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Inhibition Activity of HMG-CoA Reductase by Rice Brain Extract and Its Fractions as Anticholesterolemia *In vitro* Study

Qomariah Hasanah¹, Hasim^{1*}, Didah Nur Faridah², Dimas Andrianto¹

¹Department of Biochemistry, Bogor Agricultural University, Indonesia

²Department of Food Science and Technology, Bogor Agricultural University, Indonesia

ABSTRACT

HMG CoA reductase inhibition effect of rice bran extract and its fractions were examined. Successive extraction was done by using ethanol, followed by successive fractionation using *n*-hexane, dichloromethane, ethyl acetate, and water for fractions with highest activity. Anti HMG CoA reductase activity of the extract and its fractions were determined spectrophotometrically by NADPH oxidation, using HMG-CoA as substrate. Rice bran extract and its fractions showed more than 50% inhibitory effect on enzyme activity. The extract of rice bran showed 51.44% inhibitory effect on HMG CoA reductase activity. The water fraction from ethanol extract gave the strongest activity (64.54% inhibition). Kinetic study of the enzyme was performed on one concentrations of water fraction (0.05 mg mL⁻¹). These active fractions showed uncompetitive inhibition in Lineweaver-Burk plot analysis. Under standard condition, Km value for non-inhibitor was Km 331.45 mM and V_{max} value was 1.3 mM min⁻¹, while with inhibitor Km value was 56.57 mM and V_{max} 0.3 mM min⁻¹.

Keywords: HMG CoA reductase, Anticholesterolemia, Fractionation, Rice branx

INTRODUCTION

Coronary Heart Disease (CHD) is caused by atherosclerosis which is the thickening of blood vessel wall supplying the heart by cholesterol and triglycerides. According to the WHO in 2015, a mortality rate caused by the CHD worldwide reached 17.5 million people [1]. In Indonesia, the estimated number of CHD patients reached 2.6 million in 2013 (Center for Data and Information of Ministry of Health 2014). Death caused by CHD and stroke is expected to continue rising to 25 million deaths in 2030 [2].

HMG CoA reductase is an key enzyme that catalyzes the reduction of HMG-CoA to mevalonate coenzyme A [3]. Inhibition of HMG-CoA reductase enzyme was reported to lower intracellularly cholesterol levels. Some synthetic drugs such as; mevionolin, simvastin, pravastatin are recognized as inhibitor of the endogenous biosynthesis [4]. However, these synthetic drugs have side negative effects, such as nausea and increased blood pressure.

Some studies that used extracts of natural products as inhibitors of HMG-CoA reductase enzyme have been reported by Gholamhuseinian et al. [5] used sap extract of Majakani (*Quercus infectoria*) (84%), rose extract (*Rosa damascena*) (70%), and leaf extract of Murad (*Myrtus lommunis*) (62%), Andrianto et al. [6] used the extract of Menteng fruit peel (*Baccaurea racemosa*) (70.37%), binjai fruit peel (*Mangifera caesia* Jack) (13.84%), canistel fruit peel (*Pouteria campechiana*) (15.05%), fruit peel of santol (*Sandoricum koetjape*) (12.94%) and fruit peel of jambolan (*Syzygium cumini*) (52.29%).

Rice bran is abundant natural product derived from rice milling process. The ethanol extract of rice bran has an active component, such as; vitamin E (α , β , γ , δ -tocotrianol), tocoferol vitamin B complex as bioactive materials of anti-cholesterol [7] and as antioxidant [8,9]. Tocotrianol of rice bran also succeed in reducing 20% of total cholesterol of human blood by inhibiting the activity of HMG-CoA reductase hepatic enzymes via post-transcriptional mechanism [10].

Based on previous studies, rice bran extract has a potential as HMG-CoA reductase enzyme inhibitor agent. The focus of this study

is to evaluate the ability of rice bran extract and its fractions as anti-cholesterol agent via HMG-CoA reductase enzyme inhibition in *in vitro* setting. This natural material can be an alternative treatment for hypercholesterolemia in the future and it may have other strategic functions in the field of food and medicine.

MATERIAL AND METHODS

MATERIAL

The materials used in this study were the the rice bran of *pandan wangi* derived from a rice mill in the Situ Gede Bogor, ethanol 95%, n-hexane, dichloromethane, ethyl acetate, distilled water, HMG-CoA reductase assay kit (Sigma-Aldrich). The tools used were oven, analytical scale, Erlenmeyer, micropipette, rotary shaker, separating funnel, test tubes, volumetric flask, water bath, autoclave, rotary evaporator, microcuvette, UV-VIS spectrophotometer.

METHODS

Preparation of rice bran

A 40 mesh sample of Rice Bran was heated in an oven at 150°C for 10 min and then cooled at room temperature for 30 min [11].

Extraction of rice bran

A total of 5 g of stabilized rice bran sample were extracted with 20 ml of ethanol 95% by shaker at room temperature for 3 h [12]. The extract solution was then filtered with filter paper, while the residues were extracted back twice. Extracts were then dried with a rotary evaporator at a temperature of 50°C and then weighed.

Fractionation of ethanol extract of rice bran

The ethanol extract of red yeast rice and its bran were subsequently fractionated by liquid-liquid extraction using solvents with increasing polarity, namely n-hexane, dichloromethane, ethyl acetate and water [6]. The layers formed through fractionation were concentrated with rotary evaporator.

Phytochemical screening

Phytochemical screening were carried out on the ethanol extract of rice bran, including qualitative analysis of tannins, alkaloids, flavonoids, saponins, steroids and triterpenoids.

HMG CoA reductase assay

Anti-cholesterol activity of extracts and fractions were determined from the inhibitory activity of the HMG-CoA reductase enzyme. Samples were prepared with a concentration of 10 mg/ml in 5% DMSO solution. Pravastatin as a positive control was prepared in a solution of 50 mg/ml [6]. Three microliter of samples were added to the reaction mixture including 100 mm of potassium phosphate buffer (400 mm of KCl, 100 mg/ml of bovine serum albumin and 3.5 mm of EDTA), NADPH (250 μ m) and HMG-CoA (250 μ m) (final volume of 557 ml). Reactions were started by the addition of 3 ml of HMG-CoA reductase inhibitors (50 μ g/ml). The reaction mixture was shaken for 10 s and the absorbance was calculated every 15 sec to 6 mins. The enzyme activity was calculated by the equation:

$$Unit / mgP = \frac{\left(\frac{\Delta A_{340}}{\min_{sample}} - \frac{\Delta A_{340}}{\min_{blank}} \right)}{12.44 \times V \times 0.6 \times LP}$$

$$Inhibition(\%) = \frac{(Activity_{enzyme} - Activity_{sample})}{Activity_{enzyme}} \times 100\%$$

Kinetics analysis of HMG-CoA reductase enzyme

Study of HMG-CoA reductase enzyme kinetics was carried out on the water fraction of rice bran which was the fraction of the most active in inhibiting the enzyme HMG-CoA reductase. HMG-CoA reductase inhibition kinetics was studied with two reaction systems, the reaction system with and without inhibitor. Activity was determined by varying the substrate of HMG-CoA (0.15, 0.3, 0.45 and 0.6 mmol L⁻¹) and using a variation of the concentration of the sample (0.05 mg ml⁻¹). Model inhibition was determined by Lineweaver-Burk plot curve analysis then the Km and V_{max} were determined.

RESULTS AND DISCUSSION

Extraction and fractionation of samples

Maceration process using 95% ethanol was selected because several reference journals used ethanol. The extraction process was conducted with two repetitions to obtain high yield and to properly gain the potential of extracted active compounds. Afterwards, the extract was evaporated to rid of solvent. The yield of ethanol extract of rice bran was 4.94%. Similar results are reported by Widarta *et al.* [13], who produced 4.61% of yield from white rice bran that was extracted with ethanol. The

extract was then fractionated using solvents with increasing polarity. The different polarities were to allow the chemical compounds being extracted based on their polarities. The yield of the fractionation was 1.91% of n-hexane fraction, 1.47% of dichloromethane fraction, ethyl acetate fraction 0.73% and 1.45% of water fraction. Fractionation showed that yield of the water fraction > fraction of n-hexane > ethyl acetate fraction > fraction of dichloromethane. It indicates that chemical compounds are most abundant in the polar water fraction.

Phytochemical compounds

The water fraction of rice bran contains of tannin, flavonoid, alkaloid and triterpenoid. The results of phytochemical tests of water fraction of rice bran can be seen in Table 1.

Table 1: Phytochemical compounds of water fraction of rice bran

Chemical compound	Result	
	Rice bran	Positive control
Tannin	+	<i>Camellia sinensis</i>
Saponin	-	<i>Sapindus rarak</i>
Flavonoid	+	<i>Piper ornatum</i>
Alkaloid	+	<i>Catharanthus roseus</i>
Steroid	-	<i>Talinum paniculatum</i>
Triterpenoid	+	<i>Talinum paniculatum</i>

(+): Contain compounds; (-): Does not contain compound

Research conducted Widarta *et al.* [13], the rice bran contains of flavonoid, triterpenoid, alkaloid and saponin [14]. Research conducted Mannan *et al.* [15], found a positive bran extract contains of alkaloid, flavonoid, terpenoids and tannin.

Inhibitory activity of samples toward HMG CoA reductase

Inhibition test results showed that all samples and pravastatin as positive control had inhibition capacity on HMG-CoA reductase enzyme. The smaller the average value of the activity, the greater the inhibition capacity (Table 2).

Table 2: Inhibition activity of the HMG-CoA reductase of rice bran extracts and fractions

Sample and Control	Activity (Unit/mg)	HMG CoA Reductase inhibitory Activity (%) ^a
Pravastatin	0.23 ± 0.01	69.12 ± 1.86
Ethanol extract	0.36 ± 0.08	51.44 ± 10.78
N-hexane fraction	0.36 ± 0.01	50.42 ± 1.27
Dichloromethane fraction	0.30 ± 0.02	59.50 ± 3.41
Ethyl acetate fraction	0.33 ± 0.00	55.50 ± 0.61
Water fraction	0.25 ± 0.05	65.46 ± 6.69

^avalue shows ± standard of deviation from three replications from each sample with a concentration of 50 µg L⁻¹

The fraction of water had the highest inhibition capacity by 65.46% with the activity of 0.25 units mg⁻¹, while lowest inhibitory capacity was fraction of n-hexane with the value of the activity of 0.36 units mg⁻¹. These values indicate that the compounds that have the ability to inhibit the enzyme HMG-CoA reductase are more polar. Differences in inhibition capacity of the sample are caused by differences in the content of secondary metabolites derived from the extraction and fraction with different solvents. Differences in inhibition capacity are also indicated in a study conducted by Andrianto *et al.* [6], using *Phyllanthus acidus* sample, which fraction of ethyl has the highest inhibition capacity on HMG-CoA reductase enzyme (87.30%) while the lowest is a fraction of dichloromethane (53.98%).

According to Juliano, cited by Kim *et al.* [16], the rice bran is rich in lipids, protein, vitamin B, and dietary fiber. Other studies on rice bran extract show that rice bran has a gamma-oryzanol bioactive compounds, vitamin E (tocopherol and tocotrienol), Vitamin B and free fatty acids [7,17]. Compound that has the potential to inhibit the enzyme HMG-CoA reductase. Tocol not only acts as antioxidants but also as inhibitors compound of HMG-CoA reductase enzyme that is similar to statins work.

According to Roy and Lundry [18] rice bran has the potential to lower blood cholesterol levels because it mainly contains fiber and components that are not saponified. Components that are not saponified included oryzanol, phytosterol compounds campesterol and β-sitosterol. In a study conducted by Qureshi *et al.* [10], who were able to isolate the pure compound gamma-oryzanol and tocopherol of rice bran using chromatographic techniques, show an increasing activity of 20% hypercholesterolemia in humans by inhibiting the activity of HMG-CoA reductase hepatic enzymes via post-transcriptional mechanisms. Bioactive compounds in rice bran are able inhibiting HMG-CoA reductase, a key enzyme in the synthesis of endogenous cholesterol, through two acts of post-transcriptional, increasing degradation by controlling protein reductase and lowering the efficiency of HMG-CoA-reductase messenger of RNA translation.

Kinetics of HMG CoA reductase inhibition by the most active sample

Kinetics of HMG-CoA reductase obtained from Lineweaver-Burk equation were two reaction systems: the enzyme-substrate reaction with and without the inhibitor. Inhibitor used was rice bran water fraction which had the highest inhibition (Figure 1).

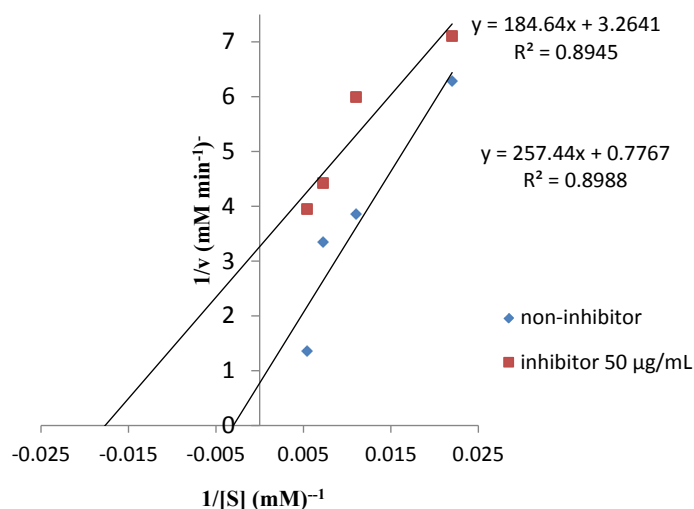


Figure 1: Lineweaver-Burk plot in enzyme kinetics test

The analysis of the kinetics was studied by varying HMG CoA substrate. Base on Lineweaver-Burk, system without inhibitor expresses the equation of $y=257.44 \times +0.7767$ with V_{\max} values of 1.3 mM min^{-1} and K_m of 331.45 mM while the system with inhibitor expresses the equation of $y=184.64 \times +3.2641$ with V_{\max} values of 0.3 mM min^{-1} and K_m of 56.57 mM .

V_m and K_m values decreased. It indicates that the inhibitors are non-competitive. The presence of inhibitors decreased the value of V_m . Inhibitors can disrupt the conformation of enzymes which caused suboptimal binding of enzymes and substrates in forming products. In addition, K_m that indicates the binding affinity of enzyme-substrate also decreased. Decreasing K_m shows great binding affinity of enzyme to substrate, where to achieve maximum speed a small substrate concentration is required.

According to Roy and Lundry [18], oryzanol potentially reduces cholesterol by acting as a competitive inhibitor of the absorption and synthesis. Kinetics test of the HMG CoA reductase enzyme is also conducted by Gholamhuseinian et al. [5] Who used three samples that were sap extract of *Quercus infectoria*, flower extract of *Rosa damascena* and leaf extract of *Myrtus lommunis* that showed the mechanism as a non-competitive inhibitor of HMG-CoA reductase enzyme? The different types of mechanism of this inhibition are caused by the type of solvent used and the purity of extracts [19].

CONCLUSION

The best inhibitory activity toward HMG CoA reductase was demonstrated by water fraction of rice bran, giving inhibitory activity value of 65.46%. The kinetics of inhibition of red yeast rice toward HMG CoA reductase showed an uncompetitive inhibition. Rice bran has a great potential as an alternative for anticholesterolemia.

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