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Protective effect of two Thai pigmented rice cultivars against H_2O_2-induced oxidative damage in HT-29 cell culture

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Abstract

Radicals derived from exogenous and endogenous sources are considered to be the principal cause of genetic damage. Exogenous and endogenous radicals participate in the reactive oxygen species (ROS) formation, which leads to damages in the DNA, RNA, proteins and lipids. However, dietary compounds, mainly from pigmented rice, are an essential source of antioxidants that help protect cells from damage. This study seeks to determine the antioxidant properties and cytoprotective effect of two Thai pigmented rice extracts namely the glutinous black rice (native name: Neaw dum moa37) and red rice (native name: Hom gradung-nga57) on H_2O_2-induced damage in HT-29 cells. The bioactive compound contents, as well as antioxidant activities of both rice extracts, were investigated. The protective effect of rice extracts on H_2O_2-induced damage was executed following the co-incubation method. HT-29 cells were exposed to H_2O_2 and different rice extract concentrations for 3 h and an MTT assay was used to measure the viability of the cell. The ROS level was determined using the 2′,7′-dichlorofluorescin diacetate (DCF-DA). The result showed that glutinous black rice extract contained significantly higher contents of all analysed antioxidants and activities than red rice extract. Glutinous black rice showed a higher cytotoxic effect compared to red rice. At the non-toxic concentration of both rice extracts, the HT-29 cells were guarded against the H_2O_2 induced oxidative stress. Besides, the intracellular ROS accumulation result from H_2O_2 exposure was significantly reduced in the presence of rice extracts for both glutinous black rice and red rice compared to control. Hence, this study has demonstrated the potential properties of both pigmented rice extracts in alleviating H_2O_2-mediated damage in HT-29 cells.

1. Introduction

It is well-documented that free radicals involve in a large number of human ailments such as inflammations, cardiovascular and cancer diseases (Pham-Huy *et al.*, 2008). Most notably, the development of cancerous cells in the body is considered to be due to error accumulation in the genome over time (Golemis *et al.,* 2018). Radicals derived from exogenous and endogenous sources are considered to be the principal causes of genetic damage (Azad *et al.,* 2008). Endogenous and exogenous radicals participate in ROS formation that can lead to damages in RNA, DNA, lipids and proteins. Dietary compounds, notably from pigmented rice, are a significant source of antioxidants that can nullify free radicals as well as have anti-cancer properties (Choi *et al.*, 2013). Rice is considered to be an excellent source of phytochemicals which includes phenolic acids, flavonoids, anthocyanins, proanthocyanidins, tocopherols, tocotrienols, γ-oryzanol, and phytic acid (Goufo and Trindade, 2014). Previous research has shown that a higher phenolic content, antioxidant activities and varieties of health-promoting phytochemicals exists in pigmented rice compared to non-pigmented rice (Sompong *et al.*, 2011). Pigmented and non-pigmented rice can be easily identified depending upon their bran colour. Pigmented rice are much darker while non-pigmented rice is lighter in appearance. The colour ranges from red and purple to black due to the presence of the colour pigments in the pericarp or aleurone layer. Pigmented rice are usually produced by removing only the hull, the outermost layer keeping most of the nutritional value intact (unpolished rice). In contrast, non-pigmented rice is produced by milling and polishing the rice kernel. They contain a large amount of antioxidants (Yawadio *et al.*, 2007), protecting our body cells from damages arising from free radicals that might be having harmful metabolic effects.

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Several studies have demonstrated that rice bran extract of pigmented rice exhibits cytoprotective effects on oxidative induced damage (Jittorntrum *et al.*, 2009; Tan *et al.,* 2016) as well as induced cellular antioxidant enzymes (Khammanit *et al.,* 2017). Furthermore, there is a rise in the consumption of unpolished pigmented rice because of its contribution to oxidative stress reduction. This study aimed to evaluate the antioxidant properties and cytoprotective effects on H_2O_2-induced damage in HT-29 cells of two Thai pigmented rice extracts (glutinous black rice and non-glutinous red rice) derived from Southern Thailand, Pattani local rice variety. The results obtained from this study might lead to a profound understanding of the potential health benefits of local rice variety.

2. Materials and methods

2.1 Sample preparation

Approximately 20 g of rice grains were placed in a 250 mL beaker with 100 mL of 80% methanol. The mixture was treated with an ultrasonic probe for 40 mins, 20 kHz and 40% amplitude with a temperature not exceeding 35°C. The same extract was extracted again for 24 hrs, 1000 rpm at room temperature with an electric shaker. Then filtrate suspension was centrifuged at 8000 rpm for 15 mins. The rice extract was made into powder by using the centrifugal evaporator so that the methanol can be removed entirely from the sample, which might interfere with the cell assays due to its toxicity. In order to prepare a stock solution of 100 mg/mL, the rice extract powder was dissolved entirely in media with 5% DMSO. The sample was then serially diluted using culture media.

2.2 Culture

HT-29 human colon cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in McCoy's 5A (IWAKATA and Grace mod.) with L-glutamine media supplemented with a 10% fetal bovine serum (FBS) and 1% of penicillin/streptomycin. The culture took place in a humidified incubator maintained at 37°C with air and CO_2 supply of 95% and 5% respectively.

2.3 Effect of rice extracts on cytotoxic

The seeding of cells occurred at a density of 1×10^5 cells (100 µL)/well onto 96 well plates and was maintained for 24 hrs before the experiment. The cells were then exposed to 100 µL of different concentrations of rice extracts (0 to 10 mg/mL) for 24 hrs. Completed media were used instead of rice extracts for control. The spent media were removed after incubation for 24 hrs, and 100 µL of MTT solution (0.5 mg/mL) was added to each well and incubated for 3 hrs. After that, 100 µL of DMSO was introduced into an individual well to solubilise formazan completely. The plate reading was carried out using a microplate reader at a wavelength of 570 nm with a reference wavelength of 630 nm. The results were expressed as the percentage of viable cells in comparison with the control (% cell viability), and IC_{50} was calculated.

2.4 H_2O_2 -induced oxidative stress in HT-29 cells

The different concentrations of H_2O_2 were exposed to cells to induce the formation of ROS, which is the precursor of highly oxidising, tissue-damaging radicals to obtain a suitable concentration of oxidant agent (H_2O_2). This was carried out so that, the particular concentration of H_2O_2 can be used in the subsequent assay to find rice extract effects on H_2O_2-induced cell death. The culture of HT-29 cells was carried out for 24 hrs using 96-well plates before the induction of oxidative stress. The culture media were removed and PBS was used to wash the cell. After that, 100 µL of H_2O_2 was added in the concentration range of between 0.3 to 10 mM. After 3 hrs of incubation, the viability of the cells was monitored with an MTT assay. (Note: The control sample was not treated with H_2O_2).

2.5 Cytoprotective effect of rice extracts on H_2O_2-induced oxidative stress in HT-29 cells

The co-incubation method was used to examine the protective effect of rice extracts on H_2O_2-induced cell death. The incubation of cells occurred in 100 µL of culture medium containing 0.5 mM of H_2O_2 (the concentration of H_2O_2 derived from the above experiment) with various non-toxic concentrations of the rice extracts for 3 hrs (0.31-2.5 mg/mL for glutinous black rice and 0.31-5 mg/mL for red rice). PBS was used to wash the cell, which is followed by the subsequent addition of 100 µL of fresh medium. Finally, the MTT assay was used to measure the viability of the cell.

2.6 Effect of rice extracts on intracellular ROS level

The plating of cells was carried out using 96-well plates at a density of 1×10^5 cells per well and incubated for 24 hrs. The incubation of cells occurred in 100 µL of culture medium containing 0.5 mM of H_2O_2 with different non-toxic rice extract concentrations for 3 hrs (0.31-2.5 mg/mL for glutinous black rice and 0.31-5 mg/mL for red rice). This is followed by the removal of the medium and the washing of cells with phosphate-buffered saline (PBS). Subsequently, 100 µL of 10 µM DCFH-DA was introduced to the cells, with 30 mins incubation in the dark. The fluorescence (corresponding to the radical species oxidised DCF) were measured by spectrofluorometer: λ excitation = 488nm, λ emission =

525nm. Positive control was performed in each experiment by treating the cells with 100 µM H_2O_2 for 20 mins before adding DCFH-DA to the cells and negative control with a completed media instead of rice extracts.

2.7 Analysis of antioxidant contents

The Folin Ciocalteu reagent was used to determine the total phenolic of the extracts following the method of (Aguilar-Garcia *et al.,* 2007) while the aluminium chloride colourimetric method (Zhishen *et al.*, 1999) was utilised to determine the total flavonoid content of the sample. The pH differential method was used to establish the total anthocyanin content, which is based on the structural changes in chemical forms of anthocyanin and absorbance measurements at pH 1.0 and 4.5 respectively (Finocchiaro *et al.,* 2010).

2.8 Analysis of antioxidant activities

DPPH radical scavenging activity in the sample was determined by using the protocol of (Butsat and Siriamornpun, 2010) while ABTS radical scavenging activity of the sample was determined according to the study of (Choi *et al.*, 2007).

2.9 Statistical analysis

All determinations were conducted in triplicate, and analyses of data were carried out using ANOVA on SPSS software. The difference between the two samples in the cytotoxic experiment was determined using a T-test. The significant differences between means were determined using Duncan's multiple range tests. The P-value considered as statistically significant is P-value less than 0.05.

3. Results and discussion

3.1 Antioxidant contents of glutinous black and red rice

The antioxidant content of both rice is provided in Table 1. The glutinous black rice had a significantly higher content of all analysed compounds and antioxidant activities than the red rice. The total polyphenol content (TPC) for glutinous black and red rice were 568.25±16.06 mg GAE/100 g grain and 433.61±12.89 mg GAE/100 g grain, respectively. Generally, in terms of rice variety, glutinous black rice variety possessed significantly higher polyphenol content than the red rice variety in any extraction method (Yodmanee *et al.,* 2011). Total flavonoid content was 2534.08±23.67 mg quercetin equivalent/100 g grain for the glutinous black rice and 1798.19±84.4 mg quercetin equivalent/100 g grain for the red rice. The anthocyanin content was relatively lower in red rice compared to black rice varieties. Anthocyanin content for glutinous black rice was 358.68±5.35 mg cyanidin 3-O-glucoside equivalent/100 g grain and 4.82±0.39 cyanidin 3-O-glucoside equivalents/100 g grain for red rice. With regards to the antioxidant activities, the red rice variety was less than black rice. The percentage inhibition of DPPH for glutinous black rice was 74.26±1.19% and 65.60±3.63% for red rice. The percentage inhibition of ABTS was 65.60±2.49% for glutinous black rice and 61.19±1.44 for red rice.

3.2 The effect of rice extracts on cytotoxic

The cytotoxic of both rice extracts were evaluated using the MTT assay and treatment of HT-29 cells was carried out using different rice extracts concentrations (0 -10 mg/mL) for 24 hrs to identify non-toxic concentrations for future experiments. The doses approximate to IC_{50} were used to examine the cytoprotective effect of both rice extracts. As illustrated in Figure 1, the viability of the cell after exposure to the two pigmented rice variety extracts for 24 hrs in a concentration-dependent manner, HT-29 cells significantly decreased ($P<0.05$). At 10 mg/mL concentration, glutinous black rice extract induces cell death with cell viabilities drop to 15% while red rice, cell viabilities drop to 43%. The IC_{50} value of glutinous black rice was 2.76 mg/mL and 4.66 mg/mL for red rice indicating that glutinous black rice had a significantly higher ($P<0.05$) cytotoxic effect on HT-29 cells compared to red rice. When the concentration of the rice extract increased, it is assumed that all bioactive compounds in the extract will also increase simultaneously, mainly phenolic compounds and flavonoids. The cytotoxic effect found in the present study was probably due to the pro-oxidant properties of polyphenol and flavonoids. The pro-oxidant activities of phenolic compounds were based on the generation of phenoxyl radicals in the presence of transition metal ions and reacted with oxygen to generate O^-_2 and H_2O_2.

Table 1. Total antioxidant contents and activities of glutinous black rice and red rice

Rice varieties	TPC (mg GAE/100 g grain)	TFC (mg quercetin /100 g grain)	TAC (mg Cy-3-G /100 g grain)	DPPH (% of inhibition)	ABTS (% of inhibition)
Glutinous black rice	568.25±16.06^A	2534.08±23.67^A	358.68±5.35^A	74.26±1.19^A	65.60±2.49^A
Red rice	433.61±12.89^B	1798.19±84.4^B	4.82±0.39^B	65.60±3.63^B	61.19±1.44^B

Values are presented as mean±SD. Values with different superscript within the same column are significantly different ($P<0.05$).

Consequently, an increase in the cellular level of ROS may induce toxicity to cells (Eghbaliferiz and Iranshahi, 2016), lipid peroxidation, DNA damage and apoptosis in normal and cancer cells. Moreover, the higher cytotoxic effect of glutinous black might be due to the higher content of anthocyanin. In this study, the total anthocyanins content of glutinous black rice was higher than the of red rice (Table 1). Anthocyanins are believed to be antioxidants, and some studies have suggested that they might modulate the cellular redox reactions and activate apoptosis (Hou *et al.*, 2005). Therefore, the non-toxic concentration range of rice extract was 0.31-2.5 mg/mL for glutinous black rice and 0.31-5 mg/mL for red rice.

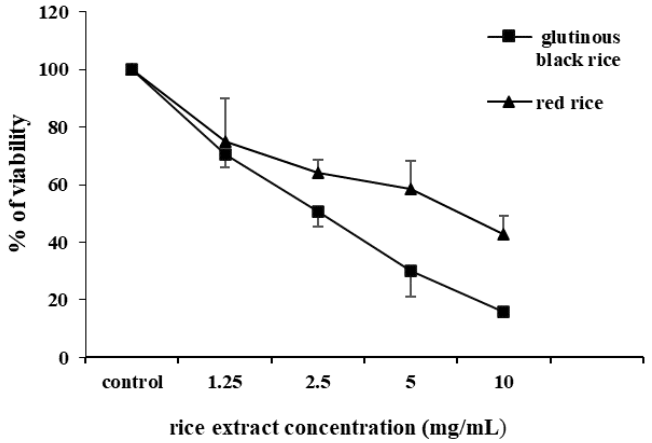

Figure 1. The cytotoxic effect of rice extracts on HT-29 Cells. The treatment of HT-29 cells with different concentrations of rice extract for 24 hrs and cell viability measurement using MTT assay. Data were mean±SD (N=3).

3.3 H_2O_2 induced oxidative stress in HT-29 Cells

The suitable concentration of H_2O_2 induced oxidative stress in HT-29 cells was determined to examine the cytoprotective activity of rice extracts on HT -29 cells. The different H_2O_2 concentrations (0.3, 0.5, 1, 5 or 10 mM) were treated with HT-29 cells for 3 h, and the viability of the cells was determined using MTT assay. As illustrated in Figure 2, as the concentration of H_2O_2 increased from 0.3 mM -10 mM, the cell viability reduced significantly. Approximately 50% of cell viability was achieved by using 0.5 mM of H_2O_2 concentration which was used in successive experiments to examine the effect of rice extract on the oxidative induced damaged cells.

3.4 Cytoprotective effect of rice extracts on H_2O_2 - induced oxidative stress in HT-29 cells

The effects of rice extracts (glutinous black rice and red rice) on H_2O_2 induced oxidative stress in HT-29 cells were studied to examine the protective aspect of pigmented rice. 0.5 mM of H_2O_2 was used to induce oxidative stress in the experiment. H_2O_2 are renowned as

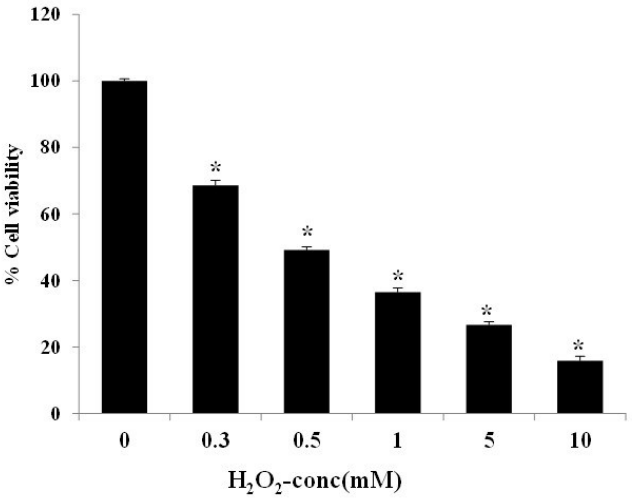

Figure 2. Effect of H_2O_2 -induced oxidative stress in HT-29 cells. Different H2O2 concentrations (0.3, 0.5, 1, 5, 10 mM) were used to treat HT-29 cells for 3 hrs, and measurement of cell viability was carried out with MTT assay. Data were means ± SD (N=3). * P<0.05 compared to control.

the precursors of highly oxidising radicals as their molecules can produce a highly reactive hydroxyl radical ($OH^{\bullet}$) in the presence of Fe^{2+} and Cu^{2+} (transition metals) through Fenton reaction (Azad *et al.*, 2008) which is toxic to many biological systems *in vivo* and *in vitro* models.

HT-29 cells were exposed to H_2O_2 (0.5 mM) and various non-toxic concentrations of rice extracts (0.31-2.5 mg/mL for glutinous black rice and 0.31-5 mg/mL for red rice) for 3 hrs and an MTT assay was used to measure the cell viability. The protective effect of both rice extracts was provided in Figure 3. Due to the oxidative stress caused by H_2O_2, the lower concentrations of both rice varieties were unable to prevent cell death. Nonetheless, when the non-toxic higher concentration of both rice extracts was exposed together with 0.5 mM of H_2O_2, cell viability increased significantly. For the red rice extract at concentrations of 0.63, 1.25, 2.5 and 5 mg/mL, the cell viability increased significantly compared to the control. The viability of the cells increased to 66% at a concentration of 5 mg/mL of red rice extract compared to the control (47%) (cell was challenged only 0.5 mM of H_2O_2). On the other hand, only two concentrations of glutinous black rice (1.25 and 2.5 mg/mL) out of (0.31, 0.63, 1.25 and 2.5 mg/mL respectively) were able to prevent the cells from the oxidative induced damage as demonstrated in cell viability, which increased to 60% at concentration 2.5 mg/mL compared to control. This indicates that the cytotoxic effect was attenuated in the presence of rice extract, which might be due to the antioxidant activities of phenolic compounds including quercetin, cyanidin, peonidin and procyanidins, which contained rice extract. Furthermore, red rice seems to have more effect than glutinous black rice as the lowest concentration of rice

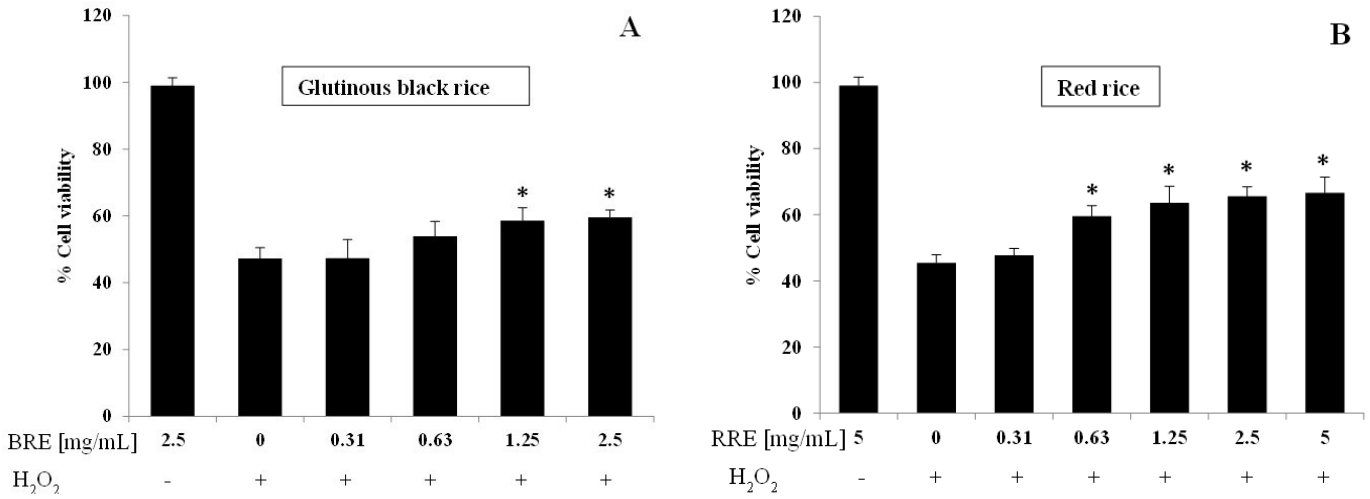

Figure 3. Effect of A: Glutinous black rice and B: Red rice extracts on H_2O_2 -induced cell damage in HT-29 cells. HT-29 cells were treated with 0.5 mM of H_2O_2 with various concentration of rice extract (0-5 mg/mL). Cell viability was measured after 3 hrs of incubation by MTT assay. Data were mean±SD (N=3). * P<0.05 compared to control (without sample). BRE; glutinous black rice extract, RRE; red rice extract

extract for protection was 0.63 mg/mL while glutinous black rice was 1.25 mg/mL. This is because the significant phytochemical components in the rice were distinct. Although, the result showed that glutinous black rice extract contained significantly high analysed compounds and antioxidant activities than red rice (Table 1), some active compounds contained in the red rice differs in amount compared with glutinous black rice. These compounds include catechins, proanthocyanins, γ-oryzanol and carotenoids. Notably, oligomeric procyanidin, which are the primary flavonoids found in red rice, has been reported to be highly effective in free radical scavenging activity (Oki *et al.,* 2002). There are several proposed mechanisms that phenolic compounds notably flavonoids can protect against injury caused by ROS. An instance is the direct scavenging of ROS, antioxidant enzymes activation and metal chelating activity (Prochazkova *et al.*, 2011). In this study, the possible mechanism in cytoprotective effect is direct scavenging of ROS since the incubation time of rice extract together with an oxidising agent was just 3 hrs which might not be enough to trigger antioxidant enzymes at a cellular level. The antioxidant activities of active compounds in the plant have been studied, and the results demonstrated that the addition of the extract could increase cytoprotective capacity. Kim *et al.* (2012) evaluated the cytoprotective and antioxidant effects of a polymeric procyanidin fraction (Fpol) from defatted grape seeds in PC12 cells. They found that PC12 cells had resistance against oxidative damage after pre-treatment with Fpol. Mok *et al.* (2014) found that the increase in anthocyanin concentration reduced the HLE-B3 cells death by oxidative stress induced by H_2O_2. Zhang *et al.* (2011) also found that the anthocyanin from the Chinese bayberry extract protected the β (INS-1) cell death from oxidative stress caused by H_2O_2 where 1mM of the H_2O_2 reduced the viability of INS- 1 cell to almost 60% compared to the control. Meanwhile, INS- 1 cells pre-treatments with 0.5 and 1 mM of anthocyanin protected the death of cells from oxidative stress caused by H_2O_2. As their results suggested, anthocyanins upregulated HO-1 Expression via PI3K/Akt and ERK1/2 activation, HO-1 expression is considered as one of the vital components of the cellular defence system against oxidative stress.

3.5 Effect of rice extracts on intracellular ROS level

The elevated ROS level obtained from endogenous and exogenous sources includes hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^{\bullet-}$), superoxide anion ($O_2^{\bullet-}$), and hydroxyl ($OH^{\bullet}$) radicals induced lipid peroxidation or DNA oxidation modifying cellular biomolecules. Consequently, there is an increased risk of genetic modification which resulted in carcinogenesis.

2′,7′-dichlorofluorescein, a dye specific for detection of ROS was used in this study to determine the modulation of ROS by rice extracts in HT-29 cells after treating with the different non-toxic concentration of rice extracts together with 0.5 mM H_2O_2 (represent of an exogenous source of free radical). The principle in this method, DCFH-DA is transferred across the cell membrane and hydrolysed with intracellular esterase to create non-fluorescent 2′,7′dichlorofluorescein (DCFH) and further transformed to a highly fluorescent 2′,7′ dichlorofluorescein (DCF) in the presence of ROS. The DCF fluorescence intensity corresponds directly to the number of ROS formed, allowing the exact estimation of intercellular ROS levels. The results of the present study demonstrate a significant decrease in the level of ROS as the concentration of both the rice extracts increased (Figure 4). In terms of rice varieties, glutinous black rice extracts with concentration (1.25 and 2.5 mg/mL) and red rice extracts (1.25, 2.5 and 5 mg/mL) reduced the

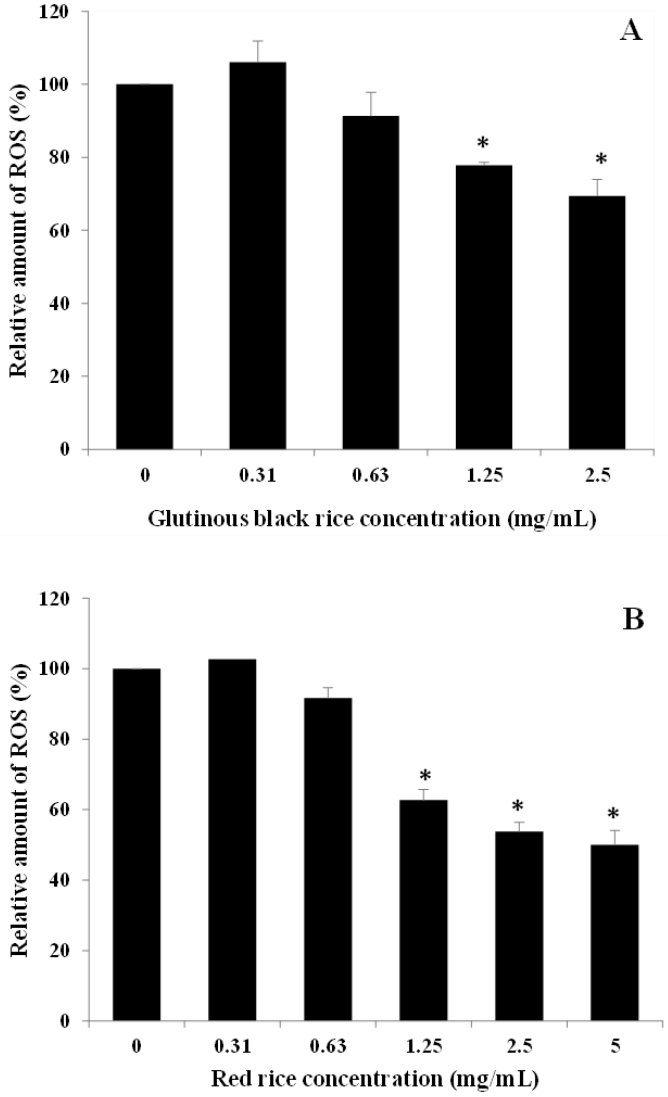

Figure 4. Effect of A: Glutinous black rice and B: Red rice extracts on intracellular ROS. HT-29 Cells were treated with 100 µl of culture medium containing 0.5 mM of H_2O_2 together with 0 to 5 mg/mL rice extract for 3 hrs ROS was measured by 10 µM DCFH-DA. The relative amounts of ROS (%) was shown as mean±SD (N = 3). * P<0.05 compared to control (without sample).

intercellular ROS level significantly (P<0.05) compared to the control. Therefore, both rice extracts can modulate the ROS produced within the cell, which may cause genetic damage. Furthermore, the result of this study also confirms the cytoprotective experiment that the H_2O_2 induced reduction of HT -29 cells viability whereas the presence of rice extract increased the cell viability compared to control (expose to H_2O_2 only). Since a high level of ROS is the cause of cell death (Circu and Aw, 2010), the reduction of ROS by rice extract may increase the cell viability in the experiment.

4. Conclusion

Glutinous black rice extract contained significantly higher contents of all analysed antioxidants and activities than red rice extract. Glutinous black rice has a significantly higher cytotoxic effect on HT-29 cell compared with red rice. At non-toxic concentration, the cytoprotective effect against oxidative induced damage by H_2O_2 was observed in the presence of rice extract both glutinous black rice and red rice. In addition, both pigmented rice extracts were able to reduce the level of ROS under oxidative damage.

Conflict of interest

The authors declare no conflict of interest.

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