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Article

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Abstract

Inflammation is commonly treated using non-steroidal anti-inflammatory drugs, analgesics, corticosteroids, and immunosuppressive agents. This study evaluated the anti-inflammatory activity of a novel serine protease inhibitor, RFIP1, isolated from *Rhamnus frangula*, using a xylene-induced ear inflammation model in male *BALB/c* albino mice. Fifty mice were randomly divided into five groups: Group A (control), Group B (xylene-induced inflammation treated with saline, 0.1 mL), Group C treated with dexamethasone (1.25 mg/kg body weight), Group D treated with RFIP1 (2.4 mg/kg body weight), and Group E treated with RFIP1 (4.8 mg/kg body weight). All treatments were administered intravenously via the tail vein for six consecutive days. Xylene induction caused a significant increase in neutrophil serine protease (NSP) expression and elevated serum levels of the pro-inflammatory mediators interleukin-1β, interleukin-6, tumor necrosis factor-α, and nitric oxide. Treatment with Dexamethasone and low-dose RFIP1 significantly reduced NSP expression and cytokine levels. Notably, administration of RFIP1 at 4.8 mg/kg produced a significant dose-dependent reductionr in NSP expression and inflammatory mediators compared with both Dexamethasone and the lower RFIP1 dose. These findings suggest that RFIP1 possesses potent anti-inflammatory activity and may represent a promising therapeutic candidate for inflammatory conditions.

Keywords: male BALB mice; serine proteases; gene expression; Interleukin-1ß; Interleukin-6; Nitric oxide; lipid peroxidation; TNF-α & DEXA

1. Introduction

Protease inhibitors (PIs) constitute an important class of biomolecules with wide applications in biotechnology and medicine. These molecules regulate proteolytic activity and serve as valuable tools for understanding protein-protein interactions and enzymatic mechanisms. In addition, protease inhibitors have attracted considerable attention due to their therapeutic potential in the management of various pathological conditions, including inflammatory, infectious, and metabolic diseases.

Among the different classes of protease inhibitors, serine protease inhibitors represent one of the most extensively studied groups. These inhibitors display considerable structural and functional diversity and are classified into several families based on their molecular structure and mechanism of action [1]. One of the most prominent families is the Serpin (serine protease inhibitor) family, which consists of a large group of proteins characterized by a unique inhibitory mechanism. Serpins inhibit target proteases by forming stable covalent complexes, leading to irreversible enzyme inactivation [2,3]. A distinctive feature of serpins is the presence of a conserved reactive center loop (RCL), which acts as a substrate mimic that attracts proteases and ultimately results in their inhibition [4].

Another important group of serine protease inhibitors is the Kazal-type inhibitors, which are relatively small proteins containing one or more Kazal domains [5]. These inhibitors bind to serine proteases and block their catalytic activity by occupying the enzyme active site, thereby preventing

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substrate binding. Kazal-type inhibitors are widely distributed among different organisms, including animals and plants [6].

Similarly, Kunitz-type inhibitors are small proteins characterized by the presence of one or more Kunitz domains. These inhibitors interact with serine proteases and suppress their enzymatic activity by blocking the catalytic site. Kunitz-type inhibitors have been identified in a wide range of organisms, including animals, plants, and microorganisms. In legumes such as Glycine max (soybean), a distinct class known as Bowman-Birk inhibitors has been reported. These small proteins effectively inhibit serine proteases by binding to their active sites and preventing substrate interaction. Bowman–Birk inhibitors have attracted significant attention due to their potential therapeutic applications, particularly their anticancer properties [6].

Another important protease inhibitor is Alpha-2-macroglobulin, a large plasma protein that functions as a broad-spectrum inhibitor of multiple protease classes. This protein inhibits proteases by entrapping them within a large molecular complex, thereby preventing their enzymatic activity. Alpha-2-macroglobulin plays essential roles in various physiological processes, including immune regulation, inflammation, and tissue repair [7].

Natural products have long been recognized as important sources of bioactive compounds with therapeutic potential. In this context, extracts obtained from *Rhamnus frangula* have been reported to exhibit various biological activities, including antioxidant, antimicrobial, and free radical scavenging properties [8,9]. Moreover, this plant has been identified as a potential source of bioactive molecules such as protease inhibitors and has traditionally been used as a natural laxative in herbal medicine [8].

Inflammatory responses are regulated by several intracellular signaling pathways, including the Mitogen-activated protein kinase pathway, the NF-κB signaling pathway, and the JAK-STAT signaling pathway [10]. Dysregulation of these signaling pathways has been associated with numerous pathological conditions, including inflammatory disorders, autoimmune diseases, metabolic syndromes, and cancer.

The MAPK family includes several kinases such as extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK), and p38 MAP kinase. These proteins belong to the serine/threonine kinase family and are activated in response to inflammatory cytokines and cellular stress signals. Activation of MAPK cascades leads to phosphorylation events that regulate key cellular processes such as cell proliferation, differentiation, and programmed cell death [10].

The NF-κB signaling pathway also plays a crucial role in the regulation of inflammatory responses. Under normal conditions, NF-κB remains inactive in the cytoplasm through its association with inhibitory proteins known as IκB. Upon stimulation by cytokines, microbial components, or other inflammatory signals, IκB is degraded, allowing NF-κB to translocate into the nucleus where it regulates the expression of genes involved in inflammation, immune responses, and apoptosis [10].

Another key regulatory pathway is the JAK–STAT signaling pathway, a highly conserved mechanism that transmits signals from extracellular cytokines and growth factors to the nucleus to regulate gene expression. Activation of receptor-associated Janus kinases (JAKs) results in the phosphorylation of signal transducers and activators of transcription (STATs). The phosphorylated STAT proteins subsequently dimerize and translocate to the nucleus, where they regulate the transcription of genes involved in immune responses, inflammation, and cell growth [11].

Therefore, the present study aimed to evaluate the anti-inflammatory activity of a novel serine protease inhibitor (RFIP1) isolated from *Rhamnus frangula* using a Xylene-induced ear inflammation model in mice and to investigate its effects on the expression of neutrophil serine proteases and pro-inflammatory cytokines.

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2. Materials and Methods

2.1. Chemicals

TRIzol™ LS Reagent 100 ML was purchased from Thermo Fisher Scientific (Waltham, US), ezDNase™ENZYME 50 RXNS and Power UP ™ SYBR™ Green Master Mix were purchased from Thermo Fisher Scientific (Waltham, US). Regular oligo synthesis 0.05 moles were purchased from Humanizing Genomics macrogen (Seoul, Korea). Super Script™III Reverse transcriptase, random primer and dNTP Mix, RNase OUT™ Recombinant Ribonuclease Inhibitor and Uracil-DNA Glycosylase (UDG) from Thermo Fisher scientific also used. Serum TNF-α, IL-6, and IL-1β were measured by ELISA kits using the BioTek Epoch 2 microplate reader. All kits were purchased from My BioSource (San Diego, USA). The Nitrite Assay Kit was purchased from Sigma Aldrich (USA).

2.2. RFIP1 Extraction and Purification

Leaves of *Rhamnus frangula* were collected from a local market in Riyadh, Saudi Arabia, and taxonomically authenticated by a botanist at King Saud University. The protease inhibitor RFIP1 was extracted and purified from the crude leaf extract using 30-75% ammonium sulfate precipitation, 50-90% ethanol fractionation, and heat treatment at 70 °C, followed by Sephadex G-50 gel filtration chromatography. Active fractions were monitored at 280 nm, analyzed by SDS-PAGE, pooled, concentrated, and stored at 4 °C, resulting in a homogeneously purified protein with an apparent molecular weight of ~22.5 kDa.

2.3. Animal and Xylene-Induced Ear Swelling

Fifty male *BALB/c* mice weighing 18-20 g were obtained from the animal facility of the College of Pharmacy at Qassim University, Buraydah, Saudi Arabia. The experiment was conducted at the animal house of the Department of Biology, College of Science, Qassim University.

The animals were housed under controlled laboratory conditions (22 ± 2 °C, relative humidity 55 ± 10%, and a 12 h light/dark cycle) with free access to standard pellet diet and water.

The mice were randomly divided into five groups (n = 10 per group) according to the protocol described by Fei et al. (2017) [10]:

- Group A (Negative control): received normal saline (0.1 mL).
- Group B (Xylene model group): received normal saline (0.1 mL) followed by induction of ear inflammation.
- Group C: treated with Dexamethasone (1.25 mg/kg body weight).
- Group D: treated with purified RFIP1 (2.4 mg/kg body weight).
- Group E: treated with purified RFIP1 (4.8 mg/kg body weight).

All treatments were administered intravenously through the tail vein once daily for six consecutive days using a restrainer.

One hour after the final treatment, mice in groups B, C, D, and E received 50 µL of Xylene applied to both the anterior and posterior surfaces of the right ear lobe to induce acute ear inflammation.

Two hours after xylene application, the animals were anesthetized and sacrificed by cervical dislocation. Blood samples were collected and divided into two portions. One portion was collected in heparinized tubes for immediate RNA extraction, while the other portion was centrifuged to obtain serum for biochemical analysis.

Ear tissue samples were collected using a 9-mm biopsy punch and preserved in 10% neutral buffered formalin for histo-pathological examination.

2.4. Quantitative Real-Time PCR

Total RNA was extracted from whole blood leukocytes samples using TRIzol LS Reagent according to the manufacturer's instructions. RNA concentration and purity were determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific) and then complementary DNA (cDNA)

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was synthesized using SuperScript III Reverse Transcriptase and random primers. Quantitative real-time PCR was performed using a Bio-Rad CFX Connect Real-Time PCR System with SYBR Green Master Mix (RR820A, TaKaRa). All qRT-PCR reactions were performed in duplicate and consisted of an initial DNA polymerase activation step for 2 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15s and annealing/extension for 1 min at 60 °C.

Relative gene expression levels of neutrophil serine proteases were calculated using the 2-ΔΔCt method, and expression levels were normalized to the housekeeping gene β-actin. The primer sequences used for amplification of Cathepsin G, Neutrophil elastase, and Proteinase-3 are listed in Table 1.

Table 1. Forward and reverse primer sequences of (NE, PR3 and CG) [11].

Species	Gene name	Primer	Primer sequence (5′-3′)
Mouse	Cts g	F	AGAAGACTTCGTCCTAACAGCA
		R	CCTTTCTCGCATTTGGATGTTGT
Mouse	Neu Elast	F	CAGGAACTTCGTCATGTCAGC
		R	AGCAGTTGTGATGGGTCAAAG
Mouse	Proteinase 3	F	CCCACTCTCGGCCTTATGTG
		R	CGAATCTCGGGTGGATCAGG

2.5. Statistical Analysis

Data were expressed as mean ± standard error (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test using SAS statistical software (SAS Institute Inc., Cary, NC, USA, copyright©1998). Differences were considered statistically significant at $P \leq 0.05$ [12].

2.6. Histopathological Analysis

Ear tissue samples fixed in 10% formalin were processed using standard histological techniques and stained with hematoxylin and eosin (H&E) [13]. Histopathological changes in the ear tissues were examined under a light microscope to evaluate inflammatory cell infiltration and tissue damage.

3. Results

3.1. Molecular Analysis

3.1.1. Neutrophil Serine Proteases (NSPs) Gene Expression

The mRNA expression levels of neutrophil serine proteases (NSPs), including Cathepsin G (CG), Neutrophil Elastase (NE), and Proteinase-3 (PR3), were analyzed using quantitative real-time PCR (qRT-PCR) across all experimental groups. Each group included ten biological replicates, and expression levels were normalized to the negative control (Group A). Results are presented as fold changes relative to control, expressed as mean ± SEM.

Xylene treatment (Group B) significantly upregulated all three NSP genes, with approximately 20-fold increases, indicating a strong inflammatory response ($p < 0.05$). In comparison, Dexamethasone (1.25 mg/kg, Group C) caused only a modest reduction in NSP expression. Conversely, RFIP1 treatment produced a dose-dependent inhibitory effect, with the higher dose (4.8 mg/kg, Group E) resulting in the greatest suppression of gene expression.

Fold-change ratios of Groups C, D, and E relative to the Xylene-treated group (B) further highlight that RFIP1 exerts a stronger inhibitory effect on NSP expression than Dexamethasone, suggesting its potential as a potent anti-inflammatory agent.

The mRNA expression levels of neutrophil serine proteases (NSPs), including Cathepsin G (CG), Neutrophil Elastase (NE), and Proteinase-3 (PR3), were assessed using quantitative real-time PCR (qRT-PCR) across the experimental groups (n = 10 per group). Gene expression was analyzed relative

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to the negative control (Group A), and fold changes were calculated with mean ± standard error of the mean (SEM).

In the Xylene-induced model group (Group B), all three NSPs exhibited a significant upregulation compared to controls, indicating a strong inflammatory response. Administration of Dexamethasone (DEXA) or RFIP1 reduced NSP gene expression, with the degree of inhibition correlating with the dose of RFIP1.

3.1.2. The Cathepsin G (CG) Gene Expression

The mRNA expression of Cathepsin G (CG) was significantly elevated in the Xylene-treated group (B), showing an 18.48-fold increase compared to the negative control (A), indicating strong transcriptional activation in response to inflammation. This indicates a strong transcriptional activation of CG in response to Xylene-induced ear inflammation, reflecting the recruitment and activation of neutrophils at the inflammatory site. Elevated CG expression is consistent with enhanced proteolytic activity, which contributes to tissue remodeling and the inflammatory response.

Treatment with Dexamethasone (1.25 mg/kg, Group C) produced a modest reduction, lowering CG expression to 16.67-fold (−1.81× relative to Group B). In contrast, RFIP1 demonstrated a dose-dependent inhibitory effect. Administration of 2.4 mg/kg RFIP1 (Group D) reduced CG expression to 5.3-fold (−13.18×), while 4.8 mg/kg RFIP1 (Group E) further decreased expression to 4.08-fold (−14.39×), representing the most effective dose in suppressing the inflammatory response (Figure 1, Table 2). This suggests that RFIP1 effectively modulates neutrophil serine protease activity and can counteract inflammatory gene expression in a dose-dependent manner.

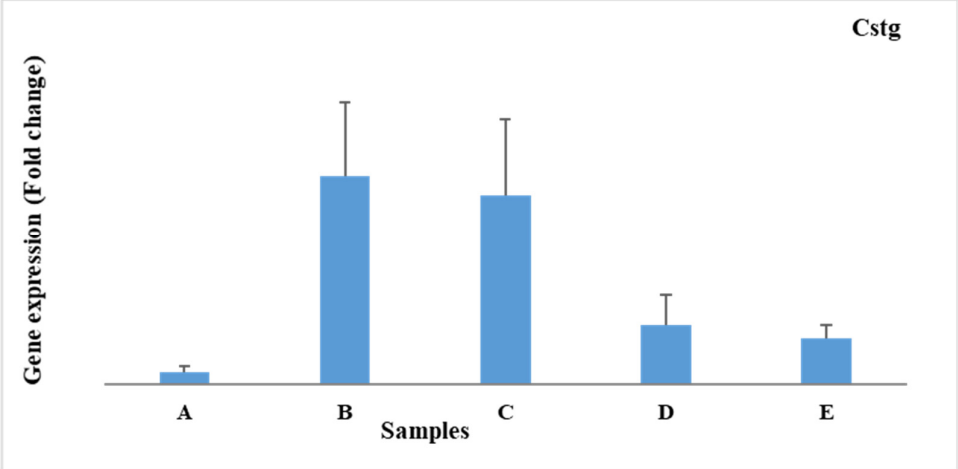

Figure 1. Relative mRNA expression levels of Cathepsin G (CG) in different experimental groups determined by qRT-PCR. **A:** Negative control, **B:** Xylene-treated mice, **C:** Xylene-DEXA treated mice, **D:** 2,4 mg xylene-RFIP1 treated mice, **E:** 4.8 mg xylene-RFIP1 treated mice.

Table 2. Effect of treatment on of CG gene expression, *compared with B.

Group	Dose mg/kg	Fold change (CG)	Change of expression *
Control negative		1.10	-
Xylene model	-	18.48	-
Dexamethasone	1.25 mg/kg	16.67	-1.81 times
RFIP1	2.4 mg/kg	5.3	-13.18 times
RFIP1	4.8 mg/kg	4.084	-14.39 times

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The fold-change analysis highlights that RFIP1 at both doses is more potent than Dexamethasone in reducing CG expression, supporting its potential as a novel anti-inflammatory agent. These results suggest that RFIP1 may interfere with neutrophil activation and protease-mediated tissue damage, making it a promising candidate for mitigating acute inflammatory responses.

3.1.3. The Neutrophil Elastase (NE) Gene Expression

The mRNA expression of Neutrophil Elastase (NE) was significantly elevated in the Xylene-treated group (B), showing a 12-fold increase compared to the negative control (A). This increase reflects heightened neutrophil activation and proteolytic activity at the site of inflammation, which contributes to tissue damage and the progression of the inflammatory response.

Treatment with Dexamethasone (1.25 mg/kg, Group C) moderately reduced NE expression to 7.43-fold (−4.57× relative to Group B), indicating partial suppression of neutrophil activity in this acute inflammation model. In contrast, RFIP1 displayed a pronounced dose-dependent inhibitory effect on NE expression. Administration of 2.4 mg/kg RFIP1 (Group D) decreased NE levels to 2.34-fold (−9.66×), while the higher dose, 4.8 mg/kg RFIP1 (Group E), further reduced NE expression to 1.15-fold (−10.85×), effectively normalizing NE expression close to baseline levels (Figure 2, Table 3).

These results demonstrate that RFIP1 more effectively suppresses NE expression than Dexamethasone, supporting its potential role as a potent anti-inflammatory agent.

Table 3. Effect of treatment on the NE expression, * compared with B.

Group	Dose mg/kg	Fold change (NE)	Change of expression %*
Control negative	-	1.25	
Xylene model	-	12	
Dexamethasone	1.25 mg/kg	7.43	-4.57 times
RFIP1	2.4 mg/kg	2.34	-9.66 times
RFIP1	4.8 mg/kg	1.15	-10.85 times

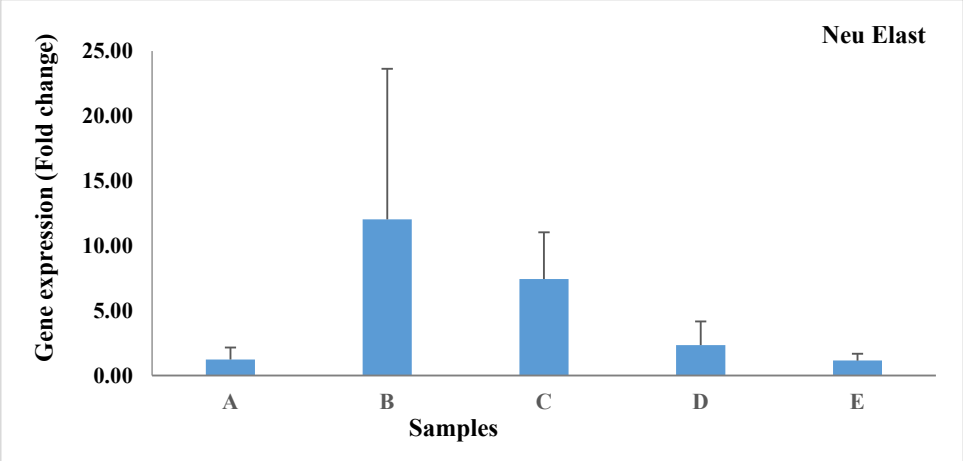

Figure 2. Relative mRNA expression levels of Neutrophil Elastase (NE) in different experimental groups determined by qRT-PCR. A: Negative control, B: Xylene-treated mice, C: Xylene-DEXA treated mice, D: 2,4 mg xylene-RFIP1 treated mice. E: 4.8 mg xylene-RFIP1 treated mice.

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3.1.4. Proteinase-3 (PR3) Gene Expressions

The mRNA expression of Proteinase-3 (PR3) was significantly elevated in the Xylene-treated group (B), showing a 4.48-fold increase compared to the negative control (A). This indicates that PR3 is transcriptionally upregulated during Xylene-induced ear inflammation, reflecting neutrophil activation and contributing to the inflammatory protease cascade.

Treatment with Dexamethasone (1.25 mg/kg, Group C) produced a modest reduction in PR3 expression to 3.88-fold (−0.6× relative to Group B), suggesting only partial suppression of PR3 transcription under acute inflammatory conditions. In contrast, RFIP1 demonstrated a dose-dependent inhibitory effect. Administration of 2.4 mg/kg RFIP1 (Group D) decreased PR3 expression to 1.58-fold (−2.9×), and the higher dose, 4.8 mg/kg RFIP1 (Group E), further reduced expression to 1.17-fold (−3.31×), approaching baseline levels.

These results indicate that RFIP1 effectively suppresses PR3 expression in a dose-dependent manner, achieving greater inhibition than Dexamethasone (Figure 3, Table 4). The findings highlight RFIP1's potential to modulate neutrophil serine protease activity and mitigate inflammatory responses in acute tissue injury.

Table 4. Effect of treatment on of PR3 expression, * compared with B group.

Group	Dose mg/kg	Fold change (PR3)	Change of expression *
Control negative	-	1.1	-
Xylene model	-	4.48	-
Dexamethasone	1.25 mg/kg	3.88	-0.6 times
RfIP1	2.4 mg/kg	1.58	-2.9 times
RfIP1	4.8 mg/kg	1.17	-3-31 times

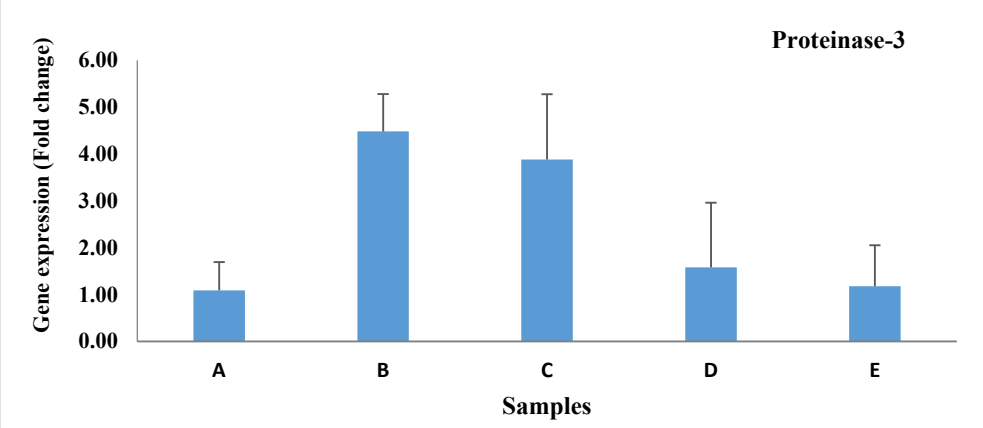

Figure 3. Relative mRNA expression levels of Proteinase-3 (PR3) in different experimental groups determined by qRT-PCR. A: Negative control. B: Xylene-treated mice. C: Xylene-DEXA treated mice. D: 2,4 mg Xylene-RFIP1 treated mice. E: 4.8 mgXylene-RFIP1 treated mice.

While various serine proteases are produced by different immune cell types, neutrophil serine proteases (NSPs) including Neutrophil Elastase (NE), Proteinase-3 (PR3), and Cathepsin G (CG) are of particular interest due to their key role in the regulation of non-infectious inflammation. Real-time RT-PCR analysis demonstrated that the mRNA expression levels of all three NSPs were substantially elevated in the blood of Xylene-treated mice (Group B) compared to negative controls (Group A), confirming the robust inflammatory response (Figures 1–3). Specifically, Xylene treatment caused

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over 18.48-fold increase in CG, 12-fold increase in NE, and 4.48-fold increase in PR3, highlighting the differential magnitude of NSP activation during acute inflammation.

Treatment with Dexamethasone (1.25 mg/kg) moderately suppressed the expression of all three NSPs, indicating partial inhibition of neutrophil activation. In contrast, RFIP1 exhibited a pronounced, dose-dependent inhibitory effect, with the higher dose (4.8 mg/kg) nearly completely suppressing NE and PR3, and reducing CG expression by approximately 78% relative to Xylene-treated mice. These findings demonstrate that RFIP1 effectively downregulates NSP expression in a dose-dependent manner, suggesting that increasing the inhibitor concentration can substantially reduce neutrophil protease production and, consequently, repress inflammatory processes.

3.2. Cytokines Results

The obtained data in Table 5& Figure 9 showed that treatment of healthy mice with 50-µL smearing of Xylene on both the anterior and posterior surfaces of the right ear lobe for an hour resulted in a significant (($p \leq 0.01$) rise (45.3%) in the levels of serum interleukin-1ß when this group was compared to the corresponding value of the control animals' group (61.3 vs 42.2 respectively). In contrast, the i.v. injection of mice with both DEXA (1.25mg/Kg b.wt.) and RFIP1 (2.5mg/kg b.wt.) separately for six successive days resulted in a low-significant decline (-13.2% and -15.5% respectively) in the serum interleukin-1ß level (53.2 Vs 61.3 and 51.8 vs 61.3) when was compared to the model animals' group. Interestingly, the treatment of healthy mice with an i.v. dose of RFIP1 (4.8mg/kg b.wt.) for 6 consecutive days revealed a high-significant ($p \leq 0.01$) decline (-23%) in the elevated serum interleukin-1ß level (47.2 vs 61.3) in comparison to model animals 'group (Table 5 and Figure 4).

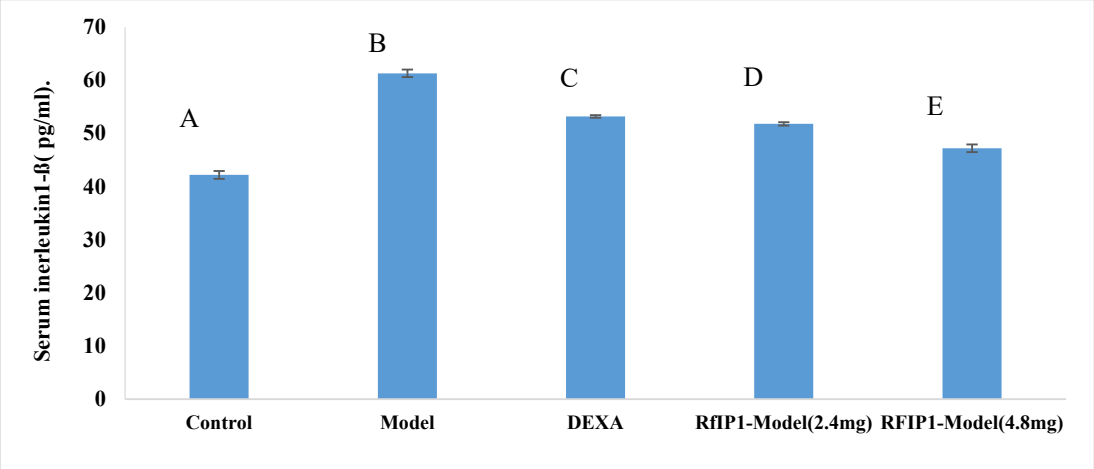

Figure 4. Means of serum Interleukin-1ß (pg/mL) of adult male albino mice *BALB/c.*

Table 5. Mean values of serum pro-inflammatory cytokines and nitric oxide of model and anti-inflammatory treated animals' groups as compared to normal control animals' group.

Groups	Parameters	Interleukin-1ß (pg/ml)	Interleukin-6 (pg/ml)	TNF-α(pg/ml)	NO (nmol/µL)
Control	M ±SE	42.2±0.740^{A}	35.2±0.067^{A}	92.4±0.927^{A}	17.1±0.707^{A}
Model	M ±SE	61.3±0.702^{B}	53.6±0.901^{B}	124.8±0.123^{B}	26.6±0.412^{B}
	% Change A	**45.3%**	**52.3%**	**35.1%**	**55.6%**
DEXA	M ±SE	53.2±0.243^{C}	43.6±0.431^{C}	109.2±0.320^{C}	22.4±0.832^{C}
	% Change B	**-13.2%**	**-18.7%**	**-12.5%**	**-15.8**
RFIP1 Model (2.4mg/kg bwt.)	M ±SE	51.8±0.321^{C}	46.4±0.322^{C}	101.4±0.304^{C}	24.6±0.101^{C}
	% Change B	**-15.5%**	**-13.4%**	**-18.8%**	**-7.5%**

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RFIP1 Model (4.8mg/kg bwt.)	M ±SE	47.2±0.732^{A}	39.8±0.112^{A}	95.8±0.107^{A}	19.2±0.345^{A}
	% Change B	**-23%**	**-25.8%**	**-23.2%**	**-27.8%**

The obtained data in Table 5 & Figure 5 showed that treatment of normal mice with 50-µL smearing of Xylene on both the anterior and posterior surfaces of the right ear lobe for an hour resulted in a significant (($p \leq 0.01$) rise (**52.3**%) in the levels of serum interleukin-6 when this group was compared to the corresponding value of the control animals' group (53.6vs 35.2 respectively). In contrast, the i.v. treatment of mice with both DEXA (1.25mg/Kg b.wt.) and RFIP1 (2.5mg/kg b.wt.) separately for six successive days resulted in a low-significant decline (-18.7% and -13.4% respectively) in the serum interleukin-6 level (43.6 vs 53.6 and 46.4 vs 53.6) when was compared to the model animals' group. Although, the treatment of control mice with an i.v. dose of RFIP1 (4.8mg/kg b.wt.) for 6 consecutive days revealed a high-significant ($p \leq 0.01$) decline (-**25.8**%) in the elevated serum interleukin-6 level (39.8 vs 61.3) in comparison to model animals 'group (Table 5 and Figure 5).

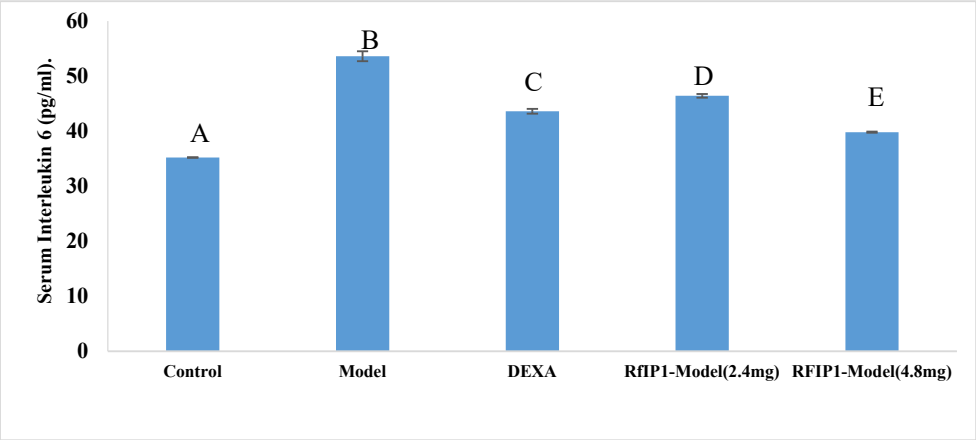

Figure 5. Means of serum Interleukin-6 (pg/mL) of adult male albino mice *BALB/c*.

The obtained data in Table 5 & Figure 6 showed that treatment of control mice with 50-µL smearing of Xylene on both the anterior and posterior surfaces of the right ear lobe for an hour resulted in a significant (($p \leq 0.01$) rise (35.1%) in the levels of serumTNF-α when this group was compared to the corresponding value of the control animals' group (124.8 vs 92.4 respectively). In contrast, the i.v. treatment of mice with both DEXA (1.25mg/Kg b.wt.) and RFIP1 (2.5mg/kg b.wt.) separately for six consecutive days resulted in a low-significant decline (-12.5% and -18.8% respectively) in the serum TNF-αlevel (109.2 vs 124.8 and 101.4 vs 124.8) when was compared to the model animals' group. Although, the treatment of control mice with an i.v. dose of RFIP1 (4.8mg/kg b.wt.) for 6 consecutive days revealed a high-significant ($p \leq 0.01$) decline (-23.2%) in the elevated serum level of TNF-α (95.8 vs 124.8) in comparison to model animals 'group.

The obtained data in Table 5 & Figure 7 showed that treatment of control mice with 50-µL smearing of Xylene on both the anterior and posterior surfaces of the right ear lobe for an hour resulted in a significant (($p \leq 0.01$) rise (55.6%) in the levels of serum nitric oxide when this group was compared to the corresponding value of the control animals' group (26.6 vs 17.1 respectively). In contrast, the i.v. treatment of mice with both DEXA (1.25mg/Kg b.wt.) and RFIP1 (2.5mg/kg b.wt.) separately for six consecutive days resulted in a low-significant decline (-15.8% and -7.5% respectively) in the serum nitric oxide level (22.4 vs 26.6 and 24.6 vs 26.6) when was compared to the model animals' group. Although, the treatment of control mice with an i.v. dose of RFIP1 (4.8mg/kg b.wt.) for 6 consecutive days revealed a high-significant ($p \leq 0.01$) decline (-27.8%) in the elevated serum level of nitric oxide (19.2 vs 26.6) in comparison to model animals 'group (Table 5 and Figure 7).

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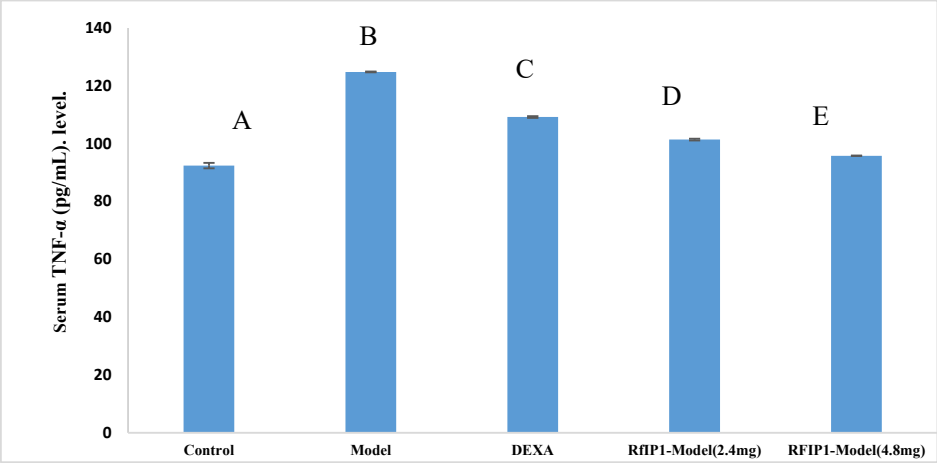

Figure 6. Means of serum TNF-α (pg/mL) of adult male albino mice *BALB/c.*

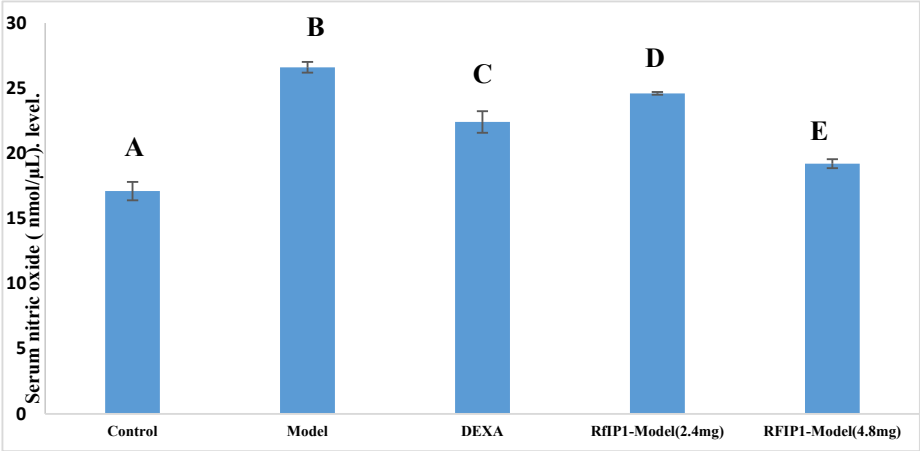

Figure 7. Means of serum nitric oxide level (nmol/µL) of adult male albino mice *BALB/c*.

3.3. Histopathological Results

Histopathological examination of ear pinna of the control group revealed normal histological appearance of the keratinized epidermal layer, dermal structures with conjunctive tissue and vessels, as well as adnexal structures with normal architecture and thickness without any sign of inflammatory changes (Figure 8A). Meanwhile, the DEXA-induced model group showed the maximum thickness compared to the negative control group and other groups. In comparison with other groups, the epithelium was thickened, exhibited cytoplasmic vacuolation in the basal and spinous layer (Figure 8B). The dermis shows dermatitis represented in extensive mast cell infiltration (Figure 8C), as well as broadening tissue space, edema, discrete and fractured striated muscle fiber in the ear tissue of Xylene-induced model group (Figure 8D). In the groups pretreated with dexamethasone or RFIP1, these histopathological alterations were reduced to different degrees, especially the group pretreated with high protein (Figures 8E,F).

Photomicrograph 8A was a section of Ear pinna of control group showing normal histological appearance of ear pinna; keratinized epidermal layer (E), dermal structures with conjunctive tissue and normal adnexal structures (A) and elastic cartilage (C). While, photomicrograph 8B was a section of Ear pinna of DEXA group, showing thickened epithelium with cytoplasmic vacuolation in the basal and spinous layer (arrow).

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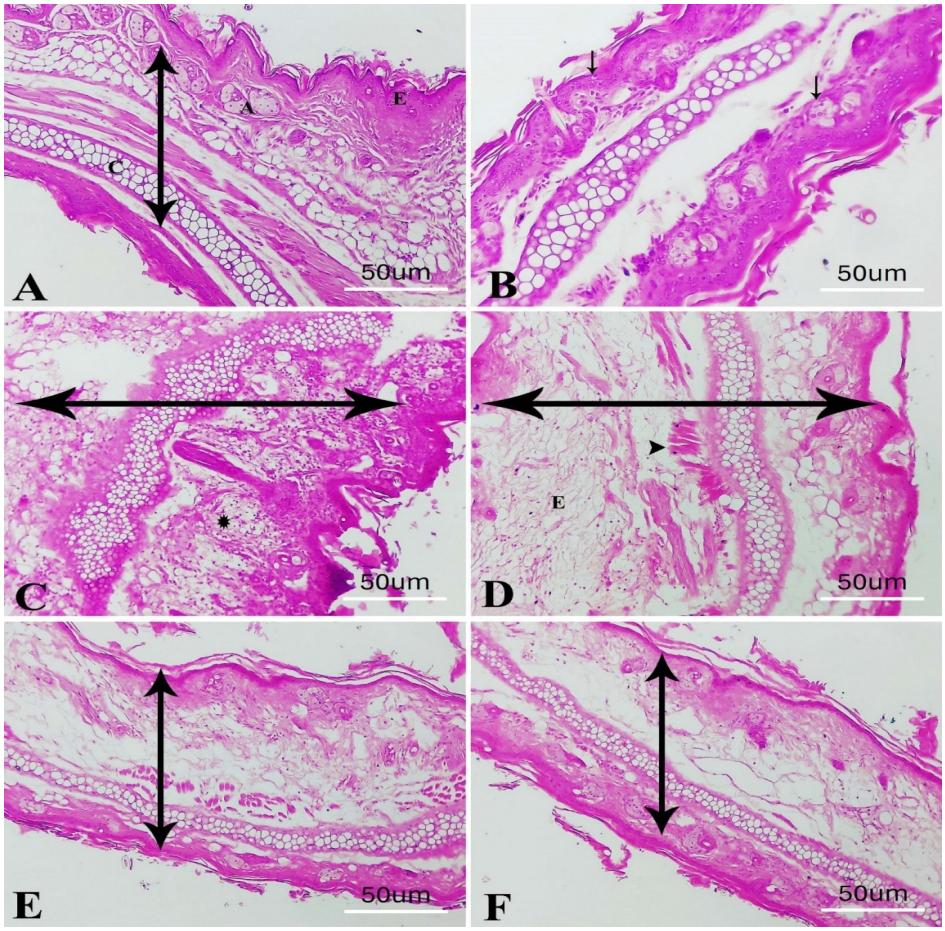

Figure 8. Ear pinna of control (A), DEXA group (B), toxic (C- D) and treated (E, F) groups,.

Photomicrograph 8C was a section of Ear pinna of group treated with Xylene showed diffuse mast cell infiltration (asterisk) in the dermis. (D) increased dermal thickness with ear tissue edema (E) and discrete breaks in the striated muscle fiber (arrowhead). While, photomicrograph 8E was a section of Ear pinna of treated group with RFIP1 at low dose (E), showing reduced dermal thickness, edema, and inflammatory cells but the transverse muscle rupture was not effectively reduced. On the contrary, photomicrograph F was a section of Ear pinna of treated group with RFIP1 at higher dose (F), showing marked restoration of normal histological appearance of ear pinna (H& E X 200).

4. Discussion

Inflammation is a tightly regulated process orchestrated by the activation and recruitment of various immune cells, including macrophages, neutrophils, T cells, and B cells. These cells mediate tissue remodeling and defense through the secretion of inflammatory mediators such as cytokines, chemokines, and complement proteins. Among these, cytokines are critical regulators of immune responses, controlling processes such as adhesion molecule expression, cell growth, apoptosis, and immunoglobulin production [14,15]. Dysregulation of cytokine production can therefore exacerbate inflammation and contribute to tissue damage and disease progression.

Reactive oxygen species (ROS) interact with nitric oxide (NO) to form reactive nitrogen species (RNS), generating oxidative-nitrosative stress. While acute inflammation represents a protective host response, chronic or uncontrolled inflammation can lead to pathologies including cardiovascular diseases, arthritis, multiple sclerosis, and cancer. Thus, suppressing excessive inflammatory mediators is crucial for preventing tissue damage and systemic complications [15,16].

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The xylene-induced ear edema model was employed in this study as a well-established acute inflammatory model with predictive value for evaluating anti-inflammatory agents. Xylene exposure led to fluid accumulation, neurogenic inflammation, and activation of resident immune cells such as mast cells, macrophages, and neutrophils. This resulted in redness, swelling, and edema of the ear tissue [17]. Molecular analyses demonstrated a significant upregulation of neutrophil serine proteases (NSPs), including Cathepsin G (CG, 18.48-fold), Neutrophil Elastase (NE, 12-fold), and Proteinase-3 (PR3, 4.48-fold), highlighting the role of neutrophil activation in acute inflammation. The increase in NSPs likely reflects NF-κB pathway activation, which promotes the transcription of anti-apoptotic genes such as Bcl-2 and Bcl-xL, sustaining neutrophil survival and prolonging inflammatory activity [18,19].

Consistent with these molecular changes, serum analyses revealed significant increases in IL-1β, IL-6, TNF-α, and nitric oxide in xylene-treated mice, reflecting heightened systemic inflammation. The rise in cytokines aligns with previous studies demonstrating xylene's ability to induce oxidative stress and activate the NF-κB signaling pathway, which in turn stimulates cytokine production and amplifies inflammatory responses [11,17].

Histopathological examination corroborated these findings. Xylene exposure induced epidermal hypertrophy, cytoplasmic vacuolation in the basal and spinous layers, mast cell infiltration, dermal edema, and fractured striated muscle fibers (Figure 8 C–D). These observations are consistent with prior reports highlighting xylene-induced tissue injury and hyperplasia, reflecting its effects on both cellular morphology and tissue architecture [17–19].

Dexamethasone (DEXA), a synthetic corticosteroid, serves as a benchmark anti-inflammatory agent due to its potent immunosuppressive properties [21]. In this study, DEXA administration (1.25 mg/kg) resulted in reduced NSP gene expression (CG by 1.81-fold, NE by 4.57-fold, PR3 by 0.6-fold) and decreased serum levels of IL-1β, IL-6, TNF-α, and NO. The anti-inflammatory effects of DEXA are mediated through multiple mechanisms, including inhibition of T-cell proliferation via CTLA-4 signaling, suppression of innate immune cell activation, and downregulation of miR-324-3p, which reduces translation of NSP proteins [22–25]. These molecular changes correlate with histopathological improvements, such as reduced dermal edema and immune cell infiltration. DEXA's effects are thus consistent with both transcriptional and translational modulation of inflammatory mediators, as supported by prior studies [10,15,26–29].

RFIP1, a natural protease inhibitor purified from *Rhamnus frangula* leaf extract, demonstrated dose-dependent anti-inflammatory activity. RFIP1 administration reduced NSP activity and expression (especially at 4.8 mg/kg), decreased pro-inflammatory cytokines, and ameliorated histopathological damage. Its mechanism appears to involve direct inhibition of NSPs, preventing their proteolytic activity and secondary activation cascades. This concept aligns with previous findings regarding dipeptidyl peptidase I (DPPI), which activates NSP zymogens. Inhibiting these proteases effectively reduces NSP-mediated tissue damage and cytokine release [30,31]. Notably, while RFIP1 did not suppress NSP expression as profoundly as DEXA, its targeted protease inhibition led to significant reductions in systemic inflammation markers, supporting its potential as a natural anti-inflammatory agent with fewer systemic side effects [32,33].

The combined molecular, cytokine, and histopathological findings indicate that RFIP1 effectively reduces inflammation through multiple levels: by modulating protease activity, decreasing cytokine production, and restoring tissue integrity. These results highlight RFIP1 as a promising therapeutic candidate for inflammation, particularly in contexts where steroid use is limited by side effects.

In conclusion, both DEXA and RFIP1 mitigated xylene-induced acute inflammation, but through distinct mechanisms: DEXA acts primarily via broad immunosuppressive and transcriptional regulation, while RFIP1 exerts protease-targeted effects, resulting in decreased NSP activity, cytokine levels, and tissue damage. The findings underscore the potential of natural protease inhibitors as complementary or alternative anti-inflammatory therapies.

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5. Conclusions

In conclusion, RFIP1, a serine protease inhibitor isolated from *Rhamnus frangula*, demonstrated significant anti-inflammatory activity in a xylene-induced mouse ear inflammation model. RFIP1 treatment markedly reduced the gene expression of neutrophil serine proteases (CG, NE, and PR3) and decreased serum levels of pro-inflammatory cytokines including IL-1β, IL-6, TNF-α, and nitric oxide. These findings suggest that RFIP1 may serve as a promising natural therapeutic candidate for the management of inflammatory disorders. Further studies are required to elucidate its molecular mechanisms and potential clinical applications.

Author: Contributions All authors contributed to the study conceptualization and design. H.F.G., I.B.A., M.R.A. performed the experiments and I.B.A. and H.F.G. the statistical analysis. All authors contributed to the analysis and interpretation of the data. M.R.A. wrote the draft of the manuscript under supervision of I.B.A and H.F.G., and all authors were involved in the reviewing and editing of the submitted article. I.B.A and H.F.G supervised the study and had a major role in the project administration and funding acquisition. We gratefully acknowledge Prof. Medhat Rehan Ramadan and Doaa Galal EL-Sahra for their excellent technical assistance, which was instrumental to the success of this study and histopathological analysis respectively.

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Abbreviations

DEXA	Dexamethasone.
CRL	Conserved reactive site loop.
MAPK	mitogen-activated protein kinase.
PIs	Protease inhibitors.
ELISA	Enzyme-linked immunosorbent assay.
JNK	c-Jun N-terminal kinases.
NF-κB	Nuclear factor kappa-B.
JAK	Janus kinase.
ANOVA	Analysis of variance.
NSPs	Neutrophil Serine Proteases.
CG	Cathepsin G
I.V.	intravenous.
NE	Neutrophil Elastase
PR3	Proteinase-3
TNF-α	Tumor necrosis factor-α.
NO	Nitric oxide.
IL-1β	Interleukin-1β.
IL-6	Interleukin-6.

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