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# Estimation of the Anti-inflammatory Effect of Coumarin Derivatives

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## Abstract

Background: Inflammation is the body's natural defense mechanism for removing noxious stimuli. According to the benzopyran substitutions, Coumarins are extensively dispersed in nature and have varied biological effects. Purpose: The purpose of this study is to estimate the anti-inflammatory effect of some Coumarin derivatives using RAW 264.7 macrophage cell lines including the inhibitory effect of these derivatives on cyclooxygenase-2 enzyme, interleukin-1β, and interleukin-6. Methods: In the present investigation, RAW 264.7 cells were treated with varying concentrations (0.15-100μM) of three Coumarin derivatives. After 72 hours, the half-maximal inhibitory concentration (IC50) was determined using the MTT assay to assess the cytotoxicity of these derivatives. The levels of cyclooxygenase-2, interleukin-1β, and interleukin-6 were also evaluated using the ELISA technique. Results: The half–maximal inhibitory concentrations for C1, C2, C3, and Diclofenac were 32 μM, 25.09 μM, 12.2 μM, and 30.42 μM, respectively. At concentrations 2.5, 5, 7.5, and 10 mg/ml of these derivatives, cyclooxygenase-2 activity was decreased significantly by C2 and C3 more than the Diclofenac (Reference compound) ($p<0.05$). C2 and C3 significantly reduced the level of interleukin-1β induced by lipopolysaccharide compared with the lipopolysaccharide alone ($p<0.05$). The inhibitory effects of C1, C2 and C3 were significantly more potent on interleukin-6 level induced by lipopolysaccharide compared with lipopolysaccharide alone ($p<0.05$).Conclusions: C2 and C3 had better anti-inflammatory effects than Diclofenac as they had a significantly higher inhibitory effect on the cyclooxygenase-2 activity than the inhibitory activity of Diclofenac and significantly inhibited interleukin-1β and interleukin-6 induced by lipopolysaccharide compared with the lipopolysaccharide (LPS) alone.

**Keywords:**

RAW264.7, Coumarin, Anti-inflammatory, Cyclooxygenase-2, Interleukins.

## Introduction

Inflammation is the body's′ natural response against noxious stimuli [1], it is an integrated and complicated reaction to irritants, infections, immunologically-mediated stimuli, toxins, or other assaults [2].

Small secreted proteins, cytokines, are produced by almost all cells to control and impact immune response [3, 4]. According to their function, cytokines can be organized into four groups: Cytokines that increased inflammation and facilitate natural immunity, such as interleukin (IL)-1, IL-6, IL-8, interferon-α, and tumour necrosis factor, cytokines that associated with allergy, such as IL-4, IL-5 and IL-13 [5], cytokines with immunoregulatory activity, such as IL-10, IL-12, transforming growth factor-β, and interferon-γ [6], and cytokines that acts as growth factors for blood cells (hematopoietic growth factors), such as IL-3, IL-7, and Granulocyte-macrophage colony-stimulating factor [7]. Under normal circumstances, cyclooxygenase (COX)-2 expression is low but increased in sites of inflammation [8].

Several cell types, including fibroblasts, endothelial cells, mononuclear macrophages, and vascular smooth muscle cells, were able to secrete COX-2 up to about 8–10 times the usual amount when triggered by proinflammatory cytokines [9]. Long-term use of steroidal and non-steroidal anti-inflammatory drugs is associated with adverse reactions.

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Therefore, it is crucial to develop natural plant chemicals to substitute conventional anti-inflammatory medications in the therapeutic management of inflammation-related disorders that have favorable curative benefits with few harmful side effects [10].

Oxazole is an important heterocyclic compound [11]. Through diverse non-covalent bonds, Oxazole rings, with one nitrogen atom and one oxygen atom, Oxazoles quickly engage physiologically with many enzymes and receptors, resulting in a different biological properties [12, 13]. Oxazoles are often found in both natural and manufactured bio- and photoactive chemicals [14]. Coumarins are a large family of benzopyrones (1, 2-benzopyrones or 2H-1-benzopyran-2-ones). The basic structure of Coumarin consists of oxygen-containing heterocycles with a typical benzopyrone framework [15]. Coumarins are widely distributed in nature with different biological activities according to the benzopyran substitutions [16, 17].

Lipoxygenase, cyclooxygenase enzymes, and prostaglandin inhibition as well as neutrophil-dependent superoxide anion formation are thought to be underlie Coumarin's anti-inflammatory properties. The current study aimed to evaluate the anti-inflammatory effect of some Coumarin derivatives using RAW 264.7 macrophage cell lines including the inhibitory effect of these derivatives on cyclooxygenase-2 enzyme,( IL)-1β, and IL-6[18]. In Conclude, two Coumarin derivatives had better anti-inflammatory effects than Diclofenac as they had a significantly higher inhibitory effect on the cyclooxygenase-2 activity than the inhibitory activity of Diclofenac and significantly inhibited( IL)-1β and IL-6 induced by lipopolysaccharide (LPS) compared with the( LPS) alone[19, 20].

## Materials & Methods:

The RAW264.7 macrophage cell line was purchased from American Type Culture Collection (ATCC), USA. They are raised from the Abelson leukaemia virus-transformed cell line of BALB/c mice.

The freezing media was purchased from Elabscience, USA. MTT dye (3-(4, 5-dimethylthiazole-2-yl)-2,5diphenyltetrazolium bromide) was purchased from Invitrogen, Germany. Tryban blue stain was purchased from Thermo Scientific, USA. Dulbecco's PBS(1x) Phosphate buffer saline and Fetal bovine serum were purchased from Capricorn, GmbH.

Penicillin /Streptomycin solution (100x), RPMI 1640 medium, and Trypsin –EDTA (0.05%) in DPBS(1x) were purchased from Capricorn, GmbH. Mouse IL-1β, Mouse IL-6, and Mouse PTGS2/ cyclooxygenase -2 (prostaglandin) ELISA kits were purchased from Elabscience, USA. The preparation of Coumarin derivatives was done at the Department of Pharmaceutical Chemistry, College of Pharmacy, Mustansiriyah University.

### -Preparation of derivatives and cell line culture:

Firstly, a 10 mM concentration of Coumarin derivatives was prepared in 1ml Dimethyl Sulfoxide (DMSO), then filtered with a 0.22µm Millipore filter to remove any particles. These derivatives included:

C1: (E)-3-(2-(2-(1-pyridin-2-yl) ethylidene) hydrazineyl) oxazole-4-yl)-2H-chromen-2-one.

C2: (E)-3-(2-(2-(1-pyridin-4-yl) ethylidene) hydrazineyl) oxazole-4-yl)-2H-chromen-2-one.

C3: (E)-3-(2-(2-(1-(6-bromopyridin-3-yl) ethylidene) hydrazineyl) oxazole-4-yl)-2H-chromen-2-one.

All derivatives (C1, C2, and C3) had been drawn by using ChemDraw (v.19.1) and transformed by the Swiss ADME to SMILE. The permeability through GIT and blood-brain barrier of the small molecule have been measured using BOILED-EGG [21].The RAW264.7 cells were generally cultured in RPMI1460 media,10%fetal bovine serum (FBS),and 1%pencillin /streptomycin at 37 ºC and 5% $CO_2$ using 75 $cm^3$ cell culture flasks.

### -Treatment of RAW264.7 cell by Coumarin derivatives

The RAW264.7 cells were treated with different concentrations from (0.15-100µM) of the three Coumarin derivatives.

### -Determination of half–maximal inhibitory concentration ($IC_{50}$) Value

The $IC_{50}$ is the minimum concentration of the extract that decreases the viability of the cells that were incubated for 72 hours by 50% [22]. The MTT assay was utilized to estimate the effects of Coumarin derivatives (C1, C2, and C3) on RAW264.7 cell viability. The $IC_{50}$ test was done after 72 hours to determine the toxicity of these derivatives on RAW 264.7 cells.

### Evaluation of the inhibitory effect of Coumarin derivatives on COX-2 enzyme in RAW 264.7 cells:

Using a Mouse PTGS2/COX-2 (Prostaglandin Endoperoxide synthase 2) ELISA Kit according to the manufacturer's instructions, the ability of the three Coumarin-synthetic derivatives to inhibit the conversion of arachidonic acid to Prostaglandin H2 was evaluated. The reaction was initiated by adding 100µL of working solution for Biotinylated Detection Ab to each well of the plate, which was then incubated at 37 °C for 90 minutes. The plate was then covered and incubated at 37°C for one hour. Each well received 350µ L of wash buffer and was immersed for one minute[23].

Then, the solution was dried with absorbent paper, and the rinsing procedure was repeated three times. In the subsequent phase, 100 µL of HRP Conjugate working solution was added to each well. The plate was covered and incubated for 30 minutes at 37°C before being washed five times. Then, 90µ L of the Substrate Reagent

was added to each well, and the plate was covered and incubated at 37°C for approximately 15 minutes. Before measuring optical density, preheat the Microplate Reader for about 15 minutes. After the addition of 50µ L of stop solution, the optical density of each well was immediately measured at 450 nm using a microplate reader [24].

### Estimation of (IL)-1β and IL-6 in the macrophage cell line RAW 264.7:

The levels of pro-inflammatory markers (IL-1β and IL-6)were measured to determine whether Coumarin derivatives could inhibit pro-inflammation in RAW 264.7 cells. Using ELISA kits, secreted (IL)-1β and IL-6 were measured in cell culture supernatants following centrifugation at 5000 g for 3 minutes at 4°C to remove insoluble material. Briefly, the cells were incubated with 100µ L of derivatives at the above-mentioned concentration for 90 minutes at room temperature while being shaken in a 96-well plate. Then, the solution was rinsed four times with 1X wash solution, and any remaining wash buffer was removed by blotting with clean paper towels after the final wash.

Then, 100µ L of 1X Biotinylated IL-1β detection antibody for the (IL)-1β experiment and IL-6 detection antibody for the IL-6 experiment were added to each well and incubated for one hour at room temperature with stirring.

The solution was then disposed, and the washing process was repeated. Each well was incubated for 45 minutes at room temperature with 100µ L of 1X HRP-conjugate working solution, after which the solution was discarded and the wash was repeated. After adding 90µL of Substrate Reagent, the plate was covered and incubated at 37°C for approximately 15 minutes. After preheating the Microplate Reader for approximately 15 minutes, measure the optical density. Finally, the reaction was terminated by adding 50µ L of the stop solution to each well, and the absorbance was promptly measured at 450 nm.

All statistical analysis of derivatives was done in Prism (version 8.1). Nonlinear curve fitting (Prism Pad 8.1) was used to calculate the $IC_{50}$. One-way ANOVA with Tukey (prism pad 8. 1) was used to compare results from several groups within the same plate for COX-2 activity, (IL)-1β, and IL-6. Significant results were defined as having a probability level of p 0.05.

## Results:

Figures 1, 2, 3, and 4 demonstrated the $IC_{50}$ for C1, C2, C3, and Diclofenac respectively in RAW 264.7 cell line, the $IC_{50}$ of C1, C2, C3, and Diclofenac were 32 µM, 25.09 µM, 12.2 µM, and 30.42 µM, respectively.

These concentrations have been used for all subsequent experiments.

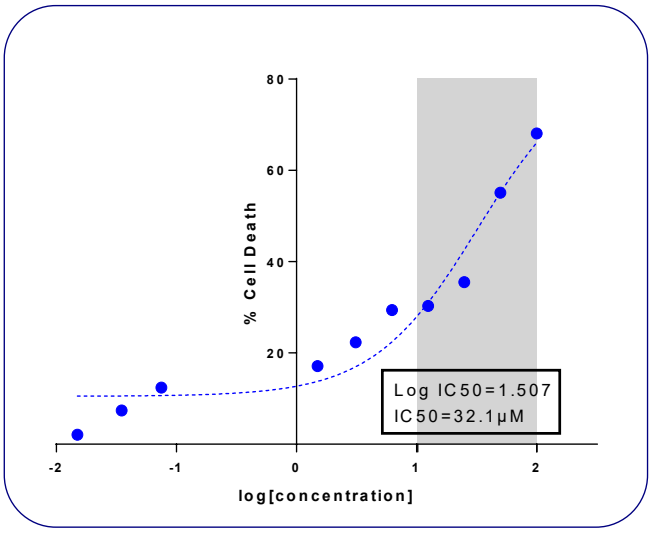


**Figure 1:** Dose-response curves of $IC_{50}$ for C1

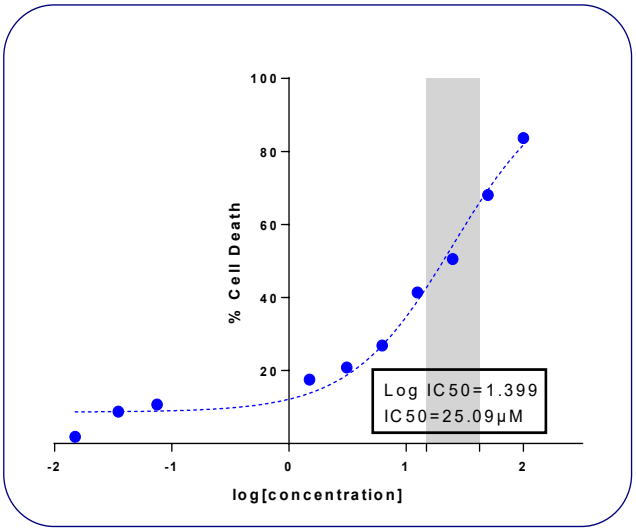


**Figure 2:** Dose-response curves of $IC_{50}$ for C2

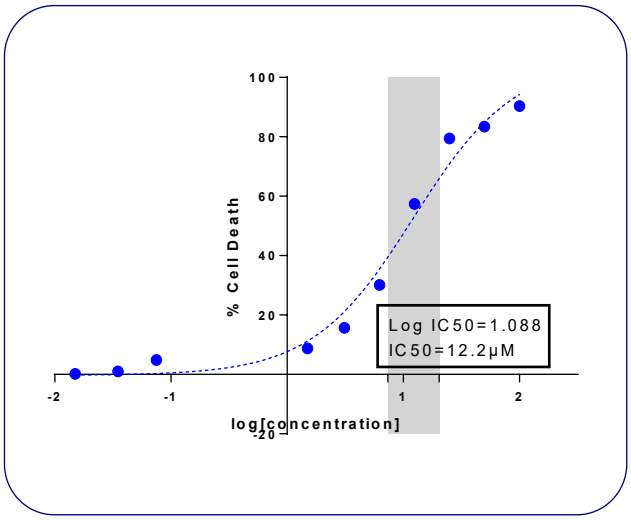


**Figure 3:** Dose-response curves of $IC_{50}$ for C3

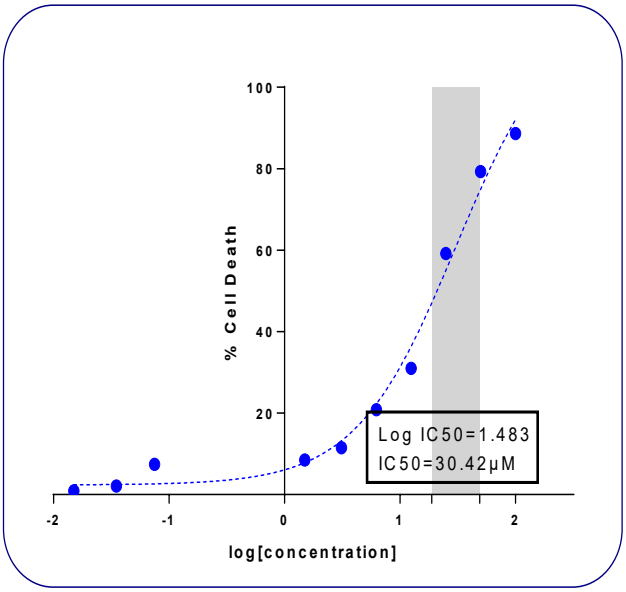


**Figure 4:** Dose-response curves of $IC_{50}$ for Diclofenac

Figure 5 show the estimate of coumarin derivatives' COX-2 enzyme-inhibiting potency in RAW 264.7 cells. Diclofenac (a COX-2 inhibitor) was used as a positive control, and COX-2 activity was considerably reduced by C2 and C3 at dosages of 2.5, 5, 7.5, and 10 mg/ml (P 0.005, 0.005, 0.01 and 0.05 respectively).

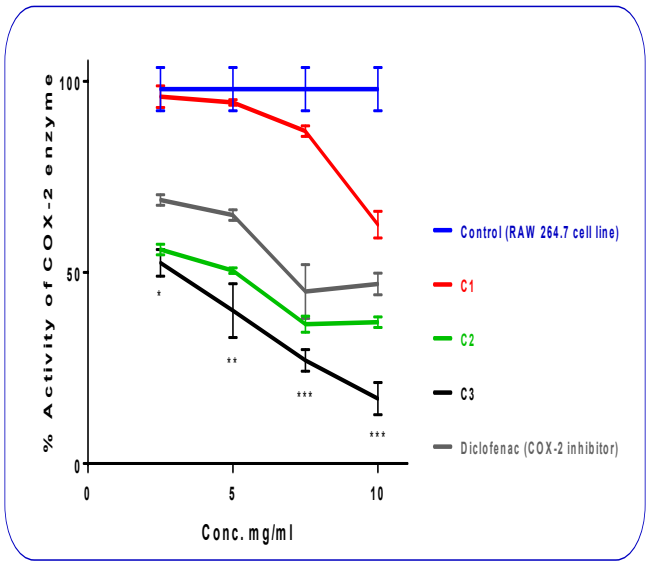


**Figure 5:** Measuring the inhibitory effect of C1, C2, and C3 derivatives on COX-2 activity with a Cyclooxygenase activity assay kit. Using Prism Pad 8.1 software, the results depict the mean percentage of activity standard deviation from three independent investigations. ***P< 0.005, **p< 0.01, *P< 0.05.

According to( IL)-1β in RAW 264.7 macrophages cell line, RAW 264.7 cells were incubated for 24 hours with or without LPS (100 ng/mL) in the presence of various concentrations of C1, C2, and C3 and 30µM Diclofenac. As shown in Figure 6, C2 and C3 significantly reduced the level of( IL)-1β induced by LPS compared to LPS alone (P 0.005).

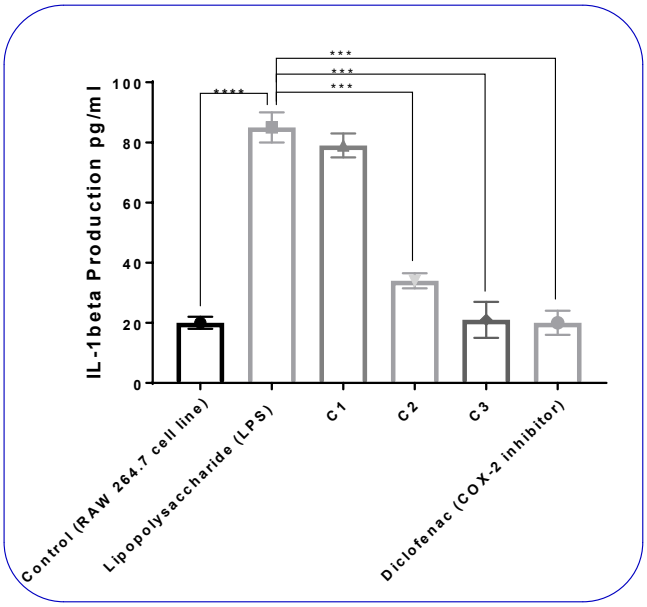


**Figure 6:(** IL)-1β secretion in RAW 264.7 cells stimulated with LPS. Three replicates' values are expressed as mean standard deviation. In comparison to control cells. (One-way ANOVA followed by Scheffe's multiple range tests utilising Prisim pad 8.1). ***p 0.005, in comparison to the LPS-only cohort. Scheffe's test indicates that means with distinct letters are significantly different (p 0.05).

While, the inhibitory effects of derivatives C1, C2 and C3 were significantly more potent on IL-6 mean level induced by LPS compared with LPS alone (P <0.05, <0.005, <0.005, respectively) as shown in figure 7.

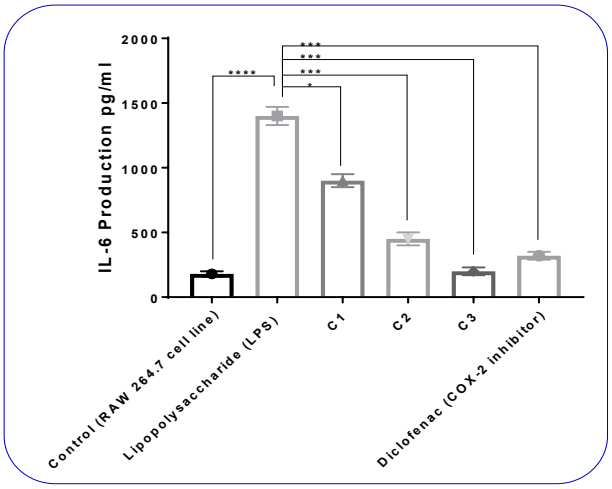


**Figure 7:** IL-6 secretion in RAW 264.7 cells stimulated with LPS. Three replicates' values are expressed as mean standard deviation. In comparison to control cells. (One-way ANOVA followed by Scheffe's multiple range tests utilising Prisim pad 8.1). *p 0.05 and ***p 0.005 were observed in comparison to the LPS-alone group. Scheffe's test indicates that means with distinct letters are significantly different (p 0.05).

## Discussion:

Inflammation is an essential response to any harmful stimuli [25]. This study was one among others that tried to explore the anti-inflammatory effects of synthetic Coumarin derivatives *in vitro* cell culture [26]. The $IC_{50}$ of C1, C2, C3, and Diclofenac in RAW 264.7 Cell Line were

32 μM, 25.09 μM, 12.2 μM, and 30.42 μM, respectively. In comparison, Daina *et al* [21]. revealed that compared to the reference Celecoxib, two new Coumarin derivatives including thiazoline and thiazolidinone moieties had $IC_{50}$ values ranging from 0.31 to 0.78 μM [27]. In another study, Murine macrophage RAW 264.7 cells were stimulated with LPS and treated with glabralactone (Coumarin derivatives) with a concentration of 0–20μM for 30 min, the $IC_{50}$ value was 11.6 μM [28]. Four novel Coumarin derivatives were tested for anti-inflammatory effects on LPS and oxalate crystal-induced *in vitro* models. The best $IC_{50}$ of these derivatives was 8.5 μM [29]. The current study revealed that at the concentrations of 2.5, 5, 7.5, and 10 mg/ml, C2 and C3 had a significantly higher inhibitory effect on the COX-2 activity than the inhibitory activity of Diclofenac [30]. This agreed with another study that was done by Daina *et al* [21]. as two Coumarin derivatives including thiazoline and thiazolidinone moieties were created and tested for their anti-inflammatory effects *in vitro* had a strong affinity toward the COX-2 isoenzyme [23]. In another study that was done by Vidhya Thomas *et al* [31], derivatives of 4-hydroxy- 3-(2- (2-[2- [(substituted phenyl)methylidene]hydrazin-1- yl]-1,3- thiazol-5- yl)-1-phenylethyl)-2H-chromen- 2-one (4a-j) had an inhibitory effects on the COX-2 with significant anti-inflammatory activity [32]. In contrast, Dunya AL-Duhaidahawi *et al* [33]. revealed that the *in vitro* investigation of the anti-inflammatory effect of 4-hydroxy Coumarin derivatives did not show a significant association between the Coumarin derivatives and the inhibition of COX-2. C2 and C3 significantly reduced the mean level of( IL)-1β induced by LPS compared with the LPS alone [24]. This was consistent with another that was done by Jin-Kyu Kang *et al.* [34] who looked at how 6-methyl coumarin affected the levels and synthesis of (IL)-1β in LPS-stimulated RAW264. 7 cell. 6-methyl Coumarin cells showed a concentration-dependent reduction in IL-1 expression; at 500 μM, the highest treatment concentration,( IL)-1β expression was reduced by 80.6%, respectively [35]. The same results were obtained in another study that was done by Kumar *et al*[11], four novel methyl Coumarin derivatives were synthesized and tested in vitro, one out of these four derivatives caused inhibition of( IL)-1β expressions [29]. The inhibitory effects of C1, C2 and C3 were significantly more potent on IL-6 mean level induced by LPS compared with LPS alone group. This agreed with the results of another study that was done by Jin-Kyu Kang *et al.*[34] who stimulated macrophages with LPS to conduct an *in vitro* experiment. In 6-methyl Coumarin cells, IL-6 expression levels considerably dropped in a concentration-dependent manner; the highest treatment concentration, 500μM, significantly reduced IL-6 expression levels by 73.1% [34]. The same results were obtained in other studies that were done by Chaoyu Mu *et al.* [36] and included a series of novel 7-substituted Coumarin derivatives [36], and Liu *et al.* [37] and included a series of new phenyl-pyrazoline-Coumarin derivatives [37].

## Conclusion

Preparations and synthesis of the proposed investigated derivatives were successfully accomplished. Two of the analysed derivatives (C2 and C3) inhibited COX-2 activity substantially more effectively than Diclofenac (a COX-2 inhibitor serving as a positive control). C2 and C3 inhibit( IL)-1β induced by LPS significantly more than LPS alone. All of the analysed derivatives had substantially more potent inhibitory effects on the LPS-induced mean level of IL-6 than LPS alone. The results of the present investigation suggest that C2 and C3 may serve as valuable starting points for the development of anti-inflammatory agents derived from synthetic derivatives.

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