

## RESEARCH ARTICLE

## Thymoquinone Modulates Local MMP-9, IL-10, and IgG in Sciatic Nerve Crush Injury Animal Model

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### Abstract

**BACKGROUND:** Interleukin (IL)-10 is involved in Wallerian degeneration after peripheral nerve crush injury. Oral thymoquinone was previously observed to decrease local immunoglobulin-G (IgG) in a crush-injured rat model. No study has evaluated the pathway of various thymoquinone dosages on local IgG and IL-10 in this injury.

**METHODS:** This experimental study used 126 *Rattus norvegicus* Wistar rats that were divided into 18 groups: six groups received a placebo, the other six groups received thymoquinone at 100 mg/kg/day and the last six groups received thymoquinone at 250 mg/kg/day, respectively. Rats were sacrificed at 12, 18, 24, 5x24, 6x24, and 7x24 hours. Matrix metalloproteinase-9 (MMP-9), IL-10, and local IgG levels were assessed by Enzyme-Linked Immunosorbent Assay (ELISA). The nuclear factor KappaB

(NF-κB) expressions on Schwann cells were examined by flow cytometry. Path analysis was performed using SmartPLS.

**RESULTS:** The path analysis showed that 100mg/kg/day of thymoquinone significantly decreased NF-κB expression. However, NF-κB did not affect local MMP-9, and MMP-9 had no significant relationship with local IL-10 and IgG. Thymoquinone 250 mg/kg/day also significantly inhibited NF-κB expression, decreased local MMP-9, and, in turn, decreased local IL-10 and IgG.

**CONCLUSION:** Administration of oral thymoquinone 250 mg/kg/day decreases local IgG and IL-10 levels via suppressing NF-κB expression and MMP-9 levels.

**KEYWORDS:** thymoquinone, crush injury, IgG, IL-10, MMP-9, NF-κB

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### Introduction

Crush injury is an injury to the peripheral nerve induced by severe trauma and compression by a blunt object.(1) About 2.8% of trauma patients exhibited peripheral nerve damage.(2) The sciatic nerve is the peripheral nerve of the

lower extremity most frequently injured by trauma, with a prevalence rate of 30%, while fracture and perioperative events cause 7.9-75% and 5-15%, respectively.(3,4) Several sensory and motor functional rehabilitation treatments for peripheral nerve damage, including surgery, medication, electrical stimulation, and herbal treatment, show unsatisfactory results. Recovery from peripheral nerve

crush injuries is frequently inadequate and can take several months and years.(5-7)

Wallerian degeneration, axonal regeneration, and functional reinnervation of end organs are the three processes necessary for recovery from a peripheral nerve crush injury. Wallerian degeneration must occur before axonal regeneration since it creates a milieu favorable to axon development and end-organ reinnervation.(1,8) This degeneration involves the production of matrix metalloprotease 9 (MMP-9) by Schwann cells and macrophages, which compromises the blood-nerve barrier, allowing macrophage infiltration and subsequent myelin degradation.(9,10) Distal-lesion immunoglobulin G (IgG) localization findings characterized the involvement of adaptive immune response in Wallerian degeneration. A prior study found that local IgG findings negatively correlated to neuronal regeneration.(11) Schwann cells induce a C-C motif chemokine ligand 2 (CCL-2) regulatory signal that stimulates M2 macrophage polarization. These M2 macrophages will increase the expression of interleukin (IL)-10, a cytokine that regulates the inflammatory response. IL-10 can further inhibit MMP-9 synthesis, reduce macrophage infiltration and myelin destruction, increase immunoglobulin production, and limit T-cell activation and differentiation to suppress the adaptive immune system. (9,10,12-14) Meanwhile, nuclear factor kappaB (NF- $\kappa$ B) activation during neuroinflammation also induces the release of tumor necrosis factor (TNF)- $\alpha$  and different ILs, including IL-6 and IL-1 $\alpha$ , while reducing the secretion of IL-10.(15,16)

Thymoquinone is one of the herbal treatments studied to enhance motor performance following sciatic nerve crush injury.(17) Thymoquinone has an anti-inflammatory feature on Schwann cells and macrophages. The anti-inflammatory effect results from decreased NF- $\kappa$ B activation and cytokine production suppression.(18) Our earlier study showed that crush-injured rats treated with thymoquinone significantly decreased local IgG antibody levels.(19) A study previously employed 100 mg/kg of thymoquinone as a successful oral dosage in a Wistar rat model of neuropathic pain.(20) Another study indicated that the maximal oral intake of thymoquinone for rats of both genders is 250 mg/kg.(15) To the best of our knowledge, there has been no research addressing the pathway mechanism of 100 mg/kg/day and 250 mg/kg/day of thymoquinone on local MMP-9, IL-10, and IgG levels, critical components of the immune system in peripheral nerve damage, hence we conducted this study to analyze the mechanism.

## Methods

### Animals Models

One hundred twenty-six male Wistar rats (*Rattus norvegicus*) aged 18 to 20 weeks and weighing 300 to 350 grams were maintained under Institutional Animal Care and Ethics Committee standards. All rats received food and drink *ad libitum* and were identically kept in a cage to minimize environmental confounders. The rats were separated into three groups: Group A (A1, A2, A3, A4, A5, and A6 subgroups) that received corn oil as a placebo treatment, Group B (B1, B2, B3, B4, B5, and B6 subgroups) that received 100 mg/kg/day of thymoquinone, and Group C (C1, C2, C3, C4, C5, and C6 subgroups) that received 250 mg/kg/day of thymoquinone. Each subgroup consisted of 7 rats.

The Sciatic Functional Index (SFI) was evaluated on all rats the day before the surgery. Prior to the crush injury treatment, 100 mg/kg of ketamine and 10 mg/kg of xylazine was injected intraperitoneally into the rats to induce anesthesia.(21) After making a small incision and carefully separating the surrounding tissue, the sciatic nerve was identified 10 mm above the left sciatic nerve's trifurcation. Kelly hemostatic forceps were used to compress the nerve for 30 seconds.(22) The procedure had been approved by the Health Research Ethical Committee of Airlangga University's Faculty of Medicine in Surabaya (No. 4/EC/KEPK/FKUA/2021).

### Thymoquinone Preparation

The thymoquinone was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, United States) with a purity of 98%. It was dissolved in corn oil before being administered to rats. Thymoquinone was administered orally daily using an orogastric tube. As soon as the anesthesia from the procedure wore off, the group-specific dose was administered once daily until sacrificed.

### Animal Termination and Sample Preparation

Animal euthanasia was performed at the end of the observation period and performed by administering an overdose of pentobarbital (300mg/kg) intraperitoneally.(21) Each subgroup had a different termination time. Subgroup code 1, 2, 3, 4, 5, and 6 was terminated 12, 18, 24, 5x24, 6x24, and 7x24 hours after the crush injury, respectively. Samples of injured sciatic nerve tissue were taken 5 mm distal to the lesion based on the procedure reported by a previous study.(23)

### Measurement of MMP-9, IL-10 and IgG

The 600 L PRO-PREP solution was used to homogenize the sciatic nerve tissue. In addition, 20-30 minutes of incubation at  $-20^{\circ}\text{C}$  in a refrigerator caused cell lysis. For five minutes, centrifugation was done at 13,000 rpm and  $4^{\circ}\text{C}$ . The sample's supernatant was then poured into a 1.5 mL tube. Enzyme-Linked Immunosorbent Assay (ELISA) was used for all protein measurement procedures based on manufacturer instruction (Elabscience, Wuhan, China). The optical density value on each well was calculated using a micro-plate reader on 450nm.(24)

### Measurement of NF- $\kappa$ B on Schwann Cells

The sciatic nerve was crushed, and 1-2 mL of phosphate buffered saline (PBS) was added. The nerve section was ground to a homogeneous suspension and continued with the addition of 10 mL of PBS to rinse off the grind. The cell suspension was collected by first filtering using a 200-mesh cell strainer. The centrifugation on 300 g was conducted for 5 minutes at room temperature, and the supernatant was removed. Cells were rinsed with PBS and combined with 0.1% bovine serum albumin (BSA) 2 times. This process was repeated several times until the final sample concentration was  $10^6$  cells/ $\mu\text{L}$ . The sample required for flow cytometry was 50  $\mu\text{L}$  and homogenized with NaCl 0.9% followed by 50  $\mu\text{L}$  PBS. As much as 2.5  $\mu\text{L}$  PerCP/Cyanine 5.5 anti-rat CD45 Antibody (Biolegend, San Diego, CA, USA) was then added to the tube.

After 15 minutes of incubation in the dark at room temperature, 1 mL of lysing solution was added and slowly mixed. The tubes were incubated in the dark at room temperature for another 10 minutes before being centrifuged at 1200 rpm for 5 minutes. Cytofix/cytoperm solution 250  $\mu\text{L}$  was then added to the tube, which was then incubated for 20 minutes at  $4^{\circ}\text{C}$  in a dark room. One mL of perm wash reagent was added to the tube and then centrifuged at 1200 rpm for five minutes, and the supernatant was removed. A 2.5  $\mu\text{L}$  rat NF- $\kappa$ B p65 antibody was added. The tube was incubated at  $4^{\circ}\text{C}$  for thirty minutes, centrifuged at 1200 rpm per minute for another five minutes, and then the supernatant was removed. Formaldehyde 1% was added to 500  $\mu\text{L}$ . The tubes were incubated at  $4^{\circ}\text{C}$  for thirty minutes. The readings were conducted using the BD FACSCalibur™ Flow Cytometer (BD, Franklin Lakes, NJ, USA).

### Statistical Analysis

Descriptive data was presented in table and line graphs. Data distribution normality was evaluated with the Shapiro-Wilk test. As the analysis showed that the data were not normally

distributed, this study would present the data as the median (min-max). The analysis was performed with SPSS Statistics for Windows, Version 25.0 (IBM Corporation, Armonk, NY, USA). This study performed path analysis using multiple linear regression tests to determine the pathway mechanism. Path analysis was conducted with SmartPLS.

## Results

Median data of NF- $\kappa$ B, MMP-9, and IL-10 level in each termination period was presented in Table 1 and Figure 1. Rats that were given 100 and 250 mg/kg of thymoquinone had lower NF- $\kappa$ B levels compared to rats that were given placebo at 12 hours, 18 hours, and 24 hours after injury, suggesting that the administration of 100 and 250 mg/kg of thymoquinone were able to decrease NF- $\kappa$ B levels. MMP-9 levels in the group that were given 100 and 250 mg/kg of thymoquinone were lower than the placebo group with a similar pattern, except at 18 hours. Likewise, the groups that were given 100 and 250 mg/kg of thymoquinone also showed decreased IL-10 levels than the placebo group, except at 18 hours, the level where the placebo group sharply decreased.

Path analysis using a linear regression test showed that oral administration of 100 mg/kg/day of thymoquinone significantly decreased NF- $\kappa$ B expression ( $\beta=-0.476$ ;  $p=0.007$ ). However, NF- $\kappa$ B did not significantly affect MMP-9 ( $\beta=-0.200$ ;  $p=0.188$ ). This level did not have significant association to local IgG ( $\beta=-0.174$ ;  $p=0.236$ ) nor IL-10 ( $\beta=0.216$ ;  $p=0.165$ ) (Figure 2).

Similar to 100 mg/kg/day thymoquinone, 250 mg/kg/day of orally administered thymoquinone also significantly decreased NF- $\kappa$ B expression. The inhibition of NF- $\kappa$ B expression could reduce MMP-9 level significantly ( $\beta=-0.328$ ;  $p=0.046$ ). The decreased level of MMP9 will lead to decreased level of local IgG ( $\beta=-0.485$ ;  $p=0.001$ ) and IL-10 ( $\beta=-0.418$ ;  $p=0.023$ ) (Figure 3).

## Discussion

This study demonstrated that 100 and 250 mg/kg of thymoquinone significantly lowered NF- $\kappa$ B expression in Schwann cells. In the subsequent path analysis, however, it was determined that in Schwann cells, only decreased NF- $\kappa$ B induced by 250 mg/kg of thymoquinone could further significantly lower the MMP-9 level. This observation is consistent with the finding that decreased MMP-9

**Table 1. The NF-κB expression, MMP-9 level, and IL-10 levels among rats in each group.**

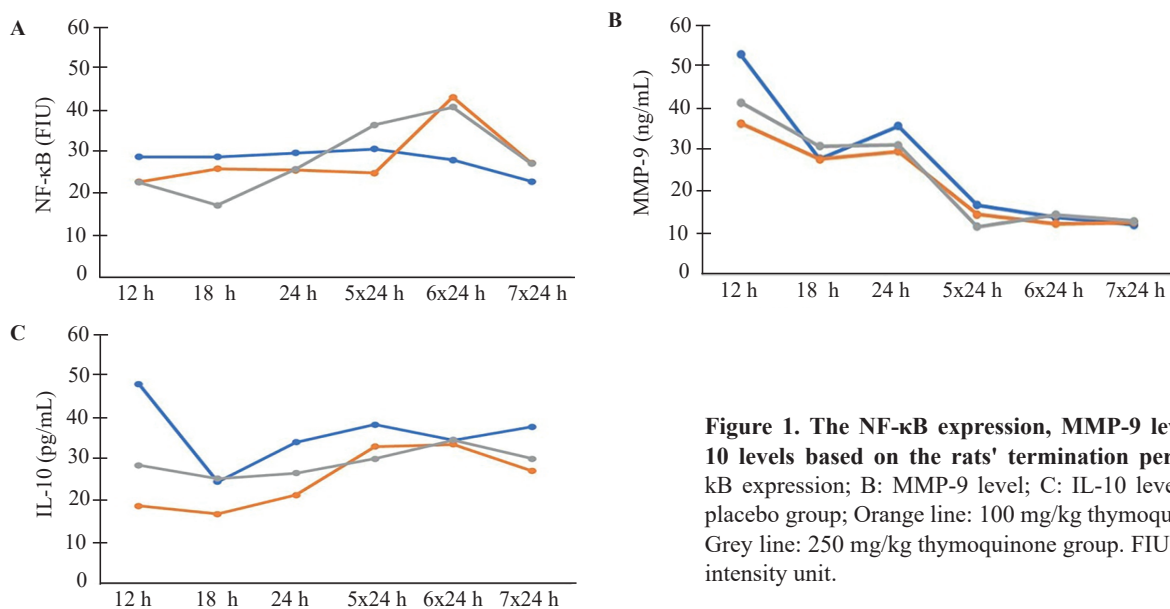
Termination Period	Variable	Median (Min – Max)		
		Placebo	100 mg/kg Thymoquinone	250 mg/kg Thymoquinone
12 hours	NF-κB (FIU)	28.36 (24.76– 34.57)	22.47 (17.78 – 30.23)	22.34 (18.65 – 30.31)
	MMP-9 (ng/mL)	52.77 (34.46 – 99.29)	36.28 (17.72 – 41.32)	41.32 (26.10 – 65.46)
	IL-10 (pg/mL)	46.24 (23.27 – 64.34)	18.10 (6.10 – 34.81)	27.44 (17.28 – 41.67)
18 hours	NF-κB (FIU)	28.39 (17.47 – 31.91)	25.57 (22.88 – 38.89)	16.94 (14.07 – 19.28)
	MMP-9 (ng/mL)	27.83 (19.86 – 39.59)	27.68 (18.86 – 51.52)	30.88 (26.77 – 39.49)
	IL-10 (pg/mL)	23.63 (13.21 – 32.52)	16.18 (8.13 – 29.47)	24.39 (7.88 – 48.02)
24 hours	NF-κB (FIU)	29.32 (24.58 – 37.18)	25.25 (16.70 – 48.26)	25.48 (22.88 – 62.64)
	MMP-9 (ng/mL)	35.66 (17.65 – 50.09)	29.56 (14.10 – 45.53)	31.07 (23.75 – 76.88)
	IL-10 (pg/mL)	32.77 (16.26 – 44.97)	20.58 (18.04 – 44.46)	25.66 (12.45 – 46.75)
5x24 hours	NF-κB (FIU)	30.27 (26.18 – 51.40)	24.58 (21.10 – 34.29)	35.92 (31.62 – 63.21)
	MMP-9 (ng/mL)	16.70 (12.26 – 71.17)	14.51 (12.46 – 15.27)	11.59 (8.78 – 15.89)
	IL-10 (pg/mL)	36.84 (22.10 – 71.93)	31.76 (7.37 – 59.96)	28.96 (22.61 – 35.06)
6x24 hours	NF-κB (FIU)	27.63 (23.29 – 50.940)	42.54 (40.68 – 55.23)	40.16 (37.52 – 56.23)
	MMP-9 (ng/mL)	13.84 (11.23 – 24.97)	12.31 (10.01 – 15.70)	14.45 (11.69 – 18.46)
	IL-10 (pg/mL)	33.28 (28.71 – 99.06)	32.27 (23.12 – 42.43)	33.28 (26.42 – 41.41)
7x24 hours	NF-κB (FIU)	22.58 (17.00 -30.78)	26.89 (22.67 -57.25)	26.79 (19.46 – 50.94)
	MMP-9 (ng/mL)	12.00 (9.96 – 15.74)	12.61 (8.68 – 16.07)	12.92 (10.21 – 15.43)
	IL-10 (pg/mL)	36.33 (15.50 – 44.72)	26.17 (17.02 – 35.06)	28.96 (22.61 – 35.06)

FIU: Fluorescent intensity unit.

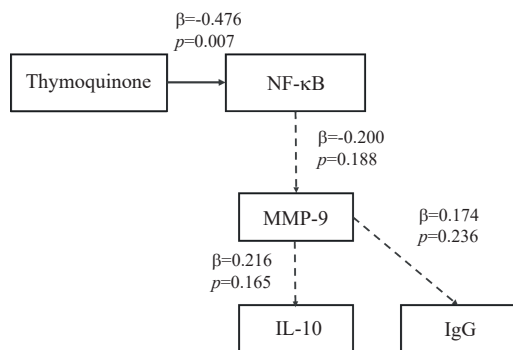
expression or production results from NF-κB inhibition.(25) This drop in the MMP-9 level also resulted in a substantial reduction in IgG levels.

Schwann cells and macrophages are two crucial innate immune cells implicated in Wallerian degeneration.(26) As an initial response to crush injury, Schwann cells have been discovered to activate the transcription factor NF-κB. Activated NF-κB induces pro-inflammatory cytokines such as cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and prostaglandin E synthase-1, while in Schwann cells, it can promote TNF-α and boost

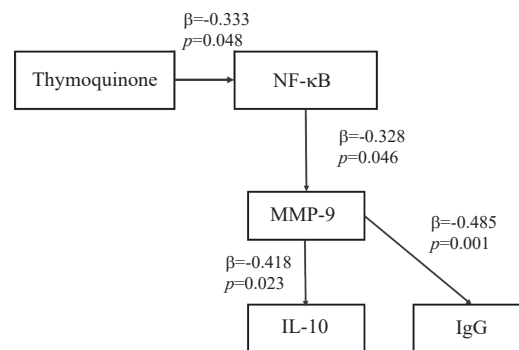
the activity of MMP-9. Thymoquinone decrease NF-κB-dependent neuroinflammation in microglia by reducing iNOS protein levels, NF-κB inhibitor phosphorylation, and NF-κB DNA binding.(15) In lowering the phosphorylation of NF-κB, thymoquinone can inhibit interleukin-1 receptor-associated kinase 1 (IRAK1) either directly or indirectly by suppressing the ubiquitylation of IRAK1 and TNF receptor-associated factor 6 (TRAF6) complex. IRAK1 is a critical component between toll-like receptors (TLR) stimulation as tissue injury response and subsequent NF-κB activation in macrophages.(27)



**Figure 1. The NF-κB expression, MMP-9 level, and IL-10 levels based on the rats' termination period.** A: NF-κB expression; B: MMP-9 level; C: IL-10 level. Blue line: placebo group; Orange line: 100 mg/kg thymoquinone group; Grey line: 250 mg/kg thymoquinone group. FIU: Fluorescent intensity unit.



**Figure 2. Pathway analysis for the oral administration of 100 mg/kg/day of thymoquinone.** Solid arrow: statistically significant; Dashed arrow: statistically insignificant.



**Figure 3. Pathway analysis for the oral administration of 250 mg/kg/day of thymoquinone.** Solid arrow: statistically significant; Dashed arrow: statistically insignificant.

The NF- $\kappa$ B-mediated neuroinflammation mechanism was believed to be thymoquinone-induced activation of the Nrf2/ARE signaling.(15) Another study believed that thymoquinone acts at two levels in the NF- $\kappa$ B signaling pathway: directly interacting with the p65 subunit and suppressing TNF-induced inhibitor of NF- $\kappa$ B kinase (IKK) activation.(28) The potential inhibition of Schwann cell NF- $\kappa$ B expression by oral thymoquinone 250 mg/day given as soon as possible favors nerve regeneration. A prior study found that despite suppressing NF- $\kappa$ B activation, this would be followed by a significant increase in motor neuron regeneration on day 65 after nerve injury.(29)

As previously mentioned, MMP-9 is a fascinating member of the MMP family that is predominantly discovered in injured adult Schwann cells. Other cells, including endothelial and immune cells, release MMP-9 (gelatinase B) in response to damage to regulate blood-nerve barrier breakdown, immune cell recruitment, glial activation, de- and re-myelination, and also pain. Due to MMP-9 modulation of myelin protein degradation and macrophage migration into injured sciatic nerves, MMP-9 knocked-out mice are notably protected from peripheral Wallerian degeneration.(30) Similar to our findings, prior studies have shown that thymoquinone may inhibit MMP-9 synthesis in several settings, including neuroblastoma cell lines but not in vascular smooth muscle cells.(31,32) Specifically, one study discovered that treatment with thymoquinone in a polycystic ovary rat model blocked NF- $\kappa$ B-mediated reductions in MMP-9 levels, lowering its activity.(33) This result was comparable to our study finding in 250 mg/kg of thymoquinone administration on crush injuries.

This study demonstrated that thymoquinone 250 mg/kg/day decreased local IgG levels via decreasing MMP-9 levels. It is believed that MMP-9 has a role in the recruitment of macrophages to the lesion site. M1 macrophages generate

the cytokines IL-23 that induce autoantibodies.(34,35) The presence of autoantibodies in the deteriorated nerve is indicated by the presence of IgG antibodies in the distal section of the damaged nerve six days after crush injury.(36) This local finding of IgG negatively correlates with neuron regeneration, as elevated local IgG concentrations impair nerve regeneration.(11) Administration of 250 mg/kg of thymoquinone was also demonstrated to significantly reduce IL-10 levels by inhibiting NF- $\kappa$ B expression and decreasing MMP-9 levels. IL-10 is a potent broad-spectrum that release anti-inflammatory cytokine implicated in various inflammatory and autoimmune diseases.(37) It protects the nerve by reducing neuroinflammation apoptosis via NF- $\kappa$ B suppression promoting tissue recovery such as axon regeneration and improving of motor and sensory function.(38,39) However, thymoquinone administration in our study decreases the IL-10 level instead of increasing it. The previous study's finding might explain our results. They observed that various injuries type could create distinct IL-10 characteristics, in which more prolonged exposure may induce a more robust response, resulting in a higher amount of IL-10. Meanwhile, the IL-10 level was significantly decreased in partial ligation of the sciatic nerve group.(40) This study only has short observation period and limited examined parameter, hence it is necessary to also examine other related parameters that might be related, such as IL-23 or IL-6.

## Conclusion

Both oral administration of 100 and 250 mg/kg thymoquinone significantly decreases NF- $\kappa$ B expression. However, only oral administration of 250 mg/kg/day of thymoquinone, that were given immediately after peripheral nerve crush injury,

can significantly further decreases local IgG and IL-10 levels via inhibiting NF- $\kappa$ B expression and MMP-9 levels. More comprehensive studies with different types of injury might be helpful to confirm our results. Future pathway analysis is required and expected to include other variables that may play a role in the adaptive immune system, such as apoptotic regulators.

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## Authors Contribution

VB, AHB, and MHM were involved in the research's planning and supervision. VB, JN, and PBN, IKS, and MA participated in sample processing and measurement. VB and PBN participated in data processing, statistical analysis and computations, as well as table and figure compilation. VB, AHB, JN, IKS, MA, and N contributed in drafting the manuscript. VB, AHB, JN, IKS, MA, PBN, and N contributed to the discussion of the results and critical suggestions for the manuscript.

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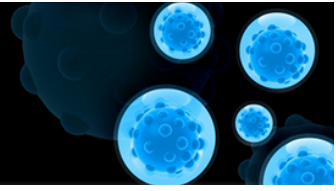
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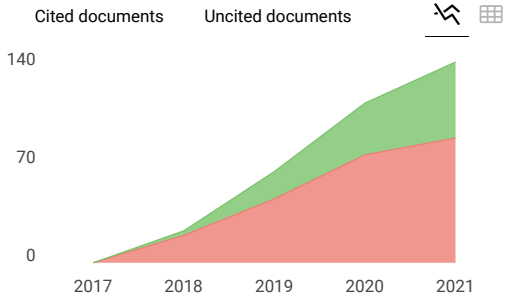
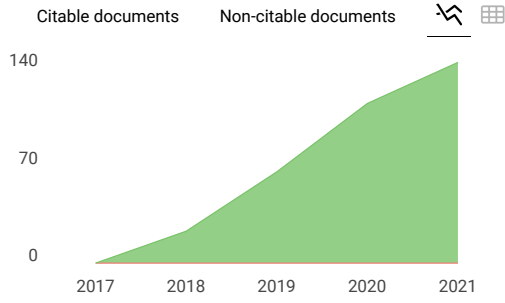
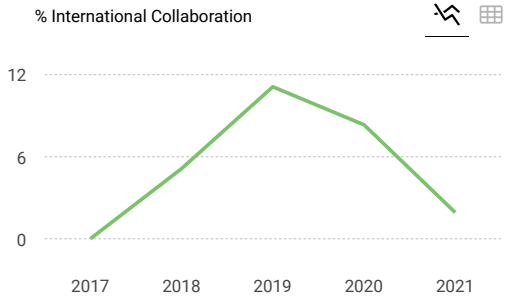
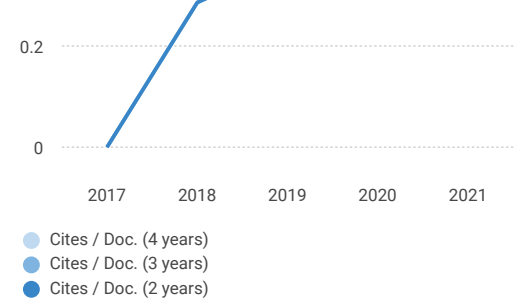
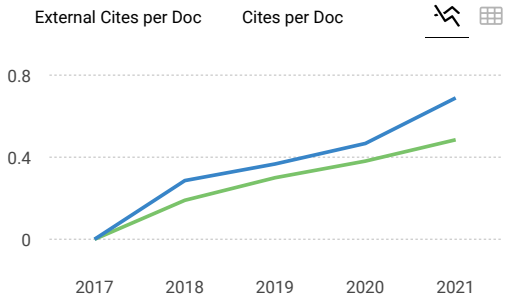
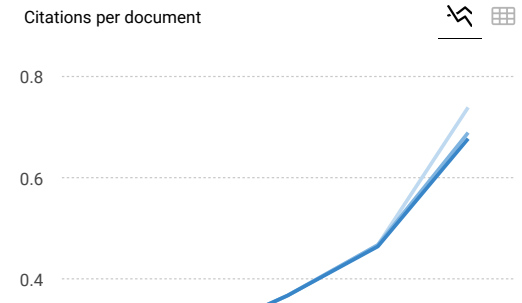
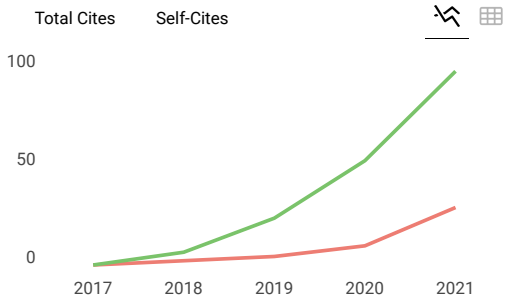
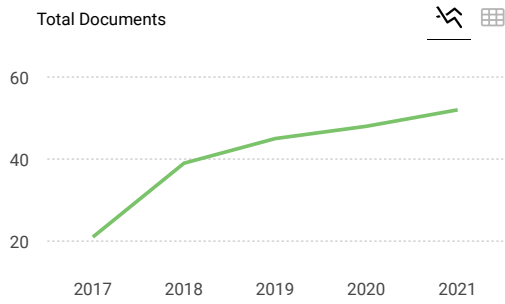
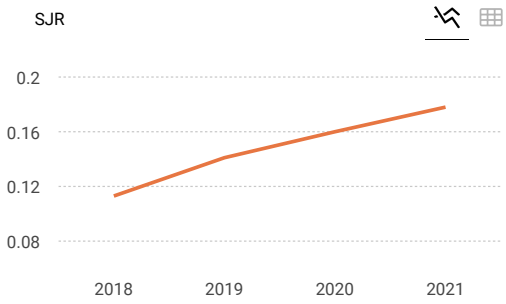
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