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Research article

Cold-pressed rice bran oil-in-water emulsion delivery system with anti-inflammatory activity in THP-1 macrophages

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Abstract

Importance of the work: Cold-pressed rice bran oil has been widely consumed, however its pathway involving anti-inflammatory activity has not been elucidated.

Objectives: To investigate the potential of CPRBO emulsion as a delivery system and its anti-inflammatory effects after *in vitro* digestion.

Materials & Methods: Commercial CPRBO was purchased from a local supermarket in Thailand. The CPRBO emulsion was fabricated by homogenizing technique. The cytotoxicity was tested through the MTT assay. The anti-inflammatory activities of digested CPRBO emulsion were evaluated by quantitative real-time polymerase chain reaction and western blot analysis.

Results: CPRBO from Khao Dawk Mali 105 rice variety contained phytochemicals (including gamma-oryzanol, phytosterols and alpha-tocopherol) and had a suitable fatty acid profile. The CPRBO emulsion had particle sizes of approximately 166 nm and remained stable for 4 wk at 4°C. The emulsification efficiency was higher than 99%. The emulsion withstood the gastric phase of digestion with no significant increase in particle size and polydispersity index. However, these parameters were significantly increased in the small intestinal phase due to the activity by lipid-digesting enzymes and bile extracts, suggesting that the CPRBO emulsion remained stable until reaching the small intestine. The digested CPRBO emulsion significantly suppressed pro-inflammatory cytokine gene expression via inhibition of NF-κB p65 expression and phosphorylation.

Main finding: In addition to reviewing the pathway involving anti-inflammatory effects, this study demonstrated the potential applicability of CPRBO-in-water emulsion as a delivery system for lipophilic bioactive compounds.

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Introduction

Rice bran, which is a potential by-product of the rice milling process, is a known source of edible oil (Van Hoed et al., 2006). Rice bran oil (RBO) has a good fatty acid profile along with phytochemicals, such as phytosterols, gamma-oryzanol, tocopherols, and tocotrienols (Saikia and Deka, 2011). In many countries, RBO is considered a "heart oil" because of its hypocholesterolemic effect (Vissers et al., 2000) and ability to lower the risk of cardiovascular diseases (Sohail et al., 2017). Specifically, because the cold-pressing technique preserves phytochemicals, it has been used to produce cold-pressed rice bran oil (CPRBO) for consumption as a nutraceutical (Siger et al., 2008).

Emulsion-based delivery systems have attracted increasing attention as a means of encapsulating lipophilic bioactive compounds (such as vitamins A and D, antioxidants, phytosterols, conjugated linoleic acid and omega-3 fatty acids) and allowing their controlled release (McClements and Li, 2010). One such delivery system is oil-in-water emulsion, in which oil droplets coated with a thin layer of emulsifier are dispersed in the aqueous phase (McClements et al., 2007). The ability of such a system to control the release of bioactive compounds at a target organ is vital for maximizing their bioavailability and bioactivities. For example, silica nanoparticles were used to encapsulate curcumin and demonstrated the gastrointestinal stability of the delivery system, where over 80% of the encapsulated curcumin was retained in the simulated gastric conditions and emulsion droplet destabilization caused the release of approximately 60% of the curcumin (Tikekar et al., 2013).

Innate immunity is determined critical for host defenses against infectious pathogens and plays a key role in regulating inflammatory diseases, such as allergies, autoimmune disorders and inflammatory bowel disease (Turvey and Broide, 2010). Gut-associated lymphoid tissues, such as the Peyer's patches of the small intestine, cecal patches and colonic patches, are some of the largest immune-responsive sites (Kobayashi et al., 2019). The largest population of macrophages is found in the small intestine, being enriched in the lamina propria close to the epithelial layer (Hume et al., 1984). As most food is digested in the small intestine, the ability to control the release of bioactive compounds (especially lipid-soluble substances) at this site (where they will be absorbed) is important to maximize their bioavailability and health-promoting effects. The bioavailability of liposome-encapsulated fisetin was 47-fold higher than that of the free molecule, resulting in significantly improved anticancer efficacy (Seguin et al., 2013).

Although the anti-inflammatory activity of CPRBO has been established (Hunthayung et al., 2019; Pattananandecha et al., 2019; Settharaksa et al., 2014), the pathway involving this effect is yet to be elucidated. Therefore, the current study investigated this by determining the effects of a cold-pressed rice bran oil (CPRBO) oil-in-water emulsion on anti-inflammatory pathways in lipopolysaccharide (LPS)-stimulated THP-1 macrophages. Additionally, the effectiveness of the emulsion was examined as a delivery system by testing its stability under simulated *in vitro* gastrointestinal digestion. The findings should contribute knowledge on the immunomodulatory mechanisms of CPRBO.

Materials and Methods

Chemicals and reagents

Commercial CPRBO from the Khao Dawk Mali 105 rice cultivar (KDML105) was purchased from a local supermarket in Thailand. Porcine pancreatic alpha-amylase (EC 3.2.1.1) (A3176), porcine gastric mucosal pepsin (EC 3.4.23.1) (P7000), porcine pancreatic pancreatin (P7545), porcine bile extract (B8631), phorbol 12-myristate 13-acetate (PMA) and LPS (*Escherichia coli* O111:B4) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemical reagents used were of analytical grade. Deionized water was used in preparing all solutions.

Chemical characterization of cold-pressed rice bran oil

Acid value and free fatty acid content

The acid value was determined according to the American Oil Chemists' Society Official Method Cd 3d-63. The CPRBO was weighed into an Erlenmeyer flask containing 125 mL of an isopropyl alcohol/toluene mixture (1:1, volume per volume). Then, 1 mL of 1% phenolphthalein was added; thereafter, the sample was titrated with 0.1 N KOH until the solution turned faint pink. The acid value (measured in milligrams of KOH per gram of sample) was calculated using Equation 1 (AOCS, 1997):

$$\text{Acid value} = \frac{(A\text{-}B) \times \text{Normality of KOH} \times 56.1}{\text{gram of sample}} \quad (1)$$

where A and B are the volumes (measured in milliliters) of 0.1 N KOH used for titrating the sample and blank (solvent mixture), respectively.

The free fatty acid (FFA) content, expressed as the oleic acid percentage, was calculated using Equation 2 (AOCS, 1997):

% Free fatty acid as oleic = Acid value / 1.99 (2)

Total phenolic content

The total phenolic content (TPC) was measured using the Folin-Ciocalteu method with slight modifications. In brief, 0.1 mL of CPRBO (10 mg/mL in dimethyl sulfoxide) was mixed with 2 mL of 0.02 mg/mL Na_2CO_3 for 3 min. Then, 0.1 mL of Folin-Ciocalteu reagent was added and the mixture was left in the dark for 30 min. Finally, the absorbance at 750 nm was recorded. Gallic acid was used as the standard, and the results were expressed as milligrams of gallic acid equivalents per gram of oil (mg GAE /g oil, (Thanonkaew et al., 2012).

Gamma-oryzanol content

After weighing 0.02 g of CPRBO into a 25 mL volumetric flask and adding *n*-heptane to the mark, the absorbance at 315 nm was measured. The gamma-oryzanol content (measured in grams per 100 g of sample) was determined using Equation 3 (AOCS, 1997):

$$\text{Gamma-oryzanol (g/100 g sample)} = 25 \times \frac{1}{W} \times A \times \frac{1}{E} \quad (3)$$

where W is the CPRBO weight (measured in grams), A is the absorbance at 315 nm and E is the specific extinction coefficient ($\varepsilon^{1\%} = 359$).

Phytosterol, fatty acid and alpha-tocopherol contents

The phytosterol content and fatty acid composition were determined using gas chromatography with a flame ionization detector and mass spectrophotometry, respectively. The alpha-tocopherol content was analyzed using high-performance liquid chromatography. All analyses were performed according to ISO/IEC 17025-certified in-house methods of the Thai Food and Drug Administration laboratory of the Institute of Food Research and Product Development, Kasetsart University, Bangkok, Thailand.

Characterization of cold-pressed rice bran oil-in-water emulsion

Oil-in-water emulsion preparation

The oil-in-water emulsion was prepared by homogenizing 5% (weight per weight, w/w) of CPRBO to a 95% (w/w) aqueous phase containing 1% (w/w) Tween 80 in 5 mM phosphate buffer (pH 7.0), using a high-speed blender (13,500 rpm) for 2 min. The coarse emulsion was immediately passed through a high-pressure homogenizer (35 MPa) for three passes (Klongdee et al., 2012), and the final CPRBO emulsion was stored at 4°C.

Free oil content

The non-emulsified oil content was measured using the methods of Thanasukarn et al. (2004) and Zang et al. (2019), with minor modifications. A standard curve was plotted using the values of absorbance at 341 nm versus the different CPRBO concentrations (on a percent weight for weight basis, %w/w) in colorless mineral oil. The linear equation derived from the curve was used to determine the free oil concentration in all test samples (Thanasukarn et al., 2004). To measure the free oil content, 16 g of emulsion was vortex mixed with 4 g of mineral oil for 3 min. The mixture was incubated at ambient temperature for 60 min and centrifuged at 1,000×*g* for 20 min. The top layer was carefully collected and centrifuged at 15,000×*g* for 20 min and the absorbance at 341 nm of the supernatant was recorded and calculated as % free oil (Zang et al., 2019).

Creaming index

In brief, 10 mL of freshly prepared CPRBO emulsion was transferred into a glass test tube (16 mm × 150 mm) and this was stored at 4°C for 4 wk. Then, the height of the cream layer at the top of the test tube and that of the total emulsion were measured and used to calculate the creaming index based on Equation 4 (Charoen et al., 2011):

$$\text{Creaming index} = \frac{\text{Height of cream layer}}{\text{Height of total emulsion}} \times 100 \quad (4)$$

Particle size distribution

The particle size distribution of the emulsion diluted in 5 mM phosphate buffer (pH 7.0) was determined using dynamic light scattering at 25°C with a Zetasizer Nano-ZS (Zen 3600; Malvern Instruments Ltd.; Worcestershire, UK) (Sawadikiat et al., 2015).

In vitro gastrointestinal digestion

First, 5 mL of CPRBO emulsion mixed with simulated salivary fluid and an alpha-amylase solution (1,500 U/mL) was incubated in a shaking water bath at 37°C for 2 min. Then, 10 mL of this ‘oral phase’ mixture together with simulated gastric fluid and a pepsin solution (25,000 U/mL) was adjusted to pH 3 and incubated at 37°C for 2 hr. Finally, 20 mL of this ‘gastric phase’ mixture together with simulated intestinal fluid, fresh bile (160 mM) and a pancreatin solution (800 U/mL based on trypsin activity) was adjusted to pH 7 and incubated in a shaking water bath at 37°C for 2 hr. Then, the reaction was stopped by immersing the mixture in liquid nitrogen immediately (Minekus et al., 2014). At the end of each

gastrointestinal phase, samples were collected for particle size distribution measurement. The digested CPRBO emulsion was stored at –20°C for further study.

Investigation of the immunomodulatory mechanisms of digested cold-pressed rice bran oil-in-water emulsion

THP-1 cell culture and macrophage differentiation

The human monocytic leukemia cell line THP-1 (American Type Culture Collection; Rockville, MD, USA) was grown in RPMI 1640 culture medium (Hyclone; Marlborough, MA, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco; Gaithersburg, MD, USA) at 37°C in a 5% CO_2 humidified incubator. THP-1 with passage numbers 12–25 was used throughout the study. THP-1 monocytes were differentiated into macrophages via treatment with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) for 48 hr. Then, the differentiated cells were gently washed twice with culture medium (without PMA) and rested for another 48 hr in culture medium to obtain THP-1 macrophages (Chanput et al., 2010).

Cytotoxicity assay

THP-1 macrophages (5×10^5 cells/mL in a 96-well plate) were exposed to up to 500 µg/mL digested CPRBO emulsion for 2 hr. The cytotoxicity was determined using MTT (3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Chanput et al., 2012).

Pro-inflammatory cytokine gene analysis

THP-1 macrophages (1×10^6 cells/mL in a 12-well plate) were simultaneously stimulated with 100 ng/mL LPS and digested CPRBO emulsions (50 µg/mL or 100 µg/mL) in culture medium for 2 hr. The expression of the pro-inflammatory cytokine genes (*tumor necrosis factor-alpha* (*TNF-α*), *interleukin* (*IL*)*-1beta* (*IL-1β*), *IL-6*, *IL-8*) was analyzed using real-time quantitative polymerase chain reaction (RT-qPCR). *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used as the housekeeping gene and non-stimulated cells were used for normalization (ΔΔCt) of the data. The results were expressed as the relative fold change (Chanput et al., 2010).

Western blot analysis

THP-1 macrophages (1×10^6 cells/mL in a 6-well plate) were simultaneously stimulated with 100 ng/mL LPS and treated with 100 µg/mL digested CPRBO emulsion for 30 min, 1 hr or 2 hr. At the indicated times, the cell pellets were collected and suspended in 200 µL of lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 1X protease inhibitor, pH 8.0) and incubated on ice for 30 min. Thereafter, the mixture was vigorously vortexed for 10 s and centrifuged at 10,000×*g* for 20 min. Then, 50 µg of whole-cell protein were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a 0.45-µm polyvinylidene difluoride membrane. Nonspecific binding sites on the membrane were blocked using 5% skim milk dissolved in TBST (10 mM Tris, 150 mM NaCl, 0.1%Tween 20, pH 7.5) at ambient temperature for 1 hr. Thereafter, the membrane was incubated with either rabbit anti-nuclear factor-kappaB (NF-κB) p65 antibody (diluted 1:1,500; Thermo Fisher Scientific; Waltham, MA USA) or rabbit anti-phospho-NF-κB p65 antibody (Ser536) (diluted 1:1,500; Cell Signaling Technology; Danvers MA, USA) at 4°C overnight. Then, the membrane was washed three times with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted 1:5,000; Abcam; Cambridge, UK) at ambient temperature for 1 hr. After another three washes with TBST, the immunoreactive bands were visualized using an enhanced chemiluminescence substrate (PerkinElmer; Groningen, the Netherlands). Beta-actin was used as the loading control. The protein bands were quantified using the NIH ImageJ software (National Institutes of Health; Bethesda, MD, USA).

The chemical composition of CPRBO was reported based on duplicate determination. All assays were performed in four independent replicates conducted with duplicates ($n = 4$) and values were presented as mean ± SD.

Statistical analysis

The data were analyzed using one-way analysis of variance and Duncan's post hoc test in the IBM Statistical Package for the Social Sciences (SPSS) software version 26 (SPSS Inc.; Chicago, IL, USA). The tests were considered significant at $p < 0.05$.

Results and Discussion

Chemical characteristics of cold-pressed rice bran oil

The quality of any oil can be determined by its acid value, with the acid value of KDML105-derived CPRBO being 2.42 mg KOH/g oil (Table 1) that complied with the CODEX

standard of edible cold-pressed oils not being higher than 4 mg KOH/g oil (AOCS, 1997). The TPC in CPRBO was 8.11 mg GAE/g oil (Table 1), similar to that reported in CPRBO from non-colored Thai rice (8.75–9.26 mg GAE/g oil) according to (Mingyai et al., 2017). The current CPRBO contained 20,466.46 parts per million (ppm) gamma-oryzanol, similar to the average amount (20,000 ppm) reported in another study (Patel and Naik, 2004). Consistent with (Mingyai et al., 2018), the CPRBO contained approximately 8,800 ppm of total phytosterols, of which beta-sitosterol was determined to be the most abundant. CPRBO consisted of saturated and unsaturated fatty acids, with palmitic acid, *cis*-9-oleic acid and linoleic acid being predominant (Table 2). The fatty acids composition and their concentrations were similar to those reported in other studies (Gopala Krishna et al., 2006; Mingyai et al., 2017).

Table 1 Chemical characteristics of cold-pressed rice bran oil (CPRBO)

Constituent	CPRBO
Acid value (mg KOH/g oil)	2.42±0.31
TPC (mg GAE/g oil)	8.11±0.12
γ-Oryzanol (ppm)	20,466.46±277.01
α-Tocopherol (ppm)*	272.85±8.41
Total phytosterols (ppm)*	8,779.35±91.57
- Campesterol	1,308.00±1.27
- Stigmasterol	1,623.15±48.44
- β-Sitosterol	5,848.25±138.66

TPC = total phenolic content; GAE = gallic acid equivalents; ppm = parts per million

* data previously published in Hunthayung et al. (2019).

Values are presented as mean ± SD.

Table 2 Fatty acid profile of cold-pressed rice bran oil (CPRBO)

Constituent	CPRBO
Saturated fatty acids (g/100 g oil)	
- Myristic acid (C14:0)	0.25±0.01
- Palmitic acid (C16:0)	18.21±0.03
- Stearic acid (C18:0)	2.06±0.01
- Arachidic acid (C20:0)	1.02±0.01
- Behenic acid (C22:0)	0.27±0.01
- Lignoceric acid (C24:0)	0.44±0.01
Monounsaturated fatty acids (g/100 g oil)	
- Palmitoleic acid (C16:1)	0.24±0.02
- cis-9-Oleic acid (C18:1)	43.15±0.08
- cis-11-Eicosenoic acid (C20:1)	0.51±0.00
Polyunsaturated fatty acids (g/100 g oil)	
- Linoleic acid (C18:2)	28.31±0.08
- alpha-Linolenic acid (C18:3)	1.10±0.00

Values are presented as mean ± SD.

Cold-pressed rice bran oil emulsion stability

The CPRBO was successfully fabricated to an oil-in-water emulsion (Fig. 1A). After 1 d of preparation, the CPRBO emulsion had a mean particle size of 166.3 nm, which did not change significantly after 4 wk of storage at 4°C (Table 3). The polydispersity index (PDI) was 0.08–0.16. PDI sharpening at 1 wk of storage occurred probably due to Oswald ripening (Zeng et al., 2017). However, the PDI value of the CPRBO emulsion during storage period was less than 0.2, indicating that the emulsion was stable with a monomodal size distribution (Aditya et al., 2014). The emulsion stability was supported by a creaming index of 0%, which did not increase significantly even after 4 wk of storage. No phase separation was observed during the storage period.

The emulsification efficiency was based on the change in absorbance attributed to the solubilization of non-emulsified oil (any free CPRBO) in the colorless mineral oil (Thanasukarn et al., 2004; Zang et al., 2019). On day 1 of storage, the free oil concentration was 0.55% and no significant increase was observed during storage (Table 3). The results showed that the formulation of the CPRBO emulsion using the homogenizing technique produced an emulsification efficacy higher than 99%. Oil-in-water emulsion delivery systems can be applied in beverages owing to their stability and ability to encapsulate bioactive lipophilic compounds (Öztürk, 2017). A water-in-oil-in-water emulsion co-loaded with curcumin and catechin had higher than 90% encapsulation efficiency, which improved the complex stability (Aditya et al., 2015).

Fig. 1 Cold-pressed rice bran oil emulsion: (A) before; (B) after *in vitro* digestion

Table 3 Stability of cold-pressed rice bran oil (CPRBO) emulsion up to 4 wk at 4°C

Storage time	Size (nm)	Polydispersity index	Creaming index (%)	Free oil (%)
1 d	166.3±2.0[a]	0.124±0.031[ab]	0.0±0.0[a]	0.55±0.27[a]
1 wk	166.9±1.7[a]	0.080±0.037[b]	0.0±0.0[a]	0.56±0.24[a]
2 wk	166.4±4.5[a]	0.116±0.036[ab]	0.0±0.0[a]	0.59±0.33[a]
4 wk	168.7±4.8[a]	0.157±0.023[a]	0.0±0.0[a]	0.60±0.31[a]

Values (mean ± SD) within each column superscripted with different lowercase letters are significantly ($p < 0.05$) different.

Digested cold-pressed rice bran oil emulsion stability

The digestibility and, hence the stability of the emulsion was examined by passing it through *in vitro* gastrointestinal digestion that simulated the human digestive tract. Fig. 1B shows the CPRBO emulsion after *in vitro* digestion. The mean particle size and PDI of the emulsion remained constant from the initial phase up to the gastric phase ($p \geq 0.05$), as shown in Table 4, indicating that there was no flocculation of oil droplets under the gastric or low pH conditions, likely because of the high emulsification efficiency of Tween 80. The nonionic structure of Tween 80 prevents the aggregation of oil droplets, allowing the formation of highly stable emulsions under acidic conditions (Zhang et al., 2015). Significant increases in the mean particle size and PDI were observed in the small intestinal phase of digestion. The lipid digestion of the CPRBO emulsion was followed by the measurement of the FFA released after exposure to digestion. The amount of released FFA increased from 1.35 to 12.84% as oleic acid, implying that the delivery system had destabilized and could thereby facilitate the release of lipophilic bioactive compounds and the digestion of lipids (Mun et al., 2007). Pancreatic lipase is a lipid-digesting enzyme that is surface-active and may compete with other molecules located at the oil droplet interface, leading to oil droplet degradation and lipid digestion (Hur et al., 2009). Furthermore, emulsion destabilization might be caused by the displacement of Tween 80 by lipolytic products, such as monoglycerides, diglycerides and FFAs, resulting from the hydrolysis of triglycerides by lipid-digesting enzymes (van Aken et al., 2011). Similar findings were reported in a study that demonstrated a large increase in the particle sizes of emulsions and nanoemulsions and their multimodal distribution after their simulated small intestinal digestion, which were probably due to the presence of mixed micelles or coalesced oil droplets (Salvia-Trujillo et al., 2019). Unfortunately, the chemical composition of the digested CPRBO emulsion was not able to be measured due to the isolation technique of bioactive compounds from the digesta. Gamma-oryzanol in the digested CPRBO emulsion was detected at 121.3 ppm.

Table 4 Particle size distribution and polydispersity index of cold-pressed rice bran oil emulsion during *in vitro* gastrointestinal digestion

Gastrointestinal phase	Mean particle size (nm)	Polydispersity index
Initial	163.4±3.3[b]	0.138±0.026[b]
Oral	160.2±3.7[b]	0.144±0.035[b]
Gastric	155.3±5.6[b]	0.167±0.020[b]
Small intestinal	197.0±29.2[a]	0.353±0.095[a]

Values (mean ± SD) within each column superscripted with different lowercase letters are significantly ($p < 0.05$) different.

Immunomodulatory mechanisms of the digested cold-pressed rice bran oil emulsion

Cytotoxicity

The viability of THP-1 macrophages incubated with various concentrations of digested CPRBO emulsion (0–500 µg/mL) for 2 hr was higher than 90% at all concentrations relative to the control of non-treated cells (set as 100%) (Fig. 2).

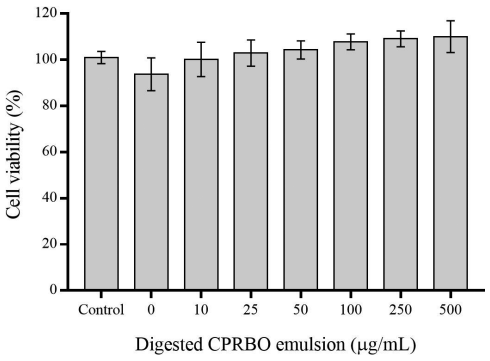

Fig. 2 Cell viability of THP-1 macrophages incubated with digested cold-pressed rice bran oil (CPRBO) emulsion at concentrations of 0–500 µg/mL for 2 hr, where error bars indicate ± SD

Effects on pro-inflammatory cytokine gene expression

The expression of pro-inflammatory cytokine genes was investigated. Due to the correlation between gene expression and cytokine production (Tao et al., 2009; Chanput et al., 2010; Bastin et al., 2021), reduction of the pro-inflammatory cytokine gene could be used as a marker for anti-inflammatory activity. LPS, which has been identified as an endotoxin from *Escherichia coli* (O111:B4), was used to stimulate the up-regulation of pro-inflammatory cytokine genes in THP-1 macrophages. The expression levels of the *TNF-α*, *IL-1β*, *IL-6* and *IL-8* genes were initially upregulated by 10 pg/mL LPS (Hunthayung et al., 2019). The LPS detected in the digested CPRBO emulsion was 7,019.56 pg/mL. Therefore, the maximum concentration of digested CPRBO emulsion tested in this study was 100 µg/mL, based on the LPS presence in the sample, which was quantified using a Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific; Waltham, MA, USA). The effect was investigated of digestive fluid and digested CPRBO emulsion on pro-inflammatory cytokine gene expression. The results showed that both the digestive fluid and digested CPRBO emulsion did not significantly upregulate the expression of pro-inflammatory cytokine genes, compared to non-stimulated cells. After co-stimulation with LPS for 2 hr, the digested CPRBO emulsion significantly decreased the expression of *TNF-α*, *IL-6*, and *IL-8* but had no significant effect on *IL-1β* (Fig. 3). The suppression of the pro-inflammatory cytokine genes indicated that the digested CPRBO emulsion possessed anti-inflammatory activity. The CPRBO emulsion before digestion could not be included in THP-1 assay due to the presence of LPS at 34.62 µg/mL emulsion. Tween 80 is 'generally recognized as safe' (GRAS) by the American Food and Drug Administration (FDA) with low-toxicity *in vitro* (in a preliminary study to the current) and *in vivo* (Jiao, 2008). As Tween 80 was used as an emulsifier to form the CPRBO emulsion, it was tested for pro- and anti-inflammatory activity at 0–20 mg/mL. There was no evidence of a change in gene expression for both conditions (data not shown).

Many studies have reported on the anti-inflammatory activity of rice phytochemicals. For example, an *in vitro* gastrointestinal digested CPRBO in a niosome delivery system exhibited anti-inflammatory activity in THP-1 macrophages, which was attributed to its ability to polarize the M1 phenotype toward the M0 phenotype (Hunthayung et al., 2019). Gamma-oryzanol (1 µM or 0.6 ppm) significantly reduced the expression of *IL-1β*, *IL-6* and *TNF-α* in THP-1 macrophage cells at the inflamed stage (Shibata and Chanput, 2018). In the current study, the gamma-oryzanol in the digested CPRBO emulsion at the maximum tested concentration (100 µg/mL) was 0.01213 ppm; therefore, any anti-inflammatory activity might not only arise from the gamma-oryzanol. Furthermore, the combination of lipidomics and gene expression indicated that among the lipophilic compounds in rice bran oil, phytosterols play a vital role to mitigate inflammatory situation in THP-1 monocytic cell model (Jom et al., 2021). Thus, the anti-inflammatory activity might result from either phytosterols or phytosterols and gamma-oryzanol.

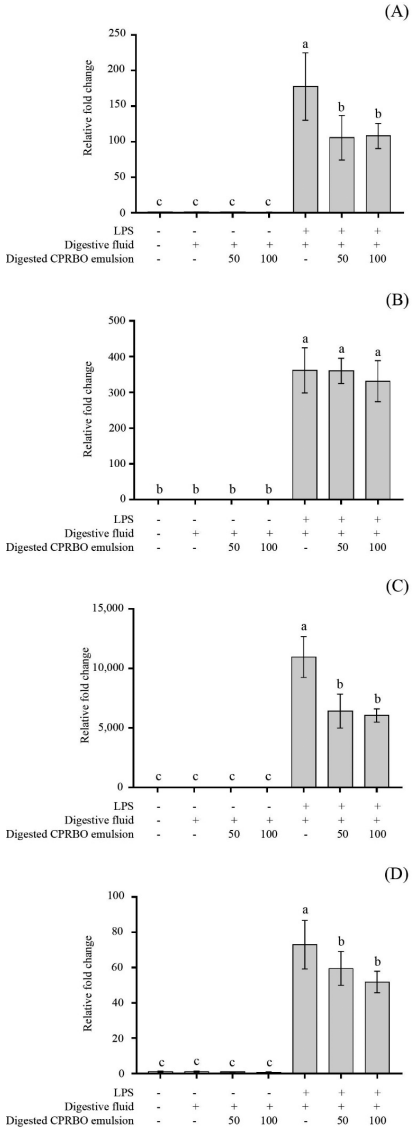

Fig. 3 Pro-inflammatory cytokine gene expression in THP-1 macrophages simultaneously stimulated with 100 ng/mL LPS and digested cold-pressed rice bran oil (CPRBO) emulsion for 2 hr: (A) *TNF-α* gene; (B) *IL-1β* gene; (C) *IL-6* gene; (D) *IL-8* gene, where gene expression is expressed as relative fold change towards GAPDH expression, columns with different lowercase letters are significantly ($p < 0.05$) different and the error bars indicate ± SD.

In addition, gamma-oryzanol has been shown to inhibit nitric oxide (NO) production in LPS-stimulated RAW 264.7 murine macrophages (Nagasaka et al., 2007; Saenjum et al., 2012; Settharaksa et al., 2014). The bioactive compounds in the CPRBO that might possess anti-inflammatory activity have been identified as phytosterols, vitamin E and polyunsaturated fatty acids, such as linoleic acid (C18:2) and alpha-linolenic acid (C18:3) (Ng and Ko, 2012; Pauls et al., 2018; Walloschke et al., 2010). From these combined findings, it could be concluded that the digested CPRBO emulsion exerted its anti-inflammatory activity at the transcriptional level through the bioactivity of its phytochemicals, especially phytosterols. These lipophilic bioactive compounds might be released from the oil droplets in the small intestine where macrophages are mostly located.

Effects on NF-κB (p65) activation and phosphorylation

NF-κB has been identified as a protein transcription factor that consists of two Rel family proteins: NF-κB1 (p50) and RelA (p65); only p65 contains C-terminal domains that activate the transcription of inflammation-related genes (Blackwell and Christman, 1997). In the current study, two forms of protein expression; NF-κB p65 and phosphorylated NF-κB p65 (p - NF-κB p65) were performed at different time points. In non-stimulated cells, NF-κB p65 was strongly expressed, while phosphorylation of NF-κB p65 occurred only slightly. The expression of NF-κB p65 and its phosphorylation were markedly stimulated at 30 min and 1 hr but declined at 2 hr after the LPS treatment. The digested CPRBO emulsion significantly reduced the protein expression of NF-κB p65 but did not completely inhibit at every time point. The expression of phosphorylated NF-κB p65 was significantly suppressed in the LPS+digested CPRBO emulsion at 30 min and 1 hr, compared to single LPS stimulation. (Fig. 4). These results were similar to another study that showed that NF-κB p65 expression was partially suppressed and the phosphorylation of NF-κB p65 was totally inhibited by the tested compound (Albrahim et al., 2020).

In cells, LPS is recognized by Toll-like receptor 4 that then triggers myeloid differentiation primary response gene 88 (MyD88) activation and thereby initiates the regulation of the NF-κB pathway (Lu et al., 2008). The phosphorylation of inhibitory-binding protein kappa B-alpha (IκB-α) leads to its degradation, which then induces NF-κB p65 phosphorylation and its translocation from the cytoplasm to the nucleus, where it binds to the promoter region and activates the transcription of inflammatory genes (Pålsson-McDermott and O'Neill, 2004). Therefore, LPS-treated THP-1 macrophages strongly express phosphorylated NF-κB p65. Since the anti-inflammatory mechanism of CPRBO has not been reported, the current study was initiated to investigate whether the digested CPRBO emulsion affected NF-κB p65 expression and phosphorylation. From the results, the digested CPRBO emulsion reduced the expression and phosphorylation of NF-κB p65 more clearly within 1 hr of exposure, which consequently downregulated the expression of pro-inflammatory cytokine genes. Thus, the current study revealed the pathway involving the anti-inflammatory activity of CPRBO, that is, via the reduction of NF-κB p65 expression and inhibition of phosphorylation, confirming the M1 polarizing activity (Hunthayung et al., 2019). Similarly, red ginseng marc oil acted as a potent anti-inflammatory agent because its phytosterols (campesterol, stigmasterol, sitosterol) inhibited NF-κB p65 phosphorylation and translocation in LPS-stimulated RAW264.7 macrophages (Bak et al., 2012). In addition, β-sitosterol and gamma-oryzanol were shown to have anti-inflammatory activity via inactivation of NF-κB p65 following pro-inflammatory signals (Nagasaka et al., 2007; Valerio and Awad, 2011). In conclusion,

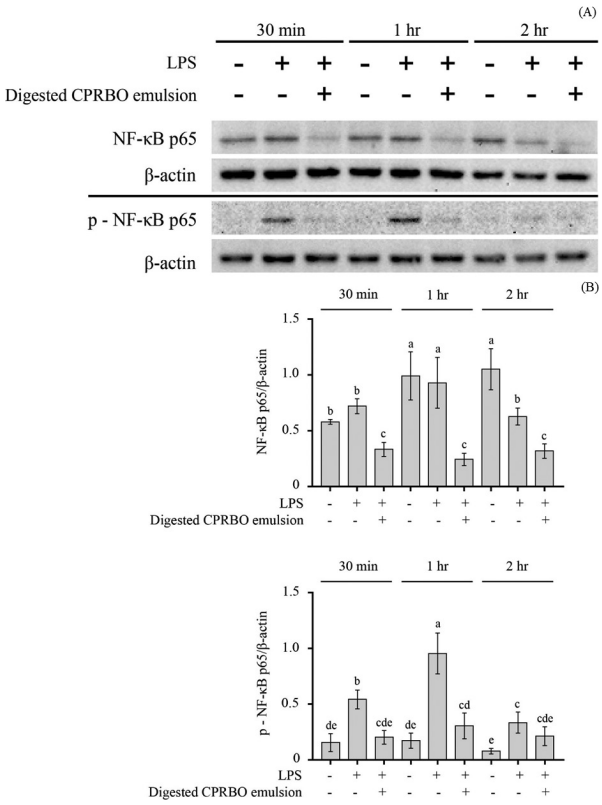

Fig. 4 Protein expression of NF-κB p65 and its phosphorylation (p-NF-κB p65) in THP-1 macrophages simultaneously stimulated with LPS (100 ng/mL) and digested cold-pressed rice bran oil (CPRBO) emulsion (100 µg/mL) for different periods, with beta-actin as the loading control: (A) Western blot analysis; (B) densitometric analysis for NF-κB p65; and p-NF-κB p65, where data shown relative to control, columns with different lowercase letters are significantly ($p < 0.05$) different and the error bars indicate ± SD; the Western blot bands shown are representative.

KDML105-derived CPRBO was successfully fabricated as a stable oil-in-water emulsion. Its resistance to gastric digestion and its destabilization in the small intestine make it a promising delivery system for lipophilic bioactive compounds. The anti-inflammatory activity resulted from its inhibition of both NF-κB p65 expression and phosphorylation and subsequent suppression of pro-inflammatory cytokine gene expression.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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