

ORIGINAL ARTICLE

## Administration of neem (*Azadirachta indica* A. Juss) leaf extract decreases TNF- $\alpha$ and IL-6 expressions in dextran sodium sulfate-induced colitis in rats

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### ABSTRACT

**Objective:** We aimed to determine the neem leaf extract's effect on Tumor Necrosis Factor-alpha (TNF- $\alpha$ ) and Interleukin-6 (IL-6) expressions in dextran sodium sulfate (DSS)-induced colitis rats.

**Materials and Methods:** In the first phase of the study, colitis was induced by DSS administration in the case group and compared to the control group. In the second phase, 84 colitis rats were divided into groups I, II, and III receiving 7.8 mg/day of mesalazine, 100 mg/200 gm body weight, and 200 mg/200 gm body weight neem leaf extract, respectively.

**Results:** TNF- $\alpha$  and IL-6 expressions were significantly increased in the case group compared to the control group. TNF- $\alpha$  and IL-6 expressions were decreasing in all groups receiving treatment. Group III showed an earlier decrease compared to group II. TNF- $\alpha$  and IL-6 expressions in group III were comparable with group I since the second week. This condition was observed in the 4th week between group II and group I.

**Conclusion:** It can be concluded that neem leaf extract decreased the expression of TNF- $\alpha$  and IL-6 in DSS-induced colitis.

### ARTICLE HISTORY

Received May 08, 2020

Revised November 10, 2020

Accepted November 14, 2020

Published December 04, 2020

### KEYWORDS

*Azadirachta indica*; Colitis;  
Interleukin-6; Neem;  
Tumor necrosis factor- $\alpha$



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### Introduction

Inflammatory bowel disease (IBD) is a chronic, incurable disease affecting the gastrointestinal tract. IBD consists of ulcerative colitis and Crohn's disease [1–3]. The prevalence of IBD has changed globally in the last two decades. The chronic duration of medication and surgeries involved in the disease has made the disease-burden associated with IBD much higher [4]. The prevalence of IBD is increased substantially from 79.5 per 100,000 people in 1990 to 84.3 per 100,000 people in 2017. But, the death rate decreased from 0.61 per 100,000 people in 1990 to 0.51 per 100,000 people in 2017. The North American region reported the highest prevalence of IBD cases, particularly the USA, with 164.5 cases per 100,000 people. IBD often occurs in the second to fourth decade of life. Risk factors for IBD consist of smoking, lifestyle choices, discontinued breastfeeding, enteric infections, appendectomy, and air pollution.

A study reported a shift in IBD prevalence with a male to female ratio of 3:3.9 [1]. The colon's disrupted epithelial barrier makes intestinal microbiota able to invade the deeper part of the colon. This, along with the dysregulated immune system (increased pro-inflammatory cytokines, such as IL-6 and TNF- $\alpha$ ), will trigger inflammation and leads to colitis [5].

There are several options for treating colitis: anti-inflammatory and immunosuppressive agents, biologic drugs, and surgery. Generally, the chronic utilization of high dose anti-inflammatory drugs is mandatory to reduce inflammation and maintain that condition. Complementary and alternative medicines are expected to give a better outcome along with minimal adverse reaction. *Azadirachta indica* A. Juss, popularly known as neem, has been used for a long time in Ayurveda, Unani, and Homoeopathic medicine. Many parts of the neem tree, especially the leaf, fruit,

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**How to cite:** Ruslie RH, Darmadi D. Administration of neem (*Azadirachta indica* A. Juss) leaf extract decreases TNF- $\alpha$  and IL-6 expressions in dextran sodium sulfate-induced colitis in rats. *J Adv Vet Anim Res* 2020; 7(4):744–749.

and bark, are extracted and used as medicine. Those are believed to possess activities that support the anti-inflammatory mechanism and as an immune-stimulant [2,3].

Over time, research has shown that *A. indica* is rich in a wide range of compounds. Some of the compounds also have various pharmacological potentials. Oil extracts are the most typical used form of neem, and its in-depth phytochemical analysis has confirmed the presence of triterpenes, flavonoids, and saponins in a relatively high level. Simultaneously, other components, such as catechins and nimbins, are found to be present in lower amounts. Out of all these compounds, triterpenes possess the highest form of therapeutic use. In particular, triterpene has shown to have antipyretic, fungicidal, antihistamine, and antiseptic properties. Flavonoids are also found in neem. The flavonoids function as inhibitors in prostaglandin biosynthesis, endoperoxides, and enzymes like protein kinases and phosphodiesterases. A particular set of glycoproteins named neem leaf glycoprotein (NGLP) developed from the leaf of the neem tree. This set of proteins showed that when tested on mammalian subjects, there was immunomodulatory activity, providing the potential to restrict tumor growth by modulating local and systemic immunity [6].

Previous studies have proven the role of neem extract as an anti-inflammatory agent. According to a study by Patel et al. [7], Neem extract is effective in decreasing IL-6 and TNF- $\alpha$  expressions in rats with colorectal cancer. Neem leaf extract is proven to reduce the circulating pro-inflammatory cytokines, including cyclooxygenase-2 (COX-2), IL-1, IL-6, TNF- $\alpha$ , and Interferon  $\gamma$  (IFN $\gamma$ ) in rats with oral squamous cell carcinoma [8]. Neem is expected to be an alternative or adjuvant therapy for colitis patients because of its anti-inflammatory activity. This study aimed to determine neem leaf extract's effect on pro-inflammatory cytokines, especially TNF- $\alpha$  and IL-6 expressions in dextran sodium sulfate (DSS)-induced colitis rats.

## Materials and Methods

### Ethical approval

All procedures were conducted according to the Helsinki Declaration. The Institutional Ethics Committee had approved this study of the Universitas Sumatera Utara, Medan, Indonesia (no: 35/TGL/KEPK/FK USU-RSUP HAM/2018).

### Animals and study design

An experimental study was conducted at the Pharmaceutical Laboratory, Biological Laboratory, and Anatomical Pathology Laboratory of Universitas Sumatera Utara, Medan, Indonesia, from June 2019 to September 2019. There were two phases in this study. There were

14 healthy male rats (*Mus musculus*) (Charles River, Inc., Kanagawa, Japan) aged 6–8 weeks and weighing 30 gm in the first phase. The rats were kept at 20°C–25°C with a controlled 12 h light/dark cycle. Laboratory-standardized cages were used to keep the animals with *ad libitum* access to food and water. They were divided into case and control groups. DSS was induced into the rats in the case group for five cycles (70 days) to create colitis, while those in the control group were left without intervention. After completing DSS induction, all rats were sacrificed, and samples (colon) were collected. The samples were then fixated in phosphate-buffered saline with 10% formalin for histopathological examination and immunohistochemical analysis of TNF- $\alpha$  and IL-6.

The second phase contained 84 rats. All of them were treated like rats in the first phase. They were divided into three groups (group I, II, and III). Group I received 7.8 mg of mesalazine daily, group II received 100 mg/200 gm body weight (bw) of neem leaf extract twice daily, and group III received 200 mg/200 gm bw of neem leaf extract twice daily [9]. On days 7, 14, 21, and 28, seven rats from each group were sacrificed, and their colon samples were collected. The samples were subjected to histopathological examination and immunohistochemical analysis, as described earlier.

### Induction of colitis

In this study, 5% of DSS (MP Biomedicals LLC) was administered in five cycles to induce colitis. Each cycle consisted of DSS administration for 7 days, followed by distilled water administration for 7 days. After five cycles in 70 days, the rats were sacrificed or treated with either mesalazine or neem leaf extracts. DSS is a common chemical substance used to induce colitis in experimental studies. It acts by damaging the epithelial barrier and causing secondary inflammatory response marked by increased pro-inflammatory cytokines production such as IL-1 $\beta$ , IL-6, IL-12, IL-18, and TNF- $\alpha$  [10].

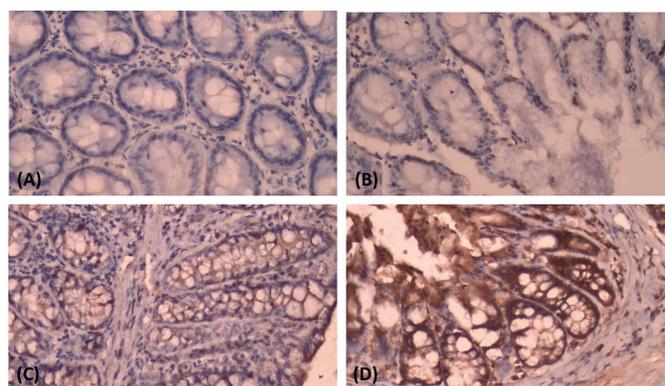
### Immunohistochemical analysis of pro-inflammatory cytokines

Colon samples were embedded in paraffin and cut into slides. The next step was to deparaffinize the slides, get them rehydrated, and then heated on the microwave with 0.01 M citrate buffer (pH 6.0) for 30 min. Endogen peroxidase activity was blocked with 3% hydrogen peroxide for 10 min and then washed with sulfate salt buffer. The specimens were incubated overnight at 4°C, then immune-stained with primary antibody (rabbit polyclonal IgG to bind the mice TNF- $\alpha$  and IL-6) (Wuhan Fine Biotech Co., Ltd., China) in a concentration of 1 mg/ml diluted by 1:600. The primary antibody was detected by

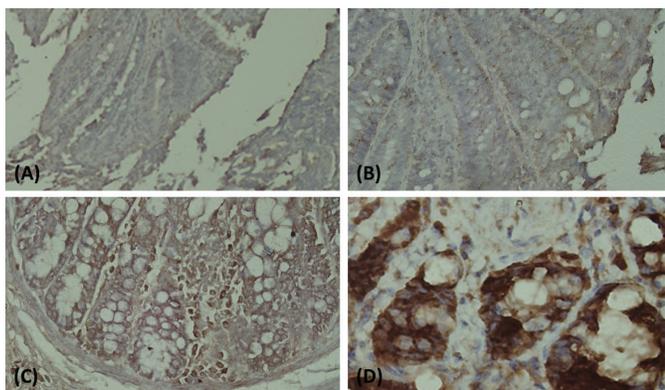
avidin–biotin–peroxidase solution (ScyTek Laboratories, Inc., West Logan, UT), and the signal was visualized using diaminobenzidine (ScyTek Laboratories, Inc., West Logan, UT). The slides were then counterstained with hematoxylin and assessed by two blinded experienced pathologists from the Anatomical Pathology Department of Universitas Sumatera Utara. The slides were categorized as 0 for 0%–15%, 1 for 15%–25%, 2 for 26%–50%, and 3 for 51%–100% stained cells for TNF- $\alpha$  (Fig. 1) and IL-6 (Fig. 2). The negative result was confirmed by scales 0 and 1, while the positive result was by scales 2 and 3 [11].

### Statistical analysis

Fisher’s exact test was used to determine DSS induction’s effect on TNF- $\alpha$  and IL-6 expressions between the case and control group in the first phase of this study. The same



**Figure 1.** Immunohistochemical analysis of TNF- $\alpha$  results. From upper left to lower left slides in clockwise order showing category 0–3 of TNF- $\alpha$  expressions. (Magnification: 400x optical power).



**Figure 2.** Immunohistochemical analysis of IL-6 results. From upper left to lower left slides in clockwise order showing category 0–3 of IL-6 expressions. (Magnification: 400x optical power).

test was also used to determine neem leaf extract’s effect on TNF- $\alpha$  and IL-6 expressions among the three interventional groups. Statistical analysis was carried out at a 95% confidence interval. The  $p$ -value of  $< 0.05$  was considered significant.

### Results

After the induction of DSS, all the rats in the group showed an increase in TNF- $\alpha$  and IL-6 expressions. Contrary to that, there were no rats in the control group that showed expressions of those pro-inflammatory cytokines. This effect was statistically significant, with a  $p$ -value of 0.001 for each cytokine (Table 1). It could be inferred that DSS had successfully induced colitis in this study sample.

The effect of neem leaf extract on TNF- $\alpha$  and IL-6 expressions in DSS-induced colitis rats was analyzed using Fisher’s exact test. We also compared the effect of mesalazine as standard therapy. The expression of TNF- $\alpha$  was decreasing overtime in both group II and group III. The decrease was observed earlier in group III, and it was comparable to group I since the 14th day. On the other hand, the reduction of TNF- $\alpha$  expression in group II was the slowest. It reached comparable expression with group I on the 28th day. Interestingly, TNF- $\alpha$  expression was absent in group I since the 1st week of treatment with mesalazine (Table 2).

Similar to the above-mentioned results, the expression of IL-6 decreased after 28 days of intervention in all groups. Again, IL-6 expression started to decline on the 14th day. It was comparable to the decrease in group I. IL-6 expression decreasing in group II was slower than group I and III and showed a similar result to the group I since the 4th week. IL-6 expression was absent in rats treated with mesalazine since the 2nd week (Table 3).

### Discussion

Ulcerative colitis is an idiopathic, chronic, and incurable inflammatory disease affecting the colon. The main characteristic is recurrent inflammation resulting in an unpredictable course. This disease is common in industrialized countries, particularly in urban areas. In the last two decades, its incidence has been rising in Asia and the

**Table 1.** The effect of DSS induction on TNF- $\alpha$  and IL-6 expressions.

Variables	Case group	Control group	$p$ -value
TNF- $\alpha$ expression, n (%)			
0 and 1	0 (0.0)	7 (100.0)	0.001*
2 and 3	7 (100.0)	0 (0.0)	
IL-6 expression, n (%)			
0 and 1	0 (0.0)	7 (100.0)	0.001*
2 and 3	7 (100.0)	0 (0.0)	

\* $p < 0.05$ .

**Table 2.** The effect of neem leaf extract on TNF- $\alpha$  expression and its comparison to mesalazine.

Days	Group I n (%)		Group II n (%)		$p$ Group I vs. II	Group III n (%)		$p$ Group I vs. III
	2 & 3	0 & 1	2 & 3	0 & 1		2 & 3	0 & 1	
7	0 (0)	7 (100)	7 (100)	0 (0)	0.001*	6 (85.7)	1 (14.3)	0.005*
14	0 (0)	7 (100)	6 (85.7)	1 (14.3)	0.005*	3 (42.9)	4 (57.1)	0.192
21	0 (0)	7 (100)	5 (71.4)	2 (28.6)	0.021*	2 (28.6)	5 (71.4)	0.462
28	0 (0)	7 (100)	2 (28.6)	5 (71.4)	0.462	1 (14.3)	6 (85.7)	1.000

\* $p < 0.05$ .**Table 3.** The effect of neem leaf extract on IL-6 expression and its comparison to mesalazine.

Days	Group I n (%)		Group II n (%)		$p$ Group I vs. II	Group III n (%)		$p$ Group I vs. III
	2 & 3	0 & 1	2 & 3	0 & 1		2 & 3	0 & 1	
7	1 (14.3)	6 (85.7)	7 (100)	0 (0)	0.005*	5 (71.4)	2 (28.6)	0.021*
14	0 (0)	7 (100)	6 (85.7)	1 (14.3)	0.005*	4 (57.1)	3 (42.9)	0.070
21	0 (0)	7 (100)	5 (71.4)	2 (28.6)	0.021*	2 (28.6)	5 (71.4)	0.462
28	0 (0)	7 (100)	3 (42.9)	4 (57.1)	0.192	1 (14.3)	6 (85.7)	1.000

\* $p < 0.05$ .

Middle East, with a rate of 0.15–6.5 per 100,000 people. The peak incidence of ulcerative colitis occurs at 20–39 years and 70–79 years. The prevalence of ulcerative colitis is found to be more common in males than females, but there is a study reporting a shift in its prevalence to female dominance [1–3,12]. The high prevalence in high-income countries is caused by the delayed or low level of exposure to common antigens during childhood, altering the host's immune response. Another reason is due to more affordable access to diagnostic tools in those countries [1].

Ulcerative colitis is believed to result from dysregulated immune functions consisting of innate and adaptive immune systems [1–3]. Other contributing factors for colitis incidence are colonic abnormal bacterial composition and reduced biodiversity [5,13,14]. Those conditions are triggered by unhealthy behaviors such as smoking, lifestyle choices, and air pollution [1]. The combination of dysregulated immune function and colonic bacteria invasion triggers inflammation and leads to colitis [5]. Cytokines play an important role in inflammation. After an injury occurs, pro-inflammatory cytokines, such as IL-1, IL-6, and TNF- $\alpha$ , are produced abundantly. During the first phase of an injury, IL-6 starts to increase, followed by TNF- $\alpha$ . The level of TNF- $\alpha$  remains in the same current during the injury process [15]. In this study, rats from the group of cases received DSS to induce colitis. The induction was successfully marked by the expression of TNF- $\alpha$  and IL-6 in all rats in the case group compared to none in the control group.

Mesalazine is being used as the drug of choice for IBD and is utilized to maintain patients' remission status with ulcerative colitis. Alternative treatments include corticosteroids, immunomodulatory drugs, biological agents,

small molecular therapies, and immunosuppressants [12,16]. However, they only provide an effectiveness of 80% [2,3]. We need adjuvant or alternative treatment to give a better outcome. Neem (*A. indica* A. Juss) is an ever-green tree belonging to the *Meliaceae* family. It is native to India and Burma and widely cultivated in the Indian sub-continent [17,18]. Neem has been used in Ayurveda, Unani, and Homeopathy medicines because of its anti-bacterial, anti-malarial, hepatoprotective, anti-inflammatory, and chemotherapeutic properties [19,20]. A neem tree has abundant liminoid terpenoids known as azadiractoids, which have anti-inflammatory properties [6]. We expected that neem extract might be used as an adjuvant or alternative treatment for patients with colitis. Therefore, we tried to determine the neem leaf extract's effect on TNF- $\alpha$  and IL-6 expressions in DSS-induced rats. We used two different measures of neem leaf extracts, 100 mg/200 gm bw and 200 mg/200 gm bw. We also compared its effect on mesalazine as the standard therapy.

A study by Schumacher et al. [19] found that 13.2% neem leaf extract satisfactorily decreases nuclear factor- $\kappa$ B, which will inhibit the production of TNF- $\alpha$ . This finding is observed in the leukemic cell model. Another study confirms the anti-cancer effect of neem extract. The study is conducted on rats with oral squamous cell carcinoma. This effect is mediated by reducing pro-inflammatory cytokines, including IL-6 and TNF- $\alpha$ , after neem extract's administration [8].

Ghatule et al. [3] showed that the neem leaf extract given to acetic acid-induced colitis rats significantly decreases inflammation and mucosal damage. The positive effect is comparable with sulfasalazine as the standard treatment.

The study used 50% neem leaf extract as much as 1 ml/100 gm bw once daily for 14 days. Neem oil showed an anti-inflammatory effect on acute and chronic inflammation, as described by Jagadeesh et al. [2]. Neem oil also has low ulcerogenic potential compared to indomethacin as a controlled drug in managing inflammation. A study by Gautam et al. [21] in trinitrobenzene sulfonic acid-induced colitis rats showed a significant improvement in the disease course after treated with neem leaf extract. It confirms that neem leaf extract has anti-bacterial, antioxidant, anti-inflammatory, and immunomodulatory activities.

In cigarette smoke- and lipopolysaccharide-induced chronic obstructive pulmonary syndrome rats, the neem leaf extract has shown a protective effect. It decreases the release of pro-inflammatory cytokines, mainly IL-6 and TNF- $\alpha$ . It also modulates the production of reactive oxygen species in the study population [17]. In asthma, another respiratory system's inflammatory disease, the neem leaf extract is also found to be beneficial. In the experimental model of asthma in rats, TNF- $\alpha$ , as one of the central pro-inflammatory cytokines, decreases after receiving a methanolic extract of neem leaf [18].

In contrast to previous studies, Dewanti et al. [20] reported that neem leaf extract increased the expression of TNF- $\alpha$ . This result comes from their research in rats with *Candida albicans* infection treated with aqueous neem leaf extract. The expression of TNF- $\alpha$  is also positively correlated with the dose of neem leaf extract [20].

We found that neem leaf extract had anti-inflammatory properties in DSS-induced colitis rats. Both experimental doses decreased the expression of TNF- $\alpha$  and IL-6 in the study subjects, but the decrease was earlier in group III. Also, TNF- $\alpha$  and IL-6 expressions were comparable with the group I since the 2nd week for group III and since the 4th week for group II. Also, rats in group I showed the earliest decrease of TNF- $\alpha$  and IL-6, which confirmed the mesalazine's superiority as the drug of choice in colitis [22].

These experiments would most likely give more insight into neem leaf extracts as an anti-inflammatory agent. In one study on neem leaf extract [23], it was concluded that IL-10, an anti-inflammatory cytokine, was significantly increased in DSS-induced colitis rats that received neem leaf extract as therapy. As this study evaluated neem leaf extract's effect on the pro-inflammatory cytokines, TNF- $\alpha$ , and IL-6, there was no evaluation on the anti-inflammatory cytokines as the previous study had conducted, simultaneously. Further studies are expected to assess the cytokines' pro- and anti-inflammatory effects for a better understanding of the neem leaf's pharmacological properties.

## Conclusion

In conclusion, neem leaf extract has proven to decrease the expression of TNF- $\alpha$  and IL-6 in DSS-induced colitis. The

decrease was more significant when given in a higher dose. Further studies regarding the approximate dose of neem leaf extract for anti-inflammation in this population is mandatory. Another research to determine neem leaf extract's anti-inflammatory effect combined with mesalazine is essential to decide on its role as adjuvant or alternative therapy.

## Acknowledgments

We would like to thank all the pharmaceutical, biological, and anatomical pathology laboratories' staff for their work and cooperation during this study.

## Conflict of interest

The authors declare that they have no conflict of interest.

## Authors' contribution

Both authors contributed equally to designing the study, collecting, analyzing, interpreting data, and preparing the manuscript.

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