Secondary metabolites profiles and antioxidant activities of germinated brown and red rice

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Abstract. The research aims to investigate the effect of germination on the secondary metabolite profiles and antioxidant activity of brown and red rice. The germination was performed by using a simple laboratory-scale machine that was designed and optimized to provide conditions that support the germination process. The germination was carried out for 2 days in dark conditions at 26°C and 99% humidity. Analysis of the secondary metabolite profile of ungerminated and germinated rice was performed using LC-MS. The antioxidant activities of ungerminated and germinated rice were done by using DPPH method. The results showed that the profiles of secondary metabolites of brown and red rice changed after germination. Some peaks were found to be induced in the germinated rice. However, some peaks were also loss during germination. The antioxidant activity of brown rice was slightly increased due to the germination, from 11.2% to 22.5%. Meanwhile the antioxidant activity of red rice was decreased after germination, from 73.8% to 60.0%.

1. Introduction
Rice is a staple food of most Indonesians, of which white rice is the most common consumed of rice type, due to the taste and texture of the cooked rice [1]. However, high starch content of the white rice may cause chronic diseases such as type 2 diabetes [2]. Unlike white rice, the consumption of pigmented rice and unpolished rice is known to prevent the risk of degenerative diseases such as type 2 diabetes [3]. Based on research conducted by Qureshi, et al., it has been demonstrated that bran layer of the pigmented and unpolished rice contains γ-oryzanol, tocopherol and tocotrienol which have the hypoglycemic effect [4]. In addition, pigmented rice contains a pigment called anthocyanin, naturally [5]. However, the consumption level of pigmented and unpolished rice is still lower than the white rice [1].

Recently, many studies have reported advantages and health benefits of the germination process in grains and legumes [6]. Germination is an effective process for increasing nutrient content in grains and cereals [7]. With germination, the texture of the rice will soften during the soaking [6]. Research conducted by Saikusa et al. and Kim et al. showed that levels of gamma amino butyric acid (GABA) and antioxidant activity of germinated brown rice are higher than ungerminated brown rice [2,8,9]. In addition, germinated brown rice have many benefits that can be used as antihypertensive agents and can reduce the risk of some chronic diseases, such as diabetes and cardiovascular disease [9].

Nevertheless, most of the studies on germinated rice were more focused on brown rice, while of the pigmented rice are still limited. Therefore, this study was conducted to determine the effect of
germination on two different rice varieties on the secondary metabolite profiles and antioxidant activities.

2. Methods

2.1 Materials
Two varieties of unpolished rice (brown rice (Pandan Wangi) and red rice (Cempo Merah)) were bought from Javara Indonesia (Jakarta, Indonesia). Sodium hypochlorite, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and analysis grade methanol, UHPLC grade acetonitrile (ACN), water, and acetic acid were purchased from Merck (Darmstadt, Germany).

2.2. Rice germination
The germination process was performed in a lab-scale sprouting machine that was made and optimized to provide conditions that support the germination process, based on the research by Aisyah, et al. (2013) [10]. The temperature (25-30°C) was maintained by a heating mat with thermostat (Hyndoor 12 V), placed under the machine. Humid air was created by a fog generator (24 V) with a fan attached to the sprouting machine to distribute the fog homogeneously. Fog generator produces fog every 3 hours with a duration of 5 minutes.

Sample preparation was performed according to Ti et al. (2014) and Ekowati, et al. with modifications [1,11]. The unpolished rice were subjected to surface-sterilizing, soaking and germination, sequentially. 40 g unpolished rice were soaked in 200 ml 0.07% sodium chlorite for 30 minutes then rinsed with aquadest. The sterilized rice were soaked in 200 ml of aquadest at room temperature for 24 hours. The unpolished rice were germinated for 2 days in the absence of the light at 26°C with 99% relative humidity. The germinated rice are then dried in an oven at 40°C for 6 hours (until moisture content ≥ 10%). Furthermore, the rice were milled using high energy milling HEM-E3D with 20:1 ball to powder ratio (BPR) and on-off timer 1 min. The rice powder were sieved with a mesh size of 70 mesh then stored at -4°C.

2.3. Rice Extraction
The dried powder of un-germinated and germinated rice were extracted using Ti et al. method with modifications. The extraction was performed in water bath-ultrasonicator (W-211, Japan) for 30 minutes. 0.2 gram of dried rice powder was extracted with 25 ml of 80% methanol (v/v). The mixture was centrifuged at 2500 rpm for 10 minutes and filtered. The residue is re-extracted with the same procedure. The filtrate was concentrated using a rotatory vacuum and then dissolved with 80% methanol until it reached the final volume of 10 ml for further analysis.

2.4. Determination of Secondary Metabolites Profiles with UPLC-ESI-QTOF
Ungerminated and germinated rice extracts were analyzed using the UPLC-ESI-QTOF Xevo ToF-1 instrument, which consisted of an Ultra Performance Liquid Chromatography (UPLC) section connected by high-resolution mass spectroscopy with the ionization technique of Electron Spray Ionization (ESI) and mass analyzer Quadrupole Time-of-Flight (Q-TOF) in positive mode. The column used was C-18 ACQUITY UPLC® BEH Shield RP18 (1.7 μm VanGuard™ Pre-Column 3/Pk (2.1x5 mm). The solvents used were solvent A: water; solvent B: acetonitrile, with a ratio of 30:70, with a flow rate of 0.200 ml/min. The sample volume was 7.5 μL. The mode of operation for mass spectroscopy is ESI (+); capillary voltage: 3.0 KV; cone voltage: 60 V; low collision energy (CE): 6.0 V; acquisition range: 100-1000 Da.

2.5 Determination of Antioxidant Activity
The method used for antioxidant activity in this research was DPPH method, according to Lee et al. (2003) with modifications. 5 mL of DPPH (10.7 ppm) was added to 1 ml of sample and then incubated
for 10 minutes in the dark. Absorbance was measured using UV-Vis Spectrophotometry at 517 nm wavelength. Percentage of antioxidant activity was calculated using the following formula bellow.

\[
\text{Antioxidant Activity} = \frac{\text{Abs. DPPH control} - \text{Abs. DPPH of test}}{\text{Abs. DPPH control}} \times 100\% 
\]

Abs. DPPH control: absorbance of DPPH prior to reacting with the sample
Abs. DPPH of test: absorbance of DPPH after reacting with the sample

3. Results and Discussion

3.1. Secondary Metabolites Profiles of Ungerminated and Germinated Rice

The RP-UHPLC-MS chromatogram showed that the germination changed the profile of secondary metabolites of both brown and red rice. There were a total of eleven compounds in ungerminated brown rice (BR) extract and twelve compounds in germinated brown rice (GBR) extract (Figure 1). Although the peak number was not so different, some peaks were identified only in germinated brown rice extract, while some peaks were found in ungerminated brown rice extract only (Table 1). Peaks 4, 6, 7 and 13 were found in the brown rice extract. However, those peaks cannot be detected in the germinated brown rice. Interestingly, peaks 8, 9, 11 and 16 were only can be found in the germinated brown rice (Figure 1). It seems that some compounds were induced during germination of brown rice, while some of them were loss.

![Figure 1](image-url)

**Figure 1.** RP-UHPLC–MS profile of 80% (v/v) methanol extracts of ungerminated (BR) and germinated (GBR) brown rice. Peak numbers refer to compounds in Table 1.
The identification of compounds in ungerminated and germinated brown rice was performed using comparison of spectra data resulting from mass spectroscopy with literature data. The retention time ($t_R$), mass per charge (m/z) of the parent and fragment ions were spectral data used to identify the peak of the compound in the extract (Table 1). However, most of the peaks (unknown compounds) cannot be identified due to the limited published literature data. Peaks of phenolic acids and flavonoids glycosylated might be tentatively determined based on the fragment ions, including hydrocaffeic and caffeic acid. It can be seen that some phenolic acid compounds found in the brown rice were not present after germination process.

Table 1. Compounds Tentatively Identified by RP-UHPLC- MS in Ungerminated and Germinated Brown Rice

| Peak no. | Tentative compounds | Retention time (min) | m/z (parent ion) | Distribution peaks in extracts |
|----------|---------------------|----------------------|------------------|-------------------------------|
| 1        | Unknown             | 0.95                 | 178.0679         | √                             |
| 2        | Unknown             | 1.14                 | 526.7372         | √                             |
| 3        | Hydrocaffeic acid   | 1.82                 | 467.9528         | √                             |
| 4        | 6'-O-feruloylsucrose| 2.04                 | 519.9201         | -                             |
| 5        | Flavonoid glycosilated 1 | 2.35      | 495.9638         | √                             |
| 6        | Hydrocaffeic acid   | 2.48                 | 183.9990         | -                             |
| 7        | Hydrocaffeic acid derivative 2 | 3.06      | 515.9164         | -                             |
| 8        | Unknown             | 3.26                 | 416.8736         | -                             |
| 9        | Caffeic Acid        | 4.17                 | 181.9169         | √                             |
| 10       | Unknown             | 5.05                 | 377.0094         | -                             |
| 11       | Flavonoid glycosilated 2 | 6.34      | 495.1987         | √                             |
| 12       | Unknown             | 6.83                 | 395.9943         | √                             |
| 13       | Unknown             | 8.74                 | 602.9640         | -                             |
| 14       | Unknown             | 8.82                 | 550.2068         | -                             |
| 15       | Unknown             | 10.51                | 522.1942         | √                             |
| 16       | Unknown             | 12.05                | 424.0126         | -                             |
| 17       | Unknown             | 13.07                | 596.0142         | -                             |

The similar fact was observed in the red rice. The chromatograms of UPLC-ESI-QTOF of red rice extracts show that germination changed the secondary metabolites profiles of red rice. In general, some peaks were not present in the extract after germination process. There were a total of seventeen peaks in ungerminated red rice extract, while only ten peaks were found in germinated red rice extract (Figure 2). Seven peaks, including peaks 6, 7, 8, 10, 12, 14 and 17 were detected in ungerminated red rice only. After germination, those peaks were not detected in the red rice extract. Interestingly, different from the brown rice, there were no peaks induced after germination process. On the other hand, some compounds were washed out during the germination process. This fact was in line with the previous study stated that some secondary metabolites, including anthocyanin, were loss during germination of pigmented rice [6].
Figure 2. RP-UHPLC−MS profile of 80% (v/v) methanol extracts of ungerminated (RR) and germinated (GRR) red rice. Peak numbers refer to compounds in Table 2

Table 2. Compounds Tentatively Identified by RP-UHPLC- MS in Ungerminated and Germinated Red Rice

| Peak no. | Tentative compounds                  | Retention time (min) | m/z (parent ion) | Distribution peaks in extracts |
|---------|-------------------------------------|----------------------|-----------------|------------------------------|
|         |                                     |                      |                 | RR  | GRR |
| 1       | Unknown                             | 0.95                 | 178.0533        | √   | √   |
| 2       | Unknown                             | 1.16                 | 526.6950        | √   | √   |
| 3       | Derivative hidroksicaffeic acid     | 1.82                 | 467.9148        | √   | √   |
| 4       | 6'-O-feruloylsucrose                | 2.04                 | 519.8772        | √   | √   |
| 5       | Flavonoid glycosylated              | 2.35                 | 495.9059        | √   | √   |
| 6       | Unknown                             | 2.48                 | 521.9023        | √   | -   |
| 7       | Derivative hidroksicaffeic acid     | 3.04                 | 515.8702        | √   | -   |
| 8       | Unknown                             | 4.01                 | 616.8730        | √   | -   |
| 9       | Unknown                             | 4.90                 | 658.8033        | √   | √   |
| 10      | Unknown                             | 6.14                 | 302.9856        | √   | -   |
| 11      | Flavonoid glycosylated              | 6.58                 | 577.8850        | √   | √   |
| 12      | Unknown                             | 7.17                 | 378.9936        | √   | -   |
| 13      | Unknown                             | 8.33                 | 602.9144        | √   | √   |
| 14      | Unknown                             | 9.95                 | 522.1513        | √   | -   |
Peak no.  | Tentative compounds       | Retention time (min) | m/z (parent ion) | Distribution peaks in extracts |
---      | ---                        | ---                  | ---              | ---                           |
15       | Unknown                    | 11.35                | 423.9173         | √                              |
16       | Pelargonidin 3,5-diglucoside | 12.27               | 595.9575         | √                              |
17       | Unknown                    | 13.06                | 647.9299         | √                              |

3.2 Antioxidant Activity of Ungerminated and Germinated Rice

The antioxidant activity of brown and red rice changed after germination. In general, the percentage of antioxidant activity of red rice was higher than brown rice (Figure 3). The germination increased slightly the antioxidant activity of brown rice, from 11.2% to 22.5%. However, the antioxidant activity of red rice was decreased after germination, from 73.8% to 60.0%. This data was in line with the change of the secondary metabolites profile of the brown and red rice during germination. Moreover, the decrease of antioxidant activity of red rice during germination might be explained by the fact that the varying antioxidant activity of extract depends on the content of the phenolic compounds of which in the pigmented rice are anthocyanin, a group of phenolic compound that is water soluble [12]. In addition, phenolic compounds in rice are found in the soluble outer layer so that the soaking can decrease the content of the compound, especially in red rice [1]. This is also supported by previous research state that germination of pigmented rice without removing the hull of rice can preserve the antioxidant compound and its antioxidant activity [6].

![Figure 3](image.png)

Figure 3. Antioxidant activity (%) of ungerminated and germinated brown and red rice

4. Conclusion

Germination changed the secondary metabolite profiles and antioxidant activities of brown and red rice. The 2 day-germination induced some peaks in brown rice, while some peaks were loss during germination of brown and red rice. In general, the antioxidant activities of ungerminated and germinated red rice were higher than that of brown rice. The antioxidant activity of brown rice increased slightly during germination, while the antioxidant activity of red rice was decline after germination.

5. References

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