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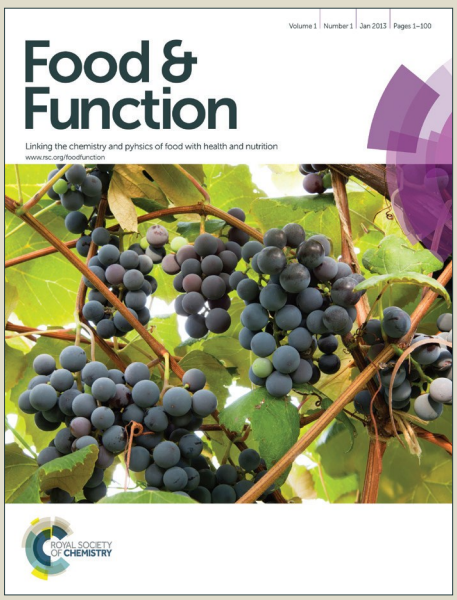

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The anti-infective activity of punicalagin against *Salmonella enterica* subsp. *enterica* serovar Typhimurium in mice

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Abstract: Punicalagin, a major bioactive component of pomegranate peel, has been proven to have antioxidant, antiviral, anti-apoptosis, and hepatoprotective properties. The aim of this study was to investigate the anti-infective activity of punicalagin in a mouse model. C57BL/6 mice were initially challenged with *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) and then treated with punicalagin. Food and water consumption and body weight were recorded daily. On day 8 post infection, mice were sacrificed to examine pathogen counts in tissues, hematological parameters, cytokines levels, and histological changes. Compared to mice only infected with *S.* Typhimurium, punicalagin-treated mice had more food consumption and less weight loss. Higher survival rate and lower counts of viable *S.* Typhimurium in feces, liver, spleen, and kidney were found in punicalagin-treated mice. The enzyme linked immunosorbent assay showed that the levels of IL-6, IL-10, IFN-γ in serum and spleen and TNF-α in serum, spleen and liver were reduced by punicalagin. Moreover, more neutrophils and higher neutrophil-to-mononuclear cell ratios in punicalagin-treated mice were observed. Histological examination showed that punicalagin protected cells in liver and spleen from hemorrhagic necrosis. It is concluded that punicalagin has a benificial effect against *S.* Typhimurium infection in mice. The anti-infective property, together with other nutritionally beneficial effects, make punicalagin a promising supplement in human food or animal feeds to prevent disease associated with *S.* Typhimurium.

1. Introduction

Salmonella enterica subsp. *enterica* , a group of enteropathogen to humans, usually cause gastroenteritis and sometimes systemic infection through consumption of contaminated foods [1]. In China, *Salmonella enterica* subsp. *enterica* has been the most common cause of the bacterial foodborne diseases [2]. *Salmonella enterica* subsp. *enterica*, also ranked among the leading causes of bacterial foodborne disease in developed countries such as the United States, Europe, and Australia [3-5].

Antimicrobial drugs such as cefoxitin, tetracyclines, and ampicillin are used to treat systematic infections caused by *Salmonella enterica* subsp. *enterica*. However, drug-resistant *Salmonella enterica* subsp. *enterica* have emerged due to widespread usage of these antibiotics in human and animal, presenting a huge challenge for treating *Salmonella* infections. Studies also reported that drug-resistant *Salmonella*, especially the multiple drug resistant *strains* have been found in various food including meat, raw chicken, milk, etc. [6-8]. These strains in food pose the risk of causing foodborne disease for human and made it more difficult to treat *Salmonella enterica* subsp. *enterica* infections. Therefore, there is a continuous demand for developing alternative strategies to prevent and treat infections caused by *Salmonella*. Recently, natural products, especially polyphenols, have gained an increasing attention due to their bacteriocidal or bacteriostatic activity. Many studies have reported that natural compounds, such as chlorogenic acid, essential oil, nobiletin and tangeretin, show antimicrobial activity against various foodborne pathogens,

including *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* and *Listeria monocytogenes* [9-11].

Pomegranate (*Punica granatum L.*), one of the richest sources of polyphenols and flavonoids, has been used in traditional Chinese medicine as therapy for a variety of ailments such as dysentery, diarrhea, ulcers, microbial infections, and hemorrhage [12, 13]. Punicalagin, a major bioactive component of pomegranate, has been demonstrated to exhibit antioxidant, antiviral, anti-apoptosis, and hepatoprotective properties [14-16]. Punicalagin has also been reported to inhibit several pathogens *in vitro* [17]. However, the anti-infective activity of punicalagin against *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) *in vivo* has rarely been investigated. Therefore, the aim of this study was to explore the anti-infective effects of punicalagin in a mouse infection model.

2. Material and methods

2.1. Reagents

Punicalagin was purchased from Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China). The enzyme linked immunosorbent assay (ELISA) kits of interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) were purchased from Xinle Bioscience Co., Ltd (Shanghai, China). Bicinchoninic acid (BCA) protein assay kit was from Beijing CoWin Bioscience Co., Ltd. (Beijing, China). Luria-Bertani (LB) broth, Xylose lysine desoxycholate (XLD) agar and tryptic soy agar (TSA) were obtained from Beijing

Land Bridge Technology CO., LTD. (Beijing, China). Gentamycin, ampicillin and anti-protease cocktail were purchased from the Sigma Chemical Co. (St. Louis, Mo, USA). All solvents and chemicals used in the study were of analytical grade.

2.2. Strains and culture conditions

S. Typhimurium SL1344 containing a green fluorescent protein plasmid has been constructed previously in our lab. Before experiment, overnight cultures were prepared by cultivation cells at 37 ℃ for 12 h in LB broth containing ampicillin (50 μg/ml). Cells were recovered by centrifugation at 13,400 g for 5 min, then washed and re-suspended in phosphate buffer saline (PBS, pH=7.4). The bacterial density was adjusted to an OD_{600} value of 0.5 using a SmartSpec™ Plus Spectrophotometer (Biorad, California, USA). The cell suspensions were diluted with PBS to the desired concentration for the following assay.

2.3. Animals

Sixty C57BL/6 mice (male, 20 ± 2 g), provided by the Laboratorial Animal Center of Xi'an Jiaotong University (Xi'an, Shaanxi, China), were maintained in specific pathogen-free (SPF) condition. All mice were housed in an air-conditioned room at 22 ± 3 ℃ with a relative humidity of 30 – 60%, a 12-h light/dark cycle, and fed with tap water and the standard laboratory rodent diet. All animal experimental protocols were approved by the Northwest A&F University Animal Care and Use Committee (Yangling, Shaanxi, China). Mouse was humanely sacrificed by isofluorane before blood and organs were collected. All efforts were made to minimize suffering.

105 **2.4. *S.* Typhimurium infection**

106 The method described by Choi *et al.* [18] was followed with some modifications.
107 Briefly, mice were randomly divided into four groups: Group I: 0.9% normal saline –
108 treatment (NS), Group II: *Salmonella*-infected (*Sal*), Group Ⅲ: *Salmonella*-infected +
109 250 μg/ml punicalagin (*Sal* + 250 μg/ml) and Group Ⅳ: *Salmonella*-infected + 500
110 μg/ml punicalagin (*Sal* + 500 μg/ml). Each group contained 15 mice. Before the mice
111 were infected with *S.* Typhimurium, a total of 100 μl of streptomycin (5 mg/ml) was
112 administered via gavage needle for three days. Then, mice in the Group Ⅱ, Group
113 Ⅲ and Group Ⅳ were inoculated with 100 μl of *S.* Typhimurium (approximately
114 10^7 CFU) via gavage needle, whereas the Group I was fed with 0.9% normal saline.
115 After bacterial infection, animals in the Group III or Group IV were orally
116 administered with 100 μl of the punicalagin (250 μg/ml or 500 μg/ml) every 24 h
117 during the entire experimental period; while Group I and Group II animals were fed
118 with 100 μl of 0.9% normal saline. Throughout the experiment, mice had access to
119 water and food *ad libitum*.

120 **2.5. Parameters investigated**

121 Only live animals were used for determining the *S.* Typhimurium cell counts in
122 organs, serum chemistry, hematology and cytokines analysis, and the number of live
123 animals for each group at the end of experiment were as follows: 10 in NS group, 7 in
124 *Salmonella*-infected group, 8 in *Sal* + 250 μg/ml punicalagin group, and 12 in *Sal* +
125 500 μg/ml punicalagin group.

126 2.5.1. Body weights, food and water consumption

Animal body weights and the amount of food and water consumed by each animal were recorded once on day 0, 1, 2, 3, 4, 5, 6, 7 and 8. Mean body weights were calculated. Food and water consumption were calculated as g/animal/day.

2.5.2. Measurement of bacterial counts in feces and tissues

Fecal samples were collected at 0, 1, 2, 3, 4, 5 and 6 days after *S.* Typhimurium was administered. At day 8 post-infection, the mice were sacrificed, and the kidney, liver, and spleen were aseptically taken. Fecal samples and a part of tissue were weighed and homogenized in sterile PBS (1:10, w/v). The numbers of the bacteria per gram of feces or organs were determined. Serial dilutions were prepared and 100 μl aliquots were plated onto XLD and TSA plates (containing 50 μg/ml ampicillin), which were subsequently incubated overnight at 37 ℃ for 24 h. Typical colonies on XLD plates were counted. For TSA plates, the number of luminous colonies was calculated at 254 nm under fluoresence. Counts on XLD and TSA plates were averaged.

2.5.3. Biochemical and hematological analysis

Blood was collected and used for routine hematological and serum biochemistry analysis. The hematological analysis was performed using an automated hematology analyzer. The hematological parameters included total erythrocyte count (red blood cells, RBC) and leukocyte (white blood cells, WBC) differential counting (neutrophil, monocyte, eosinophil, and basophil).

For the chemistry analysis, blood was allowed to coagulate and serum was separated after centrifugation. Serum chemistry parameters including alanine

aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), creatinine (Crea), uric acid (Ua), total bilirubin (TBIL), total protein (TP), copper (Cu), sodium (Na), and potassium (K), were determined by an automated analyzer.

2.5.4. Cytokines determination

Serum was obtained as described previously and detected for IL-6, IL-10, TNF-α and IFN-γ with the ELISA kit according to the manufacturer's instructions.

For organs, spleen and liver (50 mg) were homogenized in 500 μl ice-cold PBS (containing anti-protease cocktail). Then, samples were centrifuged at 4,000 g for 10 min. The supernatant was obtained and stored at -80℃ until used. The concentration of protein was measured by a commercially available BCA protein assay kit and using bovine serum albumin as a standard. The levels of IL-6, IL-10, TNF-α and IFN-γ were measured using ELISA kits. The concentration of cytokine in organ was calculated as pg/(ml·prot).

2.5.5. Histopathology analysis

The method described by Kim *et al*. was followed with slight modification [19]. The cecum, liver, spleen and kidney of the mice were fixed with 4% paraformaldehyde in PBS. The tissues were rinsed with water, dehydrated with ethanol, embedded in paraffin, sectioned into 4 μm, mounted onto glass slides, dewaxed using xylene and ethanol, and stained with hematoxylin and eosin Y (H&E). Histological changes were observed under a light microscope at 200× magnification.

2.6. Statistical analysis

All data were analyzed by one-way analysis of variance using GraphPad Prism 5 (GraphPad Software, Inc., California, USA). Results were shown as the mean ± standard deviation (SD). Means were compared using Duncan's multiple range test and differences were considered statistically significant at $P < 0.05$.

3. Results

3.1 Body weights, food and water consumption

As shown in Figure 1A, *S.* Typhimurium challenge caused significant weight loss in mice in group II compared with mice in group I. Punicalagin (500 μg/ml) treatment prevent weight loss and no statistical differences was observed between day 7 and day 0 ($P > 0.05$). However, punicalagin at 250 μg/ml had no significant effect on preventing weight loss of mice. In group II and group III, body weight of mice at day 7 was significant different from mice at day 0 i ($P \leq 0.05$).

Figure 1B shows the food consumption of mice in each group during 7 days. Between day 0 and day 3, food consumption in each group showed no statistical difference. However, food consumption differed among groups at day 3 after *S.* Typhimurium was administered. Compared with group II, the amount of food consumed by mice in group IV was larger. But the food intake of mice in group IV is less than those in group I. The amount of food consumed by mice in group III showed no difference from mice in group II ($P > 0.05$).

In addition, it is found that water consumption of mice in group II was more than that in group I. Moreover, water consumption of mice in punicalagin-treated group

showed no difference from mice in group I. At day 7, the amount of water consumption in mice in two punicalagin-treated groups was significant different from mice in group II ($P < 0.05$) (Figure 1C).

3.2. Survival of mice in different groups

The survival curves of mice in different groups are shown in Figure 1D. Eight mice in group II died during 8 days, whereas 3 in group IV and 7 in group III. Mortality of mice between group II and group III was no significantly different, but mortality of mice in each group differed from that in group IV ($P \leq 0.05$).

3.3. *S.* Typhimurium cell counts in organs and feces

Figure 2 A, B, C showed the number of bacterial cell counts in liver, spleen, and kidney of mice in each group. *S.* Typhimurium counts in the liver, spleen, and kidney of the mice in group III and group IV were significantly lower than those in group II. Significant reduction of cell counts (from 5.21×10^6 to 1.27×10^6 cfu in liver, from 2.64×10^7 to 5.56×10^6 cfu in spleen, and from 3.92×10^6 to 3.83×10^5 cfu in kidney) were observed at day 8 post *S.* Typhimurium challenged.

S. Typhimurium cell counts in feces of mice was determined for 5 days post-infection (Figure 2D). From day 2 to 5 post-infection, the pathogen counts in feces were significantly lower in mice in group III and group IV when compared with mice in group II ($P < 0.05$).

3.4. Serum chemistry

As shown in Table 1, increases of ALT, AST, BUN, Ua, and LDH were observed in mice in group II compared to control group, whereas levels of TBIL, TP, ALP, and

Crea were decreased. In group III and IV, levels of ALT, AST, and LDH were significantly lower than those in group II, however no significant change was observed in other parameters compared with mice in group II. In addition, no significantly difference was found in the levels of K, Na, or Cu in serum between mice with and without *S.* Typhimurium challenge ($P > 0.05$).

3.5. Hematology

Hematological parameters are shown in Table 1. After mice were infected with *S.* Typhimurium, symptom of paratyphoid was observed by the evidence of the reduction of WBC, NEUT and EO. After treatment with punicalagin, especially at 500 μg/ml, levels of WBC, NEUT, LYMPH and EO in mice were increased, compared with mice in group II; however, WBC and LYMPH counts were significantly lower in the punicalagin-treated group than in the uninfected group.

3.6. Cytokines in serum, liver and spleen

Serum level of cytokines IL-6, IL-10, TNF-α and IFN-γ were assayed at 8 days post-challenge for their. Mice treated with punicalagin showed lower levels of IL-6, TNF-α, IFN-γ and IL-10 compared with those mice in group II (Figure 3A, B, C, and D). The serum level of IL-6, TNF-α, IFN-γ and IL-10 decreased from 82.35±2.07 (group II) to 64.53±4.34 pg/ml (group IV) from 140.73±3.62 to 71.32±6.34 pg/ml, from 191.73±23.99 to 126.26±13.37 pg/ml and from 162.88±6.33 to 116.32±54.4199 pg/ml, respectively.

As shown in Figure 4A, higher levels of IL-6 in spleen were found in mice in group II than those in group I. For mice in group III and group IV, no significant

difference in the level of IL-6 existed compared with mice in group I. Similar results were observed for IL-10, TNF-α and IFN-γ (Figure 4B, C, D). For liver, no difference was observed in the levels of IL-6, IL-10, and IFN-γ among different groups. However, there was significant difference in the levels of TNF-α in liver between mice in group II and group IV (Figure 4A, B, C, D).

3.7. Histopathology

Histopathological changes of mice in different groups are shown in figure 5. Mice in group II were lethargic and showed histological damage in the liver and spleen. Moreover, liver injuries such as necrosis and hemorrhage were found (Figure 5). The spleen showed enlargement and extensive hemorrhagic necrosis (Figure 5). However, liver and spleen in mice in group III and IV showed minimal histological damage. In addition, no specific abnormal findings were observed in the kidney in mice that infected or uninfected with *S.* Typhimurium.

4. Discussion

Foodborne disease caused by *Salmonella enterica* subsp. *enterica* is a serious global health problem, with consequences ranging from self-limiting gastroenteritis to typhoid fever. Antibiotics are the most important therapy to treat human salmonellosis. However, due to fast development of resistant or multi-resistant strains after widespread use of antibiotics [20, 21], interest for searching for natural compounds as alternative treatment has increased significantly [22]. In this study, we investigated the effects of punicalagin against *S.* Typhimurium infection in a mouse model. It is shown

that punicalagin, in a dose-dependent manner, reduced the clinical manifestations, inflammation and tissue damage and increased the survival of infected mice.

There are two type of immune system: innate immune system and acquired immune system. Innate immune system is vital for the control of pathogens after infection, as well as for facilitating the development of acquired immune responses [23]. *S. Typhimurium* can enter into body through contaminated food, and challenge innate immune system and acquired immune responses. However, *S.* Typhimurium has the ability to evade innate immune responses. Once *S.* Typhimurium overcomes the innate immunity, it encounters the acquired immune responses. When *S.* Typhimurium reached into the intestine, it can colonize and overload in the gastrointestinal tract, which finally cause acute inflammatory response [24]. The process initiates the diffusion of fluid and leads to loosening of the tight junctions among intestinal epithelial cells. Then, the bacteria can take advantage of the process and then disseminate from the intestine to other tissues which will cause tissue injury and systematic disease [25]. We found a lower *S.* Typhimurium burden in spleen, liver, and kidney in punicalagin-treated mice, which showed that punicalagin inhibited the bacterial translocation from intestine to liver and spleen. This was also confirmed by less tissue (liver and spleen) destruction in mice treated with punicalagin. In addition, we previously reported that punicalagin reduced the *S.* Typhimurium invasion of HT29 cells [26]. This indicates that punicalagin might strengthen the tight junction of epithelial cells, which can decrease *S.* Typhimurium translocation and as a result reduce liver and spleen injury and mortality.

Liver damage usually occurs after *S.* Typhimurium infection. Serum AST, ALT and ALP could indicate liver toxicity and activities of AST and ALT are commonly used as biochemical markers for liver damage. In this study, we demonstrated that mice in punicalagin-treated group had a significant improvement in parameters associated with liver function. Previous studies of Lin *et al*. (1998, 2001) [14, 27] found that administration of punicalagin significantly prevented CCl_4 (or acetaminophen) induced elevation of AST, ALT and ALP in mice. All these finding indicated that punicalagin exhibited a hepatoprotective effect.

Leukocyte plays an important role in treating infections. There are two major types of white cells providing immunity to infection: germ-ingesting cells (neutrophils and monocytes) and lymphocytes. During acute inflammation, neutrophils are recruited to inflammatory sites to defend against invading pathogens [28]. Meanwhile, granulopoiesis is up-regulated by the inflammatory stimulus. After neutrophils migrate to the site of infection, the functions of neutrophils, such as phagocytosis and intracellular killing, are activated. The processes are regulated by various cytokines and chemokines [29]. And neutrophil functions can be impaired by many factors, leading to secondary bacterial infections. It is suggested that both the number and biological functions of neutrophils are important in controlling bacterial replication and invasion. We found more neutrophils and higher neutrophil-to-mononuclear cell ratios in punicalagin-treated mice, which enhanced the host resistance to *S.* Typhimurium infection. These indicate that punicalagin may stimulate certain cells to secrete cytokines to promote myeloid cell proliferation and neutrophil maturation.

When the neutrophils reach the inflammatory site, phagocytosis and intracellular killing activities are initiated. It is reported that phagocytic activity and the generation of free radicals can be up-regulated in response to *S.* Typhimurium stimulation in polyphenols treated mice [30]. We did not explore whether punicalagin has similar effects on the phagocytic activity and the generation of free radicals and remains to be determined in the future.

Cytokines are important mediators of inflammation. It is well known that increased pro-inflammatory cytokines (such as IFN-γ, TNF‑α.) will amplify the inflammatory cascade and result in tissue damage in patients or mice after *S.* Typhimurium infections. The down-regulation of pro-inflammatory cytokine activity and/or up-regulation of anti-inflammatory cytokine activity are useful to reduce the level of destruction caused by *S.* Typhimurium. It is reported that natural substances and probiotics reduced the injury caused by *S.* Typhimurium through lowering levels of the pro‑inflammatory cytokine [31-33]. In this study, we observed that the levels of TNF‑α, IL-6 and IFN‑γ in serum, spleen and liver of the *S.* Typhimurium infected mice were decreased by punicalagin administration. The RT‑PCR assay also confirmed that the genes expression of pro‑inflammatory cytokines (TNF‑α, IL-6 and IL-1β) in spleen and liver were reduced by punicalagin (data were not shown). This indicates that punicalagin exhibited beneficial function against *S.* Typhimurium infection in mice partly by activating innate immune cells. IL-10, which is produced by a variety of cells, is able to counter-regulate both the production of other cytokines and macrophage activation. Pie *et al*. [34] found that IL-10 is not involved in protection

but rather reflects severity of disease. We found that the levels of IL-10 in serum and spleen of the *S.* Typhimurium infected mice were decreased by punicalagin treatment. This indicated that punicalagin could alleviate the damage caused by *Salmonella*. In addition, hepatocellular injury is not due to the inducing agent itself but to the inflammatory cells that have been attracted by the stressed hepatocytes. *S.* Typhimurium induces a stress situation in hepatocytes with subsequent release of chemokines followed by accumulation of inflammatory cells and subsequent hepatocellular damage. In this study, we observed that only TNF-α in liver was increased after *Salmonella* infection. This can be caused by the immune cells that infiltrate the liver after infection, which agreed with histopathological findings in liver.

It is reported that punicalagin was hydrolyzed to ellagic acid in the gut, which was then metabolized by the colon microbiota to form the urolithin (urolithin-A and urolithin-B) [35]. Urolithins can accumulate in the intestine up to μM concentrations. It is also reported that urolithins can inhibit quorum sensing (QS) controlled biofilm formation and motility of *Yersinia enterocolitica* [36]. *Salmonella* is a Gram-negative facultative intracellular bacterium, and the main autoinducers, N-Acylhomoserine lactones, were also produced by *Salmonella* as well as most of Gram-negative pathogenic bacteria including *Yersinia enterocolitica* and *Pseudomonas aeruginosa* [37]. We previously found that punicalagin inhibited the QS system of *Salmonella in vitro* [26]. However, whether or not urolithins can exhibit any direct effect against *Salmonella* infection *in vivo* was still unknown and warrant further investigation.

Studies reported that punicalagin at the dose of 5-30 mg/kg had hepatoprotective and neuroprotective activity [14, 27, 38]. Compared to those reports, the doses used in this study were much lower (about 1.25-2.50 mg/kg). Punicalagin at 250 μg/ml (about 1.25 mg/kg) had no effect on body weight, food and water consumption of mice compared with mice in group II. This may be caused by the fact that the concentration of punicalagin in serum was not high enough to restore the changes in mice caused by *S.* Typhimurium infection.

In conclusion, punicalagin protected mice from *S.* Typhimurium-induced death and prevented bacterial translocation to the liver and spleen. In addition, punicalagin decreased levels of inflammatory cytokines, less tissues damage and improved blood parameters. These findings indicate that punicalagin has the potential to be developed as an alternative strategy to prevent or treat *S.* Typhimurium infections.

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Figure legend

Figure 1 Effect of punicalagin on body weights (A), food (B) and water consumption (C) and the survival (D) of mice after *S.* Typhimurium challenge. NS: 0.9% normal saline-treatment, *Sal*: *Salmonella*-infected, *Sal* + 250 μg/ml: *Salmonella*-infected + 250 μg/ml punicalagin and *Sal* + 500 μg/ml: *Salmonella*-infected + 500 μg/ml punicalagin. Data are presented as mean ± SD. * $P < 0.05$, ** $P < 0.01$.

Figure 2 *S.* Typhimurium cell counts in liver (A), spleen (B), kidney (C) and feces (D) of mice treated or untreated with punicalagin. NS: 0.9% normal saline-treatment, *Sal*: *Salmonella*-infected, *Sal* + 250 μg/ml: *Salmonella*-infected + 250 ug/ml punicalagin and *Sal* + 500 μg/ml: *Salmonella*-infected + 500 μg/ml punicalagin. Data are showed as mean ± SD. * $P < 0.05$, ** $P < 0.01$.

Figure 3 (A) IL-6, (B) IFN-γ, (C) IL-10 and (D) TNF-α level in sera of mice fed with 0.9% normal saline or punicalagin for 8 days followed by *S.* Typhimurium challenge. Each vertical bar represents the mean ± SD; * $P < 0.05$, ** $P < 0.01$. NS: 0.9% normal saline-treatment, *Sal*: *Salmonella*-infected, *Sal* + 250 μg/ml: *Salmonella*-infected + 250 μg/ml punicalagin and *Sal* + 500 μg/ml: *Salmonella*-infected + 500 μg/ml punicalagin.

Figure 4 The levels of IL-6 (A), IFN-γ (B), IL-10 (C) and TNF-α (D) in liver and spleen of mice fed with 0.9% normal saline or punicalagin for 8 days after *S.*

Typhimurium infections. Each vertical bar represents the mean ± SD; * $P < 0.05$, ** $P < 0.01$. NS: 0.9% normal saline-treatment, *Sal*: *Salmonella*-infected, *Sal* + 250 μg/ml: *Salmonella*-infected + 250 μg/ml punicalagin and *Sal* + 500 μg/ml: *Salmonella*-infected + 500 μg/ml punicalagin.

Figure 5 Histological examination of liver, spleen and kidney of mice nontreated or treated with punicalagin for 8 days after challenge with *S.* Typhimurium. NS: 0.9% normal saline-treatment, *Sal*: *Salmonella*-infected, *Sal* + 250 μg/ml: *Salmonella*-infected + 250 μg/ml punicalagin and *Sal* + 500 μg/ml: *Salmonella*-infected + 500 μg/ml punicalagin.

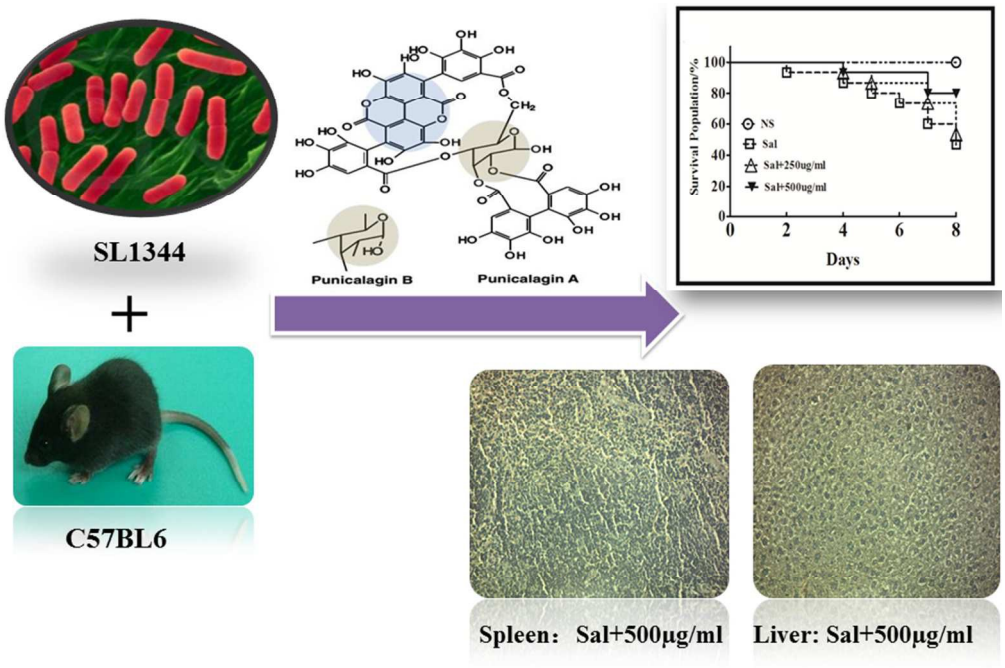
Table 1 Effect of punicalagin on hematological and serum chemistry parameters in mice in different groups.

		Groups[1]			
Parameter	Units	NS	*Sal*	*Sal*+250ug/ml	*Sal*+500ug/ml
ALT	U/L	69.75±7.89	847.33±96.41^{++}	796.50±33.50^{++}	527.67±22.84^{**++}
AST	U/L	313.25±23.60	1221.33±77.67^{++}	1044.00±27.00*$^{++}$	832.00±29.61**$^{++}$
TBIL	μmol/L	10.08±0.34	13.33±2.03^{+}	9.15±0.35*	10.23±1.00
TP	g/L	73.50±1.19	62.67±3.76	58.50±0.5^{+}	61.00±5.57^{+}
ALP	U/L	166.25±6.42	99.67±14.52^{+}	67.50±7.50^{++}	116.33±27.74
BUN	mmol/L	10.04±0.53	17.39±4.27	12.62±2.98	11.96±0.41
Crea	μmol/L	61.20±2.03	47.67±2.85	52.00±4.00	57.67±6.67
Ua	μmol/L	119.20±7.97	266.67±23.85^{++}	270.50±9.50^{++}	198.33±36.33^{+}
LDH	U/L	1686.00±118.76	3973.33±525.51^{++}	2526.00±456.00**	2091.75±216.52**
K	mmol/L	6.07±0.10	5.65±0.08	5.99±0.24	5.68±0.33
Na	mmol/L	149.30±0.93	150.33±1.45	153.50±1.3	153.10±1.90
Cu	mmol/L	26.43±0.64	26.70±0.49	26.10±0.7	26.87±0.20
WBC	10^3/cm	9.53±1.24	2.79±0.51^{++}	3.98±1.37^{++}	4.17±0.16^{++}
RBC	10^6/cm	10.86±0.37	9.49±0.26	8.23±0.14^{++}	9.05±0.58^{+}
NEUT	10^9/L	0.32±0.01	0.16±0.02^{++}	0.25±0.01^{**++}	0.30±0.01**
LYMPH	10^9/L	5.56±0.34	1.5±0.27^{++}	2.24±0.22^{++}	3.06±0.27^{*++}
MONO	10^9/L	0.06±0.01	0.75±0.02^{+}	0.53±0.23	0.41±0.18
EO	10^9/L	0.51±0.03	0.02±0.01^{+}	0.08±0.01^{+}	0.39±0.16*
BASO	10^9/L	0.56±0.09	0.03±0.02^{++}	0.02±0.01^{++}	0.09±0.05^{++}

[1] NS: 0.9% normal saline-treatment, *Sal*: *Salmonella*-infected, *Sal* + 250 μg/ml: *Salmonella*-infected + 250 μg/ml punicalagin and *Sal* + 500 μg/ml: *Salmonella*-infected + 500 μg/ml punicalagin.

* Compared with the group that only infected with *S.* Typhimurium.

+ Compared with the NS group that only administered with 0.9% normal saline.



Graphicle abstract
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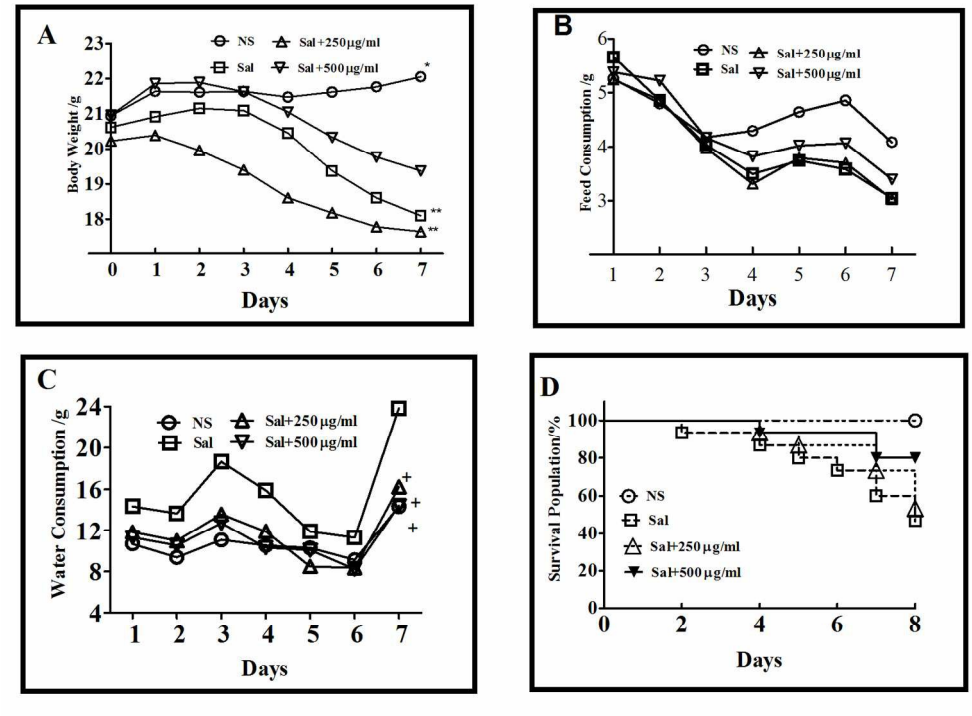

Figure 1 Effect of punicalagin on body weights (A), feed (B) and water consumption (C) and the survival (D) of mice after S. Typhimurium challenged. NS: 0.9% normal saline – treatment, Sal: Salmonella-infected, Sal + 250 μg/ml: Salmonella-infected + 250 μg/ml punicalagin and Sal + 500 μg/ml: Salmonella-infected + 500 μg/ml punicalagin. Data are presented as mean ± SD. * $P < 0.05$, ** $P < 0.01$.
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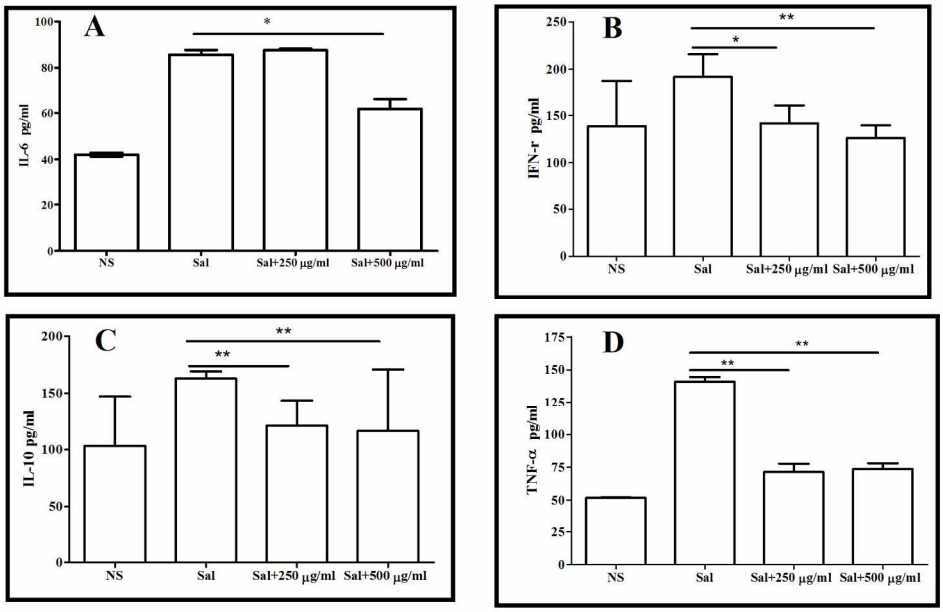

Figure 3 (A) IL-6, (B) IFN-γ, (C) IL-10 and (D) TNF-α level in sera of mice fed with 0.9% normal saline or punicalagin for 8 days followed by S. Typhimurium challenge. Each vertical bar represents the mean ± SD; * $P < 0.05$, ** $P < 0.01$. NS: 0.9% normal saline – treatment, Sal: Salmonella-infected, Sal + 250 µg/ml: Salmonella-infected + 250 µg/ml punicalagin and Sal + 500 µg/ml: Salmonella-infected + 500 µg/ml punicalagin.
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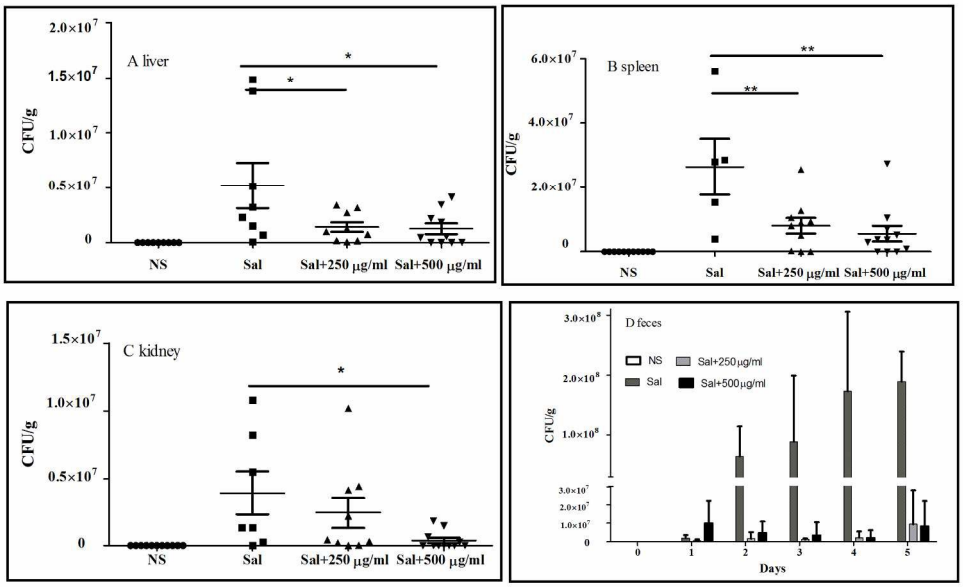

Figure 2 S. Typhimurium in liver (A), spleen (B), kidney (C) and feces (D) of mice treated or untreated with punicalagin. NS: 0.9% normal saline – treatment, Sal: Salmonella-infected, Sal + 250 μg/ml: Salmonella-infected + 250 ug/ml punicalagin and Sal + 500 μg/ml: Salmonella-infected + 500 μg/ml punicalagin. Data are showed as mean ± SD. * $P < 0.05$, ** $P < 0.01$.
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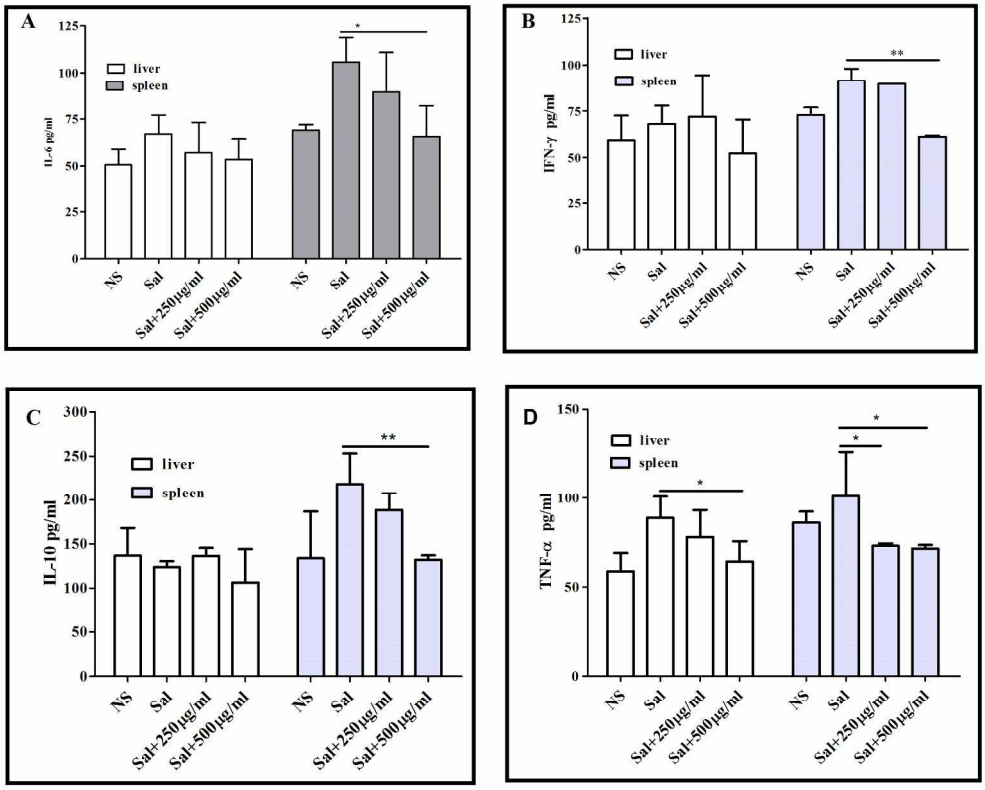

Figure 4 The levels of IL-6 (A), IFN-γ (B), IL-10 (C) and TNF-α (D) in liver and spleen of mice fed with 0.9% normal saline or punicalagin for 8 days after S. Typhimurium infections. Each vertical bar represents the mean ± SD; * P < 0.05, ** P < 0.01. NS: 0.9% normal saline – treatment, Sal: Salmonella-infected, Sal + 250 μg/ml: Salmonella-infected + 250 μg/ml punicalagin and Sal + 500 μg/ml: Salmonella-infected + 500 μg/ml punicalagin.
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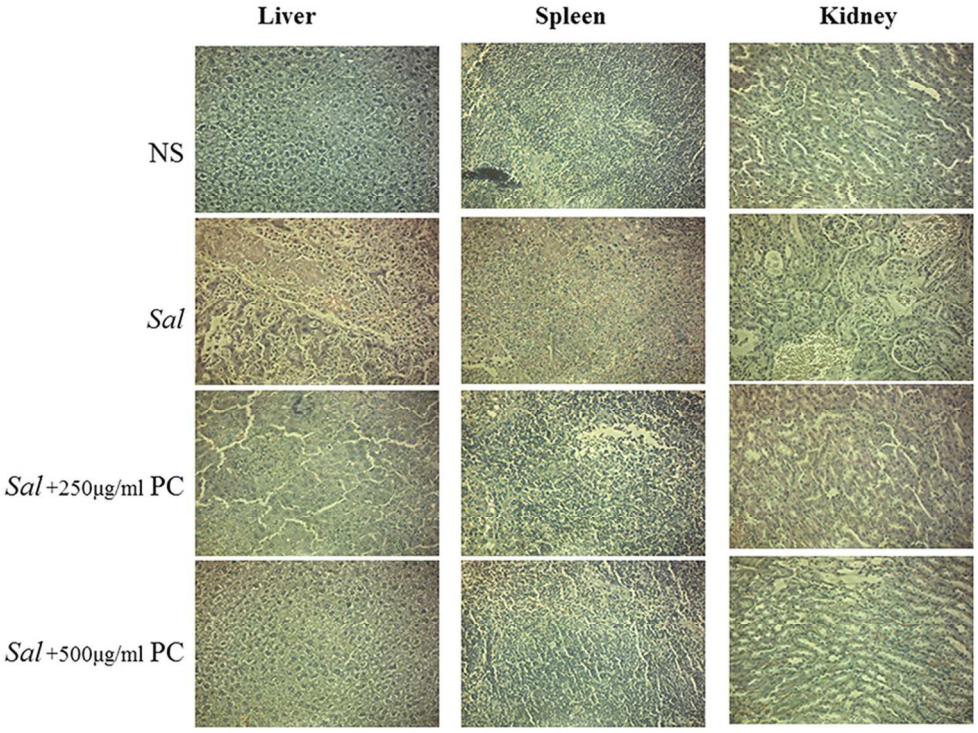

Figure 5 Histological aspect of liver, spleen and kidney of mice nontreated or treated with punicalagin for 8 days after challenged with S. Typhimurium. NS: 0.9% normal saline – treatment, Sal: Salmonella-infected, Sal + 250 μg/ml: Salmonella-infected + 250 μg/ml punicalagin and Sal + 500 μg/ml: Salmonella-infected + 500 μg/ml punicalagin.
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