

Revealing the Therapeutic Potential of *Nigella Sativa* Extract in *Aspergillus Niger*-Induced Otitis Externa: Anti-Inflammatory and Antioxidant Properties

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Abstract

Otitis externa (OE), a fungal infection of the external auditory canal, often involving *Aspergillus niger*, can lead to OE externa, posing severe health risks. Establishing an animal model of OE induced by *Aspergillus niger*, this study aimed to evaluate the therapeutic potential of *Nigella sativa* (NS) extract, specifically its effect on inflammation and antioxidant activity in this OE animal model. The detection of *Aspergillus niger* on the 3rd day post-induction confirmed its thriving presence within the ear canal. NS extract, containing thymoquinone, significantly reduced the expression of IL-1 β and TNF- α proteins. Notably, NS extract at 5 and 10 % showed comparable effects, surpassing the efficacy of topical miconazole. Evaluation of MDA protein expression, indicative of oxidative damage, revealed a significant decrease with NS extract treatment, especially at 5 % dosage. NS extract exhibited a notable decrease in subunit p50 and p65 proteins expression, particularly at the 5 % concentration. In conclusion, NS extract displayed promising anti-inflammatory effects on *Aspergillus niger*-induced otitis externa by modulating various inflammatory markers and pathways. These findings highlight the potential therapeutic role of NS extract in addressing inflammation associated with OE.

Keywords: Otitis externa, Anti-inflammation, Antioxidant, NF-kB pathway, *Aspergillus niger*

Introduction

Otitis externa (OE) caused by *Aspergillus niger* is a condition known as otomycosis, which refers to a fungal infection of the external auditory canal [1]. The prevalence of OE caused by *Aspergillus niger*, a common fungal pathogen, is a significant concern, with *Aspergillus* species being implicated in approximately 9 to 30 % of OE cases worldwide [2,3]. *Aspergillus niger* is particularly prevalent, accounting for about 75 % of otomycosis cases, a form of OE caused by fungal infection [4]. This condition can lead to invasive external otitis, especially in immunocompromised individuals, and may pose a severe and potentially life-threatening risk [5]. OE also can present with symptoms such as unilateral otalgia, persistent otorrhea, pruritus, tinnitus, and progressive hearing loss due to fungal debris in the ear canal. The management of *Aspergillus niger*-induced OE presents challenges, especially when the infection becomes chronic or recurrent, leading to prolonged treatment and potential resistance to conventional therapies [5].

The current treatment for otitis externa (OE) typically used antifungal medications and anti-inflammatory drugs [1-6]. Topical antibiotic drops and pain control are the mainstay of uncomplicated OE treatment, with the goal of suppressing chronic inflammation. Commonly used topical antibiotics and antifungal for OE include polymyxin B, neomycin, hydrocortisone, ofloxacin, ciprofloxacin, and miconazole [5-7]. Pain management often involves the use of acetaminophen or nonsteroidal anti-inflammatory drugs (NSAIDs). However, topical antibiotics may lead to the development of antimicrobial resistance and local hypersensitivity reactions. Additionally, prolonged use of topical antibiotics can disrupt the normal flora of the ear canal, leading to secondary fungal infections [8-10]. Therefore, there is a growing interest in exploring natural remedies and plant extracts with potential therapeutic effects against such infections and inflammation.

OE triggers an inflammatory response in the ear canal, leading to the release of reactive oxygen species (ROS) and subsequent lipid peroxidation [11]. It was recognized that nuclear factor- κ B (NF- κ B) signaling pathway played an important role in regulating inflammatory response and oxidative stress. Convincing data has shown that the levels of pro-inflammatory cytokines and ROS along with NF- κ B activity were significantly elevated in the inflammation rats induced by fungal infections [12]. Activation of NF- κ B resulted in the transcription of several proinflammatory cytokines including tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) and interleukin-1 β (IL-1 β) [13].

Nigella sativa extract (NS) have a lot of bioactive ingredients which has been widely researched for its anti-oxidative, anti-apoptotic, anticancer, and anti-inflammatory effects [14-17]. NS and its active component, thymoquinone, have been shown to modulate inflammation and immune responses, reduce the expression of TNF- α , and downregulate the levels of IL-1 β [16,18,19]. Thymoquinone has been found to have anti-inflammatory, analgesic, and immunomodulatory effects, and it can reduce the expression of TNF- α , IL-1, and NF κ B, which are involved in the inflammatory process [18,20]. Previous study reported that thymoquinone a major compound of NS extract inhibits proinflammatory cytokine interleukin-6 (IL-6) by blocking the p38/MAPK and ERk1/2 pathways [21,22]. By inhibiting inflammation potentially contribute to the resolution of otitis externa. Therefore, in this study aims to evaluate the effect of NS on the *Aspergillus niger*-induced otitis externa model focusing on the inflammation pathway.

Materials and methods

Ethics statement

Animal procedures were conducted following approval from the Animal Ethics Committee of the Universitas Islam Sultan Agung Semarang (Central Java, Indonesia) under number

43/AEC/Biomedik/2023. Animals were sourced and housed in the Animal Facility Stem Cells and Cancer Research Indonesia (Central Java, Indonesia).

Extraction of *Nigella sativa* extract

Nigella sativa seeds were collected from Semarang in Central Java Indonesia in May 2023 (Latitude -7.6565111 and Longitude 109.129500). They were rinsed with tap water followed by distilled water to remove the dirt on the surface. The dried seeds were blended until small pieces and sieved with a mesh size of 120 mesh. The 500 g of NS seeds was extraction in a maceration apparatus with 5 L 98 % ethanol for 24 h. The filtrated was then evaporated under rotary vacuum evaporator (IKA) and the crude extract was store in refrigerator 4 °C [23,24]. The NS extract were stored at 4 °C until further analysis.

Aspergillus niger-induced otitis externa animal model

Thirty healthy male Wistar rats (250 ± 25 g) CV = 10 % were fed ad libitum and reared at 28 °C and a photoperiod of 12 h. After a week of acclimatization, rats were randomly divided into the following 5 groups: Healthy, negative control, positive control (topical miconazole), NS extract 5 % and NS extract 10 %. Each group consisted of 6 rats. Otitis externa was induced in the animal model by injuring the ear canal (2 mm) and inducing it with *Aspergillus niger*, suspension doses was 1 mg/50 μ L in NaCl on day 1. On the 2nd day, the ear canal was swabbed with KOH to detect hyphae under a microscope. After hyphae were observed, the animal was treated with NS extract every day until day 14. On day 15, the animal was sacrifices and ear tissue was collected to further analysis.

Protein expression analysis by western blot

The ear was lysed in RIPA buffer and protein concentration was measured using Pierce BSA CBB Assay. Aliquots of 10 μ g total protein were mixed with 2 \times Laemmli buffer (Biorad) with ratio 1:1, boiled, and separated on 10 % SDS-PAGE gels, transferred to Polyvinylidene Fluoride (PVDF) membranes. Then blocked with 5 % Bovine Serum Albumin (BSA) (Sigma Aldrich, Louis St, MO) in Phosphate Buffered Saline with Tween (PBST) (Sigma Aldrich, Louis St, MO) for 1 h. IL-1B antibodies (Santa Cruz Biotechnology) and TNF- α antibodies (Santa Cruz Biotechnology) were applied in blocker overnight at dilutions of 1:1,000 separately, after which membranes were washed, incubated with HRP-conjugated secondary antibody (GeneTex Biotechnology), washed again, incubated with ECL reagent and exposed to chemiluminescence. ECL chemiluminescence was captured using the Invitrogen I-Bright ChemiDoc Imaging System [25-28].

Collagen density analysis

Isolated ear tissues were immediately fixed in 10 % formalin for a preserve tissue architecture. Following fixation, tissues were dehydrated using a graded ethanol series and embedded in paraffin to facilitate sectioning. Paraffin-embedded tissues were sectioned at a thickness of 5 μ m using a microtome. Tissue sections were deparaffinized in xylene and rehydrated through a graded ethanol series to prepare them for staining. The Masson Trichrome staining protocol was employed to visualize collagen fibers. Stained tissue sections were examined under a light microscope equipped with appropriate filters. Images were captured using a digital camera for subsequent analysis. Collagen density was quantified using ImageJ analysis software. Regions of interest were selected, and the percentage of collagen area was calculated relative to the total tissue area [29].

Statistical analysis

Statistical analyzes were accomplished with software SPSS 22.0 (SPSS Inc., Chicago, IL, USA). All data are presented as mean \pm standard deviation (SD). Data analysis used one-way ANOVA and continued with the Least Significant Difference (LSD) test with p -value < 0.05 .

Results and discussion

OE animal model validation

Aspergillus niger is known to be associated with otomycosis, a fungal infection of the external auditory canal. It can contribute to chronic otitis externa, particularly in cases of invasive external otitis, which can be severe and potentially life-threatening. In this study we made an animal model of otitis externa induced by *Aspergillus niger*. The detection of *Aspergillus niger* on the 3rd day of the study provided clear evidence of the fungus's thriving presence within the ear canal (**Figure 1**).

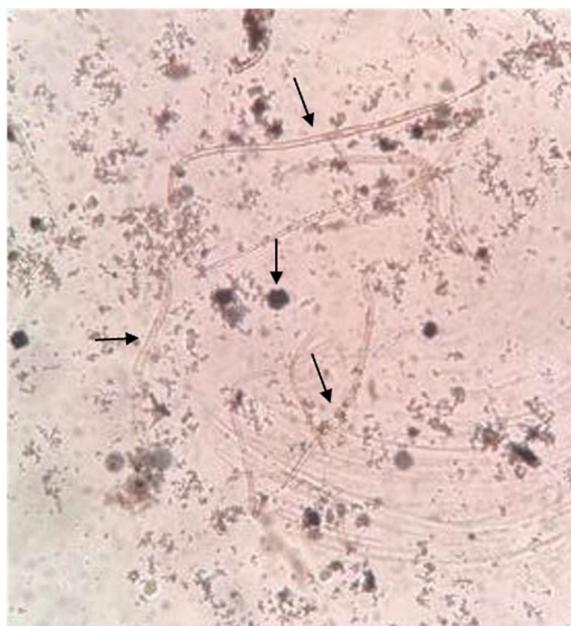


Figure 1 *Aspergillus niger* colonies in the ear canal on day 3 after induction. Black arrows indicated *Aspergillus niger*, magnification $\times 400$.

NS extract inhibited inflammation marker on OE animal model

The administration of NS extract containing thymoquinone has demonstrated a significant reduction in the expression of IL-1B and TNF- α proteins (**Figures 2(A) - 2(C)**). NS extract decreased IL-1B (**Figure 2(B)**) and TNF- α (**Figure 2(C)**) protein expression level, with a strongest doses was NS extract 5 %, 49.37 ± 2.70 and 15.87 ± 2.35 , respectively. The topical application of NS extract in 5 and 10 % gel formulations demonstrated no significant difference in effect between the 2 doses. However, when compared to the positive control, topical miconazole, the extract showed a markedly significant difference, indicating its greater potential in suppressing these inflammatory markers. The lack of significant difference between the 5 and 10 % doses implies that the lower dose may be as effective as the higher dose, offering potential benefits in terms of safety and cost-effectiveness. Furthermore, to explore the mechanism underlying NS extract inhibit inflammatory cytokines, we explored the possible mechanism through ROS pathway.

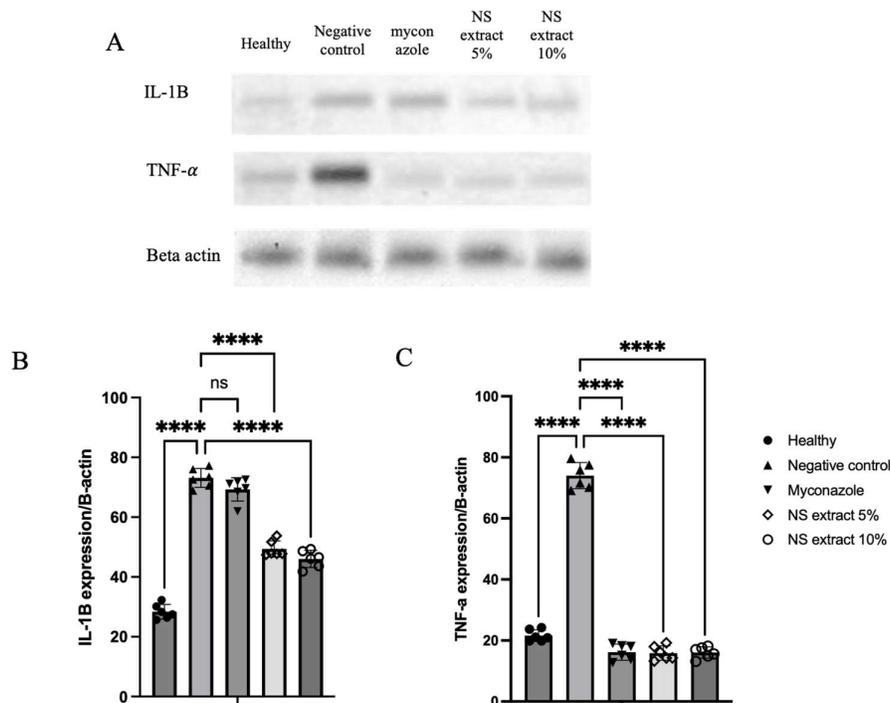


Figure 2 Western blot analysis. (A) Protein levels of IL-1B and TNF- α in the ear. Relative protein expression was quantified using Image J. (B) Relative protein expression of IL-1B. (C) Relative protein expression of TNF- α . The healthy group received no intervention, while the negative control group was induced with OE and did not receive any treatment. The miconazole group served as the positive control, receiving topical miconazole. The NS extract 5 and 10 % groups were administered NS extract in gel form. Values are expressed as mean \pm SD. *** p < 0.03 and **** p < 0.001 vs. negative control.

NS extract decreased MDA protein expression on OE animal model

The increased MDA levels, indicative of oxidative damage, are associated with elevated levels of pro-inflammatory markers, reflecting an augmented inflammatory response. Previous study reported that decreased MDA levels and reduced expression of inflammation markers such as COX-2 and TNF- α , indicating a potential interplay between oxidative damage and inflammatory responses. In this study we evaluated the MDA protein expression on the NS extract-treated OE. The NS extract significantly decreased MDA protein expression level. The NS extract decreased the strongest MDA expression at a 5 % NS dose up to 10.93 ± 13.81 (**Figures 3(A) - 3(B)**). Furthermore, to explore the mechanism underlying NS extract inhibit inflammatory cytokines and ROS through MDA suppression, we explored the possible mechanism through NF- κ B pathway.

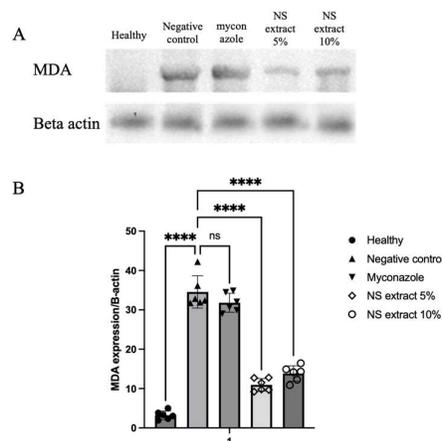


Figure 3 Western blot analysis. (A) Protein levels of MDA in the ear. Relative protein expression was quantified using Image J. (B) Relative protein expression of MDA. The healthy group received no intervention, while the negative control group was induced with OE and did not receive any treatment. The miconazole group served as the positive control, receiving topical miconazole. The NS extract 5 and 10 % groups were administered NS extract in gel form. Values are expressed as mean \pm SD. **** $p < 0.001$ vs. negative control.

NS extract decreased subunit p50 and p65 protein expression on OE animal model

The NS extract demonstrated a robust decrease in the expression levels of the subunit p50 and p65 proteins expression in the OE model (Figures 4(A) - 4(C)). The reduction was particularly pronounced at the NS 5 % dosage. This finding suggests that the NS extract, at the specified concentration, exerts a significant inhibitory effect on the expression of these key proteins expression associated with the inflammatory response in the OE model. The observed correlation underscores the potential anti-inflammatory properties of the NS extract, specifically at the 5 % concentration, and its possible relevance in the context of OE and related inflammatory conditions.

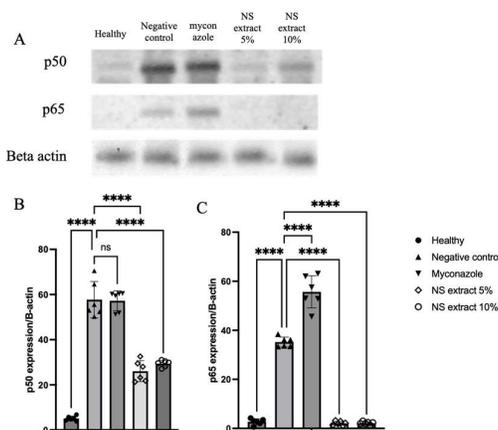


Figure 4 NF-kB pathway analysis by western blot and qRT-PCR. (A) Protein levels of sub unit p50 and p65 in the ear. Relative protein expression was quantified using Image J. (B) Relative protein expression of p50. (C) Relative protein expression of p65. The healthy group received no intervention, while the negative control group was induced with OE and did not receive any treatment. The miconazole group served as the positive control, receiving topical miconazole. The NS extract 5 and 10 % groups were administered NS extract in gel form. Values are expressed as mean \pm SD. **** $p < 0.001$ vs. negative control.

NS extract induced collagen density on OE animal model

The administration of NS extract at a 5 and 10 % concentration resulted in a significant increase in collagen density in doses-dependent manner compared to the negative control. Histological analysis revealed a notable augmentation in collagen fibers within the affected ear tissue, suggesting a positive impact on tissue repair and regeneration. The negative control group exhibited a substantial decrease in collagen density up to 6.47 ± 1.23 %. Comparatively, the positive control group treated with myconazole demonstrated a collagen density increase comparable to that observed with NS extract at a 10 % concentration (**Figures 5(A) - 5(B)**). This suggests that the efficacy of NS extract, particularly at a higher dose, matches the collagen-promoting effects of myconazole, a standard antifungal agent.

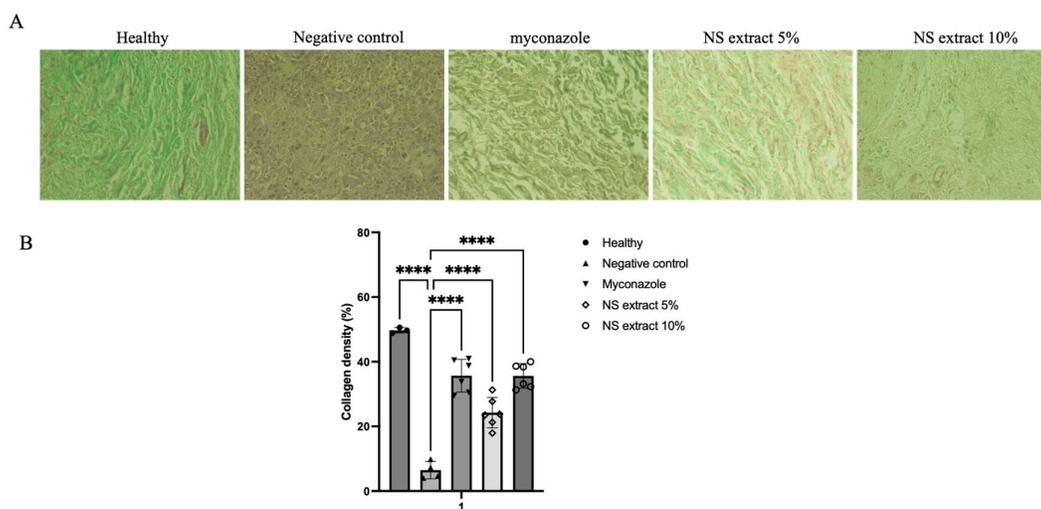


Figure 5 Collagen density was evaluated using Masson's trichrome staining. (A) OE treated with the miconazole and NS extract showed greater collagen deposition and organization as compared to negative control. (B) The percentage of collagen density. The healthy group received no intervention, while the negative control group was induced with OE and did not receive any treatment. The miconazole group served as the positive control, receiving topical miconazole. The NS extract 5 and 10 % groups were administered NS extract in gel form. Values are expressed as mean \pm SD. **** $p < 0.001$ vs. negative control.

Otitis externa (OE), commonly known as swimmer's ear, is an inflammatory condition of the external auditory canal, often induced by bacterial or fungal infections [1,6]. The presence of live bacteria in the exudates of perforated acute otitis media has been associated with a vigorous production of proinflammatory cytokines, regardless of the causative agent [7]. This heightened cytokine response accompanies AOM with membrane rupture, reflecting the significant inflammatory triggers associated with bacterial induction in the middle ear [8]. Previous study reported that *Aspergillus niger* have been isolated from middle ear fluids of immunodeficient patients with chronic suppurative otitis media, further highlighting the potential impact of fungal infections on the inflammatory processes in the ear [2]. The NF- κ B pathway, a key regulator of immune and inflammatory genes in response to infection, has been implicated in the pathogenesis of OE. Both canonical and non-canonical NF- κ B activation have been shown to contribute to the proliferative response of the middle ear mucosa during fungal infection, reflecting the intricate interplay between NF- κ B signaling and the inflammatory processes associated with OE [11,12]. the induction of OE by fungal agents, is closely linked to the activation of inflammatory cytokines and the

NF- κ B pathway [12,30-33]. Understanding the complex interplay between infectious triggers, inflammatory cytokine responses, and the regulatory role of NF- κ B is crucial for elucidating the pathophysiology of OE and may offer potential targets for therapeutic interventions. However, currently topical antibiotics for OE treatment may lead to the development of antimicrobial resistance and local hypersensitivity reactions. Therefore, in this study we elucidated the effect of NS extract on *Aspergillus niger*-induced OE model focusing on the inflammatory responses and NF- κ B pathway.

In this study we found that NS extract, particularly its active component thymoquinone, has demonstrated significant anti-inflammatory and antioxidant effects through the regulation of the NF- κ B pathway on OE model. This study correlated with previous study that thymoquinone dose- and time-dependently reduced the synthesis of TNF- α , IL-1 β , and COX-2, and mediated its effects through reducing the activity and transcription of NF- κ B on pancreatic cancer cells [34]. Similarly, in a mouse model of allergic airway inflammation, thymoquinone was found to inhibit the *in vivo* production of pro-inflammatory mediators, including PGD₂, PGE₂, and TNF- α , through its anti-inflammatory properties [20]. Furthermore, thymoquinone has been shown to reduce the expression of inflammatory factors such as IL-1 β , TNF- α , IFN- γ and IL-6, while alleviating the increase of COX-2 in skin cells induced by TPA, a potent NF- κ B activator [35]. In this study we also found that NS extract induced collagen density in dose-dependent manner. This phenomenon supported by previous study that NS seed extract, demonstrated its ability to modulate collagen cross-linking, collagenase, and elastase activities, indicating its potential in improving collagen density [36]. Additionally, NS and its active compound, thymoquinone, have been reported to accelerate wound healing by stimulating tissue growth, which involves the synthesis of collagen, essential for increasing tissue permeability [37,38]. Furthermore, the effect of NS extract in reducing inflammation and severity in collagen-induced arthritis mice suggests its potential in modulating collagen density in the context of inflammatory conditions [39]. In this study, the 5 % dose of NS extract was more potent compared to the 10 % doses in a otitis externa model. The effects of different doses of NS extract can be influenced by various factors, including the pharmacokinetics of the compounds, bioavailability, and their specific interactions with the molecular pathways involved in the regulation of p50/p65 and TNF- α [40].

These findings collectively suggest that NS extract, exerts its anti-inflammatory effects by suppressing the NF- κ B pathway, leading to reduced expression of key inflammatory mediators. The modulation of the NF- κ B pathway by NS presents promising therapeutic potential in the management of inflammatory conditions, including otitis externa, by targeting the expression of pro-inflammatory cytokines and enzymes involved in the pathogenesis of the disease. Further research and clinical studies are warranted to explore the translational potential of NS extract in the treatment of otitis externa and other inflammatory disorders. The limitation of this study, we found that the 5 % dose of NS extract was more potent compared to the 10 % dose in the OE model. This highlights the need for further investigation into the optimal dosing and potential biphasic effects of NS extract.

Conclusions

In conclusion, the NS extract strongly inhibit inflammatory cytokine including TNF- α , IL-1 β , and COX-2 through MDA ana NF- κ B pathway. The establishment of the *Aspergillus niger*-induced otitis externa animal model and the subsequent evaluation of NS extract's therapeutic effects provide valuable insights into the potential management of this inflammatory condition.

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