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Salvadora persica L leaf fraction inhibits inflammation by targeting active IL-1β release and GSDMD cleavage in LPS-stimulated THP-1 cells

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Abstract

Salvadora persica L (Sp) plant has been widely used in traditional African medicine to treat inflammation pulmonary diseases such as chronic obstructive pulmonary disease and asthma. However, the underlying mechanism remains incomprehensive. NLRP3 is the inflammasome complex proteins located in the cytoplasm and the primary key activator of chronic inflammation diseases via hypersecretion of Interleukin-1β (IL-1β) and Gasdermin D (GSDMD) cleavage. This present study aims to clarify the effects of flavonoid-rich fraction from Sp leaves (S1TF7) against IL-1β and GSDMD released through NLRP3 activated pathway in LPS-stimulated THP-1 cell. High-performance liquid chromatography (HPLC) analysis was performed to identify and quantify the major flavonoids components in S1TF7. Then, RT-PCR and Western blot (Wb) analysis were used for pharmacology tests. HPLC analysis results reveal two flavonoids with high content, rutin = 148.78±0.17µg/mg being the highest, followed by quercetin = 127.16±0.78µg/mL (Table 2). In LPS-stimulated THP-1 cells, RT-PCR analysis results showed that S1TF7 significantly inhibits the mRNA expression ofIL-1β active, GSDMD cleavage and NLRP3 activation in a dose-dependent manner ($p < 0.05$). The results of WB analysis also showed that S1TF7 decreases the protein level of active IL-1β, GSDMD cleavage and NLRP3 activation in a dose-dependent manner ($p < 0.5$).Furthermore, rutin (50 µg/mL)

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showed a similar effect to that of S1TF7 at 200 µg/mL ($p > 0.5$).The potential inhibitory effect of S1TF7 against the production of inflammatory cytokines, IL-1β and GSDMD could be associated with high content of rutin and quercetin present in Sp leaves. In vivo model and clinical studies are necessary in order to produce improved phytomedicine without toxicity..

Keywords: *Salvadora persica* L; inflammation; IL-1β; GSDMD; NLRP3.

1. Introduction

Inflammation is the first reaction of the innate immune system in response to the various exogenous and endogenous aggressors (microbial infections and toxins) (Pinkerton et al. 2017). However, excessive inflammation induces chronic inflammation associated with NLRP3 inflammasome. Studies show that NLRP3 is the inflammasome complex proteins located in the cytoplasm and the primary key activator of chronic inflammatory diseases such as chronic obstructive pulmonary disease (COPD) severe asthma and Covid-19 (Jin et al. 2023; Kaufmann et al. 2024; Kim et al. 2015; Osei et al. 2020). Previous studies reported that NLRP3 activation promotes host system immunity in response to various exogenous and endogenous aggressors. Nevertheless, excessive stimuli of microbial infections and toxins lead to NLRP3 assembled inflammasome complex that induces the caspase-1 activation. Hence, caspase-1 active induces the maturation of IL-1β directly into its active form (Kelley et al. 2019). Uncontrolled or excess IL-β production plays a pivotal factor in developing chronic inflammatory diseases such as COPDand neutrophilic asthma (Kim et al. 2015; Theofani et al. 2019). Several studies reported, NLRP3 assembled at caspase-1 induces inflammation and programmed cell death, called pyroptosis, by releasing IL-1β and GSDMD (Kesavardhana, Malireddi, and Kanneganti 2020; Yuk, Silwal, and Jo 2020; Zheng et al. 2020).

The NLRP3 is activated through two signal pathways (Sollberger et al. 2014; Theofani et al. 2019). In response to the pathogens, signal one (inactive) pathway induces the secretion of NLRP3 component, pro-Interleukin-1β (pro-IL-1β) and pro-Interleukin-18 (pro-IL-18) through toll-like receptors/Nuclear Factor-kappa B pathway. In signal two (active) pathway, NLRP3 component assembled in complex-Apoptosis-associated Speck-like protein containing a Caspase (ASC/protein) induced the pro-caspase1 into caspase1-mature. The caspase1-active induces the hypersecretion of IL-1β active and GSDMD cleavage mature that increase the risk of chronic inflammation and pyroptosis (Im and Ammit 2014; Kelley et al. 2019; Yuk et al. 2020; Zheng et al. 2020). Pyroptosis is a cell programmed death which is different from other cell programmed death such apoptosis and necrosis (Bergsbaken, Fink, and Cookson 2009; Kesavardhana et al. 2020). Also, excessive mitochondrial activity and mitochondrial dysfunction leads to the generation of reactive oxygen species (mtROS) that activates the hypersecretion of caspase1 directly or via NLRP3 activation, which are the promoters of chronic inflammation and pyroptosis (Harijith, Ebenezer, and Natarajan 2014; Li et al. 2018; Yu et al. 2024).Furthermore, the hypersecretion of IL-1β active and GSDMD cleavage was reported as the high-risk factor that causes neutrophilic inflammation and pyroptosis, which are common in COPD, neutrophilic asthma, and Covid-19 cases (Gao et al. 2015; Hosseinian et al. 2015; Mason 2020; Queiroz et al. 2020).

Recently, the MCC950 compound was reported to promote NLRP3 inhibition and reduce IL-1β secretion greatly, but it promotes lung infection risk (El-Sharkawy, Brough, and Freeman 2020). The treatment of respiratory diseases linked with the secretion of IL-1β release, neutrophilic asthma, chronic obstructive pulmonary diseases, and Covid-19 is too expensive. Therefore, further study is essential to discover potent anti-inflammatory components through IL-1β inhibition associated with NLRP3 that may have less side effects and be less expensive in treatment. Previous studies reported that

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medicinal plants are sources of drugs used in treating inflammatory diseases, such as asthma, COPD, and Covid-19 (Leu, Chen, and Guh 2019; Lv, Chen, and Wang 2017; Owis et al. 2020). Phytochemistry study of the Sp revealed rutin, catechin, gallic acid, chlorogenic acid, catechin, caffeic acid, quercetin and biochanin (Noumi et al. 2017; Saeed et al. 2013). Also, flavonoid-rich of Sp significantly inhibits the protease of Covid-19 (Owis et al. 2020).

However, the effect of Sp leaves against inflammation has not been clarified yet. Therefore, we hypothesized that the flavonoid-rich fraction of Sp leaves (S1TF7) could inhibit inflammation by targeting the release of active IL-1β and cleavage of GSDMD stimulated by the activation pathway of NLRP3 in THP-1 cells. These inhibitory effects could be justified by the high content of flavonoid compounds in Sp leaves.

The present study aimed to: i) Identify and quantify the major flavonoids components in the flavonoid-rich-fraction of Sp leaves (S1TF7), ii) Evaluate the effects of S1TF7 on IL-1β active, GSDMD cleavage and NLRP3 activation in LPS stimulated THP-1 cells.

2. Materials and Methods

2.1. Plant Material

Salvadora persica (Salvadoraceae) leaves were obtained from Abdou Moumouni University of Niamey, Republic of Niger. The voucher specimen (N^0S.persica-2015/Niger) was deposited at the herbarium of the Biology Laboratory of Niamey University 10662, Niger.

2.2. Flavonoid-rich fraction extraction

Dried powder of Sp leaves (500g) was dissolved in distilled water (w/v: 1g/10mL), and refluxed for one hour. After filtration, water extract was subjected to the chromatography column which contained resin D101, and eluted with a stepwise gradient of 10% and 20% (v/v) of ethanol in distiller water to remove impurities. After subjecting 70% ethanol/water, the flavonoid-rich fraction from Sp leaf (S_1TF_7) was obtained, and all solvents were evaporated. The dried of the S_1TF_7 was pre-stored at -20° C until use.

2.3. HPLC-DAD analysis

The standard references such as rutin and quercetin compounds obtained from Nat-Inst (Beijing China) were used to characterize the components in S_1TF_7. In this study, the identification was performed using High-performance liquid chromatography and a Diode Array Detector (HPLC-DAD, Shimadzu) analysis on the Chromsil^{TM} C18 column (4.6 mm × 250 mm, 5 µm, SinoChrom, Dalian, China).

Thus, the identification was stabilized by the comparison of the retention time and peak area of each of the compounds with their standards. The 0.1% aqueous formic acid solution (A) and acetonitrile (B) were used as the mobile gradient phases. The flow rate at 1mL/min, injection volume (10 µL), and column oven temperature (30 °C) were used. The wavelength detected was set at 330 nm. The condition of separation was established from 0 to 25min, 14.5%-15% B; 25min-26min, 15%-17% B; 26min-30min, 17%-20% B; 30min-37min, 20%-24% B; 37min-37.01min, 24%-26% B; 37.01min-50min, 26%-28% B; 50min-55min, 28% B; 55-60min, 14.5%B and 60-65min, 14.5% B. And at 280-330 nm, wavelengths were recorded.

2.4. Cell culture

We have received the Human monocyte cells line (THP-1) from Shanghai Institutes for Life Science (Shanghai-China). The cells were incubated in RPMI-1640 medium with 10% of fetal bovine serum and cultured under a 5% CO2 atmosphere and 37°C during all experiments.

2.5 Preparation of different cell-groups and treatments

We have seeded the THP-1 Cells into different six-well plates (2×10^{6} per well or approximately 80% of confluence) containing culture medium.

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We have determined the dose of lipopolysaccharides (LPS) that significantly induced the secretion of inflammatory mediators and pyroptosis in THP-1 cell. Thus, we made three groups of LPS (0.2, 2, and 4 µg/mL) for 6 hours of incubation and those not treated with LPS as placebo or blank.

Furthermore, we made five groups of cells: group-1 (blank or untreated cell) as normal cell in culture medium without LPS and drug treatments, group-2 (LPS cell-group or negative control) is the stimulated cells with 2 µg/mL of LPS only for 6 hours, group-3 and group-4 (S_1TF_7 cell-groups) are the pretreated cells with S_1TF_7 at 50 and 200 µg/mL respectively for 12 hours and group-5 (rutin as positive control) are then treated cells with rutin at 10 µM for 12 hours. Then, group-3, group-4 and group-5 were stimulated with 2 µg/ml of LPS and incubated for 6 hours again. At the end of the incubation at 37 °C and 5% CO_2, all cell-groups were collected for further analyses.

2.6. Real-time PCR analysis

For PCR analysis, the total RNA quantity of each cell group was extracted and purified in Trizol reagent (Ambion, Life Technologies, and USA) following the manufacture's procedure. One (1 µl) of RNA purity was used to obtain cDNA using a premix Script RT Master Mix reverse transcription kit (Tsingke, Biological Technology, China). The program condition was set as follows: 95˚C - 3 min followed by 40 cycles of 95˚C - 30 sec, 60˚C - 30 sec, 72˚C - 30 sec, and extension step of 65˚C - 5 sec. The detection was performed using a CFX Maestro system (Bio-Rad Laboratories, Inc 2017). The target mRNA expression was measured via the following formula: 2-ΔΔCt (Leu et al. 2019). All target gene expressions were normalized using actin, as an internal control. All target gene expressions were normalized using actin, as an internal control.In table 1, the primer fragments were used for PCR analysis.

Table 1. Primer fragments

Targets	Forward fragments: 5' ----------3'	Reverse fragments : 5' ----------3'
Actin	GACAGGTGCAGAAGGAGATTACT	TGATCCACATCTGCTGGAAGGT
NLRP3	TCCTCGGTACTCAGCACTTAATC	CGTGAGGTTGCAGTTGTCTAAT
IL-1β	AAACAGATGAAGTGCTCCTTCCAGG	TGGAGAACACCACTTGTTGCTCCA

2.7. Extraction of protein and Western-blot Analysis

For Western-blot analysis (Wb), each of the cell groups was analyzed in a radio immuno precipitation (RIPA) buffer (Beyotime, Shanghai China). The BCA protein kit protocol (Beyotime, Shanghai, China) was used to measure protein concentration. Then, each group of a sample with an approximately 30 µg of protein was subjected to SDS-PAGE (10%) at 120 V for 100 min. And it was transferred to the PVDF membranes (Millipore Bedford, MA, USA) at 250 mA ice bath film for 90 min. The membranes were then blocked using TBST solution container 5% of BSA for 2 hrs. The specific primary antibodies include anti-NLRP3 (1:1000), anti-IL-1β(1:1000), anti-AMPK (1:1000), and anti-actin (1:1000) (Beyotime, Shanghai-China) were used to incubate the membranes at 4°C overnight. The membranes were then washed with mixture of tris-buffered saline (TBST) and were incubated again with conjugated secondary antibody for 75 min at room temperature. The band density variation was determined as fold changes compared with the control in the blot after

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normalized to β-actin. Quantity Ones 4.4.1 (Bio-Rad Laboratories) was performed to calculate the level expression of proteins.

2.8. Statistical analysis

GraphPad Prism 8.4.3 (686) software was used for statistical analysis. Values were presented as mean ± Standard deviation (n ≥ 3). T-test was used when the comparison was between the two groups. One-way ANOVA was performed for more than two groups. Statistical differences were defined at $p < 0.05$ (# or *), $p< 0.01$ (## or**), $p< 0.001$ (### or ***).

3. Results

3.1. Content of Rutin and Quercetin in S_1TF_7

By the HLPC analysis method, the presence of rutin and quercetin was respectively detected at RT = 29.11/29.18 min and at RT = 50.60/50.80 min in the S_1TF_7 fraction (Fig.1 and Fig.2). Moreover, as shown in Table 2, these two flavonoids have higher content, where rutin = 148.78±0.17µg/mg was highest, followed by quercetin = 127.16± 0.78 µg/mL. These results reveal a high rutin content in this fraction.

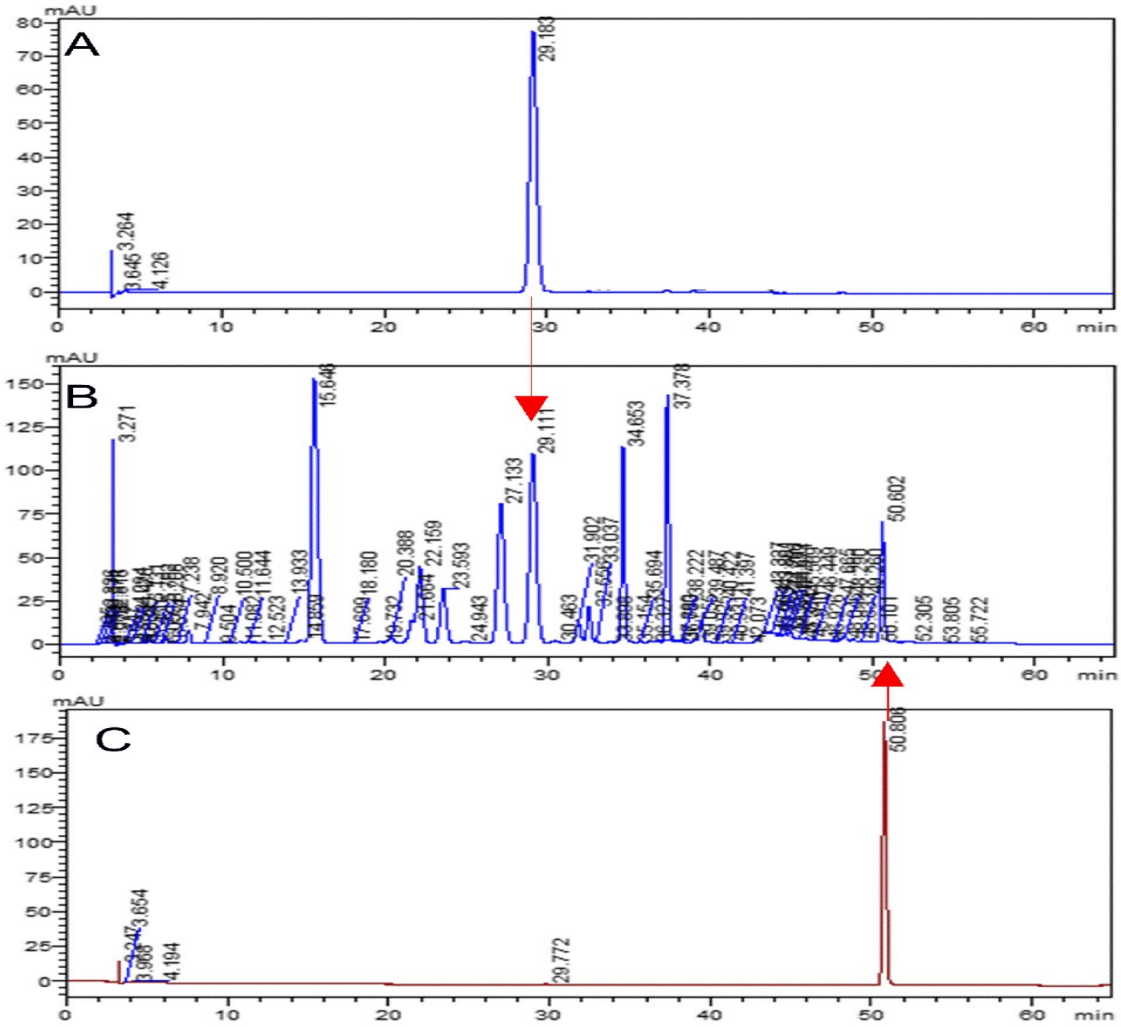

Fig.1 High-performance liquid chromatography (HPLC-LC-20AD, Shimadzu) analysis. **A** = Chromatogram of rutin standard (Rt = 29.18 min), **B** = Chromatograms of S_1TF_7 (rutin identified at Rt = 29.11 min and quercetin at Rt = 50.60 min), **C** = Chromatogram of quercetin standard (Rt = 50.80 min). The identification was based on comparing each compound's retention time and peak area with their standard reference. Detection wavelength (280 nm).

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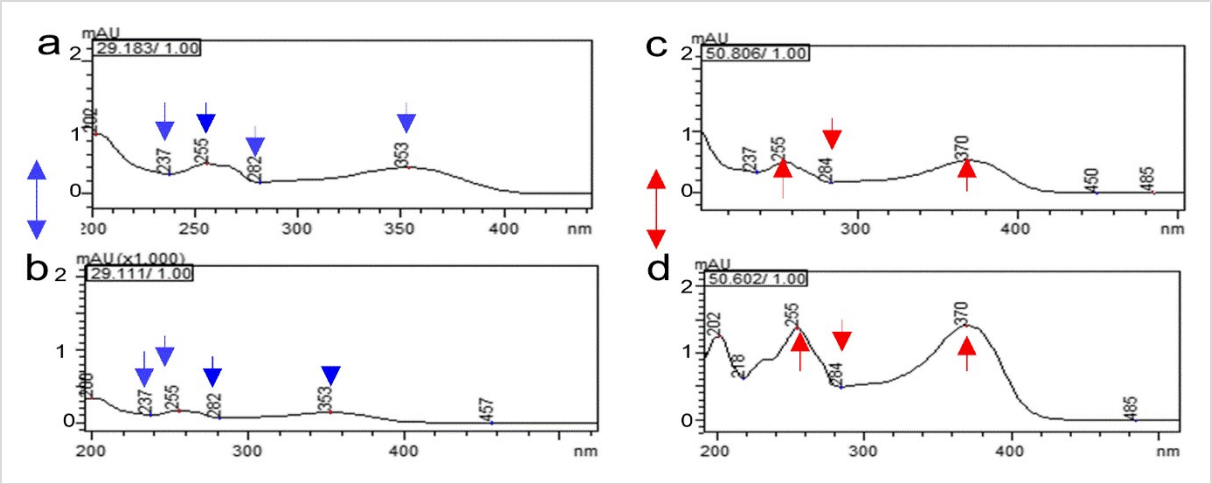

Fig. 2: UV-Spectra of rutin and quercetin in Flavonoid-rich Sp leaves. The identification was based on comparing the absorbance of UV-Spectra and retention time (RT). a = absorbance of rutin standard (RT = 29.18 min), b = absorbance of rutin present in S_1TF_7 (RT = 29.11 min), c =absorbance of quercetin standard (RT = 50.80 min), d = absorbance of quercetin present in S_1TF_7 (RT= 50.60 min). The detection wavelength at 280 nm.

Table 2. Contents of rutin and quercetin in Flavonoid-rich fraction from Sp leaves (µg/mg)

Compounds	Rutin	Quercetin
S1TF7	148.78 ± 0.17	127.16 ±0.78

Rutin and quercetin were quantified using the calibration plot of standards (rutin, y = 13640x - 35262, r^2 = 0.9996 and quercetin, y = 4125.5x - 16852, r^2 = 0.9988).

3.2 LPS induces IL-1β and GSDMD production through NLRP3 activation in THP-1 cells

In PCR analysis, we observed significant increase in level of mRNA NLRP3 expression (Fig.3A) and mRNA IL-1β expression (Fig.3B) with dose-dependent manner when they are compared to the control respectively ($p< 0.05$). Between both dosesof LPS (2 µg/mL and 4 µg/mL), the difference is not significant for the expression of NLRP3 and IL-1β mRNA with $p > 0.05$ (Fig.3A-B). On the other hand, Wb analysis shows a significant increase in GSDMD cleavage protein level in LPS cell groups compared to the control group ($p< 0.001$; Fig.3C). The results reveal that from 2 µg/mL LPS induces significant effects on the activation of NLRP3, active IL-1β and cleavage of GSDMD (Fig.3). Thus, the dose of 2 µg/mL was used to stimulate the production of active IL-1β and the cleavage of GSDMD via activation of the NLRP3 pathway in the THP-1 cell for the pharmacological test.

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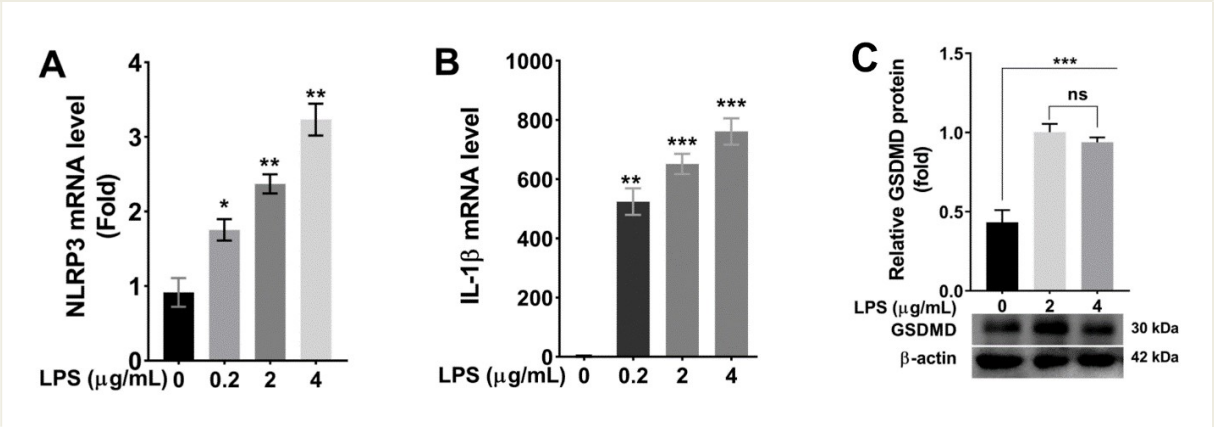

Fig 3. Activation of Inflammasome and cytokines in LSP-stimulated THP-1 cells. The cells were pretreated with different doses (0.2, 2 and 4 µg/mL) of LPS for 6 hours. Cells without LPS treatment were considered as control. In RT-PCR analysis, LPS showed a significant increase in relative mRNA levels of NLRP3 (A) and IL-1β (B) in a dose-dependent manner. Wb analysis was performed to evaluate the expression level of GSDMD cleavage protein (C). Results are means ± STD (n=3); * $p< 0.05$, ** $p < 0.01$ and *** $p< 0.001$ versus control.

3.3 Effect of S_1TF_7 against IL-B and GSDMD production in LSP-stimulated THP-1 cells

In RT-PCR results, the group pretreated only with LPS showed hyper-expression of IL-1β mRNA compared to the untreated group ($p< 0.001$; Fig 4.A). However, the cell groups pretreated with S_1TF_7significantly inhibited IL-1β mRNA expression compared with the LPS group ($p < 0.05$; Fig. 4A). Furthermore, the groups of cells pretreated with S_1TF_7 showed a decrease in IL-1β mRNA expression in a dose-dependent manner with a significant difference between the doses of the Sp leaves fraction ($p < 0.05$; Fig 4.A). Consistent with the results of the S_1TF_7 effect on LPS-stimulated cells, cells treated with rutin (50 µg/mL) showed a significant decrease in IL-1β mRNA level compared to the control group ($p < 0. 0.001$; Fig 4.A). On another hand, the western blotting analysis showed the excessive release of protein IL-1β active in response to LPS significantly decreased in dose-dependent by S_1TF_7 with a more substantial inhibitory effect at 200 µg/mL ($p< 0.001$; Fig 4.B). However, the treated cell-group with rutin (50µg/mL) shows the most significant inhibitor effect on the overproduction of protein IL-1β active than S_1TF_7 ($p < 0.05$; Fig 4.B).

Furthermore, the effects of S_1TF_7 and rutin on GSDMD release in LPS-stimulated THP-1 cells evaluated using Wb analysis. Thus, we observed a significant release of protein GSDMD cleavage in the LPS-cells group ($p < 0.001$; Fig 4.C). In contrast, S_1TF_7 cell-groups showed significant inhibitor effects on the protein expression of GSDMD cleavage in a dose-dependent manner (Fig 4.C). Besides, rutin significantly inhibits the overproduction of the protein GSDMD cleavage ($p< 0.001$; Fig 4.C) similar to S_1TF_7 at 200 µg/mL (Fig 4.C). These results indicate that $S_1TF_7$and rutin significantly inhibited the hypersecretions of mature IL-1β and GSDMD induced in LPS-stimulated THP-1 cells ($p < 0.05$).

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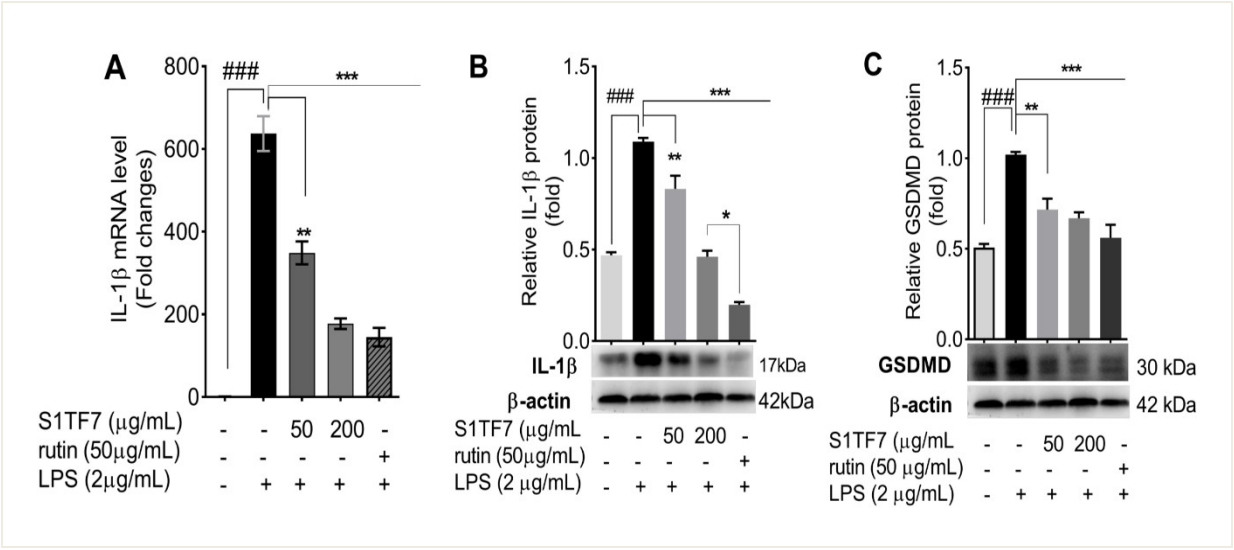

Fig 4. Inhibitory effects of S_1TF_7 and rutin on over-production of IL-1β and GSDMD in LPS-stimulated THP-1 cells. The cells were pretreated with diluted concentrations (50 and 200 µg/mL) of S_1TF_7 or rutin (50 µg/mL) for 12 hours. Then, the pretreated cell groups were exposed with 2 µg/mL of LPS and incubated for 6 hours again. Cells without LPS or samples were considered as blank. Cells treated with only LPS were considered as negative control. The RT-PCR analysis was used to measure the mRNA expression of IL-1β (**A**). The western blotting assay was performed to measure the protein expressions of IL-1 β active (**B**) and GSDMD cleavage (**C**). The values are mean ± STD (n=3). ### $p< 0.001$ versus control. *$p< 0.05$, **$p < 0.01$ and *** $p < 0.001$ versus LPS-cell group.

3.4. Flavonoid-rich fraction inhibits NLRP3 activation in LSP-stimulated THP-1 Cell

In the present RT-PCR investigation, the mRNA expression of NLRP3 has significantly increased in response to LPS-stimulated cell-group when compared to untreated cells ($p< 0.001$; Fig 5A). In Fig 4, western blot results confirmed that the levels of protein NLRP3 highly increased in LSP cell-group when compared to untreated cell-group ($p < 0.001$). However, the S_1TF_7 significantly inhibited the mRNA expression of NLRP3 in a dose-dependent manner when compared to LPS cell-group ($p< 0.01$ at 50 µg/mL and $p < 0.001$ at 200 µg/mL; Fig 5A). In contrast, the S_1TF_7 groups showed a significant inhibition effects on protein NLRP3 expression in a dose-dependent manner ($p< 0.5$ at 50 µg/mL and $p < 0.01$ at 200 µg/mL, respectively). Moreover, rutin (50µg/mL) showed similar effect with S_1TF_7 at 200 µg/mL ($p> 0.05$; Fig 5B).

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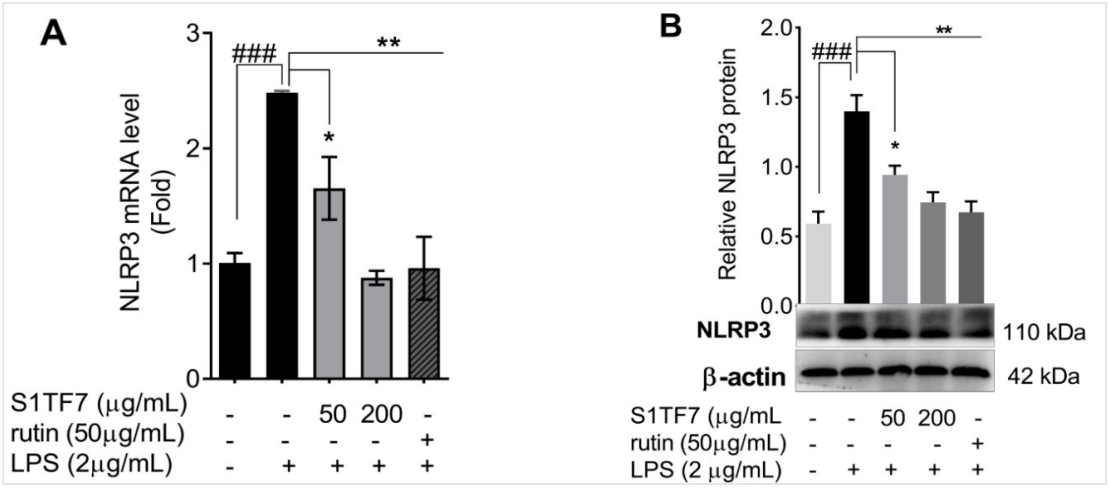

Fig 5. Inhibitor effects of Flavonoid-rich fraction from Sp leaves on NLRP3 activation in LPS-stimulated THP-1 cells. The relative mRNA level change of NLRP3 was determined by RT-PCR analysis (A). The Wb analysis was used to measure the protein level change of NLRP3. Values are means ± STD (n=3). Results are mean ± STD (n=3). ###$p < 0.001$ versus control. * $p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ against LPS-cell group.

4. Discussion

In consistence with previous studies (Muhammad et al., 2018), this study identified the rutin and quercetin flavonoid compounds in the S1TF7 (Fig.1 and Fig.2). These results revealed a high rutin and quercetin content in this fraction (Table 1). Several studies reported medicinal plants' flavonoid components have anti-inflammatory effects (Dias et al. 2020; Lim et al. 2018; Zhao et al. 2019). Moreover, rutin is a natural flavonoid compound from plants with anti-asthmatic effects (Lv et al. 2017) and anti-inflammatory activities on inflammasomes such as NLRP3, ASC, and caspase1(Aruna, Geetha, and Suguna 2014).

On the author hand, we evaluate the effects of S_1TF_7 and rutin on hypersecretion of NLRP3, IL-1β active, and GSDMD cleavage induced by LPS in THP-1. A previous study reported that ATP induces activation of NLRP3 followed hypersecretion of IL-1β and GSDMD (Zito et al. 2020). Like in some previous studies (Shi et al. 2015), the present study showed that LPS (2 µg/mL) induces a significant increase in the mRNA and protein of NLRP3 expression in THP-1 cells at $p < 0.01$ when they are compared to the control, respectively (Fig.3). Our results proved that LPS at 2 µg/mL stimulated THP-1 might also induces the activation of NLRP3 (Fig.5) that leads to the overproduction of IL-1β active and GSDMD cleavage (Fig.3 and Fig.4). The present result is in line with the LPS in SiHa cells, Caski cells (He et al. 2017), and Hela cells (Shi et al. 2019).

In contrast, the present study proved significant hypersecretion ($p< 0.001$) of IL-β active and GSDMD cleavage have a considerable decrease in a dose-dependent by S_1TF_7 with the best inhibitory effect at 200 µg/mL ($p< 0.001$; Fig.4A-C). Also, rutin exhibited the same trend effect with S_1TF_7 but with a more potent effect on IL-1β than S_1TF_7 ($p< 0.05$; Fig.4B). In agreement with previous works (Gao et al. 2015; Kim et al. 2015; Osei et al. 2020) , this study found that the inhibitory effect of S_1TF_7 on IL-1β active can inhibit neutrophil inflammation and COPD. In line with previous clinical trial (Van De Veerdonk and Netea 2020), potent inhibition effect on hypersecretion of IL-1β can be used to block excessive IL-1β production in Covid-19 treatment. This significant inhibition effect of S_1TF_7 on IL-1β ($p< 0.001$) can be associated with the rutin compound (Fig.1) per with rutin effect reported in Fig.4A-B and previous studies (Lim et

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al. 2018). Moreover, our results proved that S_1TF_7 and rutin suppress pyroptosis via inhibited GSDMD cleavage expression ($p< 0.001$; Fig.4C). This inhibitory effect of rutin and S_1TF_7 on GSDMD can be attributed to the ant-inflammatory activities of flavonoids in line with previous studies (Hussain et al. 2020; Owis et al. 2020).

Besides, when the cells were pretreated with S_1TF_7 before they were exposed to LPS, the mRNA and protein expressions of NLRP3 (Fig.5a-b) decreased dose-dependently with significant inhibitory effects at 200 µg/mL at ($p< 0.01$). Similar results observed at 50 µg/mL of rutin (Fig.5.a-b). Previous studies reported that rutin inhibits NLRP3 assembled to caspase1(Aruna et al. 2014). Our results reveal that the significant inhibitory effects of S1TF7 against NLRP3 activation and release of IL-1βand GSDMD cleavage could be associated with a higher rutin content present in the Sp fraction (Fig1 and Table 2).

5. Conclusion

This study proved that Sp leaves inhibited inflammation and pyroptosis by directly inhibiting the cleavage of IL-1β, GSDMD associated with the NLRP3 activation pathway. These results demonstrate the mechanism of action of Sp leaves against inflammation induced by the NLRP3 complex pathway. These effects could be explained by the high quantity of rutin in Sp leaves. Bio-guided studies, particularly *in vivo*, are necessary to discover new anti-inflammatory molecules without adverse effects.

Authors Contributions: Conceptualization: Issa I.I, Ag-Arya M and Chao Y. Supervision: Chao Yu. Methodology and Investigation: Issa I.I, Jin L and Cao W. Data Analysis and Writing-original draft preparation: Issa I.I. All authors have read and agreed with the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author and first author. The data are not publicly available due to intellectual property/confidentiality issues.

Conflicts of Interest: The Authors declare that the authors have no conflict of interest.

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