Tropical Journal of Pharmaceutical Research December 2017; 16 (12): 2943-2951
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v16i12.19

Original Research Article

Anti-oxidant and anti-inflammatory effects of rice bran and green tea fermentation mixture on lipopolysaccharide-induced RAW 264.7 macrophages

Yun-Hee Rhee[1], Chun Hee Rhee[2], Phil-Sang Chung[1,3], Jin-Chul Ahn[1]
[1]Beckman Laser Institute Korea, Dankook University, Cheonan 31116, [2]Enzyme LAPA. Co., Buldang 955, Cheonan 31114, [3]Laser Translational Clinical Trial Center, Dankook University, Cheonan 31116, Republic of Korea

For correspondence: **Email: jcahn@dankook.ac.kr; **Tel:** +82-41-550-3889; **Fax:** +82-41-559-7838*

Sent for review: 21 June 2017 *Revised accepted: 28 November 2017*

Abstract

Purpose: *To investigate the anti-inflammatory and anti-oxidant properties of an enzyme bath of Oryza sativa (rice bran) and Camellia sinensis O. Kuntz (green tea) fermented with Bacillus subtilis (OCB).*
Methods: *The anti-oxidant effects of OCB were assessed by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay and flow cytometry. The anti-inflammatory effects of OCB were assessed by a nitric oxide (NO) assay. Enzyme-linked immunosorbent assay and real-time polymerase chain reaction were used to quantify expression of pro-inflammatory cytokines in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. The major compounds of OCB were identified using high performance liquid chromatography (HPLC) analysis.*
Results: *OCB had no cytotoxic effect on LPS-stimulated macrophages or peripheral blood mononuclear cells up to 1 mg/mL. OCB displayed anti-oxidant effects comparable to those of ascorbic acid and reduced reactive oxygen species (ROS) levels in target cells. OCB treatment of LPS-stimulated mavrophages decreased nitric oxide (NO), NO synthase (iNOS), cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2) and key pro-inflammatory cytokine expressions, suggesting that OCB acts as an anti-oxidant and anti-inflammatory agent by reducing ROS levels and inhibiting pro-inflammatory mediators. The main effector compounds in OCB were epicatechin gallate, cathechin, synigrin acid, epicathechin, epigallocatechin gallate, rutin, and isoquercetin, which are known anti-oxidants.*
Conclusion: *OCB fermentation product may be used as synergistic adjuvant therapy for inflammatory diseases.*

Keywords: *Rice bran, Green tea, Bacillus subtilis, Enzyme bath, Anti-oxidant, Anti-inflammation*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Enzyme baths have traditionally been used in Asian countries to treat various neurological disorders. More recently, enzyme baths were officially used at the 11th Winter Olympics Games of 1972 in Sapporo, Japan to reduce athlete fatigue. Enzyme bath is made in tubs through the fermentation of sawdust or rice bran using microbial enzymes. This fermentation process generates heat that, when combined with useful fermentation metabolites, improves the

physiology of the human body by augmenting blood circulation and immune function. Although well known for its beneficial properties on body condition, the physiological properties of enzyme bath have only been poorly studied. One way that enzyme baths may stimulate repair is through modulation of the immune system. Inflammation is a normal physiological response to counteract the assault of chemicals, microbial pathogens. Macrophages are major immune cells that mediate this inflammatory response by responding to cytokines and secreting pro-inflammatory mediators including nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6. However, an unbalanced or prolonged inflammatory state can lead to progressive tissue damage. Indeed, prolonged inflammatory conditions are implicated in the development of chronic diseases such as cancer and neurodegenerative disorders. Given the relationship between inflammation and tissue repair, the effects of an enzyme bath on inflammation and macrophage activation warrants investigation.

In this study, we examined the effect of metabolites of rice bran, green tea, and microbial enzymes from fermentation on oxidative stress and pro-inflammatory cytokine production in cultured cells. Various components of the enzyme bath are well known for their biological properties. Rice bran is a source of antioxidants, vitamins, fatty acids, protein, carbohydrates, and other critical minerals [1-3], Polyphenols of green tea are recognized for their anti-cancer or anti-inflammation properties [4-7]. We examined the cytotoxicity of OCB extract on both primary peropheral blood mononuclear cells (PBMCs) and RAW 264.7 macrophages. We purchased the product of fermented *Oryza sativa* (rice bran) and *Camellia sinensis O*. Kuntz (green tea) fermented from *Bacillus subtilis* (Enzyme LAPA Co,) and extracted with ethanol (OCB; Figure 1). We characterized the anti-oxidant potency of OCB using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and examined its protective effect against oxidative damage from reactive oxygen species (ROS) in lipopolysaccharide (LPS)-induced RAW 264.7 macrophages. We also measured the expression of inducible NO synthase (iNOS), TNF-α, IL-6, and cyclooxygenase-2 (COX-2) by real-time polymerase chain reaction (PCR), the expression of NO by NO detection assay, and the expression of PGE_2 by enzyme-linked immunosorbent assay (ELISA). Finally, we identified key components in the fermentation products of OCB using high-performance liquid chromatography (HPLC). Together these data elucidate a mechanism for the therapeutic properties of enzyme bath and suggest that it might be used for the treatment of inflammatory disorders.

EXPERIMENTAL

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were purchased from WelGENE Co. (Daejeon, Korea). The enzyme immunoassay kit for PGE_2 was obtained from Cayman (Ann Arbor, MI, USA). The glutathione (GSH)/glutathione disulfide (GSSG) ratio detection assay kit was purchased from Abcam (Cambridge, MA, USA). LPS and 2',7'-dichlorofluorescin diacetate (DCFH-DA) were purchased from eBioscience (Campbell, CA, USA). The Griess reagent system was purchased from Promega (Madison, WI, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DPPH, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of ethanol extract from OCB

A mixture of 1220 g *O. sativa* and 450 g *C. sinensis* O. Kuntz was fermented with 600 mL *B. subtilis* culture fluid at 30 °C in a fermentator for 7 days. After fermentation, filtered supernatant from OCB fluid was removed and concentrated using a rotary evaporator (Eyela, Tokyo, Japan). The powder from OCB fluid was extracted with 70 % ethanol for 3 days at room temperature. The crude ethanol extract for OCB was evaporated, and the yield was measured to be 38.6 %. The lyophilized powder was dissolved in 10 % dimethyl sulfoxide and filtered through a 0.2-μm syringe filter.

Cells

RAW 264.7 cells, a murine macrophage cell line, were purchased from American Type Culture Collection and maintained in DMEM supplemented with 5 mg/mL glucose, 10 % FBS, 1 % penicillin-streptomycin at 37 °C with 5 % CO_2. PBMCs were isolated as described by Panda et al. [8]. Five week-old ICR mice were purchased from Orient Bio (Suwon, Korea) and raised following guidelines from the Ethics Committee of Dankook University (DKU15-026). Mice were euthanized using CO_2 and rinsed liberally with 70 % ethanol. The mouse femurs and tibiae were removed and carefully cleaned of soft tissues.

Figure 1: Photograph of *Oryza sativa*, *Camellia sinensis O. Kuntz, and Bacillus subtilis*

The marrow was harvested by inserting a 20-gauge syringe needle and flushing with minimum essential medium-alpha containing 20 % FBS. Cells were inoculated and incubated in a 25 cm^2 flask for 72 h at 37°C with 5 % humidified CO_2. Non-adherent PBMCs were removed and loaded for cell viability assessment under OCB.

Cell viability assay

The cytotoxic effect of OCB on RAW 264.7 cells and PBMCs was evaluated by the MTT assay. Briefly, cells were seeded at a density of 5×10^3 cells/well in a 96-well plate for 24 h. Cells were then treated with various concentrations of OCB with or without 1 µg/mL LPS. After 24 h, 5 mg/mL MTT was added to each well, and cells were incubated until formazan was constituted. The formazan was dissolved in MTT lysis solution (20 % SDS, 50 % dimethylformamide), and absorbance at 450 nm was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Cell viability was calculated as cell viability in the OCB treated group versus that in the untreated control group. Each experiment was repeated three times.

DPPH assay

DPPH solution with methanol at 0.1 mM was added to various concentrations of OCB, using ascorbic acid as a positive control. After incubating for 30 min in dark, the absorbance of the samples was measured at 517 nm using a microplate reader. All data were converted to scavenging radical activity (S) using Eq 1.

$$S (\%) = \{(Ab – As/Ab)\}100 \quad (1)$$

where Ab is the absorbance of blank and As is the absorbance of ascorbic acid- or OCB-treated samples. Each experiment was carried out in triplicate.

Analysis of ROS

Cells were pre-incubated for 1 h in presence or absence of 1 µg/mL LPS before the addition of OCB followed by addition of 10 µg/mL, DCFH-DA. After a 20-min incubation, the suspensions were washed with PBS and assayed with a flow cytometer (FACsort systems, Becton-Dickinson, Bedford, MA, USA).

Glutathione (GSH) assay

Cells were treated same as well as ROS anaylsis, and followed by measurement of total GSH and glutathione disulfide (GSSG) using a GSH/GSSG ratio detection assay kit. The assay was performed according to the manufacturer's instructions and repeated three times.

Nitrite assay

The production of NO by LPS-activated macrophages was measured with the Griess reagent system. Briefly, RAW 264.7 cells (1×10^4 cells/well) were seeded onto 96-well plates and incubated for 24 h. The cells were then treated with various concentrations of OCB with or without 1 µg/mL LPS for 24 h, and the supernatant was removed. Fifty microliters of supernatant from OCB-treated wells was mixed with the same volume of Griess reagent (1 % sulfanilamide and 0.1 % naphthylethylenediamine solution), and then incubated for 10 min at room temperature. Absorbance at 550 nm was read using a spectrophotometer (TECAN, Männedorf, Switzerland). The NO level was estimated as the percentage of absorbance of the sample relative to that of control.

Real-time PCR

RAW 264.7 cells (1×10^6 cells/well) were plated in 6-well plates and incubated for 24 h. Cells

were then treated with various concentrations of OCB with or without 1 μg/mL LPS for 24 h. After washing twice with PBS, total RNA was extracted using the TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The PCR template was prepared using the Quantitect SYBR green PCR kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions and quantitative PCR was performed using the Applied Biosystems 7500 (Life Technologies). Primer sequences are shown in Table 1. Quantitative analysis compared the ΔCT ratio of primers versus that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control.

Table 1: Primer sequences

Primer	Sequence
Inducible nitric oxide species (iNOS)	AGTGGTGTTCTTTGCTTC
	GCTTGCCTTATACTGGTC
Tumor necrosis factor alpha (TNF-α)	ACGGCATGGATCTCAAAGAC
	AGATAGCAAATCGGCTGACG
Cyclooxygenase type-2 (COX2)	GGTCTGGTGCCTGGTCTG
	CTCTCCTATGAGTATGAGTCT GC
Interleukin-6 (IL-6)	CTTCCATCCAGTTGCCTTCTT
	ACGATTTCCCAGAGAACATGT

Prostaglandin E_2 assay

Using supernatant collected from the NO assay, PGE_2 concentrations with or without OCB in LPS-induced RAW 264.7 cells were measured using a specific enzyme immunoassay kit according to the manufacturer's instructions.

High performance liquid chromatography (HPLC)

OCB components were analyzed using Agilent 1260 model with a pump (G1311C), auto sampler (G1329B), column (G1316A), and UV detector (G1314F) purchased from Agilent (Santa Clara, CA, USA). We determined the HPLC conditions and separated the active ingredients of rice bran and green tea with reference to previous studies [9,10]. The analysis conditions are described in Table 2.

Statistical analysis

The results are presented as mean ± standard deviation from three or more independent experiments. Data was compared using the Student's *t*-test and significant p-value was*p < 0.1, **p < 0.05, and ***p < 0.001. Prism® program VER 5.03 (GraphPad, La Jolla, CA, USA) was used for statistical analysis of the data.

RESULTS

Cytotoxicity effect of OCB on macrophages

The viability of RAW 264.7 macrophages under various concentrations of OCB was similar in the presence and absence of LPS (Figure 2). We also observed no cytotoxic effects of OCB on mouse PBMCs in the presence or absence of LPS.

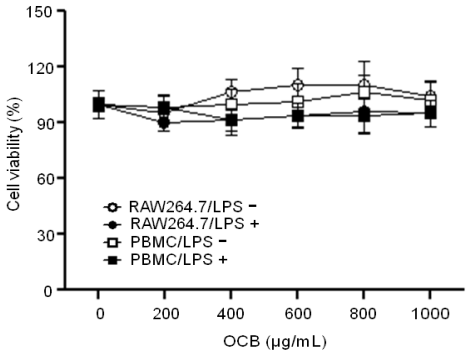

Figure 2: Cytotoxicity of OCB on LPS induced RAW 264.7 macrophages

Table 2: HPLC analysis conditions

Detector	UV detector
Wavelength.	310nm
Column	Aminex® HPX-87H Ion Exclusion column (300 × 7.8 mm, 9μm)
Mobile phase	10mM H_2SO_4 in water (isocratic)
Running time	30min
Flow rate	0.5mL/min
Injection volume	10 μl
Temperature	40 ^oC

Anti-oxidant potency of OCB

DPPH assay, commonly used in natural product anti-oxidant studies, is based on the idea that hydrogen donors are antioxidants and is used to assess the free radical scavenging ability of compounds.

We used ascorbic acid, a known free radical scavenger, as a positive control. We found that OCB demonstrated anti-oxidant activity in a dose-dependent manner (Figure 3A). Furthermore, we found that 500 µg/mL OCB had greater radical scavenger potency than 1 mg of ascorbic acid ($p = 0.0045$).

Effect of OCB on ROS production in LPS-induced macrophages

LPS treatment increases cellular oxygen uptake and induces the release of intracellular ROS. To determine whether OCB can alter LPS-induced intracellular redox states, we examined ROS production levels. LPS treatment alone significantly increased ROS levels from 66.6 % to 72.9 % in RAW 264.7 macrophages (Figure 3 B). However, OCB reduced LPS-induced ROS production in a dose-dependent manner from 68.9 % to 56.6 %.

Effect of OCB on GSH in LPS-induced macrophages

GSH exists in either a reduced or oxidized (GSSG) form in cells. The enzyme GSH reductase recycles GSSG by converting it to GSH with the simultaneous oxidation of β-nicotinamide adenine dinucleotide phosphate (β-NADPH2).

We assessed how OCB treatment affects expression of the two forms of GSH in RAW 264.7 macrophages lysates using GSH/GSSG detection assay. We found that OCB increased levels of GSH in a dose-dependent manner (Figure 3 C).

Effect of OCB on NO production in LPS-induced macrophages

To test the effects of OCB on NO production in RAW 264.7 macrophages, the cells were treated with various concentrations of OCB in presence of 1 µg/mL LPS for 24 h. The culture media was collected, and NO production was measured by the accumulation of nitrite in the cell culture media. We found that LPS induced a marked accumulation of NO in culture media, whereas OCB significantly reduced NO production at all dose from 62.5 to 500 µg/mL (Figure 4 A).

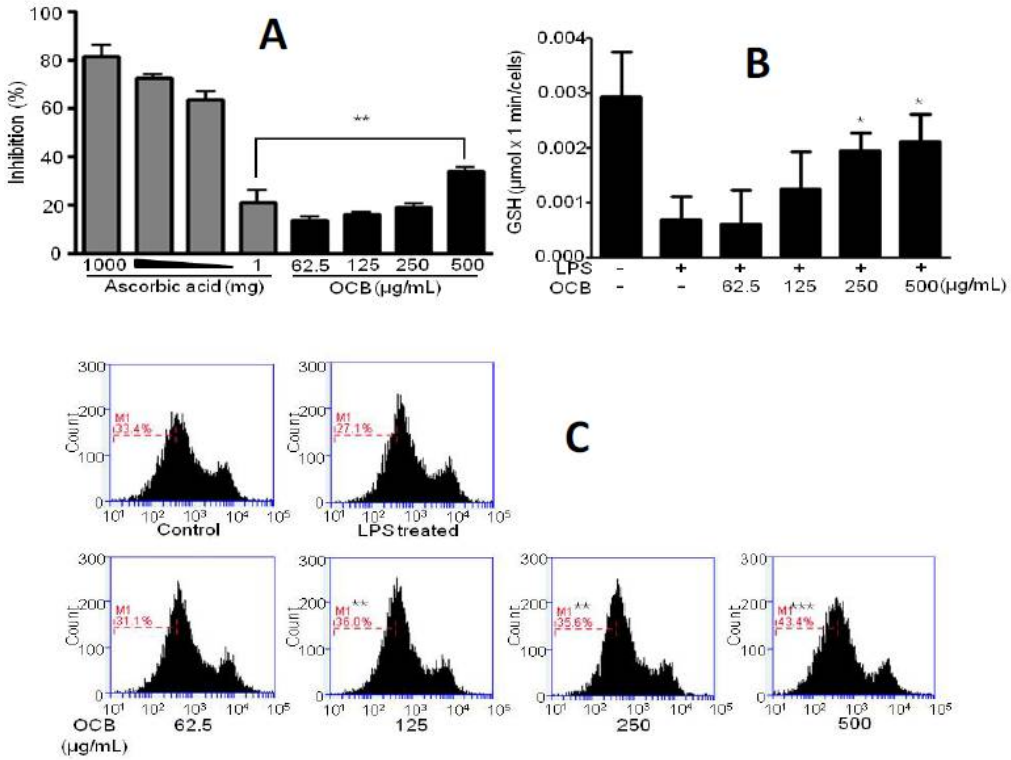

Figure 3: Anti-oxidant effect of OCB on LPS-induced macrophages. (A) DPPH assay, all data was converted into percentage of scavenging radical by the following Eq 2. ; LPS treatment alone significantly increased ROS levels from 66.6 % to 72.9 % in RAW 264.7 macrophages (C). However, OCB reduced LPS-induced ROS production in a dose-dependent manner from 68.9 % to 56.6 % analysis by flow cytometer. (B) GSH assay. Data was compared using the student's *t*-test and significant *p*-value was*$p < 0.1$, **$p < 0.05$, and ***$p < 0.001$

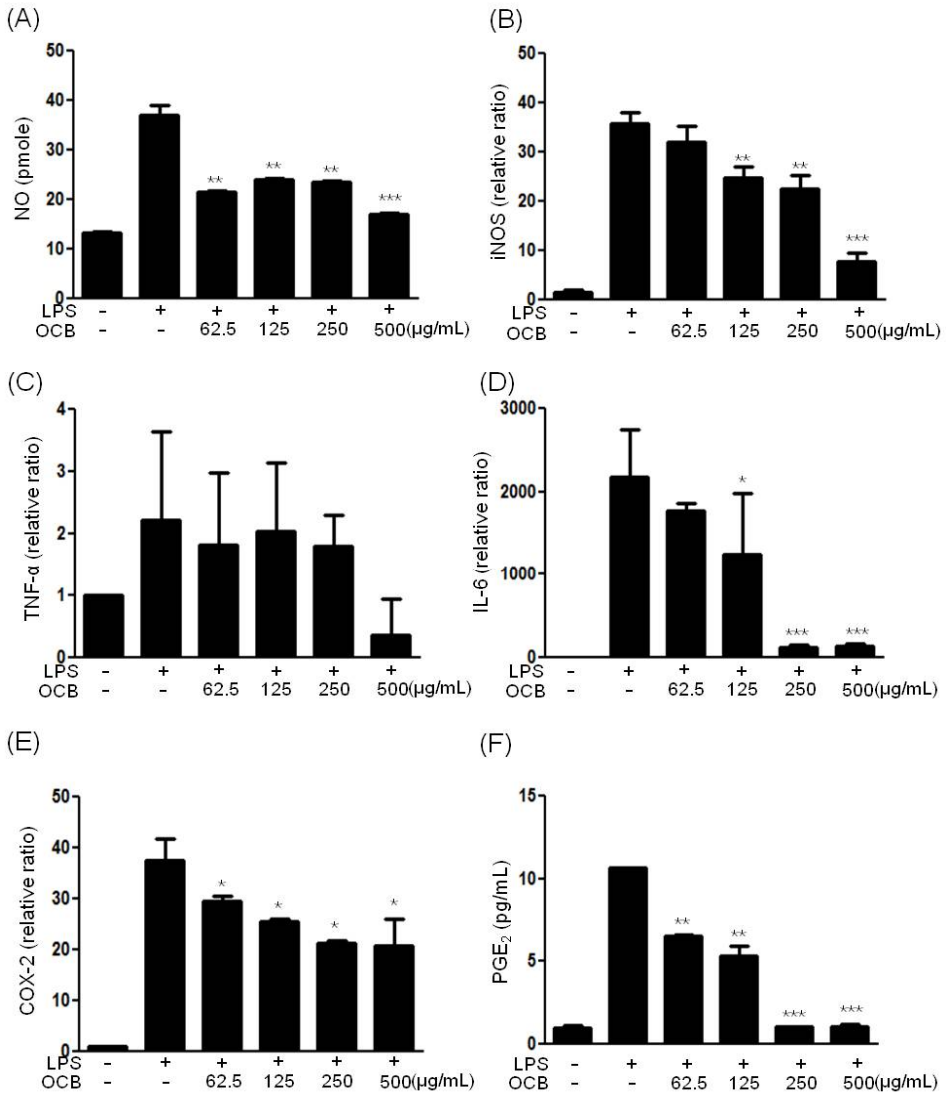

Figure 4: Effects of OCB on levels of (A) NO, (B) iNOS, (C) TNF-α, (D) IL-6, (E) COX-2, and (F) PGE_2 in LPS-induced macrophages. Data were compared using Student's *t*-tests, and significance is indicated as $*p < 0.1$, $**p < 0.05$, $***p < 0.001$

Effect of OCB on pro-inflammation cytokines in LPS-induced macrophages

To determine the effect of OCB on pro-inflammatory cytokine expression in LPS-induced RAW 264.7 macrophages, mRNA and protein levels of iNOS, TNF-α, IL-6, COX-2, and PGE_2 were measured by quantitative PCR and ELISA, respectively. OCB treatment reduced the transcriptional levels of iNOS (Figure 4B), IL-6 (Figure 4 D), and COX-2 (Figure 4 E) and the protein level of PGE_2 (Figure 4 F). OCB treatment also reduced the transcriptional level of TNF-α, but this was not statistically significant (Figure 4 C).

OCB constituents

Using HPLC, we isolated 9 leading compounds from the OCB product. The HPLC conditions are described in Table 2. As shown in Figure 4, the compounds isolated with their separation time in parentheses were ECG (8.985 min), cathechin (11.861 min), suringin acid (17.507 min), epicathechin (18.260 min), EGCG (18.602 min), rutin (24.705 min), isoquercetin (25.605 min), tolirin (67.848 min), and linoleic acid (87.911 min). The contents are described in Table 3.

DISCUSSION

Although enzyme bath reportedly increases activity of the immune system and have been used as a folk remedy for various skin disease including atopic dermatitis, acne, psoriasis, and blood circulation, there has been little scientific inquiry into the mechanisms underlying their physiological benefits. We hypothesized that

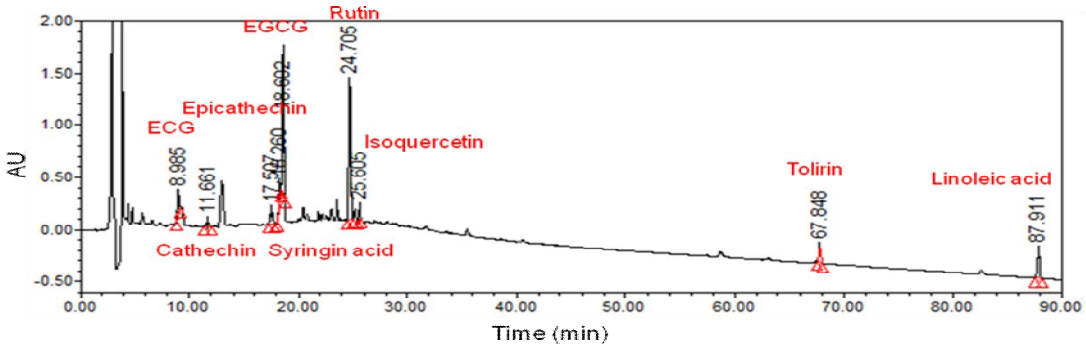

Figure 5: HPLC chromatogram of OCB components using an Agilent 1260 system and the analysis conditions described in Table 2

enzyme bath inhibits production of free radicals and regulates the activation of pro-inflammatory factors in tissues.

Table 3: Major compounds of OCB based on HPLC analysis

Major compound	Content (mg/g)
ECG	0.397
Cathechin	0.439
Syringin acid	1.663
Epicathechin	1.088
EGCG	8.516
Rutin	1.699
Isoquerctin	1.218
Tolorin	5.331
Linoleic acid	18.221

To determine whether the ethanol extraction of OCB is cytotoxic, we performed cell viability analysis using cultured primary PBMCs and RAW 264.7 macrophages. We found that OCB extract had no cytotoxic effects on PBMCs or RAW 264.7 macrophages regardless of the presence of LPS. As oxidative stress related to ROS can induce inflammation and degrade intracellular proteins, lipids, and nucleic acids [11], we assessed the ability of OCB to reduce oxidative stress in target cells using DPPH assay, flow cytometry, and GSH assay. In the DPPH assay, OCB showed a potency as a free radical scavenger as well as ascorbic acid. OCB also attenuated LPS-stimulated ROS production.

Glutathione in its reduced from is a tripeptide composed of L-cysteine, L-glutamic acid and glycine. It is the smallest intracellular thiol-containing protein in all cells and prevents cell damage caused by ROS such as free radicals and peroxides. We found that LPS reduced GSH activity, whereas OCB treatment reversed this effect in a dose-dependent manner. Thus, OCB appears to attenuate the intracellular depletion of GSH in its reduced form.

ROS and pro-inflammatory cytokines act synergistically through an ROS/RNS-cytokine-transcription factor regulatory loop, thereby augmenting the inflammatory response and exacerbating tissue damage [11,12]. Excessive production of NO and prostaglandins induced by iNOS is considered to be the most prominent molecular mechanism in the inflammatory process [13]. We observed that the production of NO and iNOS in LPS-induced murine macrophages was reduced by OCB treatment. During inflammation, macrophages have three major functions: antigen presentation [14], phagocytosis [15], and immunomodulation [15,16], which is mediated through the production of various cytokines and growth factors. The inflammatory process is usually tightly regulated, involving both signals that initiate and maintain inflammation and signals that resolve inflammation. An imbalance between these two types of signals leaves inflammation unchecked, resulting in cellular and tissue damage. We found that OCB dose-dependently inhibited the expression of TNF-α, IL-6, COX-2, and PGE_2. Furthermore we identified, key components in the OCB extract using HPLC.

Compared to the yields of active ingredients in OCB extract in other reports, we demonstrated that products from OCB fermentation could be extracted on the order of milligrams rather than micrograms. Ahmad et al. reported that ethanol was most efficient for ECG or EGCG extraction from green tea. According to their HPLC analysis, ECG yield was 0.17 ± 0.002 mg/g and EGCG yield was 1.635 ± 0.18 mg/g on average [17].

We also found significant amounts of linolenic acid (18.221 mg/g), ECG (0.397 mg/g), EGCG (8.516 mg/g), and tolorin (5.331 mg/g) in the OCB extract. These compounds are well-known anti-oxidants [9, 18], and the extraction efficiency in our experiments was more than double that of previous experiments. This increased extraction efficiency likely results from the fermentation process using microorganisms. Ultimately, these results demonstrate that the reduction of

inflammatory cytokines by OCB plays a crucial role in regulating inflammation through ROS suppression (Figure 6) and that using fermentation could allow higher extraction efficiency.

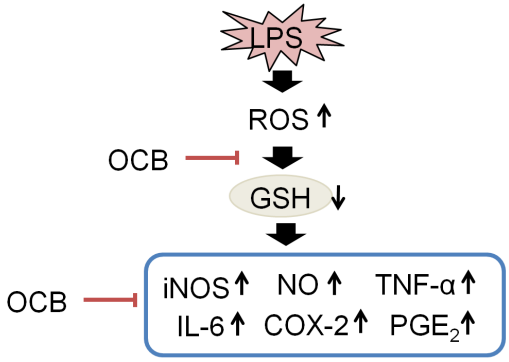

Figure 6: A schematic illustration of antioxidant and anti-inflammation activities of rice bran and green tea fermentation

CONCLUSION

The findings of this work suggest that the fermentation of rice bran and green tea using *B. subtilis* is capable of poducing high yields of compounds and can critically modulate pro-inflammatory factors via GSH and ROS regulation. Thus, the product of OCB fermentation may be used as a synergistic adjuvant therapy for inflammatory diseases by acting as a radical scavenger and ROS inhibitor.

DECLARATIONS

Acknowledgement

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by Republic of Korea Ministry of Education (no. 2017R1D1A1B03030060), a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by Ministry of Health & Welfare, Republic of Korea (grant no. HI15C1524), and Research Fund of Dankook University in 2016.

Conflict of interest

The authors declare that they have no competing interests with regard to this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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