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# Mitigation of LPS-Induced Inflammatory Responses Via Inhibition of NF-kB and MAPK Signaling Pathways by Betanin, a Coloring Agent

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**Abstract** Betanin has been approved for use in food and pharmaceutical products as a natural red colorant. Recently, potential health benefits of betanin and betanin-rich foods have been discussed. This study aimed to investigate the attenuation of LPS-induced inflammatory responses through inhibition of NF-kB and MAPK signaling pathways by betanin. The results show that betanin at 1-50 μM exerted an anti-inflammatory effect on inhibiting LPS-induced intracellular nitric oxide (NO), prostaglandin E2 (PGE2), and interlukin 6 (IL-6). In addition, betanin decreased iNOS and COX-2 protein expression. Western blotting analysis indicated that betanin inhibited the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) protein expression and also inhibited the MAPK signaling pathway. Above all, anti-inflammatory effects of betanin are attributed to its inhibition of inflammatory gene expression through the blocking of the NF-kB and MAPK signaling pathways. Taken together, these findings suggest that betanin is a potential anti-inflammatory mediator for inflammation-related disease treatment.

***Keywords:*** *Anti-inflammatory effect, betanin, lipopolysaccharide (LPS), NF-kB, MAPK, RAW264.7 macrophage*

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## 1. Introduction

The inflammation is a complex response of body's tissues to harmful stimuli, such as irritants, pathogen, bacteria and viruses, and is a protective response involving the action of various immune cells, such as natural-killer cells, neutrophiles, and macrophage [1]. Therefore, inflammation is a defense mechanism that is vital to health [2]. Macrophages are major inflammatory and immune effectors cells, originating from blood monocytes that leave the circulation to differentiate in different tissues [3]. Upon stimulation of macrophages with interferon-γ, pro-inflammatory cytokines, and bacterial lipopolysaccharides (LPS), the downstream intracellular signaling cascades are activated, leading to secretion of pro-inflammatory cytokines, chemokines, NO, and PGE2, as well as the expression of inflammatory proteins [4,5]. However, excessive activation of macrophages has damaging effects, which leads to multiple organ/tissue dysfunction syndrome and certain chronic diseases [5]. Therefore, modulation of activation of macrophages is an effective strategy for controlling the inflammatory response.

Betanin belongs to the betalain family that presents ubiquitously in plants, such as beet root. Betanin is the only betalain approved for use in food and pharmaceutical products as a natural red colorant by the Food and Drug Administration (FDA) in the USA [6]. Recently, the use of natural bioactive compounds to prevent and attenuate chronic diseases has attracted much attention. Among these compounds, the biological activity of betanin has been reported. For example, betanin has shown antioxidant [6], heptoprotective [2], antihypertensive [7] and antiadipogenic [8] properties. Given that betanin has shown some biological effects, it is likely to have an anti-inflammatory property. However, no specific studies have investigated the anti-inflammatory effects of betanin. Taking this background into account, the aim of this study is to investigate the anti-inflammatory effect of betanin on LPS-induced murine macrophages. Moreover, we explore the molecular mechanism underlying these effects.

# 2. Materials and Methods

## 2.1. Chemicals

Betanin was purchased from Tokyo Chemistry Industry (Tokyo, Japan). It is sold under the name "betanin" which is described as red beet extract diluted with dextrin. Dimethyl sulfoxide (DMSO) was purchased from Aldrich Chemical (Milwaukee, WI, USA). All chemicals were of analytical reagent grade.

## 2.2. Measurement of Cell Viability

The tetrazolium dye colorimetric test (MTT assay) was done to determine cell survival. The dye MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was purchased from PanReac AppliChem (Darmstadt, Germany). RAW264.7 cells (BCRC number: 60001) were purchased from the Bioresources Collection and Research Center (Shin-chu, Taiwan) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 1.5 g/L sodium bicarbonate, and 4.5 g/L glucose, and were maintained in humidified 5% CO2/95% air at 37°C. The culture media containing betanin (1-50 μM) were added to each of the 96 wells, and the cells were incubated in humidified 5% $CO_2$/95% air at 37°C for 24 h, with untreated cells served as control. Fifty μL of a 0.1% MTT solution was added, and the cells were incubated for a further 1 h, and then the formazan dye was solubilized by addition of DMSO. The optical density of each well was determined at 550 nm using an ELISA reader (Thermo Scientific Multiskan GO, Waltham, MA, USA) [9].

## 2.3. Inhibitory Action of Betanin on NO and PGE2 Production in RAW 264.7 Cells

Nitrite levels in the cultured media, which reflect intracellular nitric oxide synthase activity, were determined by the Griess reaction. Briefly, cells were pretreated with betanin for 1 h, and then LPS (0.1 μg/mL) was added to the medium and incubated for 24 h. Then, the growth medium was mixed with the same volume of Griess reagent; absorbance of the mixture at 550 nm was determined using an ELISA reader (Thermo Scientific Multiskan GO, Waltham, MA, USA).

For the measurement of PGE2 production, the cells were pretreated with betanin for 1 h, and then LPS (0.1 μg/mL) was added to the medium and incubated for 18 h. The PGE2 in the cultured media was determined by using the PGE2-monoclonal enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions. The absorbance of the mixture at 405 nm was determined using an ELISA reader (Thermo Scientific Multiskan GO, Waltham, MA, USA) [10].

## 2.4. Western Blot

The protein expression of IL-6, iNOS, COX-2, NF-kB, and MAPKs was measured by Western blot. In brief, the RAW264.7 macrophage cells were treated with betanin for 1 h, and then LPS (0.1 μg/mL) was added to the medium and incubated for 18 h, 24 h, 18 h, 1.5 h, and 6 h for IL-6, iNOS, COX-2, NF-kB, and MAPKs protein expression, respectively. The cells were washed with ice-cold phosphate buffer saline (PBS), and then the cells were treated with lysis buffer. Cellular lysates were centrifuged at 10,000 xg at 4°C for 10 min. The supernatants were collected and the protein contents were determined by using the BCA protein assay kit (Piece, Pockfold, IL, USA). Each sample, which contained 50 μg protein, was separated on 8% or 12% sodium dodecyl sulfate (SDS)–polyacrylamide gels. After electrophoresis, gels were transferred to a nitrocellulose membrane. After washing with distilled water, the membrane was incubated with 5% albumin in PBST (0.1% Tween-20 in PBS, pH 7.4) for 30 min and was then immunoblotted with rabbit monoclonal anti-iNOS antibody (#13120) (1:1000) (Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal anti-COX-2 antibody (#12282) (1:1000) (Cell Signaling Technology, Danvers, MA, USA), anti-IL-6 antibody (#12912S) (1:1000) (Cell Signaling Technology, Danvers, MA, USA), anti-NF-kB antibody (#8242) (1:1000) (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-JNK (AF6318), rabbit anti-pJNK (AF3319), rabbit pp38 (AF4001), rabbit anti-p38 (AF6456), rabbit anti-ERK (AF0155), rabbit pERK (AF1015) (Affinity Biosciences, Cincinnati, OH, USA) (1:1000), and β-actin antibody (#4970) (1:1000) (Cell Signaling Technology, Danvers, MA, USA). Blots were then incubated with anti-rabbit immunoglobulin G (IgG) antibody conjugated to horseradish peroxidase (Sigma, St. Louis, MO, USA). Blots were developed using the Western Bright Enhanced Chemiluminescence (ECL) plus kit (Advansta Inc., Menlo Park, CA, USA), exposed to Gel Electrophoresis Documentation-Multi-function Gel Image system (Topbio Co., Taiwan, ROC). The relative expression of proteins was quantified densitometrically using the Image J software and calculated according to the reference bands of β-actin [11].

## 2.5. Statistical Analysis

All data were presented as means ± SD. and ANOVA was conducted by using the SPSS software (SPSS Inc., Chicago, IL, USA). Significant differences between means were determined by Duncan's multiple range tests at a level of $p < 0.05$.

# 3. Results

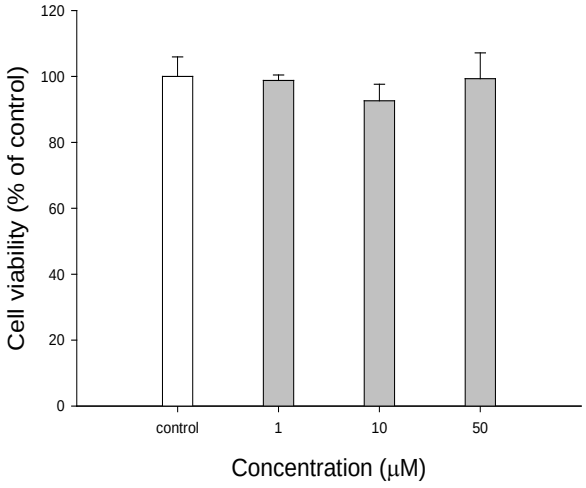
Beet root is a rich source of a group of red and yellow pigments known as betalains, which are a class of natural pigments including reddish purple betacyanins and yellow betaxanthins [12].Betanin is the most abundant betacyanin component, accounts for 75-95% of the total betalains commonly used as a colorant for food products [12,13]. Clearly, betanin is partly responsible for the red pigment in the roots of beet root.

The effects of different concentrations of betanin on cell viability, NO production, and on LPS-induced cell survival, NO production, and PGE2 production in RAW 264.7 cells were shown in Figure 1. Figure 1A shows the cytotoxicity of betanin on RAW 264.7 cells measured by

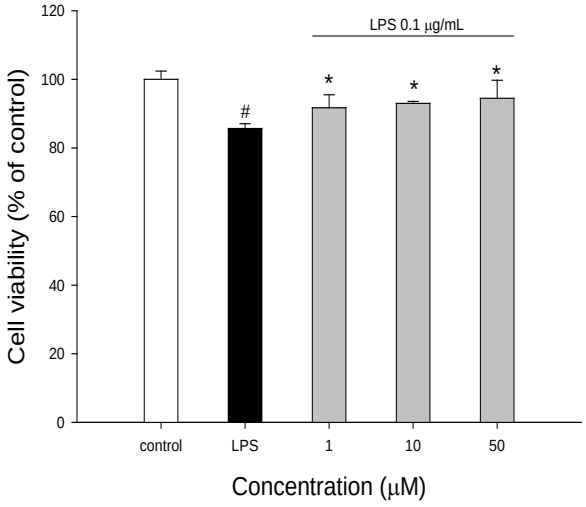
MTT assay. The viability of the cells treated with betanin at different concentrations (1-50 μM) was > 90%, indicating that betanin shows no cytotoxicity to RAW 264.7 cells under the test concentrations. In addition, NO production of RAW264.7 cells treated with betanin at 1-50 μM was <0% (data not shown). This finding implies that betanin did not affect NO production in RAW 264.7 cells. Figure 1B shows the effect of betanin on cell viability stimulated with LPS in RAW 264.7 cells. The cell viability generally exceeded 91% in the range of 1-50 μM, indicating that betanin shows no cytotoxicity to LPS-treated RAW 264.7 cells under the test concentration. The inhibitory effect of betanin on NO production in RAW264.7 cells stimulated with LPS was determined (Figure 1C). The NO production increased remarkably when 0.1 μg/ml of LPS was added to RAW264.7 cells, compared to the control without LPS. NO production from LPS-stimulated RAW264.7 cells decreased with increasing concentration of betanin in the range of 1-50 μM, indicating that betanin showed a significantly inhibitory effect on NO production in the LPS-stimulated RAW 264.7 cells. Figure 1D shows the effect of betanin on PGE2 production in LPS-induced RAW264.7 cells. LPS-induced cells produced a high level of PGE2, compared to the control. Betanin showed remarkable inhibition of PGE2 production in LPS-induced cells under the test concentration. These results show that betanin was capable of inhibiting PGE2 production in LPS-induced RAW264.7 cells at concentrations ranging from 1-50 μM.

Figure 2 shows the effect of betanin on iNOS and COX-2expression in LPS-induced RAW264.7 cells. As shown in Figure 2A, the LPS in the culture induced a marked increase in iNOS expression, compared with the control. In addition, when the cells were pretreated with betanin, the LPS-induced increase in iNOS expression was suppressed in a concentration dependent manner. Meanwhile, betanin in the range of 1-50 μM also decreased COX-2 expression, in a dose dependent manner, in LPS-induced RAW264.7 cells (Figure 2B). These results imply that betanin may down-regulate the iNOS and COX-2 expression in LPS-induced RAW264.7 cells. In addition, to determine whether betanin can regulate proinflammatory mediators, the transcription level of proinflammatory cytokine in LPS-induced RAW264.7 cells was measured. As shown in Figure 3, the cells pretreated with betanin showed significantly reduced expression of interleukin 6 (IL-6) at 10 and 50 μM, compared with that of cells induced only with LPS. However, betanin at 1 μM did not affect the transcriptional level of IL-6 in LPS-induced cells.

NF-κB, a transcription factor, is considered to be a regulator of innate immunity, which contributes to overall mitochondrial function. Therefore, we further investigated the effect of betanin on NF-κB in LPS-induced RAW264.7 cells. As depicted in Figure 4, the nuclear protein level of NF-κB increased by 48 % in the LPS-induced cells, as compared to the unstimulated cells, whereas the betanin at 25 μM showed a 24% decreased in nuclear protein level of NF-κB relative to the untreated cells. Clearly, betanin in treated cells demonstrated comparatively strong inhibitory effects on transcriptional level of NF-κB as compared with only LPS-induced cells, suggesting that betanin prevented the LPS-induced nuclear transcriptional level of NF-κB in RAW 264.7 cells.




(A)

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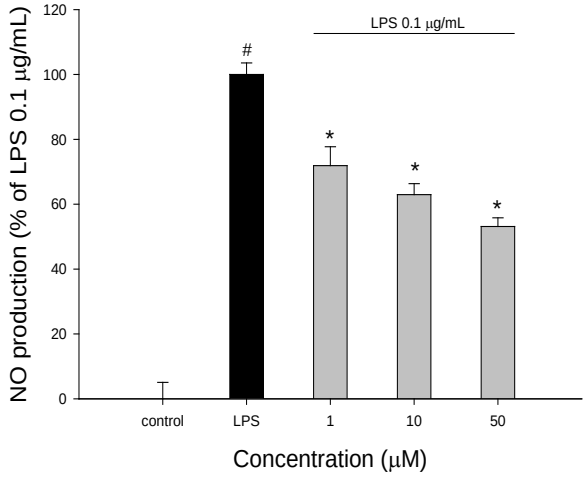


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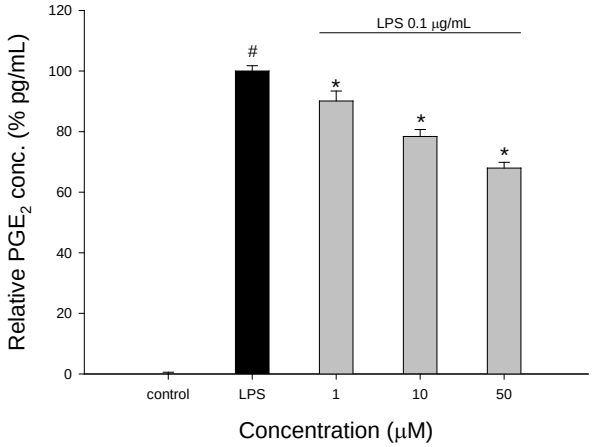
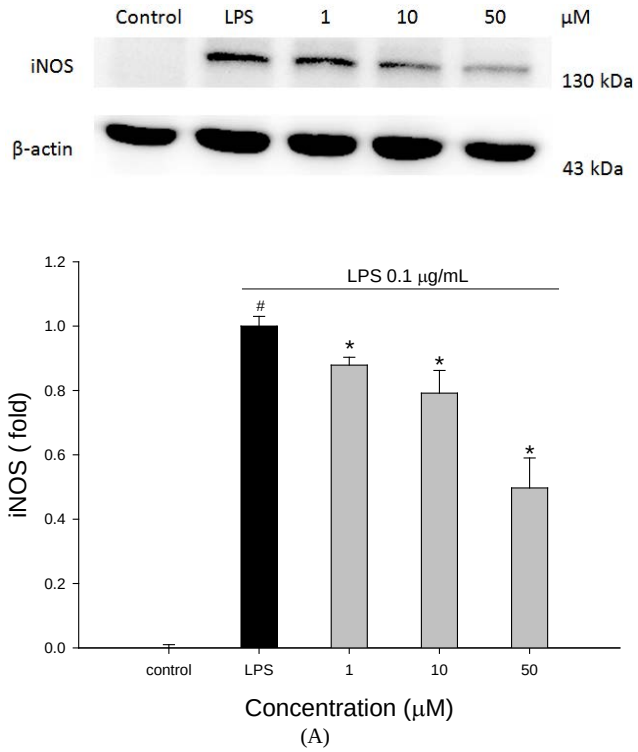
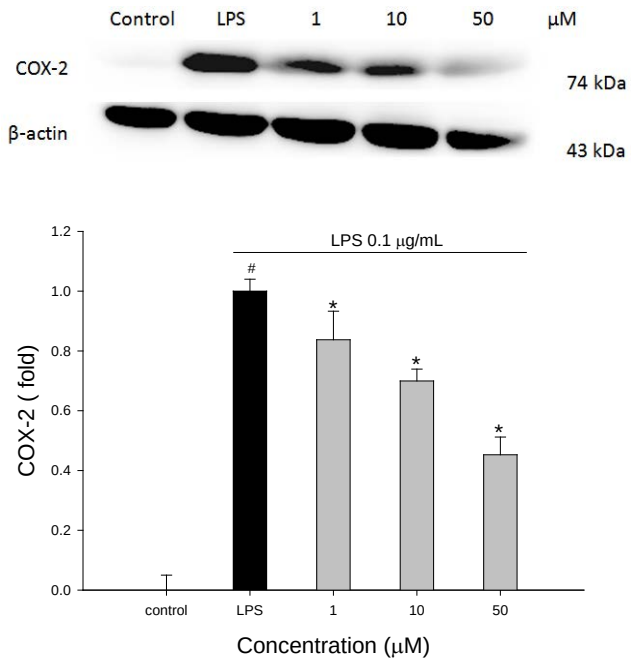



(D)

**Figure 1.** The effects of different concentrations of betanin on cell viability, and on LPS-induced cell survival, NO production, and PGE2 production in RAW 264.7 cells. (A) The effects of different concentrations of betanin on cell viability. The cells were treated with different concentrations of betanin for 24 h; (B) The effect of different concentrations of betanin on LPS-induced cell survival. The cells were pretreated with different concentrations of betanin for 1 h, and then LPS (0.1μg/mL) was added to the medium and incubated for 24 h, and then the cell survival was measured by MTT assay; (C) The effect of different concentrations of betanin on LPS-induced NO production in RAW264.7 cells. The cells were pretreated with different concentrations of betanin for 1 h, and then LPS (0.1μg/mL) was added to the medium and incubated for 24 h; (D) The effect of different concentrations of betanin on LPS-induced PGE2 production in RAW 264.7 cells. The cells were pretreated with different concentrations of betanin for 1 h, and then LPS (0.1 μg/mL) was added to the medium and incubated for 18 h. Data are presented by means ± SD (n=3). # $p < 0.05$, compared to the control. * $p< 0.05$, compared to the cells treated with LPS.




(A)




(B)

**Figure 2.** The effects of betanin on iNOS and COX-2 expression in LPS-induced RAW264.7 cells. (A)The cells were treated with different concentrations of betanin for 1 h and exposure to LPS 0.1 μg /ml for 24 h; (B) Inhibition of LPS-Induced COX-2 protein expression in RAW264.7 cells by betanin. The cells were treated with different concentrations of betanin for 1 h and exposure to LPS 0.1 μg /ml for 18 h. Data are presented by means ± SD (n=3). # p<0.05, compared to the control; *p<0.05, compared to the cells treated with LPS

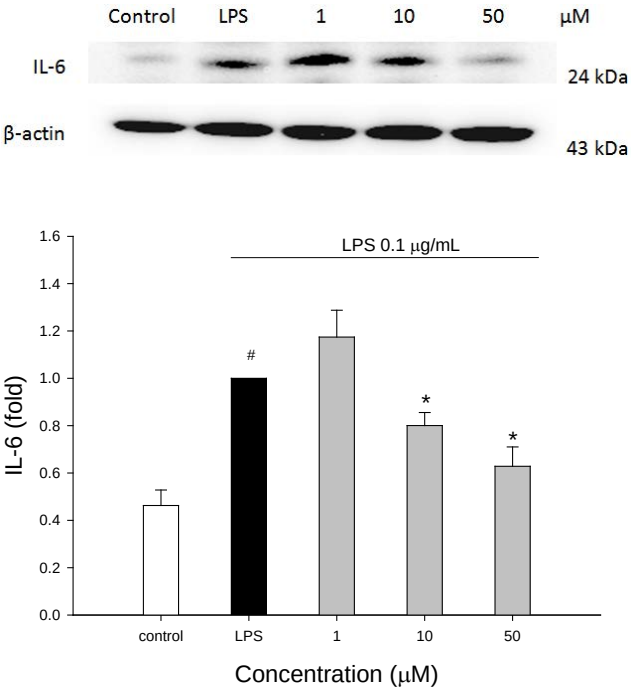



**Figure 3.** The effects of betanin on IL-6 protein expression in LPS-induced RAW264.7 cells. The cells were treated with different concentrations of betanin for 1 h and exposure to LPS 0.1 μ g /ml for 18 h. Data are presented by means ± SD (n=3). # p<0.05, compared to the control; *p<0.05, compared to the cells treated with LPS.

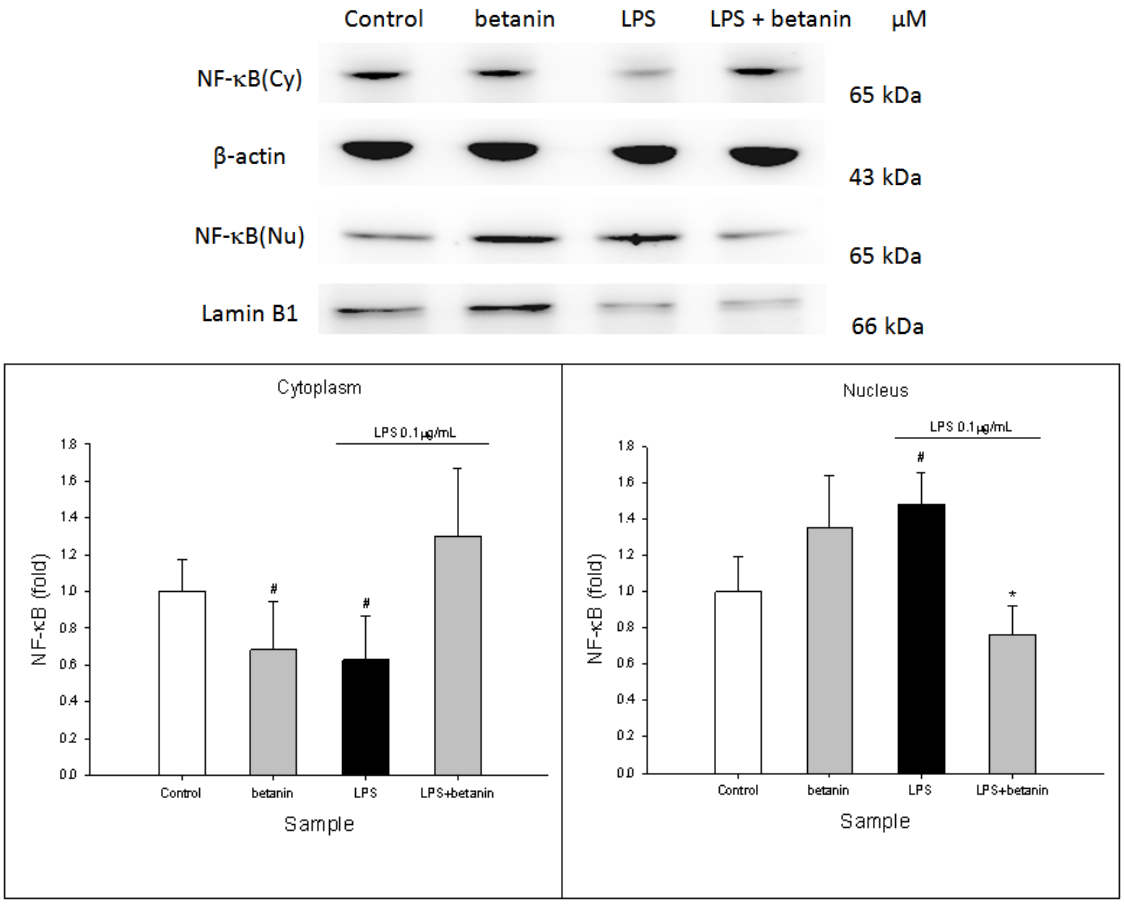



**Figure 4.** The effects of betanin on NF-kB signaling pathways. The cells were treated with different concentrations of betanin for 1 h and exposure to LPS 0.1 μ g /ml for 1.5 h. Data are presented by means ± SD (n=3). # p<0.05, compared to the control; *$p$<0.05, compared to the cells treated with LPS

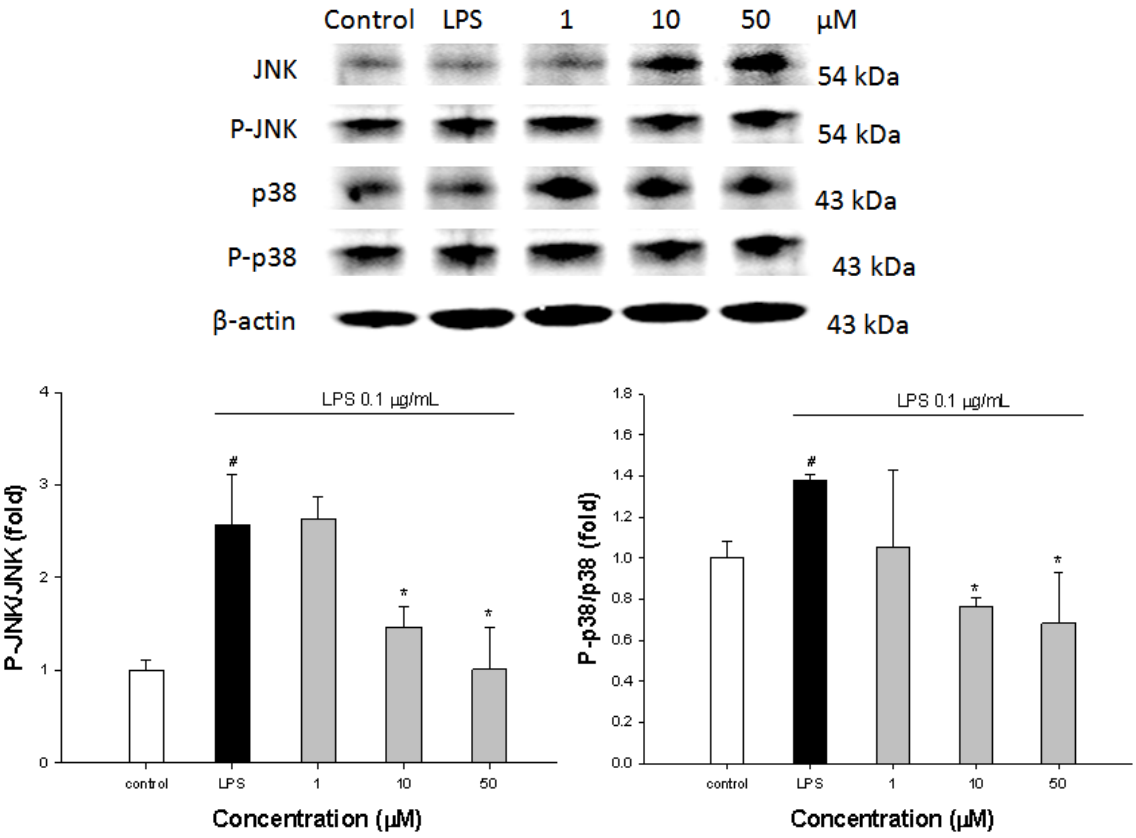



**Figure 5.** The effects of betanin on JNK and p38 signaling pathways. The cells were treated with different concentrations of betanin for 1 h and exposure to LPS 0.1 μg /ml for 6 h. Data are presented by means ± SD (n=3). # $p$<0.05, compared to the control; *$p$<0.05, compared to the cells treated with LPS

To evaluate the molecular mechanism underlying the anti-inflammatory effects of betanin, the effects of betanin on the activation of MAPKs by Western blotting analysis was determined. Figure 5 shows the effects of betanin on pJNK and pp38 signaling pathways. LPS-induced cells produced a high ratio of pJNK/JNK and pp38/p38, compared to the control, indicating that LPS treatment alone significantly induced the phosphorylation of JNK and p38 in RAW264.7 cells. In contrast, betanin at 10 and 50 μM demonstrated marked inhibition of pJNK/JNK and pp38/p38 ratio in LPS-induced cells, however, did not affect ERK phosphorylation (data not shown), suggesting that the activation of MAPK signaling pathway was significantly reduced by10 and 50 μM betanin treatment in LPS-induced cells.

## 4. Discussion

Betanin has been largely used to color food and pharmaceutical products. In addition to its soluble red pigment, in several studies it was shown that betanin demonstrated multibiological effects [2] [6,7,8]. In the present work, we explore for the first time the anti-inflammatory effect of betanin on LPS-induced murine macrophages and the underlying mechanism action of betanin on RAW264.7 cells induced by LPS. In several studies it was shown that betalains are effective free radical scavengers.

Lipopolysaccharide (LPS), an endotoxin, activates downstream intracellular signaling cascades, leading to the production of several inflammatory mediators, such as NO and PGE2 and pro-inflammatory cytokines to promote inflammatory response [4]. In the present study, exposure of macrophages to LPS resulted in a significant upregulation of iNOS expression and an increase in nitrite/nitrate levels, as an indicator of NO [3]. As expected, betanin significantly inhibited LPS-induced iNOS expression and reduced NO production in a concentration-dependent manner, thus, confirming the inhibitory effect of NO. COX-2 is an important inflammatory mediators involved in the inflammatory process. Upon stimulation of macrophages with LPS, over expression of COX-2 leads to the production of PGE2, and TXB2, which are inflammatory mediators that cause organ dysfunction and thereby induce tissue damage [3]. Likewise, LPS significantly induced COX-2 expression. However, betanin concentration-dependently decreased LPS-induced COX-2 expression, compared with the cells treated with LPS alone (Figure 2B). In addition, COX-2 is the key enzyme in the conversion of arachidonic acid to PGE2. Apparently, the results showed that betanin down-regulated LPS-induced COX-2 expression. These observations may explain why betanin inhibited LPS-induced PGE2 production in RAW 264.7 cells. Regulating the protein expression of iNOS and COX-2 has been considered as an effective strategy to alleviate inflammatory diseases [14]. According to the data from Figures 2A and 2B, betanin at 1-50 μM treatment remarkably suppressed LPS-induced iNOS/COX-2 expression, indicating that betanin may down-regulate iNOS and COX-2 expression, leading to a reduction in NO and PGE2 production in macrophage cells. In addition to investigating the effect of betanin on inflammatory cytokine production, the levels of IL-6 in RAW264.7 macrophages were also determined. The levels of IL-6 in LPS-induced cells were markedly higher than those in the untreated control. When LPS was co-incubated with betanin, the levels of this cytokine decreased remarkably in a betanin concentration-dependent manner. It is well known that IL-6 is considered as an important pro-inflammatory cytokine which leads to various kinds of inflammatory related diseases [14]. The results showed that betanin obviously decreased the production of NO, PGE2 and IL-6, and significantly inhibited expression of iNOS and COX-2 in LPS-induced cells, which accelerated the production of NO and PGE2, respectively, suggesting that betanin may modulate immune activity through anti-inflammatory effects [4].

To elucidate the mechanism action underlying the anti-inflammatory effect of betanin, the effects of betanin on the NF-κB and MAPK signaling pathways were further investigated. The results showed that the treatment of LPS significantly increased the expression of NF-κB, compared with that in untreated controls, confirming the activation of the NF-κB pathway [4]. However, pretreatment with betanin significantly attenuated the activation of NF-κB to nucleus compared to the cells treated with LPS alone. NF-κB is a key signaling pathway responsible for regulating the transcription of pro-inflammatory cytokine and mediators, such as IL-6, IL-1β, NO, and PGE2. In addition, upon stimulation of macrophages to LPS, IκB undergoes phosphorylation, allowing NF-κB to translocate to the nucleus, thereby binding to the promoter of pro-inflammatory genes, such as iNOS and COX-2 amongst others [3]. These results imply that pretreatment of betanin reduced LPS-induced inflammatory response in RAW264.7 macrophages in part due to inhibition of NF-κB signaling pathway.

Recently MAPKs are among the most ancient signal transduction pathways, which coordinately regulate gene expression, apoptosis, mitosis, metabolism, motility, survival, apoptosis, and differentiation [15]. In mammals, 14 MAPKs have been characterized into seven groups, however, by far the most extensively studied groups of mammalian MAPKs are focused on ERK1/2, JNKs, and p38 isoforms [15,16]. Previous studies have shown that JNK and p38 pathways are mainly related to stress and apoptosis of cells, whereas the ERK signaling pathways is closely related to cell proliferation and differentiation and plays an important role in the cell signal transduction network [17]. In addition, many studies reported that each MAPK has different effects on the expression of inflammatory cytokines in the immune and inflammatory response [4]. For example, LPS-induced NO production and iNOS expression are related to JUN and p38 [4,18]. In addition, LPS-induced expression of COX-2 is related to ERK and p38 [19]. Given that betanin inhibited the protein expression of iNOS, COX-2 and IL-6 in LPS-induced cells, it is also possible that MAPKs pathways may be regulated by betanin in LPS-induced cells. Results in Figure 5 show that the expression of phosphorylated of JNK and p38 significantly increased

in the presence of LPS alone compared to the control. However, betanin significantly inhibited p38 and JNK activated by LPS but did not affect ERK phosphorylation (data not shown). These findings implied that anti-inflammatory effect of betanin may be in part through inhibition of the phosphorylation of JNK and p38 MAPK, thereby inhibiting the MAPK signaling pathway [4].

Epidemiological evidences have shown that phytochemicals found in various parts of plants lead to reduction in the risk of some diseases [20]. Betanin, being a bioactive compound present in red beetroot, comprises a phenolic and cyclic amine groups, both of which are good donors of electron that endow it with exceptional high biological activities [12]. It has been reported that there is a strong correlation between radical scavenging and anti-inflammatory activity [21]. Therefore, we speculated that the exceptionally high anti-inflammatory effects of betanin mat be associated with its H-donation and electron-donation ability. However, this speculation requires further study.

## 5. Conclusions

In summary, betanin showed an anti-inflammatory effect, *in vitro*. The levels of pro-inflammatory cytokines mediators and corresponding protein expression were suppressed through regulation of NF-κB/MAPKs signaling pathways. These novel findings may provide an anti-inflammatory background in the efficacies of betanin, which may be a potential alternative approach to prevention of inflammation or related diseases. However, further *in vivo* testing is required to confirm the relevance of these results.

## Credit authorship contribution statement

**Ming-Jen Sheu:** Conceptualization; Review & Editing.
**Shih-Ying Chen:** Formal analysis; Investigation.
**Charng-Cherng Chyau:** Methodology.
**Ying-Chun Lin:** Review & Editing.
**Pin-Der Duh**: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Writing - Original draft; Writing -Review & Editing.

## Conflicts of Interest

The authors declare no conflict of interest.

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