



# *Annona muricata* Extract Reduces Inflammation via Inactivation of NALP3 Inflammasome

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## Abstract

*Annona muricata* (AM) is a herb traditionally used to treat various human ailments. The mechanistic effect of *Annona muricata* extract on NALP3 inflammasome activation is not well understood. **Objective:** The present study investigates the inhibitory effects of *Annona muricata* extract on NALP3 inflammasome activation and its role in sepsis prevention. **Methods:** Sepsis was induced in mice by intraperitoneal injection of *Escherichia fergusonii*. Mice were treated with *Annona muricata* extract. Its effect on liver was assessed both histologically and biochemically. Lipid peroxidation, level of IL-1 $\beta$ , TNF- $\alpha$  was measured colorimetrically and using Elisa kit in liver homogenates. The expression levels of IL-1 $\beta$ , TNF- $\alpha$ , caspase-1 and NALP3 genes were measured using RT-PCR. **Results:** AM extract significantly minimized the inflammation by decreasing the level and the expression levels of IL-1 $\beta$  and TNF- $\alpha$  in a time-dependent manner. Significant decrease in the gene expression level of caspase-1 and NALP3 was observed. Histopathologically, normal architecture of the liver of infected mice was regained after the herb treatment. **Conclusion:** *Annona Muricata* can act as a potent therapeutic agent in treating various NALP3 associated inflammatory disorders.

**Keywords:** *Annona muricata*, Cytokines, *Escherichia fergusonii*, NALP3 Inflammasome

## 1. Introduction

*Annona muricata* L., commonly named as graviola, soursop or guanabana, belongs to the Annonaceae family<sup>1</sup>. It is cultivated throughout all tropical and subtropical regions of the world<sup>2</sup>. It is an evergreen, erect tree of large yellow flowers, shiny roundish dark green leaves and huge oval green fruit<sup>3</sup>. *Annona* species, especially *Annona muricata* (AM), have been well-known for their richness in Annonaceous Acetogenin compounds (AGEs)<sup>4</sup>. Phytochemical estimation of AM leaves revealed the existence of alkaloids<sup>5,6</sup>, flavonol triglycosides<sup>7</sup>, megastigmanes<sup>8</sup>, cyclopeptides<sup>9</sup> and phenolic compounds<sup>10</sup>. In addition, AM leaves contain numerous minerals such as K, Na, Ca, Mg and Cu and Mg that are essential for human health.

An array of human illness and disorders are traditionally cured by various parts of the AM tree. Anciently, the fruit was used to treat arthritic pain,

rheumatism, dysentery, diarrhea, fever, parasites, worms and skin rushes<sup>11</sup>. The leaves are active against diabetes, cystitis, rheumatism, and headaches<sup>12-14</sup>. Seeds were used as anti-helminthic against various internal and external parasites and worms<sup>15</sup>.

The isolated AGEs from the different part of AM trees have been proven to have anti-proliferative effects against various cancer cell lines by inducing apoptosis through the mitochondrial-mediated pathway up-regulating Bax and down-regulating of Bcl-2 gene expression and by arresting cell cycle in the G1 phase<sup>16,17</sup>. Aqueous and methanolic extracts of AM leaves revealed anti-oxidative activities against H<sub>2</sub>O<sub>2</sub>-induced cellular toxicity<sup>18</sup> and antihypertensive effect through peripheral mechanisms as Ca<sup>2+</sup> antagonist<sup>19</sup>. AM extracts also showed anti-nociceptive activities in vivo<sup>20</sup> as well as anti-bacterial activities against various types of gram-positive and gram-negative bacteria including *Escherichia* species<sup>21</sup>. AM leaves extracts exhibited anti-inflammatory effect

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where it protected against Complete Freund's Adjuvant (CFA)-induced arthritis and xylene-induced ear edema and led to a reduction in the tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin 1 beta (IL-1 $\beta$ ) levels in rodents<sup>22</sup>.

IL-1 $\beta$ , an inflammatory cytokine that is considered as an endogenous pyrogen, harbors variety of roles such as mediator of inflammatory responses, cell proliferation, differentiation and apoptosis. Synthesis and processing of IL-1 $\beta$  is under tight control. Immature IL-1 $\beta$  (pro-IL-1 $\beta$ ) is stored in intracellular vesicles that are cleaved by caspase-1 to produce the mature cytokine. IL-1 $\beta$  production is induced via innate Pathogen Recognition Receptors (PRRs) through NF- $\kappa$ B and Mitogen Activated Protein (MAP) kinase signaling pathways<sup>23</sup>. Cleavage of pro-IL-1 $\beta$  is mediated by a multi-protein complex known as 'inflammasome'. Inflammasomes are activated in response to pathogen- and danger-associated molecular patterns (PAMPs and DAMPs). They consist of a sensor protein of the NOD-like receptor family (NLR), an adaptor protein apoptosis-associated speck-like protein containing CARD (ASC) and a downstream effector caspase-1. NALP3 is one of the best-characterized inflammasome. It serves as a scaffold for recruitment of pro-caspase-1 and induce its auto-proteolytic activation. In turn, caspase 1 cleaves pro-IL-1 $\beta$  and pro-IL-18 into active cytokines IL-1 $\beta$  and IL-18<sup>24-26</sup>.

Cellular injuries, bacterial infections and various toxic substances induce NALP3 gene expression and complex activation<sup>27-29</sup>. NALP3 activation is through a two-signal model, a priming signal and an activation signal. The priming signal may be provided by either endogenous cytokine such as TNF- $\alpha$  or by bacterial infection leading to upregulation of NALP3 and pro-IL-1 $\beta$  through NF- $\kappa$ B signaling pathway. Different stimuli including K<sup>+</sup> efflux, ATP, toxins, Reactive Oxygen Species (ROS) mediate the activation of NALP3 and production of mature IL-1 $\beta$  in the second signal<sup>30-32</sup>.

Recent studies on NALP3 inflammasome showed that it plays an essential role in *Escherichia coli* infection, a gram-negative enteric bacterium that can modulate innate responses<sup>33</sup>. *Escherichia fergusonii* is one of the *Escherichia* species that evolved from *Escherichia coli* and that is considered as a non-pathogenic bacterium.

However, recent studies showed that it induces immunological responses both in animals and in humans and it is resistant to a broad spectrum of antibiotics<sup>34</sup>.

Although the anti-inflammatory effects of AM leaves extracts have been widely reported, their effect on NALP3 inflammasome activation is not well documented. In this study we investigate the effects of AM aqueous extract on NALP3 activation and IL-1 $\beta$  regulation in Balb/c mice infected with *Escherichia fergusonii*.

## 2. Materials and Methods

### 2.1 Reagents

Agarose was purchased from Peqlab (USA), chloroform from Fluka (USA), acetylsalicylic acid (aspirin), ethanol, bovine serum albumin, ortho-phosphoric acid, sodium chloride, potassium chloride, Thiobarbituric Acid (TBA), Trichloroacetic Acid (TCA), potassium phosphate monobasic/basic and formaldehyde were purchased from Sigma-Aldrich (USA). Coomassie brilliant blue G250 was purchased from Fisher Scientific (USA). PMSF was purchased from Roche Diagnostics (Switzerland). NaCl was purchased from Serum Products. TNF $\alpha$  and IL-1 $\beta$  ELISA kit (cat no. ab100747 and ab100705 for IL-1 $\beta$  respectively) were from Abcam (Cambridge, UK). RNeasy Mini Kit, Quantitect Reverse Transcription Kit, QuantiFast SYBR Green RT-PCR Kit were purchased from Qiagen (Cambridge, UK).

### 2.2 Preparation of the Water Extract of *A. muricata* Leaves

Sterilized and shade air-dried *Annona muricata* leaves were purchased from USA from Nallife Company. The leaves were of Thailand origin. Sixty grams of powdered AM leaves were soaked in 200 ml of boiling distilled water for 30 min. Filtrate were extracted a second time with an additional 200 ml of boiling distilled water for 30 min. The combined filtrates were then freeze-dried yielding a 2.43 g of brown, sticky precipitate that was stored at 4°C. Aliquot of extract residues were weighed and suspended in deionized distilled water before use.

## 2.3 Animals

Healthy female Balb/c albino mice (25–30 g) used in this study was obtained from the animal house at Beirut Arab University. The animals were housed under standard laboratory conditions (humidity  $55 \pm 5$  %, temperature  $22 \pm 2$  °C, 12/12 h light/dark cycle) and had access to food and water ad libitum throughout the study. Mice were randomly divided into six experimental groups of 6 mice each. The control group received only distilled water. The aspirin group received 100 mg/kg of aspirin. The AM group received 100 mg/kg of AM extract. The *E. fergusonii* group were intra-peritoneally injected with  $1.5 \times 10^8$  CFU bacterium. The *E. fergusonii* + aspirin group were pretreated with aspirin at 100 mg/kg 2 hours prior to intraperitoneal injection with  $1.5 \times 10^8$  CFU of *E. fergusonii*. The *E. fergusonii* + AM group received 100 mg/kg of AM extract 2 hours prior to intraperitoneal injection with  $1.5 \times 10^8$  CFU of *E. fergusonii*. The animals were sacrificed 6, 24 and 72 hours after treatment, their livers were used for molecular analysis and histological studies.

**Ethical Consideration:** All experimental procedures performed on mice were approved by the Institutional Review Board (IRB) Committee at Beirut Arab University. The animals were taken care of in accordance with the recommendations provided in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences.

## 2.4 Induction of Peritonitis

Peritonitis was induced by injecting  $1.5 \times 10^8$  CFU of *Escherichia fergusonii* ATCC35469 that was a generous gift from Al-Hayat Hospital. The bacterium was identified by 16SrRNA sequencing. Bacteria were cultured in Luria Bertani (LB) broth at 37°C. They were harvested at the mid-log phase and washed twice with sterile saline before injection.

## 2.5 Paraffin Tissue Processing and Hematoxylin and Eosin Staining

Pieces of the isolated livers were washed with PBS, fixed using 10% formaldehyde for 18 hours, dehydrated through series of graded alcohol, cleared in xylene and embedded in molten paraffin wax. Tissue blocks were sectioned (4  $\mu$ m thickness) using microtome (14274

microtome HM340E), deparaffinized and stained with Hematoxylin and Eosin. The sections were examined with a Zeiss Primo light microscope with Axio Vision software (400x magnification) and photomicrographed using AxioCam camera for analysis.

## 2.6 Tissue Homogenization

Liver tissues were homogenized in sterilized PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4 containing 1mM protease inhibitor PMSF). The homogenate was then centrifuged for 20 minutes at 12000 g at 4°C. The supernatant was stored at –80°C for later use.

## 2.7 Determination of Malondialdehyde Concentration

Lipid peroxidation was established by measuring Malondialdehyde (MDA) in the liver homogenates using thiobarbituric acid. The preparation is based on the method described previously<sup>35</sup>. In brief, thiobarbituric acid react with MDA at low pH and high temperature resulting in a pink adduct that absorbs at 532 nm.

## 2.8 Measurement of Inflammatory Cytokines

The levels of IL-1 $\beta$  and TNF- $\alpha$  were measured using the Mouse ELISA Kit for IL-1 $\beta$  and TNF- $\alpha$  according to the manufacturers’ protocol. In brief, immobilized antibody against the cytokine measured is bound to a 96 well plate. Samples and standards (100  $\mu$ l) are added to each well. After incubation followed by washing (3x, 300  $\mu$ l washing solution), biotinylated anti-mouse IL-1 $\beta$  or TNF- $\alpha$  beta antibody is added. HRP conjugated streptavidin is next added to wells after washing unbound biotinylated antibody. TMB substrate solution is next added. The yellow color developed after adding the stop solution was measured at 450 nm and is proportional to the amount of bound cytokine.

## 2.9 Quantitative Real-time Polymerase Chain reaction (qRT-PCR) Analysis of Gene Expression

Total RNA were isolated from the liver homogenates using the RNeasy Plus Mini Kit according to manufacturer’s recommendations. RNA concentration was determined

by measuring the absorbance at 260 nm using a spectrophotometer. RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit according to manufacturer's recommendations. Quantitative real-time PCR were carried out using QuantiFast SYBR Green PCR Master Mix in triplicates and was performed using the QiagenRotor-Gene Q. The thermal cycling conditions were optimized as follow: 10 seconds at 95°C followed by 45 cycles of amplification (5 seconds at 95°C and 30 seconds at 57°C). The relative expression of each gene was calculated using the comparative threshold cycle method normalized to GAPDH gene.

The sequences of the primers used are as the following: GAPDH (forward:5'-TGGTGCTCAGTGTAGCCCAG-3' and reverse:5'-GGACCTGACCTGCCGTCTAG-3'); TNF- $\alpha$  forward: 5'-GAAGTTCCCAAATGGCCTCC-3', and reverse:5'-TCTATGGCCCAGACCCTCAC-3'); IL-1 $\beta$  (forward: 5'-TGGGCCTCAAAGGAAAGA-3', and reverse: 5'-GGTGCTGATGTACCAGTT-3'); NALP3 (forward: 5'-ATTACCCGCCCCGAGAAAGG-3, and reverse: 5'-CTGTGTGGATCTTTGCTGCGA-3');Caspase-1 (forward: 5'-CGTGGAGAGAAACAAGGAGTG-3 and reverse: 5'-AATGAAAAGTGAGCCCCTGAC-3').

Relative gene expression was calculated using the  $2^{-\Delta\Delta CT}$  Gene Dosage Ratio formula (GDR) where the difference between mean Cycle threshold (Ct) of gene of interest and mean cycle threshold of GAPDH gene in control samples are subtracted from their differences in test sample according to the following equation.

$$\Delta\Delta Ct = (mCt \text{ gene of interest} - mCt \text{ GAPDH}) \text{ test sample} - (mCt \text{ gene of interest} - mCt \text{ GAPDH}) \text{ control sample}.$$

## 2.10 Statistical Analysis

All statistical analyses were performed using Graph Pad Prism Ver 7. The data are expressed as mean  $\pm$  standard deviation (n = 3). In all tests, the minimum criterion for the statistical significance was  $p < 0.05$ . Statistical analysis of the obtained results was analyzed using One-way ANOVA. P-values of less than 0.05 were considered significant.

## 3. Results

### 3.1 Effect of AM Extract on Liver

Histopathology of the livers of normal control mice showed typical hepatolobular architecture, consisting of a central vein with radiating cords of hepatocytes separated by sinusoids. The hepatocytes are polygonal in shape, with central, lightly stained nuclei and prominent nucleolus. The cytoplasm is regularly distributed (Figure 1a).

However, the livers of mice treated with aspirin or AM extract showed deranged architecture of the hepatocyte with mild cloudy swelling and vascular congestion (Figure 1).

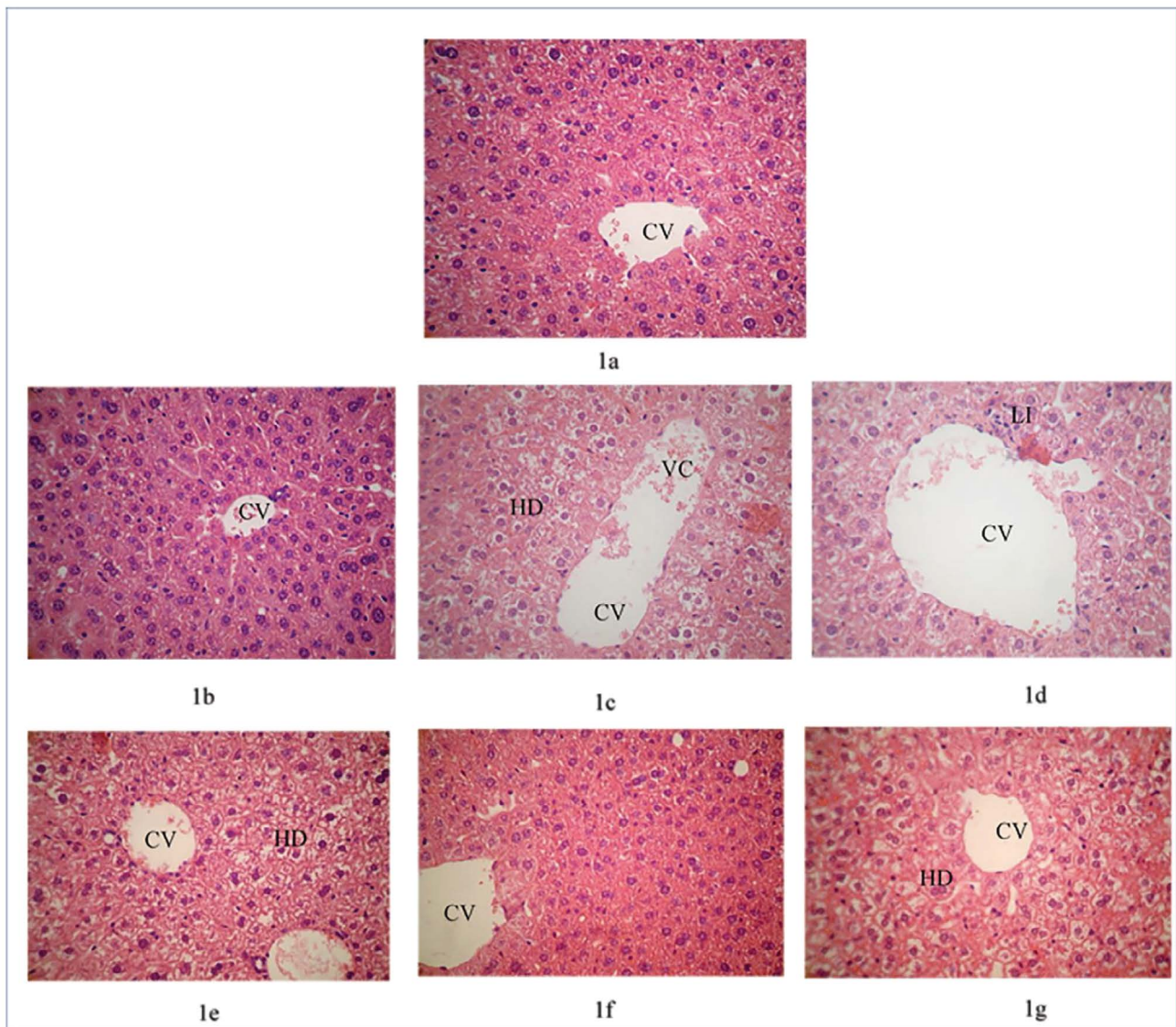
On the other hand, liver of mice injected with *E. fergusonii* showed moderate vascular congestion with slight leukocyte infiltration after 6 hours (Figure 2a). However, after 24 hours, deranged sinusoidal arrangement with dilatation, multiple bi-nucleated cells with undistinguished nucleoli, low to moderate vascular congestion, and hydropic degeneration were observed (Figure 2b). Non-occluding thrombosis and fibrin accumulation were seen after 72 hours (Figure 2c).

Liver of mice treated with aspirin prior to bacterial infection and sacrificed after 6 hours showed mild hepatosteatosis (Figure 3a). Those sacrificed after 24 and 72 hours showed lower leukocytes infiltration and vascular congestion with diminutive hydropic degeneration (Figures 3b and 3c).

On the other hand, treating infected mice with AM extract showed great anti-inflammatory effects. It reduced the vascular congestion, the leukocyte infiltration and the hydropic degeneration after 6 hours (Figure 3d). Liver of mice sacrificed after 24 and 72 hours appeared almost normal (Figure 3e and 3f).

### 3.2 Effect of AM Extract on MDA Levels

MDA levels were determined in the liver homogenates of all groups after 6, 24 and 72 hours. As shown in Figure 4, both aspirin and AM extract had no significant effect on MDA levels compared to normal



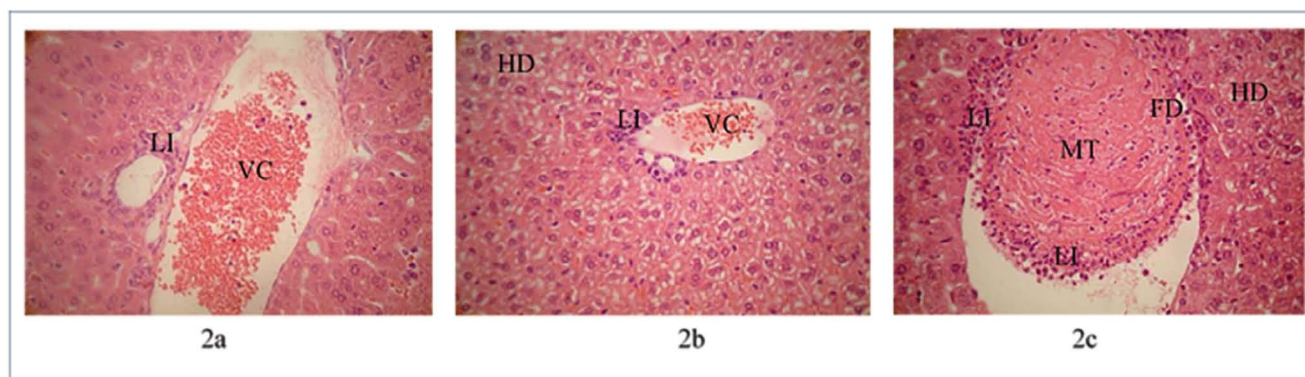
**Figure 1.** Liver sections from control groups. Figure 1a shows normal architecture of the liver with the central vein (CV) (1a). Figures 1b, 1c and 1d show sections of liver from mice that received aspirin (100 mg/kg), and sacrificed after 6, 24 and 72 hours. After 6 hours, a deranged sinusoidal arrangement with dilatation and slight vascular congestion is observed (1b). Mild Hydropic Degeneration (HD) and Vascular Congestion (VC) were observed after 24 hours (1c). After 72 hours, Leukocytes Infiltration (LI) was seen in addition to the mild HD and VC (1d). Figures 1e, 1f and 1g show sections of liver from mice that were pre-treated with 100 mg/kg AM extract and sacrificed after 6, 24 and 72 hours. Moderate HD was observed after 6 hours (1e) and slight fat deposition were seen after 24 hours (1f). HD persisted after 72 hours (1g) (H and E x400).

control. However, the MDA level in liver of mice infected with *E. fergusonii* exhibited an increase in MDA levels implying significant ROS generation. A significant decreased in MDA level was observed in mice that were pretreated with aspirin or AM extract two hours prior to bacterial injection (Figure 4a and 4b). Hence, AM extract protected the liver from ROS

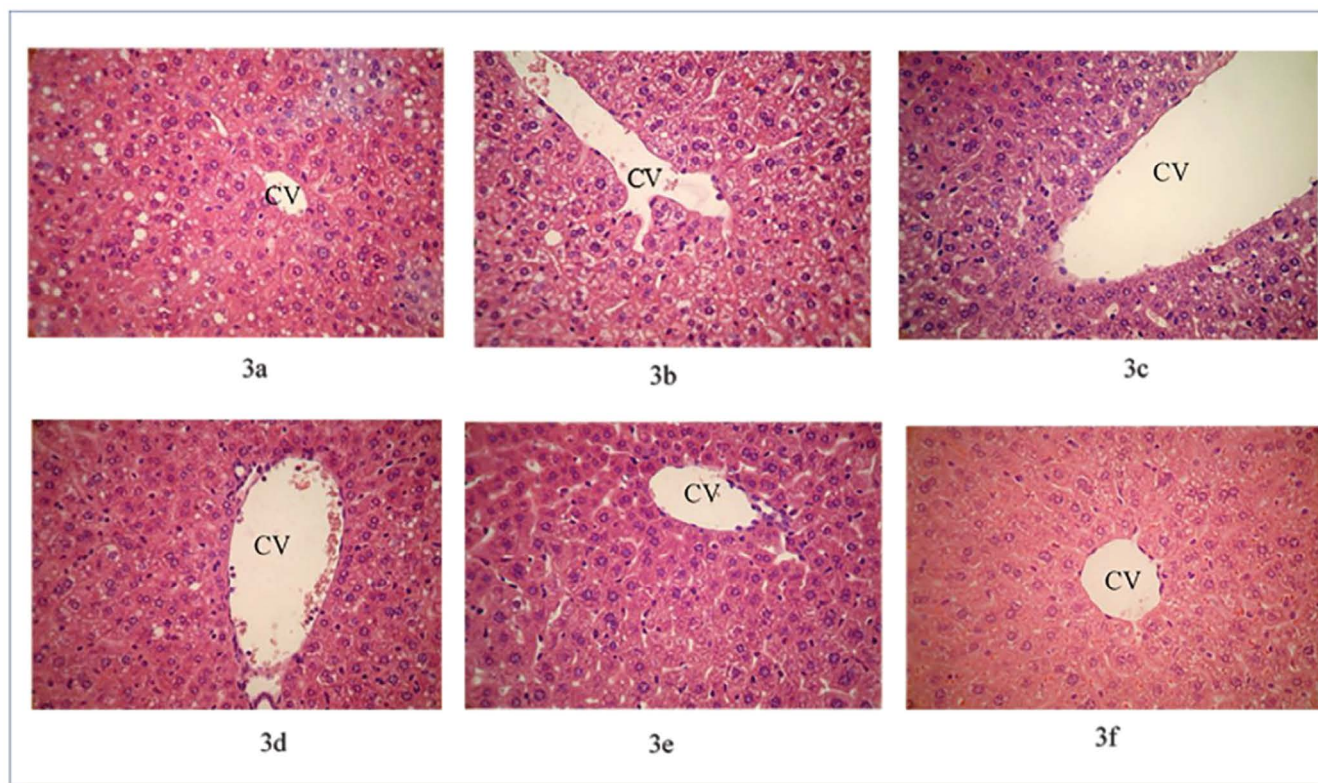
production and alleviating the hepatic necrosis seen in untreated infected mice (Figure 4).

### 3.3 Effect of AM Extract on TNF- $\alpha$ and IL-1 $\beta$ Production in Balb/c mice

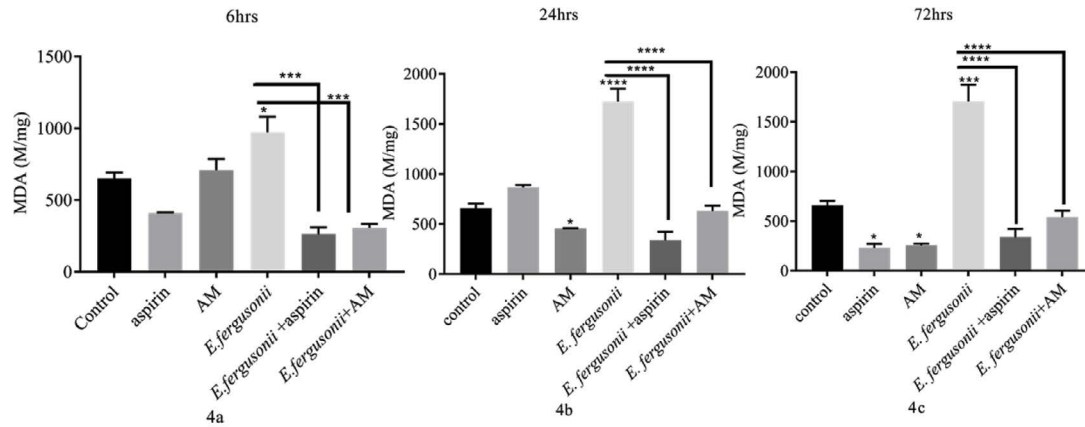
Aspirin had no significant effect on TNF- $\alpha$  release. The level of TNF- $\alpha$  increased in liver of mice treated with AM Extract alone within 24 hours, then returned to normal.



**Figure 2.** Sections of liver from mice intra-peritoneally injected with  $1.5 \times 10^8$  *E. fergusonii* and sacrificed after 6, 24 and 72 hours. Severe Vascular Congestion (VC) and Leukocytes Infiltration (LI) were observed after 6 hours (2a). After 24 hours, mild hydropic degeneration, vascular congestion, and leukocytes infiltration were observed (2b). Macroscopic non-occluding thrombus (MT) with massive lymphocytes infiltration surrounding the thrombus, Fibrin Deposit (FD) and Hydropic Degeneration (HD) were seen after 72 hours (2c) (H and E x400).



**Figure 3.** Sections of liver from mice pre-treated with 100 mg/kg aspirin 2 hours prior to *E. fergusonii* infection and sacrificed after 6 hours (3a), 24 hours (3b) and 72 hours (3c). After 6 hours, mild hepatosteatosis consisting of mixed macrovascular and microvascular fat accumulation were observed (3a). After 24 hours, mild cloudy swelling, vascular congestion and mild inflammation were seen (3b). After 72 hours, significant decrease in the hydropic degeneration and inflammation was observed (3c). Figures 3d, 3e and 3f show sections of liver from mice that received 100 mg/kg AM extract, 2 hours prior to bacterial infection and sacrificed after 6, 24 and 72 hours respectively. After 6 hours, mild vascular congestion and mild inflammation were observed (3d). Significant improvement of the cellular degeneration and better sinusoidal arrangement were seen after 24 hours (3e). Liver architecture returned back to normal after 72 hours (3f) (H and E x400).



**Figure 4.** The effect of AM extract on MDA levels in liver homogenates of Balb/c mice. The levels of MDA were measured after 6 hours (4a), 24 hours (4b) and 72 hours (4c). Data are expressed as mean  $\pm$  SD (n = 3). Statistical analysis was performed using One-way ANOVA. \*p < 0.05, \*\* < 0.01, and \*\*\* < 0.001.

However, the level of TNF- $\alpha$  significantly increased in mice infected with *E. fergusonii* compared to normal mice in a time dependent manner. The levels returned to normal upon treatment with aspirin or AM extract after 6 hours (Figure 5a, 5b, 5c).

As for IL-1 $\beta$  level, aspirin had no significant effect on IL-1 $\beta$  production. Interestingly, AM extract induced a significant increase in IL-1 $\beta$  level after 6 hours then returned to less than normal after 72 hours. Bacterial infection induced a significant release of IL-1 $\beta$  after 6 and 72 hours post infection. Treating infected mice with aspirin or AM extract led to a significant decrease in IL-1 $\beta$  production (Figure 5d, 5e and 5f). Therefore, AM extract minimized hepatic necrosis induced by *E. fergusonii* infection through the decreasing level of the innate immune cytokines IL-1 $\beta$  and TNF- $\alpha$ .

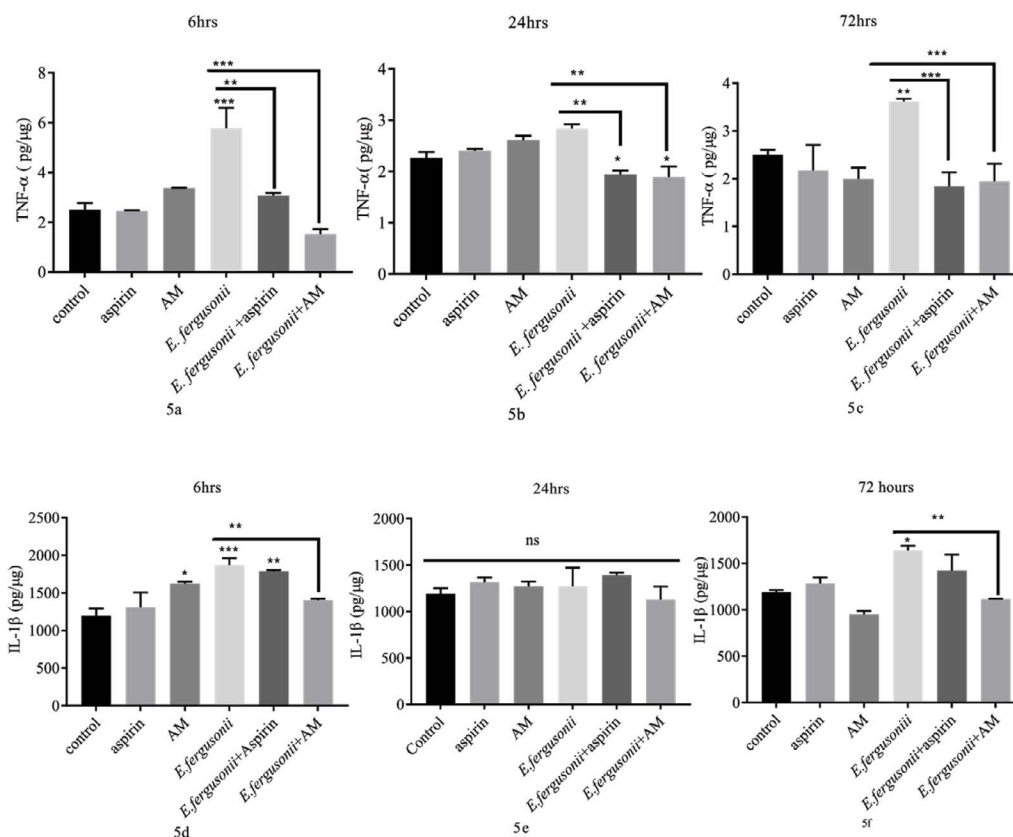
### 3.4 AM Extract Down-Regulates Inflammasome-related Gene Expression in Infected Balb/c Mice

The effect of AM extract on the expression of TNF- $\alpha$  that activate the priming signal of NALP3 and on the expression of downstream signaling genes in the NALP3-mediated response to *E. fergusonii* (IL-1 $\beta$ , caspase-1 and NALP3) was studied. Aspirin alone had no significant effect on TNF- $\alpha$  expression. AM extract significantly up regulated the expression level of TNF- $\alpha$  and caspase-1 after 24 hours. These results corroborate with the

up-regulation of IL-1 $\beta$  production and consistent with the hepatic necrosis observed after 6 hours (Figures 5 and 6). As expected, *E. fergusonii* infection up-regulated the expression levels of TNF- $\alpha$ , IL-1 $\beta$ , caspase-1 and NALP3. Treatment of these mice with both aspirin and AM extract led to the down-regulation in the expression levels of these genes. These results showed that AM extract greatly reduces NALP3 inflammasome pathway that was activated in response to *E. fergusonii* infection.

## 4. Discussion

NALP3 inflammasome is well known for its involvement in various diseases including bacterial inflammation. It induces the overproduction of different cytokines involved in the innate inflammatory response<sup>36</sup>. Gram-negative bacterial infection leads to the release of IL-1 $\beta$  through caspase-1 activation, a downstream effector of NALP3 signaling. Overproduction of IL-1 $\beta$  leads to chronic inflammation and sepsis. Much attention is given nowadays to natural therapies that can act as specific NALP3 inhibitors<sup>25</sup>. *Annona muricata* possesses anti-inflammatory and anti-bacterial effects. Its involvement in NALP3 signaling is not well established yet. This study investigated the effect of AM extract on NALP3 inflammasome in *E. fergusonii*-induced septic peritonitis model.



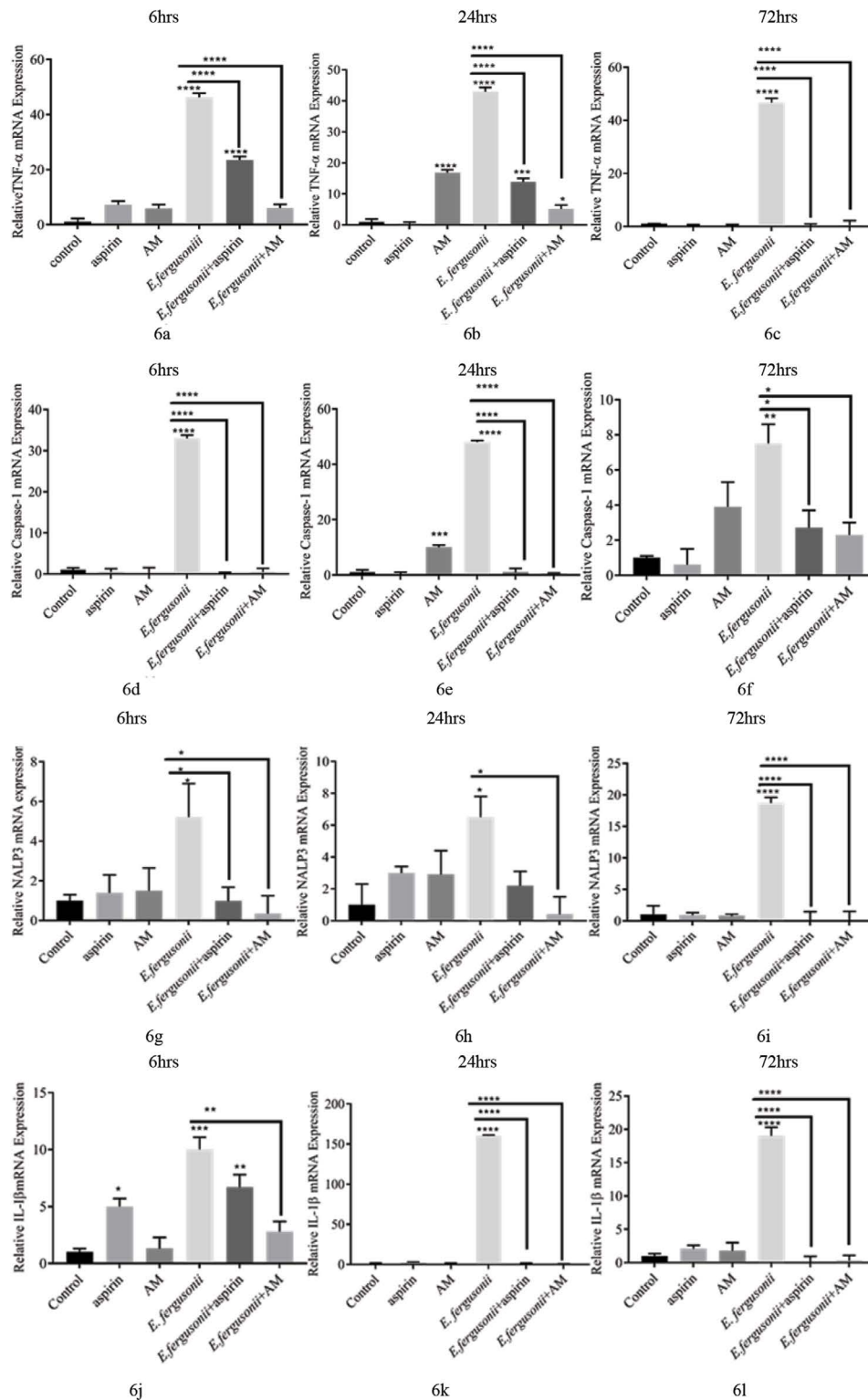
**Figure 5.** The effect of AM extract on TNF- $\alpha$  and IL-1 $\beta$  levels in liver homogenate of Balb/c mice. TNF- $\alpha$  and IL-1 $\beta$  levels were measured in the liver homogenates of mice sacrificed after 6 hours (4a, 4d), 24 hours (4b, 4e) and 72 hours (4c, 4f) after treatment. Data are expressed as mean  $\pm$  SD (n = 3). Statistical analysis was performed using One-way ANOVA \*p < 0.05, \*\* < 0.01, and \*\*\* < 0.001.

*E. fergusonii* induced symptoms of sepsis was confirmed macroscopically, microscopically and biochemically. Shivering, crouched position and ruffled fur were main characteristics seen in septic mice. A mortality rate of 44% was obtained in infected untreated mice 24 hours post-infection (data not shown). Signs of infection were observed 6 hours post-infection and subsided after 24 hours if the mouse survived. The signs completely disappeared after 72 hours. The disappearance of septic features could be due to activation of innate immune responses. Aspirin treatment led to 23% a decrease in mortality rate. On the other hand, pre-treatment with AM extract minimized all features of sepsis with 0% mortality rate. Therefore, AM extract possesses strong antibacterial effect.

Macroscopically, livers of mice infected with *E. fergusonii* displayed global necrosis with white patches throughout. None were observed in control mice

given either aspirin or AM extract. Treating infected mice with aspirin led to a reduction in the observed necrosis. However, pre-treatment with AM extract completely protected the liver from the damage induced by *E. fergusonii* (data not shown). Furthermore, the histological examination confirmed our macroscopic examinations. AM extract significantly lessened the global hepatic necrosis seen in infected untreated mice in a time dependent manner. It prevented thrombosis after 72 hours. This prevention was not observed with aspirin treatment. Thrombosis formation is due to the role of neutrophils that are the central components of innate immune system<sup>37</sup>. They prevent bacterial dissemination by Neutrophil Extracellular Traps (NET) that promotes the formation of immune thrombosis. Thrombosis traps the circulating bacteria, avoid tissue attack and lessen the surviving bacteria in spleen and liver<sup>38</sup>. AM extract was able to inhibit the formation of immune thrombosis





**Figure 6.** Effect of AM extract on the expression of TNF $\alpha$ , IL-1 $\beta$ , NALP3, and caspase-1 genes in liver of Balb/c mice. Expression levels of treated and controls were normalized to their respective GAPDH. Fold expression was determined relative to the control. All bars represent mean of three determinations  $\pm$  SEM. (\*), (\*\*), (\*\*\*) , (\*\*\*\*) correspond to p value < 0.05, 0.01, 0.001, and 0.0001 respectively. Asterisks drawn upwards represent inter-categorical statistical significance

induced by *E. fergusonii* and eliminated its existence in the liver. Moreover, AM extract reduced the vascular congestion and leukocytes infiltration observed in untreated infected mice 6 hours post infection, leading to normal hepatic architecture after 24 hours. This confirms the antibacterial effect that AM extract possesses.

Biochemically, AM extract eliminated hepatic necrosis by minimizing the levels of reactive oxygen species that induce membrane damage. MDA is the products of lipid peroxidation and the major indicator of ROS-dependent tissue damage in numerous organs including liver<sup>39,40</sup>. AM extract caused a significant decrease in MDA levels in infected mice confirming its anti-oxidative protective role. The hepato-protective effect of AM extract was reported in several studies and it protects DNA against H<sub>2</sub>O<sub>2</sub>-induced toxicity<sup>41</sup>. AM is rich in anti-oxidant (vitamin E and C), contains high superoxide dismutase, catalase activity<sup>42</sup> and elevated GSH levels<sup>43</sup>. All these enzymatic and non-enzymatic anti-oxidants lowers MDA levels. ROS secretion was essential for NALP3 activation<sup>44</sup> and ROS inhibitors reduce the activity of NALP3 at the activation signal<sup>45</sup>.

The levels of the inflammatory markers IL-1 $\beta$  and TNF- $\alpha$  levels were significantly high 6 hours post-infection in untreated infected mice. They decreased within 24 hours then rose again at 72 hours. These results are attributed to the innate physiological response. In this study, we proved that AM extract down-regulated the innate inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  in the *E. fergusonii* infected hepatic tissues in a time dependent manner. The level of these biochemical parameters is consistent with the histopathological results (Figure 2 versus Figure 5). Therefore, hepatic protection is conferred through reduction of the inflammatory cytokines expression.

During bacterial infection, activated Kupffer cells secrete the inflammatory mediator TNF- $\alpha$  that primes NF- $\kappa$ B and activates caspase-1 to secrete IL-1 $\beta$ <sup>30</sup>. AM extract down-regulated TNF- $\alpha$  leading to the inhibition of IL-1 $\beta$  production at the priming and activation stages of NALP3 pathway. The levels of NALP3, TNF- $\alpha$ , caspase-1, and IL-1 $\beta$  gene expression were assessed in this study. IL-1 $\beta$  significantly increased at 6, 24 and 72 hours post-bacterial infection.

AM administration to normal mice also led to a significant increase in the level of IL-1 $\beta$  at 6 hours and then decreased afterwards. An increase in gene expression level of caspase-1 and TNF- $\alpha$  was also observed at 24 hours and then decreased. Metabolism of the AM active ingredients in the liver could have induced the increase in IL-1 $\beta$  at 6 hours (Figure 5) which in turn led to the activation of gene expression of caspase-1 and TNF- $\alpha$  (Figure 6). These changes are consistent with the slight hepatic changes observed with AM extract treatment at 6 hours.

## 5. Conclusions

AM extract was able to prevent the release of IL-1 $\beta$  via inhibition of NALP3 activation at the priming and activation signals. It was able to attenuate oxidative stress as shown by lower MDA levels. AM extract down-regulated NALP3 activity making this herb an efficient antibacterial and anti-inflammatory agent. Our results offer a novel inflammasome-associated mechanism for *Annona muricata* that can be considered as therapeutic agent in treating NALP3 associated inflammatory disorders.

## 6. Acknowledgement

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