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ORIGINAL ARTICLE

Anti-inflammatory Effect of Procumbenoside B from *Justicia spicigera* on Lipopolysaccharide-Stimulated RAW 264.7 Macrophages and Zebrafish Model

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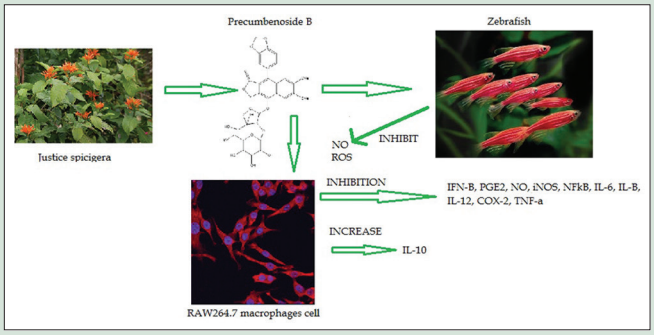
ABSTRACT

Background: *Justicia spicigera* is widely used in the Mexican traditional medicine for the treatment of inflammation. **Objectives:** The purpose of this study was to investigate the anti-inflammatory effect of procumbenoside B (PB) from *J. spicigera* on pro-inflammatory mediators. **Materials and Methods:** Lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages and Zebrafish model were used to assess the potential anti-inflammatory effects of PB; its structure was elucidated on the basis of spectroscopic data. The production of inflammatory mediators such as interferon-β, prostaglandin E2, inducible nitric oxide synthase (iNOS), nuclear factor-kappa B (NF-κB) p65, interleukin-6 (IL-6), IL-1β, IL-12, cyclooxygenase (COX-2), tumor necrosis factor-α, and anti-inflammatory IL-10 was measured using enzyme-linked immunosorbent assay. NO production was measured using Griess reagent. **Results:** In LPS-induced-inflammatory response in RAW264.7 macrophage cells, PB strongly inhibited secretion of all pro-inflammatory mediators test and increased the production of IL-10 and blockade of NF-κB. In addition, PB suppressed LPS-stimulated nitric oxide and reactive oxygen species generation in a zebrafish model. **Conclusion:** These results indicated that the anti-inflammatory effects of PB may be attributed to the downregulation of iNOS and COX-2 through the suppression of NF-κB signaling pathway in RAW264.7 macrophages.

Key words: Anti-inflammatory, cytokines, *Justicia spicigera*, macrophage model, procumbenoside B, zebrafish model

SUMMARY

- Justicia spicigera was evaluated for its anti-inflammatory activity in LPS-stimulated RAW 264.7 macrophages and a zebrafish embryos model
- J. spicigera demonstrated anti-inflammatory effect in the current study by enhance pro-inflammatory mediators in macrophages
- J. spicigera exhibited potential protective effect in the zebrafish embryos
- Against reactive oxygen species and nitric oxide
- J. spicigera could be considered as a candidate potential anti-inflammatory drug for the treatment of inflammatory diseases



Abbreviations used: 4-Amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM-DA), Cyclooxygenase-2 (COX-2), 2,7-dichlorofluorescein (DCH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dimethylsulphoxide (DMSO), Dulbecco's modified eagle's medium (DMEM), Fetal bovine serum (FBS), Granulocyte-macrophage colony-stimulating factor (GM-CSF), Interferon-β (IFN β), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Interleukin-1B (IL-1b), Layer chromatography (TLC), Lipopolysaccharide (LPS), Matrix metalloproteinases-3 (MMP-3), Matrix metalloproteinases-13 (MMP-13), Nitric oxide (NO), Nitric oxide synthase (iNOS), Nuclear factor-kappa B (NF-βB), Prostaglandin E2 (PGE2), Reactive oxygen species (ROS), Tumor necrosis factor-β (TNF-β).

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INTRODUCTION

The inflammatory process is normally triggered by different stimuli as noxious chemical, microbiological, and physical which lead to the production of a variety of inflammatory mediators such as pro-inflammatory cytokines (interleukin-6 [IL-6], IL-8, IL-1b, tumor necrosis factor-α [TNF-α], and granulocyte-macrophage colony-stimulating factor), mediating enzymes (matrix metalloproteinases-3 [MMP-3], MMP-13, cyclooxygenase-2 [COX-2], and inducible nitric oxide synthase [iNOS]), and chemokines (nitric oxide [NO] and prostaglandin E2 [PGE2]).[1] In addition, macrophages play a very important role as immune cells which regulate inflammation and host defense by secretion of pro-inflammatory cytokines and chemokines and the expression of inflammatory proteins.[2] A excessive production of these inflammatory cytokines and mediators can lead to organ dysfunction syndrome results in the development of chronic inflammation, such as psoriasis, and rheumatoid arthritis.[2] Thereby, regulation of macrophage activation is a recommended strategy to prevent these diseases.

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Mexican traditional medicine is very important in rural zones as an alternative to resolve health problems. Among them, *Justicia Spicigera,* which is widely used in the treatment of cancer, pain, fever,[3] dysentery,[4] and scabies,[5] is very popular in the traditional Chinese medicine to treat cancer, pain, and fever.[6] In addition, it is one of the main herbs in Jian-er syrup, a Chinese herbal medicine preparation.[7] Leaves contain compounds that change color of green to blue of with air exposure.[8] The genus *Justicia* contains aryl-naphthalene lignans that have a considerable interest for their anti-lipidemic and antitumor activities which are considered for the development of new drugs.[9,10] In a previous report found that kaempferitrin mainly contributed to the immunostimulatory effect[11] and antidiabetic activity.[12] Ethanol extract possesses effect against intestinal parasites.[13] Methanol extract also showed moderate inhibition on carrageenan-induced hind paw edema assay.[14] Furthermore, ethanol extract revealed the presence of allantoin, kaempferitrin, O-sitosterol-3 β-glycoside, cryptoxanthin,[15] rutin, quercetin-3-β-O-rutinoside, linarin, neoponcirin, and hesperidin.[16]

Zebrafish (*Danio rerio*) recently being is used as an alternative model organism for *in vivo* experiments due to its numerous advantages such as physiological similarity with mammals, low cost, large clutch, and small size.[17] Initially, zebrafish was used in molecular genetics and biology; however, recent studies have demonstrated the potential of this organism to be used as a model for pharmacological and toxicological studies. Currently, zebrafish embryos are widely used in the study of small molecules and drugs, which can be easily absorbed through gills and skins when dissolved in the bathing media, which is not possible in adult fish.[18] In addition, due to transparent nature of zebrafish, embryos can be monitored through fluorescence-based assays.[19] Taking into account all the advantages that present zebrafish embryos, in this study, we used it as an animal model to evaluate as support of the anti-inflammatory effect of procumbenoside B (PB).

Therefore, this study examined the anti-inflammatory effect of PB in RAW 264.7 macrophage cells and zebrafish model

MATERIALS AND METHODS

Materials

Quantitation ELISA kit of IL-10, IL-12p40 IL-6, IL-1b, TNF-α, NO, and PGE2 was purchased from R and D (R and D Systems, Minneapolis, MN, USA). Griess reagent, protease inhibitor cocktail, lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4-Amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM-DA) (DAF-FM-DA), 2,7-dichlorofluorescein (DCH-DA), 2-phenoxyethanol, and 2-phenoxyethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). NE-PER nuclear and cytoplasmic extraction reagent from Thermo Fisher Scientific (IL, USA), nuclear factor-kappa B (NF-κB) transcription factor assay kit from Abcam (Cambridge, UK), DMEM, penicillin, glutamine, and other products used for culture cells were acquired to Gibco.

Plant material

Leaves of *J. spicigera* Schlechtendal (*Acanthaceae*) were collected in Hidalgo State, Mexico. The plant was identified and authenticated by Biol Aurora Gonzalez. A voucher herbarium specimen (6783) is kept at the herbarium of the Escuela Nacional de Ciencias Biologicas, IPN-Mexico.

Isolation of procumbenoside B

The air-dried whole plants of *J. spicigera* (3 kg) were milled and then extracted two times with MeOH under reflux. After filtration, the MeOH extract was concentrated under reduced pressure to yield a dark brown residue (90 g). This residue was chromatographed on a silica gel column (Merck, 230-400) using n-hexane/EtOAc (4:2) to obtain five fractions (PB1-PB5). Each fraction (50 ml) was monitored by thin layer chromatography (TLC); fractions with similar TLC patterns were combined. Each fraction was monitored for its anti-inflammatory effect. PB2 was separated by column chromatography and eluted with a gradient of CH_2Cl_2:MeOH (50:1-4:1) to yield four fractions (PB2-1-PB2-4). Fraction PB4-3 was chromatographed on silica gel column chromatography eluted with $CHCl_3$–MeOH (5:1) to yield six fractions (PB41-PB46) among them PB43 was active subfraction which was separated by filtration on Sephadex LH-20 with a gradient solvent of $CHCl_3$–MeOH, to obtain 1 (87 mg).

Cell cultures

RAW264.7 cells line were obtained from the American Type Collection (Rockville, MD, USA) and cells were cultured at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 2 mM glutamine in a humidified incubator 5% carbon dioxide atmosphere. Methanol extract was dissolved in dimethyl sulfoxide.

Cell viability test

RAW264.7 cells were seeded into 96-well plates, in DMEM supplemented with 10% FBS and incubated for 1 h with various concentrations of EJS PB (140, 280, 420, and 560 μg/ml) and stimulated con 20 ng/ml of lipopolysaccharide (LPS) for 4 h. After, the relative cell viability was determined by 0.5 mg/ml of MTT test. Hence, supernatants were replaced with culture medium containing MTT reagent and incubated overnight. Cell viability was assessed by determination of absorbance at 570 nm using a microplate reader.

Appraisal of cytokine response by ELISA

RAW264.7 cells were pretreated with various concentrations of PB or 1 μM of dexamethasone (DM) for 1 h and stimulated with 20 ng/ml of LPS for 4 h. DM is used as a control, which is a glucocorticoid that suppresses LPS-induced pro-inflammatory cytokine expression at 0.5 mg/ml in 50 ml PBS. The supernatant was collected to assess the levels of IL-10, IL-6, IL-1β, IL-12, and TNF-α, using enzyme-linked immunosorbent assay, and the manufacturer's instructions were followed to assess the cytokine response.

Assessment of nitric oxide, inducible nitric oxide synthase, prostaglandin E2, and interferon-β

RAW264.7 macrophages at a density of 2×10^5 cells/ml were cultured in a 96-well plate for 24 h. Then, the cells were pretreated with different concentrations of PB (25, 50, and 100 μg/ml) for 1 h and after were treated with 1 μg/ml of LPS for 16 h more. The supernatant was collected for the interferon-β (IFN-β), NO, and PGE2 tests. For NO assay, the supernatant (100 μl) was mixed with 100 μl of Griess reagent in a 96-well plate and incubated for 15 min at room temperature, and the NO concentration was determined at 520 nm and their concentration was calculated using a calibration standard curve constructed using sodium nitrite dissolved in DMEM. The concentration of IFN-β and PGE2 in the supernatant was measured using an ELISA kit. Activity of iNOS was measured using an ELISA kit according to the manufacture instructions, and values of iNOS are expressed as activity units per milligram protein.

Nuclear proteins extraction

RAW264.7 cells at a density of 2 × 10 5 cells/ml were cultured in a 100 mm dish for 24 h. Then, the cells were pretreated with different concentrations of PB (25, 50, and 100 μg/ml) for 1 h, then were treated with 1 μg/mL of LPS for 30 min. The macrophages were harvested; subsequently, cytosolic and nuclear fractions were prepared using an

NE-PER nuclear and cytoplasmic extraction reagent according to the manufacturer's protocol.

Nuclear factor kappa B ELISA test

The activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) which is a protein complex that controls transcription of DNA was evaluated using NF-κB transcription factor assay kit according to the manufacturer's protocol.

Zebrafish embryos

Twenty fishes were kept in a 5 L acrylic tank at 14/10 h light/dark cycle at 28.5°C and fed three times a day with fish food supplemented with live *Artemia salina*. Embryos were obtained from natural spawning induced by turning on the light in the morning. Then, within 30 min, embryos were collected.

Reactive oxygen species production

Generation of ROS production in the inflammatory zebrafish model was determined by oxidation-sensitive fluorescent assay dye, 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) as the substrate. Zebrafish larvae were transferred to 96-well plates and treated with DCH-DA solution (20 μg/ml) and incubated for 1 h at 28.5°C in the dark. Then, Zebrafish larvae were rinsed with fresh embryo media and anaesthetized with 2-phenoxyethanol (1/500 dilution) followed to observation and photographed using a CoolsNAP-Procolor and a digital camera Olympus Japan. The fluorescence intensity of each larva was quantified using the ImageJ software.

Evaluation of generation of intracellular nitric oxide in zebrafish embryo

Zebrafish embryos were collected and arranged in 12-well plates. Each well contained 12 embryos during 7–8 h postfertilization (hpf) in 2 ml of the embryo medium. Then, it was added 10 μg/ml LPS dissolved in the embryo medium and incubated for 24 hpf at 28.5°C to induce inflammation. Later zebrafish embryos were rinsed with fresh embryo media. NO production in the inflammatory zebrafish model was analyzed using the oxidation-sensitive fluorescent probe dye, NO production in the inflammatory zebrafish model was analyzed using the oxidation-sensitive fluorescent probe dye, DAF-FM DA. In the presence of dioxygen, transformation of DAF-FM DA by NO generates highly fluorescent triazole derivatives. Zebrafish embryos were treated with a solution 5 μM of DAF-FM-DA and incubated in the dark at 28.5°C for 1 h. After incubation, the embryos were transferred fresh embryo media and anesthetized using 2-phenoxyethanol (1/500 dilution) before observation under a fluorescence microscope (CoolsNAP-Procolor and a digital camera Olympus Japan). DM was used as positive standard (0.5 μg/ml).

Statistical analysis

All data are showed as the mean ± standard deviation differences between the variables were reported using one-way analysis of variance, $P = 0.05$ or less was considered as statistically significant. The statistical analysis was performed using SPSS statistics program (Version 22, SPSS Inc., Chicago, IL, USA). Triplicate assays were performed by different samples.

RESULTS

Identification of compound procumbenoside B

Repeated chromatography on silica gel and Sephadex LH-20 of the methanol crude extract led to the isolation of PB. The structure of the known compound was characterized by comparison with the spectroscopic data published in the literature. PB was previously isolated from the whole plant of *J. procumbens*[20,21] and characterized by comparison of their NMR spectral data including ^{1}H and ^{13}C NMR, with the published literature data.[21] The structure of PB (1) is shown in Figure 1.

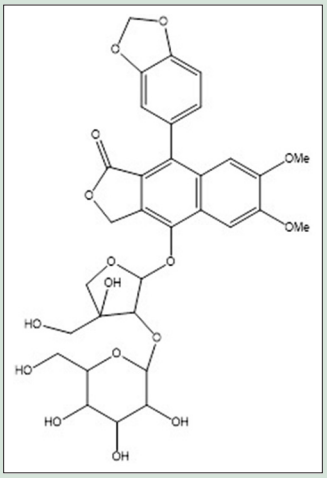

Figure 1: Procumbenoside B isolated from methanol extract of *Justicia spicigera*

Cytotoxicity of procumbenoside B

The cell cytotoxicity of PB was determined in RAW264.7 macrophages. These were treated with different concentrations of PB (0, 140, 280, 420, and 560 μg/ml) for 24 h, and then cell cytotoxicity was measured. The results indicated that PB did not exhibit cytotoxicity to RAW264.7 macrophage cells at the doses tested [Figure 2].

Measurement of anti-inflammatory cytokines interleukin-10

Exposure of RAW264.7 cell to LPS showed significantly inhibition of the levels of IL-10 when compared with the untreated cells. After treatment of PB the levels of cytokine IL10 increase [Figure 3a].

Measurement of pro-inflammatory cytokines interleukin-12, interleukin-6, interleukin-1β, and tumor necrosis factor-α production

IL-12, IL-6, IL-1β, and TNF-α are pro-inflammatory cytokine secreted in the inflammatory response being found at high levels in chronic and acute inflammatory diseases. Therefore, we investigated whether PB possesses potential anti-inflammatory effects in LPS-stimulated RAW264.7 macrophages. LPS treatment significantly increased the levels of IL-6, IL-1β, TNF-α, and IL-12 as shown in Figure 3b-e, respectively, when compared with the untreated cells. However, after pretreatment for 1 h with PB, the levels of these cytokines in the supernatant of RAW264.7 macrophage cells were significantly ($P < 0.05$) reduced in a dose-dependent manner.

Effect of procumbenoside B on LPS-induced nitric oxide, inducible nitric oxide synthase, prostaglandin E2, and interferon-β production

The levels of NO, iNOS, and PGE2 IFN-β secreted by the RAW264.7 macrophages were significantly ($P < 0.05$) increased by LPS as shown in Figure 4a-c, respectively, when compared with the untreated cells.

However, pretreatment with PB (25, 50, and 100 μg/ml) remarkably decreased the expression of iNOS, NO, PGE2, and IFN-β in RAW264.7 macrophages in a dose-dependent manner. These results indicate that PB decreased the PGE2 and NO production [Figure 4] by inhibiting the expression of iNOS in LPS-stimulated RAW264.7 cells.

Effect of procumbenoside B on the lipopolysaccharide-induced activation of transcription factor nuclear factor kappa B in RAW264.7

Some intracellular signaling pathways play an important role in the inflammation as NF-κB transcription factor which is LPS activated in RAW264.7 cells macrophages. NF-κB DNA-binding activity was measured using the Elisa kit for the transcription factor p65. NF-κB DNA-binding activity was increased in the LPS-treated macrophages when compared with the untreated cells ($P < 0.05$). NF-κB DNA binding activity was significantly ($P < 0.05$) inhibited in the cells pretreated with PB at 100 μg/ml when compared with DM used as positive control. The results are shown in Figure 4d. These date suggest that PB improves the inflammatory response through the suppression of NF-κB activation.

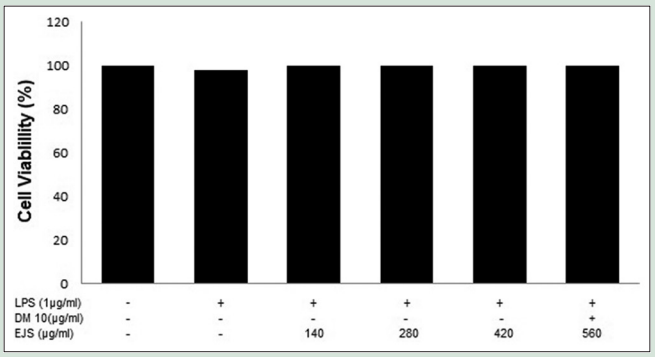

Figure 2: Effect of procumbenoside B (140, 280, 420, and 560 μg/ml) on the viability of RAW264.7 cells. Data represent the mean ± standard deviation of three independent experiments. Compared with lipopolysaccharide group *$P < 0.05$

Effects of procumbenoside B on intracellular reactive oxygen species generation in zebrafish

Previous studies have reported that zebrafish larvae have been widely used for the determination of the anti-inflammatory activity against LPS-stimulated inflammation *in vivo*.[22] Intracellular ROS generation can be detected using oxidation-sensitive fluorescent assay dye, DCF-DA as the substrate. The group treated only with LPS (10 μg/ml) produced a fluorescence image [Figure 5]. Figure 5 shows the effect in the zebrafish model of PB on LPS-induced intracellular ROS generation. Figure 5 shows a micrograph with typical fluorescence of a zebrafish taken as control, which was subjected to neither LPS nor PB. While Figure 5 shows LPS treatment group, generated a fluorescence image, which indicated ROS generation. However, when the zebrafish larvae were given a pretreatment with PB at dose of 25 and 100 μg/ml before LPS administration showed a significant ($P < 0.05$) reduction in ROS levels in zebrafish [Figure 5]. Thus, pretreatment with PB and LPS in a dose-dependent manner significantly inhibited ROS generation [Figure 5b].

Effect of procumbenoside B on lipopolysaccharide-induced nitric oxide production

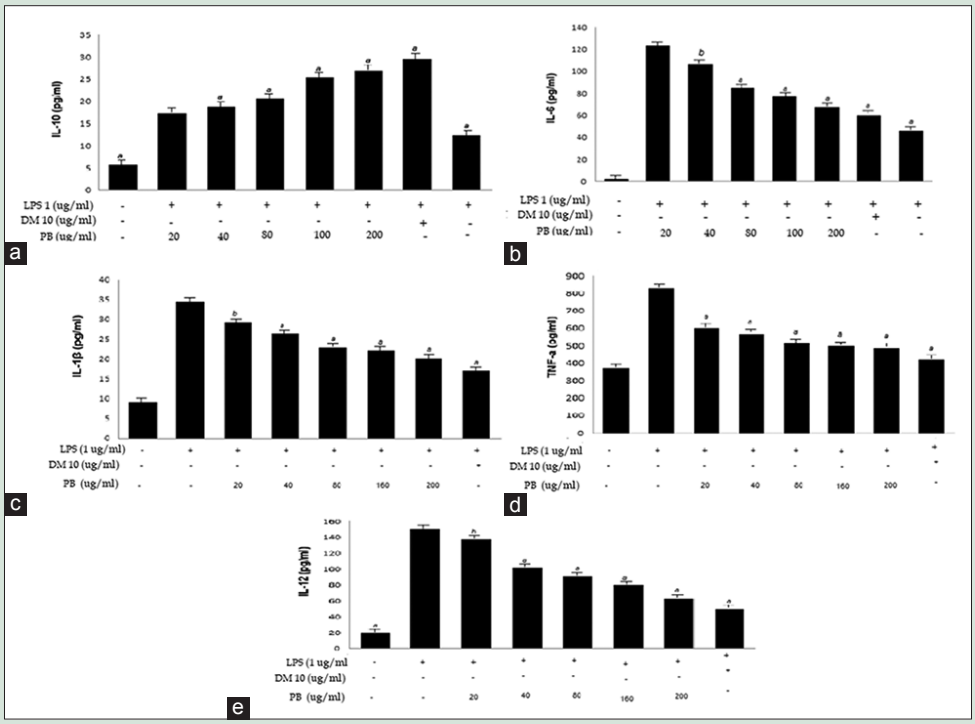

Figure 3: Effect of PB on LPS-induced inflammatory cytokines expression. Cells were treated with LPS and PB for 4 h, IL-10 (a), IL-6 (b), IL-1b (c), TNF-a (d) and IL-12 (e) production in the supernatant were detected using ELISA kits. Data represent the mean ± SD of three different assays and differences between mean values were assessed by one-way ANOVA. Compared with LPS group, ap< 0.05, bp< 0.01. Dexamethasone (DM)

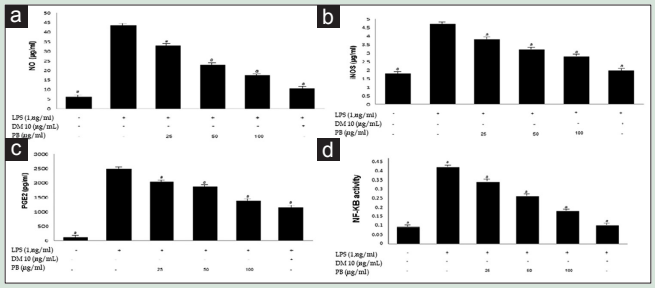

Figure 4: Effects of PB on NO (a), iNOS (b), PGE2 (c) and NF-kB (d) production in RAW264.7 cells. Each column represents the mean ± SD of three independent assay. One-way ANOVA was used to discriminate the effects of PB at each dose point. Compared with LPS group ap<0.05. Dexamethasone (DM)

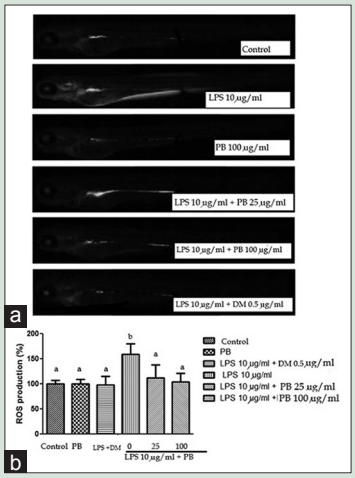

Figure 5: Protective effect of PB against on LPS-induced ROS generation in zebrafish larvae. In (a) the ROS levels were determined by fluorescence microscope and image analysis. In (b) the percentage generation by ROS. Assays were performed in triplicate and the results are expressed as mean ± standard deviation values compared with LPS group $^{a}P < 0.05$, $^{b}P < 0.01$. PB: Procumbenoside B; LPS: Lipopolysaccharide; ROS: Reactive oxygen species

in zebrafish embryo

The effect of PB on LPS-induced NO production is shown in Figure 6. Survival rate of zebrafish embryo was slightly decreased after treatment of LPS and also a significantly increased yolk sac edema size was perceived with LPS exposure. In addition, the level of intracellular NO in zebrafish after LPS stimulation was significantly elevated compared with the non-LPS treated group [Figure 6a]. However, pretreatment of zebrafish embryos with PB decreases NO generation in the dose of 25 and 100 μg/ml [Figure 6a]. LPS and PB reduced NO production dose dependently [Figure 4b]. In this study, DM was used as a standard anti-inflammatory agent. Finding suggested that PB significantly attenuated the increase in NO levels generated by LPS in the zebrafish model compared to the LPS group.

DISCUSSION

For decades, much attention has been focused in finding new, effective, and safe anti-inflammatory agents in vegetables and fruits due to their negligible adverse effects. *J. spicigera* has been used in traditional medicine to treat diabetes, as well as in various anti-inflammatory processes.

LPS is a glycolipid constituent of the external membrane of Gram-negative bacteria, which can stimulate a systemic inflammatory response inducing the expression of pro-inflammatory cytokines and the production of NO in mouse macrophages.[23] Induction of NO production is a defense mechanism against invading microorganisms; nevertheless, the excess production of NO can lead to tissue damage.[24] IL-12, IL-6, IL-1β, and TNF-α are cytokines known as pro-inflammatory are produced in inflammation induction affecting chronic and acute inflammatory diseases. Biological functions as immunity, differentiation, and regulation of cell proliferation are associated with these cytokines which recruit other immune cells to inflammatory sites.[25] TNF-α is a pro-inflammatory mediator which can mediate other pro-inflammatory mediator as IL-6 and COX2. However, IL-10 is a cytokine with a potent effect of anti-inflammatory and several immunomodulatory effects including the inhibition of TNF-α.[26] IL-10 can be expressed in macrophages, monocytes, mastocytes, and Th2 cells and regulatory T-cells.[27]

In this study, anti-inflammatory effect of cytokine IL-10 significantly ($P < 0.05$) increased after PB treatment. IL-12 is an inflammatory mediator that plays an important role in the differentiation of T-cells to Th1 cells profile and IL-12 is secreted by phagocytic in response to the bacteria which contributes to inflammation and phagocytic cell activation.[28] IL-12 production is induced to LPS through the downregulation of NF-κB binding activity in RAW264.7 cells.[29] The results indicated that PB treatment significantly ($P < 0.05$) decreased the secretion of IL-6, IL-12, and TNF-α and attenuated LPS-induced nuclear translocation of NF-κB, suggesting that PB supports the anti-inflammatory activity by inhibiting NF-κB activation.

INOS is an isoform of the reaction of arginine deamination that generates large quantities of NO and is one of the key enzymes associated with inflammation[30] due to participate in the conversion of arachidonic acid to PGH2, as a precursor of PE2, in activated macrophages. A previous study determined that overexpression of iNOS can contribute to increase PGE2 and NO production. Decrease of iNOS was consistent with the date of PGE2 and NO obtained in this study.

Finally, NF-κB is a transcription factor, which participates in transcriptional regulation of gene expression in response to immunity and inflammation. Activation of NF-κB is related to the ubiquitination and phosphorylation, following with proteasome-mediated degradation of NF-κB-bound IκB kinase.[31] NF-κB is essential for the expression of pro-inflammatory cytokines, COX-2, and iNOS.[30] Therefore, an assay was conducted to analyze translocation to the nucleus of NF-κB in LPS-stimulated RAW264.7 macrophages and results of the treatment with LPS increased the translocation of NF-κB, whereas treatment with PB in a dose-dependent manner markedly suppressed their translocation to the nucleus. Considering that NF-κB is translocated to the nucleus and links to the region of genes COX2, IL-6, iNOS, IL-1β, and TNF-α,[32] can assume that PB inhibited pro-inflammatory cytokines production through the suppressed NF-κB signaling pathway in LPS-induced RAW264.7 cells, other studies are currently underway to confirm this.

It is known that increased ROS level induces oxidative stress, resulting in the development of a variety of physiological and biochemical lesions. Thus, cellular damage deteriorates the metabolic function producing inflammation of tissues and cell death.[33] Our results demonstrate that LPS-treated zebrafish significantly increase ROS levels. However, PB markedly inhibits LPS-induced ROS generation. These data show that PB alleviates inflammation by suppressing ROS generation-induced LPS treatment.

Activated macrophages express iNOS, which is responsible for prolonged and high-level production of NO. Aberrant release of NO can

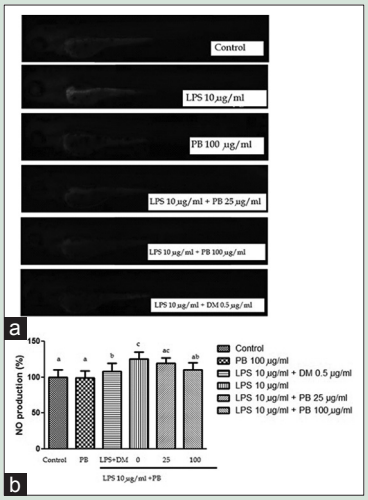

Figure 6: Inhibitory effect of PB against LPS-induced NO generation in zebrafish embryos. Different superscript letters (a-c) indicated significant differences $P < 0.05$ among groups. (a) The NO level was measured after staining with DAF-FM-DA, the results were quantified using the ImageJ software. (b) The data are presented as means with standard deviations of three replicates. PB: Procumbenoside B; LPS: Lipopolysaccharide; NO: Nitric oxide; DAF-FM-DA: Diamino-fluorophore 4-amino-5-methylamino-2′,7′-dichlorofluorescein diacetate

lead to tissue injury and amplify inflammation. Thus, NO inhibitors are indispensables for avoid inflammatory disorder. In this study, we utilized a zebrafish model to determine the support of anti-inflammatory effect of PB *in vivo*. NO generation in zebrafish was measured using a fluorescent assay dye. As shown in Figure 6a, the NO level in zebrafish was elevated after LPS treatment compared with the control. Therefore, LPS increased NO generation similar to that in RAW264.7 macrophage cells. NO production in both models was significantly inhibited in the presence of PB. Our results indicated that PB has a protective effect against the toxicity generated by LPS exposure in zebrafish embryos demonstrated that could improve the toxicity of LPS by inhibiting intracellular NO and ROS generation *in vivo* in a zebrafish model.

CONCLUSION

In this study, we demonstrate that PB significantly causes inhibition in the expression of the pro-inflammatory mediators, NO and iNOS, pro-inflammatory cytokines, including IL-12, IL-6, IL-1β, PGE2, and TNF-in LPS-stimulated RAW264.7 macrophages. Furthermore, level of the anti-inflammatory cytokine, IL-10, increased after PB treatment. In addition, PB ameliorates the toxicity of LPS by inhibiting intracellular ROS and NO generation *in vivo* in a zebrafish model conferring protection against inflammation generated by chemical and physical damage in the zebrafish embryo assay. The antiinflammatory mechanism of PB may be due to its ability to inhibit the production of pro-inflammatory mediators through the suppression of NF-κB. Thus, PB may be considered as a candidate potential anti-inflammatory drug for the treatment of inflammatory disease.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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