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Research Article

The effect of 1.3 bis(p-Hydroxyphenyl)urea compound on IL-6, IL-1β, TNF-α and COX-2 protein expression on λ-Carrageenan-induced rats

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Abstract

Objectives: This study aims to determine the protein expression of IL-6, IL-1β, TNF-α, and COX-2 using an immunohistochemistry method based on the anti-inflammatory activity of intraplantar carrageenan solution in male rat paws.

Materials and methods: Animals were provided with 1.3 bis(p-Hydroxyphenyl)urea at 12.5, 25, 50, 100, and 200 mg/kg BW. This was administered approximately one hour before the 1% carrageenan induction. As a control measure, diclofenac sodium and CMC sodium 0.5% was similarly administered. After 360 mins, all of the rats were sacrificed, and the inflammatory tissue in the rat's paw was acquired. The immunohistochemistry preparations were carried out to observe the expression of IL-6, IL-1β, TNF-α, and COX-2.

Results: The result showed a decrease in the expression of IL-6, IL-1β, TNF-α, and COX-2, all groupsgiven 1.3 bis(p-Hydroxyphenyl)urea and diclofenac sodium compared to the control group CMC sodium 0.5% CMC sodium 0.5%. From the results of the mean difference test, it can be seen that the IL-1β expression atdoses of 50, 100, and 200 mg/Kg BW was not significantly different from that of diclofenac sodium ($p > 0.05$). Meanwhile, the expression of COX-2, TNF-α, and IL-6 at doses of 100 and 200 mg/Kg BW was not significantly different from that of diclofenac sodium ($p > 0.05$).

Conclusion: The 1.3 bis(p-Hydroxyphenyl)urea has anti-inflammatory activity by decreasing the percentage of IL-6, IL-1β, TNF-α, and COX-2 protein expression in rat's paw inflammation tissue.

Keywords

anti-inflammatory, IL-6, IL-1β, TNF-α, Cox-2, 1.3 bis(p-Hydroxyphenyl)urea

PENSOFT

Introduction

Inflammation is the immune system's response to pathogens, damaged cells, toxic compounds, or irradiation. By producing cytokines and mediators of inflammation, the inflammatory process serves as the primary defense mechanism for health (Medzhitov 2010; Chen et al. 2018). Following infection, cells, particularly macrophages, dendritic cells, and mast cells, secrete various cytokines, chemokines, lipid mediators, and bioactive amines (Lawrence 2010). The inflammatory response in the body is characterized by the presence of numerous mediators, including pro-inflammatory cytokines such as Interleukin-1 (IL-1), Tumor Necrosis Factor (TNF), interferon (INF)-c, Interleukin-6 (IL-6), Interleukin-12 (IL-12), and Interleukin-1 (IL-1), in addition to Nitric Oxidase and COX-2 (Setia and Tjitaresmi 2014). Almost all inflammatory processes result in the activation and infiltration of tissue macrophages and blood monocytes. This activation results in numerous changes in cells, including the production of TNF, IL-1, and IL-6, which are pro-inflammatory cytokines with various effects on the host. These include the induction of fever, an acute phase response in the liver characterized by leukocytosis and the production of acute-phase proteins such as C-Reactive Protein (CRP), and the differentiation or activation of T cells, B cells, and macrophages (Ishartadiati 2009; Warsinah 2017).

1.3 bis(*p*-Hydroxyphenyl)urea is one of the modified *p*-aminophenol compounds that have analgesic and anti-inflammatory activity (Purnomo 2016; Waruwu et al. 2021). A previous study demonstrated that the compound 1.3 bis(*p*-Hydroxyphenyl)urea inhibited inflammation in carrageenan-induced rat paws while having fewer toxic side effects (Waruwu et al. 2022). Based on the inhibition of the cyclooxygenase (COX-2) enzyme, this compound may have analgesic and anti-inflammatory properties (Purnomo 2016). In comparison, the in silico test for COX-1 and TNF-α demonstrated that 1.3 bis(*p*-Hydroxyphenyl) urea had a greater affinity for COX-1 (1CQE) and TNF-α (2AZ5) than the control compounds dexamethasone and diclofenac. This compound may be developed into an anti-inflammatory agent (Harahap et al. 2021). This study aims to determine the expression of COX-2, TNF- α, IL-1, and IL-6 using an immunohistochemical method based on the anti-inflammatory activity of intraplantar carrageenan solution in male rat paws.

Materials and methods

The performance of this study was conducted based on the guidelines and approval of the Ministry of Health and the Ethics Committee. In addition, all operations were approved by the Animal Research Ethics Committees (AREC) of the University of Sumatera Utara's Faculty of Mathematics and Natural Science, Biological Department, with the approval number 0423/KEPH-FMIPA/2021, 14 July 2021. The animals were provided with 1.3 bis(*p*-Hydroxyphenyl)urea compound at 12.5, 25, 50, 100, and 200 mg/Kg BW. This was administered at approximately one hour before the 1% carrageenan induction, which was injected through the intraplantar route into the right hind leg of the rat at 0.1 ml. As a control measure, diclofenac sodium and CMC sodium 0.5% was similarly administered. After 360 mins, all rats were sacrificed, and the inflammatory tissue in the rat's paw was acquired. The principle of euthanasia in test animals before being sacrificed was for them to be anaesthetized first. Animals were handled with care without causing any fear, then animals were sacrificed with one of the techniques in a separate place from other animals, and no living animals were kept in the vicinity. Euthanasia was carried out by competent personnel, and was accompanied by a confirmation process to confirm death (BPOM RI 2014).

Reagents and chemical

λ-Carrageenan 1% (Sigma Aldrich), Formaldehid 10% (Smart Lab), Na2HPO4 (Smart Lab), CMC sodium 0.5% (Sodium-Carboxy Methyl Cellulose), diclofenac sodium (Novell), Hematoxylin and Eosin, IL-6 Antibody (Abclonal), IL-1β Antibody (Abclonal), TNF-α Antibody (Abclonal), and COX-2 Antibody (Abclonal), Triton (E. Merck), Aquades (Smart Lab), and Ethanol (Smart Lab).

Analysis of IL-6, IL-1β, TNF-α, and COX-2 expression procedures by immunohistochemistry

The immunohistochemistry preparations were carried out to observe the expression of IL-6, IL-1β, TNF-α, and COX-2. Immunohistochemical staining using peroxidase blocking with 0.3% H_2O_2 followed by washing with PBS 3 times. The surgically removed tissue was fixed in a 10% formalin buffer solution. Dehydration is done gradually. First, dehydration is carried out in 50%, 70%, 80%, 95%, and 100% alcohol solutions with the same length of time for each alcohol content of 90 minutes 2 times. Clearing, the tissue is put into a solution containing xylol for 90 minutes. Infiltration was carried out by inserting the tissue into a 90-minute paraffin solution and carried out 2 times. Infiltration was carried out in an oven at 60 °C. Embedding, tissue, and paraffin solution were put into a paraffin block mold and left for ± 3 hours or until the paraffin solidified. Sectioning, the formed paraffin blocks were cut with a rotary microtome. The tissue was cut with a thickness of 3–4 m and placed on a glass poly-L-lysine object, then left in an incubator overnight at 40 °C. Deparaffinization, sequentially put into a solution of xylol, 100% alcohol, 95% alcohol, 80% alcohol, 70% alcohol, 50% alcohol, for 90 minutes each 2 times. Peroxidase blocking with 0.3% H_2O_2 in methanol for 20 minutes. The preparations were washed with 10% Phosphate Buffer Saline (PBS) for 3× 5 minutes. Then non-specific blocking was performed with 10% normal serum for 30 minutes followed by incubation with primary antibodies at 4 °C for 18–22 hours.

The preparations were washed with 10% Phosphate Buffer Saline (PBS) 3 times for 5 minutes, dropped with secondary antibody (universal antibody) for 30 minutes. Then the preparations were washed with 10% Phosphate Buffer Saline (PBS) 3 times for 5 minutes, dropped with Chromogen 3,3-diaminobenzidine for 5–10 seconds, washed with distilled water, counterstained with Hematoxylin Mayer for 5–10 seconds followed by washing with running tap water for 10–15 minutes. Dehydration was carried out by putting in 80% alcohol, 95% alcohol, and xylol 2 times. Mounting using the E. Z mount (Lab Vision, Cat#MS-1378-PO). Increased expression of COX-2, TNF-, IL-1β, and IL-6 which is characterized by dark brown, medium brown, and purplish light brown, while those that do not express it are purple. These were calculated and analyzed using a light microscope with 400 times magnification. In addition, each preparation was observed in 6 view fields, with the average being calculated (Kim et al. 2016; Sugihartini et al. 2017; Permata and Ahmad 2019).

Immunohystochemical procedures

Statistical analysis

The results were presented as means ± SD. The statistical analysis was carried out by using SPSS edition 22.

Results and discussion

Immunohistochemistry was performed to reveal the total expression of IL-6, IL-1β, TNF-α, and COX- 2 on the connective tissue area of the wound edge using a light microscope 400 times. The expression of IL-6, IL-1β, TNF-α, and COX-2 was indicated by brown granules in the plasma and nuclear membrane. Each IHC preparation was observed in 6 fields of view, and the average was calculated and then analyzed by Oneway Anova. The results observed a decrease in the amount of expression of

COX- 2, TNF-α, IL-1β, and IL-6 in all groups given the 1.3 bis(p-Hydroxyphenyl)urea and diclofenac sodium compared to the control group CMC sodium 0.5%. The graph of the average amount of expression of COX-2, TNF-α, IL-1β, and IL-6 in mouse paws is given in Fig. 1.

After analyzing the average number of IL-6, IL-1β, TNF-α, and COX-2 proteins expression using the Oneway Anova method, it showed differences in the amount of COX-2, TNF-α, IL-1β, and IL-6 that were found. A significant difference between the test group and the negative control group was found. From the Tukey HSD mean difference test results, it can be seen that the IL-1β expression at doses of 50, 100, and 200 mg/Kg BW was not significantly different from that of diclofenac sodium ($p > 0.05$). Meanwhile, the expression of COX-2, TNF-α, and IL-6 at doses of 100 and 200 mg/Kg BW was not significantly different from that of diclofenac sodium ($p > 0.05$).

COX-2

The results showed a decrease in COX-2 expression in all groups given the 1.3 bis(p-Hydroxyphenyl)urea and sodium diclofenac compared to the control group CMC sodium 0.5%. The least COX-2 expression occurred in the diclofenac sodium group and the 1.3 bis(p-Hydroxyphenyl)urea at doses of 100 and 200 mg/Kg BW. This indicates that the 1.3 bis(p-Hydroxyphenyl)urea can inhibit inflammation by reducing the amount of COX-2 expression. Cyclooxygenase-2 (COX-2) is an enzyme whose presence is influenced by tissue stimulation. These stimuli can be cytokines, bacterial lipopolysaccharides, inflammation, or other pathological conditions (Gilroy et al. 1999). NSAIDs work primarily by blocking the COX pathway. In this pathway, most NSAIDS work reversibly by preventing the arachidonic acid from meeting with the active site of the COX enzyme so that prostaglandin biosynthesis reduces inflammation (Flood et al. 2015). The microscopic picture of COX-2 expression can be seen in Fig. 2. In injured tissue, a wound healing process be-
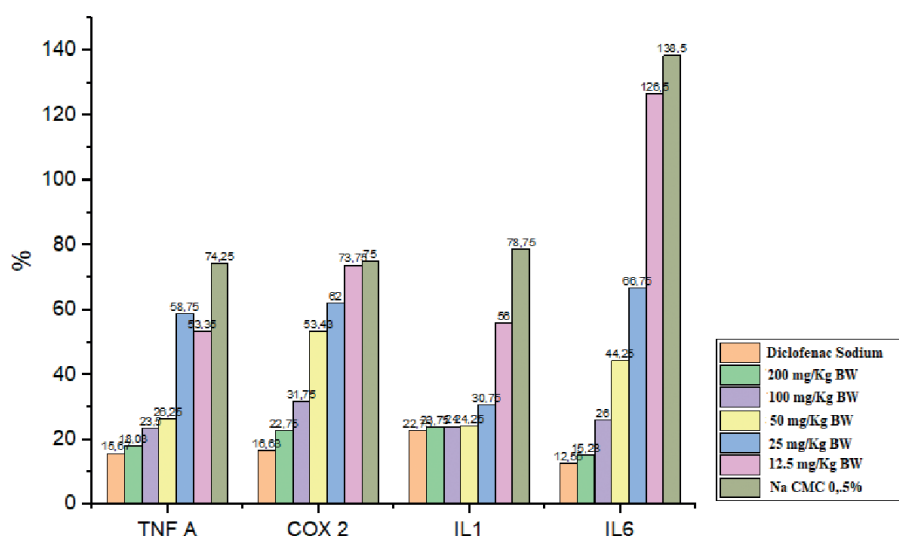

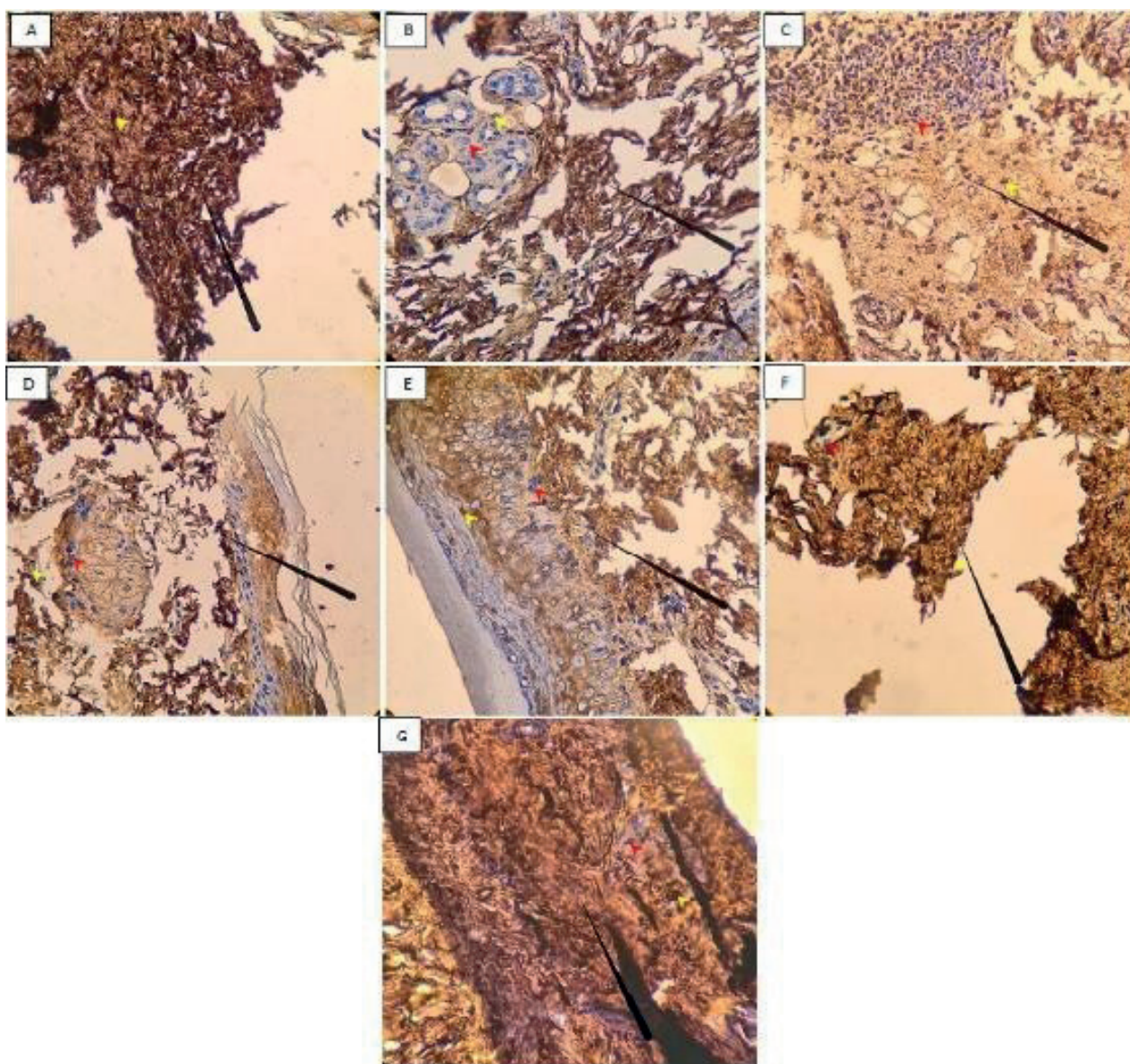**Figure 1.** Graph of observations of the average amount of COX-2, TNF-α, IL-1β, and IL-6.

Figure 2. Overview of COX-2 expression in rat paw inflammation tissue. **Notes:**   (yellow) ⅄ = Express, (red) ⅄ = Not Expressing, **A.** CMC sodium 0.5%, **B.** Diclofenac Sodium, **C.** Dose 200 mg Kg/BW, **D.** Dose 100 mg/Kg BW, **E.** Dose 50 mg/Kg BW, **F.** Dosage 25 mg/Kg BW, **G.** Dose 12.5mg/Kg BW.

gins with the formation of blood clots and is followed by an inflammatory phase and remodeling after the injury (Kumar et al. 2010). The wound healing process will not occur if there is no inflammation and will be a source of pain. Inflammation causes many substances to be released endogenously, known as inflammatory mediators. Arachidonic acid is one of the essential inflammatory mediators; arachidonic acid plays a role in the biosynthesis of prostaglandins through the cyclooxygenase pathway (Robbins and Kumar 2015).

TNF-α

The statistical test results showed that the amount of TNF-α at doses of 100 and 200 mg/Kg BW was not significantly different from that of diclofenac sodium ($p > 0.05$). Based on Fig. 3, it can be seen that there was a decrease in the amount of TNF-α expression in all groups

given the 1.3 bis(p-Hydroxyphenyl)urea and sodium diclofenac compared to the control group CMC sodium 0.5%. This indicates that the 1.3 bis(p-Hydroxyphenyl) urea can inhibit inflammation by decreasing the amount of TNF-α expression. TNF-α is an essential mediator in the inflammatory response. The pro- inflammatory cytokine TNF-α is mainly produced by immune cells such as macrophages and lymphocytes. TNF-α acts as an immune response to inflammation, infection, and tissue damage. Excessive production of TNF-α triggers a decrease in nitric oxide (NO), thereby inducing an increase in endothelin1 (ET-1), causing the proliferation of vascular smooth muscle (Barale and Russo 2020). TNF-α levels represent the severity of inflammation; the higher the TNF-α levels, the higher the severity of inflammation and vice versa; the lower the TNF-α levels, the lower the severity of inflammation and the faster healing process (Souto 2014).
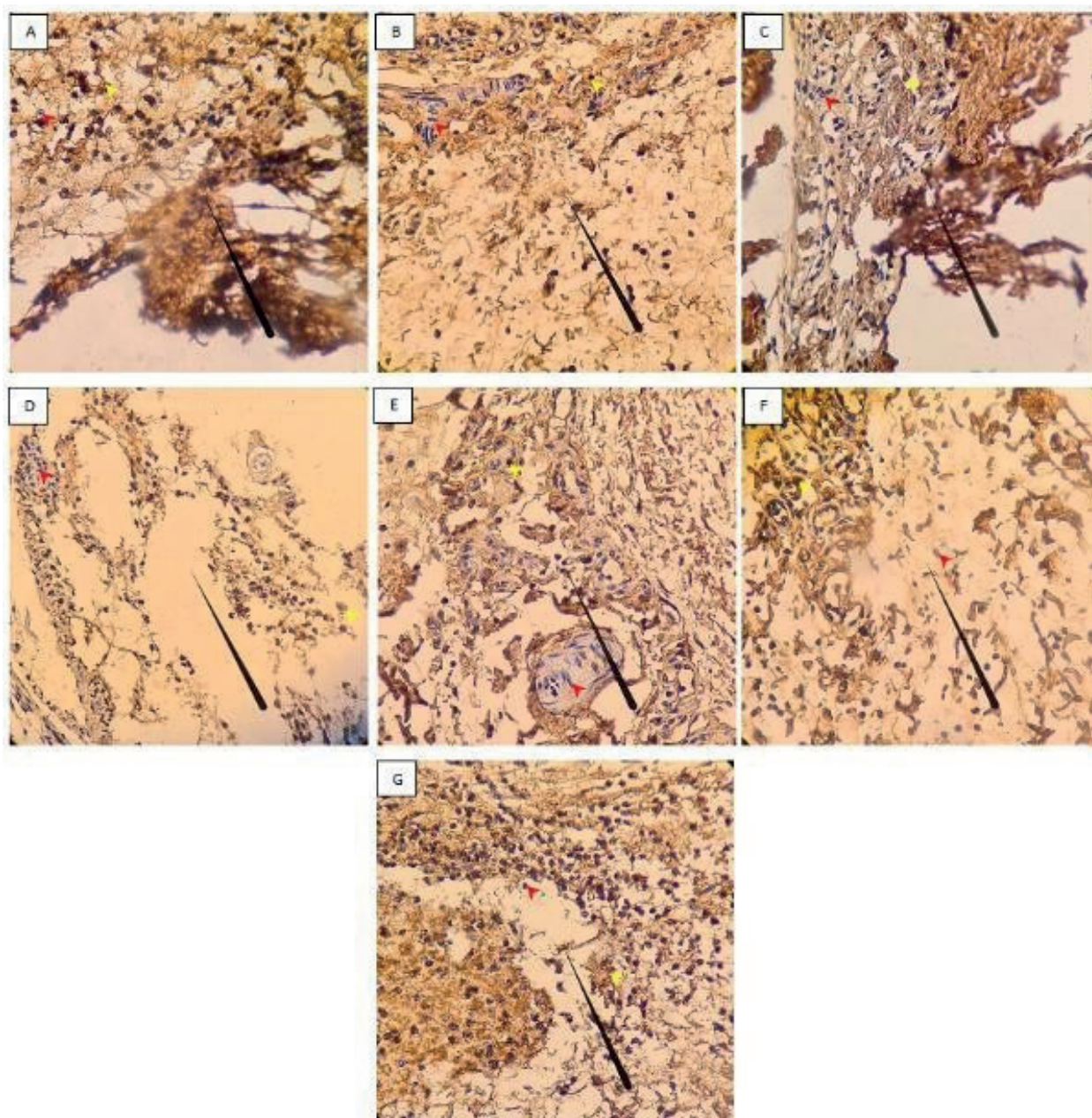
Figure 3. Overview of TNF-α expression in rat paw inflammation tissue. **Notes:**   (yellow) ⚊ = Express, (red) ⚊ = Not Expressing, **A.** CMC sodium 0.5%, **B.** Diclofenac Sodium, **C.** Dose 200 mg Kg/BW, **D.** Dose 100 mg/Kg BW, **E.** Dose 50 mg/Kg BW, **F.** Dosage 25 mg/Kg BW, **G.** Dose 12.5mg/Kg BW.

IL-1β

Based on observations, it can be seen that there is a decrease in the amount of IL-1β expression in all groups given the compound 1.3 bis(*p*-Hydroxyphenyl)urea and diclofenac sodium. Statistical test results show that the amount of IL-1β doses of 50, 100, and 200 mg/Kg BW was not significantly different from diclofenac sodium (p > 0.05). This shows that the compound 1.3 bis(*p*-Hydroxyphenyl)urea can inhibit inflammation by reducing the amount of IL-1β expression. The microscopic picture of IL-1β expression can be seen in Fig. 4. The inflammatory cytokine IL-1β, through its receptor (IL-1R) on cells and other chemotactic factors, serves as a beacon to attract neutrophils and other immune cells, thereby eliciting an inflammatory

response to tissue injury (Shi 2010). Il-1β is a critical inflammatory cytokine that is a significant mediator of the inflammatory response and upregulates the proinflammatory chemokine IL-18 (Cullen et al. 2015). Tissue injury can damage cell membranes without recognizing a cell receptor which causes the accumulation of inflammatory cytokines (Wen et al. 2012). The expression of Il-1β and IL-18 plays an essential role by acting as early mediators of inflammation (Li et al. 2019).

IL-6

Based on observations, it can be seen that there is a decrease in the amount of IL-6 expression in all groups given the 1.3 bis(*p*-Hydroxyphenyl)urea and diclofen-
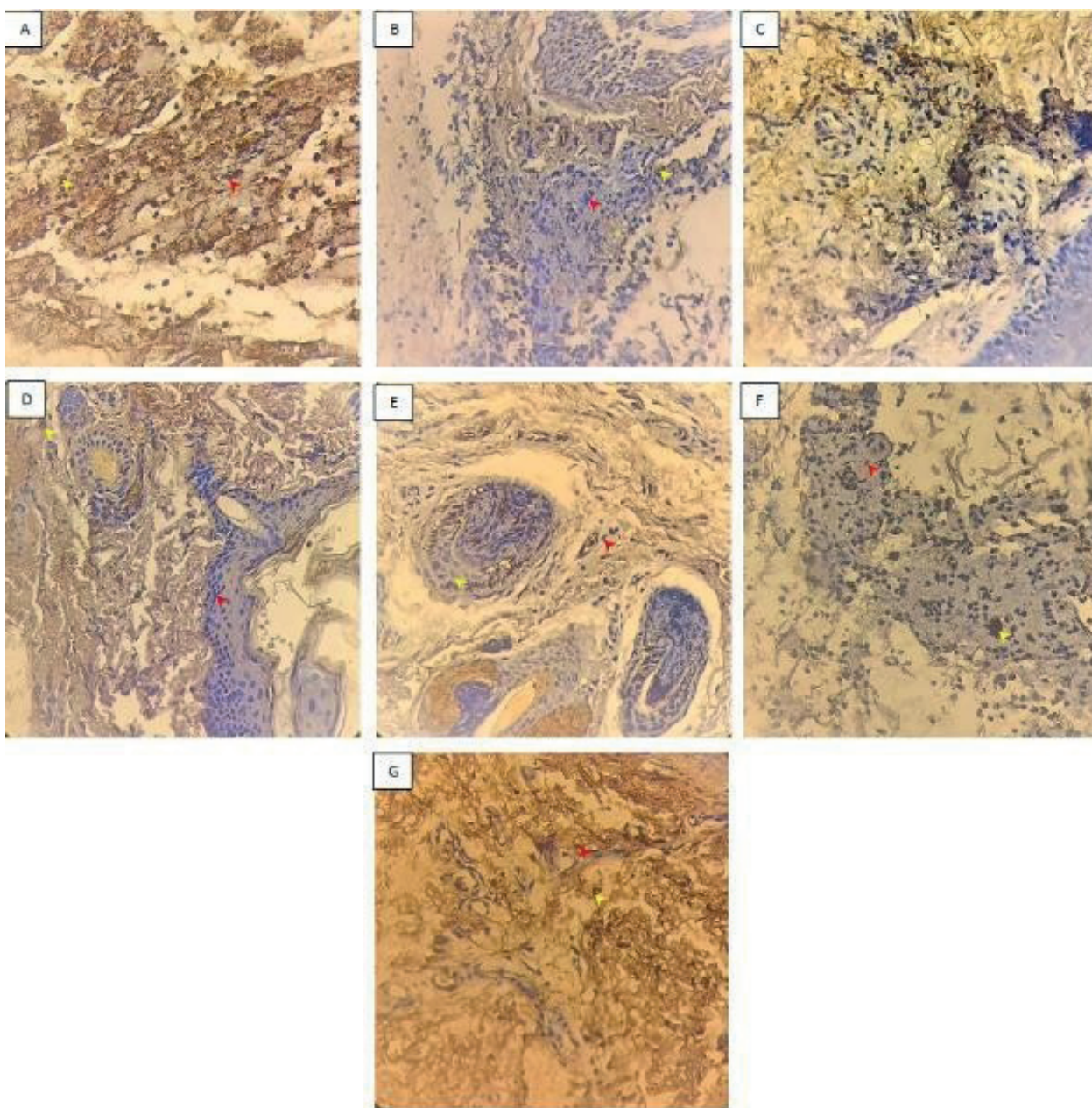
Figure 4. Overview of IL-1β expression in rat paw inflammation tissue. **Notes:** (yellow) ⋏ = Express, (red) ⋏ = Not Expressing, **A.** CMC sodium 0.5%, **B.** Diclofenac Sodium, **C.** Dose 200 mg Kg/BW, **D.** Dose 100 mg/Kg BW, **E.** Dose 50 mg/Kg BW, **F.** Dosage 25 mg/Kg BW, **G.** Dose 12.5mg/Kg BW.

ac sodium. The statistical test results showed that the amount of IL-6 at doses of 100 and 200 mg/Kg BW was not significantly different from that of diclofenac sodium (p > 0.05). This indicates that the 1.3 bis(*p*-Hydroxyphenyl)urea can inhibit inflammation by reducing the amount of IL-16 expression. IL-6 functions as a pro-inflammatory and anti-inflammatory cytokine secreted by T cells and macrophages to stimulate the body's immune response during infection. In the acute inflammatory stage, pro- inflammatory cytokines are released, including IL-6 as a marker of inflammation (Kovacs et al. 2014; Gulati et al. 2016). Macleod and Mansbridge (2015) explained that IL-6 is essential in delaying woundhealing, where IL-6 helps stimulate the formation of keratinocytes that play a role in wound healing. IL-6 is very beneficial in the wound healing process at the beginning of injury that stimulates epithelial proliferation; however, inhibition of IL-6 has proven successful in the treatment of cases of systemic inflammation (Kuhn et al. 2014). The microscopic picture of IL-6 expressioncan be seen in Fig. 5.

Conclusion

The 1.3 bis(*p*-Hydroxyphenyl)urea has anti-inflammatory activity by decreasing the percentage of COX-2, TNF-α, IL-1β, and IL-6 expression in rat paw inflammation tissue.
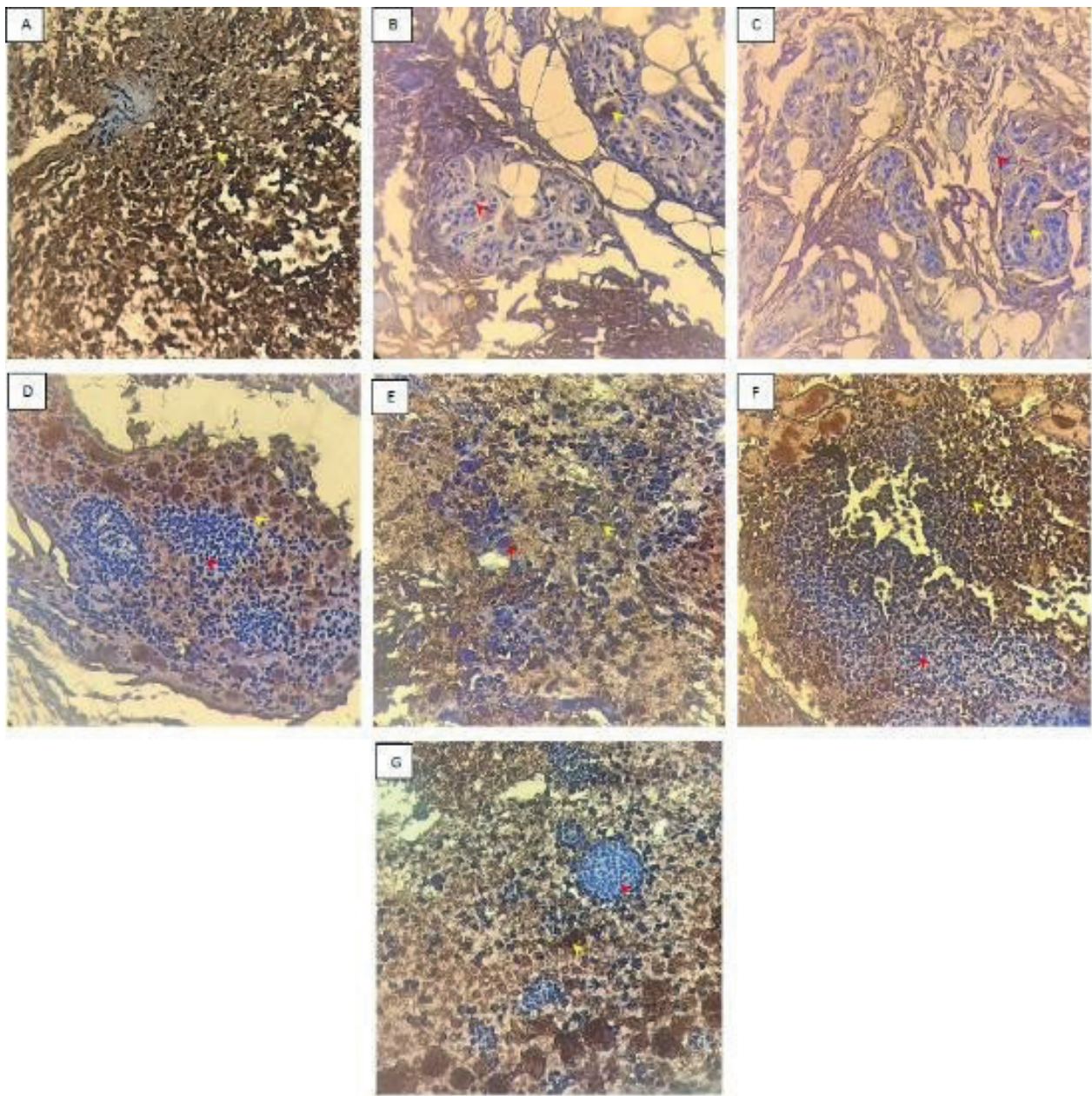
Figure 5. Overview of IL-16 expression in rat paw inflammation tissue. **Notes:** (yellow) ⅄ = Express, (red) ⅄ = Not Expressing, **A.** CMC sodium 0.5%, **B.** Diclofenac Sodium, **C.** Dose 200 mg Kg/BW, **D.** Dose 100 mg/Kg BW, **E.** Dose 50 mg/Kg BW, **F.** Dosage 25 mg/Kg BW, **G.** Dose 12.5mg/Kg BW.

Conflict of interest

The authors declare no conflict of interest in conducting this study.

Acknowledgements

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