TO EVALUATE ANTIOXIDANT ACTIVITY OF \( \gamma \) – ORYZANOL EXTRACTED FROM RICE BRAN OIL

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ABSTRACT

Rice bran oil is unique among edible oils due to its rich source of nutritionally important phytoceuticals such as oryzanol, tocotrienols, tocopherols, etc. \( \gamma \) – Oryzanol is one of the components of rice bran oil which have the potential to be used in pharmaceuticals, cosmeceutical and nutraceutical. It is present in rice bran oil at a level of 1-2\% where it serves as natural antioxidant. In the present study, \( \gamma \) – oryzanol was extracted from rice bran oil in such a way that high yield product was obtained in the end. During the process, rice bran oil was treated with alkali to form soap stock followed by its acid treatment. After this, distilled water washing was given to the extracted product which is further crystallized. The crystallization was done by passing the product through crystallization solvents followed by deep freezer incubation (4°C). These treatments gave \( \gamma \) – oryzanol in the pure form. The crystallized form of \( \gamma \) – oryzanol was quantized at 315 nm that shows 33.6\% of \( \gamma \) – oryzanol content. There are various kinds of phytosterols and ferulic esters which comprise \( \gamma \) – oryzanol. The antioxidant property of \( \gamma \) – oryzanol is regarded as the sole reason of using it under various fields such as lowering cholesterol levels in human body. This antioxidant activity was demonstrated in the present study by different methods viz., DPPH scavenging assay, nitric oxide scavenging assay & reducing power assay. The results showed that \( \gamma \) – oryzanol has very high antioxidant activity due to which it could be implemented as one of the components in human diet to fight against many diseases that arises due to high cholesterol levels such as hypercholesterolemia, arteriosclerosis etc.

KEYWORDS: Rice Bran Oil, Phytoceuticals, \( \gamma \) – Oryzanol, Crystallization, Spectroscopy, Antioxidant

INTRODUCTION

Rice bran oil (also known as rice bran extract) is extracted from the germ and inner husk of rice, commonly called rice bran. The rice bran contains 10–23\% oil and (unlike oat bran) negligible amounts of water-soluble glucans and larger amounts of insoluble dietary fiber. It is notable for its high smoke point of 232 °C (450 °F) and its mild flavor, making it suitable for high-temperature cooking methods such as stir frying and deep frying. It is popular cooking oil in several Asian countries, including Japan and China. Many people have replaced their usual cooking oils with rice bran oil due to this higher nutritional value. In the studies it has been showed that administration of rice bran oil to both animals and human helped in lowering of cholesterol level to appreciable extent (Most et al., 2005; Cicero & Gaddi 2001). Rice bran oil is a rich source of natural antioxidants which can be used as free radical scavengers. It contains about 0.1–0.14\% vitamin E components and 0.9\%–2.9\% oryzanol. It is widely recognized that many diseases are due to the oxidative stress that results from an imbalance between formation and neutralization of free radicals. Oryzanol provides hydrogen for the neutralization of those free radicals. The rice bran oil antioxidants are very efficient in reducing low density lipoprotein and total serum cholesterol.
Vitamin E is a generic term for a group of four tocopherols (α, β, γ and δ) and four tocotrienols (α, β, γ and δ), of which α-tocopherol has the highest biological activity (Duvernay et al., 2005). γ - oryzanol is a class of nonsaponifiable lipids of rice bran oil (RBO). It was thought to be single compound when initially isolated; it is now known to be a fraction containing ferulate (4-hydroxy-3-methoxy cinnamic acid) esters of triterpene alcohols and phytosterols (Arab et al., 2011). Cycloartenyl ferulate, 24-methylenecycloartanyl ferulate and campeseryl ferulate are the three major components of γ –oryzanol and account for 80% of its total content. (Patel & Naik 2004; Pan et al., 2014). For purifying oryzanol, its crystallization is preferred. Crystallization of oryzanol is usually carried out using rice bran oil or by using rice bran oil soap stock as starting material. This is generally used as final step of oryzanol recovery after preliminary purification steps. During crystallization, the parameters considered are temperature, type of the solvent or mixture of solvents along with their proportions, rate of nucleation and rate of crystal growth (Chen & Cheng 2006; Narayan et al., 2005). Oryzanol is insoluble in water and leads to a poor bioavailability for its bad absorption in intestine, which limited its application in food and medical systems. Normally, the solubility limitations could be partially overcome by applying surfactants, co-solvents or new technology (Yu et al., 2006; Kim et al., 2010). The microemulsion is chosen to evaluate the antioxidant activity of rice bran oil and rice bran oil plus γ-oryzanol. The rice bran oil plus γ-oryzanol microemulsion shows higher antioxidant activity than rice bran oil microemulsion. Thus, γ-oryzanol contributes to antioxidant activity in rice bran oil (Bernardi et al., 2011; Xu & Godber 2001). Present study is one such attempt of isolation of oryzanol from rice bran oil.

**MATERIAL AND METHODS**

Rice bran oil was used as starting material of extraction of oryzanol, which was purchased from the market at Chandigarh, India. The purchased rice bran oil was made by Ricela brand named company by physical refining of rice bran. Ricela rice bran oil contained 1L (910g) of quantity in one tetra pack. Chemicals used were high purity and procured from HiMedia Laboratory Pvt. Ltd.

**Extraction of oryzanol from rice bran oil**

Soap stock solution prepared as described by Seetharamaiah and Prabhakar in 1986 with little modification. Briefly, 1000 g of rice bran oil was taken in a clean beaker and boiled at 65°C. 20% NaOH was added to it. The solution was stirred at 20-30rpm with the help of stirrer for 20 minutes. Resultant solution was turbid in nature and known as soap stock. After the formation of soap stock, the turbid solution was transferred to separating funnel for the separation of lipids and other undesirable content from desired solution. The solution was left in the separating funnel undisturbed for overnight. After overnight settlement, two layers were formed from which upper layer contained all the lipids and fats and lower one contained oryzanol. The lower layer containing oryzanol was kept in the beaker for further processing and upper layer was stored in the 500ml of container. The lower layer containing oryzanol was taken in a beaker and added distilled water to it in 1: 0.2 ratios. The beakers were stirred continuously for 20 minutes. After 20 minutes of stirring, thick and sticky product was formed, 20% HCl was immediately added to the product which released water containing impurities and the thicker layer was taken. After HCl treatment, entire liquid was removed from the beaker and distilled water was added to it in 1: 0.5 ratios. Then, the whole content was stirred continuously for 20 minutes with the release of light pink color liquid on first washing. Then, the pink colored liquid was removed and again added distilled water in the same proportion. Stirring was done for 20 minutes and water was removed after stirring. Washing with distilled water was repeated for more than one time for completely removal of all the impurities. After the distilled water washing, the oryzanol product was placed in the refrigerator for overnight. The oryzanol containing beaker was placed over hot plate for drying with continuous stirring. Initially, the heating was done at 60°C for half an hour followed by heating at 80°C to 100°C for one and half hour. Heating was continuously done till sticky product showed an oily appearance. The oily oryzanol product was left for some time for cooling and separation of any residual oil. After half to one hour, the product was cooled down and residual oil was settled at the bottom. The residual oil was removed with the help of separating funnel and collected the product i.e. oryzanol. Then, oryzanol was crystallized by the method given by
Rao et al., (2002) in their patent, with little modification. Briefly, acetone: methanol in 3:7 ratios was added to the product. Oryzanol content was determined by spectrophotometer, where 50 mg of dried oryzanol was taken and added 10 ml hexane to it. This sample was diluted again with hexane in 1:100 ratios and absorbance was taken at 314 nm.

Following equation is implicated to determined oryzanol content (Choudhary et al., 2013): -

\[
\text{Content of oryzanol (\%)} = \frac{\text{Absorbance of sample (E)} \times \text{Volume of hexane used/ Weight of sample (W)}}{\text{Extinction coefficient of sample (358.9)}}
\]

**HPLC analysis**

Reversed phase HPLC was performed for the determination of various components of oryzanol. HPLC machine Shimadu-Class-VP V6.14 SPI and column of phenomenex, 5\(\mu\) (250*4.6) mm were used. A standard was also run along with the sample of oryzanol. Oryzanol crystals were taken as sample. The mobile phase used during the procedure was methanol: acetonitrile: acetic acid in the ratios of 52: 45:3 (by volume). Isocratic system was run at the flow rate of 1.6 ml/min. Analytes were detected at 330nm (Xu & Godber, 2000) (Yoshie et al., 2009).

**Antioxidant activity of oryzanol**

Antioxidant activity of oryzanol was measured by **in-vitro** methods i.e. DPPH scavenging activity, Nitric oxide scavenging activity and reducing power assay. The assay was conducted in triplicate with ascorbic acid as standard. The DPPH assay was performed as described by Muthal et al., 2015 with little modification. Briefly, 2mg/ml oryzanol sample was prepared in ethanol and poured into different test tube of varying concentration ranges from 10 to 100µg/ml; to this add 1ml DPPH 0.1mM solution prepared in ethanol. This mixture was shaken and incubated at 37\(^{\circ}\)C for 30 minutes. After 30 minutes OD was taken at 517 nm. Ascorbic acid was taken as positive control. DPPH scavenging activity was calculated by:

\[
A_{\text{control}} - A_{\text{test}} / A_{\text{control}} \times 100.
\]

Where \(A_{\text{control}}\) represents reading of control reaction and \(A_{\text{test}}\) absorbance of the oryzanol.

The nitric oxide scavenging activity was assayed as described in Alam et al., 2013 with little modification. 2mg/ml of oryzanol solution was prepared in methanol and 0.5 ml of different concentration ranging from 10 to 500 mg/ml poured into different test tubes. In each test tube 2 ml of solution of sodium nitropusside was added, solution was prepared by dissolving 10mM sodium nitropusside in 0.5 ml phosphate buffer saline (pH 7.4) and incubated at room temperature for 150 minutes. After incubation time, 0.5ml Griess reagent [1 ml sulfanilic acid reagent (0.33% in 20% glacial acetic acid at room temperature for 5 minutes with 1ml of naphthylenediamine dichloride (0.1% W/V)] was added and incubated for 30 minutes. Absorbance was taken at 546nm.

The nitric oxide radical inhibition calculated by equation:

\[
\% \text{ inhibition of NO radical} = \frac{[A_0 - A_1]}{A_0} \times 100
\]

Where \(A_0\) represents absorbance before and \(A_1\) represent absorbance after reaction has been take plane with Griess reagent.

The reducing power assay was performed by 2mg/ml of oryzanol sample, made in methanol and poured to each test tube in the concentration from 10 to 500 mg/ml. 2.5 ml of normal saline (0.9%) and 2.5ml of potassium ferricyanide (1%) was added and incubated at 50\(^{\circ}\)C for 20 minutes. After incubation 2.5ml of chilled TCA (10%) was added and centrifuge for 10 minutes at high speed.
2.5 ml of distilled water was added to 2.5 ml of supernatant. To this, 0.5ml of 1% ferric chloride and absorbance was taken at 700nm after 5-10 minutes. The antioxidant activity was measured by plot between absorbance and sample concentration.

**STATISTICAL ANALYSIS**

Results are presented as mean value ± standard deviation (at least three replicate experiments).

**RESULTS AND DISCUSSION**

Before the crystallization, the isolated γ – Oryzanol was collected. It appeared thick, yellowish brown and sticky (Figure-1).

**Drying of γ – oryzanol**

For the drying purpose the product was heated at various temperature from 80°C to 140°C. Initially it showed dark oily appearance (Figure 2) just after temperature reached to 138°C, it stopped boiling and appeared dark brown in color.

**Crystallization of γ – oryzanol**

After the completion of drying the product was subjected to crystallization for the purification purpose. The product was started to crystallize in 5-10 minutes after cooling at 0°C to 4°C and growth of crystals was very rapid. The crystals were creamish in color at the starting of crystallization as shown in the Figure 3. But the color of crystals was turned to white by regular washing with crystallization solvent.

**Quantization of γ – oryzanol**

γ – Oryzanol content in the product was determined by using spectrophotometric analysis at 314 nm. The absorbance of the crystallized γ – oryzanol came out to be 0.606 at 315 nm. According to the absorbance, γ – oryzanol content was 33.76% in the crystallized sample calculated with the help of formula containing extinction coefficient of γ – oryzanol.

\[
\text{Content of oryzanol (%) = } \frac{\text{Absorbance of sample (E) x Volume of hexane used}}{\text{Weight of sample (W) x Extinction coefficient of sample (359)}}
\]

Where, Absorbance of oryzanol sample: 0.606, Volume of hexane:10 ml, weight of sample: 50 mg

**HPLC analysis**

HPLC was performed for determining the components of γ – oryzanol. As shown in Figure 3 and 4 the chromatogram is showing three potent peaks indicating the presence of 1) cycloartenyl ferulate, 2) 24-methylene cycloartenyl ferulate and 3) campesteryl ferulate. Our results are in corroboration with Sakunpak et al., 2014, this study has shown the presence of 1) cycloartenyl ferulate 2) 24-methylene cycloartenyl ferulate 3) campesteryl ferulate and 4) β-sitosteryl ferulate as evaluated by LC-MS/MS. Kim et al., 2013 studied 33 variety of pigmented rice and contents varies from 3.5 to 21.0 mg/100 g with a mean of 11.2 mg/100 g. Total ten components were observed in HPLC chromatogram which is comparable to our results and highest peaks are shown by cycloartenyl ferulate, 24-methylene cycloartenyl ferulate and campesteryl ferulate.

**Antioxidant activity of oryzanol**

Our results shown in figure 5 revealed the relationship between the DPPH activity and concentration of oryzanol. The DPPH scavenging activity was found to be concentration dependent. At concentration 10µg/ml the % scavenging activity was 5.09% and as the concentration raised from 10 µg/ml to 100µg/ml the activity was increased to 60.51%. Akiyama et al., in 2005 studied free radical scavenging activity of γ – oryzanol constituents identified by NMR and MS as cycloartenyl ferulate, 24-methylene cycloartenyl ferulate, β-sitosteryl ferulate and campesteryl ferulate. They observed the scavenging activity is equal to the activity of ferulic acid, which is an active constituent of γ – oryzanol responsible for DPPH scavenging activity. Muthal et al., in 2015 observed 100 µg of γ – oryzanol and ascorbic acid exhibit 65.56% and 64.88% inhibition respectively. Figure 6 indicates nitric oxide activity of γ – oryzanol. The plot shows that as the concentration of γ – oryzanol increases during the assay, the antioxidant activity also increases which can make us to formulate that the dose of γ – oryzanol in the human diet would be effective for fighting against many diseases. The IC50 value calculated for nitric oxide assay is 80.03. Juliano et al., 2005 observed Gamma-oryzanol prevent AMVN triggered lipid peroxidation and also observed that gamma
oryzanol increase the stability of oil that can undergo lipid peroxidation, so can act as stabilizer of lipiddic materials. Voraratn et al., 2010 concluded that rice bran oil and rice bran oil emulsion have high antioxidant activities tested by DPPH and FRAP assay. Rice bran oil showed good amount of vitamin E and gamma oryzanol contents. Sengupta et al., 2015 compared antioxidant activity of medium chain fatty acid (MCFA) rich-rice bran oils in with native rice bran oil. The antioxidant activity of different MCFA rice bran oil found in the order Caprylic acid rich rice bran oil>Capric acid rich rice bran oil>Lauric acid rich rice bran oil>RBO(Rice bran oil).They also stated that high antioxidant activity of MCFA rich RBO is due to decrease level of unsaturated fatty acid by interesterification with MCFA.Tuncel et al., 2011 studied antioxidant activity of rice milling fraction and observed high antioxidant activity in the rice unpolihed rice compared with the white polished rice and also observed that 94% of gamma-oryzanol content is reduced by whitening and polishing of the rice. Figure 7 indicates reducing power activity of γ – oryzanol. This activity has similar result as that of the results of other antioxidant activities. It also shows that increasing the γ – oryzanol concentration would have positive effect on the body. When the reducing power assay was conducted, the color of the sample tubes increases with increasing concentration of oryzanol. This is believed that during reducing power assay, the antioxidant compound forms colored complex with the components of this assay. Thus, more is the antioxidant compound more will be the color of reaction mixture. If there is no antioxidant compound present then no such color will be formed in the reaction mixture. Oryzanol produced more color in increasing order showing that it has very high antioxidant activity. Pengkumsri et al., 2015 studies showed that hexane and Supercritical Fluid Extraction method resulted in yield of gamma-oryzanol compared to Hot pressed, Cold pressed methods and high antioxidant and phytochemical properties was also high in hexane extracted rice bran oil.

Figure 1

*Extracted γ – Oryzanol from rice bran oil*
Figure 2
Crystals of oryzanol

Figure 3
HPLC chromatograph of oryzanol sample
Figure 4
*HPLC chromatograph analysis of oryzanol standard*

Figure 5
*DPPH scavenging activity*

Figure 6
*Nitric oxide scavenging activity*
CONCLUSION

Gamma oryzanol extracted from the rice bran oil found posses antioxidant activity which seems to be increase with the concentration. As an antioxidative reduces the chances of generation of free radicals and avoid cell damage. The study concluded that it must be treated as good source of oryzanol and if correlates with other authors work find to be comparable. It is also noted by reviewing the literature that unpolished rice showed highest gamma oryzanol content that polished rice. Gamma oryzanol found to be other health benefit also for example it lowers the cholesterol level and reduces the risk of heart disease. It is also suggested that crystals of oryzanol can be used in the food preparation as spices.

REFERENCES

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