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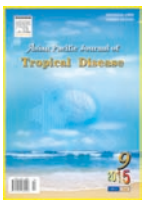

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DNA damage inhibitory effect and phytochemicals of fermented red brown rice extract

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ABSTRACT

Objective: To determine the polyphenol compounds (phenolic and flavonoid compounds), antioxidant activity [1,1-diphenylpicryl-2-picrylhydrazyl (DPPH) radical scavenging activity] and DNA damage inhibitory effect of fermented local red brown rice.
Methods: DNA nicking assay was employed to determine the antioxidant activity of the fermented rice extract. Phytochemical screening was completed using standard methods and DPPH radical assays were used to confirm the antioxidant properties of the extracts.
Results: After four days of fermentation, fermented red brown rice had more polyphenol compounds compared to unfermented counterpart. Fermented red brown rice showed greater antioxidant properties with EC_{50} value of DPPH radical scavenging of 43.00 mg extract/mL or 8 mg quercetin equivalent antioxidant activity. In addition, fermented rice extract showed DNA damage inhibitory effect to a certain extent. It protected DNA from reactive oxygen species; however, at high concentration it might induce reductive damage to DNA, whereas, unfermented red brown rice showed weak DNA damage inhibitory effect.
Conclusions: Fermented red brown rice could protect DNA from oxidative damage but might induce reductive damage to DNA at high concentrations.

1. Introduction

As a consequence of metabolism of oxygen in the human bodies, reactive oxygen species (ROS) are constantly produced. ROS is therefore defined as reactive free radicals molecules containing oxygen[1]. Several examples of ROS include hydroxyl radical ($^{\bullet}OH$), hydroxyl ion (OH^-) and superoxide anion ($O_2^{-\bullet}$)[2]. The unpaired electrons in their molecules can capture electron from other substances in order for themselves to get stabilized[3]. However, among the many types of ROS, hydroxyl radical has been determined as the most reactive among the others[4].

The amount of ROS in one's body is affected by environmental stresses. During the time of great environmental stress (*e.g.* exposure to UV light, ionizing radiation, smoking), there is a greater increase of radicals in the body. Research has shown that this ROS is dangerous and it can cause mutation, cell death, cancer and aging, not to mention many other disease that affect almost all parts of human bodies[2]. Types of damage that ROS might induce are lipid peroxidation, DNA alteration and stimulation of cell proliferation[5]. ROS can promote inactivation or loss of tumour suppressor gene or oncogene by causing mutations to these genes and therefore may lead to cancer. In addition, it might exert its effect on protein and genes that respond to cell proliferation, differentiation and apoptosis. Moreover, ROS can oxidise lipid present in cell membrane therefore causing cell damage[5].

Naturally, human body has the defence mechanism to regulate and control the amount of ROS in the body, such as enzymatic antioxidant system (superoxide dismutase)[1]. However, prolonged exposure will overwhelm body's mechanism and oxidative stress might happen. The answer to how to prevent such event and how to reduce the chances of getting under oxidative stress is to take antioxidants. Antioxidants are substances that prevent damage of biologically active molecules such as DNA, protein and lipid membrane[2]. They do this by donating one electron to the unstable radicals. They help in stabilizing the radicals and preventing them from causing damage[6]. Currently, there are a lot of research looking into rice and its potential as antioxidant source[7-9]. As a staple food of most of the countries around the world, rice has been identified as the most produced and most consumed food product. Therefore, rice may have an important role in the concentration of

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antioxidant consumed daily.

In Malaysia, fermented rice or 'tapai' is typically prepared using cooked glutinous rice, rice or tapioca tubers, with the addition of 'ragi' starters. Ragi is produced locally, and comprises of rice flour, spices, microflora inoculum and water or sugar cane juice[10]. The presence of microorganisms is reported to enhance the antioxidant activity in fermented rice, when compared to unfermented rice[11]. In the current work, the antioxidant activity of fermented local pigmented rice variety, namely, unpolished 'beras merah' or red brown rice was studied. Red brown rice selected as pigmented rice has been generally known to contain more antioxidants than white rice[12]. To date, there are no studies regarding the antioxidant activity of red brown rice of Sarawak. This study is significant as rice is a staple food for Malaysians. Through traditional biotechnology approach such as fermentation, a new product with added value (antioxidant-rich) can be produced and marketed.

The antioxidant activity of fermented rice will be determined using the DNA nicking assay, which assesses the damage caused by inducing hydroxyl radical or other radicals to DNA model[12]. The model, plasmid DNA, will give three types of forms (supercoiled, linear and supercoiled) that assist in the determination of damage. Damaged plasmid DNA no longer retain its original supercoiled conformation, and will be present either in linear or nicked form and this can be easily observed by running gel electrophoresis[13].

The hypothesis of this study was that fermented red brown rice extract would have the ability to protect plasmid DNA from damage. Assays were carried out to assess the DNA damage inhibitory effect of fermented red brown rice extract by using DNA nicking assay and to compare the phytochemicals of unfermented and fermented red brown rice.

2. Materials and methods

2.1. Plasmid extraction

Escherichia coli harbouring the pGEM-T plasmid (Promega) was cultured in Luria-Bertani broth with 100 μg/mL of ampicillin, and incubated for 24 h at 37 ℃ with shaking. The bacteria culture was harvested and the plasmid was extracted using the Promega Wizard Miniprep kit (alkaline lysis method) according to manufacturer's instruction. The precipitated pGEM-T plasmids were washed with 750 μL of cold ethanol, decanted and air dried before re-suspended in 50 μL of TE buffer and stored at -20 ℃.

2.2. Preparation of rice samples

The red brown rice used for this study was commercially sourced from the Beras Kampung Sarawak products (Empire Rice Mill Sdn Bhd, Kuching, Sarawak). The dry ragi starter was in the form of dry, white disk, 3 cm in diameter and 0.5 cm thick (Kampung Meranek, Kota Samarahan, Sarawak). Prior to usage, the ragi was ground into fine powder (< 1 mm diameter) using electric kitchen blender.

The rice was cooked based on the absorption method. Briefly, 300 g of dry rice grains were cooked in 900 mL of tap water using an electric rice cooker. The cooked rice was fluffed to separate the grains and left to cool. Fermentation was initiated by mixing ragi powder to the cooled cooked rice using ratio rice: ragi of 100:1. The mixture was transferred into glass bottles and allowed to ferment for 4 days at 30 ℃. After 4 days, the fermented rice was oven dried (24 h at 60 ℃). For control sample, the red brown rice was cooked, cooled, and immediately oven-dried using similar procedures as before, except no fermentation was involved.

2.3. Preparation of ground rice powder and extraction

Extraction method for both fermented and unfermented rice samples was done according to Plaitho *et al.* with some modifications[14]. The rice samples were ground to fine powder and approximately 5 g of powder was added with 50 mL of Milli-Q water in conical flask. The mixture was boiled to 100 ℃ with stirring using magnetic stirrer. The solution was left to cool before it was transferred into 50 mL falcon tubes and centrifuged at 5000 r/min for 15 min at 4 ℃. Supernatant was filtered using No. 4 Whatman filter paper and collected in another 50 mL falcon tube. The supernatant was topped up with Milli-Q water until 50 mL mark to replace the water loss after boiling and to obtain a concentration of 100 mg/mL. All samples were prepared in triplicates.

2.4. Determination of total flavonoid content

The total flavonoid content was determined using the method according to Chahardehi *et al.* with slight modifications[15]. Briefly, 1.0 mL of 2% aluminium trichloride in methanol and 1.0 mL of extracts were mixed and left to stand for 10 min. The solution was then shaken for 15 s and absorbance was measured using Bio Tek Synergy HT multi detection microplate reader at 415 nm against a blank consisting of 1.0 mL distilled water and 1.0 mL methanol. The total flavonoid content was calculated using standard curve with quercetin as standard and results were expressed as mg of quercetin equivalent (QE) per 100 g of extract.

2.5. Determination of total phenolic content (TPC)

The TPC was determined by Folin-Ciocalteu assay as described by Pantelidis *et al.* with slight modifications[16]. The mixture of sample solution contained 0.05 mL extract, 0.45 mL Milli-Q water, and 2.5 mL Folin-Ciocalteu reagent. The mixture was left to stand for 5 min, followed by addition of 2 mL 7.5% (w/v) sodium carbonate. After 5 min of incubation at 60 ℃, absorbance was measured using spectrophotometer at 760 nm against distilled water as blank. Phenolic content was calculated from gallic acid standard curve and results were expressed as mg of gallic acid equivalent (GAE) per 100 g of extract.

2.6. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay

Free radical scavenging capacity of the red brown rice extract was determined using DPPH according to Bhawya and Anilakumar with some modifications[17]. Different dilutions of the extract (20, 40, 60, 80 and 100 mg/mL) were prepared and 0.2 mL of each dilution was added to 1.8 mL of freshly prepared DPPH solution (0.1 mmol/

L) in methanol. The mixture was allowed to stand in dark for 30 min. Control was prepared containing same volume without extract. The absorbance was measured using spectrophotometer at 517 nm against methanol as blank and quercetin was used as standard. The scavenging activity of extracts on DPPH free radical was calculated using the following equation:

DPPH radical scavenging activity (%) = $(A_{control}–A_{sample})/A_{control}) \times 100$

Where, $A_{control}$ is the absorbance of control and A_{sample} is the absorbance of sample. All the tests were carried out in triplicates.

2.7. DNA nicking assay

DNA damage inhibition of red brown rice extracts were tested by photolysing H_2O_2 with UV radiation in the presence of pGEM-T plasmid DNA and performing agarose gel electrophoresis as described by Kalita *et al.* with slight modifications[18]. The plasmid DNA samples of 10 μL aliquots were transferred to PCR tubes and added with 10 μL of extracts with different dilutions (20, 40, 60, 80, 100 mg/mL) followed by addition of 10 μL 6% (w/v) H_2O_2. Positive control was prepared accordingly with addition of 10 μL quercetin in exchange of extracts whereas negative control was without addition of both quercetin and extracts. All the tubes were then placed directly on the surface of UV transilluminator (300 nm) for 10 min. After irradiation, 1 μL of loading buffer was mixed with 5 μL of samples and all samples were analysed by gel electrophoresis on 0.6% agarose gel stained with ethidium bromide in Tris-acetate-EDTA buffer for 60 min at 100 V.

3. Results

3.1. Polyphenols content analysis

The results of polyphenol content of the unfermented and fermented rice were as summarized. The amount of phenolic content in fermented rice, expressed as mg of GAE per 100 g of dry weight, was 206.53 ± 8.45. This amount was 5 times greater than the phenolic content of unfermented rice, which was only (43.69 ± 5.42) mg of GAE per 100 g dry weight. Gallic acid was used as the standards and the amount of phenolic contents were expressed as mg of GAE per 100 g of rice dry weight (n = 3). The flavonoid content of fermented rice was determined to be (189.45 ± 11.56) mg of QE per 100 g of dry weight. This amount was 2.5 times greater than the amount of flavonoid content in unfermented rice extract, which was only (75.38 ± 11.26) mg of QE per 100 g of dry weight. The flavonoid content was expressed as mg of QE per 100 g of dry weight (n = 3).

3.2. DPPH radical scavenging activity

Figures 1A and 1B represent the results of DPPH scavenging assay for unfermented and fermented red brown rice. The EC_{50} value (concentration of antioxidant required to scavenge 50% of DPPH free radical) of fermented rice extract was (43.00 ± 10.79) mg/mL or 8 mg QE antioxidant activity whereas unfermented rice extract was not able to reach EC_{50} (Figure 1A). The DPPH (20–100 mg/mL) value of fermented rice and unfermented rice extracts ranged from 4.50 to 14.33 mg QE antioxidant activity and from 1.67 to 5.67 mg QE antioxidant activity respectively (Figure 1B).

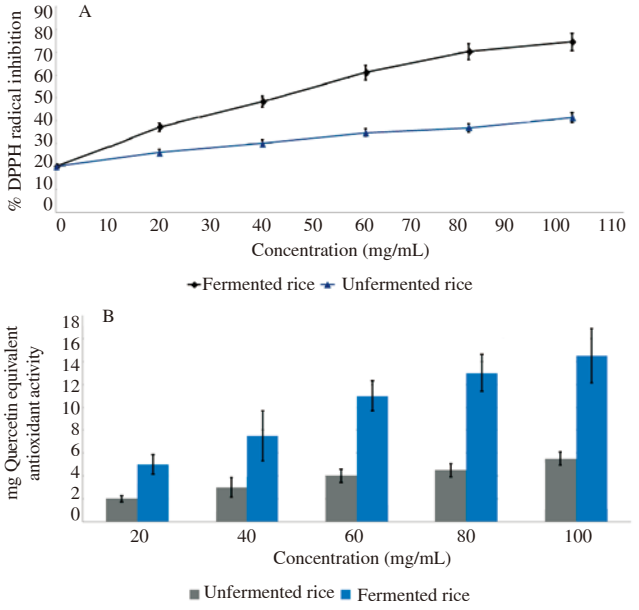

Figure 1. Antioxidant activity of unfermented and fermented rice. A: Antioxidant activity of rice extract in different concentration and values are expressed as percentage of DPPH radical inhibition ± SD (n = 3). B: Antioxidant activity of different types of rice in terms of QE antioxidant activity ± SD (n = 3).

3.3. DNA nicking assay

3.3.1. Method verification

Figure 2 shows the results of method verification. Lane 2 showed the location of supercoiled plasmid (SCP) control band. In lane 3, plasmid was irradiated on UV but it did not cause damage to plasmid DNA as the location of band was still the same as plasmid control. Water, which was the solvent used in this study, did not undergo photolysis into hydroxyl radical. This was supported by band shown in Figure 3. In lane 6, plasmid that was added with H_2O_2 was also not damaged. Lane 5 showed clearly that the plasmid had been damaged by the presence of nicked circular plasmid (NCP) bands.

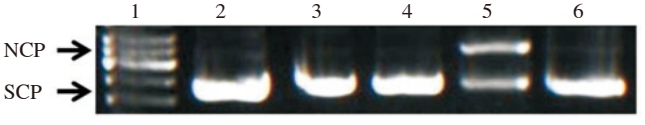

Figure 2. Verification of DNA damage method induced by UV + H_2O_2. Lane 1: DNA marker; Lane 2: Plasmid only; Lane 3: Plasmid + UV; Lane 4: Plasmid + H_2O + UV; Lane 5: Plasmid + H_2O_2 + UV; Lane 6: Plasmid + H_2O_2;

3.3.2. DNA damage inhibitory effect of unfermented and fermented red brown rice

Figures 3 and 4 show DNA nicking assay results of unfermented rice and fermented rice respectively. In Figure 3, there were bright NCP bands and few faded SCP bands observed when the concentration of unfermented rice extract was increased. In Figure 4, as the fermented rice extract concentration increased, the intensity of nicked plasmid band decreased until it disappeared at 60 mg/mL of rice extract. Once the extract concentration increased to greater than 60 mg/mL, the NCP band reappeared.

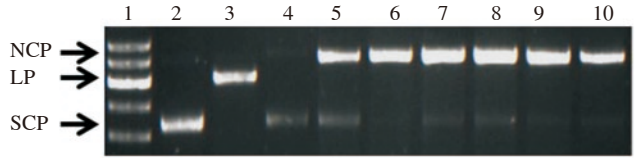

Figure 3. Effect of unfermented red brown rice extract on DNA damage induced by UV + H_2O_2.
Lane 1: DNA marker; Lane 2: Plasmid only; Lane 3: EcoR1 digested plasmid; Lane 4: Plasmid + quercetin + H_2O + UV; Lane 5: Plasmid + H_2O_2 + UV; Lanes 6–10: Plasmid + extract (20, 40, 60, 80, 100 mg/mL) + H_2O_2+ UV; LP: Linear plasmid.

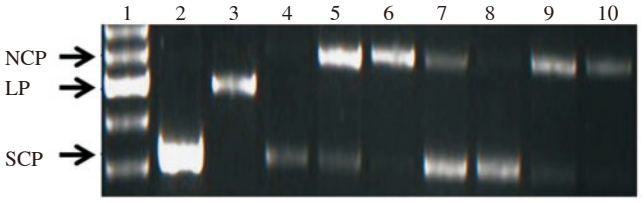

Figure 4. Effect of fermented red brown rice effect DNA damage induced by UV + H_2O_2.
Lane 1: DNA Marker; Lane 2: Plasmid only; Lane 3: EcoR1 digested plasmid; Lane 4: Plasmid + quercetin + H_2O + UV; Lane 5: Plasmid + H_2O_2 + UV; Lanes 6–10: Plasmid + extract (20, 40, 60, 80, 100 mg/mL) + H_2O_2+ UV; LP: Linear plasmid.

4. Discussion

4.1. Polyphenols content analysis

In this study, the phenolic content of fermented rice was analysed and compared with phenolic content of unfermented rice to determine any differences. Phenolic content is associated with antioxidant activity of samples[19]. This assay was carried out to compare the overall antioxidant activity of unfermented and fermented red brown rice.

Phenolic content of fermented rice was greater than that of unfermented rice. The result was as expected, in which fermented rice has higher antioxidant activity. This was because the phenolic content of fermented rice was enhanced by the fermentation process. During the course of fermentation process, microbes release hydrolytic enzymes that are used to release the phenolic compounds that are bound in plant materials[20].

Phenolic compounds fall into several different categories. One of the major classes is flavonoids compound. Therefore, total flavonoids assay was carried on the rice extracts using aluminium chloride colorimetric method[20].

The result followed similar trend as TPC, whereas fermented rice extract had higher flavonoid content than unfermented rice. This enhancement was as explained before, in which phenolic compounds amount in rice was enhanced by microbial fermentation.

4.2. DPPH radical scavenging activity

One of the mechanisms of antioxidants in preventing DNA damage is by scavenging the free radicals present in the system. DPPH radical scavenging activity assay assessed the effects of antioxidants on DPPH based on the hydrogen donating ability or scavenging ability of free radicals. DPPH radical is a stable free radical that donates hydrogen and gets reduced to diphenyl picryl hydrazine when antioxidant compound reacts with it[21]. Therefore, the scavenging abilities of both fermented and unfermented rice extracts were taken as parameter to determine their antioxidant potentials.

The graph showed that both extracts had scavenging properties but fermented rice extract possessed higher effect of scavenging free radicals than unfermented rice extract. The result further suggested that fermented rice extract exhibited better radical scavenging activity and higher antioxidant properties compared to unfermented rice extract, which was in line with study carried out by Yen *et al.*[8], Liang *et al.*[9] and Plaitho *et al.*[14]. Both extracts showed similar trend in which increasing the concentration of the extract in reaction mixture would subsequently raise the potency to scavenge free radicals resulting in an increase in antioxidant properties.

4.3. DNA nicking assay

There are not many studies using UV-photolysis of hydrogen peroxide to generate hydroxyl radical in DNA nicking assay. However, it worked well in visualizing the DNA strands breakage when coupled with hydrogen peroxide[17]. When exposed to UV, hydrogen peroxide will undergo photodecomposition and produce two hydroxyl radicals. Hydroxyl radicals generated by this reaction can cause oxidative damage to the DNA and induce breakage in DNA strands to its damaged forms. Different DNA forms have their own migration pattern. The fastest moving prominent band corresponded to the native SCP, and the slowest moving band represented the NCP. Thus, the extent of DNA breakage could be identified through the presence of specific bands on gel image. It was important to verify UV-photolysis method in the presence of H_2O_2 before carrying on further with the DNA nicking assay. In conclusion, H_2O_2 only induced DNA damage when exposed to UV. This verification showed that UV-induced hydroxyl radical DNA damage method was plausible and properly working in inducing the damage to plasmid DNA. There were several advantages of using this method over Fenton's reaction. First, it had less uncertainty than latter where ascorbic acid used as reducing agent in Fenton's reaction might inhibit hydroxyl radical[4]. Besides, this method had faster reaction time (15-minute incubation time) compared to Fenton's reaction, which required 20 to 30 min incubation at 37 ℃[4].

4.4. DNA damage inhibitory effect of unfermented and fermented red brown rice

The results of the nicking assay on unfermented rice show that it did not possess sufficient antioxidants, which could scavenge the hydroxyl radical and thus protect the DNA from oxidative damage. The result was in line with previous tests where unfermented rice had low TPC, total flavonoid content and low antioxidant activity in DPPH radical scavenging assay.

Unfermented red brown rice showed weak DNA damage inhibitory effect whereas fermented red brown showed distinctive results. The ability of fermented red brown rice extract to protect the DNA from hydroxyl radical damage was likely due to the presence of rich polyphenol compounds in fermented red brown rice after microbial fermentation. Polyphenol compounds are active antioxidants, which could scavenge the hydroxyl radicals and thus protect the DNA from oxidative damage[22]. However, once the extract concentration increased to a point beyond 60 mg/mL, it lost its DNA damage

inhibitory effect. This was in contrary to popular belief that increase in antioxidant concentration could prevent the DNA from oxidative damage. The reason of this could due to the nature of antioxidants. Antioxidants contained many loose electrons around their structure. This feature allows them to neutralize radical species by transferring the electron to radical species and stabilizing them. This mechanism is known as dissociative electron transfer. In Lu *et al.* study[23], it was found that excessive antioxidants might cause reductive damage to DNA. When the amount of radical species was scarce, excessive antioxidants might transfer their electron to nucleotides bases especially guanine base and induce it to radical guanine species which led to chemical bond breakage. Ultimately, this would induce irreversible nucleotide breakage and DNA damage.

This study demonstrated that the polyphenol content, radical scavenging ability and DNA damage inhibitory effect of Sarawak local red brown could be enhanced by microbial fermentation. The study results suggested that fermented red brown rice could be consumed as a source of natural antioxidants. However, further investigations are required to validate the detailed effects of fermented red brown rice for human consumption.

Fermented rice had higher polyphenol compounds (phenolic content and flavonoid content) than unfermented rice. TPC in fermented rice was enhanced 5 times by fermentation process. Total flavonoid content in fermented rice was enhanced 2.5 times. In addition, DPPH radical scavenging activity in fermented rice was higher than in unfermented rice. Fermented rice extract showed DNA damage inhibitory effect to a certain extent. It might protect DNA from ROS; however, at high concentration it might induce reductive damage to DNA.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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