EXAMINATION OF ANTIOXIDANT ACTIVITY AND DEVELOPMENT OF RICE BRAN OIL AND GAMMA-ORYZANOL MICROEMULSION

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ABSTRACT: The aims of this study were to examine the antioxidant activity and formulate rice bran oil and gamma-oryzanol product. Two methods of antioxidant activity examination were DPPH radical scavenging assay and Ferric Reducing Antioxidant Potential (FRAP) assay. According to our study, the outcome of free radical scavenging properties of gamma-oryzanol was demonstrated in terms of Trolox equivalent antioxidant capacity (TEAC). For DPPH assay, TEAC values were of 0.0015-0.0206 mmol/g when the concentrations were 0.0625-1.0000 mg/ml. For FRAP assay, TEAC values were of 0.0054-0.0272 mmol/g when the concentrations were 0.0680-1.0910 mg/ml. While the linear correlation between TEAC and log concentration was determined as $R^2 = 0.9929$ and 0.9975 respectively. The outcomes of free radical scavenging properties of rice bran oil, for DPPH assay, TEAC values were of 0.0059-0.0214 mmol/g when the concentrations were 8-40 mg/ml. While FRAP assay, could not examine the antioxidant activity because of the immiscibility between reagent and rice bran oil. Then microemulsion was formulated using rice bran oil and gamma-oryzanol as an active antioxidant, Cremophor and Span 80 as a surfactant and absolute ethanol as a co-surfactant. The developed formulation had high antioxidant activity with no skin irritation.

Keywords: Rice bran oil, Gamma-oryzanol, Microemulsion, Antioxidant

INTRODUCTION: Rice is an important economic crop and export product of Thailand. The by-products of rice are consisting of bran, germ, and grain. Rice bran has high nutritional value maximize to 60% such as amino acids. Rice bran oil (RBO) is the oil extracted from the germ and inner husk of rice. Rice bran oil contains a range of oils, with 18-23% of the unsaturated oil including monounsaturated and polyunsaturated oil. The fatty acids compositions of RBO are linolenic acid (omega-3, omega-6) and oleic acid (omega 9). This natural oil is rich in essential vitamin E complex; tocotrienols and gamma oryzanol. These compounds play an important role in preventing heart attack and reducing bad cholesterol level (LDL-C) in the blood. RBO is an interesting natural antioxidant and very popular as a cooking oil in several countries.

Gamma-oryzanol (Figure 1) is an extract from RBO that has high anti-oxidant activity. It is a mixture of sterol esters of ferulic acid and triterpene alcohols. The chemical structure serves as an important anti-oxidation effect caused by part of ferulic acid similar to cholesterol that affected on blood glucose levels and serum lipid parameters. It also protects the skin from Ultra-violet radiation and increase moisture to the skin as well. Therefore, the idea for application in cosmetics is both antiwrinkle and moisturizer for the skin.

Microemulsion is a novel drug delivery system for topical use. This system is suitable for lipophilic substances including RBO and gamma-
oryzanol. Moreover, the surfactant and co-
surfactant in microemulsion effect on the skin
permeation and penetration through stratum
corneum10).

In this work, the free radical scavenging
activities of RBO and gamma-oryzanol were
followed via their reaction with the stable DPPH
(2,2-diphenyl-1-picrylhydrazyl) free radical and
their ferric ions reducing antioxidant activity
potential (FRAP) assay. Results from this study
would provide a better evidence of the antioxidant
properties and development into value-added
cosmeceuticals.

MATERIALS AND METHODS:

Chemicals
2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-
Tri(2-pyridyl)-s-triazine (TPTZ), Iron (III) chloride
hexahydrate (FeCl3•6H2O) and Trolox (C15H20O4)
were from Sigma-Aldrich (St. Louis, USA).
Gamma-oryzanol was purchased from Wako Pure
Chemical Industries, Co.Ltd, Japan. Rice bran oil
was from Namseng Co.Ltd, Thailand. Absolute
ethanol, isopropranol, hydrochloric acid, glacial
acetic acid and sodium acetate trihydrate were
from Merck (Darmstadt, Germany). All other
reagents were analytical grade available.

Antioxidant activity of rice bran oil and
gamma-oryzanol

The DPPH radical scavenging and Ferric
Reducing Antioxidant Potential (FRAP) assay were
used to evaluate the antioxidant properties11-13).

DPPH radical scavenging assay

The 96-wells of a microtiter plate were divided
into 3 sets (set A-C) as follows: each well of set A
(test sample) contained 100 µl of ethanolic test
sample (concentration 25 – 400 µg/ml) and 100 µl
of the ethanolic DPPH radical; each well of set B
(blank of test sample) contained 100 µl of
ethanolic test sample and 100 µl of ethanol; each
well of set C (control) contained 100 µl ethanol
and 100 µl of the ethanolic DPPH radical. After
filling and mixing the solutions in the well, the
plate was incubated at 25°C for 30 min. The
absorbance was measured at a wavelength of 520
nm by using a microplate reader (Anthos Labtech
Instrument, Zenyth 200). %AA was calculated
from the equation below by comparing with the
standards Trolox. IC50 was obtained from the
calibration curve between % Inhibition of free
radical DPPH and the concentration of sample. Percent Inhibition of free radical DPPH was
calculated according to the formula:

% Inhibition = \( \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \)

Where \( A_{control} \) is the absorbance of the control
reaction (containing all reagents except the test
compound) and \( A_{sample} \) is the difference of
absorbance of test sample and the absorbance of
blank of test sample

Ferric-reducing antioxidant power (FRAP)
assay

The FRAP reagent was prepared from acetate
buffer (300 mM, pH 3.6), 10 mmol TPTZ solution
in 40 mM HCl and 20 mM ferrous chloride
solution in the proportion of 10:1:1 (v/v),
respectively. The FRAP reagent was fresh daily
prepared and was warmed to 37°C in a water
bath prior to use. The 96-wells of a microtiter
plate were divided into 3 sets (set A-C) as follows:
each well of set A contained 20 µl of isopropanolic
RBO (concentration 6.25 – 50 mg/ml) and 200 µl
of the FRAP reagent; each well of set B contained
20 µl of isopropanolic gamma-oryzanol (concentration
0.15625 – 2.5 mg/ml) and 200 µl of the FRAP
reagent; each well of set C (control) contained 20
µl of isopropanolic Trolox (concentration 0.01 -
0.05 mg/ml) and 200 µl of the FRAP reagent. The
absorbance of the reaction mixture was then
recorded at 595 nm after 5 min. The standard
curve was constructed using Trolox (concentration
0.01-0.05 mg/ml), and the results were expressed
as TEAC mmol equivalents per gram of the
sample. All measurements were taken in triplicate
and the mean values were calculated.

Preparation of rice bran oil and gamma-
oryzanol microemulsion

The microemulsion was prepared by using rice
bran oil and gamma-oryzanol as an active
antioxidant, cremophor and span 80 as the
surfactant and absolute ethanol as a co-
surfactant. The oil phase was mixed with the
surfactant and co-surfactant. The mixture was
titrated with water until it turned turbid. The water titration was continued until it turned clear. The pseudoternary phase diagrams (Figure 2 and 3) were constructed by plotting the amounts of water phase, oil phase, and surfactant:co-surfactant phase used in the experiment. The corresponding microemulsion regions were identified as shown as three red spots in Figure 2 and six red spots in Figure 3.

Skin irritation studies

The skin irritation studies were performed in 12 healthy volunteer in accordance with the guidelines of the Consumer Product Safety Commission. The study was approved by the Institutional Ethics Committee at Faculty of Pharmacy, Srinakharinwirot University. The selected formulations; microemulsion of RBO and RBO plus gamma-oryzanol were applied at a dose of 0.5 g to the desired area (3 x 3 cm²). The erythema index was measured by using Mexameter™ at 0, 30, 60 min, and 24 h after use.

**RESULT:**

**Antioxidant activity of rice bran oil and gamma-oryzanol**

**DPPH radical scavenging assay**

The standard curve between concentration (mg/ml) and UV absorbance of Trolox was performed by DPPH assay as shown in Figure 4. The linear correlation between % inhibition and concentration was determined as:

\[ Y = 3084.8X + 9.0124 \text{ and } R^2 = 0.9982 \]

IC₅₀ of Trolox was calculated to be 0.0133 mg/ml.

The antioxidant activity of gamma-oryzanol was performed by DPPH assay as shown in Figure 5. The linear correlation between TEAC and log concentration was determined as:

\[ Y = 0.0071 \ln (X) + 0.0205 \text{ and } R^2 = 0.9929 \]

TEAC values were of 0.0015-0.0206 mmol/g when the concentrations were 0.0625-1.0000 mg/ml.

The antioxidant activity of RBO was performed only by DPPH assay as shown in Figure 6. The linear correlation between % inhibition and concentration was determined as:

\[ Y = 1.4404X + 18.485 \text{ and } R^2 = 0.9844 \]

IC₅₀ of rice bran oil was calculated to be 21.8793 mg/ml.

**Ferric-reducing antioxidant power (FRAP) assay**

The standard curve between concentration (mg/ml) and UV absorbance of Trolox was performed by FRAP assay as shown in Figure 7. The linear correlation between % inhibition and concentration was determined as:

\[ Y = 101.86X + 0.094 \text{ and } R^2 = 0.9962 \]

IC₅₀ of Trolox was calculated to be 0.0133 mg/ml.

The antioxidant activity of gamma-oryzanol was performed only by FRAP assay as shown in Figure 8. The linear correlation between TEAC and log concentration was determined as:

\[ Y = 0.0078 \ln (X) + 0.0263 \text{ and } R^2 = 0.9975 \]
TEAC values were of 0.0054-0.0272 mmol/g when the concentrations were 0.0680-1.0910 mg/ml.

The antioxidant activity in term of TEAC values could not be examined by FRAP assay because of the immiscibility between FRAP reagent and rice brain oil.

**Preparation of rice bran oil and gamma-orizanol microemulsion**

The microemulsion of RBO and RBO plus gamma-orizanol were light-yellowish in color, odorless, and clear.

**Antioxidant activity of rice bran oil and gamma-orizanol microemulsion**

The antioxidant activities of two types of microemulsion were evaluated by using two different assays (DPPH and FRAP assay). In Table 1 the results of the monitoring of antioxidant capabilities are present. It can be seen that the antioxidant activity of microemulsion contained rice bran oil plus gamma-orizanol was higher than microemulsion contained rice bran oil alone. In addition, the antioxidant activity of microemulsion was associated with the ratio of the surfactant and co-surfactant. The tube number 6/3 (S:CoS = 2:1) and the tube number 6/2 (S:CoS = 3:1) have the highest percent of inhibition of oxidation (more than 94%).

**Skin irritation studies**

The safety of the products was observed in term of the skin irritation (edema and erythema). The developed formulation showed non-irritant to the skin with no erythema or edema in all subjects.

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**Figure 4** The correlation between concentration (mg/ml) and UV absorbance of Trolox by DPPH assay

**Figure 5** The correlation between log concentration and TEAC (mmol/g) of Gamma Orizanol by DPPH assay

**Figure 6** The correlation between concentration (mg/ml) and percent of inhibition of RBO by DPPH assay

**Figure 7** The correlation between concentration (mg/ml) and UV absorbance of Trolox by FRAP assay

**Figure 8** The correlation between log concentration and TEAC (mmol/g) of Gamma Orizanol by FRAP assay
**DISCUSSION:**

The generation of radical oxidative species involves either radical preocesses or different potential redox systems. The soluble properties of antioxidant compounds determine their effective antioxidant activities in either aqueous or lipid systems\(^1\). Therefore, the microemulsion was chosen to assess the antioxidant activity of RBO and RBO plus gamma-oryzanol, one measuring radical-scavenging activities, and the other measuring total reducing power. DPPH radical scavenging assay was estimated from their ability to eliminate free radicals of DPPH, the color was changed from purple to colorless. While FRAP assay was estimated from their power to reduce the TPTZ-Fe (III) complex to TPTZ-Fe (II) complex which is simple, fast, and reproducible\(^2\). The color was changed from colorless to violet. The measured absorbance by the DPPH and FRAP methods were represented as the percent of inhibition. The comparison with standard trolox was reported in term of a Trolox Equivalence Antioxidant Capacity (TEAC) value. The antioxidant examination of gamma-oryzanol demonstrated that TEAC value of FRAP assay were higher than those of DPPH assay. The FRAP assay was versatile and could be readily applied to both aqueous and alcohol extracts\(^3\). The limitation of this method was the false negative reaction with the SH-group compounds, such as Glutathione (GSH)\(^4\).

The formulation of microemulsion was developed by using three phases diagram: oil, water and surfactant. The ratio of surfactant: co-surfactant was varied from 2:1 to 3:1. When using S/CoS ratio 2:1, the microemulsion was performed in plane/tube number 6/3, 7/2 and 8/1. While using S/CoS ratio 3:1, the microemulsion was performed in plane/tube number 6/1, 6/2, 6/3, 7/1, 7/2 and 8/1. The developed microemulsions were light-yellowish in color, odorless, and clear. After mixing all three phases together and observed the physical properties. Then the anti-oxidation activity was performed by DPPH and FRAP assay. The results of both methods showed that microemulsion of RBO plus gamma-oryzanol gave higher TEAC value than RBO microemulsion. The highest antioxidant activity of microemulsion of tube number 6/3 was detected by DPPH assay and that of tube number 7/2 was detected by FRAP assay. The ratio of RBO, surfactant, and water was 10:60:30 and 10:70:20 in the tube number 6/3 and 7/2, respectively.

**CONCLUSION:**

In conclusion, the antioxidant activity of RBO and RBO plus gamma-oryzanol microemulsion were evaluated by DPPH and FRAP assay. The RBO, in general, showed high antioxidant activities and vitamin E complex particularly gamma-oryzanol. Relatively, the antioxidant activity both assays showed the highest in gamma-oryzanol.
Moreover, there were relative between antioxidant activities and gamma-orzyanol contents in microemulsion. The RBO plus gamma-orzyanol microemulsion showed higher antioxidant activity than RBO microemulsion. It could be concluded that gamma-orzyanol contribute to antioxidant activity in RBO plus gamma-orzyanol microemulsion.

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