Protective Effect on Oxidative DNA Damage and Antiproliferative Activity of Standardized γ-Oryzanol-Rich Extracts from Thai Purple Rice Bran

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ABSTRACT

This study was carried out to investigate the protective effect of standardized γ-oryzanol-rich extracts on oxidative DNA damage induced by Fenton reaction and antiproliferative activity against human cancer cells. Six cultivars of Thai purple rice were collected in northern Thailand. Rice bran was extracted with hexane/ethyl acetate mixture and the extract was evaporated to obtain crude rice bran oil. Each rice bran oil was further purified by column chromatography to obtain the γ-oryzanol-rich extract. The extracts contained γ-oryzanol in the range of 1.17 – 7.54 % w/w, in which GAM THOR exhibited the highest γ-oryzanol content. The extr \acts containing more than 5.0 % w/w γ-oryzanol (GAM THOR, GAM DOI MUSUR and GAM SUKHOTHAI-2) were selected to be standardized with γ-oryzanol and then the protective effect on oxidative DNA damage and antiproliferative activity against four human cancer cell lines (HT-29, HCT 116, MDA-MB-468 and PC3) were investigated. The extracts (10 µg/ml) exhibited a protective effect on oxidative DNA damage induced by Fenton reaction as compared with standard quercetin (lower than 5 µg/ml). Furthermore, all of the extracts exerted antiproliferative activity against human cancer cell lines in a dose-dependent manner. GAM THOR exhibited the highest antiproliferative activity against HT-29, HCT 116, MDA-MB-468 and PC3 with an 50% inhibition concentration value of 52.18 ± 1.21, 40.58 ± 5.69, 48.59 ± 2.40 and 51.61 ± 1.30 µg/ml, respectively. From these findings, γ-oryzanolrich extracts from Thai purple rice bran show potential as chemopreventive supplements or in nutraceuticals.

Keywords: γ-Oryzanol, Thai purple rice bran, DNA damage, Antiproliferative activity

INTRODUCTION

Reactive oxygen species and reactive nitrogen species play an important role in human cancer development (Wiseman and Halliwell, 1996). Reactive oxygen species and reactive nitrogen species have been demonstrated to possess

many characteristics of carcinogenesis effects. Reactive oxygen species, especially hydroxyl radical ($OH^{\bullet}$) / reactive nitrogen species, especially nitric oxide (NO) and peroxynitrite (ONOO-), cause structural alteration in DNA or large genomic alterations; e.g. base pair mutations, DNA-strand crosslink, DNA-strand breaking, chromosomal rearrangement, translocations and deletions (Wiseman and Halliwell, 1996; Poirier M.C., 2004). Mutagenesis by reactive oxygen species / reactive nitrogen species is relevant to cancer generated as a consequence of DNA injury and could contribute to the initiation of carcinogenesis, in addition to being important in the promotion and progression of cancer (Hoeijmakers J.H.J., 2009). It has been reported that dietary components can protect against cell proliferation in the destruction of certain reactive oxygen species that initiate carcinogenesis through oxidative damage to DNA (Williams et al., 1989).

Thai purple rice has been commonly cultivated and consumed by Thais, especially in northern Thailand, and has also been popular for health maintenance and disease prevention. It has been reported that purple rice is rich in health-beneficial phytochemicals such as anthocyanin, γ-oryzanol, tocopherols, tocotrienols and phenolic compounds. Rice bran is one of the valuable by-products of the rice milling process, particularly rich in dietary fibers, γ-oryzanol, essential vitamin E complex, tocotrienols and β-sitosterol (Chotimakorn et al., 2008). Several reports have revealed that rice bran extracts contain biologically active compounds which play a role in antioxidative, chemopreventive, anti-inflammatory, antimutagenic and anticarcinogenic activities (Hudson et al., 2000; Nam et al., 2005; Srisala et al., 2009). However, the mechanism of the active compound is uncertain and not well investigated. Therefore, studies of the active component and biological activity of Thai purple rice bran are warranted.

This study was designed to evaluate the content of γ-oryzanol isolated and purified from Thai purple rice bran and to find out whether the γ-oryzanol rich extracts exert protective effect on oxidative DNA damage induced by Fenton reaction and antiproliferative activity against human cancer cell lines.

MATERIALS AND METHODS

Chemicals and materials

Six purple rice cultivars (*Oryza sativa* L.) were collected from northern Thailand in December 2009: *SUKHOTHAI-2* (Agricultural Project, Sukhothai Airport, Sukhothai Province); *GAM DOI MUSUR, GAM THOR and GAM JADEEKHO* (Phob Pra Agricultural Extension Office, Tak Province); and *NIAW SAN PAH TAWNG* and *GAM LANPANG* (Samoeng Rice Research Center, Chiang Mai Province). HCT 116 (colorectal carcinoma cell), HT-29 (colorectal adenocarcinoma cell), MDA-MB-468 (breast adenocarcinoma cell) and PC3 (preactive oxygen speciestate carcinoma cell) were obtained from Dr. Francoise Raynaud, Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques-UMR 8601, Institute Interdisciplinaire des Sciences du Vivant des Saints-Pères, Université Paris Descartes, Paris, France. All chemicals and solvents used were analytical grade and purchased from Sigma Chemical (St. Louis, MO), Merck (Darmstadt,

Germany), Invitrogen (USA), Roche (Germany) and Vivantis (Malaysia).

Preparation and determination γ-oryzanol content of γ-oryzanol rich extracts

Fresh rice bran powder (60-mesh sieve) was obtained by rice milling process and stabilized by heating-cooling process to inactivate endogenous lipase (Malekian et al., 2000). The extraction and purification of γ-oryzanol rich extracts were carried out using an improved method with slight modifications (Xu and Godber, 1999). Fifty grams of each rice bran type was extracted with the 100 mL hexane and ethyl acetate mixture (7:3) in Soxhlet extractor for 3 h. Then, the organic solvent layer was evaporated under reduced pressure to obtain crude rice bran oil. Subsequently, crude rice bran oil was purified by classical column chromatography packed with silica gel (grade 60) to remove triglyceride and other lipids. The extract was concentrated under reduced pressure by using Rotary evaporator to obtain semi-purified γ-oryzanol rich extract. Then, γ-oryzanol rich extracts were determined for γ-oryzanol content by reversed-phase HPLC using Agilent 1100 with UV detector, the wavelength of the detector was set at 330 nm. The 150 x 3.0 mm diameter Kinetex C18 column was obtained from Phenomenex Co, Ltd. and the mobile phase consisted of methanol, acetonitrile, dichloromethane and acetic acid (50, 44, 3, and 3%, respectively), with a flow rate of 0.3 mL/min. All samples were tested in triplicate. Finally, the extracts were standardized to contain 10% w/v γ-oryzanol by adding the equivalent amount of each extract and their protective effect on oxidative DNA damage and antiproliferative activity were investigated.

Protective effect on oxidative DNA damage induced by Fenton reaction

The protective effects on oxidative DNA damage induced by Fenton reaction of γ-oryzanol-rich extracts were determined as previously described (Saenjum et al., 2010). The reaction mixture contained pUC18 plasmid DNA in PBS buffer, 8 μM of Fe(II), 25 μM of H_2O_2 and tested samples at different concentrations. After incubating at 37°C for 60 min, an aliquot solution mixture (10 μL) was loaded to a 1% agareactive oxygen speciese gel in 0.5x TBE buffer. Then, electrophoresis was carried out at 100 V for 90 min. Subsequently, the electrophoresis gels were stained with ethidium bromide for 30 min. After washing, the intensities of supercoiled and relaxed open-circular bands were determined and photographed with a gel documentation system.

Antiproliferation assay

4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate (WST-1) was used to evaluate antiproliferative activity against four human cancer cell lines. The assay principle is based on the reduction of the tetrazolium salt WST-1, producing a soluble formazan by cellular dehydrogenases. HCT 116, HT-29, MDA-MB-468 and PC3 were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% Fetal Bovine Serum, 100 units/mL penicillin. Initially, 96-well cell culture microplates were seeded with 200 μL containing cells in suspension: 5 x 10^3 cells/well for HCT 116, 8 x 10^3 cells/well for PC3

and 10^4 cells/well for HT-29 and MDA-MB-468. After 24 h incubation in culture medium, cells were treated with sample extracts at different concentrations, positive control and solvent control (cisplatin was used as a positive control). The final concentration of the sample extracts in each well was 200, 100, 50 and 25 µg/mL, respectively. Three wells containing only the same number of cells were left as the negative control on each plate. After 72 h incubation in 5% CO_2 at 37°C, WST-1 solution was added to the test wells and further incubated at 37°C for 1-2 h. The optical density was measured with a microplate reader (Beckman counter) at 490 nm to determine cell viability. The results were expressed as 50% inhibition concentration (IC_{50}, µg/mL) (Jarray et al., 2011).

Statistical analysis

All results were expressed as a mean of three replicates ± standard deviations (SD). SPSS PC software (version 16) was used for all statistical analysis; p values < 0.05 were considered to be significant.

RESULTS

As illustrated in Figure 1A, control γ-oryzanol is composed of four major peaks. Similarly, the γ-oryzanol rich extracts from Thai purple rice bran are also composed of four major peaks (Figure 1B) that are identical to those in control γ-oryzanol. Moreover, they also exhibited one unidentified major and five minor peaks.

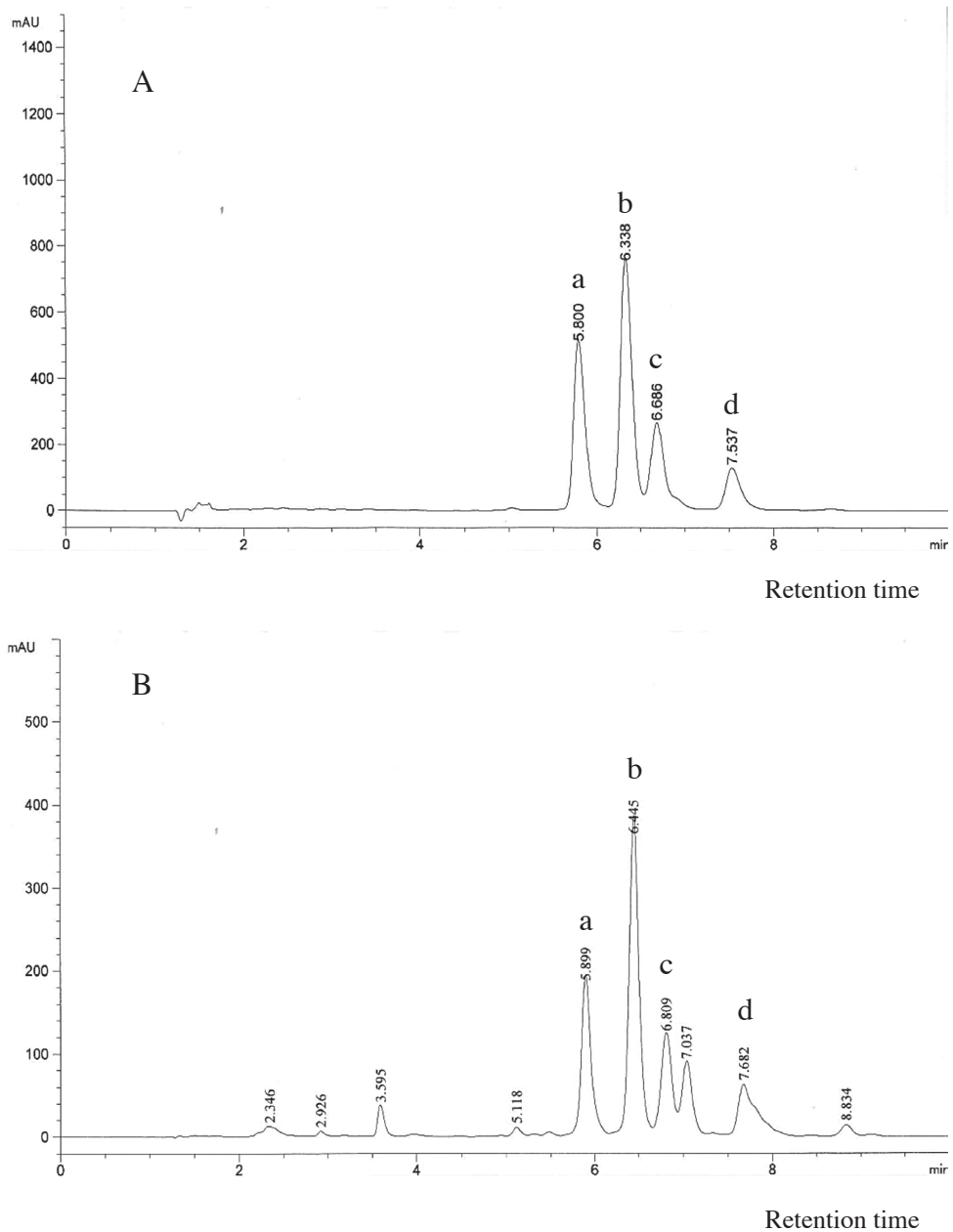

Figure 1. HPLC chromatogram of (A) standard γ-oryzanol and (B) γ-oryzanol rich extract from Thai purple rice bran: (a) cycloartenyl ferulate, (b) 24-methylenecycloartanyl ferulate, (c) campesteryl ferulate and (d) β-sitosteryl ferulate.

The results in Table 1 indicated that the γ-oryzanol content of γ-oryzanol-rich extracts from Thai purple rice bran were in the range of 1.17 – 7.54 % w/w according to the cultivar, topography and environment. Therefore, the γ-oryzanol rich extracts with γ-oryzanol content of more than 5 % w/w were selected (GAM THOR, GAM DOI MUSUR and GAM SUKHOTHAI-2) and standardized by adding the equivalent amount of each extract to give 10 % w/v γ-oryzanol before being investigated for protective effect on oxidative DNA damage and antiproliferative activity.

Table 1. Extract yield and γ-oryzanol content from different cultivars of Thai purple rice bran.

Rice cultivars	Extract yield (%w/w)	γ-Oryzanol content (%w/w extract)
GAM SUKHOTHAI-2	1.19[a]	6.65 ± 0.11[e]
GAM DOI MUSUR	1.45[d]	6.17 ± 0.09[d]
GAM THOR	1.35[c]	7.54 ± 0.19[c]
GAM LAMPANG	1.23[b]	2.65 ± 0.07[b]
GAM JADEEKHO	1.25[b]	1.17 ± 0.11[a]
NIAW SAN PAH TAWNG	1.18[a]	2.93 ± 0.05[b]

Note: Mean values within a column superscripted by the same letter are not significantly different at $p < 0.05$.

Protective effect on oxidative DNA damage induced by Fenton reaction

The protective effect of γ-oryzanol rich extracts on oxidative pUC18 DNA damage induced by Fenton reaction was assessed by agareactive oxygen speciese gel electrophoresis. The effect was compared to standard quercetin. The Fenton reaction can produce hydroxyl radicals by initiating and catalyzing the decomposition of H_2O_2 by Fe(II). Hydroxyl radicals can react to plasmid DNA to produce a relaxed open-circular form. As illustrated in Figure 2, we can observe that the control pUC18 plasmid DNA contains mostly supercoiled form and a small amount of relaxed form. At the fixed concentrations of Fe(II) (8 μM) and H_2O_2 (25 μM), the proportion of supercoiled form decreased, whereas the relaxed open-circular form increased. As shown in Table 2, the ratio between supercoiled and relaxed open-circular forms of plasmid DNA was correlated to oxidative DNA damage, which increase in relaxed opened-circular form and decrease in supercoiled form. All of γ-oryzanol rich extracts exhibited the protective activity against DNA damage induced by Fenton reaction in the same concentration (10 μg/ml), which is comparable to standard quercetin that exhibited the protective effect on oxidative DNA damage in the concentration lower than 5 μg/ml.

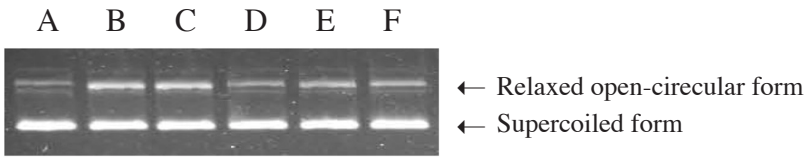

Figure 2. The electrophoresis picture indicated a protective effect of γ-oryzanol rich extracts on pUC18 plasmid DNA damage-induced by hydroxyl radicals. The conditions were treated by 8 μM Fe(II) and 25 mM H_2O_2 for 60 minutes with different concentrations of γ-oryzanol rich extracts: (A) control without Fe(II) and H_2O_2, (B) control with Fe(II) and H_2O_2 (C) 5 μg/ml, (D) 10 μg/ml, (E) 25 μg/ml and (F) 50 μg/ml.

Table 2. Ratio between supercoiled and relaxed open-circular form of pUC18 plasmid DNA.

Concentration (μg/ml)	Ratio between supercoiled and relaxed open-circular forms			
	Quercetin	GAM SUKHOTHAI-2	GAM DOI MUSUR	GAM THOR
Control (A)	3.75 ± 0.27	3.65 ± 0.18	3.74 ± 0.23	3.77 ± 0.24
5 (C)	3.71 ± 0.24	1.90 ± 0.26 *	1.45 ± 0.33 *	1.56 ± 0.22 *
10 (D)	3.72 ± 0.19	3.67 ± 0.31	3.78 ± 0.19	3.78 ± 0.31
25 (E)	3.81 ± 0.24	3.72 ± 0.16	3.75 ± 0.31	3.69 ± 0.28
50 (F)	3.77 ± 0.27	3.69 ± 0.29	3.69 ± 0.39	3.74 ± 0.19

Note: The data are expressed as the mean ± SD (n=3). The results are significantly different at $p< 0.05$.

Antiproliferation assay

Cell proliferation reagent WST-1 was used to evaluate the antiproliferative activity of standardized γ-oryzanol rich extracts. The antiproliferative activity of γ-oryzanol rich extracts on HT-29, HCT116, MDA-MB-468 and PC3 cell lines were observed after incubation at 72 h. All samples significantly decreased viability of HT-29, HCT116, MDA- MB-468 and PC3 cell lines in a dose-dependent manner. As shown in Table 3, γ-oryzanol rich extracts from *GAM THOR* exhibited the highest antiproliferative activity against all human cancer cell lines followed by *GAM DOI MUSUR* and *GAM SUKHOTHAI-2*, respectively. The antiproliferative activity of γ-oryzanol rich extracts were lower than positive control cisplatin, which is an anti-neoplastic and potent cytotoxic drug. On the other hand, the activity was better than control γ-oryzanol.

Table 3. 50% Inhibition concentration of standardized γ-oryzanol rich extracts on human cancer cell lines viability.

Sample	50% Inhibition concentration (μg/ml)			
	HCT 116	HT-29	MDA-MB-468	PC3
GAM SUKHOTHAI-2	48.29 ± 5.63^{d}	63.58 ± 3.09^{d}	89.55 ± 3.85^{d}	65.39 ± 4.81^{c}
GAM DOI MUSUR	43.43 ± 5.08^{c}	55.52 ± 2.16^{c}	76.91 ± 2.97^{c}	53.53 ± 4.43^{b}
GAM THOR	40.58 ± 5.69^{b}	52.18 ± 1.21^{b}	48.59 ± 2.40^{b}	51.61 ± 1.30^{b}
Cisplatin	2.56 ± 0.61a	5.98 ± 0.91^{a}	5.98 ± 0.88^{a}	3.83 ± 0.74^{a}
Standard γ-oryzanol	79.84 ± 6.43^{e}	88.53 ± 4.72^{e}	113.31 ± 4.32^{e}	97.45 ± 5.32^{e}

Note: Data was expressed as mean ± SD. n=3. Mean values within a column superscripted by the same letter are not significantly different at $p < 0.05$.

DISCUSSION

The γ-oryzanol rich extracts from Thai purple rice bran (*GAM THOR, GAM DOI MUSUR and GAM SUKHOTHAI-2*) contain high amounts of the bioactive compound γ-oryzanol, which showed a protective effect on oxidative DNA damage and significantly inhibited colon, breast and preactive oxygen speciestate cancer cells by suppressing cell proliferation. The γ-oryzanol contents of γ-oryzanol rich extracts from Thai purple rice bran varied from 1.17 – 7.54 % w/w according to cultivar, topography and environment. This finding is consistent with the reports of Miller et al., (2003), who reported that the variation of γ-oryzanol can depend on the genotype and environment. As shown in Figure 1(B), γ-oryzanol rich extracts are composed of five major components and five minor components. This is similar to the report described by Xu and Godber (1999), who reported that γ-oryzanol is a mixture of phytosteryl ferulate esters, which is composed of ten compounds. Four of these, cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campesteryl ferulate and β-sitosteryl ferulate, are the major components of γ-oryzanol. The γ-oryzanol determination method using reversed-phase HPLC, which was described by Xu and Godber (1999), took 30-40 minutes for stop page time. In the present study, we modified the stationary phase by using the Kinetex C18 column instead of the classical C18 column. In this method, the stop page time was reduced to 10 minutes and the flow rate was reduced from 1.4 to 0.3 ml/min, resulting in improved sensitivity and reduced solvent consumption.

It is known that iron chelators inhibit hydroxyl radicals-medicated *in vitro* DNA damage by binding Fe(II) or preventing its activity in the Fenton reaction (Kim and Park, 2004). Plasmid DNA is composed mostly of supercoiled form and a small amount of the relaxed open-circular form. Plasmid DNA is sensitive to damage caused by a variety of agents, especially oxidants or free radicals. When cleavage of one of the phosphodiester chains of the supercoiled DNA occurs, it produces a relaxed open-circular form. Therefore, determining the proportion of supercoiled and relaxed open-circular forms provides an indication of the amount of oxidative DNA damage that has occured. If γ-oryzanol rich extracts inhibit or reduce the hydroxyl radicals-associated with the Fenton reaction, the DNA strand

breakage should be decreased. In the present study, γ-oryzanol rich extracts exerted a moderate protective effect on DNA damage induced by Fenton reaction when compared to standard quercetin. In general, the mechanism of phytochemicals to prevent oxidative DNA damage induced by Fenton reaction may be either by chelating metal ions, which inhibit hydroxyl radicals generated from Fenton reaction (Halliwell and Gutteridge, 1990), or providing hydrogen atoms from their phenolic hydroxyl groups to scavenge hydroxyl radicals produced from Fenton reaction (Shahidi and Wanasundara, 1992) before attacking the supercoiled form of plasmid pUC18 DNA to produce relaxed open-circular form.

γ-Oryzanol rich extracts exhibited antiproliferative properties in a dose-dependent manner against colon (HCT 116, HT-29), breast (MDA-MB-468) and preactive oxygen speciestate (PC3) cancer cells. The antiproliferative activity of γ-oryzanol rich extracts were less than the standard cisplatin, an anti-neoplastic and potent cytotoxic drug that binds to DNA and non-DNA targets and induces cell death through apoptosis or necreactive oxygen speciesis. In fact, the γ-oryzanol is a mixture of phytosteryl ferulate esters, a non-polar compound. It is possible these phytosteryl ferulate esters can penetrate through the cell membrane due to their high lipophilicity property and then are decomposed by esterase enzyme to generate free ferulic acid and phytosterols, especially cycloartenol, 24-methylene-cycloartanol, campesterol and β-sitosterol. The results correlate with other studies that revealed that β-sitosterol inhibits the growth of HT-29 human cancer cells by activating the sphigomyelin cycle without effecting phospholipase C, a key cycle in the PKC pathway (Awad et al., 1996). β-sitosterol and campesterol inhibits the growth and initiated apoptosis of MDA-MB-231 human breast cancer cells (Awad et al., 2007). Furthermore, ferulic acid inhibits the proliferation of colonic Caco-2 cells, the result of S phase prolongation and altered cell cycle distribution (Janicke et al., 2005). The present finding indicated that γ-oryzanol rich extracts from Thai purple rice bran exhibited a protective effect on oxidative DNA damage and inhibited the proliferation of HT-29, HCT 116, MDA-MB-468 and PC3. To understand the mechanism of actions on antiproliferative activity against human cancer cell lines, further investigation at the molecular level is necessary.

CONCLUSION

This study indicates that Thai purple rice bran extracts, standardized to contain 10% w/w γ-oryzanol, clearly exhibited a moderate protective effect on oxidative DNA damage induced by Fenton reaction. Furthermore, they also exerted an antiproliferative activity against four human cancer cell lines (HCT 116, HT-29, MDA MB-231 and PC3). The results from these findings indicated that γ-oryzanol rich extract might be proposed as a dietary supplement for prevention and/or treatment of conditions that occur due to oxidative damage, especially oxidative DNA damage.

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