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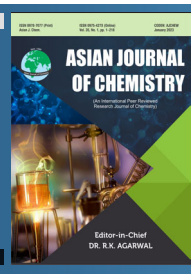

Effect of γ-Irradiated Chitosan to Enhance Antioxidant Activity of Khai Mod Rin Germinated Brown Rice

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Khai Mod Rin has been certified as NSRC95001-1-3 grower community enterprise in Hua Sai district, Nakhon Si Thammarat, Thailand. The effect of γ-irradiated chitosan concentrations and soaking time was examined for a proper conditions of germination. The standard phytochemical screening method and quantitative analysis of DPPH, ABTS, FRAP, total phenols and flavonoids, were also evaluated. The Khai Mod Rin germinated brown rice (KMR-GBR) with the best antioxidant activity and highest in total phenolic and flavonoid contents was produced after exposure to γ-irradiated chitosan (500 ppm concentration) at room temperature for 36 h germination. Phytochemicals including alkaloids, terpenoids, coumarin, flavonoids and cardiac glycosides were detected still the same as germination in water and pure chitosan. Evidently, γ-irradiation on chitosan can be used to enhance antioxidant activity during brown rice germination, which is a potential source of natural antioxidants for food processing.

Keywords: Antioxidant activity, γ-Irradiated chitosan, Khai Mod Rin rice, Germinated brown rice, Nakhon Si Thammarat.

INTRODUCTION

Khai Mod Rin, also called scientifically as *Oryza sativa* L., is the native rice certified as NSRC95001-1-3 grower community enterprise in Hua Sai district, Nakhon Si Thammarat, Thailand [1,2]. Farm management, rice production process and products derived from both of white and brown rice have been developed by educational institutes to promote competency of community participation [3-5]. Recently, the aqueous extract of Khai Mod Rin rice (KMR) showed its antioxidant activity, while antimicrobial activity was only expressed in its ethanol extract [6]. However, KMR rice exhibited negligible biological activity including both antioxidant activity and antimicrobial activity. Modification of brown rice to germinated brown rice is needed to be easier to consume and nutritional enhancement, such as vitamins, minerals, dietary fibers, essential amino acids and more bioactive compounds [7]. Risk of cancer, diabetes, cardiovascular disease and Alzheimer's disease can be reduced by changing in lifestyle with respect to diet, such as germinated brown rice and its products.

Chitosan is a biodegradable and non-toxic polysaccharide derived from chitin [8]. It can be applied in food technology, pharmaceutical and medical biomaterials due to its low allergenicity, non-toxicity, biocompatibility and biodegradability [9-12]. Chitosan and its derivatives offer greater physical properties including high surface area, porosity, tensile strength and conductivity, which can be molded to get different shapes and forms. Chitosan is natural polymers which is extracted from chitin in shrimp shell, crab shell and squid core. It contains an amino and several hydroxyl groups, which can react with free radicals exhibiting scavenging ability. Therefore, different methodologies have been used to determine chitosan and its derivatives antioxidant assays [13]. Ability of chitosan to increase significantly antioxidant contents in Sung Yod Phatthalung rice has been reported [14]. In chitosan's structure, functional groups including a primary amino group, primary and secon-

dary hydroxyl groups, glycosidic bonds and the acetamide group, are capable of being chemically modified, producing polymers with new properties and behaviours [13]. γ-Irradiation on chitosan provides plant growth promotion [15]. Most research articles focus on germinated brown rice containing high levels of γ-aminobutyric acid (GABA) stimulated by chitosan application and other treatment methods in fermentation process [16-19]. Definitely, chitosan is considered as the safety raw material and solutions for its application of food industry [20].

This work aims to assess the effect of γ-irradiated chitosan application during fermentation process whether antioxidant activity of germinated brown rice produced from the local rice *i.e.* Khai Mod Rin, can be enhanced effectively. Parameters affecting rice germination included chitosan concentration and soak-ing duration. Phytochemical compositions, *in vitro* antioxidant and free radical scavenging potential of the given brown rice germinated with γ-irradiated chitosan were also investigated.

EXPERIMENTAL

All the chemicals in analytical grade were used and most of them was supplied by Merck and Sigma-Aldrich, USA. Ethanol, methanol, HCl, H_2SO_4, iodine, potassium iodide, acetic acid, 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ) and Na_2CO_3 were purchased from Merck, USA. Ascorbic acid, gallic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich, Germany. γ-Irradiated chitosan (at sterilizing doses of 40 kGy) was supplied by Thailand Institute of Nuclear Technology (Public Organization).

Preparation of chitosan solution: Three aqueous solutions of chitosan and γ-irradiated chitosan with concentrations 300, 400 and 500 ppm in the presence of 1% acetic acid (20 mL) were prepared separately.

Preparation of germinated brown rice: A good quality brown rice, Khai Mod Rin, was selected for germination. Germinated brown rice (GRB) was prepared by varying concentrations of both chitosan and γ-irradiated chitosan and soaking time. After washing, placing 250 g of brown rice in a container, covering it with 300 mL of water and then soaking it at room temperature (not higher than 30 ºC) for 12, 24 and 36 h. The experiments were performed in triplicate. Correspondingly, chitosan and γ-irradiated chitosan solutions with different concentrations (300, 400 and 500 ppm) were used instead of water. The germinated brown rice dried at 60 ºC for 4 h was pulverized to rice flour and storing at room temperature before analysis.

Preparation of ethanolic extract: The rice flour (200 g) was soaked in 95% ethanol (250 mL) for 5-7 days. After filtration and solvent removal, the crude extract was stored in an amber bottle wrapped with aluminium foil. The results were expressed in the percentage of the crude extract.

Detection methods

Phytochemical screening of crude extracts: Components of the crude extract including alkaloids, steroids, terpenoids, coumarins, anthraquinone, flavonoids, saponins, tannins and cardiac glycosides were identified by standard methods [21]. The qualitative results are specified as (+) for the presence and (–) for the absence of phytochemicals.

Test for alkaloids: Exactly 0.2 g of crude extract was dissolved in 1 mL of 1.5% HCl. The mixture was then warmed on stream bath for 5 min and filtered. After adding Wagner's reagent, yellow precipitate was observed indicating the presence of alkaloids.

Test for steroids: The crude extract was dissolved in 1 mL of dichloromethane. Filtrate was added with 0.5 mL glacial acetic acid and conc. H_2SO_4. If colour turns blue, it composed of steroids.

Test for anthraquinone: The exact amount of the crude extract was added with 10% H_2SO_4, warmed and filtered. The filtrate was added with 0.5 mL of 10% NH_3 and red colour occurred indicates the presence of anthraquinone.

Test for terpenoid: The crude extract was dissolved in 1 mL of dichloromethane and then filtered. Concentrated H_2SO_4 was added to the filtrate to form lower layer. A brown colour at the interface suggests the presence of terpenoid.

Test for coumarin: The crude extract was dissolved in 1 mL of 50% ethanol and then filtered. The filtrate was added with 1 mL of 6 M NaOH. The presence of coumarin when its colour turns yellow.

Test for flavonoids: The crude extract was dissolved in 1 mL of 50% ethanol and filtered. A piece of magnesium chip was then added to the filtrate followed by a few drops of conc. HCl. The presence of flavonoids when its colour turns purple.

Saponin foam test: The crude extract was added with 5 mL of distilled water and warmed for 5 min. The filtrate was shaken vigorously. Frothing which persisted on warming was taken as evidence for the presence of saponins.

Test for tannins: The crude extract was dissolved in 1 mL of distilled water, warmed and filtered. Few drops of 1% ferric chloride solution were added to 2 mL of filtrate. Tannins are present as occurrence of a blue-black, green or blue-green precipitate.

Test for cardiac glycosides: The crude extract was dissolved in 2 mL of chloroform and then filtered. The filtrate was added with 1% $FeCl_3$ and acetic acid (5 drops each). conc. H_2SO_4 (0.5 mL) carefully to the mixture to form lower layer. Formation of brown colour at the interphase of the two layers was a positive test for cardiac glycosides.

DPPH antioxidant assay: Antioxidant activity was estimated by DPPH radical scavenging assay (RSA) according to the previous report [22]. Different concentrations of sample solutions (5, 10, 15, 20 and 25 mg/L) were prepared from stock solution of crude extract (500 mg/L in 95% ethanol). Briefly, an ethanolic solution of DPPH (2 mL) was added to 1 mL of different concentrations of the sample and ascorbic acid. All mixtures were incubated in a dark at room temperature for 30 min and the absorbance was read at 516 nm. The RSA of DPPH was calculated as:

$$RSA = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ and A_{sample} are the absorbance of the negative control and the sample, respectively. The IC_{50} values were

calculated from the curve of RSA (%) *versus* concentrations of ascorbic acid. The results were expressed as inhibition concen-trations at fifty percent (IC_{50}).

FRAP antioxidant assay: The reducing power was estimated according to the method as described earlier [23]. The stock solution was mixed with 4.5 mL of FRAP reagent (300 mM acetate buffer (pH 3.6) + 10 mM TPTZ + 20 mM of $FeCl_3$ = 10:1:1 by volume). The mixture was incubated at 37 ºC for 4 min. The absorbance was read at 593 nm with UV-VIS spectrophotometer. Ferrous sulfate was used as a standard and the results were expressed as mg of ferrous ion equivalents per g crude extract (mg Fe(II)/g extract).

ABTS antioxidant assay: The free radical scavenging activity (RSA) was estimated according to the literature [24]. Five sample solutions were prepared in different concentrations (5, 10, 15, 20 and 25 mg/L) from stock solution of crude extract (500 mg/L in 95% ethanol). All mixtures (1 mL of each sample solution + 2 mL of ABTS) were kept in dark. After 10 min incubation, the absorbance was measured at 734 nm. The RSA for ABTS was calculated as:

$$RSA = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ and A_{sample} are the absorbance. The IC_{50} values were calculated from the curve of RSA (%) *versus* concentrations of the sample.

Determination of total phenolic content: Total phenolic content (TPC) was estimated by Folin-Ciocâlteu colourimetric assays [25]. The sample solution (25 mg/mL, 0.3 mL) was mixed with 10-fold dilution of Folin-Ciocâlteu phenol reagent (1.5 mL). After the addition of 7.5% Na_2CO_3 (1.2 mL), the mixture was mixed well and then allowed to stand for 30 min in dark at room temperature. Absorbance at 765 nm using UV-VIS spectrophotometer was measured and recorded. Gallic acid was used as a standard and the results were expressed as mg of gallic acid per g crude extract (mg GAE/g extract).

Determination of total flavonoid content: Total flavonoid content (TFC) was estimated by aluminum nitrate colourimetric method [26]. The sample solution (1 mg/mL in ethanol, 2 mL) was added with 0.15 mL of 5% $NaNO_2$, 1 mL of distilled water, 0.15 mL of 10% $Al(NO_3)_3$ and 1 mL of 1 M NaOH. After incubation for 30 min, absorbance at 510 nm using UV-Vis spectrophotometer was recorded. Quercetin was used as a standard and the results were expressed as mg of quercetin per g crude extract (mg QE/g extract).

Statistical analysis: Data are expressed as mean ± SD from each set of triplicate measurement. For different antioxidant assays, Pearson's correlation coefficient was used to analyze the differences among phenolic and flavonoid contents of GBR with chitosan treatment for 36 h. A probability of $p < 0.05$ was considered as significant.

RESULTS AND DISCUSSION

Germinated brown rice was prepared by varying soaking duration in the presence and absence of γ-irradiated chitosan solution in a range of 300-500 ppm. The best brown rice germination (Fig. 1) was shown by soaking with 500 ppm of irradiated chitosan solution at room temperature for 36 h. The germination part of the seed is the embryo of rice grain [27]. As chitosan is a natural antimicrobial agent [28] offer no water changing every 4-6 h during longer time germination at room temperature. After drying in hot air oven at about 60 ºC for 4 h, the fine powder of rice sample was kept for extraction.

The rice sample was macerated with 95% ethanol. After solvent removal, the rice extract was filtered with Whatman No. 1 filter paper. As a result, the percentage of extract yield is shown in Table-1. Although, the yield of ethanolic extract derived from germinated brown rice sample exposed to γ-irradiated chitosan was lower than the ones exposed to water and non-irradiated chitosan. The γ-irradiated chitosan concentration in the germination process would be chosen optimally based on its antioxidant potential.

TABLE-1
PERCENTAGE OF EXTRACT YIELD FROM DRIED RICE FLOUR AFTER CHITOSAN TREATMENT FOR 36 h

Chitosan treatment	Dried rice flour (g)	Crude extract (g)	Extract yield (%)
Control (water)	200	2.77	1.38
Chitosan (ppm)			
300	200	2.46	1.23
400	200	2.77	1.38
500	200	3.55	1.78
γ-Irradiated chitosan (ppm)			
300	200	1.72	0.86
400	200	1.99	0.99
500	200	2.01	1.00

Preliminary phytochemical screening of nine constituents in the crude extracts from KMR-GBR was examined by precipitation and spectrophotometric methods, and the results were shown in Table-2. It is found that all the crude extracts consisted of alkaloids, terpenoids, coumarin, flavonoids and cardiac glycosides.

Total phenolic compounds and flavonoid contents of the rice extracts obtained from the rice treated under different concentrations of γ-irradiated chitosan and non-irradiated chitosan for 36 h soaking duration are shown in Table-3. The content of phenolic compounds in the ethanolic extracts ranged from 5.22 to 8.88 mg GAE/g, representing an approximate two-fold variation. In Table-3, Khai Mod Rin GBR stimulated by 500 ppm of γ-irradiated chitosan showed the greatest phenolic content (8.88 mg GAE/g extract), which is better than the one soaked in 500 ppm chitosan solution (7.34 mg GAE/g extract) and in water (7.14 mg GAE/g extract). Furthermore, the greatest flavonoid content of the extract from Khai Mod Rin GBR stimulated by 500 mg/L chitosan (23.45 mg QE/g extract) and γ-irradiated chitosan (25.57 mg QE/g extract) solutions was observed. Similarly in a previous work, chitosan (500 ppm) could significantly increase the total polyphenol contents (220.75 mg GAE/100 g dry weight sample) and anthocyanin contents in the grain extracts of Sung Yod Phatthalung rice [10].

The DPPH and ABTS radical scavenging activities and the reducing power of Fe^{2+} were selected to evaluate antioxidant

Fig. 1. Germinated brown rice produced after stimulating an amount of Khai Mod Rin brown rice with 500 ppm irradiated chitosan at different time durations (a) 12 h, (b) 24 h and (c) 36 h, compared to the original seed

TABLE-2
PHYTOCHEMICAL SCREENING OF THE CRUDE EXTRACTS FROM GBR AFTER CHITOSAN TREATMENT FOR 36 h

Chitosan treatment for rice germination	Phytochemicals								
	Alkaloids	Steroids	Anthraquinone	Terpenoids	Coumarins	Flavonoids	Saponins	Tannins	Cardiac glycosides
Control (water)	Found	Not found	Not found	Found	Found	Found	Not found	Not found	Found
Chitosan (ppm)									
300	Found	Not found	Not found	Found	Found	Found	Not found	Not found	Found
400	Found	Not found	Not found	Found	Found	Found	Not found	Not found	Found
500	Found	Not found	Not found	Found	Found	Found	Not found	Not found	Found
γ-Irradiated chitosan (ppm)									
300	Found	Not found	Not found	Found	Found	Found	Not found	Not found	Found
400	Found	Not found	Not found	Found	Found	Found	Not found	Not found	Found
500	Found	Not found	Not found	Found	Found	Found	Not found	Not found	Found

TABLE-3
TOTAL PHENOLIC AND FLAVONOID CONTENTS OF CRUDE EXTRACTS FOUND IN GBR AFTER CHITOSAN TREATMENT FOR 36 h

Chitosan treatment for rice germination	Total phenolic content (mg GAE/g extract)	Total flavonoid content (mg QE/g extract)
Control (water)	7.14 ± 0.09	17.73 ± 0.02
γ-Irradiated chitosan (ppm)		
300	5.67 ± 0.05	20.12 ± 0.04
400	7.34 ± 0.05	22.79 ± 0.03
500	8.88 ± 0.10	25.57 ± 0.06
Chitosan (ppm)		
300	5.22 ± 0.01	19.64 ± 0.17
400	6.21 ± 0.02	20.88 ± 0.08
500	7.34 ± 0.05	23.45 ± 0.08

activity of Khai Mod Rind GBRs stimulated by chitosan and γ-irradiated chitosan for 36 h soaking time in each extract are presented in Table-4, compared with the IC_{50} value of standard ascorbic acid (6.47 mg/L). Rising in radical scavenging capacity were concentration-dependent in both chitosan and γ-irradiated chitosan. The greatest DPPH radical scavenging strength of with a minimum IC_{50} value was noted for Khai Mod Rin GBR stimulated by γ-radiated chitosan at 500 ppm (56.25 mg/L), followed by the GBR stimulated by γ-radiated chitosan at 400 (72.88 mg/L) and 300 ppm (82.28 mg/L). Moreover, antioxidant activity can be enhanced in the presence of γ-irradiation on chitosan used as stimulant in GBR production, which is better than only chitosan application. The greatest concentration of the sample required to scavenge 50% of the ABTS free radicals was expressed in the crude extract from Khai Mod Rin GBR stimulated with 500 ppm irradiated chitosan (IC_{50} = 108.37 mg/L). Similar to the radical scavenging activity, all the crude extracts displayed concentration-dependent reducing power. The greatest reducing antioxidant power (21.42 mg Fe(II) equivalents/g extract) was recorded for the ethanolic extract from brown rice germination stimulated with 500 ppm irradiated chitosan. The results suggest a good antioxidant natural source of Khai Mod Rin brown rice in the presence of irradiated chitosan stimulant at a proper concentration during fermentation process.

TABLE-4
ANTIOXIDANT ACTIVITIES OF CRUDE EXTRACTS FROM GBR AFTER CHITOSAN TREATMENT FOR 36 h

Chitosan treatment for rice germination	DPPH IC_{50} (mg/L)	ABTS IC_{50} (mg/L)	FRAP (mg Fe(II)/g extract)
Ascorbic acid	6.47 ± 0.00	10.93 ± 0.04	–
Water	9.38 ± 0.00	132.07 ± 0.00	21.32 ± 0.05
γ-Irradiated chitosan (ppm)			
300	82.28 ± 0.00	216.51 ± 0.00	20.01 ± 0.02
400	72.88 ± 0.01	150.88 ± 0.00	20.49 ± 0.04
500	56.25 ± 0.00	108.37 ± 0.00	21.42 ± 0.05
Chitosan (ppm)			
300	90.22 ± 0.01	220.67 ± 0.00	19.96 ± 0.03
400	74.58 ± 0.00	189.46 ± 0.01	20.16 ± 0.02
500	67.13 ± 0.00	134.84 ± 0.01	20.52 ± 0.01

A statistical measure of the strength of a linear relationship between quantitative variables is called the correlation coefficient (R-value) varying from –1 to +1. The correlation of total phenolic and flavonoid contents with antioxidant activity using DPPH, ABTS and FRAP assays of ethanolic extracts from GBR with chitosan treatment for 36 h at $p < 0.05$ as indicated in Table-5. Total phenolic content and antioxidant capacity (DPPH, R = -0.775; ABTS, R = -0.957; FRAP, R = 0.868) were correlated as TPC increases; antioxidant activity also tends to rise. As a result, the relationship of TPC and DPPH values was considered more expressive than the other two antioxidant activity methods. By mean of a negative correlation, values in a series of TPC content increasing as those in the other (DPPH or ABTS) decline. Since high FRAP values for the greatest reducing antioxidant power, the correlation between TPC and FRAP values was strongly positive as values in a series of TPC content rising as those in FRAP also increase. Total flavonoid content and antioxidant activity (DPPH, R = -0.963; ABTS, R = -0.531; FRAP, R = 0.272) were related in similar fashion. The relationship of TFC and ABTS values was more pointed than the other methods. However, weak positive correlation was found between TFC and FRAP values. By comparing the correlation coefficients, phenolic and flavonoid contents are accountable for the antioxidant activity of the ethanolic extract from Khai Mod Rin GBR, when it was stimulated with 500 ppm γ-irradiated chitosan.

TABLE-5
CORRELATIONS BETWEEN ESTIMATION OF ANTIOXIDANT ACTIVITY AND PHENOLIC AND FLAVONOID CONTENTS OF GBR WITH CHITOSAN TREATMENT FOR 36 h AT $p < 0.05$

Estimation of antioxidant activity	TPC	TFC
DPPH (IC_{50})	-0.775	-0.963
ABTS (IC_{50})	-0.957	-0.531
FRAP (mg Fe(II)/g extract)	0.868	0.272

Conclusion

The germination of Khai Mod Rin brown rice in the presence of 500 ppm irradiation chitosan solution for 36 h could be enhanced its antioxidant potential. It also contained five phytochemicals including alkaloids, terpenoids, coumarins, flavonoids and cardiac glycosides. Hence, the brown rice germination process using γ-irradiated chitosan could be considered as an alternative method to promote health product.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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