

Protective Effect of (+)-Catechin Against Lipopolysaccharide Induced Inflammatory Response in RAW 264.7 Cells through Downregulation of NF- κ B and p38 MAPK

MA Sunil

Mahatma Gandhi University

vasudevan Sunitha

Mahatma Gandhi University

Prasanthkumar Santhakumaran

Mahatma Gandhi University

Mohind C. Mohan

Mahatma Gandhi University

Midhun Sebastian Jose

Mahatma Gandhi University

Radhakrishnan E.K.

Mahatma Gandhi University

Mathew Jyothis (✉ prof.dr.jyothismathew@gmail.com)

Mahatma Gandhi University <https://orcid.org/0000-0002-7900-3815>

Research Article

Keywords: Catechin, Inflammation, COX-2, TNF- α , NF- κ B, MAPK

Posted Date: March 10th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-265638/v1>

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Abstract

Catechin, a flavonol belonging to the flavonoid group of polyphenols is present in many plant foods. The present study was done to evaluate the effect of catechin on various inflammatory mediators using lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. The effect of catechin on total cyclooxygenase (COX) activity, 5-lipoxygenase (5-LOX), myeloperoxidase, nitrite and inducible nitric oxide synthase (iNOS) level, secretion of tumour necrosis factor- α (TNF- α) and interleukin-10 (IL-10) were assessed in LPS-stimulated RAW 264.7 cells. The expression of COX-2, iNOS, TNF- α , nuclear factor- κ B (NF- κ B) and p38 mitogen-activated protein kinase (MAPK) genes were also investigated. The effect was further analyzed using human PBMCs by assessing the level of TNF- α and IL-10. The study demonstrated that the inflammatory mediators such as COX, 5-LOX, nitrite, iNOS, and TNF- α were significantly inhibited by catechin in a dose-dependent manner whereas IL-10 production was up-regulated in RAW 264.7 cells. Moreover, catechin down-regulated the mRNA level expression of COX-2, iNOS, TNF- α , NF- κ B and p38 MAPK. The current study ratifies the beneficial effect of catechin as a dietary component in plant foods to provide protection against inflammatory diseases.

1. Introduction

Inflammation is an invasive phenomenon that functions during severe changes of homeostasis, for instance, injury, infection, and contaminant exposure (Ashley et al., 2012). It is classified as part of the innate immunity of a host's defensive mechanism (Yang et al., 2012). The inflammatory responses include complex interactions between various kinds of immune cells to trigger a range of cellular events (Yang et al., 2014). Macrophages are one of the main immune cells of the innate immune system. The stimulation of macrophages plays a crucial role in inflammatory responses to various infections. Macrophages can destroy pathogens immediately by phagocytosis and also by means of the secretion of different pro-inflammatory mediators, for example, reactive oxygen and nitrogen species, bioactive lipids (prostaglandins, thromboxanes, prostacyclins and other arachidonic acid-derived metabolites), metalloproteinases, cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX) and pro-inflammatory cytokines (TNF- α , IL-6, and IL-1 β). High level of the inflammatory mediators secreted by stimulated macrophages has been associated with the pathophysiology of several inflammatory diseases, such as atherosclerosis, rheumatoid arthritis, pulmonary fibrosis, chronic hepatitis, inflammatory brain diseases, cardiovascular diseases, and diabetes. Macrophages are stimulated in response to cytokines and components of bacterial pathogens invading the host (Jung et al., 2009). Lipopolysaccharide (LPS) the endotoxin originated from the outer membrane of gram-negative bacteria stimulates macrophages to secrete inflammatory cytokines, IL-1 β , TNF- α , and other inflammatory mediators, like nitric oxide (NO) by inducible nitric oxide synthase (iNOS) and prostaglandin E₂ (PGE₂) by COX-2, through activating multiple signalling pathways involves nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinases (MAPKs). Several studies have demonstrated the role of MAPKs on inflammatory response in LPS-induced macrophages. MAPKs have been shown to be involved in the regulation of COX-2 and iNOS gene expression in macrophages. Moreover, the over-expression of COX-2 and iNOS by NF- κ B activation

causes the production of PGE₂ and NO. Furthermore, NF-κB can control proinflammatory cytokines expression in response to various signals (Cheon et al., 2009). Each of the above-mentioned mediators in inflammatory signalling is now considered as an anti-inflammatory target for new drug development.

Polyphenols are a broad and diverse group of organic compounds; occurring naturally in a variety of plant foods. This group of plant metabolites includes over 8000 well-known compounds, varying from simple phenols to complex substances such as tannins (Ferguson, 2001). Polyphenols have become a developing area of attention in nutritional research in recent decades. The growing research interest in this area indicates that polyphenols consumption may play an essential role in the maintenance of human health through the control of metabolism, cell proliferation and body weight (Cory et al., 2018). Many of the polyphenols have capabilities comprising antioxidant, anti-inflammatory, anti-mutagenic, anti-carcinogenic and anti-estrogenic effects. The flavonoids are the major and best-studied group of polyphenols (Ferguson, 2001).

Catechins (flavan-3-ols) are naturally occurring flavanols belonging to the flavonoid group of polyphenols and are well-established antioxidant phytochemicals. The predominant catechins found in plants include (+)-catechin, (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin, and (-)-epigallocatechin gallate. Catechin and its derivatives are found in high concentrations in several medicinal plants and plant-based foods. Catechin is a pharmacologically active natural compound found to be effective against colorectal cancer (Weyant et al., 2001). It inhibits oxidation of low-density lipoprotein (Mangiapane et al., 1992), suppresses Kruppel-like factor 7 expression and enhances adiponectin protein expression and secretion in 3T3-L1 cells (Cho et al., 2007), and prevents human plasma oxidation (Lotito and Fraga, 1998). Transcriptomic studies in apo E-deficient mice have indicated the anti-atherosclerotic effects of catechin (Auclair et al., 2009). It was established that catechin-like compounds were effective therapeutic agents for protection against neurodegenerative diseases including HIV-associated neurocognitive disorders (Nath et al., 2012). Catechin was described as a histidine decarboxylase inhibitor, preventing the histidine to histamine conversion in stress ulcer condition (Reimann et al., 1977). This phytochemical also inhibits monoamine oxidase B (MAO-B) activity and was found to be against oxidative neurodegeneration occurring in Alzheimer's and Parkinson's diseases (Hou et al., 2005).

Plant foods such as cocoa (Gottumukkala et al., 2014), berries (Huang et al., 2012) and tea (Lee et al., 2014) are known to be rich in catechin. There are reports suggesting that these catechin rich foods had a beneficial effect on human health especially to fight against inflammatory diseases (Selmi et al., 2006; Joseph et al., 2014; Ramadan et al., 2017). *Acacia catechu* the decoction of heartwood of which is widely used as a thirst quencher in India and other Asian countries has been reported to be rich in catechin. The plant is known for its immunostimulatory properties (Ismail and Asad, 2009). Moreover, we have previously described that the butanol fraction of ethanol extract of *A. catechu* containing catechin was exhibiting anti-inflammatory activities (Sunil et al., 2019). In the present study, we have examined the effect of catechin on various inflammatory mediators such as COX, 5-LOX, nitrite, iNOS, myeloperoxidase (MPO), TNF-α and IL-10 in LPS-stimulated RAW 264.7 macrophages and also on the production of TNF-α and IL-10 in human peripheral blood mononuclear cells (PBMCs).

2. Materials And Methods

2.1 Catechin

The compound used for the present study (+)-catechin was brought from Sigma-Aldrich (USA).

2.2 Experiments in RAW 264.7 cells

2.2.1. Cell culture

The murine RAW 264.7 cell line was purchased from National Centre for Cell Sciences (NCCS), Pune, India. Cells were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco, New York, USA) supplemented with heat-inactivated fetal bovine serum (10%), penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37 °C in a humidified incubator (Thermo Fisher Scientific, USA) with 5% CO₂. The culture medium was removed every 2 days and replaced with fresh medium. Cells were utilized at 60% confluency for experimentation and acclimatized for 24 h before treatment.

2.2.2. Drug treatment

A stock solution of catechin (1 mg/mL) was made by dissolving in 0.1% dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS) (pH 7.2). For experiments, it was further diluted with DMEM to obtain the desired concentration. RAW 264.7 cells (2×10^5 cells/well) were cultured in 24-well plates overnight and were then washed with the DMEM. The macrophages were pre-treated with catechin (6.25-100 µg/mL) for 30 min, and further incubated with lipopolysaccharide (LPS) (Sigma-Aldrich, USA) (1 µg/mL) for 24 h (Jeong et al., 2014). Diclofenac sodium (Zydus Healthcare Ltd., Ahmedabad, India) at same concentrations that of catechin was used as a standard drug for anti-inflammatory experiments. After incubation, cell lysates were collected and used for *in vitro* anti-inflammatory assays.

2.2.3. Preparation of cell lysate for assays

The monolayer of RAW 264.7 cells obtained after the incubation period was washed with ice-cold PBS (pH 7.2) several times and harvested by trypsinization. The cells were then treated with lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 0.2% sodium dodecyl sulfate (SDS), 0.2% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium ethylenediaminetetraacetate, 5 µg/mL of leupeptin, 5 µg/mL of aprotinin) and the supernatant was collected after centrifugation at 5000 rpm for 10 min. The RAW 264.7 cell lysate was stored at -80 °C until utilized for anti-inflammatory assays.

2.2.4. Effect of catechin on cell viability

The RAW 264.7 macrophages (1×10^6 cells/mL) were seeded in 96-well tissue culture plates and pre-incubated for 18 h. After pre-incubation, cells were washed with culture medium, different concentrations (6.25-100 µg/mL) of catechin were added into the wells and further incubated in a humidified CO₂ (5%) incubator for 24 h at 37 °C. The cytotoxic effect of catechin was assessed using the MTT assay

(Mosmann, 1983). After incubation, 20 μ L of MTT solution (Himedia, India) (5 mg/mL in PBS) and 40 μ L of DMEM were added and again incubated for 4 h at the above-mentioned condition. After incubation, the culture medium was discarded and 100 μ L of acid-isopropanol (0.04 M HCl in isopropanol) was added to the wells. Then, 100 μ L of distilled water was added to dilute the solution and the absorbance was read at 570 nm (Manosroi et al., 2003).

2.2.5. Effect of catechin on total cyclooxygenase (COX) activity

The COX activity was assessed by a method of Jayesh et al., (Jayesh et al., 2017). Briefly, the cell lysate (100 μ L) was incubated in glutathione (5 mM/L), Tris-HCl buffer (pH 8), and hemoglobin (5 mM/L) at 25 $^{\circ}$ C for 1 min. Arachidonic acid (200 mM/L) was added to initiate the reaction and ceased by the addition of trichloroacetic acid (10%) in 1N HCl after 20 min incubation at 37 $^{\circ}$ C. The supernatant was collected from the reaction mixture by centrifugation at 3000 rpm for 10 minutes. After the addition of 200 μ L of 1% thiobarbiturate into the supernatant, it was boiled for 20 minutes and cooled. Then centrifuged for three minutes and the absorbance of the supernatant was measured using a microplate reader (Thermo Fisher Scientific, USA) at 632 nm. The percentage inhibition of the enzyme was calculated by the following formula.

$$\text{Inhibition of COX activity (\%)} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100$$

2.2.6. Effect of catechin on 5-lipoxygenase (5-LOX) activity

The lipoxygenase (LOX) activity was evaluated by a method explained by Axelrod et al., (Axelrod et al., 1981) with slight modifications. The assay reaction mixture consists of 50 μ L of cell lysates, 200 μ L of sodium linoleate, and 1.75 mL of Tris-HCl buffer (pH 7.4). The LOX activity was observed as an increase in absorbance at 234 nm that indicates the formation of 5-hydroxy-eicosatetraenoic acid. The percentage inhibition of 5-LOX was determined using the following formula.

$$\text{Inhibition of 5-LOX activity (\%)} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100$$

2.2.7. Effect of catechin on myeloperoxidase activity

The myeloperoxidase activity of RAW 264.7 cells was evaluated by a method of Bradley et al., (Bradley et al., 1982). After catechin treatment, myeloperoxidase was extracted from cell lysate by homogenization of the material in a solution of 0.5% hexadecyltrimethylammonium bromide (HTAB) (Sigma-Aldrich, USA) in 50 mM potassium phosphate buffer (pH 6.0) (Sigma-Aldrich, USA). The homogenate mixture was freeze-thawed three times with liquid nitrogen and the supernatant was obtained by centrifugation at 3000 rpm for 30 min at 4 $^{\circ}$ C. Myeloperoxidase activity was analyzed by mixing 2.9 mL of 50 mM

phosphate buffer (pH 6.0) containing 1.67 mg/mL guaiacol (Sigma-Aldrich, USA) and 0.0005% hydrogen peroxide with 0.1 mL of the supernatant. The absorbance of the reaction mixture was read at 460 nm. MPO activity was expressed in units per mL of cell lysate. One unit of myeloperoxidase activity is described as the amount of enzyme that degrades 1 μ M of peroxide per minute at 25 °C.

2.2.8. Effect of catechin on inducible nitric oxide synthase (iNOS) activity

Nitric oxide synthase activity was measured by a method explained by Salter et al., (Salter et al., 1995). The cell lysate was mixed with 2 mL of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, homogenized and the supernatant was collected by centrifugation (3000 rpm for 30 min at 4 °C). The reaction mixture was prepared by mixing 0.1 mL enzyme (sample), 0.1 mL manganese chloride, 0.1 mL oxygenated hemoglobin, 0.1 mL dithiothreitol (30 μ g), 0.1 mL tetrahydropterin, 0.1 mL L-Arginine as a substrate, and 0.1 mL NADPH and kept at 37 °C. The increase in absorbance was recorded at 401 nm. The percentage inhibition of the iNOS was calculated using the following formula.

$$\text{Inhibition of iNOS activity (\%)} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100$$

2.2.9. Effect of catechin on nitrite level

The effect of different concentrations of catechin (6.25-100 μ g/mL) on nitric oxide production was quantified in the form of nitrite concentration of cell lysate using Griess reagent. Culture supernatant (50 μ L) was mixed with 150 μ L of Griess reagent (Sigma-Aldrich, USA) and incubated for 10 min at room temperature in 96-well plate. The absorbance of the reaction mixture was read at 540 nm in a microplate reader. Sodium nitrite was utilized as the standard for the experiment (Ignácio et al., 2001).

2.2.10. Effect of catechin on cytokine secretion

The effect of catechin on the level of TNF- α and IL-10 in RAW 264.7 cells was determined by the use of ELISA kits according to instructions of the manufacturer (BioLegend, USA).

2.2.11. mRNA analysis by reverse transcriptase-polymerase chain reaction (RT-PCR)

The effect of catechin on COX-2, iNOS, TNF- α , NF- κ B, and p38 MAPK mRNA expression levels was evaluated by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). RAW 264.7 macrophages (4×10^5 cells) were pretreated with catechin (100 μ g/mL) in a 6-well plate for 30 min prior to incubation with 1 μ g/mL of LPS for 6 h. Subsequently, total RNA was isolated from cells using TRIzol reagent (Invitrogen, USA) according to the instructions of the manufacturer and stored at -70 °C until use. The cDNA was manufactured from the total mRNA using two steps RT-PCR kit (Thermo Fisher Scientific, USA) by following the manufacturer's instructions. RT-PCR was conducted in a thermal cycler (Thermo Fisher Scientific, USA) by utilizing forward and reverse primers of iNOS, COX-2, TNF- α , NF- κ B, and p38 MAPK. GAPDH primers were kept as internal control. All primers used in the experiment are listed in Table

1. The PCR products were separated on 1.5% agarose gel (containing ethidium bromide) using electrophoresis and an E-Gel imager system (Invitrogen, USA) was used to visualize the gel. The relative intensity of various bands was evaluated using a GelDoc 2000 scanner (Bio-Rad, USA). ImageJ software was used to analyze the band intensity and expressed in arbitrary units.

2.3. Experiments in human peripheral blood mononuclear cells (PBMCs)

Institutional Human Ethics Committee approved the experiments in human PBMCs (Approval No: 15/IHEC20082015).

2.3.1. Effect of catechin on cell viability

The viability of human PBMCs treated with catechin was evaluated by MTT assay (Mosmann, 1983). Briefly, blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes (CML, Biotech (P) Ltd., Kerala, India) from healthy volunteers and mononuclear cells were separated using Histopaque-1077 according to instructions of the manufacturer (Sigma-Aldrich, USA). A hundred microliters of PBMC suspension (2×10^5 cells) in DMEM with 10% FBS was incubated in the presence of various concentrations of catechin (6.25-100 $\mu\text{g}/\text{mL}$) in a 96-well plate for 24 h at 37 °C in a 5% CO₂ incubator. The culture medium without catechin was treated as control. After incubation, MTT assay was performed.

2.3.2. Effect of catechin on cytokine secretion

The human PBMCs were separated and pooled. A hundred microliters of mononuclear cell suspension (2×10^5 cells) in DMEM with 10% FBS was incubated in the presence of various concentrations of catechin (6.25-100 $\mu\text{g}/\text{mL}$) with LPS (5 $\mu\text{g}/\text{mL}$) in a 96-well plate. The cells treated with LPS alone used as a positive control. The plate was incubated at 37 °C for 24 h in a 5% CO₂ incubator and cell culture supernatants were separated by centrifugation at 2000 rpm for 20 min at 18 °C. The presence of TNF- α and IL-10 in the supernatants were analyzed by ELISA kits according to guidelines of the manufacturer (BioLegend, USA).

2.4. Statistical analysis

The results of *in vivo* studies are expressed as the mean \pm standard error of the mean (S.E.M.) for six animals. Data of *in vitro* studies are expressed as mean \pm S.E.M. of three independent experiments. One-way ANOVA with post-hoc Tukey's HSD test was used to analyze the data by means of the SPSS program (version 20). A level of $p \leq 0.05$ was regarded as statistically significant.

3. Results

3.1. Effect of catechin on the viability of RAW 264.7 macrophages

The effect of various concentrations of catechin on the viability of RAW 264.7 cells was demonstrated in Fig. 1. The viability of macrophages was assessed by MTT assay. The cell viability was not considerably altered by 24 h incubation with catechin up to 100 µg/mL. In the presence of catechin at 100 µg/mL concentration $86.06 \pm 0.43\%$ cell viability was seen.

3.2. Effect of catechin on total cyclooxygenase (COX) activity

To analyze the effect of catechin on LPS-induced COX activity in RAW 264.7 macrophages, the cells were first treated with catechin for 30 min and then incubated with LPS (1 µg/mL) for 24 h. Figure 3 shows that COX activity was significantly inhibited by the treatment of catechin in a dose-dependent manner as compared to standard drug diclofenac. Treatment of cells with diclofenac (100 µg/mL) exhibited total COX inhibition of $87.78 \pm 0.57\%$ while the catechin treatment showed $75.46 \pm 0.15\%$ inhibition at the same concentration.

3.3. Effect of catechin on 5-LOX activity

The effect of catechin on 5-LOX activity of LPS-stimulated RAW 264.7 cells is depicted in Fig. 4. Dose-Related inhibition of 5-LOX activity was observed with catechin treatment. Treatment of RAW 264.7 cells with catechin at 100 µg/mL concentration showed $90.42 \pm 0.16\%$ inhibition of 5-LOX activity. At the same concentration, diclofenac gave $98.26 \pm 0.15\%$ inhibition of 5-LOX activity.

3.4. Effect of catechin on myeloperoxidase (MPO) activity

Myeloperoxidase (MPO), an enzyme present in the intracellular granules of neutrophils, macrophages, and monocytes is employed as an indicator of inflammatory reaction. The synthesis of MPO has induced in RAW 264.7 cells with 1 µg/mL of LPS. The effect of catechin on the MPO activity of RAW 264.7 cells stimulated with LPS is shown in Fig. 5. The LPS induced MPO activity of RAW 264.7 cells was 0.00843 ± 0.00 U/mL of the cell lysate. Treatment with catechin (100 µg/mL) considerably inhibited the MPO activity to a level of 0.00118 ± 0.00 U/mL. The MPO activity with the standard drug diclofenac (100 µg/mL) was 0.00074 ± 0.00 U/mL.

3.5. Effect of catechin on inducible nitric oxide synthase (iNOS) activity

Inducible nitric oxide synthase (iNOS), one of the key enzymes producing nitric oxide is considered as a significant pro-inflammatory marker. Figure 6 shows the effect of catechin on iNOS activity of RAW 264.7 cells stimulated with LPS. Concentration-dependent inhibition of iNOS activity was observed in both diclofenac and catechin treated RAW 264.7 cells. Treatment of cells with catechin at 100 µg/mL concentration showed $78.35 \pm 0.10\%$ inhibition of iNOS activity in RAW 264.7 cells. At the same concentration, diclofenac gave $89.40 \pm 0.13\%$ inhibition of iNOS activity.

3.6. Effect of catechin on nitrite level

Nitric oxide (NO) is an intracellular mediator that performs an essential role in inflammation. The effect of catechin on NO production in LPS-stimulated RAW 264.7 cells was expressed in terms of nitrite concentration in the cell culture supernatant (Fig. 7). Stimulation of RAW 264.7 cells with LPS induced the production of nitrite up to $51.39 \pm 0.25 \mu\text{M}$ while cells without LPS stimulation produced only $7.79 \pm 0.06 \mu\text{M}$. A dose-related reduction in nitrite concentration was observed with catechin treatment. Catechin at $100 \mu\text{g/mL}$ concentration considerably inhibited the production of nitrite to $17.10 \pm 0.26 \mu\text{M}$. At the same concentration, the standard drug diclofenac-treated cells produced $12.91 \pm 0.46 \mu\text{M}$ of nitrite.

3.7. Effect of catechin on cytokine secretion by RAW 264.7 cells

The anti-inflammatory effect of catechin was further investigated by evaluating the production of the pro-inflammatory cytokine, TNF- α and anti-inflammatory cytokine, IL-10 by ELISA. Unstimulated RAW 264.7 macrophages produced $38.86 \pm 0.19 \text{ pg/mL}$ of TNF- α after 24 h of incubation. The stimulation of RAW 264.7 cells with LPS ($1 \mu\text{g/mL}$) increased the secretion of TNF- α up to $583.00 \pm 1.73 \text{ pg/mL}$. A dose-dependent significant reduction in LPS-induced TNF- α production was observed in RAW 264.7 cells after treatment with catechin. At the maximum concentration of catechin ($100 \mu\text{g/mL}$) used in the present study, TNF- α production was reduced to $86.68 \pm 0.23 \text{ pg/mL}$. In the presence of diclofenac ($100 \mu\text{g/mL}$), the LPS-stimulated RAW 264.7 cells produced $54.38 \pm 0.71 \text{ pg/mL}$ of TNF- α (Fig. 8). Catechin treatment enhanced the production of IL-10 by RAW 264.7 cells. LPS treatment of RAW 264.7 cells induced the production of IL-10 up to $91.35 \pm 0.25 \text{ pg/mL}$ while unstimulated cells produced only $30.78 \pm 0.28 \text{ pg/mL}$ (Fig. 9). A concentration-dependent augmentation in IL-10 production was observed in LPS stimulated RAW 264.7 macrophages after treatment with catechin (up to $297.98 \pm 0.27 \text{ pg/mL}$ at $100 \mu\text{g/mL}$).

3.8. Effect of catechin on COX-2, iNOS, TNF- α , NF- κ B, and p38 MAPK gene expression

In order to find out whether the inhibitory effects of catechin on inflammatory mediators occurs at the transcription level, mRNA expression levels of COX-2, TNF- α , iNOS, NF- κ B, and p38 MAPK were evaluated in RAW 264.7 cells stimulated with LPS. Figure 10 shows the effect of catechin on the mRNA level of these genes in relation to the constant expression of GAPDH mRNA level. Stimulation with LPS significantly upregulated the mRNA levels of COX-2, TNF- α , iNOS, NF- κ B, and p38 MAPK, while this effect was repressed by catechin treatment ($100 \mu\text{g/mL}$).

3.9. Effect of catechin on the viability of human PBMCs

The effect of catechin on the viability of human PBMCs was determined using the MTT assay. The viability of human PBMCs in the presence of different concentrations of catechin (6.25 – $100 \mu\text{g/mL}$) is

shown in Fig. 11. After 24 h incubation with catechin, the cell viability was not much affected. Catechin at 100 µg/mL concentration demonstrated $86.62 \pm 0.36\%$ cell viability.

3.10. Effect of catechin on cytokine secretion by human PBMCs

The effect of catechin on TNF- α and IL-10 secretion was further studied in human PBMCs. Pretreatment with different concentration of catechin (6.25–100 µg/mL) significantly suppressed the production of TNF- α by the LPS-stimulated human PBMCs (Fig. 12). Human PBMCs stimulated with LPS produced 701.32 ± 0.58 pg/mL of TNF- α while unstimulated cells produced only $88.91 \pm$ pg/mL. Catechin treatment (100 µg/mL) decreased the production of TNF- α by LPS stimulated PBMCs up to 79.85 ± 0.24 pg/mL. Consistent with the stimulatory effect of catechin on IL-10 secretion by RAW 264.7 cells, human PBMCs were also stimulated by catechin to secrete IL-10. While the treatment of human PBMCs with catechin (100 µg/mL) caused 201.18 ± 0.38 pg/mL of IL-10, the IL-10 produced by the untreated PBMC was 32.80 ± 0.14 pg/mL (Fig. 13).

4. Discussion

There is a large growing demand in the use of plant foods as natural agents to treat various inflammatory diseases. Phytochemicals like flavonoids, alkaloids, lactones, polysaccharides, glycosides, and diterpenoids present in a number of plant foods have been described to be responsible for their anti-inflammatory properties. Hence the exploration of phytochemicals from plant foods as new leads for the development of effective and safe anti-inflammatory agents is receiving much research attention. The present study demonstrated the anti-inflammatory effects of catechin in LPS-stimulated RAW 264.7 cells and human PBMCs.

During inflammation, secretion of arachidonic acid from the cellular membrane by phospholipase enzyme is considerably increased. Then through the activity of cyclooxygenase enzyme (COX-1 or COX-2), arachidonic acid is transformed into prostaglandin H₂ (PGH₂) which, in turn, is converted into a sequence of final active compounds such as PGE₂, thromboxane A₂ (TXA₂) and prostacyclin (Prostaglandin I₂ or PGI₂). Another enzyme called lipooxygenase (5-LOX) converts arachidonic acid to leukotrienes. The products of both the enzymes act as important mediators of the inflammatory process and produce various effects such as high vascular permeability, vasodilatation, and movement of leucocytes to the affected site (Martel-Pelletier et al., 2003). The elevated level of these mediators plays a significant role in the development of several chronic inflammatory diseases, for instance, rheumatoid arthritis, cardiovascular diseases, and cancer. In the present study, treatment of RAW 264.7 cells with catechin showed a significant dose-dependent decrease in the total COX and 5-LOX activity of the cell. Burnett et al., (Burnett et al., 2007) had reported that a flavonoid mixture containing catechin and baicalin could act as a dual inhibitor of COX and 5-LOX to reduce inflammation. Results of the current study demonstrated the specific role of catechin as a dual inhibitor of COX and 5-LOX.

Myeloperoxidase (MPO), a heme-containing enzyme found mainly in neutrophils, also present in monocytes, and macrophages. This peroxidase is produced in excessive amount during inflammation (Shaeib et al., 2016). The activity of MPO produces a reactive oxygen intermediate, hypochlorite from hydrogen peroxide and chloride ions (Kindt et al., 2007). Myeloperoxidase has been established as a mediator of local tissue damage and the ensuing inflammation in several inflammatory diseases. Therefore MPO is considered to be as a significant therapeutic target in the medication of different inflammatory conditions (Aratani, 2018). Treatment with catechin significantly inhibited LPS-stimulated MPO activity of macrophages. Thus the inhibitory effect of catechin on myeloperoxidase activity is an indication of the anti-inflammatory potential of catechin.

The expression of genes responsible for the production of inflammatory mediators, for instance, nitrogen and reactive oxygen species (nitric oxide, superoxide, hydrogen peroxide and peroxynitrite) are rapidly triggered by the stimulation of macrophages with LPS. Nitric oxide (NO) is a free radical that performs a crucial part in cell death and survival and produces different pro-inflammatory effects (Jung et al., 2009). It may control most phases of the development of inflammation, mainly the initial phases of transmigration of inflammatory cells to the site of inflammation. Overproduction of NO primarily by inducible nitric oxide synthase (iNOS) occurs in many inflammatory diseases, consisting of rheumatoid arthritis, atherosclerosis, septic shock, diabetes, multiple sclerosis, and transplant rejection. The expression of iNOS in macrophages is triggered by particular inducers and contributes to the pathophysiology of inflammatory diseases (Choi et al., 2014). In the present investigation, NO production and iNOS activity in LPS-stimulated RAW 264.7 cells were significantly inhibited after treatment with catechin. The result suggests that the inhibitory effect of catechin on NO production might through the inhibition of iNOS.

Tumour necrosis factor- α (TNF- α) is a strong pro-inflammatory cytokine that performs a significant role in inflammation, cell differentiation, proliferation and apoptosis (Zelová and Hošek, 2013). Macrophages are the major TNF- α producing cell and fascinatingly are also very responsive to TNF- α . The elevated level of TNF- α production has been reported to be linked with the pathogenesis of a number of diseases, such as atherosclerosis, Crohn's disease, rheumatoid arthritis, diabetes, sepsis, obesity and psoriasis. TNF- α has been demonstrated to play an essential role in regulating the production of other inflammatory mediators. It orchestrates the synthesis of a proinflammatory cytokine cascade and regarded to be a "master regulator" of proinflammatory cytokine production (Parameswaran and Patial, 2010). It has been found out that TNF- α and various other pro-inflammatory cytokines activate iNOS expression in macrophages and certain other leukocytes. It also stimulates cyclooxygenase 2 (COX-2), thereby enhances the synthesis of prostanoids PGE₂, PGF_{2 α} , and PGI₂ (Zelová and Hošek, 2013). Catechin treatment effectively reduced the production of TNF- α in RAW 264.7 cells stimulated with LPS. This result suggests that the anti-inflammatory effect of catechin might be through the inhibition of TNF- α consequently affecting the production of other inflammatory mediators.

Interleukin-10 (IL-10) is an essential immunomodulatory cytokine with an extensive effect on both innate and adaptive immune responses (Iyer et al., 2010). It is produced by lymphocytes, monocytes and

macrophages. It attracts a lot of interest owing to its anti-inflammatory properties (Grütz, 2005). IL-10 has been demonstrated to inhibit the expression of pro-inflammatory cytokines such as IL-1 α and β , TNF- α , IL-6, IL-12, and IL-18, and thereby control the inflammatory response in disease conditions (Couper et al., 2008). The current study showed that IL-10 production was considerably increased in macrophages after treatment with catechin. It can be assumed that the enhanced production of IL-10 may be contributing to the inhibitory potential of catechin on TNF- α production.

The anti-inflammatory effect of catechin was further established by investigating the mRNA level expression of inflammatory mediators such as COX-2, TNF- α , iNOS and also the expression of two important factors, NF- κ B and MAPK, involved in the inflammatory signalling pathways that generate the production of inflammatory mediators. RT-PCR analysis demonstrated that the LPS-induced mRNA levels of COX-2, iNOS, TNF- α in RAW 264.7 cells were suppressed by treatment with catechin. These results point out that the inhibitory effect of catechin on these inflammatory mediators seems to occur at the transcriptional level.

The transcription factor NF- κ B controls several features of innate and adaptive immune responses and functions as an essential arbitrator of inflammatory responses. It stimulates the expression of different pro-inflammatory genes, comprising those code for cytokines and chemokines (Liu et al., 2017). NF- κ B regulates the expression of IL-1 β , TNF- α , IL-6, iNOS and COX-2 in macrophages stimulated with LPS. So, down-regulation of activity of NF- κ B is an effective approach to control inflammatory diseases through inhibition of TNF- α , NO and PGE₂ expression (Kang et al., 2012). In the present investigation, the mRNA level expression of NF- κ B was significantly inhibited by catechin treatment. The result suggests that catechin can inhibit the production of inflammatory mediators such as TNF- α , NO and PGE₂ by suppressing the NF- κ B-mediated inflammatory gene expression.

Mitogen-activated protein kinases (MAPKs) are involved in a broad range of signalling cascades in which several extracellular stimuli activate inflammation, with the synthesis of inflammatory mediators. There are three major components of the MAPK family, comprising the p38 MAPK, the extracellular signal-regulated kinases (ERKs) and the c-Jun N-terminal kinases (JNKs) (Sun et al., 2013). MAPK activation usually overlaps with proinflammatory NF- κ B pathways (Serhan et al., 2010). The phosphorylation (activation) of MAPKs is recognized to be a component of the upstream signalling pathway for the NF- κ B activation (Kim et al., 2007). Additionally, MAPK phosphorylation is identified to perform a significant role in the up-regulation of iNOS and NO in LPS-stimulated macrophages (Zhou et al., 2008). In the present investigation, the expression of p38 MAPK was inhibited by the pretreatment of macrophages with catechin and showed the effect of catechin on MAPK signalling pathway. The p38 MAP kinase pathway like other MAPK cascades is linked with inflammation, cell differentiation, cell growth, and cell death (Kaminska, 2005). From the result of the present study, it may be assumed that the inhibition of p38 MAP kinase might have a significant effect on the inhibition of one of the downstream component of MAPK signalling pathway, NF- κ B. Because catechin can inhibit NF- κ B and p38 MAP kinase both can be considered as two possible targets of catechin.

Freshly isolated human PBMCs are used as an *in vitro* model system for studying the anti-inflammatory effect of compounds on the human immune system (Jenny et al., 2011). The effect of catechin on the viability of human PBMCs and the cytokine secretion by PBMCs were evaluated in the present study. The viability of human PBMCs was not affected by the concentration of catechin used in the experiments. Catechin treatment significantly decreased the production of TNF- α and enhanced the production of IL-10 in human PBMCs. These findings are consistent with that obtained from the studies using RAW 264.7 cells and indicate the possible anti-inflammatory effect of catechin.

5. Conclusion

The results of the present investigation demonstrate the anti-inflammatory effects of catechin. In this study, the anti-inflammatory potential of catechin was found to be a function of the inhibition of COX, 5-LOX, MPO, iNOS, NO and TNF- α production in RAW 264.7 cells stimulated with LPS. The mRNA level expression of iNOS, TNF- α , COX-2, NF- κ B, and p38 MAPK was suppressed by catechin treatment. The inhibitory effect of catechin on the production of different inflammatory mediators seems to be mediated through the inhibition of NF- κ B and MAP kinases activation. The level of anti-inflammatory cytokine IL-10 has enhanced in both RAW 264.7 cells and human PBMCs after catechin treatment. The study strongly suggests that catechin has the potential to be developed as an anti-inflammatory therapeutic drug for treating various inflammatory conditions. The use of catechin as an anti-inflammatory ingredient in nutraceutical preparations can also be suggested.

Declarations

Funding: No research grants received from funding agencies

Employment: no association with present or anticipated employment by any organization that may gain or lose financially through publication of this manuscript.

Financial interests: No stocks or shares in companies (including holdings of spouse and/or children) that may gain or lose financially through publication of this manuscript; consultation fees or other forms of remuneration from organizations that may gain or lose financially; patents or patent applications whose value may be affected by publication of this manuscript.

Non-financial interests: No non financial interests that could impart bias on the work submitted for publication such as professional interests, personal relationships or personal beliefs (amongst others).

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Tables

Table 1 List of PCR primers and their sequence used in the present study

Name		Sequence (5' to 3')
COX-2	F	GGAGAGACTATCAAGATAGT
	R	ATGGTCAGTAGACTTTTACA
iNOS	F	AATGGCAACATCAGGTCGGCCATCACT
	R	GCTGTGTGTGTACAGAAAGTCTCGAACTC
TNF- α	F	CCCAGGCAGTCAGATCATCTTC
	R	AGCTGCCCTCAGCTTGA
NF- κ B1	F	CCTAGCTTTCTCTGAACTGCAAA
	R	GGGTCAGAGGCCAATAGAGA
MAPK p38	F	CAGCTTCAGCAGATTATGCGT
	R	GTA CTGAGCAAAGTAGGCATGT

F-forward primer; R-reverse primer

Figures

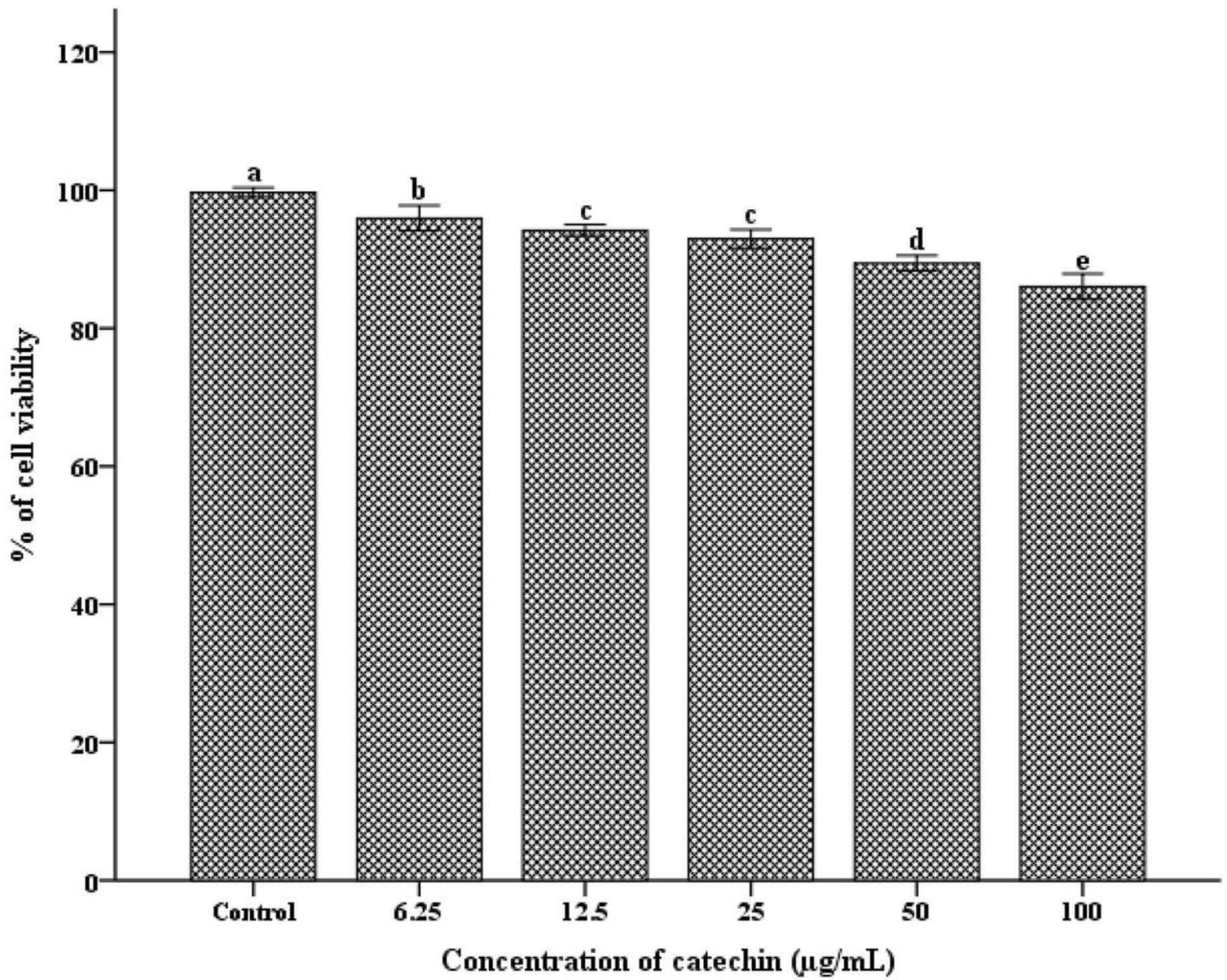


Figure 1

Effect of catechin on the viability of RAW 264.7 cells. Values represent mean \pm S.E.M. of three independent experiments; and values with similar alphabet did not differ significantly (Tukey's HSD; $p \leq 0.05$).

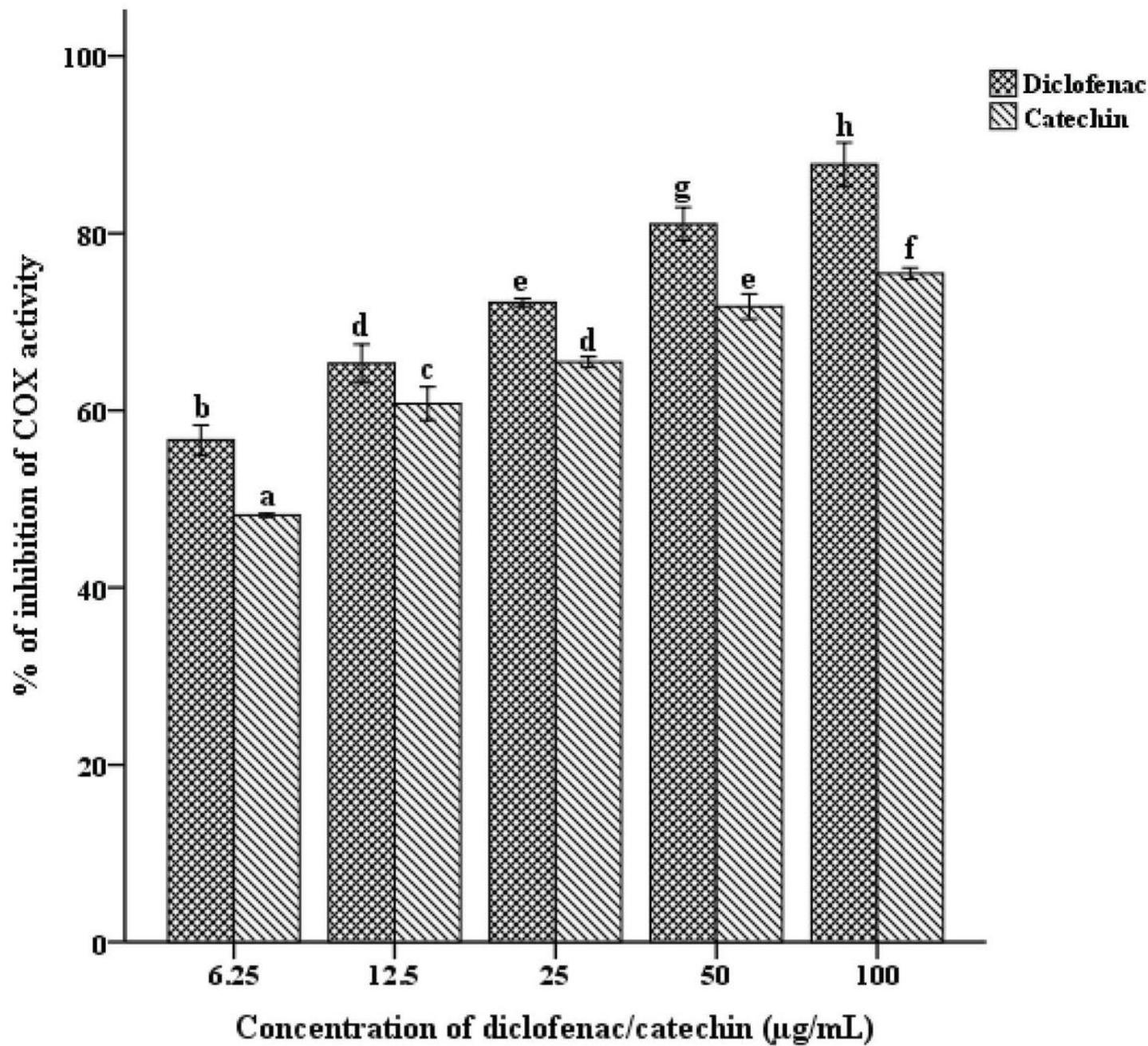


Figure 2

Effect of catechin on COX activity in RAW 264.7 cells. Values represent mean \pm S.E.M. of three independent experiments; and values with similar alphabet did not differ significantly (Tukey's HSD; $p \leq 0.05$).

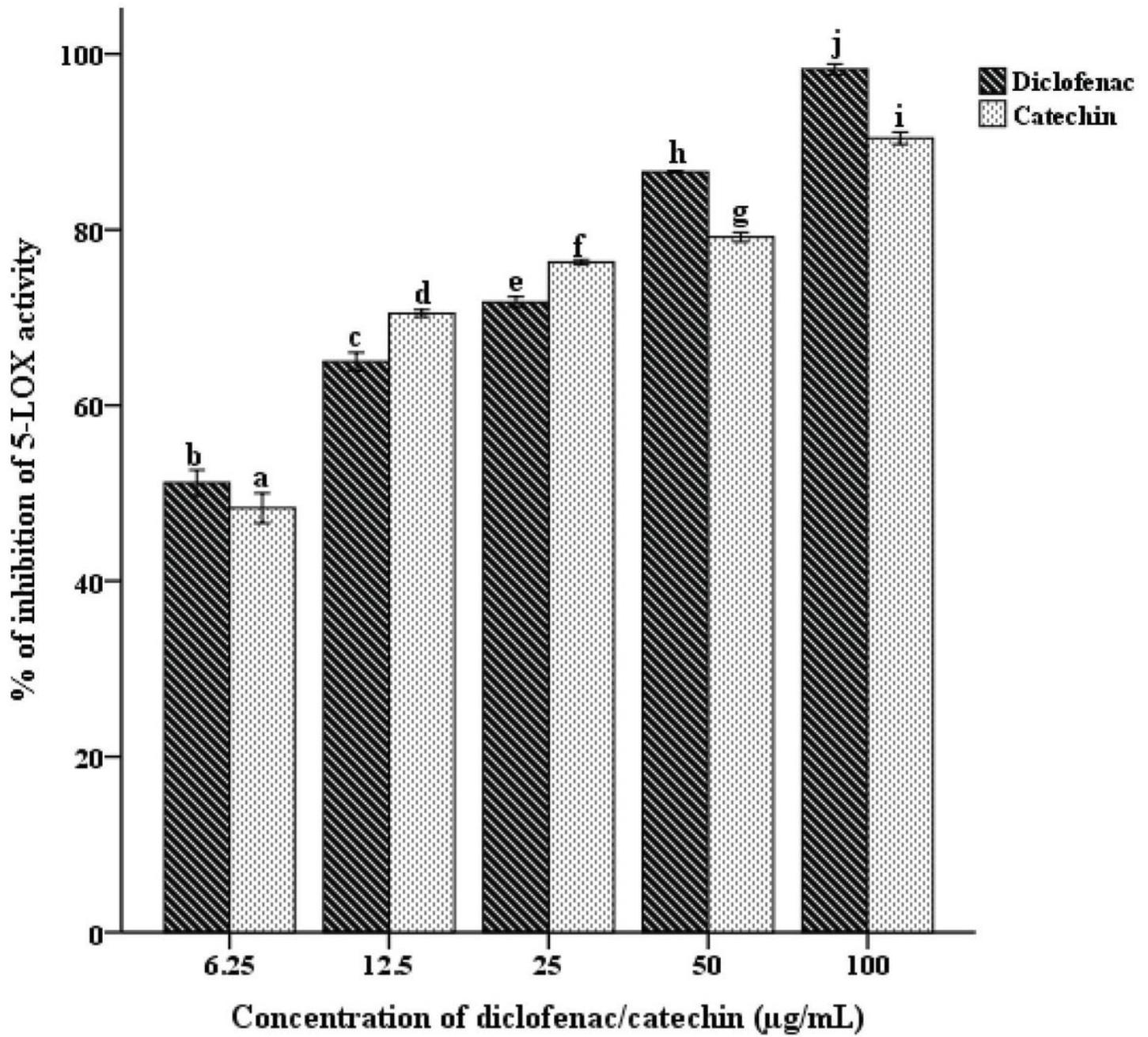


Figure 3

Effect of catechin on 5-LOX activity in RAW 264.7 cells. Values represent mean \pm S.E.M. of three independent experiments; and values with similar alphabet did not differ significantly (Tukey's HSD; $p \leq 0.05$).

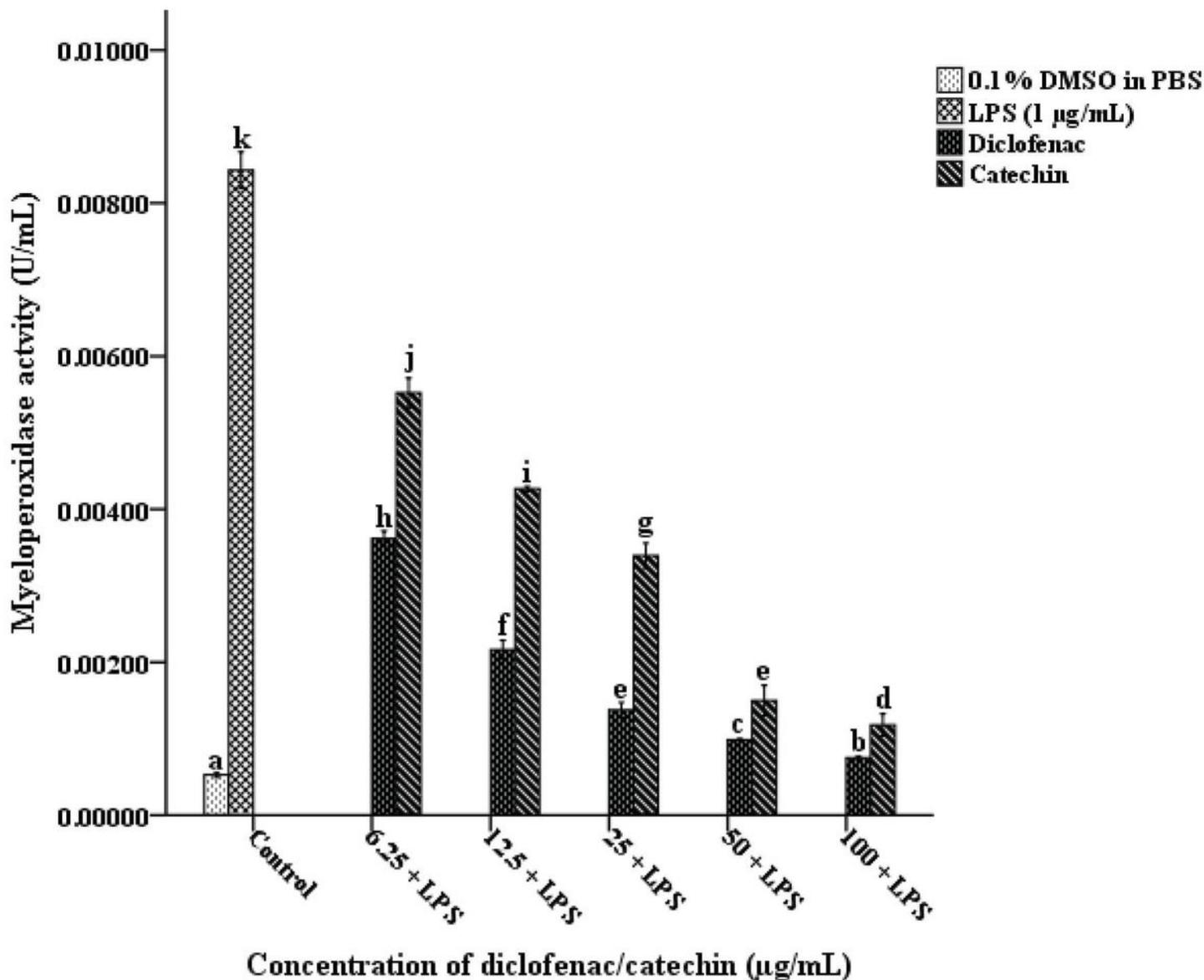


Figure 4

Effect of catechin on myeloperoxidase activity in RAW 264.7 cells. Values represent mean \pm S.E.M. of three independent experiments; and values with similar alphabet did not differ significantly (Tukey's HSD; $p \leq 0.05$).

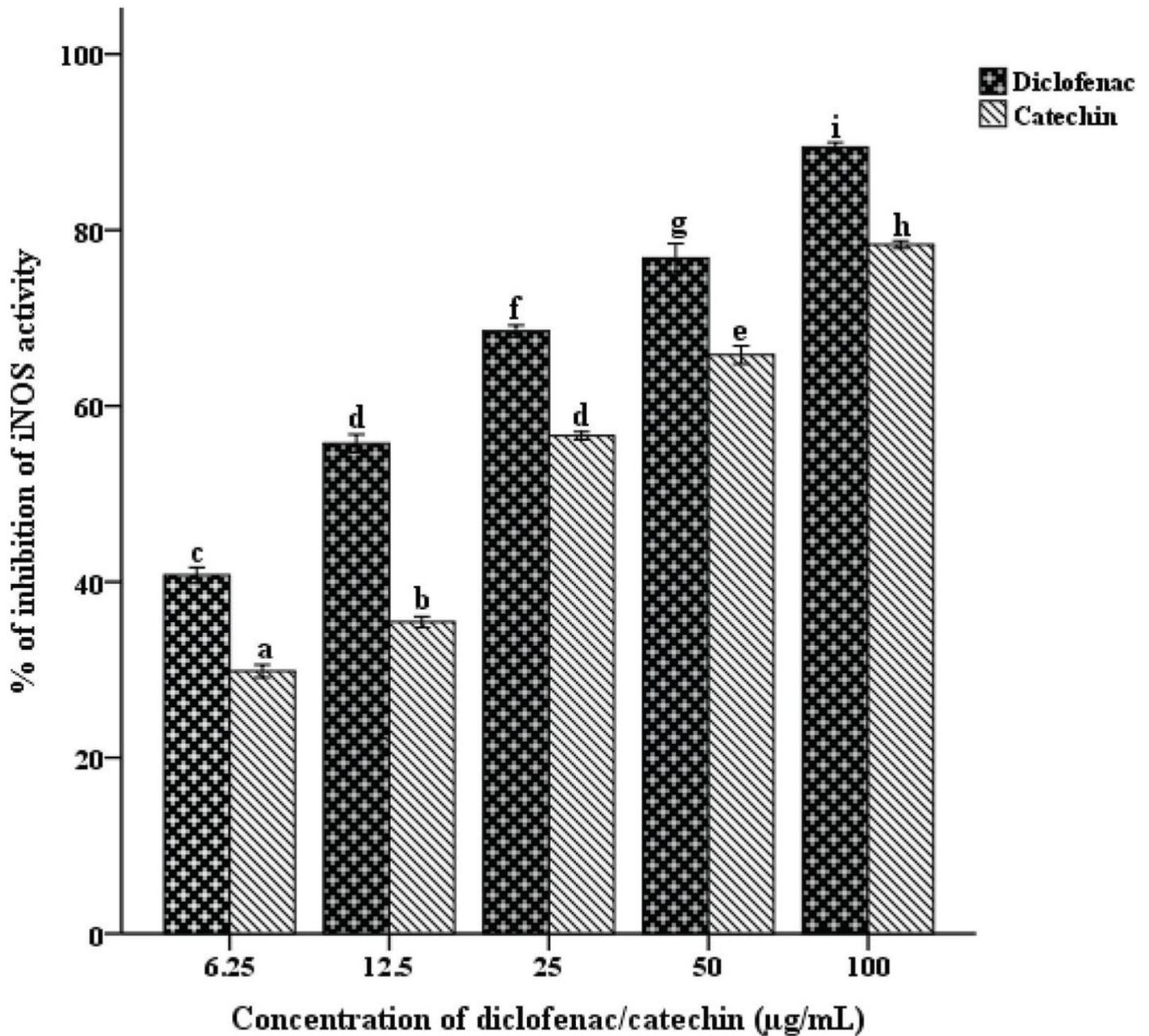


Figure 5

Effect of catechin on inducible nitric oxide synthase (iNOS) activity in RAW 264.7 cells. Values represent mean \pm S.E.M. of three independent experiments; and values with similar alphabet did not differ significantly (Tukey's HSD; $p \leq 0.05$).

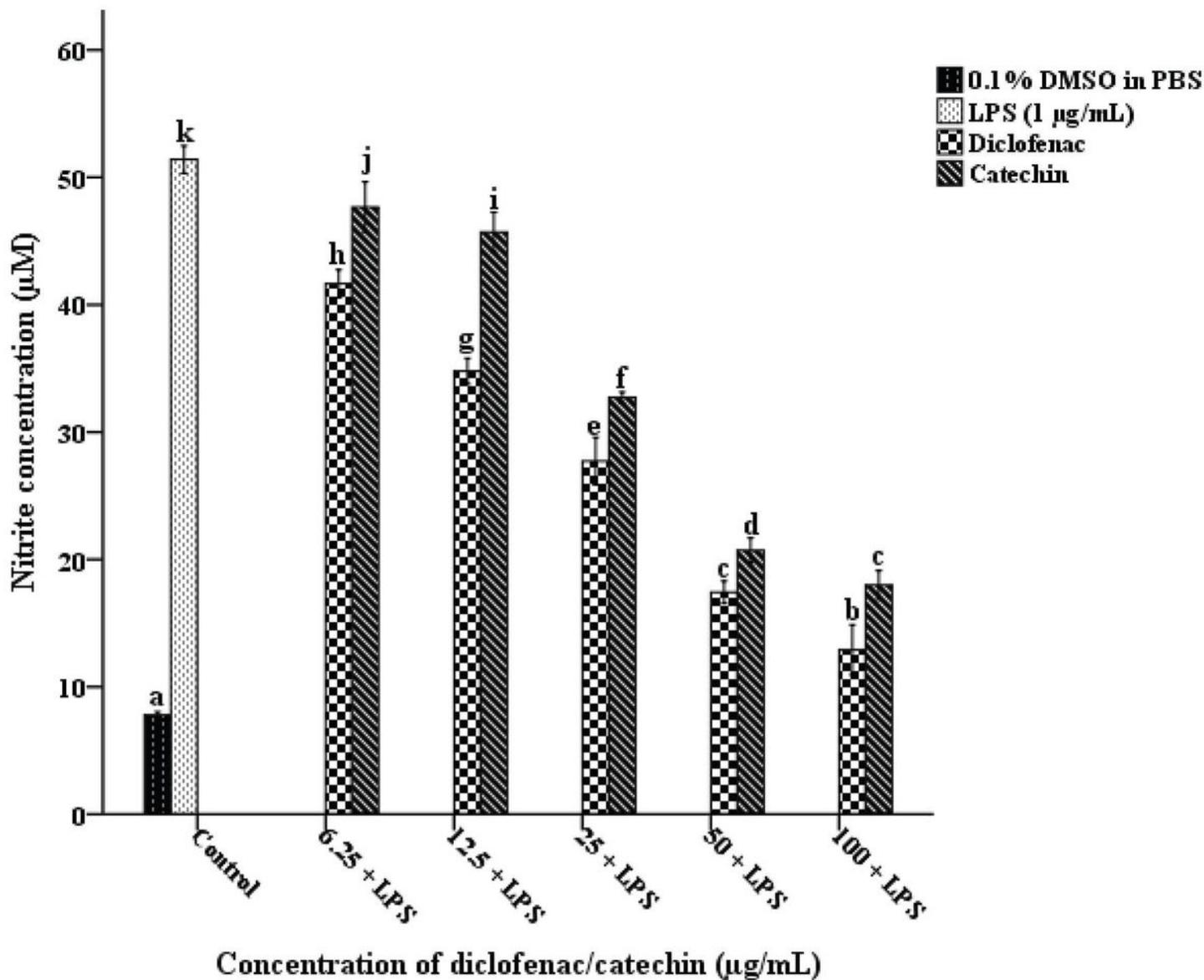


Figure 6

Effect of catechin on nitrite level in RAW 264.7 cells. Values represent mean \pm S.E.M. of three independent experiments; and values with similar alphabet did not differ significantly (Tukey's HSD; $p \leq 0.05$).

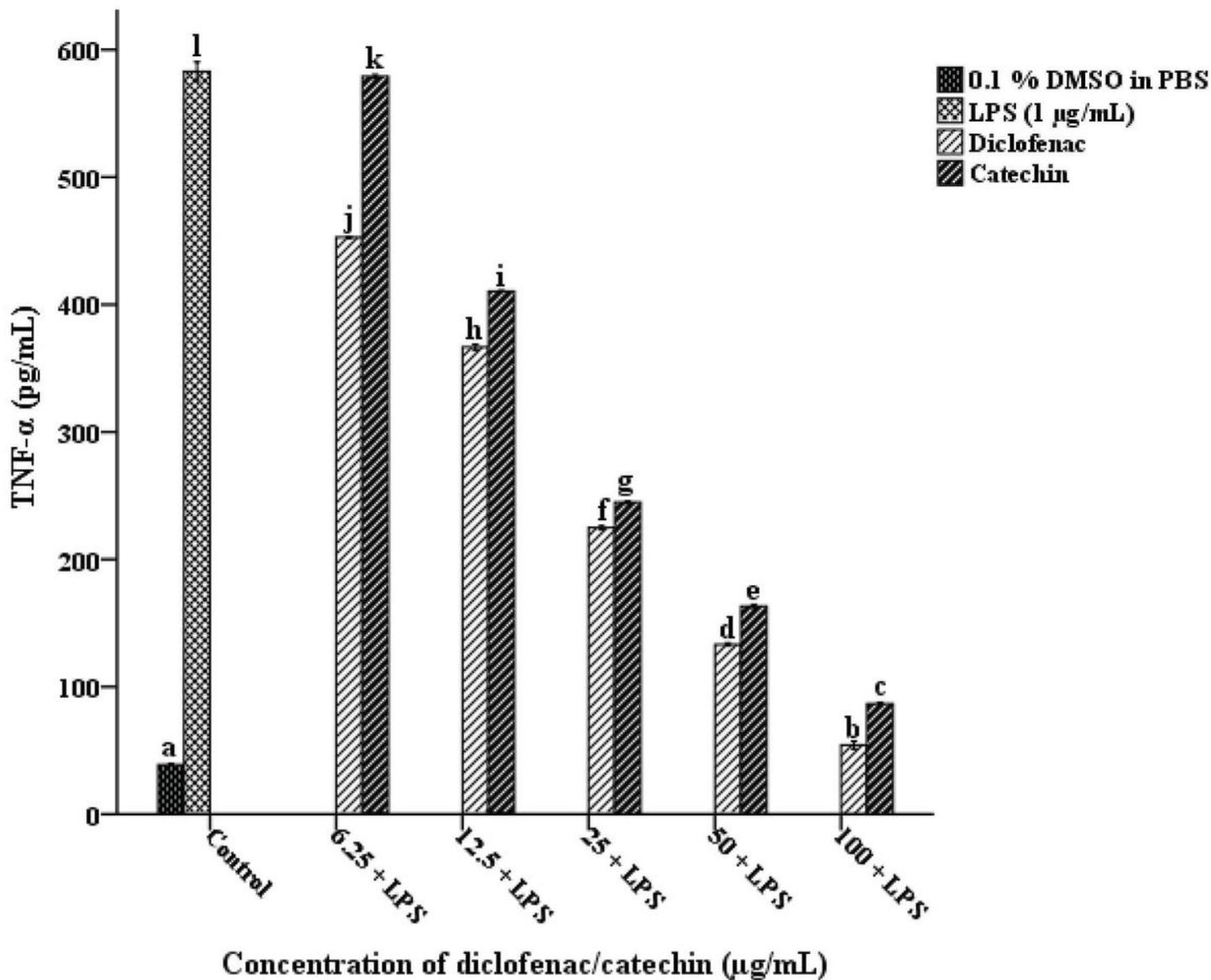


Figure 7

Effect of catechin on the secretion of TNF- α by RAW 264.7 cells. Values represent mean \pm S.E.M. of three independent experiments; and values with similar alphabet did not differ significantly (Tukey's HSD; $p \leq 0.05$).

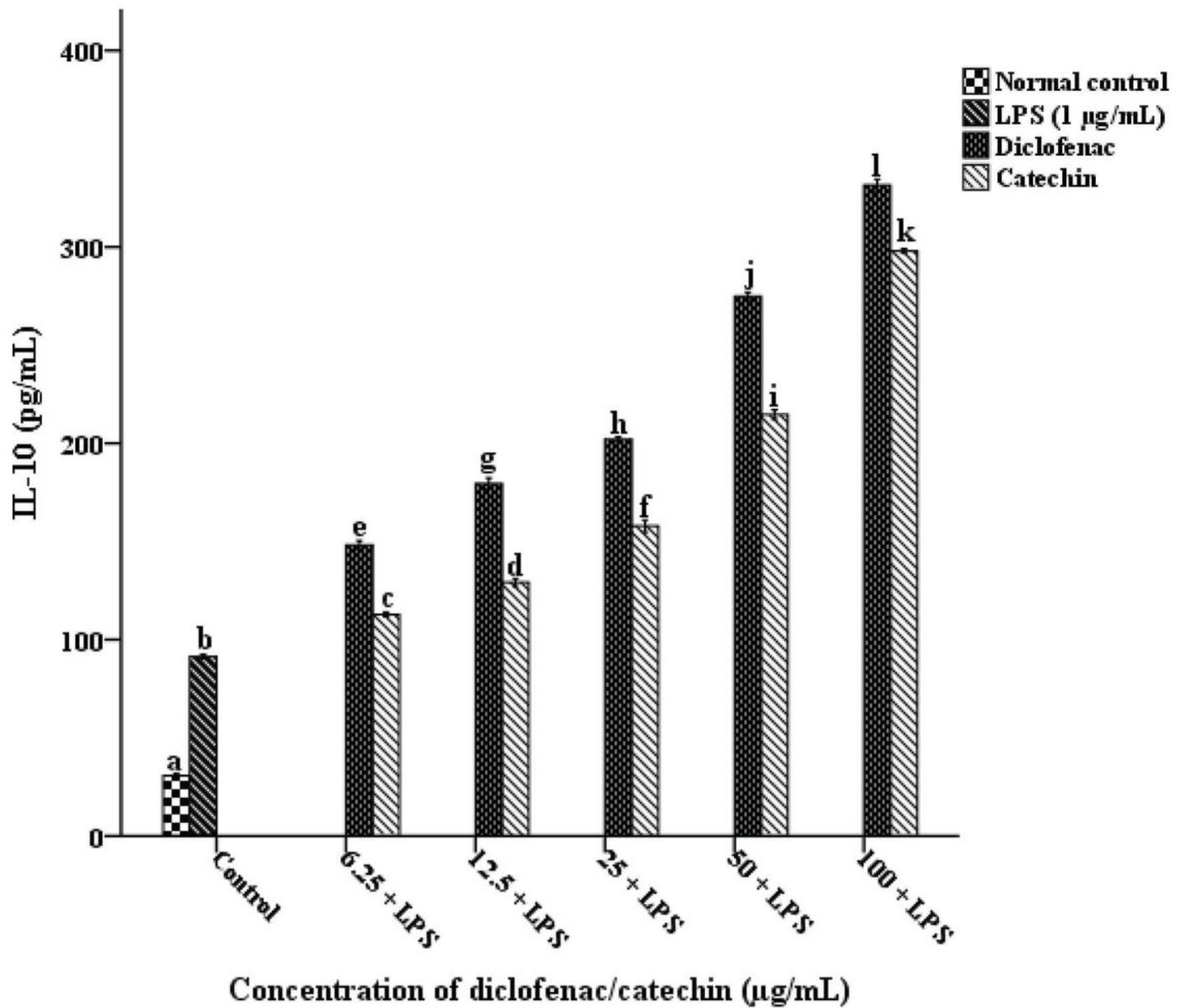


Figure 8

Effect of catechin on the secretion of IL-10 by RAW 264.7 cells. Values represent mean \pm S.E.M. of three independent experiments; and values with similar alphabet did not differ significantly (Tukey's HSD; $p \leq 0.05$).

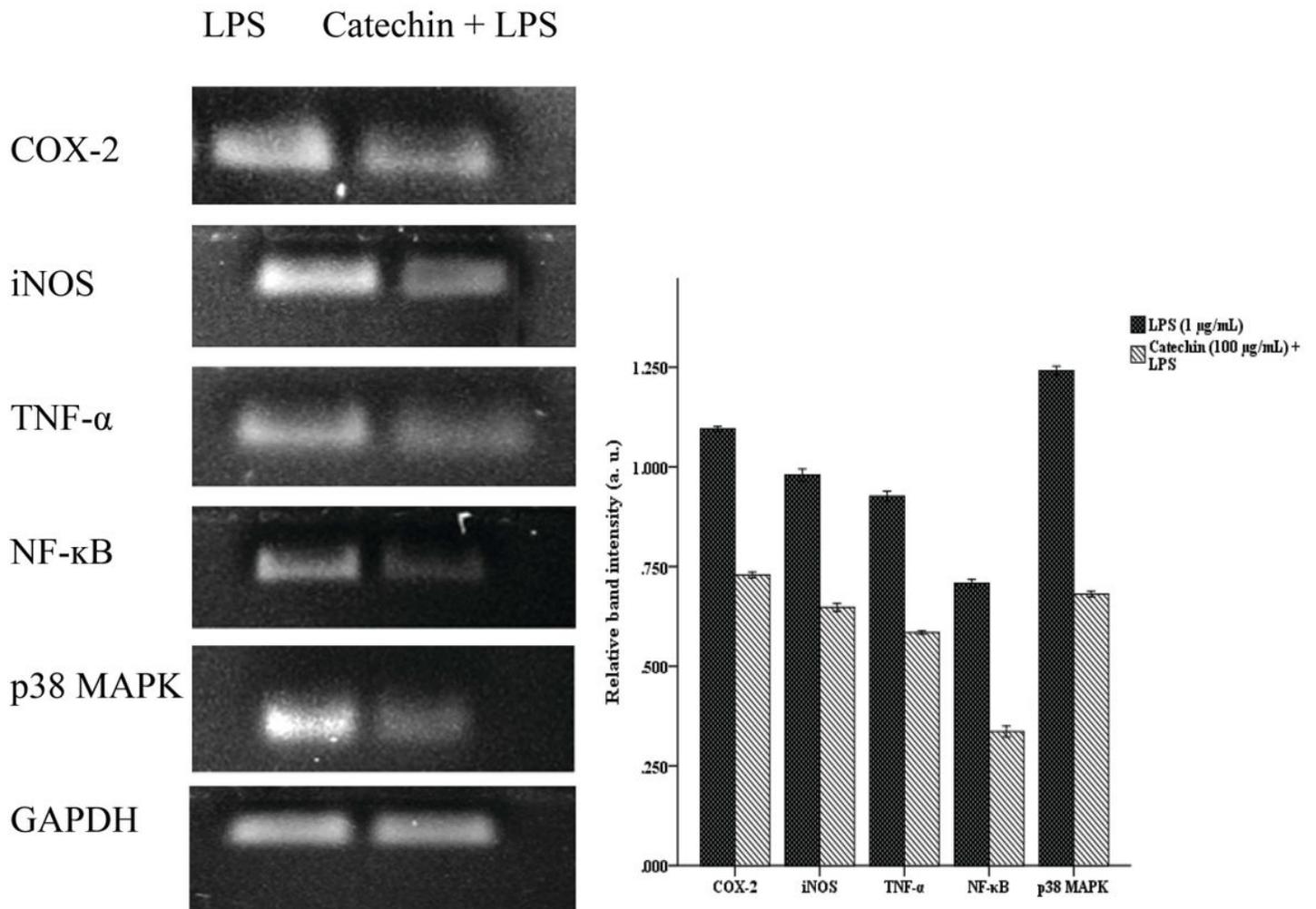


Figure 9

Effect of catechin on the mRNA expression level of inflammatory mediators such as COX-2, iNOS, TNF- α , NF- κ B, and p38 MAPK. GAPDH was kept as control.

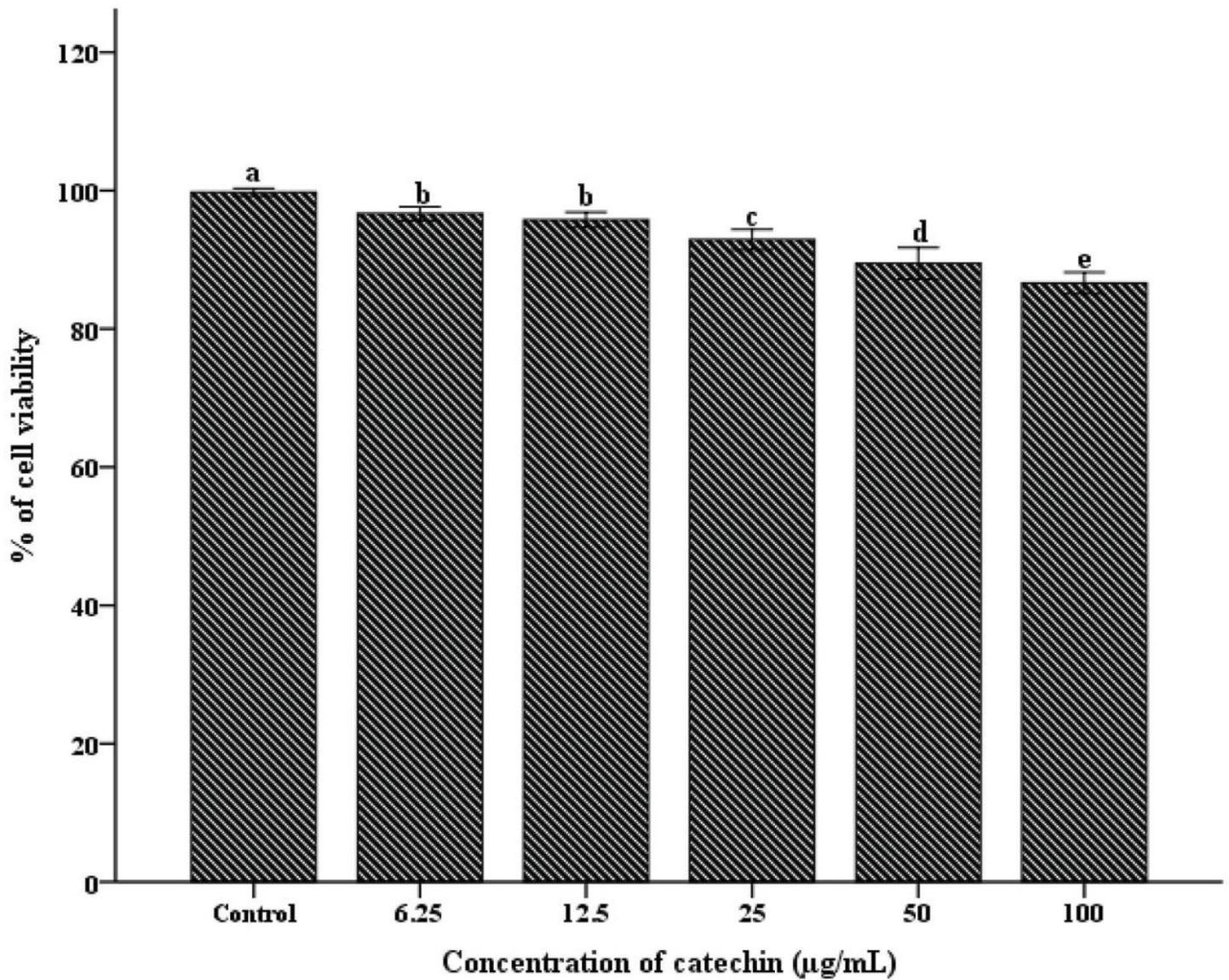


Figure 10

Effect of catechin on the viability of human peripheral blood mononuclear cells (PBMCs). Values represent mean \pm S.E.M. of three independent experiments; and values with similar alphabet did not differ significantly (Tukey's HSD; $p \leq 0.05$).

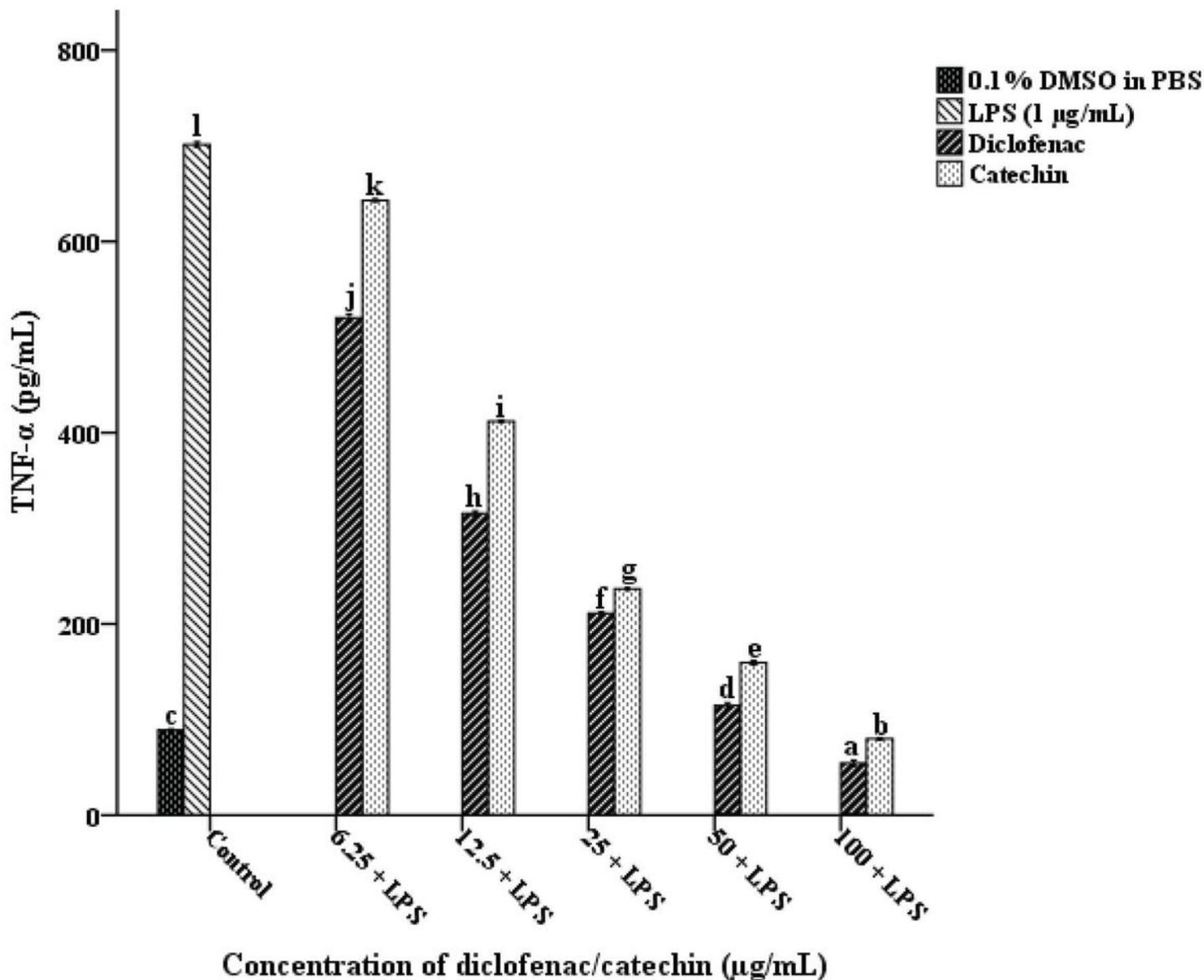


Figure 11

Effect of catechin on TNF- α secretion of by human peripheral blood mononuclear cells (PBMCs). Values represent mean \pm S.E.M. of three independent experiments; and values with similar alphabet did not differ significantly (Tukey's HSD; $p \leq 0.05$).

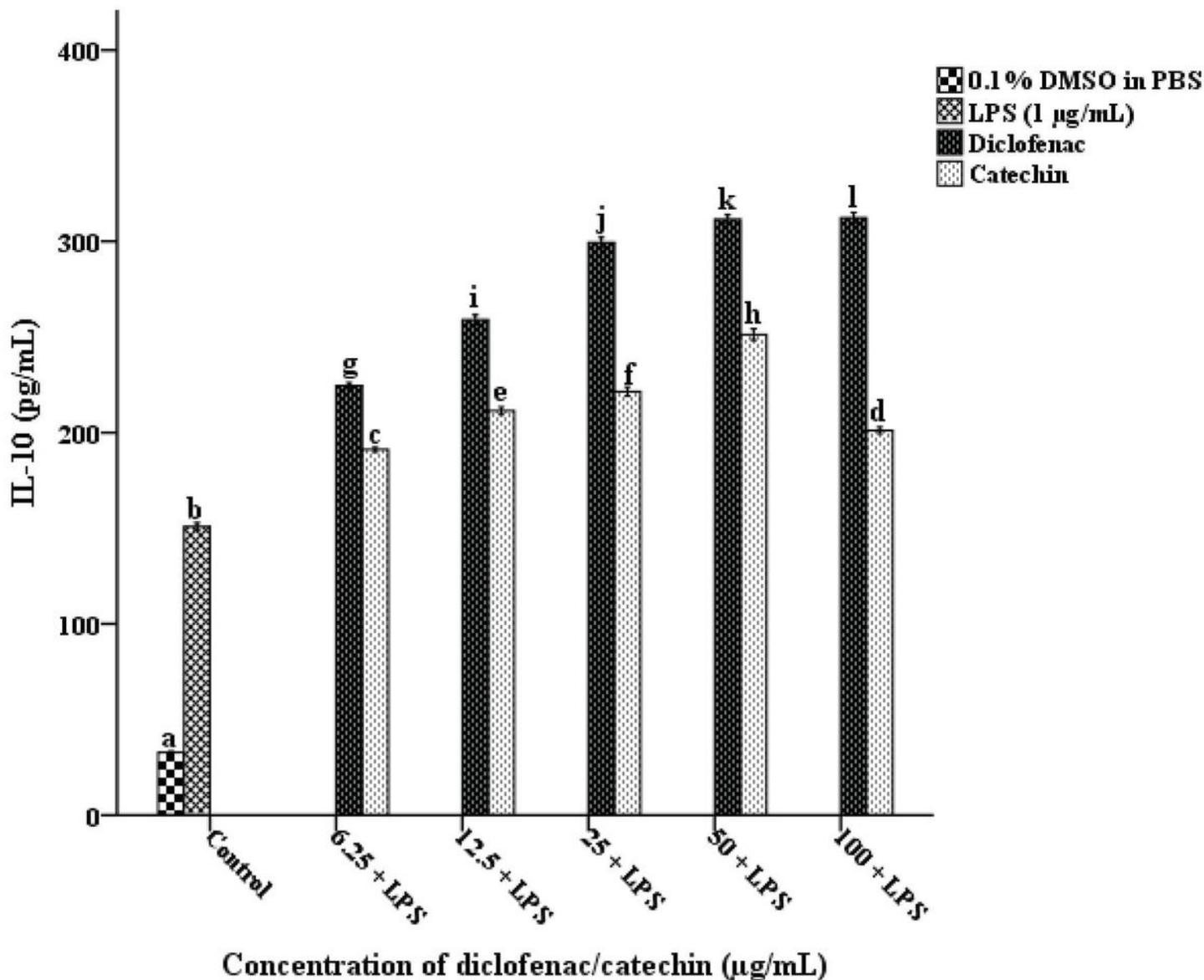


Figure 12

Effect of catechin on IL-10 secretion by human peripheral blood mononuclear cells (PBMCs). Values represent mean \pm S.E.M. of three independent experiments; and values with similar alphabet did not differ significantly (Tukey's HSD; $p \leq 0.05$).

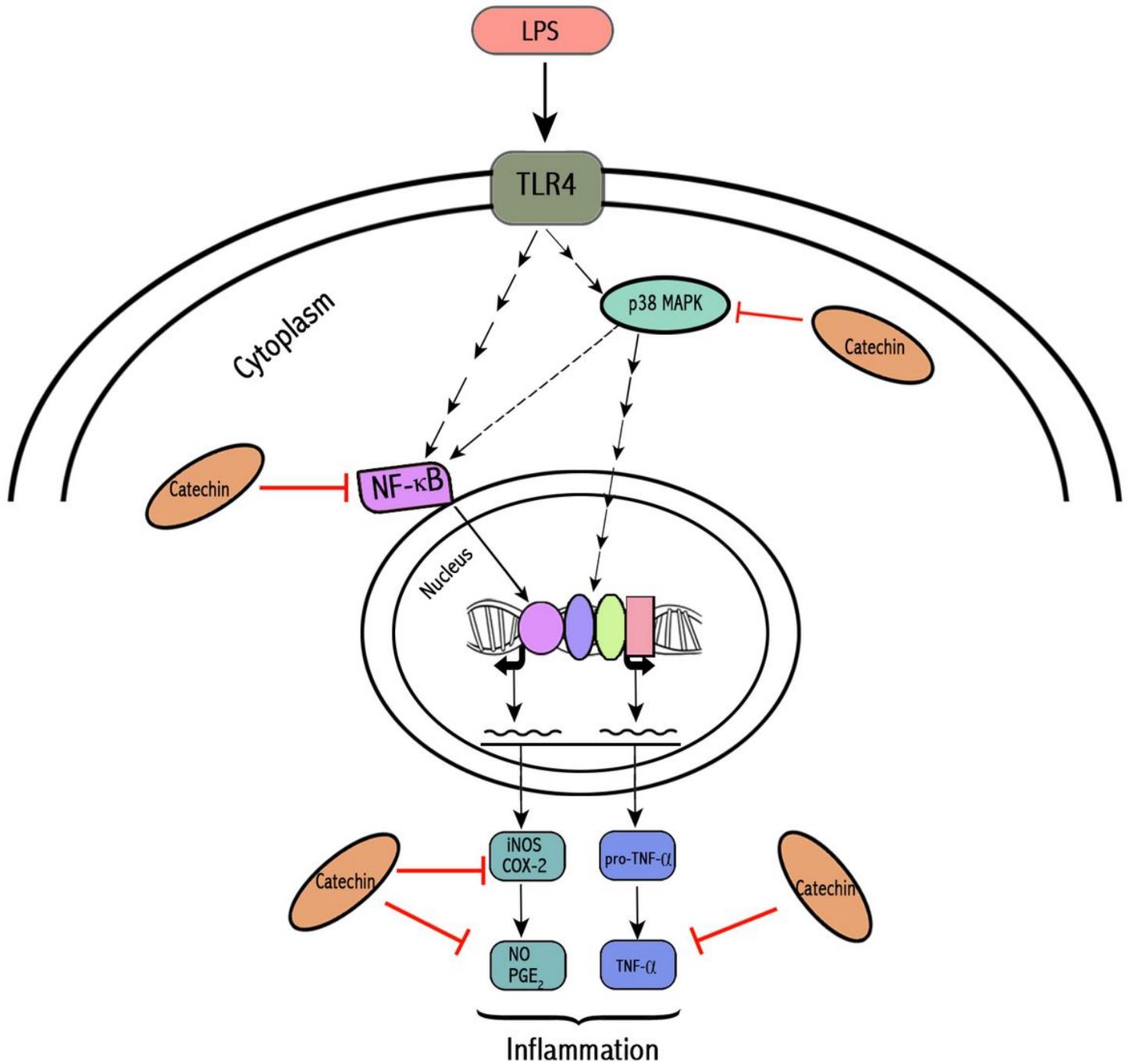


Figure 13

Schematic demonstration of anti-inflammatory activity of (+)-catechin