In-vitro Free Radicals Scavenging and Antioxidant Activity of Rice Bran Extract

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ABSTRACT

Aim: To evaluate the antioxidant activities of the γ-oryzanol isolated from rice bran oil of Indian origin in various in vitro models. Materials and Methods: The hydrogen donating ability of the hydroalcoholic extract of γ-oryzanol was measured in the presence of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical. Results: A 100 μg mL⁻¹ of γ-oryzanol and ascorbic acid exhibited 64.56 and 64.88% inhibition, respectively and the IC₅₀ values were found to be 48 and 56.50 μg mL⁻¹ for γ-oryzanol and ascorbic acid respectively. The effect of γ-oryzanol on reducing power was studied according to the reaction of Fe³⁺ to Fe²⁺. The reducing power of the extract increased with the increasing amount of the concentration. The total polyphenolic content of the γ-oryzanol was tested using Folin-Ciocalteau reagent. It was found that γ-oryzanol contained 19.31±1.79 mg of GA/g, which is significant (p<0.05) when compared to gallic acid. γ-oryzanol hydrogen peroxide (H₂O₂) radical at IC₅₀ values of 45 μg mL⁻¹ against the corresponding standards ascorbic acid (IC 50 = 35 μg mL⁻¹). Moreover, the results were observed in a concentration dependent manner. Conclusion: All the above in-vitro studies clearly designate that the hydroalcoholic isolated γ-oryzanol has a significant antioxidant activity.

Key words: Antioxidant, DPPH, γ-oryzanol, rice bran extract

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between beneficial and harmful effects of free radicals is a very key aspect of living organisms and is executed by mechanisms known as “redox regulation” (pro-oxidant-antioxidant homeostasis). The processes of “redox regulation” shields living tissues from numerous oxidative stresses formed within the cells and retain “redox homeostasis” via controlling the redox status in-vivo.40-48

Strenuous exercise causes oxygen utilization and that results into disturbance of intracellular redox regulation. The mitochondrial electron transport chains, polymorphonuclear, along with xanthine oxidase have been recognized as crucial sources of intracellular free radical generation centers during exercise. Reactive oxygen species constitute a significant defilement to the cellular antioxidant defense mechanism, like decreased storage of antioxidant such as vitamins and glutathione and increased tissue propensity to oxidative damage. Nevertheless, enzymatic and nonenzymatic antioxidants have signified great familiarization to acute and chronic exercise. The important balance between pro-oxidants and antioxidants propose that supplementation of antioxidants may be useful for physically active individuals under certain physiological conditions by imparting a larger protective margin. Research from human and animal studies proved that strenuous physical exercise may produce a state wherein the antioxidant defense mechanisms of several tissues are affected by excess reactive oxygen. A wide range of physiological and dietary antioxidants exploit in concert to elude such a stress. Regular physical activity in combination with dietary supplement that provide adequate supply of a combination of appropriate antioxidants may be anticipated to yield desirable outcome.48

Ramarathnam in 1989 first identified and reported that the components of rice bran as flavonoid, isovitexin, α-tocopherol and γ-oryzanol possess high antioxidant activity. γ-oryzanol is composed of mixture of phytosterol ferulates and comprises of cycloartenyl ferulates, 24-methylene cycloartanyl ferulate and campesteryl ferulate. This mixture has been studied for antioxidant activity and this effect has been achieved as it enables donation of electron and destroys the action of reactive oxygen species and reactive nitrogen species. γ-oryzanol blocks pyrogallol autoxidation moreover, γ-oryzanol is more effective than the synthetic antioxidants against hydroperoxide formation. Also, reports suggested that γ-oryzanol inhibits the cholesterol oxidation and prevents tissues through stabilization of cellular metabolism preventing disease formation.60 But the reports of the γ-oryzanol isolated from Indian rice bran extract are less about its antioxidant property. So, the main objective of the present study was to isolate γ-oryzanol from Indrayani rice bran of Indian origin and to study the antioxidative potencies, scavenging activities against DPPH radical, reducing power and estimation of polyphenolic contents.

MATERIALS AND METHODS

Chemicals: Standard γ-oryzanol was procured from Oryza Oil and Fat Chemical Co. Ltd. Japan. Toluene, ethyl acetate, methanol and other analytical grade reagents and chemicals were used in the study are purchased from Merck Specialties Private Limited, Mumbai, India. Double distilled water filtered via 0.45 μ filter paper was utilized in the research work. Microliter syringe (Hamilton, Bonaduz, Switzerland) was acquired from Anchrome Enterprises (I) Pvt. Ltd Mumbai, India. Precoated Thin Layer Chromatographic (TLC) silica gel plates were purchased from Merck, Bangalore, Karnataka, India, of Kieselgel 60, F-254, 0.2 mm. Precoated glass silica gel plates were utilized for preparative TLC. High-Performance Thin-Layer Chromatography (HPTLC) spectra analysis was carried out on a Linomat V (Camag, Muttenz, Switzerland). Butylated hydroxytoluene (BHT), L-ascorbic acid, gallic acid, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), potassium ferricyanide, 2-deoxyribose, trichloroacetic acid (TCA) and ferric chloride were obtained from Sigma-Aldrich Co. LLC. Mumbai, India.

Extraction and isolation of γ-oryzanol Rice Bran Oil (RBO): The extraction of rice bran oil was carried out according to previously reported method.61 Shortly, rice bran of Indrayani variety of rice was acquired from Radhe Shyam Poha mill, Pune. This bran was kept in a refrigerator before its use. Direct solvent extraction method was applied for getting crude rice bran oil from 1 kg rice bran. Petroleum ether (60-80) was used as the solvent for extraction and the solvent was properly kept for 24 h for getting more yields. This procedure was performed with same rice bran with fresh petroleum ether three times for getting more yields. These obtained three fractions were mixed with each other to get one petroleum fraction. The resultant fraction then concentrated on rotary evaporator to get oil. The percentage yield was 10.4% of petroleum ether extract.
This collected rice bran oil was kept in a refrigerator before its use. From this, 3 mL oil was mixed with 5 mL absolute ethanol. Ethanol-oil mixture was further vigorously mixed by using cyclo-mixer and kept aside for 5 min. The supernatant was collected. The same procedure was repeated four times, so that 25 mL of supernatant was obtained. All the ethanol fractions were mixed and concentrated on rotary evaporator. The resultant concentrate was used for HPTLC spotting, mixed and concentrated on rotary evaporator. The supernatant was obtained. All the ethanol fractions were precipitated and the procedure was repeated four times, so that 25 mL of supernatant or precipitate was obtained and all the ethanol fractions were further precipitated.

**Chromatography:** A Camag High-pressure Thin-Layer Chromatography (HPTLC) system equipped with an automatic TLC sampler (Linomat 5) with TLC scanner 3 and integrated software Win-Cats version 4 was used for the analysis. The HPTLC was implemented on a precoated silica gel HPTLC 60F254 (20×10 cm) plate for the quantification of γ-oryzanol. Oil (lower layer) remained in the test tube was dissolved in 5 mL ethyl acetate to verify the complete extraction of γ-oryzanol.

**In-vitro antioxidant activity of 24-mCAF isolated from rice bran oil**

**Free radical scavenging activity in 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) assay:** The antioxidant activity in terms of hydrogen donating or radical-scavenging capability using the stable radical DPPH. The DPPH solution of 0.1 mM was prepared in ethanol and from this 1 mL was added to γ-oryzanol solution in ethanol at varying concentrations ranging from 10-100 µg mL⁻¹. These test solutions were kept at 37°C for incubation for 30 min. After 30 min of incubation, the absorbance of sample solutions was measured at 517 nm using ethanol as blank. Ascorbic acid was used as positive control for the study. Lower absorbance of reaction mixture stipulates greater free radical scavenging activity. To get exact scavenging capacity of γ-oryzanol following mathematical formula has been used:

\[
\text{DPPH scavenged} (\%) = \left( \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \right) \times 100
\]

where, \(A_{\text{control}}\) indicates the absorbance of control reaction while \(A_{\text{test}}\) is for the absorbance in the presence of γ-oryzanol. The antioxidant capacity of the γ-oryzanol was manifested as IC<sub>50</sub>. Here, IC<sub>50</sub> value was described as the concentration of γ-oryzanol in µg mL⁻¹ that inhibits the formation of DPPH radicals by 50%.

**Reducing power assay:** Various concentrations of γ-oryzanol (10-100 µg mL⁻¹) of 1 mL were mixed with 2.5 mL of 0.2 M sodium phosphate buffer of pH 6.6 plus 2.5 mL of 1% potassium ferricyanide. The mixture was kept for incubation at 50°C for 20 min. After incubation, 2.5 mL of 10% trichloroacetic acid (w/v) was added followed by centrifugation at 3000 rpm for 10 min. The upper layer 2.5 mL was taken and mixed with 2.5 mL deionised water plus 0.1% of 0.5 mL of ferric chloride was added. The absorbance of final mixture was analyzed on UV spectrophotometer at 700 nm by using sodium phosphate buffer having pH 6.6 with 0.2 M strength as blank solution. Higher absorbance at 700 nm denotes higher reducing power. The assay was performed in triplicate. Reducing power is indicated in Ascorbic Acid Equivalent (ASE) with unit ASE/mL that shows amount of ascorbic acid expressed in milli-Moles those reducing power is the same than that of 1 mL of sample.

**Determination of total phenolic content:** A 0.5 mL of sample i.e., 24-mCAF was mixed with 0.5 mL of Folin and Ciocalteu’s phenol reagent. Above mixture was mixed thoroughly and was kept aside for three minutes. After 3 min, 3 mL of 2% sodium carbonate solution was added to the mixture. Final volume at 10 mL of mixture was made by adding sufficient amount of distilled water. This final mixture was allowed to stand for 2 h with intermediate shaking. The absorbance of the blue colored solution was read at 760 nm. Total phenolic content of 25-mCAF was performed in triplicate. The concentration of total phenolic compounds in the gallic acid was measured in terms of µg of gallic acid equivalent using an equation acquired from the standard gallic acid graph.

\[
\text{Absorbance} = 0.0008 \times \text{gallic acid (µg)}
\]
Hydrogen peroxide scavenging assay: The assay was done by adding 0.1 mL of 1 mM of EDTA, 0.01 mL of 10 mM FCl₃, 0.1 mL of 10 mM H₂O₂ and 0.36 mL of 10 mM deoxyribose along with 1.0 mL of different dilutions of 24-mCAF (10-100 µg mL⁻¹) dissolved in ethanol plus 0.33 mL of phosphate buffer of pH 7.4 of strength 50 mM and 0.1 mL of ascorbic acid in sequence. The mixture was kept for incubation at 37°C aside for 1 h. for the development of pink color to the incubated mixture was taken 1 mL and mixed with 1.0 mL of 10% TCA and 1.0 mL of 0.5% TBA (in 0.025 M NaOH containing 0.025% BHA). The absorbance of the final mixture was read at 532 nm. The hydroxyl radical scavenging activity of the compound is reported in terms of % inhibition of deoxyribose degradation and is calculated as per following formula:

\[ \text{H}_2\text{O}_2 \text{ scavenged (\%) = } \left( \frac{A_{\text{cont}} - A_{\text{test}}}{A_{\text{cont}}} \right) \times 100 \]

where, “A cont” is absorbance of the control reaction whereas “A test” is the absorbance in the presence of the sample of the compound.

Statistical analysis: Experimental results were expressed in Mean±SD of three measurements. The values p<0.05 recorded were considered as significant.

RESULTS AND DISCUSSION
Preparative HPTLC of petroleum extract: The method of extraction of phytochemicals from rice bran is the most significant route in the progress of pharmaceutical development of any plant species which is identified or described to have medicinal value. Preparative HPTLC is one of the simplest, robust and precise and yet highly steadfast procedure in the desire of collecting pure isolated compound from the rice bran extract. The rice bran extract revealed the brown fluorescent band and light brown band at Rf 0.72 for \( \gamma \)-oryzanol under the TLC scanner at 317 nm. The clear, bright fluorescent band was confirmed by comparing with the bands of standard \( \gamma \)-oryzanol and having same Rf value was scarped and dissolved in absolute ethanol and filtered. Ethanol was evaporated under vacuum and \( \gamma \)-oryzanol was isolated. The same procedure was repeated to get sufficient quantity of \( \gamma \)-oryzanol.

In-vitro antioxidant activity of isolated compound: A wide range of in-vitro schemes have been used to evaluate radical scavenging ability and antioxidant activity of a compound. Antioxidant capacity is broadly applied as a criterion for medicinal bioactive components⁶²,⁶³. Different artificial entities have been utilized like 1, 1-diphenyl-2-picrylhydrazyl (DPPH), OH radicals scavenging activity, Fe³⁺–Fe²⁺ reducing activity, ferrous ions (Fe²⁺) chelating activity, H₂O₂ scavenging activity. In this study, the antioxidant activity of the \( \gamma \)-oryzanol was studied. The antioxidant activity of the \( \gamma \)-oryzanol has been studied in a succession of in-vitro tests namely: DPPH, free radical scavenging, reducing power, scavenging of superoxide anion radical-generated non-enzymatic system, hydrogen peroxide scavenging and metal chelating activities.

Inhibition of DPPH radical: With this method, it was promising to conclude the antiradical power of an antioxidant by measuring of a decrease in the absorbance of DPPH⁺ at 517 nm. Color change from purple to yellow, the absorbance decreased when the DPPH⁺ was scavenged by an antioxidant during donation of hydrogen to form a stable DPPH⁺ molecule. In the radical form, this molecule had an absorbance at 517 nm, which vanished after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule. Table 1 demonstrate a significant (p<0.05) reduce in the concentration of DPPH radical due to the scavenging ability of isolated \( \gamma \)-oryzanol and the standard ascorbic acid as a reference compound, conferred the maximum activity at all concentrations. A 100 µg mL⁻¹ of \( \gamma \)-oryzanol and ascorbic acid exhibited 64.56 and 64.88% inhibition, respectively and the IC₅₀ values were found to be 48 and 56.50 µg mL⁻¹ for \( \gamma \)-oryzanol and ascorbic acid, respectively.

<table>
<thead>
<tr>
<th>Concentration (µg mL⁻¹)</th>
<th>Inhibition (%) (Extracts)</th>
<th>Inhibition (%) (Standard)</th>
</tr>
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<tbody>
<tr>
<td>10</td>
<td>22.42±0.257</td>
<td>33.14±0.150</td>
</tr>
<tr>
<td>20</td>
<td>27.14±0.144</td>
<td>48.93±0.076</td>
</tr>
<tr>
<td>40</td>
<td>44.96±0.160</td>
<td>62.93±0.117</td>
</tr>
<tr>
<td>60</td>
<td>50.10±0.163</td>
<td>70.58±0.139</td>
</tr>
<tr>
<td>80</td>
<td>61.32±0.175</td>
<td>76.87±0.127</td>
</tr>
<tr>
<td>100</td>
<td>64.46±0.122</td>
<td>81.53±0.125</td>
</tr>
</tbody>
</table>

Data is presented as the percentage of DPPH radical scavenging, Mean±SD.
Reducing ability: The reducing capability of a compound generally depends on the presence of reductants which have been demonstrating antioxidant potential by breaking the free radical chain and donating a hydrogen atom. The presence of reductants in γ-oryzanol causes the reduction of the Fe³⁺/ferricyanide complex to the ferrous form. Hence, the Fe²⁺ can be monitored by measuring the production of Perl’s Prussian blue at 700 nm. Figure 1 shows the reductive capabilities of the “γ-oryzanol” extract compared to ascorbic acid. The results on reducing power reveals the electron donor properties of γ-oryzanol thus neutralizing free radicals by forming stable products. The outcome of the reducing reaction is to conclude the radical chain reactions that may otherwise be very damaging.

Amount of total phenolic compounds: Phenolic compounds are identified as powerful chain breaking antioxidants. Phenols are very significant plant constituents because of their scavenging capacity owing to their hydroxyl groups. The phenolic compounds may provide directly to antioxidant action. It is recommended that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when ingested up to 1 g daily from a diet rich in fruits and vegetables. The total phenolic content expressed in terms of Gallic Acid Equivalent (GAE) and was found to be 19.31±1.79 mg of GA g⁻¹. The total phenolic contents were calculated using the following linear equation based on the calibration curve of gallic acid:

\[ y = 0.0025X + 0.013, \text{ } R^2 = 0.964 \]

where, \( A \) is absorbance and \( X \) is amount of gallic acid in \( \mu g \).

Inhibition of hydroxyl radical: The highly reactive hydroxyl radicals can origin oxidative damage to DNA, lipids and proteins. The effect of γ-oryzanol on the inhibition of free radical-mediated deoxyribose damage was evaluated by means of the Iron (II)-dependent DNA damage assay. The Fenton reaction creates hydroxyl radicals (OH) which demean DNA deoxyribose, using Fe²⁺ salts as an important catalytic component. Oxygen radicals may assault DNA either at the sugar or the base, giving production of large number of products. Figure 2 shows the effect of the γ-oryzanol on the Iron (II)- dependent deoxyribose damage. The γ-oryzanol was the capable of sinking DNA damage at all concentrations. (IC₅₀ = 53 µg mL⁻¹). Ascorbic acid, used as a standard was highly effective in inhibiting the oxidative DNA damage, showing an IC₅₀ = 38.50 µg mL⁻¹.

Inhibition of H₂O₂ radical: Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cell since it may give mount to hydroxyl radical in the cells. Therefore, removing H₂O₂ as well as O₂⁻ is very significant for protection of food systems. The scavenging ability of γ-oryzanol on hydrogen peroxide is shown in Fig. 3 and compared with that of ascorbic acid as standard. γ-oryzanol at 45 µg mL⁻¹ and α-tocopherol
Fig. 3: Inhibition of H$_2$O$_2$ radical by γ-oryzanol isolated from rice bran oil. Each value is expressed as Mean±SD (n = 3)

at 35 µg mL$^{-1}$ exhibited 50% scavenging activity, respectively, on H$_2$O$_2$ radical. There was statically significant correlation between γ-oryzanol value and standard (p<0.05).

CONCLUSION

According to data obtained from the present study, γ-oryzanol isolated from Indian rice bran oil extract was found to be an effective antioxidant in different in-vitro assay including reducing power, DPPH radical. Hydrogen peroxide scavenging and metal chelating activities when it is compared to standard antioxidant compounds such as BHT; γ-oryzanol plays equal role as natural antioxidant. Based on the discussion above, it can be applicable for minimizing and preventing lipid oxidation in food products, retarding the formation of toxic oxidation products, maintaining nutritional quality and lengthening the shelf life of foods and pharmaceuticals.

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