Research Article **AJAR (2021) 6:107**

American Journal of Agricultural Research (ISSN:2475-2002)

In-vitro Antioxidant Capacities and Genetic Classification of Indonesian Selected Pigmented Rice

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ABSTRACT

Rice is a world-famous cereal food divided into pigmented and non-pigmented rice. Pigmented rice is popular as healthier food than non-pigmented rice due to its potency as an antioxidant. Nevertheless, the potential of pigmented rice has not been widely studied. Indonesian selected pigmented rice protein's antioxidant potential and the non-protein compound were in-vitro studied. The antioxidant potencies were evaluated by extracting fresh seeds of nine pigmented rice (Aek Sibundong, Beureum Taleus, Gogo Niti-2, Lamongan-1, Merah SP, Merah Wangi, Mota, Ketan Hitam-2, and Super Manggis) and non-pigmented rice (IR-64) as control. Various free radical scavenging methods to determine the antioxidant activity (ABTS•+, DPPH•, OH• and O2-) were conducted. Meanwhile, the genetic classification was performed by a simple sequence repeat (SSR) marker to determine the relationship between varieties. The results showed that protein of Ketan Hitam-2 had the highest ABTS•+ radical scavenging (98.06%), followed by Beureum Taleus (42.54%). Ketan Hitam-2 protein also showed the highest OH• and O2- activities (43.49% and 6.02%, respectively). The highest DPPH• potency of the non-protein compounds also shown by Ketan Hitam-2 (32.23%) with the activity of OH• and O2- (20.63% and 14.56%, respectively). These results showed that Ketan Hitam-2 has the highest potency as an antioxidant, which could be recommended as a nutraceuticals compound.

Keywords: Antioxidant; Pigmented rice; SSR marker

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How to cite this article:
Erlin Susilowati, Addieni Zulfa Karimah, Sandy Pradipta, Bella Rhea Lavifa Sanjaya, Mohammad Ubaidillah, Tri Agus Siswoyo. *In-vitro* Antioxidant Capacities and Genetic Classification of Indonesian Selected Pigmented Rice. American Journal of Agricultural Research, 2021; 6:107.

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Website: https://escipub.com/

Introduction

Rice is a world-famous cereal food full of nutrients such as carbohydrates, protein, vitamins, and various phytochemicals [1]. Based on the pigment content, rice is divided into pigmented and non-pigmented rice. Pigmented rice mainly consists of various grain colours, namely brown, black, and purple [2]. Indonesia has several regions that grow various landraces of pigmented rice. Based on Indonesian Center for Rice Research, there are more than 2000 accessions of local rice, several of which are pigmented rice. Recently, pigmented rice has become more popular than non-pigmented rice due to its potential for healthy foods. Previous studies reported that pigmented rice has higher protein content than non-pigmented rice [3]. Moreover, its antioxidant activities and phytochemical contents are higher than non-pigmented rice [1]. The higher abundance of proteins in non-pigmented rice has excellent benefits as an antioxidant due to its capability to inhibit lipid oxidation. Furthermore, proteins have been confirmed to inactivate reactive oxygen species and scavenging free radicals [4].

Pigmented rice has been reported to have higher phytochemical content. One of the phytochemical contents found in pigmented rice is a phenolic compound. This compound has been confirmed for its role in regulating anti-inflammatory, immune system, antibacterial, and antioxidant [5]. Hydroxyl groups in the aromatic rings are responsible for these compounds antioxidant activity. Usually, the higher the phenolic compounds, the greater the antioxidant activity produced [6]. Although various researches have mentioned about the great benefits of pigmented rice, however, genetic diversity classification of pigmented rice has not been carried out extensively. Therefore, the study of this particular issue needs to be addressed.

Morphological characteristics and molecular techniques of pigmented rice can provide useful information for rice improvement. Morphological characterization can be performed based on the colour, shape, and size of the grains [7]. Molecular techniques could be performed by simple sequence repeats (SSR) markers. SSR marker is a tandem repeat of a short DNA sequence that is useful for the genetic mapping of the specified loci chromosome. Over the past 20 years, SSR has been the most frequently used markers for genotyping plants. Its capabi- lities to obtain highly informative, multi-allele can be experimentally replicated, codominant, and transferred between related species [8]. Research on the *in-vitro* antioxidant activity and genetic classification of Indonesian selected pigmented rice is essential to obtain potency information of pigmented rice as nutraceutical sources and the relationship between varieties.

Materials and methods

Plants collection and reagent

The nine varieties of pigmented rice seeds (Aek Sibundong, Beureum Taleus, Gogo Niti-2, Lamongan-1, Merah SP, Merah Wangi, Mota, Ketan Hitam-2, and Super Manggis) and non-pigmented rice seeds (IR-64) as control were cultivated with conventional plantation measure and their seeds were harvested on January 2020 at the Center of Excellence on Crop Industrial Biotechnology (PUI-PT BioTIn) in Agrotechno Park research area, University of Jember, Jember, East Java, Indonesia. The reagents used in this study, 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{\bullet+}$), 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$), 2-deoxy-D-ribose, pyrogallol, trichloroacetic acid (TCA), thiobarbituric acid (TBA), ethylenediaminete- traacetic acid (EDTA), were purchased from Sigma-Aldrich, Singapore and other supporting chemicals were analytical grade product from Merck Co., USA.

Sample preparation

Preparation of sample was carried out by extracting each variety of rice by grounding and homogenizing the fresh seed (1 g) with two solvents, phosphate buffer (10 mL, 50 mM, pH 6.8) and absolute methanol (10 mL) on a magnetic stirrer for 2 h. Phosphate buffer was used to obtain an aqueous extract with a large quantity of dissolved protein, while absolute methanol was used to extract a wide range of phenolic compounds. The mixture was cen-

trifuged with a speed of 10.000 rpm for 15 min. The supernatant was then analyzed its total protein content using the Bradford method [9] and the total phenolic method [10].

The activity of ABTS$^{\bullet+}$ Radical Scavenging

The free radical scavenging activity of the dissolved protein sample was evaluated through the ABTS$^{\bullet+}$ radical scavenging activity, using the analytical protocol described by Lalhminghlui and Jagetia [11] with slight modification. The radical cations were prepared by mixing ABTS stock solution (1 mL, 7 mM) and potassium persulfate (1 mL, 2.45 mM) followed by incubation in a dark place at room temperature about 15 h. The ABTS solution was diluted with phosphate saline buffer (0.2 M, pH 7) to produce the absorbance of 0.700-0.750 at 734 nm. The photometric assay was conducted on ABTS solution (950 µL) and sample (20 µg), followed by incubation at room temperature for 4 min. The absorbance was measured using a spectro- photometer at 734 nm. The antioxidant activity of the tested samples was calculated by using the following formula: (%) ABTS$^{\bullet+}$: $[(A_1-A_2)/A_1]$ x 100%, where is A_1 = absorbance blank, and A_2 = absorbance sample.

The activity of DPPH$^{\bullet}$ Radical Scavenging

The free radical scavenging capacity of phenolic samples was measured with DPPH radical scavenging activity assay [12]. The radical scavenging was performed by mixing the sample (20 µg) with methanolic DPPH$^{\bullet}$ solution (1mL, 2 mM). The mixture was then incubated at room temperature for 1 h, followed by measuring the absorbance at 517 nm. The antioxidant activity of the tested samples was calculated by using the following formula: (%) DPPH$^{\bullet}$: $[(A_1-A_2)/A_1]$ x 100%, where is A_1 = absorbance blank, and A_2 = absorbance sample.

The activity of Hydroxyl (OH$^{\bullet}$) Radical Scavenging

Hydroxyl radical scavenging activity was analyzed using the method described by Pavithra and Vadivukkarasi [13], with slight modification. Hydroxyl radical was generated by mixing 2-deoxyribose (28 mM, 50 µL), $FeCl_3$ (10 µL, 10 mM), ascorbic acid (100 µL, 1 mM), ethylenediaminetetraacetic acid (100 µL, 1 mM), hydrogen peroxide (10 µL, 1 mM) and sample (20 µg). The mixture was then diluted with phosphate buffer (pH 7.4) until the final volume of 1 mL and was incubated for 1 h at 37°C. Into the mixture, thiobarbituric acid (500 µL, 1%) and tri-chloric acetate (500 µL, 2,8%) were added. The mixture was incubated at 80°C for 30 min, and its absorbance was measured at 532 nm. Percentage inhibition was evaluated by com- paring the absorbance of sample and blank solution sample, by using the following formula: (%) OH$^{\bullet}$: $[(A_1-A_2)/A_1]$ x 100%, where is A_1 = absorbance blank, and A_2 = absorbance sample.

The activity of Superoxide (O_2^{-}) Radical Scavenging

Superoxide radical scavenging was analyzed using the superoxide method [14]. Superoxide radical was performed by mixing the sample (20 µg) with Tris-HCl (1.7 mL, 50 mM, pH 8.2). The mixture was then incubated at the temperature room for 10 min, followed by the addition of pyrogallol (100 µL, 10 mM) in HCl (10 mM) and measured the slope for 4 min at320 nm. Percentage inhibition was evaluated by com- paring the slope of sample and blank solution sample, by using the following formula: (%) O_2^{-}: $[(S_1-S_2)/S_1]$ x 100%, where is S_1 = slope blank, and S_2 = slope sample.

Genetic Diversity Analysis

Genetic diversity was analysed by isolating the genomic DNA from each rice's leaf tissues by the method [15]. The DNA was amplified by Polymerase Chain Reaction (PCR) method[16]. A total of 22 Simple Sequence Repeat (SSR) markers distributing 12 chromosomes were used (**Table 1**). The PCR products were run at 1.5 % gel electrophoresis and visualized by gel documentary. The DNA bands were used for scoring to construct the phylogeny tree by using the NTSYPC program. Jaccard's coefficient was used to determine the relationship between varieties.

Table 1. Primer list of simple sequence repeat (SSR) marker

Marker	Repeat motif	Primer sequence (5'-3')	Chromo-some	Al-leles	AT (˚C)
RM259	(CT)17	F TGGAGTTTGAGAGGAGGG R CTTGTTGCATGGTGCCATGT	1	9	51.35 51.35
RM431	(AG)16	F TCCTGCGAACTGAAGAGTTG R AGAGCAAAACCCTGGTTCAC	1	11	52.10 52.10
RM452	(GTC)9	F CTGATCGAGAGCGTTAAGGG R GGGATCAAACCACGTTTCTG	2	15	51.50 51.50
RM154	(GA)21	F ACCCTCTCCGCCTCGCCTCCTC R CTCCTCCTCCTGCGAC-CGCTCC	2	13	63.10 63.10
RM55	(GA)17	F CCGTCGCCGTAGTAGAGAAG R TCCCGGTTATTTTAAGGCG	3	9	51.40 51.40
RM489	(ATA)8	F ACTTGAGACGATCGGACACC R TCACCCATGGATGTTGTCAG	3	8	52.50 52.50
RM124	(TC)10	F ATCGTCTGCGTT-GCGGCTGCTG R CATGGATCAC-CGAGCTCCCCCC	4	7	61.65 61.65
RM307	(AT)14 (GT)21	F GTACTACCGACCTACCGTTCAC R CTGCTATGCATGAACTGCTC	4	10	52.45 52.45
RM161	(AG)20	F TGCAGATGAGAA-GCGGCGCCTC R TGTGTCATCAGAC-GGCGCTCCG	5	9	61.45 61.45
RM334	(CTT)20	F GTTCAGTGTTCAGTGCCACC R GACTTTGATCTTTGGTGGACG	5	5	52.30 52.30
RM162	(AC)20	F GCCAGCAAAAC-CAGGGATCCGG R CAAGGTCTTGTGCGGCTT-GCGG	6	9	60.75 60.75
RM118	(GA)8	F CCAATCGGAGCCACCGGA-GAGC R CACATCCTCCAGCGAC-GCCGAG	7	14	61.50 61.50
RM11	(GA)17	F TCTCCTCTTCCCCCGATC R ATAGCGGGCGAGGCTTAG	7	2	52.00 52.00
RM284	(GA)8	F ATCTCTGATACTCCATCCATCC R CCTGTACGTTGATCCGAAGC	8	4	51.55 51.55
RM404	(GA)33	F CCAATCATTAACCCCTGAGC R GCCTTCATGCTTCAGAAGAC	8	7	50.55 50.55
RM408	(CT)13	F CAACGAGCTAACTTCCGTCC R ACTGCTACTTGGG-TAGCTGACC	8	12	54.20 54.20
RM215	(CT)16	F CAAAATGGAGCAGCAAGAGC R TGACGACCTCCTTCTCTGTAG	9	13	52.35 52.35
RM171	(GATG)5	F AACGCGAGGACACGTACTTAC R ACGAGATACGTACGCCTTTG	10	12	53.20 53.20
RM536	(CT)16	F TCTCTCCTCTTGTTTGGCTC R ACACACCAACACGACCACAC	11	3	53.00 53.00
RM552	(TAT)13	F CGCAGTTGTGGATTTCAGTG	11	11	52.25

		R TGCTCAACGTTTGACTGTCC			52.25
RM277	(GA)11	F CGGTCAAATCATCACCTGAC	12	7	50.45
		R CAAGGCTTGCAAGGGAAG			50.45
RM19	(ATC)10	F CAAAAACAGAGCAGATGAC	12	13	48.70
		R CTCAAGATGGACGCCAAGA			48.70

*Chr: chromosome, AT: annealing temperature, F: forward primer, R: revers primer

Statistical Analysis

Values were expressed as means ±standard deviations (SD) of three replications. Data were analyzed statistically using analysis of variance (ANOVA). The significant difference between means was determined by Duncan's multiple range test (DMRT) (p<0.05). The SPSS®20.0 Package (IBM Cooperation, Chicago, USA) was used for statistical analysis.

Results

Free radical scavenging activity of pigmented rice seed protein

ABTS•+, DPPH•, OH•, and O_2^- free radical scavenging activity assays were analyzed to determine each rice seed protein's antioxidant potential. The results showed that Ketan Hitam-2 has the highest capability to inhibit radical antioxidants, followed by Beureum Taleus, in which $ABTS^{\bullet+}$ radical inhibition by Ketan Hitam-2 (98.06%), Beureum Taleus (42.54%), while the non-pigmented rice (21.90%). Ketan Hitam-2 protein also obtained the highest inhibition activity towards hydroxyl and superoxide. Hydroxyl inhibition by Ketan Hitam-2 (43.49%), Beureum Taleus (41.45%), and the non-pigmented rice (8.74%). Meanwhile, the inhibi- tion of superoxide radical by Ketan Hitam-2 (6.02%), Beureum Taleus (5.67%), while the non-pigmented rice (1.02%) (**Table 2**). These results indicated that the protein of Ketan Hitam-2 has the highest ability as an antioxidant agent.

Table 2: Free radical scavenging activity of pigmented rice seeds protein

Variety	$ABTS^{\bullet+}$ (%)	Hydroxyl (%)	Superoxide (%)
Aek Sibundong	25.62±0.21^{c}	40.27±0.60^{f}	3.91±0.37^{c}
Beureum Taleus	42.54±0.28^{h}	41.45±0.37^{f}	5.67±0.08^{c}
Gogo Niti-2	30.54±0.21^{e}	22.43±0.28^{d}	2.81±0.88^{b}
Lamongan-1	24.89±0.14^{b}	23.05±0.37^{d}	1.09±0.54^{a}
Merah SP	28.90±0.21^{d}	6.51±0.56^{a}	5.12±1.36^{c}
Merah Wangi	34.89±0.27^{g}	24.47±0.47^{e}	2.51±0.84^{b}
Mota	31.47±0.20^{f}	19.45±0.28^{c}	2.91±0.55^{b}
Ketan Hitam-2	98.06±1.25^{i}	43.49±0.85^{g}	6.02±0.56^{c}
Super Manggis	31.59±0.37^{f}	40.46±0.84^{f}	4.11±0.88^{c}
IR 64	21.90±0.28^{a}	8.74±0.32^{b}	1.02±0.75^{a}

*The protein concentration of sample (20µg/mL). All the values are expressed as the means±standard deviations (SD) of three replicates, and followed by Duncan's multiple range tests at p<0.05. The different letters in the column are expressed the data was significantly different.

Free Radical Scavenging Activity of Non-Protein Compounds of Pigmented Rice Seeds

The antioxidant activity of non-protein compunds is determined by reducing DPPH, hydroxyl, and superoxide radical methods. Ketan Hitam-2 has the highest total antioxidant activity of DPPH (32.23%), followed by Gogo Niti-2 (30.78%), Aek Sibundong (19.72%), and Mota (19.48%), while the non-pigmented rice as control (IR 64) has the lowest antioxidant activity (9.25%). In addition, the non-protein compounds of Ketan-Hitam-2 were also found to have the highest ability to scavenge the hydroxyl and superoxide (20.63%

and 14.56%, respectively), while the ability of non-pigmented rice to reduce hydroxyl and super- oxide radicals are (2.33% and 1.09%, respectively) (**Table 3**). These results showed that the non-protein compounds of Ketan Hitam-2 also have the highest ability as an antioxidant agent.

Table 3: Free radical scavenging activity of non-protein compound of pigmented rice seeds

Variety	DPPH (%)	Hydroxyl (%)	Superoxide (%)
Aek Sibundong	19.72±0.76^{d}	15.76±0.70^{d}	5.87±0.21^{b}
Beureum Taleus	14.86±0.43^{b}	12.31±0.28^{c}	6.65±0.58^{b}
Gogo Niti-2	30.78±0.12^{f}	21.85±0.48^{f}	11.43±1.12^{c}
Lamongan-1	15.68±0.38^{c}	10.53±0.77^{b}	6.43±0.14^{b}
Merah SP	22.30±0.54^{e}	16.91±0.84^{d}	9.83±0.48^{c}
Merah Wangi	19.11±1.11^{d}	13.07±0.32^{c}	6.38±0.53^{b}
Mota	19.48±0.87^{d}	12.92±0.33^{c}	7.37±0.82^{b}
Ketan Hitam-2	32.23±0.52^{g}	20.63±0.03^{e}	14.56±0.98^{d}
Super Manggis	16.16±0.32^{c}	12.95±0.87^{c}	5.21±0.89^{b}
IR 64	9.25±0.56^{a}	2.33±0.29^{a}	1.09±0.28^{a}

*The phenolic content of sample (20µg/mL). All the values are expressed as the means±standard deviations (SD) of three replicates, and followed by Duncan's multiple range tests at p<0.05. The different letters in the column are expressed the data was significantly different.

Cluster Analysis of pigmented rice seeds using SSR Markers

The morphological variation of pigmented rice seeds could be distinguished by its shape and colour in which the rice seeds of each variety were hulled to determine the shape and colour. The results showed that rice seed of each variety has various shapes and colours. Visually, the shape could be determined as slender, medium, and round. As for colour, it is varied from brown to black (**Figure 1**).

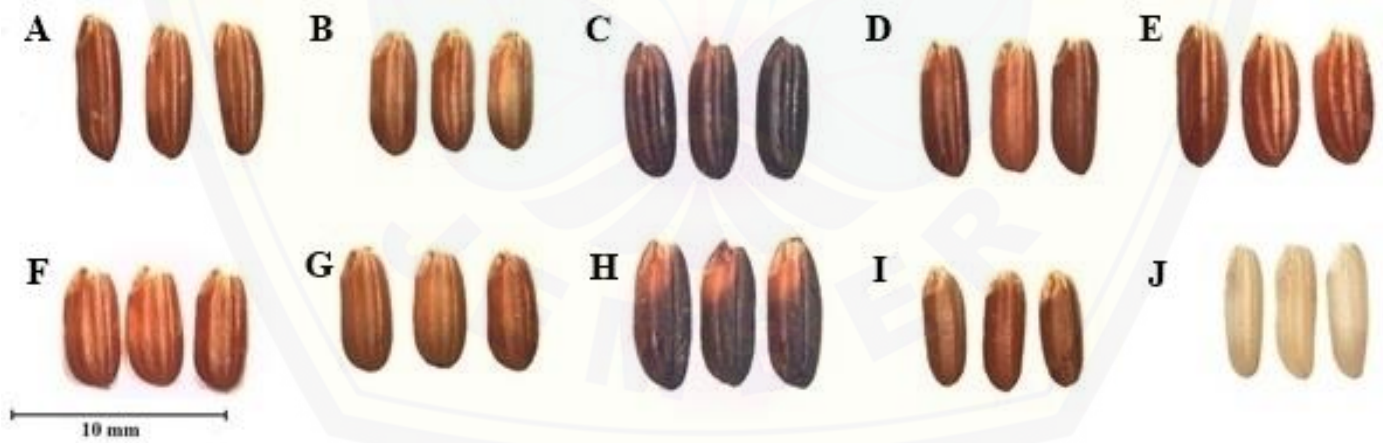

Figure 1: Variation shapes and color morphology of rice seed.
Milled grain of rice was captured by a stereo microscope (Leica EZ4HD) at 100x magnifications.
A: Aek Sibundong, B: Beureum Taleus, C: Gogo Niti-2, D: Lamongan-1, E: Merah SP,
F: Merah Wangi, G: Mota, H: Ketan Hitam-2, I: Super Manggis, J: IR-64.

The genetic diversity of rice seeds were analyzed with 22 SSR markers by using the PCR method. The data were then used to construct a phylogenetic tree. The results showed two main rice groups, namely Ketan Hitam 2 and Beureum Taleus, have the closest relationship with the index similarity of 0.39 (**Figure 2**). This analysis strengthens the close relationship between Ketan Hitam 2 and Beureum Taleus, which has a similar antioxidant capability.

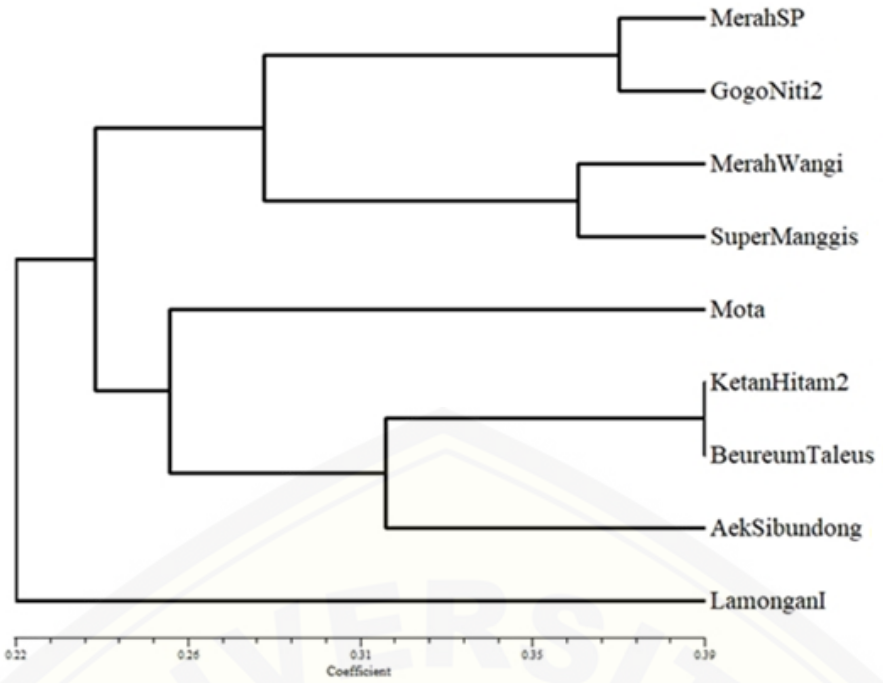

Figure 2: Phylogenetic tree of pigmented rice varieties based on SSR (simple sequence repeat) markers

Discussion

The antioxidant activity of pigmented rice seed protein is determined using ABTS, hydroxyl, and superoxide radical methods. Ketan Hitam-2 protein has the highest capability to scavenge ABTS, hydroxyl, and superoxide radicals, followed by Beureum Taleus (**Table 2**). This indicates that the protein of Ketan Hitam-2 has a significant role as a reduction agent through its electron donor to stabilize the reactive free radicals. The highest activity of antioxidants was found in Ketan Hitam-2 protein, Ketan Hitam-2 has dark pigment, which might be correlated with its highest content of protein and phytochemicals. Another study has reported that the colour differences of pigmented rice can affect its capabilities. Dark rice has a higher ability to scavenge free radicals than the red rice group. This ability was related to its protein and polyphenol content [17]. Various studies have recorded that proteins on food could play a role as an antioxidant through several mechanisms, including inhibit lipid oxidation by blocking metal access to the water-oil interface by electrostatic repulsion [18], scavenge free radicals [19], and reduce lipid hydroperoxides [20].

The antioxidant activity of non-protein compounds is determined by its ability to reduce DPPH, hydroxyl, and superoxide radicals. DPPH is a free radical and becomes stable after receiving electrons or hydrogen from non-protein compounds such as phenolic, flavonoid, carotenoid, or anthocyanin. The degree of discoloration indicates the potency of antioxidant activity [21]. Ketan Hitam 2 has the highest antioxidant activity on DPPH (**Table 3**). This was due to the ability of non-protein compounds on Ketan Hitam-2 could stabilize the DPPH radicals. The non-protein compounds of Ketan Hitam-2 also showed the highest capability to scavenge the hydroxyl and superoxide radicals. This result showed that Ketan Hitam-2 has non-protein compounds that can compete with 2-deoxy-D-ribose to react with hydroxyl radicals, resulting in a decrease of malondialdehyde formation [22]. Hydroxyl Radical is the most reactive free radical and interacts with nitrogenous bases purine and pyrimidine DNA, causing biological damage [23]. Hydroxyl radicals are generated from the Fenton reaction ($Fe^2 + H_2O_2 \rightarrow Fe^3 + + OH^- + OH^*$), then the resulting hydroxyl reacts with 2-deoxy-D-ribose forming malondialdehyde, which gives a pink colour [24].

The non-protein compounds of Ketan Hitam-2 also have the highest ability to scavenge superoxide radicals (**Table 3**). Superoxide anion is

one of the reactive oxygen species (ROS), which highly reactive and causes uncontrolled reactions and oxidative damage to functional groups [23]. Based on the results of antioxidant activity assessments of the non-protein compounds, it can be seen that the pigmented rice has higher antioxidant activities compared to the non-pigmented rice. It might be due to the content of the non-protein compounds (phenolic and flavonoid) in pigmented rice is higher than the non-pigmented rice [25]. Another study has reported that phenolic and flavonoids com- pounds can decrease the oxidative rate of organic materials by transferring hydrocarbon atoms or electrons to radical molecules [26].

Based on the antioxidant activities of protein and non-protein compounds, Ketan Hitam-2 shown the highest antioxidant activity, followed by Beureum Taleus. In this study, the genetic classification was analyzed using 22 SSR markers. The results showed two main rice groups, namely Ketan Hitam 2 and Beureum Taleus have the closest relationship (**Figure 2**). The result of analysis also shown that Ketan Hitam-2 and Beureum Taleus have similar properties. Various studies have reported that genetic diversity could affect antioxidant capacity [27]. The SSR has polymorphic genetic information, which can produce very high allelic variations [28]; thus, it can identify the physiological relationship and biochemical pro- perties. Between these two pigmented rices, Ketan Hitam-2 shown higher antioxidant activity than Beureum Taleus; thus, it can be concluded that Ketan Hitam 2 has greater efficacy as an antioxidant compares to Beureum Taleus.

Acknowledgement

This research was supported by the Ministry of Research, Technology, and BRIN and DIKTI, The Republic of Indonesia. SKIM Penelitian Magister.

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