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Flavonoids from *Boldoa purpurascens* inhibit proinflammatory cytokines (TNF-α and IL-6) and the expression of COX-2

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Abstract

The flavonoids comprise a large class of plant metabolites distributed in food plants. These compounds have antioxidant, antitumor, antiallergic and anti-inflammatory effects. The molecular mechanisms of their biological activities remain to be clearly understood. We investigated the *in vitro* anti-inflammatory potential of a flavonoid mixture and isolated compounds from the leaves of *Boldoa purpurascens*. Our results provide direct evidence of the anti-inflammatory effects of the mixture, which are mediated by the inhibition of the proinflammatory cytokines TNF- α and IL-6 as well as the modulation of the expression of COX-2.

Keywords

Boldoa purpurascens; Nyctaginaceae; anti-inflammatory activity; flavonoids; proinflammatory cytokines; murine macrophages

INTRODUCTION

The beneficial effects of flavonoids have been attributed to their antioxidant and anti-inflammatory properties (Kim *et al*., 2004; Scalbert *et al*., 2005). The effects of flavonoids, including quercetin, on a variety of inflammatory processes and immune functions have been extensively reviewed (Camuesco *et al*., 2004; Comalada *et al*., 2005; Nair *et al*., 2002; 2004). Cytokines are involved in the pathogenesis of chronic inflammatory diseases and their secretion is modulated by several factors including oxidative stress (Calamia, 2003; Taylor *et al*., 2004). Tumor Necrosis Factor alpha (TNF-α) is a multifunctional cytokine that regulates the growth, proliferation, differentiation, and viability of activated leukocytes. TNF-α also triggers the cellular release of other cytokines, chemokines, or inflammatory mediators and displays antiviral and antimicrobial effects (Aggarwal, 2000; Wajant *et al*., 2001; Dempsey *et al*., 2003; Hsu *et al*., 1995). The present study was undertaken to investigate the direct effect of pure flavonoids and a flavonoid mixture obtained from a traditionally used extract from leaves of *Boldoa purpurascens*, the safety of which has been demonstrated (Hernandez et al., 2016), on the secretion of the proinflammatory cytokines TNF- α and IL-6 and the gene expression of COX-2.

MATERIALS AND METHODS

Extraction of the flavonoid mixture and isolation of the compounds

The extraction of the flavonoid mixture from the leaves of *Boldoa purpurascens*, the isolation and structural elucidation of its constituents, and the preparation of the aglycon, was performed according to Hernández *et al*. (2017). Four flavonoids have been reported, two of which are included in the present study, i.e. 3,4',5-trihydroxy-6,7-methylenedioxyflavone-3-O-[α-L-rhamnopyranosyl-(1"'-2")]-β-D-glucopyranoside (compound **2**); and 3,4',5-trihydroxy-6,7-methylenedioxyflavone-3-O-α-L-rhamno-pyranosyl-(1"'-2")-β-D-xylopyranoside] (compound **4**) (Fig. 1).

Evaluation of the inhibitory activity on proinflammatory cytokines (TNF-α and IL-6) production for murine macrophages

Macrophage culture

C57BL/6 mice, 8 weeks age, were obtained from the mice facility of INDICASAT. The animals were kept at constant temperature (25 °C) with free access to chow and water in a room with a 12 h light/dark cycle. The experiments were performed in strict accordance with the recommendations in the Institutional Animal Welfare Committee

guidelines.The protocol was approved by the Institutional Animal Care and Use Committee of INDICASAT AIP (IACUC-15-001). Peritoneal macrophages were obtained after 5 days of administration of 2 mL of thioglycolate 3% (i.p.), and washed with cold RPMI medium (Gibco). Cells were plated in 96-well plates in RPMI culture medium supplemented with 10% fetal bovine serum at a concentration of $2x10^5$ cells / well. They were then cultured for two hours at 37 °C in a CO_2 atmosphere (5%). The unbound cells were removed and adherent cells were treated with the compounds at concentrations of 50, 100 and 200 µM dissolved in DMSO, one hour before being stimulated with 10 ng/mL of LPS (0111: B4, *E coli*) (In vivoGen). After six hours, the supernatants were collected and the concentrations of TNF-α and IL-6 were determined by the ELISA method. Cells cultured in the presence of DMSO were used as negative controls.

Cell viability assay

After the supernatants were collected, 100 µL of methyl thiazol tetrazolium (MTT, Sigma-Aldrich) (0.5 mg/mL) was dissolved in RPMI medium and added to each well. Cells were incubated at 37 °C for a minimum of 2 hours, supernatants were removed, and formazan crystals dissolved in 100 µL of 0.04 M HCl in isopropanol. Absorbance was measured at 570 nm in an ELISA plate reader. The percentage of cell viability was calculated as follows: **%Viability= [(Abs sample) x 100%]/ (Abs control).**

Unstimulated cells, cultured in the presence of 10% FBS and 0.5% DMSO, represented 100%.

Inhibition of COX-2 expression

Macrophage culture

The macrophages were obtained according to the procedure previously described. The cells were then seeded in RPMI culture medium supplemented with FBS (10%) at a concentration of $2×10^6$ cells / well in a 6-well plate and cultured for two hours at 37 °C in a CO_2 atmosphere (5%). The unbound cells were removed and the adhered ones were pre-treated for one hour with the tested compounds (concentration of 100 µM) and then stimulated with LPS (1 µg/mL) for three hours.

Extraction of RNA and real-time PCR

After three hours of LPS stimulation, the total RNA was extracted by lysing the cells with trizol (Life Technology Corporation: Invitrogen and Applied Biosystems). An amount of 2 µg of total RNA was taken and reverse transcription was performed using the reverse transcription kit (Life Technology Corporation: Invitrogen and Applied

Biosystems) to obtain the complementary DNA. Finally, the polymerase chain reaction was performed in real time using an ABI 7500 (Applied Biosystems) and using SYB Green (Applied Biosystems). The amplification conditions employed were as follows: 95 °C (10 min), 40 cycles of 95 °C (15 s) and 60 °C (60 s) and HPRT was used as the reference gene. Analysis of gene expression levels was performed by the 2-ΔΔCT method (Livak and Schmittgen, 2001). The primer sequence used for reverse transcription was as follows: for HPRT F: 5'-GCTGGTGAAAAGGACCTCT-3 'and R: 5'-CACAGGACTAGAACACCTGC-3'; COX-2 F: 5'-CGTGGTCACTTTACTACGAG-3 'and R: 5'-AGGTACATAGTAGTCCTGAGC-3'.

Statistical analysis

Results were analyzed by using a statistical software package (GraphPad Prism 6). Data are presented as mean ± S.D. Statistical analysis was performed by unpaired t test. A difference between groups was considered to be significant if $P < 0.05$ (*, $P < 0.05$; **, $P < 0.01$).

RESULTS AND DISCUSSION

Evaluation of the inhibitory activity on proinflammatory cytokines (TNF-α and IL-6) production for murine macrophages

Lipopolysaccharide is one of the major components of the outer surface of gram negative bacteria and one of the most studied Toll-like receptor ligands (Duthie *et al.*, 2011). It is a potent activator of cells from the immune system including monocytes, endothelial cells and macrophages. The latter have a fundamental role in the inflammatory response because, when activated, release a wide variety of factors, including nitric oxide, prostaglandins and proinflammatory cytokines (TNF-α, IL-1β, IL-6). In order to evaluate the anti-inflammatory activity of the flavonoid mixture, pure compounds **2** and **4** as well as the aglycon we analyzed the ability of these compounds to modulate the secretion of the proinflammatory cytokines TNF -α and IL-6 in LPS-activated macrophages.

For compound **2**, no significant differences were observed with respect to the control at doses of 50 and 200 µM, but only at a dose of 100 µM, and therefore it did not show a dose-dependent behavior (Fig. 2). In the cell viability assay (Fig. 3, no cytotoxicity was observed at this dose.

In the IL-6 release inhibition assay (Fig. 4), compound **2** did not inhibit the production of this mediator. Similar results were observed for compound **4**.

Studies carried out by Ribeiro *et al*. (2015) demonstrated that the anti-inflammatory activity of flavonoids is related to their chemical structural features, among which the presence of a planar ring system within the molecule; unsaturation in the C-ring at the C2-C3 positions; the number and position of the hydroxyl groups on rings A and B, particularly on C-5 and C-7 of ring A and C-3 'and C-4' on ring B. In addition, the absence of hydroxyl groups in the B ring eliminates anti-inflammatory activity. In view of this, flavonoids with C-3 'and C-4' hydroxyl groups (quercetin and luteolin) have a greater inhibitory effect on TNF-α release than those with a single OH group in the ring B (genistein) (Comalada *et al*., 2006). Taking into account this structure-activity relationship, both, compound **2** and compound **4** isolated from *Boldoa purpurascens* (Fig. 1) show the C2-C3 unsaturation as well as a C-4' hydroxyl group; it is expected that these compounds will have a moderate inhibitory activity on TNF-α release.

In relation to the aglycone, this partially inhibited IL-6 production at doses of 200 μM; however, the aglycon was toxic to cells by causing 40 -50% of death, so, this inhibitory effect might be due to cell death. In addition, it has been reported that in flavonoids the aglycone has a higher cytotoxicity than their glycosides, and in the case of methylenedioxyflavonols, cytotoxicity is higher when the compound has a double bond in the C2-C3 position and an OH group at the C-3' position (Orlikova *et al*., 2014).

A higher anti-inflammatory effect was observed for the flavonoid mixture, which significantly inhibited the production of TNF- α and IL-6. Inhibion of IL-6 occurred at a concentration of 200 μM, while TNF-α production was inhibited at all concentrations tested. This inhibitory effect was not due to cytotoxicity because at the concentrations assessed, the mixture did not interfere with cell viability. The blend contains nine different flavonoids from *Boldoa purpurascens*.

Inhibition of COX-2 expression

Fig. 5 shows the inhibition of COX-2 expression, expressed as a relative intensity with respect to the control. Both the aglycone and the flavonoid mixture produced a statistically significant decrease in COX-2 mRNA levels. According to the results obtained previously in the cellular viability assays, the aglycone was cytotoxic, so this COX-2 inhibition might be due to cell death. This experiment does not allow the quantification of cytotoxicity, since it is necessary to perform the lysis of the cells to obtain the total RNA. However, the above experiments have already shown the cytotoxic effect of the aglycone.

In relation to the mixture of flavonoids, an inhibitory effect of COX-2 was observed at the transcriptional level, but not its constituents **2** and **4**. This might be due to the

presence of other flavonoid-type compounds, which have not yet been isolated or characterized.

During inflammation, cells of the immune system can be activated by recognizing a pathogenic endotoxin, the lipopolysaccharide (LPS), by Toll-like receptor 4. This event causes a signaling pathway that culminate in the activation of NF-κB and the subsequent transcription of genes related to the inflammatory process, such as *iNOS* and *COX-2* (Heredia *et al*., 2016). There are three distinct isoforms of COX: COX-1 is present in all tissues and produces prostaglandins involved in physiological processes such as the protection of gastric epithelium, maintenance of renal flow and platelet aggregation through production of thromboxanes. COX-2, which is an inducible isoform, is released in response to inflammatory stimuli such as cytokines (TNF-α), interleukins, growth factors and other inflammatory mediators. There is also a third isoform (COX-3), and two partial isoforms, COX-1a and b detected in the cerebral cortex and in the human heart (Goetz *et al*., 2017). It is well established that flavonoids have a mechanism of action similar to NSAIDs and that they inhibit the activity or gene expression of COX (Ribeiro *et al*., 2015).

CONCLUSION

The results obtained for the mixture of flavonoids isolated from *Boldoa purpurascens* suggest that its anti-inflammatory effect involves the inhibition of COX-2 at the transcriptional level and the release of proinflammatory cytokine such as TNF-α and IL-6. This will allows to identify some of the flavonoids present in the mixture as candidates for the development of a finished dosage form which can be employed in the treatment of inflammatory processes once *in vivo* assays have been performed.

Conflict of Interest

There are no conflicts of interest.

Acknowledgements

The Special Fund for Research on the University of Antwerp is kindly acknowledged for providing a joint-PhD fellowship to Y. Hernández Ortega.

References

Aggarwal BB. 2000. Tumour necrosis factor receptor associated signaling molecules and their role in activation of apoptosis. JNK and NF-kB. *Ann. Rheum. Dis*. **59**: 6–16.

Calamia KT. 2003. Current and future use of anti-TNF agents in the treatment of autoimmune and inflammatory disorders. *Adv. Exp. Med. Biol.* **528**: 545–549.

Camuesco D, Comalada M, Rodríguez-Cabezas ME, Nieto A, Lorente MD, Concha A, Zarzuelo A, Galvez J. 2004. The intestinal anti-inflammatory effect of quercitrin is associated with an inhibition in iNOS expression. *Br.J. Pharmacol*. **143**: 908–918.

Comalada M, Camuesco D, Sierra S, Ballester I, Xaus J, Galvez J, Zarzuelo A. 2005. *In vivo* quercitrin antiinflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF-kappaB pathway. *Eur. J. Immunol.* **35**: 584–592.

Dempsey PW, Doyle SE, He JQ, Cheng G. 2003. The signaling adaptors and pathways activated by TNF superfamily. *Cytokine Growth Factor Rev*.**14**: 193–209.

Dong D, Yin L, Qi Y, Xu L, Peng J. 2015. Protective effect of the total saponins from *Rosa laevigata* Michx fruit against carbon tetrachloride-induced liver fibrosis in rats. *Nutrients*. **7**: 4829-4850.

Duthie M, Windish H, Fox C, Reed S. 2011. Use of defined TLR ligands as adjuvants within human vaccines. *Immunol. Rev*. **239**: 178-196.

Goetz M, Vargas PK, Lupepsa AC, Baller E, Nobre GC. 2017. Cyclooxygenase biology in renal function-literature review. *Revista Colombiana de Nefrología*. **4**: 1.

Heredia B, Leyva-López N, Gutiérrez-Grijalva E, Ambriz-Perez D. 2016. Flavonoids as Cytokine Modulators: A Possible Therapy for Inflammation-Related Diseases. *Int. J. Mol. Sci.* **17**: 921.

Hernandez Ortega Y, González Mosquera DM, Galapththi S, Tamargo Santos B, Sierra González G, Foubert K, Pieters L, Buc Calderon P, Apers S. 2016. Safety assessment of a traditionally used extract from leaves of *Boldoa purpurascens*. *J. Ethnopharmacol*. **192**: 302-308

Hernández Ortega Y, Foubert K, Vanden Berghe W, Chirumamilla CS, Pieters L, González Mosquare DM, Apers S. 2017. Flavonol glycosides from the leaves of *Boldoa purpurascens* and their anti-inflammatory properties. *Phytochemistry Letters*. **19**: 71-76.

Hsu H, Xiong J, Goeddel DV. 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-kB activation. *Cell.* **81**: 495–504.

Kim HP, Son KH, Chang HW, Kang SS. 2004. Antiinflammatory plant flavonoids and cellular action mechanisms. *J. Pharmacol. Sci*. **96**: 229–245.

Kumar RE, Schwartz SA. 2002. The flavonoid, quercetin, differentially regulates Th-1 (IFNgamma) and Th-2 (IL4) cytokine gene expression by normal peripheral blood mononuclear cells. *Biochim. Biophys. Acta*. **1593**: 29–36.

Livak KJ, Schmittgen TD. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $\mathbf{2^{-\Delta\Delta C_T}}$ Method. Methods. **25** (4): 402-408.

Nair HK, Rao KV, Aalinkeel R, Mahajan S, Chawda R, Schwartz SA. 2004. Inhibition of prostate cancer cell colony formation by the flavonoid quercetin correlates with modulation of specific regulatory genes. *Clin. Diagn. Lab. Immunol*.**11**: 63–69.

Nair MP, Kandaswami C, Mahajan S, Chadha KC, Chawda RH, Nair N, Orlikova B, Legrand N, Panning J, Dicato N, Diederich. 2014. Anti-inflammatory and anticancer drugs from nature. *Cancer. Treat. Res*. **159**: 123-143.

Ribeiro D, Freitas M, Tomé SM, Silva A, Laufer S, Lima J, Fernandes E. 2015. Flavonoids inhibit COX-1 and COX-2 enzymes and cytokine/chemokine production in human whole blood. *Inflammation*. **38**: 858-870.

Scalbert A, Johnson IT, Saltmarsh M. 2005. Polyphenols: anti-oxidants and beyond. *Am. J. Clin. Nutr*. **81**: 215S–217S.

Taylor PC, Williams RO, Feldmann M. 2004. Tumour necrosis factor alpha as a therapeutic target for immune-mediated inflammatory diseases. Curr. *Opin. Biotechnol*. **15**: 557–563.

Wajant H, Henkler F, Scheurich P. 2001. The TNF-receptor-associated factor family: scaffold molecules for cytokine receptors, kinases and their regulators. *Cell. Signal*. **13**: 389–400.

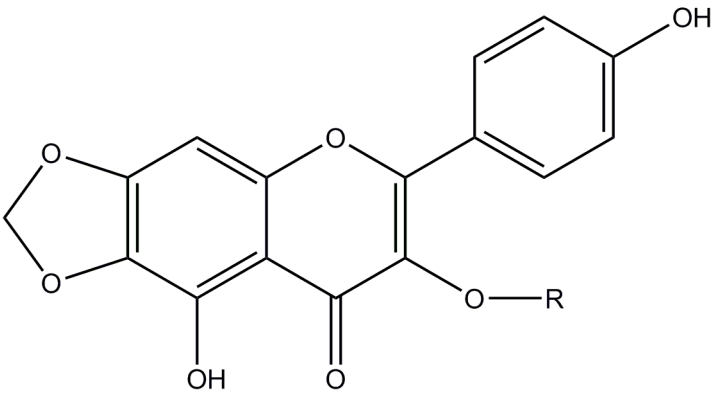

Figure 1:

2 R = α-L-rhamnopyranosyl-(1”’-2”)]-β-D-glucopyranoside

4 R = α-L-rhamnopyranosyl-(1”’-2”)-β-D-xylopyranoside

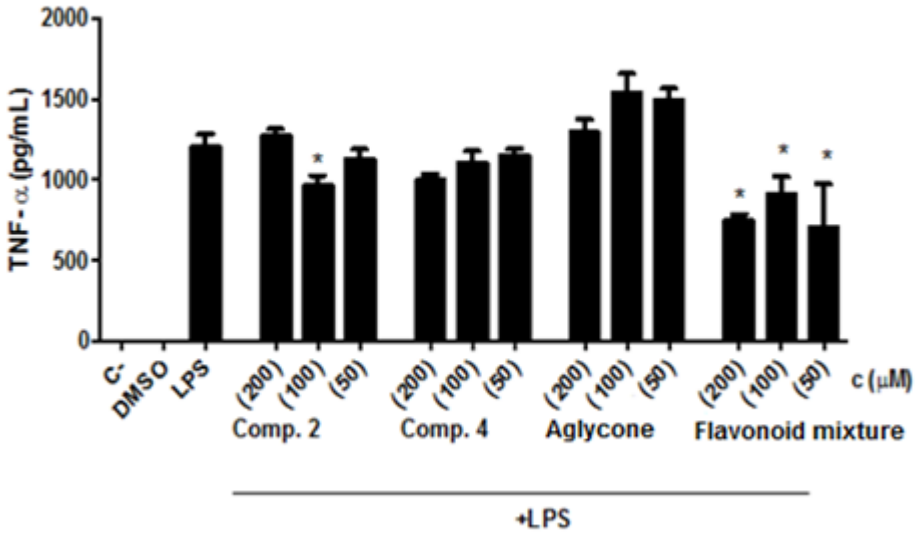

Figure 2: Inhibitory effect of flavonoids compounds on TNF-α release.

Peritoneal macrophages from C57Bl/6 mice were pre-treated with different concentrations of compounds and then stimulated with 10 ng/mL of LPS. Supernatants were collected after 6 hours of stimulus and levels of TNF were determined by the Elisa method. Results represent mean ± S.D. from stimuli performed in triplicates and are representative of two different experiments. *, $P < 0.05$ compared with LPS stimulus alone.

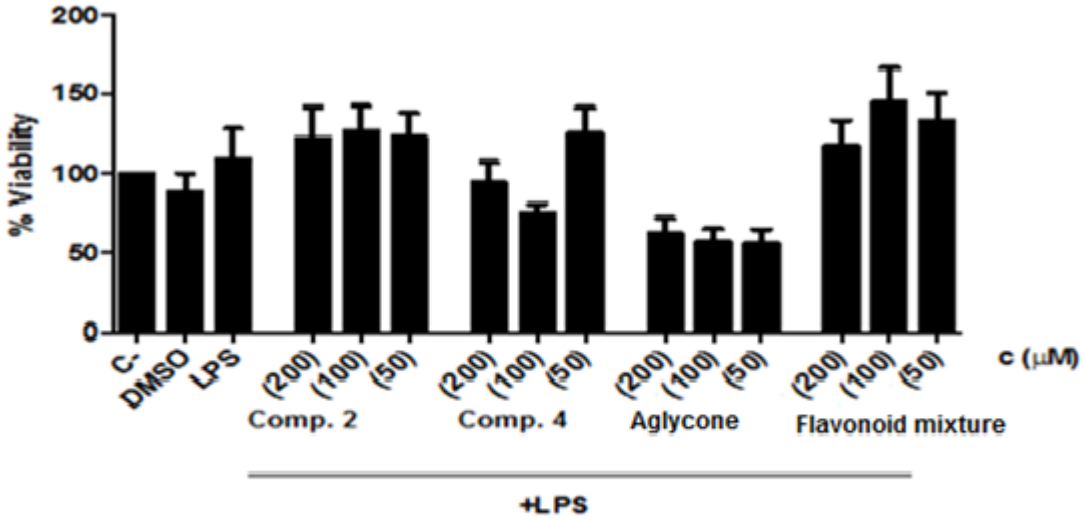

Figure 3: Cell viability in the MTT method

Peritoneal macrophages from C57Bl/6 mice were pre-treated with different concentrations of compound**s**. After 6h, supernatants were collected and cell viabilities were assessed by a MTT assay. Results represent means ± S.D.. from stimuli performed in triplicate.

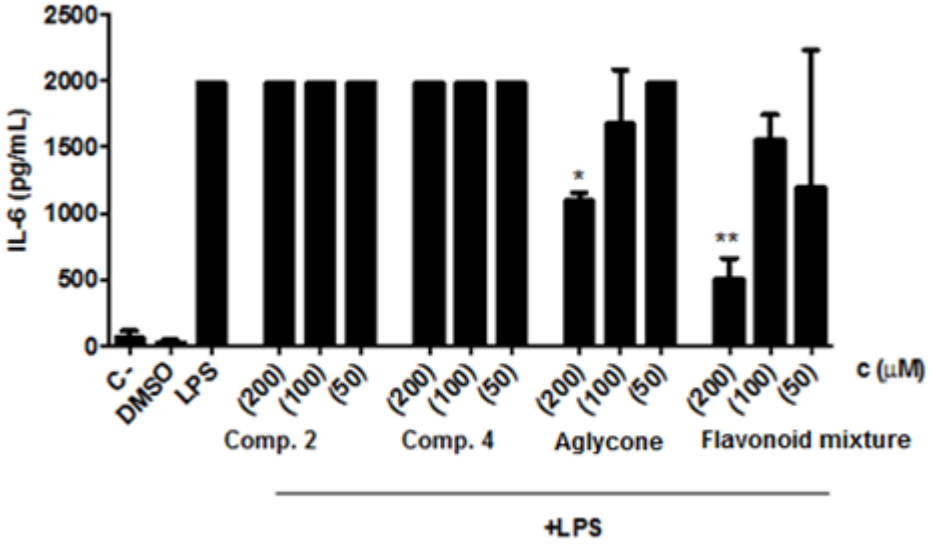

Figure 4: Inhibitory effect of flavonoids compounds on IL-6 release

Peritoneal macrophages from C57Bl/6 mice were pre-treated with different concentrations of compounds and then stimulated with 10 ng/mL of LPS. Supernatants were collected after 6 hours of stimulus and levels of IL-6 were determined by the Elisa method. Results represent mean ± S.D. from stimuli performed in triplicates and are representative of two different experiments. *, $P < 0.05$; **, $P < 0.01$ compared with LPS stimulus alone.

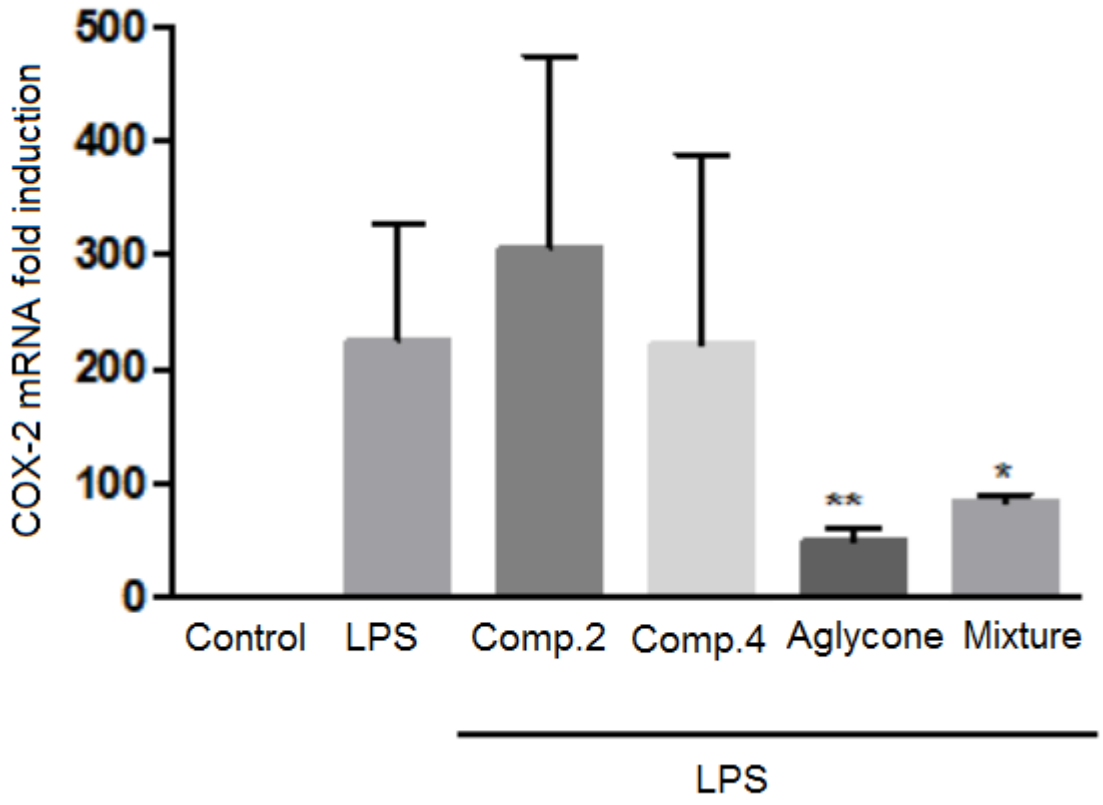

Figure 5: Expression of COX-2 induced by LPS

Peritoneal macrophages were treated for 3 hours with compounds (100 µM) and stimulated with LPS (1 µg/mL). The amount of mRNA for COX-2 was determined by quantitative PCR. Results were normalized to HPRT expression and are presented as fold induction of mRNA expression relative to control samples. Results represent means ± S.D. from stimuli performed in duplicates and are representative of two different experiments. *, $P < 0.05$; **, $P < 0.01$, compared with LPS stimulus alone.