red rice. Rice bran is a by product of rice milling process that contains protein, dietary fiber, and natural antioxidant [2]. Pigmented rice contains procyanidine, anthocyanine and other phenolic compounds with antioxidative activities such as tocopherols, tocotrienols, oryzanols and phytosterols [3] that has many health benefits like enhancing immunity, reducing cancer risks and hepatoprotective benefits. While many previous studies have exhibited the bioactivities of rice bran from pigmented rice in regards to inhibiting activities of tyrosinase, also antialergic, anti-inflammatory, anti-mutagenic and anti-glycation activities [2].

β - sitosterol is the major sterol in rice oil much as in common vegetable oils, and more

than 75% of rice bran-oil sterols are esterified and collectively known as oryzanol. γ -oryzanol, which contains sterols and ferulic acids called cycloartanyl ferulate or triterpene alcohol ferulate, has anti-cancer properties and is unique to the rice plant [4].

Further research reported cycloartenol trans-ferulate (m/z 601), 24-methylenecycloartanol trans-ferulate (m/z 615), campesterol trans-ferulate (m/z 575), and sitosterol trans-ferulate (m/z 589) were the major components, representing up to 90 % in the control brown rice and averaged of 75 % in the wild rice samples. Indeed, the amounts of 24-methylenecycloartanol trans-ferulate (249 $\mu\text{g/g}$), cycloartenol trans-ferulate (171 $\mu\text{g/g}$), and total γ -oryzanol (688 $\mu\text{g/g}$) obtained in the present study for long grain regular brown rice are in agreement with those reported by [5].

While [6] noted that from four different rice varieties (Super Kernel, 386, 385 and Basmati) of Pakistan contained campesterol, stigmasterol, β -sitosterol, and Δ -5-avenasterol

as the major fractions of the rice bran oil sterol. Three different rice varieties of Iran (Khazar, Alikazemi, and Hashemi), all contained β -sitosterol, campesterol, Δ -5-avenasterol, Δ -7-avenasterol, cholesterol dan brassicasterol [7]. Furthermore [8] observed that sitosterol, campesterol, Δ -5-avenasterol and stigmasterol were the main contributor of sterol components of rice bran oil of selected rice varieties in Pakistan.

A study on Thai Black non glutinouse rice bran Riceberry results that Thai Black contains four phytosterols namely 24-methylene-ergosta-5-en- 3β -ol, 24-methylene-ergosta-7-en- 3β -ol, fucosterol and gramisterols. Also found in Thai Black are triterpenoids consisting of cycloeucalenol, lupenone and lupeol that are found in 2 fractions that displayed strong activities in anti leukemia poliferations at an IC_{50} = 2.80 and 32.89 μ g/mL [3].

Previous researches by [9] was conducted on the antioxidant characteristics of several varieties of North Sulawesi rice, indicated that crude extract of the rice bran of the red rice variety had better antioxidant characteristics compared to other varieties (Cigeulis and Superwin) which has shown by the DPPH scavenging radical activity of $88.29 \pm 5.62\%$ with the lowest IC_{50} value (26.26 ± 0.95 μ g/ml) and highest total antocyanine content (68.61 ± 1.98 mg/g). By understanding the bioactive component profiles especially the stigmasterol content of native rice bran from Minahasa, North Sulawesi, Indonesia, therefore the importance of this research as the foundation for further applied development researches in conjunction with health and functional food of rice bran bioactives. Therefore, the objective of this research was to isolate and elucidate stigmasterol phytosterols from native red variety rice bran from the Minahasa Regency of North Sulawesi, Indonesia.

Methodology

Rice Bran Sterol Isolation

Rice bran extraction was carried out according to the method of [10] that was slightly modified. Red rice variety of rice bran flour samples at one kg each were macerated and extracted with 3000 ml of 70% ethanol three times and kept overnight at room temperature.

The ethanol extracts were then fractionated with organic solvents (hexane, ethyl acetate and n-butanol) in accordance with their polarity level. Each extract was prefiltered with Whatman paper No. 42 and then evaporated by rotary evaporator (Buchi rotavapor) under vacuum to obtain the hexane, ethyl acetate and n-butanol crude extract of rice bran. Sterol isolation from 20 mg rice bran hexane crude extract was conducted by chromatography using silica gel 60 Merck as the stationary phase and hexane-ethyl acetate for the eluent at a ratio of 9:1 with a 5% stepwise.

Sterol Infrared Spectrum

Infrared spectrum was taken with Fourier Transform Infrared Spectrophotometer (FTIR)- Shimadzu 8400. The liquid sample is placed between two KBr pellets with the help of capillary tube. Each pellet is made of 0.2 mm thickness and it is placed in the path of the sample beam. The spectra are recorded from 4000 to 450 cm^{-1} , the number of scans being 256 at a resolution of 4 cm^{-1} , scan speed is 0.20 cm/s.

Sterol Structure Elucidation - 1H NMR, ^{13}C NMR

Sterol structure elucidation was performed by Nuclear Magnetic Resonance (NMR) - JEOL JNM-ECA 500 spectrometer (500Mhz), JEOL Tokyo, Japan, with Chloroform-D ($CDCl_3$) as a solvent and tetramethylsilane (TMS=0 ppm) as an internal standard. Chemical shifts are given on a δ scale (ppm).

Result and Discussion

Sterol Isolation

Chromatograph results of hexane rice bran sterols extract was indicated by a single spot visible under 365nm UV. Thin layer chromatograph results can be viewed in figure 1 below.

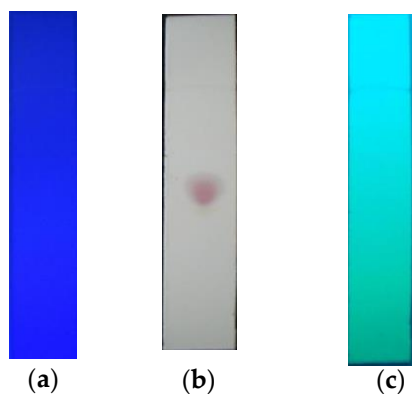


Figure 1. Thin Layer Chromatograph Results of Rice Bran Sterol (a) Under UV 254 nm (b) Under UV 365 nm (c) Sprayed with 10% H₂SO₄ in ethanol

Rice Bran Sterol Infrared Spectrum

Rice bran sterol infrared spectrums are shown in figure 2.

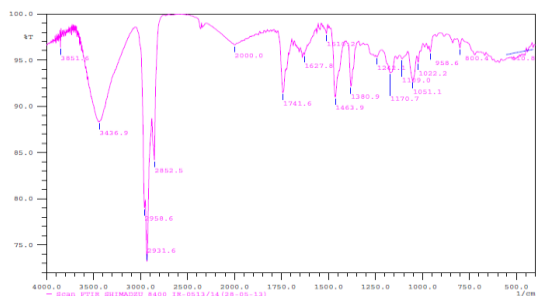


Figure 2. Infrared Spectrum of Rice Bran Sterol

Table 1. Interpretations of Rice Bran Sterol Infrared Spectrums

Absorptions (cm ⁻¹)	Peak Appearance	Intensity	Most Probable Functional Groups
3436,9	broad	moderate	OH
2931,6	sharp	high	CH sp ³
2958,6	sharp	high	CH sp ³
2889,1	sharp	high	CH sp ³

Sterol Structure Elucidation by ¹H NMR, ¹³C NMR

The ¹H-NMR spectrum results on rice bran sterol is shown in figure 3 below.

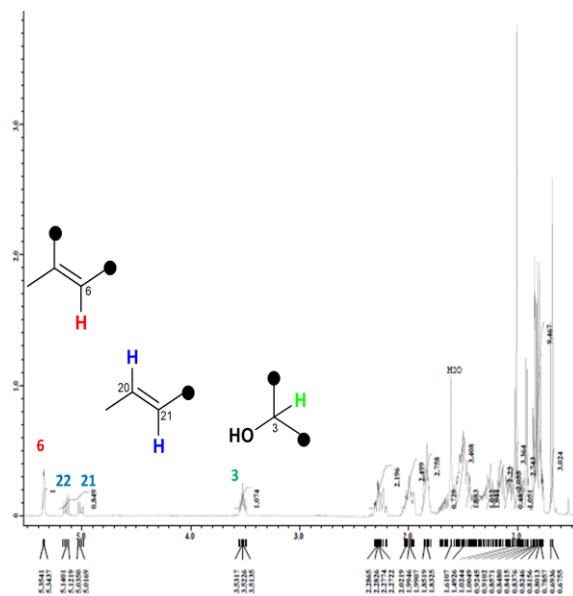


Figure 3. Rice Bran Sterol ¹H-NMR

¹³C-NMR spectrum of rice bran sterol is shown in figure 4.

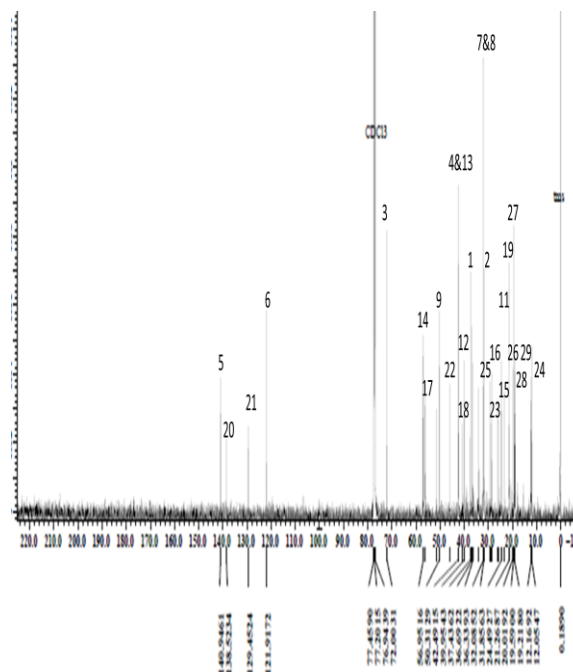


Figure 4. ¹³C-NMR Spectrum of Rice Bran Sterol

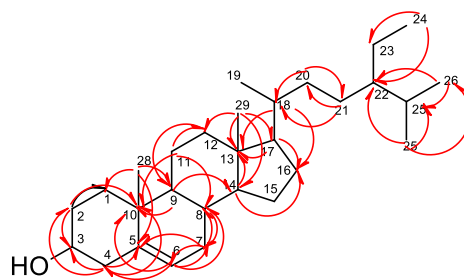
¹H-, ¹³C- and 2D-NMR chemical shifts of rice bran sterol lipid is illustrated in Table 2.

Table 2. ^1H -, ^{13}C - and 2D-NMR of Rice Bran Sterol

No	$\delta_{\text{C}} 125$ MHz (ppm)	δ_{H} (Int., mult., $J=\text{Hz}$) 500 MHz (ppm)	HMBC		COSY
			2J	3J	
1	37,4	1,00 (2H, m)	-	-	
2	31,8	1,83 (2H, m)	1,3	10	
3	72,0	3,52 (1H, m)	-	-	4
4	42,3	2,28 (2H, m)	3,5	2,6	3
5	140,9	-	-	-	
6	121,9	5,34 (1H, d, $J=5,2$ Hz)	7	4,10	
7	32,0	1,48 (2H, m)	6,8	5	
8	32,0	1,48 (2H, m)	7	-	
9	50,3	0,92 (1H, d)	-	14	
10	36,6	-	-	-	
11	21,2	1,48 (2H, m)	12	10	12
12	39,9	1,99 (2H, m)	-	9,14	11
13	42,3	-	-	-	
14	56,9	1,07 (1H, m)	13	16	
15	24,4	1,49 (2H, m)	-	-	
16	28,4	1,24 (1H, m)	-	13	
17	56,2	1,05 (1H, m)	13,16	-	
18	40,6	1,24 (1H, m)	13,16	-	
19	21,2	1,48 (3H, m)	-	-	
20	138,5	5,00 (1H, q, $J=$	18,21	-	
21	129,4	5,15 (1H, q, $J=$	20	-	22
22	46,0	0,92 (1H, d)	-	-	21

23	25,6	1,17 (2H, m)	-	-	
24	12,1	0,69 (3H, s)	23	22	
25	29,3	1,66 (1H, m)	-	-	
26	20,0	0,82 (3H, d, $J=$ Hz)	25	22,27	27
27	19,6	1,00 (3H, s)	25	22,26	26
28	18,9	0,82 (3H, s)	10	1,5,9	
29	12,2	0,69 (3H, s)	13	12,14,17	

By deducing the infrared spectrum results and the spectrums of the nuclear magnetic resonance (NMR), it is suggested that rice bran sterol lipid structure would appear as in Figure 5.

**Figure 5.** HMBC of isolated rice brand sterol lipid

According to the chemical shift data of ^1H - and ^{13}C -NMR on Table 3, rice bran sterol is in comparison to stigmasterol, structure-wise results of rice brand sterol compares to other sterol.

Based on the values of the chemical shifts in Table 3 it can be concluded that rice bran sterol chemical shifts are similar to those of stigmasterol. The variance that occurred in shifts of these two compounds are most like due to the differences in solvents used during identification process via NMR and the differences in the NMR facilities that was utilized, therefore it is assumable that sterols isolated from Minahasan red rice bran from North Sulawesi is indeed the IUPAC stigmasterol with a structure as in Figure 6.

Table 3. ^1H - dan ^{13}C -NMR Data of Rice Bran Sterol and Stigmasterol

No	δ_{C} 125 MHz (ppm)		δ_{H} (Int., mult., J=Hz) 500 MHz (ppm)	
	Stigma-sterol	Rice Bran Sterol	Stigma-sterol	Rice Bran Sterol
1	37,6	37,4		1,00 (2H, m)
2	32,1	31,8		1,83 (2H, m)
3	72,1	72,0	3,51 (1H, tdd, J= 4,5; 4,4; 3,8 Hz)	3,52 (1H, m)
4	42,4	42,3		2,28 (2H, m)
5	141,1	140,9		-
6	121,8	121,9	5,31 (1H, t, J=6,1 Hz)	5,34 (1H, d, J=5,2 Hz)
7	31,8	32,0		1,48 (2H, m)
8	31,8	32,0		1,48 (1H, m)
9	50,2	50,3		0,92 (1H, d)
10	36,6	36,6		-
11	21,5	21,2		1,48 (2H, m)
12	39,9	39,9		1,99 (2H, m)
13	42,4	42,3		-
14	56,8	56,9		1,07 (1H, m)
15	24,4	24,4		1,49 (2H, m)
16	29,3	28,4		1,24 (2H, m)
17	56,2	56,2		1,05 (1H, m)
18	40,6	40,6		1,24 (1H, m)
19	21,7	21,2	0,91 (3H, d, J= 6,2 Hz)	1,48 (3H, m)
20	138,7	138,5	4,98 (1H, m)	5,00 (1H, q, J=

21	129,6	129,4	5,14 (1H, m)	5,15 (1H, q, J=
22	46,1	46,0		0,92 (1H, d)
23	25,4	25,6		1,17 (2H, m)
24	12,1	12,1	0,83 (3H, t, J=7,1 Hz)	0,69 (3H, s)
25	29,6	29,3		1,66 (1H, m)
26	20,2	20,0	0,82 (3H, d, J= 6,6 Hz)	0,82 (3H, d, J= Hz)
27	19,8	19,6	0,80 (3H, d, J= 6,6 Hz)	1,00 (3H, s)
28	18,9	18,9	0,71 (3H, s)	0,82 (3H, s)
29	12,2	12,2	1,03 (3H, s)	0,69 (3H, s)

- a) Spectrometers Varian Unity plus 600 MHz
b) Spektrometer JEOL 500 MHz

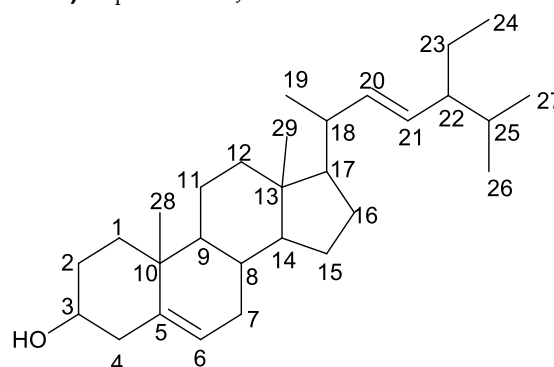
**Figure 6.** Rice Bran Stigmasterol of Minahasan Red Rice Variety

Figure 2 showed the presences of OH groups indicated by $3436,9\text{ cm}^{-1}$ absorption. While absorption regions of $2931,6$ to $2852,5\text{ cm}^{-1}$ indicates the presence of single bond CH. Furthermore, a more comprehensive interpretation of rice bran sterol lipid infrared spectrum is shown in Table 1. The absorption of OH group of 4,4-dimethylsterol from rice bran sample was determined at 3440 cm^{-1} , and [11] also observed a similar absorption at 3465 cm^{-1} for most probable OH group in unheated commercial rice bran oil and decrease to 3432 cm^{-1} which believe due to the lack of

hydroperoxide formation during heat treatment.

The $^1\text{H-NMR}$ of rice bran sterol in figure 3 indicated the presence of 3 hydrogen signals with double bonds that appeared in shift area of δ_{H} 5,34; 5,14 and 4,98 ppm. Hydrogen signal at δ_{H} 5,34(H-6) ppm is assumed to be ethylenic carbon methyn that is attached to a double bonded quaternary carbon as illustrated on the fragment with the hydrogen indicated in red, while two hydrogen signals with the same multiplicity in δ_{H} 5,14(H-22) and 4,98(H-21) ppm, therefore it is assumed to be vicinal hydrogen atoms, adjacent hydrogen atoms at a distance of 3 bonds (vicinal hydrogen-hydrogen). The single hydrogen signal on δ_{H} 3,52 ppm indicated the presences of a hydrogen signal bonded to an oxygenated carbon.

$^1\text{H-NMR}$ and ^{13}C spectrums of rice bran sterol shows that the sterol lipid has 29 carbon atoms, 48 hydrogen atoms and 1 oxygen atom. Molecular compound is identified as $\text{C}_{29}\text{H}_{48}\text{O}$ and considered to have 2 double bonds and 4 cyclic structures. $^{13}\text{C-NMR}$ spectrum in Figure 4, indicates 29 carbon signals, that consisted of 25 single bond carbons ($\text{C } sp^3$) in chemical shift areas under 80 ppm and 4 double bonded carbons ($\text{C } sp^2$) within the chemical shift areas above 120 ppm. Carbon signals ($\text{C } sp^2$) are shown by carbon signals above 120 ppm as such δ_{C} 141,1(C-5); 138,5(C-22); 129,4(C-21) and 121,8(C-6) ppm, while signals below 75 ppm are single bond carbons ($\text{C } sp^3$) which consisted of oxygenated carbon as in δ_{C} 72,0(C-3), further other carbons incorporating single bonds to other carbons are : δ_{C} 12,1(C-24); 12,2(C-29); 18,9(C-28); 19,6(C-27); 20,0(C-26); 21,2(C-11&C-19); 24,4(C-15), 25,6(C-23), 28,4(C-16), 29,3(C-25) 31,8(C-2), 32,0(C-7&C-8), 36,6(C-10), 37,4(C-1), 39,9(C-12), 40,6(C-18), 42,3(C-4&C13), 46,0(C-22), 50,3(C-9), 56,2(C-17) and 56,9(C-14) ppm. From the $^{13}\text{C-NMR}$ data it is likely that the isolated compound would be a sterol with 2 double bonds since it was apparent that the compound had 4 carbons with double bonds. The 29-carbon 4-desmethylsterol is one type of 4-desmethyl phytosterol or commonly known as 4 – desmethylsterol are made up by 24α ethyl epimers (sitosterol and stigmasterol), and stigmasterol is Δ -22, $24\text{-}\alpha$ -ethylcholesterol [12-

13]. Similar results were observed by [2], phytosterol found in Thai dark purple glutinous rice bran extract cultivar Luem pua are campesterol, β -sitosterol, stigmasterol, and four isomeric sterol which are 24-methylene-ergosta-5-en- 3β -ol, 24-methylene-ergosta-7-en- 3β -ol, fucosterol, and gramisterol.

β -cytosterols are known for their biological activities as in decreasing blood cholesterols, anti-inflammation, and anti diabetic, while stigmasterols found in rice bran oil are known for their effects as anti-inflammation and their cytotoxic activity towards cancer cells [14, 15, 16]. Understanding the phytochemical composition and structures of rice bran oil, especially the compounds contributing to biological activities, will place these results as the foundation for the application of phytosterols of rice bran in its future developments in functional food.

Conclusion

The present study showed that a single spot was found in TLC analysis and it is believed as sterol of red rice bran extract, and further analysis using FTIR indicated that the absorption of OH group was observed at 3436.9 and the CH single bond were in the range of 2931.6 to 2852.5 cm^{-1} . The spectra of $^1\text{H-NMR}$, $^{13}\text{C-NMR}$ and 2D-NMR confirmed that the substance from native red rice bran variety extract is stigmasterol.

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