

Anti-inflammatory Activity of Rice Phytosterols and γ -oryzanol in THP-1 Macrophages

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ABSTRACT

Immune modulating effects of three bioactive compounds found in rice bran oil; β -sitosterol, campesterol and γ -oryzanol, were investigated using THP-1 macrophage cell model. β -sitosterol and campesterol belong to a group of phytosterols, while γ -oryzanol is not the case. γ -oryzanol is usually found in conjugated forms, which uniquely exists only in rice bran oil. Cytotoxicity was performed to examine toxicity phytosterols and γ -oryzanol to provide proper working concentrations for gene expression analysis. A range of phytosterols and γ -oryzanol concentrations from 1-12 μ M was assayed. The results showed that cytotoxicity was observed as the concentration of phytosterols and γ -oryzanol increased. Concentrations up to 2 μ M could provide cell viability more than 85%. This was the case for all three compounds at both resting and inflamed stage. β -sitosterol, campesterol and γ -oryzanol were tested as individuals at 2 μ M or as combinations at the final concentration of 2 μ M. At resting stage, none of the compounds showed pro-inflammatory activity. At inflamed stage, β -sitosterol showed pro-inflammatory activity, while γ -oryzanol showed a tendency to inhibit expression of pro-inflammatory cytokine genes. For the mixture of β -sitosterol, campesterol and γ -oryzanol, the results showed that immune modulating activities were not depended on types of phytosterols, but rather final concentrations.

Key Words: phytosterol, γ -oryzanol, THP-1 macrophages, immune modulation, inflammation

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INTRODUCTION

The immune system is a host defense system to remove foreign matters or abnormal cells in a living body, which is precisely controlled by a close cooperation with cells, tissues and organs. Immune system is divided in two parts, innate and adaptive immunity. Innate immunity is the first response for infection. It is non-specific and momentary reaction mediated by various white blood cells, for instance, monocytes, macrophage, dendritic cells, and natural killer cells. They release various kinds of cytokines as signals to promote or to inhibit inflammation. Cells belong to adaptive immunity can memorize information about antigens from the first invasion, which make them work quicker and stronger upon the second infection. Modulating the immune system is an important factor to keep the body protective system to function properly.

Phytosterol is a sterol from plants which is one kind of phytochemicals. Phytosterol is widely known as bioactive compounds which can decrease blood cholesterol level by dietary intake (Katan *et al.*, 2003 and Demonty *et al.*, 2008). Recently, immune modulating functions of phytosterols have been reported. (Demonty *et al.*, 2008 and Alappat *et al.*, 2010) However, the mechanisms of these effects are still unclear. β -sitosterol is the most popular phytosterol because it shows the highest content in many kinds of plant oils (Sugano *et al.*, 1997). γ -oryzanol, which is ferulate ester with phytosterol or triterpene, has been also shown to function as an immune modulating compound. It has been reported to be much more absorbed into small intestine than β -sitosterol (Sugano *et al.*, 1997). Rice bran oil is a unique source of phytochemicals, not only plentiful phytosterols, but also γ -oryzanol. However, there are few reports on immune modulating effects of γ -oryzanol and few reports about the interaction among phytosterols. Therefore, the immune modulating effects of phytosterols found in rice bran oil, β -sitosterol, campesterol, and that of γ -oryzanol were studied using THP-1 macrophage cell model.

MATERIALS AND METHODS

1. THP-1 cell culture

The human monocyte THP-1 cell line was purchased from American Type Culture Collection ATCC[®]. Cell culture and differentiation THP-1 monocyte into macrophage were performed according to the protocol used by Chanput *et al.*, 2010. RPMI-1640 with L-glutamine (Invitrogen, England) was used as a culture medium.

2. Preparation of medium containing phytosterols

γ -oryzanol (Wako Pure Chemical Industries Ltd.), β -sitosterol (TAMA BIOCHEMICAL Co., Ltd) and campesterol (TAMA BIOCHEMICAL Co., Ltd) were dissolved in absolute ethanol (Merck, Germany) and kept at -20 °C until use. A vial of stock solutions was further dissolved in culture media

until reaching designated concentrations. The final concentration of ethanol in stimulating medium was kept maximally at 0.5%.

3. Treatment of THP-1 macrophage by phytosterols

3.1 Resting stage

THP-1 macrophage at resting stage in 24-well plate was treated with designated concentrations of phytosterols or γ -oryzanol and 0.5% ethanol in culture medium for 3 h. Untreated cells were used as a negative control.

3.2 Inflamed stage

THP-1 macrophage at resting stage in 24-well plate was pre-treated with designated concentrations of phytosterols or γ -oryzanol and 0.5% ethanol in culture medium for 3 h. Then medium was removed and cells were stimulated with stimulating medium containing 100 ng/mL LPS for 6 h.

4. Cytotoxicity assay

Cytotoxicity of phytosterols and γ -oryzanol was tested on THP-1 macrophage in both resting and inflamed stage using MTT assay. After incubation of THP-1 macrophage with phytosterols for 3 h, 3-(4,5-Dimethylthylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT; Sigma, United States) solution was added in each well. The final concentration of MTT was 0.5 mg/mL. Then cells were incubated for 2 h at 37 °C. After removal of the medium, the cells were lysed by 100 μ L of extraction solvent (dimethyl sulfoxide (DMSO; Fisher Scientific, England) and ethanol were mixed by 1:1). Then absorbance was detected at a wavelength of 570 nm by a microplate reader.

5. Gene expression assay

RNA was extracted from macrophage by following manufacture protocol of Thermo® GeneJET RNA purification kit. RNA purification was performed following manufacture protocol of RNase-free DNase I. Concentration of RNA solution was detected by Thermo® Nanodrop 2000/2000c. Then cDNA was synthesized from RNA solution by following manufacture protocol of Thermo® RevertAid First Strand cDNA Synthesis Kit. Expression of pro-inflammatory cytokine genes; IL-1 β , IL-6, IL-8, TNF- α and NF- κ B, anti-inflammatory cytokine gene; IL-10, and housekeeping gene GAPDH were determined by real time-PCR. 0.5 μ L of cDNA solution was mixed with 5 μ L iTaq® Universal SYBR Green Super mix, 4.3 μ L RNase-free water, 0.1 μ L primer forward solution and 0.1 μ L primer reverse solution in 96-well PCR plate. First, as initialization step, plate was heated to 95°C for 5 minutes. As annealing and extension step, the reaction temperature was changed to 95°C for 30 seconds, 60°C for 30 seconds, 75°C for 30 seconds and 78°C for 10 seconds. This step was repeated 40 times.

6. Statistical analysis

The experiment design was CRD (completely randomized design) with 2 independent replications for treatment by phytosterol and two duplications for gene expression analysis. One-way analysis of variance (ANOVA) and Dunnet post-hoc test were used to determine the significant differences between groups. Statistical significance was accepted with $p < 0.05$.

RESULTS AND DISCUSSION

1. Effect of phytosterols and γ -oryzanol on cell viability of THP-1 macrophage.

Each compound was tested their cytotoxicity up to 12 μM . The result showed that cell viability was decreased at high concentrations. More than 85% of cell viability was maintained up to 2 μM of individual β -sitosterol, campesterol or γ -oryzanol (data not shown). Combination between β -sitosterol, campesterol and γ -oryzanol, was also tested at 2 μM as the final concentration. Two compounds were mixed at a ratio of 1:1 or a combination of 3 compounds, each of them was kept at 0.66 μM . More than 85% of cell viability could be observed (data not shown). Similar finding was found in inflamed stage (data not shown). Thus, rice phytosterols; β -sitosterol, campesterol and γ -oryzanol, at concentration of 2 μM were selected for gene expression analysis.

2. Phytosterols and γ -oryzanol as immune modulating agents on THP-1 macrophage.

2.1 Resting stage

The objective to use THP-1 macrophages at resting stages is to investigate whether rice phytosterols and γ -oryzanol have pro-inflammatory activity. Compared with the control (cells incubated with culture medium and 0.5% ethanol), expression of IL-1 β and TNF- α gene was significantly reduced by phytosterols and γ -oryzanol (Figure 1A). Expression of IL-6 and NF- κB gene could not be observed. Therefore, it can be concluded that β -sitosterol, campesterol and γ -oryzanol do not have pro-inflammatory activity.

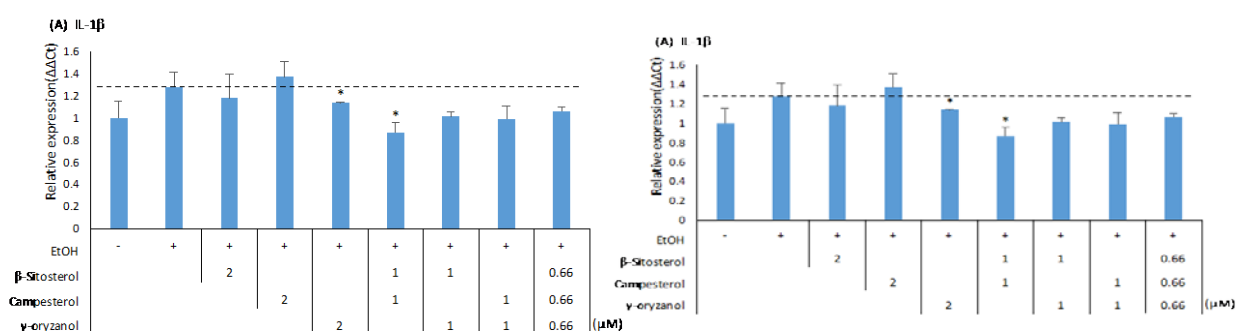


Figure 1 Effect of β -sitosterol, campesterol and γ -oryzanol on gene expression of THP-1 macrophages at resting stage. (A) Interleukin-1 β , (B) Tumor necrosis factor- α . * $p < 0.05$ with respect to ethanol control.

2.2 Inflamed stage

At inflamed stage, THP-1 macrophages stimulated with 100 ng/ml LPS were used as a positive control. Expression of IL-1 β , IL-6, TNF- α , IL-8, IL-10 and NF- κ B genes were highly up-regulated after LPS stimulation (Figure 2). Pre-treated THP-1 macrophages with individual β -sitosterol, campesterol or γ -oryzanol for 3 hours, then incubated with LPS for 6 hours could stimulate expression of IL-1 β , IL-6 and TNF- α genes (Figure 2A-C). Anti-inflammatory cytokine IL-10 gene was strongly promoted by 1 μ M of β -sitosterol or campesterol, approximately 330% compared to the positive control (Figure 2E). For summary of individual treatments, β -sitosterol promoted gene expression of not only pro-inflammatory cytokines (IL-1 β , IL-8 and TNF- α) but also anti-inflammatory cytokine IL-10. Campesterol has the smallest effect among the three phytosterols but slightly promote expression of IL-10 gene significantly. γ -oryzanol showed the biggest tendency among the three to inhibit expression of pro-inflammatory cytokines, especially TNF- α gene was significantly inhibited by 1 μ M of γ -oryzanol.

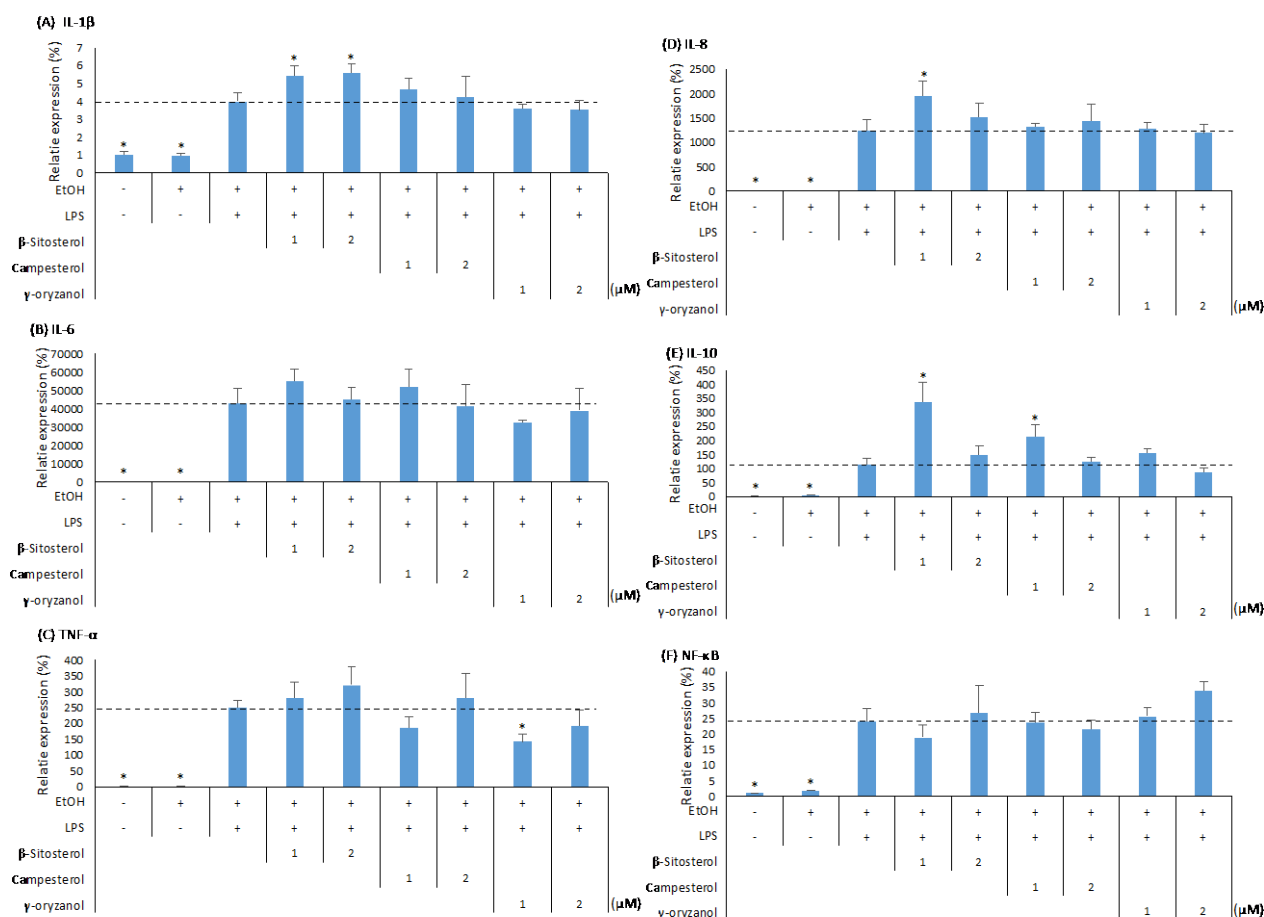


Figure 2 Effect of individual β -sitosterol, campesterol or γ -oryzanol on gene expression of THP-1 macrophages at inflamed stage. (A) Interleukin-1 β , (B) Interleukin-6, (C) Tumor necrosis factor- α , (D) Interleukin-8, (E) Interleukin-10, (F) Nuclear factor-kappa B. * $p < 0.05$ with respect to positive control by LPS.

Interestingly, mixture of two compounds of which the final concentration was 1 μM (0.5 μM β -sitosterol + 0.5 μM campesterol, 0.5 μM β -sitosterol + 0.5 μM γ -oryzanol and 0.5 μM campesterol + 0.5 μM γ -oryzanol) promoted gene expression of IL-1 β (Figure 3A). IL-6 gene expression was inhibited only mixture of 1 μM β -sitosterol and 1 μM γ -oryzanol (Figure 3B). For TNF- α , several treatments (1 μM β -sitosterol + 1 μM campesterol, 1 μM β -sitosterol + 1 μM γ -oryzanol, 1 μM campesterol + 1 μM γ -oryzanol and 0.33 μM β -sitosterol + 0.33 μM campesterol+ 0.33 μM γ -oryzanol) inhibited gene expression compared with positive control (Figure 3C). Only 1 μM β -sitosterol + 1 μM campesterol promoted gene expression of IL-10 (Figure 3E) and there is no significant difference observed among treatments on IL-8 and NF- κ B (Figure 3D and F). As summary of these results, mixture of two kinds from phytosterols and γ -oryzanol had an effect on gene expression induced by LPS. However, tendency of these effects seemed to depend not only choices of compounds but also final concentration. Three pro-inflammatory genes, IL-1 β , IL-6 and TNF- α , exhibited pro-inflammatory effect from 1 μM + 1 μM mixture but anti-inflammatory effect from 0.5 μM + 0.5 μM mixture, comparatively. Interestingly, mixtures of more than two compounds often showed different tendency to modulate expression of inflammatory cytokines, compared with individual phytosterol or γ -oryzanol (Figure 2 and 3). In addition, even individual compounds showed different tendency, mixture of two compounds showed similar tendency following total concentration. Gene expression of IL-10 at inflamed stage was influenced only mixture of 1 μM β -sitosterol and 1 μM Campesterol.

It seems that γ -oryzanol showed a tendency of among three compounds to inhibit expression of pro-inflammatory cytokines, especially TNF- α is significantly inhibited by 1 μM of γ -oryzanol. It has been reported that γ -oryzanol could have a function to inhibit NF- κ B activation of macrophage and inhibit following inflammation cascades (Nagasaka *et al.*, 2007). While, there are several contrast reports about pro- and anti-inflammation activity of β -sitosterol. Anti-inflammatory effect of β -sitosterol for murine macrophages was reported (Valerio *et al.*, 2011). There are two hypotheses to explain mechanisms of phytosterols and γ -oryzanol influencing expression of inflammatory cytokine genes. First one is up- or down-regulation of TLR4 receptor pathway (Valerio *et al.*, 2013). Second one is inhibit NF- κ B activation and following pro-inflammatory signals (Nagasaka *et al.*, 2007). Stimulation of TLR4 results in activation of NF- κ B. First, activated TLR4 by recognition of lipopolysaccharide or inflammatory cytokines, which are signal of infection, remove an inhibitory subunit I κ B bounding with NF- κ B. Then free NF- κ B translocates to the nucleus to up-regulate transcription of cytokines to induce and promote inflammation (Lu *et al.*, 2008). Phytosterols and γ -oryzanol was considered to affect both or either of two factors, TLR4 or NF- κ B. In both cases, how β -sitosterol affected those is still unclear. We need to clarify the point of action in the mechanism for farther application of phytosterols and γ -oryzanol as immune modulating compound

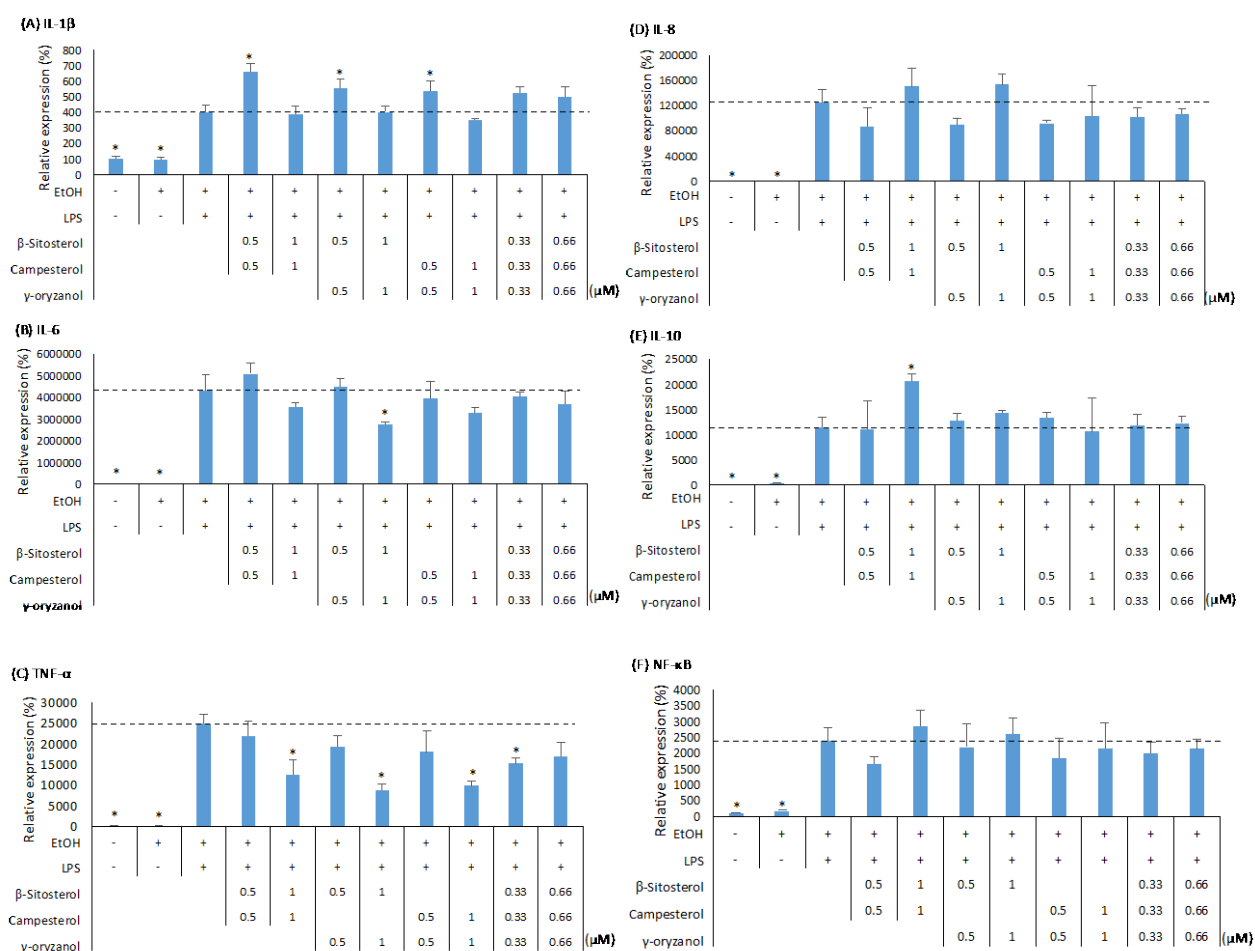


Figure 3 Effect of combination of β -sitosterol, campesterol and γ -oryzanol on gene expression of THP-1 macrophages at inflamed stage. (A) Interleukin-1 β , (B) Interleukin-6, (C) Tumor necrosis factor- α , (D) Interleukin-8, (E) Interleukin-10, and (F) Nuclear factor-kappa B.

* $p < 0.05$ with respect to positive control by LPS.

Seventeen treatments of inflamed stage were analyzed using PCA cluster analysis, in which they were divided into four groups (Figure 4). Group 1 contains treatment no.1 and 2, which do not contain LPS. Group 2 contains treatment no.3 (LPS treatment) and also treatment no.4-7. Group 3 consists of treatment no. 10, 12, 14, 16 and 17, while treatment no. 8, 9, 12, 13 and 15 was clustered in group 4. PCA cluster analysis shows that treatments in group 3 and 4 are different from LPS treatment, in which they seem to exhibit anti-inflammatory and pro-inflammatory activity, respectively. From this information, mixture of phytosterols and γ -oryzanol has been considered to have interactions to each other. It is because results of mixture did not reflect the characteristic that is derived from gene expression assay by individual phytosterol or γ -oryzanol. Regardless of kind of molecular from three compounds, mixture had characteristic effect depending on final total concentration.

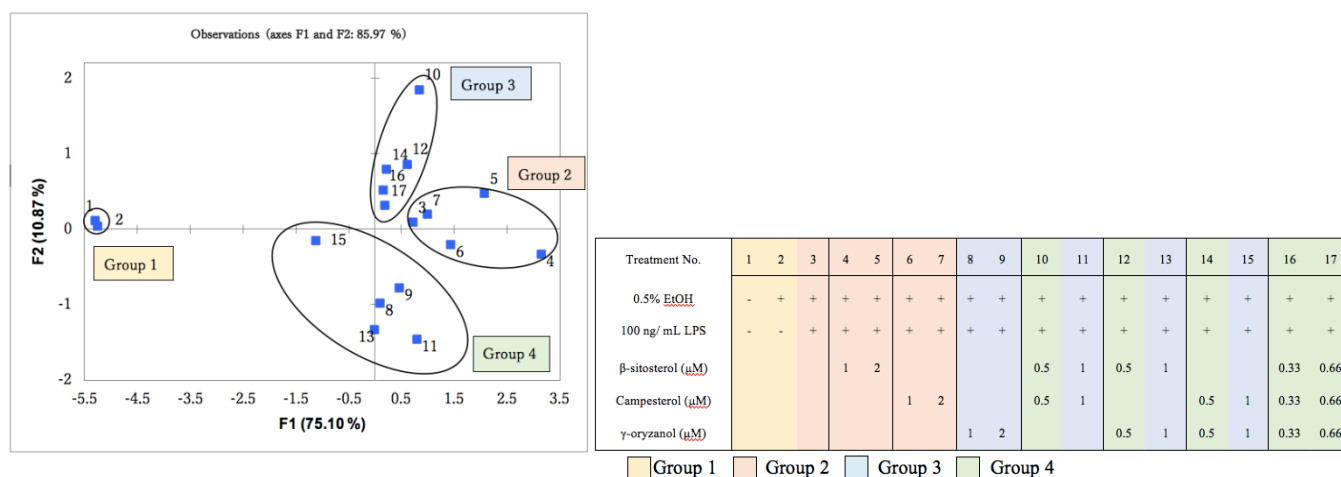


Figure 4 Principal component analysis (PCA) of gene expression at the inflamed stage.

CONCLUSION

For resting stage of THP-1 macrophage, there were no difference on gene expression of inflammatory cytokines by treatment of β -sitosterol, campesterol and γ -oryzanol. Among individual β -sitosterol, campesterol and γ -oryzanol, immune modulating effect of each compound on LPS-stimulated macrophage was different. β -sitosterol showed most pro-inflammatory effect and γ -oryzanol showed most anti-inflammatory effect. PCA analysis showed immune modulating effects of these treatments on six genes, including pro-inflammatory cytokines, anti-inflammatory cytokines and NF- κ B, could be divided into three groups. They are “no effect group”, “pro-inflammatory effect group” and “anti-inflammatory effect group”. Types of compound and concentration can affect them. *Ex vivo* and *in vivo* experiments should be performed to draw definite conclusion on immune modulation of rice phytosterols and γ -oryzanol.

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