

Exploring the anti-inflammatory effects of *Nigella sativa* on cyclooxygenase-2 through the nuclear factor-kappa B pathway in an *Aspergillus niger*-induced otitis externa mouse model

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ABSTRACT

Inflammatory condition of the external ear canal is called otitis externa (OE). This condition is often associated with microbial infections such as *Aspergillus niger*. This research aims to assess the anti-inflammatory activities of the *Nigella sativa* (NS) extract in an *A. niger*-induced OE mouse model. Mice with *A. niger*-induced OE were treated with NS, and the cyclooxygenase-2 (COX-2), p50, and p65 protein expression were evaluated using Western blot analysis and gene expression assays, respectively. Histological examination was performed to assess the infiltration of inflammatory cells in the external ear canal tissues. Administration of NS extract significantly reduced the expression levels of COX-2, as well as the subunits p50 and p65 genes, in a dose-dependent manner. Histological analysis revealed a notable reduction of inflammatory cell infiltration in NS extract-treated mice, with higher doses yielding greater reductions. NS extract effectively suppresses the expression of key pro-inflammatory cytokines and genes, indicating its therapeutic potential in managing inflammatory conditions of the external ear canal.

Key words: Anti-inflammation, *Aspergillus niger*, *Nigella sativa*, otitis externa

INTRODUCTION

Otitis externa (OE), also known as swimmer's ear, is an inflammation that affects the outer canal of the ear.^[1] It is characterized by pain, swelling, and discharge, often exacerbated by moisture or microbial infections. *Aspergillus niger*, a ubiquitous fungus found in the environment, is a

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common pathogen known to induce OE.^[2] Its ability to thrive in warm and humid conditions makes it a frequent culprit in fungal ear infections, leading to persistent inflammation and discomfort.

The pathogenesis of OE involves a complex interplay of inflammatory mediators. On activation by microbial stimuli such as fungal infections, the nuclear factor-kappa B (NF- κ B) translocates into the nucleus and triggers cyclooxygenase-2 (COX-2) transcription,^[3-7] thus propagating the inflammatory cascade.^[8] The dysregulation of this pathway can lead to prolonged inflammation and tissue damage in the external ear canal.^[9]

Antimicrobial medicines, corticosteroids, and analgesics are frequently used to treat OE and reduce symptoms.^[10] However, challenges such as antibiotic resistance, side effects of corticosteroids, and limited efficacy against fungal pathogens such as *A. niger* pose significant hurdles in achieving optimal outcomes for patients with OE.^[11,12] *Nigella sativa* (NS), also called black seed or black cumin, has garnered attention for its potent anti-inflammatory properties and diverse therapeutic benefits.^[13-15] Studies have shown that NS exhibits anti-inflammatory effects by modulating various signaling pathways, including NF- κ B, thereby reducing the production of the inflammatory enzyme COX-2.^[13,16,17] Since the NS, anti-inflammatory activities target the key inflammatory pathways mediated by COX-2 and NF- κ B. Administration of NS in OE cases might alleviate the inflammation and improving treatment outcomes. Therefore, we aim to investigate the potential of NS in reducing inflammation in OE induced by *A. niger* and present a novel approach toward enhancing therapeutic strategies for this common inflammatory ear condition.

METHODS

Ethical clearance statement

All animal-related experimental methods were conducted in conformity with the ethical standards and guidelines stated by the Institutional Animal Care and Use Committee (IACUC) at Faculty of Medicine Universitas Islam Sultan Agung, Semarang, Central Java, Indonesia, under IACUC Protocol Number 278B/Komisi Bioetik/2023/EC.

Extraction of *Nigella sativa* extract

NS seeds were obtained in May 2023 from Semarang in Central Java, Indonesia (coordinates: latitude - 7.6565111, longitude 109.129500). The cleaned seeds were dried, ground into small fragments, and sifted through a 120-mesh sieve. A maceration apparatus was used to extract 500 g of NS seeds for 24 h using 5l of 98% ethanol. The extract was then filtered and concentrated using IKA rotary vacuum evaporator. The crude extract was stored at 4°C in a refrigerator until further analysis.

Aspergillus niger-induced otitis externa animal model

Thirty male Wistar rats, weighing 250 ± 25 g with a coefficient of variation of 10%, were maintained under *ad libitum* feeding conditions at 28°C with a 12-h photoperiod. After a 1-week acclimatization, the rats were randomly allocated into five groups: healthy, negative control, positive control (treated topically with miconazole), NS extract 5%, and NS extract 10%, each comprising six rats. OE was induced in the rat model by creating a 2-mm injury in the ear canal and inoculating it with *A. niger* at a dose of 1 mg/50 μ L in NaCl on day 1. On the following day, KOH was used to swab the ear canal for hyphae detection under a microscope. On observing hyphae, the animals received daily treatment with NS extract until day 14. On day 15, the animals were euthanized, and ear tissue samples were collected for further analysis.

Protein expression analysis by Western blot

The ears were lysed using RIPA buffer to extract proteins, and their concentrations were determined with the Pierce BSA CBB Assay. Next, 10 μ g of individual sample protein underwent 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis gel separation and transferred onto polyvinylidene fluoride membrane. The membrane was blocked for 1 h in 5% bovine serum albumin solution (Sigma-Aldrich) and incubated overnight with COX-2 antibodies (sc-19999) at a 1:1000 dilution. The membranes were washed and treated with a horseradish peroxidase-conjugated secondary antibody from GeneTex Biotechnology. The chemiluminescent signal was detected using the ECL reagent and the Invitrogen iBright ChemiDoc Imaging System.^[18]

p50 and p65 gene expression

Rat ear tissue was subjected to total RNA extraction using TRIzol (Invitrogen). SuperScript II (Invitrogen) was used to synthesize the first-strand cDNA from 1 μ g of total RNA. Reverse transcription was performed using SYBR No. ROX Green I dye (SMOBIO Technology, Inc.) in PCRmax Eco 48. Specific primers [Table 1] were used to quantify the mRNA amount of the p50 and p65 genes. The cycle threshold (Ct) was used to measure gene expression. The data were analyzed based on 2- $\Delta\Delta$ Ct method for data analysis.^[19-21]

Statistical analysis

The results are reported as mean $\pm$ standard deviation to determine differences between groups; a one-way analysis of variance was used, followed by the least significant difference test. All statistical tests were used at a 5% significance level using SPSS 22.0 software (SPSS Inc. Chicago, IL).

RESULTS

Administration of NS in the *A. niger*-induced OE mouse model resulted in a notable inhibition of COX-2 protein expression

within the external ear canal. A significant decrease of COX-2 proteins in the NS-treated group compared to the control group was observed in Western blot results [Figure 1a]. The results are also supported by a quantitative assessment, which indicates a significant reduction in COX-2 protein expression levels ($P < 0.05$) [Figure 1b].

The NS exhibited downregulation of the subunits p50 and p65 genes expression in the OE model [Figure 2a and b]. Notably, the reduction was most prominent at the 10% concentration of NS. This observation indicates that the NS extract, particularly at the specified concentration, effectively inhibits the expression of these critical proteins and genes associated with the inflammatory response in the OE model.

Administration of NS reduces the inflammatory cell count compared to the negative control group, demonstrating a dose-dependent response [Figure 3a and b]. This effect was assessed through histological examination utilizing hematoxylin and eosin staining. Quantitative analysis of

HE-stained tissue sections revealed a notable decrease in the infiltration of inflammatory cells in the external ear canal tissues of mice treated with NS extract, with the most pronounced reduction observed at higher doses. Specifically, mice receiving higher doses of NS exhibited a greater decrease in the number of infiltrating inflammatory cells compared to those treated with lower doses.

DISCUSSION

The study investigating the administration of NS in an *A. niger*-induced OE mouse model revealed significant inhibitory effects on COX-2 protein expression within the external ear canal. This inhibition was correlated with a substantial suppress in the abundance of COX-2 compared to the control group. Moreover, the study highlighted a dose-dependent response, with higher concentrations of NS leading to more pronounced reductions in inflammatory markers and gene expression associated with the inflammatory response.

Previous research findings support and complement these results, shedding light on the broader anti-inflammatory and pain-relieving effects of NS. Studies have demonstrated that bioactive compounds in NS act as potent anti-inflammatory agents by regulating key pathways involved in inflammation control.^[13] In addition, investigations into the antibacterial efficacy of NS seed oil have shown promising results against bacteria commonly isolated in otitis media and externa, further emphasizing its therapeutic potential in combating inflammatory conditions.^[16,17]

Previous research has demonstrated that NS active components, thymoquinone, had anti-inflammatory activities by lowering levels of pro-inflammatory mediators, including interleukin-6, tumor necrosis factor- α , interleukin-1 β , and prostaglandin E2, as well as COX-2 production inhibition.^[22] The observed reduction in p50 and p65 expression suggests inhibition of NF- κ B activation,

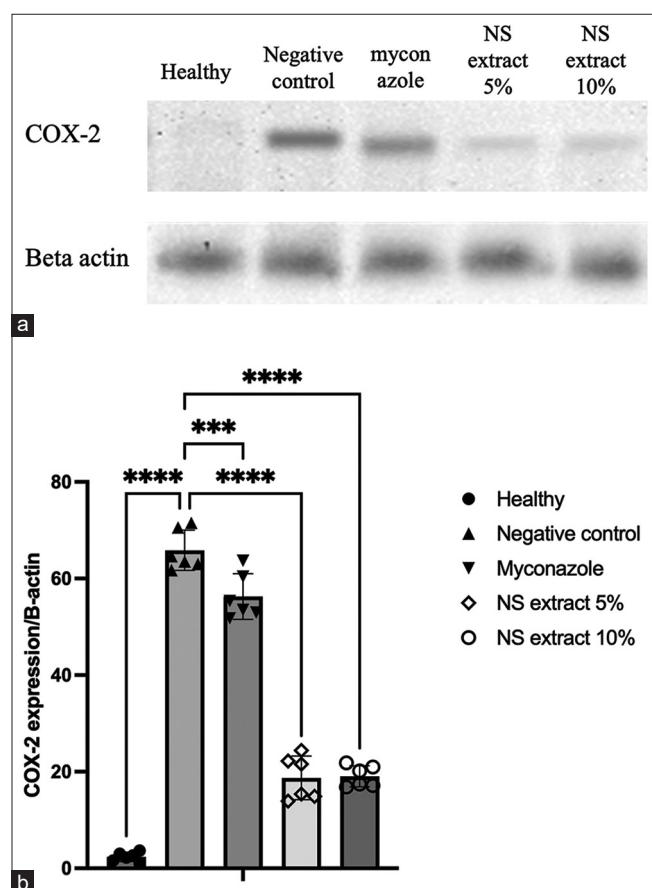


Figure 1: (a) Representative bands from Western blot analysis of cyclooxygenase-2 (COX-2) and β -actin in the external ear canal tissues of otitis externa mice model (b) Quantification of Western blot analysis presented as the percentage ratio of COX-2 protein bands relative to β -actin (data in mean $\pm$ standard deviation, $n = 6$, **** = statistically significant difference). NS: *Nigella sativa*, COX-2: Cyclooxygenase-2

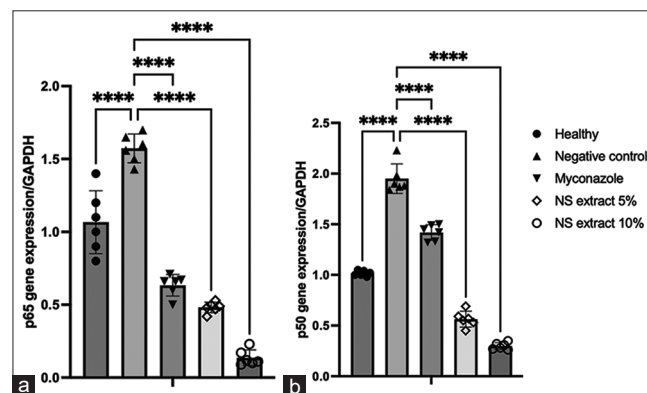


Figure 2: (a) Relative gene expression level of p65 and (b) Relative gene expression level of p50 (data in mean $\pm$ standard deviation, $n = 6$, **** = statistically significant difference vs. negative control). NS: *Nigella sativa*

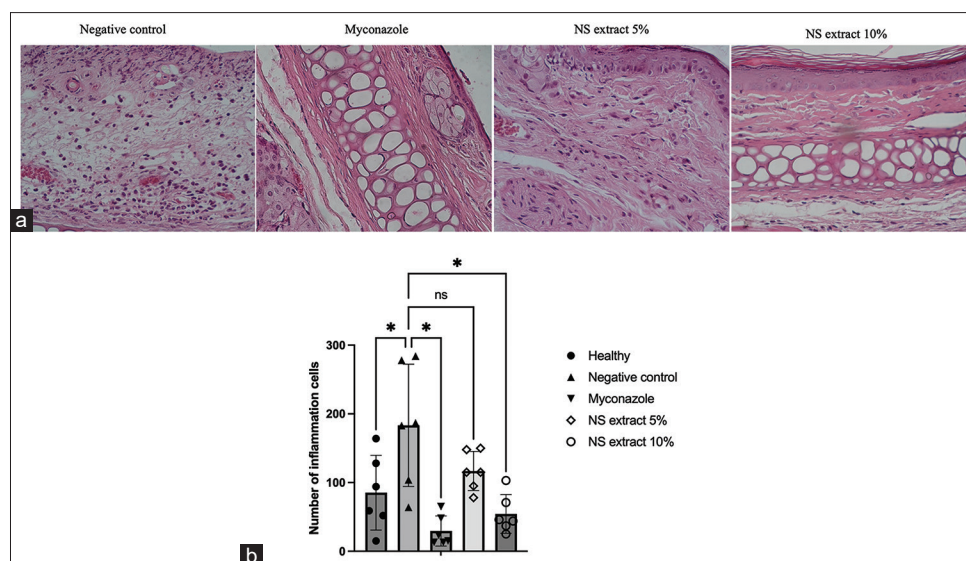


Figure 3: (a) Histological analysis of HE-stained tissue sections showing the effect of *Nigella sativa* (NS) on the infiltration of inflammatory cells. (b) Quantification of inflammatory cells in the external ear canal tissues of mice with otitis externa treated with NS. (Data in mean $\pm$ standard deviation, $n = 6$, * = statistically significant difference). NS: *Nigella sativa*

Table 1: Primer sequence

Gene	Sequence
p50	Forward: TGGACAGCAAATCCGCCCTG Reverse: TGTTGTAATGAGTCGTCATCCT
p65	Forward: TGAACCGAAACTCTGGCAGCTG Reverse: CATCAGCTTGCGAAAAGGAGCC

leading to a decreased production of pro-inflammatory mediators and a diminished inflammatory response in the OE model. This mechanism is consistent with previous studies that have demonstrated NS's ability to inhibit NF- κ B signaling and suppress inflammation in various disease contexts.^[23-25] Thymoquinone in NS suppresses the activation of I κ B α kinase, which is required for the phosphorylation and degradation of I κ B α , and thymoquinone acts as an inhibitory protein that sequesters NF- κ B in the cytoplasm. It achieves this by inhibiting I κ B α kinase, hence stopping NF- κ B from being released and moving to the nucleus. Moreover, NS and thymoquinone can activate the Nrf2 antioxidant pathway, which can suppress NF- κ B activation by reducing oxidative stress.^[22]

HE analysis further corroborates the anti-inflammatory property of NS, revealing a dose-dependent reduction in the infiltration of inflammatory cells within the external ear canal tissues. This observation suggests that NS not only targets specific molecular pathways but also attenuates the cellular infiltration characteristic of inflammatory responses. This comprehensive suppression of inflammation at both the molecular and cellular levels underscores the therapeutic potential of NS in managing OE and related inflammatory conditions. Our findings are consistent with previous research demonstrating the anti-inflammatory capacity of NS in various disease models. Studies have

elucidated the ability of NS to modulate NF- κ B signaling, inhibit pro-inflammatory cytokine production, and attenuate inflammatory cell infiltration in conditions such as asthma, arthritis, and colitis.^[16,25] The present study extends these observations to the context of OE, providing further evidence of NS's efficacy as a potent anti-inflammatory agent.

In conclusion, our results highlight the promising therapeutic potential of NS in the management of OE by targeting key inflammatory pathways and cellular processes. The optimal dosage of NS against OE was 10%, exhibiting strong anti-inflammatory properties. Future studies elucidating the precise molecular mechanisms behind the anti-inflammatory actions of NS, as well as clinical trials evaluating its efficacy in human OE, are warranted. NS holds great promise as a natural therapeutic agent for alleviating inflammation and improving the clinical outcomes of OE and other inflammatory disorders.

CONCLUSION

This study's findings highlight NS's anti-inflammatory effects in reducing COX-2 expression through inhibition of the NF- κ B pathway in a mouse model of *A. niger*-induced OE. These results offer valuable insights into the therapeutic potential of NS as a natural remedy for treating inflammation associated with OE.

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Conflicts of interest

There are no conflicts of interest.

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