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Anti-hypercholesterolemic agent from Indonesian rice bran

Syaikhul Aziz[1,2], Elfahmi[1], Andreanus Andaja Soemardji[1], Sukrasno[*1]

[1]School of Pharmacy, Institut Teknologi Bandung, West Java-40132, Indonesia.
[2]Departement of Pharmacy, Faculty of Science, Institut Teknologi Sumatera, Lampung-35365, Indonesia.

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ABSTRACT

This research was conducted to determine the total oryzanol content of various extracts and bioassay-guided isolation of active anti-hypercholesterolemic agent from Indonesian rice bran. Hot solvent extraction was done by reflux method using single and binary solvents, namely hexane, chloroform, ethyl acetate, dichloromethane, isopropanol, acetone, hexane-ethyl acetate (1:1, v/v), hexane-isopropanol (1:1, v/v), and chloroform-ethyl acetate (1:1, v/v). TLC densitometric was used to quantitatively analyze the total oryzanol content in various extracts. The hexane extract was selected for fractionation and evaluated for their anti-hypercholesterolemic activity. The structure of an isolated compound was determined on the basis of NMR and Mass spectroscopy. The total oryzanol content was obtained using various solvents ranging from 27.92 to 43.59 mg/100g rice bran, in which hexane extract showed the highest amount (43.59 $\pm$ 3.36 mg/100g rice bran). β-sitosterol has been isolated from rice bran hexane extract, which shows a significant decrease in serum total cholesterol level. In summary, hexane is the best solvent to extract oryzanol compound using reflux method, and β-sitosterol was suspected to be responsible for anti-hypercholesterolemic activity in addition to existing compound such as oryzanol that contained in the rice bran.

*Corresponding Author

Name: Sukrasno
Phone:
Email: sukras@fa.itb.ac.id

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INTRODUCTION

Rice bran (Rb) is the outermost layer of rice seed, a by-product of the rice milling process. Nowadays, the utilization of Rb has been widely studied due to its valuable composition, which is rich in minerals, vitamins, proteins, lipids, and crude fiber (Rosniyana *et al.*, 2007). Furthermore, it has been recognized in numerous pharmacological properties, such as anti-oxidative, anti-microbial, anti-allergenic, anti-carcinogenic, anti-hypercholesterolemic, and anti-diabetic (Friedman, 2013). Oryzanol is one of the unique constituents contained in Rb, in which it was suspected to have an influence on wide range Rb activities (Patel and Naik, 2004). In general, it can be extracted from Rb by organic solvent (hot and cold extraction), supercritical fluid extraction, mechanical extraction, microwave-assisted extraction and modification of these techniques to improve their extractability (Imsanguan *et al.*, 2008; Jesus *et al.*, 2010; Thanonkaew *et al.*, 2012; Trinovita *et al.*, 2017).

Previous research on Rb and oryzanol has revealed to have cholesterol-lowering properties in animals and humans (Ausman *et al.*, 2005; Berger *et al.*, 2005; Mäkynen *et al.*, 2012; Wilson *et al.*, 2007).

However, the research on the exploration of other hypocholesterolemic active compounds from Rb is still limited. Hence, we interested in exploring the possibility of another compound from rice bran that having biological activity, particularly to reduce total cholesterol. The present study was conducted to determine the oryzanol content of various extracts and isolate the anti-hypercholesterolemic compound from the Indonesian white rice bran.

MATERIALS AND METHODS

General materials

All chemicals and solvents were obtained from JT-Baker, Merck, Sigma-Aldrich, TCI, and WAKO. Diagnostic kits for estimating serum total cholesterol (TC), triglyceride (TG), and high-density lipoprotein cholesterol (HDL-c) were purchased from Rajawali Nusindo, Indonesia. Each separation step was monitored by thin-layer chromatography (TLC). The visualization was done under UV light and sprayed reagent (H_2SO_4 10%). Electrothermal (Thermo Fisher®) was used for determining the melting point, and the results were not corrected. Mass spectra were recorded on Waters Xevo QToF spectrometer. The 1D and 2D-NMR spectra were recorded on Agilent spectrometer in $CDCl_3$.

Rice bran extract preparation

Fresh white rice bran was collected from a local rice mill in Banten province - Indonesia. Rb was separated from the unwanted material through 20 mesh sieve and was then stored in the refrigerator before use. In order to determine the total oryzanol level from Rb extracts, 5 g of Rb was extracted by reflux extraction for 30 minutes with 50 ml of single and binary solvent, namely hexane, chloroform, ethyl acetate, dichloromethane, isopropanol, acetone, hexane-ethyl acetate (1:1, v/v), hexane-isopropanol (1:1, v/v), and chloroform-ethyl acetate (1:1, v/v). After filtering through filter paper, the solvents were evaporated in a vacuum rotary evaporator. In order to *in vivo* assay and isolation step, to the best solvent that produces the high content of oryzanol was employed on a larger scale.

Determination of total oryzanol content

Total oryzanol content was analyzed by TLC densitometric method as has been described by (Sakunpak *et al.*, 2014) with some modification. Precoated silica gel F_{254} aluminum TLC plate was used as a stationary phase, and hexane-ethyl acetate-formic acid (80:20:1, v/v) was used as mobile phase. A series of standard oryzanol solutions were prepared from 25 to 175 μg/ml to construct a calibration curve. 4 μl of standard solutions and sample solutions (4 %, w/v) were applied as a dot with 10 mm distance between the dot. After the TLC was developed, the TLC plate was dried at room temperature. Total oryzanol was quantified by direct densitometric scanning at 320 nm without derivatization. The calibration curve was made by plotting peak areas against an amount of standard oryzanol which applicated on the TLC plate. The data were expressed an average $\pm$ standard deviation.

Anti-hypercholesterolemic assay

The experiment was conducted under ethical approval from the Animal Research Ethics Committee - Institut Teknologi Bandung with registered number 03/KEPHP-ITB/09-2017. Male Wistar rats (*Rattus novergicus*) weighed 150-200 g were obtained from animal laboratory - School of Pharmacy, Institut Teknologi Bandung. They were kept in the cage at room temperature and 12 h of light/dark cycle. The rats were then divided into eight groups randomly, that is a normal group, control group, simvastatin group, and five test groups. Every group had free access to the standard chow (Ratbio®) and water during the experimental period. The hypercholesterolemic rat models were induced by daily gavage administration of 0.5 ml/100 g body weight of cocktail containing in palm oil which consists of 2% cholesterol, 1% cholic acid (CA), and 0.6% propylthiouracil (PTU) for 30 days period. After cocktail-induced hypercholesterolemic administration, the animals were treated as below:

Normal group: Not treatment

Control group : 2% cholesterol + vehicle

Simvastatin group : 2% cholesterol + simvastatin 10 mg/kg body weight

Test group : 2% cholesterol + (F2 / F3 / F4 / F5 / F6) 200 mg/kg body weight

The fasting blood samples were collected at the beginning of induction, after induction, and 4 days after treatment. At the end of the experiment, the rats were anesthetized and sacrificed. The serum TC, TG, and HDL-c were determined by commercial diagnostic kit analysis using UV spectrophotometer (Microlab 300), and the low-density lipoprotein cholesterol (LDL-c) was determined by (Friedewald *et al.*, 1972) formula. The data were stated as mean $\pm$ standard deviation. Statistical assessment was performed by the Kruskal-Wallis test, followed by the Mann-Whitney test for the differences between the means, in which p-value < 0.1 is considered statistically different.

Fractionation of Rb hexane extract

Rb hexane extract (80 g) was subjected to column chromatography using silica gel 60 (0.062-0.2 mm) and gradient elution using hexane-ethyl acetate to give 6 combined fractions (F1 - F6), in which F2 - F6 was used as a sample for an anti-hypercholesterolemic assay. F5 (0.46 g) was subjected to centrifugal thin layer chromatography (CTLC) with silica gel GF_{254} and gradient elution using hexane-ethyl acetate to give 9 sub-fractions (F5a - F5i). F5d (0.12 g) was further purified by CTLC with isocratic elution using hexane-ethyl acetate (9:1), followed by recrystallization with ethanol to give compound **1** (18.2 mg).

White solid, m.p. 134.3 – 135.8 ^{0}C. ESI-MSMS *m/z* = 413.22 [M-H]+. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ ppm: 3.52 (1H, m, H-3), 5.35 (1H, m, H-6), 0.68 (3H, s, H-18), 1.00 (3H, s, H-19), 0.92 (3H, d, *J*= 6.5 Hz, H-21), 0.81 (3H, d, *J*= 7 Hz, H-26), 0.83 (3H, d, *J*= 7 Hz, H-27), 0.85 (3H, t, H-29). ^{13}C-NMR ($CDCl_3$, 125 MHz) δ ppm: 37.4 (C-1), 31.8 (C-2), 71.9 (C-3), 42.4 (C-4), 140.9 (C-5), 121.9 (C-6), 32.1 (C-7), 32.0 (C-8), 50.3 (C-9), 36.6 (C-10), 21.2 (C-11), 39.9 (C-12), 42.5 (C-13), 56.9 (C-14), 24.5 (C-15), 28.4 (C-16), 56.2 (C-17), 12.0 (C-18), 19.6 (C-19), 36.3 (C-20), 18.9 (C-21), 34.1 (C-22), 26.2 (C-23), 46.0 (C-24), 29.3 (C-25), 19.2 (C-26), 20.0 (C-27), 23.2 (C-28), 12.1 (C-29). The ^{1}H and ^{13}C NMR data were identical with published structure (Rahimifard *et al.*, 2018) .

RESULTS AND DISCUSSION

Prior to the isolation of anti-hypercholesterolemic compound from rice bran extract, a preliminary study was made on solvent extraction using reflux method at ambient pressure. The comparison of the extraction yield and total oryzanol content from each solvent was presented in Table 1. The results showed that all solvent could extract oryzanol, in which its content ranged from 27.92 to 43.59 mg/100 g Rb. Hexane has been found to be the best solvent to extract oryzanol by reflux method than others. Therefore, hexane extract was continued for further separation.

Rb hexane extract was separated using column chromatography techniques and gave 6 combined factions (F1-F6) as described in Figure 1. Due to the number of F1 fraction is too small, then F1 does not carry out further investigation. F2 – F6 fraction was then continued to investigate their anti-hypercholesterolemic activity. Based on the TLC profiles, the oryzanol compound is mostly found in the F4 fraction with an estimated level of 0.90% (w/w) relative to its fraction.

Most of the previous research on hypercholesterolemic activity was conducted to preventive action, by designing the administration of induction substances and treatment on the same day until the specified day (Ausman *et al.*, 2005; Wilson *et al.*, 2007). In this study was designed to conduct curative action of Rb fraction on hypercholesterolemia condition, by forming the animal model first and then continued for the treatment. The animal model was formed by exogenous and endogenous induction simultaneously. Exogenous induction was done by cholesterol feeding, while the endogenous induction was done by administering of CA and PTU. Table 2 shows the difference in lipid profile between normal and induced group after 30 days of induction. The statistical calculation showed that the induced group has been increased five-fold on TC and six-fold on LDL-c level compared to the normal group ($p<0.1$), whereas there was no significant difference on TG and HDL-c level ($p>0.1$). These conditions were predicted due to the role of endogenous induction. CA administration will be lead the inhibition of cholesterol conversion into bile acid and increase the absorption of exogenous cholesterol (Einarsson and Grundy, 1980), in order that it can decrease cholesterol elimination and an increase in the TC level. Whereas for PTU administration will be expected to decrease the expression of LDL-c receptors by reducing the thyroid hormone synthesis (Liberopoulos and Elisaf, 2002; Rizos, 2011) so it makes the LDL-c levels will be increased.

The observation of TC remittent levels was performed after 4 days of treatment to evaluate anti-hypercholesterolemic activity from each fraction of the Rb hexane extract. Prior to observing the other groups results, the experimental method was validated by an observed standard cholesterol-lowering drug (simvastatin group). According to our experiment, it was exhibited the decrease in TC level compared to the control group. Therefore, the experimental method is acceptable to determine anti-hypercholesterolemic activity.

Based on the experiment in TC levels (Figure 2), F2 and F5 fractions exhibited statistically different to the control group ($p<0.1$), with the decreasing percentage of 38.72 $\pm$ 2.73 and 37.32 $\pm$ 4.02 % respectively. Hence, it can be deduced that F2 and F5 fractions are supposed to have anti-hypercholesterolemic activity. The simvastatin group as a standard drug with a 38.53 $\pm$ 6.78 % of decrease compared to the F2 and F5 fractions show no difference ($p>0.1$), so it can be concluded that F2 and F5 fractions have anti-hypercholesterolemic activity as well as simvastatin. In addition, all fractions of Rb extract do not affect to other lipid profiles directly, such as TG, HDL-c, and LDL-c.

Table 1: Extraction yield and total oryzanol content using different solvents by reflux method

Solvent	Dielectric constant (20 ^{0}C)	Yield (%) w/w	Total oryzanol content (mg/100g rice bran)
Hexane	1.90	9.25 ± 0.50	43.59 ± 3.36
Chloroform	4.80	9.71 ± 0.41	27.92 ± 4.45
Ethyl Acetate	6.02	10.57 ± 0.20	37.39 ± 0.18
Dichloromethane	9.10	8.54 ± 0.82	33.64 ± 4.53
Isopropanol	18.30	10.51 ± 0.64	33.91 ± 6.39
Acetone	20.60	9.91 ± 0.30	36.46 ± 0.55
Hexane-Ethyl Acetate (1:1)	3.96	8.78 ± 0.20	35.11 ± 2.27
Chloroform-Ethyl Acetate (1:1)	5.41	9.71 ± 0.23	32.96 ± 7.11
Hexane-Isopropanol (1:1)	10.10	9.65 ± 0.23	32.16 ± 6.70

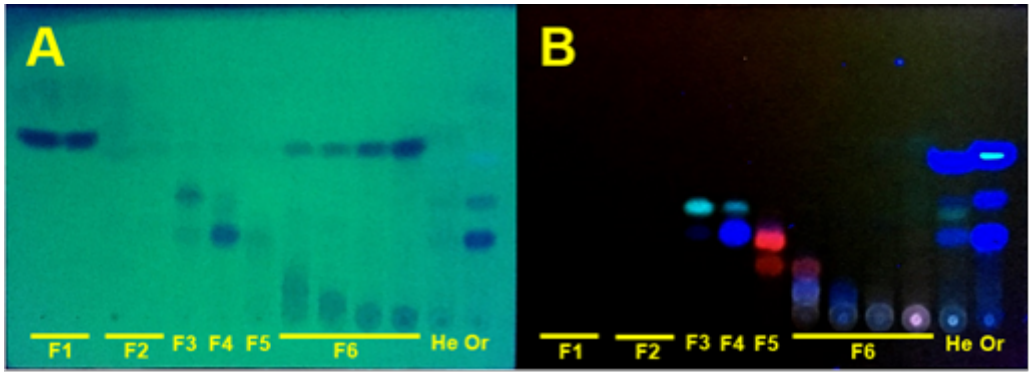

Figure 1: The TLC profiles of rice bran fraction under short and long-wave UV (A and B), stationary phase silica gel F_{254} and mobile phase hexane-ethyl acetate-formic acid (80:20:1,v/v). Fraction number (F1-F6), hexane extract (He), and standard oryzanol (Or).

Table 2: Lipid profiles: Percentage of increasing of the hypercholesterolemic rat model

Group	TC	TG	HDL-c ^{a}	LDL-c
Normal rats	31.96 ± 13.13	30.74 ± 42.05	8.34 ± 10.64	50.80 ± 28.49
Induced rats	162.76 ± 54.91*	25.95 ± 49.46	2.59 ± 24.05	309.93 ± 149.10*

The asterisks indicates significant differences to the normal group (p<0.1). ^{a} indicates the percentage of decreasing.

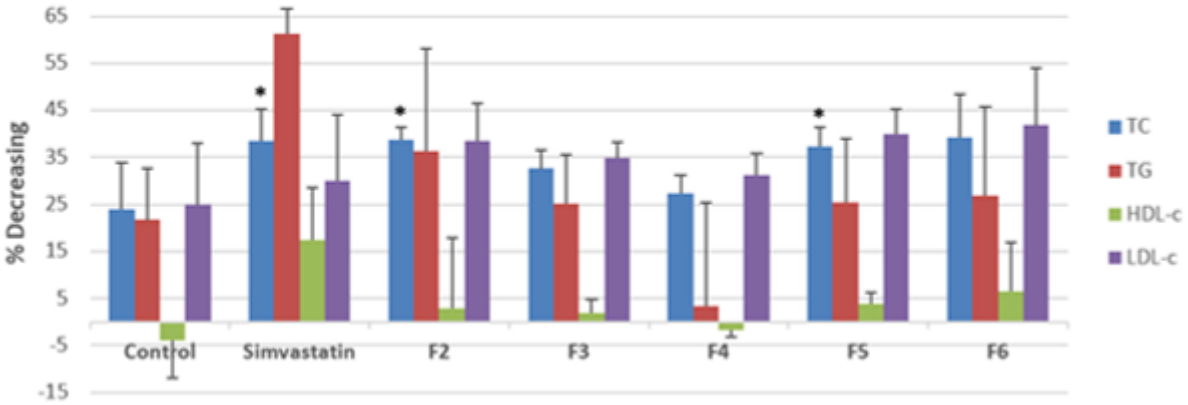

Figure 2: Lipid profiles after receiving rice bran extract fraction. The asterisk indicates the existence of significant differences compared to the control group (p<0.1).

A compound **1** has been successfully isolated from F5 fraction. Based on ^{1}H-NMR spectra exhibited the presence of an olefinic proton at δ 5.35, a carbon proton that it is attached to the oxygen at δ 3.52, six proton signals at δ 0.68, 0.81, 0.83, 0.85, 0.92, and 1.00 were assigned to the methyl groups. In addition, the other chemical shift in the region δ 0.10 to 2.30 as overlap signals were assigned to aliphatic groups. The ^{13}C-NMR spectra showed 29 signals of carbon. These carbon signals can be classified as methyl, methylene, methine or quaternary carbon by gHSQCAD spectra. The gHSQCAD spectra exhibited the presence of 6 methyl groups, 11 methylene groups, 9 methine groups. The absence correlation of 3 signals carbon (δ 36.6, 42.5, and 140.9) to proton spectra indicated the presence 3 quaternary carbon. In addition, the signals at δ 140.9 and 121.9 are recognized for olefinic carbon, and the signal at δ 71.9 is known for the carbon that is bonded to the oxygen. Figure 3 shows the relationship between ^{1}H and ^{13}C spectra that happened in long-range coupling. Based on the physical properties and spectroscopic data, compound **1** was suggested to be β-sitosterol.

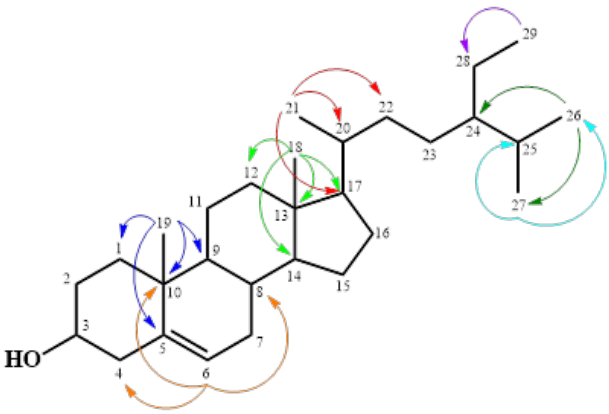

Figure 3: The gHMBCAD correlations of compound 1

β-sitosterol is one of phytosterol that found in the plant, in which, it has an almost similar structure and function to human's cholesterol. Numerous studies have been reported that β-sitosterol administration has the potential to reduce cholesterol levels (Sugano *et al.*, 1977; Lei *et al.*, 2017; Suganya *et al.*, 2017). Its cholesterol-reduction mechanism has several theories, but the most widely proposed was to reduce dietary cholesterol absorption and interfere with intestinal transport (Dumolt and Rideout, 2018). Furthermore, the reduction of dietary cholesterol absorption is possible due to the cholesterol displacement by β-sitosterol in bile acid micellar formation (Jesch and Carr, 2006), thereby limiting cholesterol uptake in the small intestine and increasing fecal excretion. The other suggested mechanisms are related to interfering with protein expression that plays a role in intestinal transport, one of which has been proved is the inhibition of Niemann-Pick C1-like 1 (NPC1L1) expression, an intestinal cholesterol transmembrane protein that can take cholesterol from the intestines into the enterocytes (Jesch *et al.*, 2009).

CONCLUSIONS

Hexane was the best solvent to extract oryzanol using reflux method. The presence of phytosterol (β-sitosterol) compound in rice bran as depicted from our result, was suspected of being responsible for reduced serum total cholesterol.

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