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Original Article

In Vitro and In Vivo Antibacterial Activity of *Punica granatum* Peel Ethanol Extract against *Salmonella*

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Punica granatum is commonly used in Korea as a traditional medicine for the treatment of pathogenic bacteria. In this study, we investigated the *in vitro* and *in vivo* antimicrobial activity of *P. granatum* peel EtOH extract (PGPE) against 16 strains of *Salmonella*. The minimal inhibitory concentrations of PGPE were in the range of 62.5–1000 x03BCg mL^{-1}. In addition, the *in vivo* antibacterial activity of the PGPE extract was examined in a *S. typhimurium* infection mouse model. Mice were initially infected with *S. typhimurium* and then with PGPE. The extract was found to have significant effects on mortality and the numbers of viable *S. typhimurium* recovered from feces. Although clinical signs and histological damage were rarely observed in the treated mice, the untreated controls showed signs of lethargy and histological damage in the liver and spleen. Taken together, the results of this study indicate that PGPE has the potential to provide an effective treatment for salmonellosis.

1. Introduction

Salmonella enterica, which are Gram-negative bacterial pathogens capable of infecting humans and animals, cause significant morbidity and mortality worldwide [1]. *S. enterica* serovar *typhimurium* is a clinically important intracellular bacterial pathogen that causes food poisoning and gastroenteritis in millions of people worldwide each year [2]. The Centers for Disease Control (CDC) estimates that there are nearly 1.4 million food-borne *Salmonella* infections annually in the USA [3]. This bacterium infects the intestinal tract and causes systemic infection of various organs such as the liver and spleen [4].

Fluoroquinolones and tetracyclines are the antibiotics most commonly used to treat *Salmonella*, and until recently most strains were susceptible to these drugs. However, a high incidence of *Salmonella* strains resistant to commonly prescribed antibiotics has recently been reported in Korea and other countries [5, 6], and the increased appearance of antibiotic resistant strains of *Salmonella* further exacerbates this problem [7]. One major concern to public health has been the global dissemination of *S. typhimurium* Definitive Type 104, which is commonly resistant to five or more antimicrobial agents [8–11]. The rise in antibiotic-resistant pathogens has led to the development of new therapeutic agents that are effective against these bacteria. Recently, there has been considerable interest in the use of plant materials as an alternative method to control pathogenic microorganisms [12], and many compounds of plant products have been shown to be specifically targeted against resistant pathogenic bacteria [13].

Punica granatum, which belongs to the family of Punicaceae, is commonly known as pomegranate, grenade, granats and punica apple [14]. *Punica granatum* has been used extensively as a traditional medicine in many countries [15] for the treatment of dysentery, diarrhea, helminthiasis, acidosis, hemorrhage and respiratory pathologies [16, 17]. In addition, *P. granatum* is reported to have antioxidant

Table 1: List of *Salmonella* strains used in this study.

Strain	Serotypes	Origin	Resistant antibiotics
JOL 380	*S. typhi* ATCC 19943	Human	—
JOL 381	*S. paratyphi* A	Human	—
JOL 386	*S. enteritidis*	Chicken	—
JOL 387	*S. typhimurium*	Cattle	—
JOL 388	*S. typhimurium*	Cattle	—
JOL 389	*S. typhimurium*	Pig	AM, C, G, S, TIC
JOL 407	*S. enteritidis*	Chicken	—
JOL 408	*S. typhimurium*	Pig	—
JOL 409	*S. dublin* ATCC 39184	Cattle	—
JOL 410	*S. derby* ATCC 6960	Pig	—
JOL 411	*S. choleraesuis* ATCC 7001	Pig	AM, SXT
JOL 419	*S. gallinarum*	Chicken	CF, G, SXT
JOL 420	*S. gallinarum*	Chicken	CF, CIP, NA
JOL 421	*S. gallinarum*	Chicken	G, NA, S
JOL 422	*S. gallinarum*	Chicken	—
JOL 423	*S. gallinarum* ATCC 9184	Chicken	AM, AMC, C, G, S

AM, ampicillin; AMC, amoxicillin/clavulanic acid; C, chloramphenicol; CF, cephalothin; G, sulfisoxazole; NA, nalidixic acid; S, streptomycin; SXT, trimethoprim/sulfamethoxazole; TIC, ticarcillin.

[18, 19], anti-atherosclerotic [20, 21], antibacterial [22, 23] and antiviral [24] properties. The constituents of *P. granatum* include gallocatechins, delphinidin, cyanidin, gallic acid, ellagic acid, pelargonidin and sitosterol, which are very well known for their therapeutic properties [25].

Punica granatum peel is used to treat infections found in human sexual organs as well as mastitis, acne, folliculitis, pile, allergic dermatitis, tympanitis, scalds, diarrhea, dysentery and as an antioxidant [26]. In addition, it is reported that the extracts of *P. granatum* have antimicrobial activity against *Salmonella* [23]. However, to date, no studies regarding the antimicrobial activity of *P. granatum* peels have been conducted. Therefore, the goal of this study is to evaluate the antimicrobial activity of the EtOH extract of *P. granatum* peel using various *in vitro* and *in vivo* models.

2. Methods

2.1. Bacterial Strains and Culture Medium. *Salmonella typhi* (ATCC 19943), *S. dublin* (ATCC 39184), *S. derby* (ATCC 6960), *S. choleraesuis* (ATCC 7001) and *S. gallinarum* (ATCC 9184) were used in this study (Table 1). In addition, this study included local isolates of *S. enteritidis, S. typhimurium, S. gallinarum* and *S. paratyphi* A, which were provided by the National Veterinary Research and Quarantine Service, Republic of Korea. Bacterial strains were suspended in Mueller Hinton broth (MHB, Difco, USA) and then incubated at 37°C for 20 h. Mueller Hinton agar (MHA, Difco) was used for the agar diffusion method and minimal inhibitory concentration (MIC). *Salmonella typhimurium* (JOL 389) was used for *in vivo* assays in mice.

2.2. Extraction of Plant Material. *Punica granatum* peel was purchased from an Oriental drug store, Daehak Hanyakkuk (Iksan, Korea), and then authenticated by Dr D.Y. Kwon. A voucher specimen (no. 06-022) was deposited in the Laboratory of Herbalogy, College of Pharmacy, Wonkwang University, Iksan, Korea. Next, the *P. granatum* peel was air-dried in the dark at room temperature and then ground into a powder using a mechanical grinder. Approximately 500 g of the powdered materials were then boiled in 1500 mLof EtOH for 3 h. The solvent was then removed under reduced pressure in a rotary evaporator (N-1000S, EYELA, Japan) and dissolved in water or 50% dimethyl sulfoxide (DMSO, Sigma, USA) prior to use.

2.3. High-Performance Liquid Chromatography Analysis. The High-performance liquid chromatography (HPLC) system consisted of a Shimadzu LC-6A model (Shimadzu, Tokyo, Japan), with a column of ODS-C18 (4.6 × 250 mm, 5 μm) and a detection of SPD- 6AV with a sensitivity of 0.04 AUFS and a wavelength of 254 nm. Elution was carried out at a flow rate of 0.8 mL/min under a linear gradient of acetonitrile (solvent A) and H_2O with 1% formic acid (solvent B) from 5% A to 100% A in 50 min. The *P. granatum* peel EtOH extract (PGPE) was dissolved in a mixture of methanol and water ($6:4\,v\,v^{-1}$), and 20 μlL was injected into the HPLC. The presence of gallic acid and ellagic acid was confirmed by the same retention time of their standards (Sigma Chemical Co, St Louis, USA) (Figure 1). The obtained chromatogram is shown in Figure 2 [27].

2.4. Antimicrobial Resistance Testing. The resistance of the various *Salmonella* strains to different antimicrobial agents was determined using the disk-agar method standardized by the Clinical and Laboratory Standards Institute [28]. The quality control strain used was *Enterococcus faecalis* ATCC 29212.

2.5. Disc Diffusion Method. The antibacterial activities of the isolates on the different extracts were tested using the disk-agar method described by the Clinical and Laboratory Standards Institute Standards and by using a modified agar-well diffusion method [28, 29]. Briefly, sterile paper discs (6 mm; Toyo Roshi Kaihsa, Japan) were loaded with 20 μL of PGPE (varying concentrations: 100, 200 and 500 μg) dissolved in 50% DMSO and then left to dry for 18 h at 37°C in a sterile room. The bacterial suspensions were then diluted to a turbidity of approximately 0.5 McFarland (~1.5×10^8 CFU mL^{-1}), and then further diluted to obtain the final inoculum. Next, the MHA was poured into Petri dishes and inoculated with 100 μlL of the suspension containing 1×10^5 CFU mL^{-1} of bacteria. Ampicillin (Sigma Chemical Co) was used as the positive control and discs treated with 50% DMSO were used as the negative control. The plates were then placed in an incubator (Vision Co, Seoul, Korea) at 37°C for 24 h, after which the diameter of the zone of inhibition around each of the discs was measured and recorded. Each experiment was performed in triplicate.

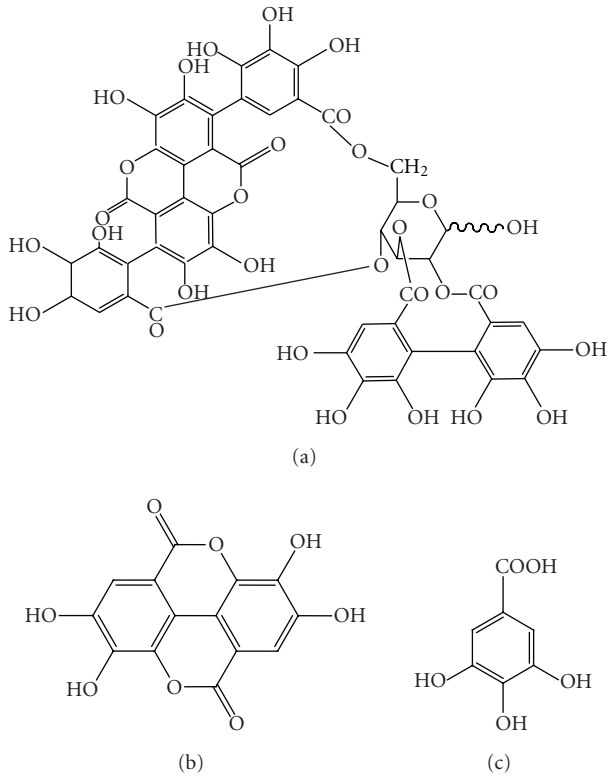

Figure 1: The chemical structure of punicalagin (a), ellagic acid (b) and gallic acid (c).

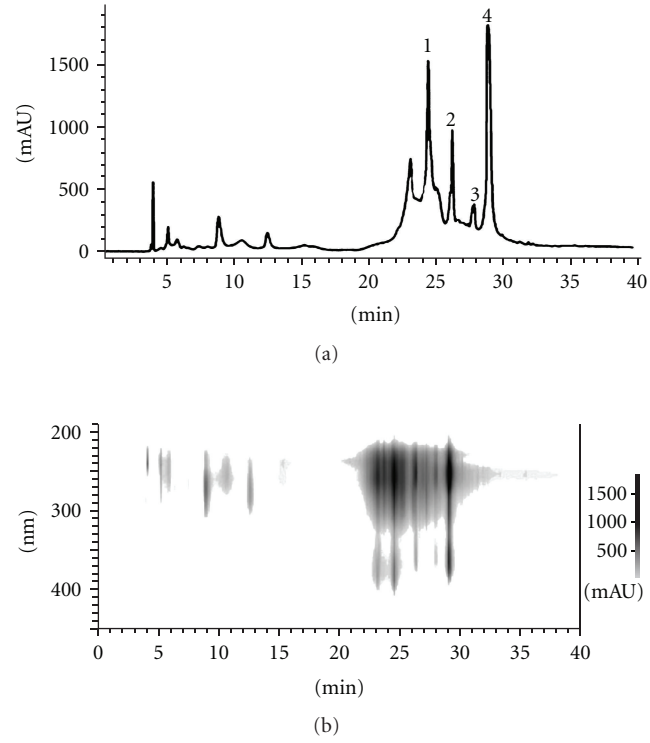

Figure 2: HPLC analysis of PGPE (a) and 3D HPLC analysis (b). (1) and (2) punicalagin isomers; (3) gallic acid; (4) ellagic acid.

2.6. Determination of MICs. The MIC values were determined for microorganisms that were found to be sensitive to PGPE during the disc diffusion assay. To accomplish this, the microorganism inocula were prepared from 12-h broth cultures and the suspensions were then adjusted to a turbidity of 0.5 McFarland. Susceptibility tests were then conducted using the standard broth micro dilution method in accordance with the CLSI guidelines [30] in MHB with an inoculum of $\sim 5 \times 10^4$ CFU mL^{-1}. The MHB was then supplemented with serial dilutions of *P. granatum* peel of EtOH extracts ranging from 3.9 to 2000 μg mL^{-1} and ampicillin concentration was ranging from 0.03 to 250 μg mL^{-1}. The lowest concentration of PGPE capable of inhibiting visible growth after 24 h of incubation at 37°C was then recorded as the MIC [30].

2.7. Animals. Mice were obtained from Da Mool Science (Deajeon, Korea). All mice experiments in this study were approved by the Wonkwang University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care. Fifteen male Balb/c mice (15–17 g) aged between 5 and 6 weeks were used for all *in vivo* experiments. They were kept in a temperature-controlled room under a 12 h light 12 h dark cycle. Animals had free access to commercial solid food (SCF Co. Ltd, Korea) and water *ad libitum*, and were acclimatized for at least 1 week prior to beginning the experiments.

2.8. In Vivo Assay Using Mice. Mice were divided into the following groups: control (CON), *Salmonella*-infected (SI) and *Salmonella*-infected + PGPE (SIPG). Each treatment group contained five mice. Throughout the experiment, mice were provided with water that contained streptomycin (5 mg mL^{-1}) in order to reduce the level of facultative anaerobic bacteria that normally colonize the mouse intestine [31]. The inhibition of the growth of test organisms in mice was then determined by monitoring *S. typhimurium* in the feces of the mice. Briefly, *S. typhimurium* (JOL 389) was grown overnight in Luria–Bertani broth (Difco), centrifuged, washed in phosphate-buffered saline (PBS) and then diluted into 20% sucrose to achieve a final concentration of 1×10^5 CFU. The SI and SIPG groups exclusively were then inoculated using gavage needle orally with approximately 10^5 CFU of *S. typhimurium* in a 0.1 mL volume. One hour after infection, animals in the SIPG group were orally administered 5 mg (using gavage needle) of the PGPE daily, whereas CON and SI animals were not. Fecal samples were then collected 0, 1, 2, 3, 4, 5 and 6 days after the bacterial suspensions were administered and the numbers of the bacteria per gram of feces were determined. Aliquots (100 μl) of fecal suspensions were serially diluted in PBS and then plated on duplicate *Salmonella-Shigella* agar plates (Difco), which were subsequently incubated overnight at 37°C. Typical colonies were then counted on plates that contained between 30 and 300 colonies [32], after which confirmation of *S. typhimurium* was performed by a PCR

Table 2: Antimicrobial activity (as inhibition zone diameters) of PGPE and ampicillin (APCL) against 16 strains of *Salmonella*.

Strain	Serotypes	Diameter of clear zone (mm)			
		PGPE			APCL[a]
		100 μg	200 μg	500 μg	10 μg
JOL 380	*S. typhi* ATCC 19943	13.3 ± 1.1	16.3 ± 1.5	17.3 ± 1.1	34.2 ± 1.0
JOL 381	*S. paratyphi* A	14.3 ± 0.5	15.6 ± 1.1	18.6 ± 1.1	28.5 ± 1.1
JOL 386	*S. enteritidis*	9.0 ± 1.0	12.6 ± 1.5	14.3 ± 1.1	31.1 ± 0.3
JOL 387	*S. typhimurium*	9.6 ± 0.5	11.6 ± 0.5	14.6 ± 0.5	27.5 ± 1.0
JOL 388	*S. typhimurium*	11.0 ± 1.0	14.0 ± 1.0	15.0 ± 1.0	26.0 ± 1.0
JOL 389	*S. typhimurium*	9.0 ± 1.0	12.0 ± 1.0	12.6 ± 0.5	ND
JOL 407	*S. enteritidis*	9.3 ± 1.1	12.3 ± 0.5	14.6 ± 0.5	26.7 ± 1.2
JOL 408	*S. typhimurium*	8.6 ± 1.1	10.6 ± 0.5	14.0 ± 2.0	30.2 ± 0.5
JOL 409	*S. dublin* ATCC 39184	8.0 ± 0.0	10.0 ± 0.0	13.3 ± 0.5	27.0 ± 1.1
JOL 410	*S. derby* ATCC 6960	10.0 ± 0.0	12.0 ± 0.0	14.3 ± 0.5	25.6 ± 1.1
JOL 411	*S. choleraesuis* ATCC 7001	11.6 ± 0.5	15.6 ± 1.0	16.0 ± 0.0	11.2 ± 1.0
JOL 419	*S. gallinarum*	12.3 ± 0.5	16.0 ± 1.0	16.6 ± 1.1	27.3 ± 1.5
JOL 420	*S. gallinarum*	16.0 ± 0.0	16.0 ± 0.7	17.6 ± 0.5	25.8 ± 1.1
JOL 421	*S. gallinarum*	11.3 ± 0.5	15.0 ± 0.0	16.3 ± 0.5	25.2 ± 0.3
JOL 422	*S. gallinarum*	12.0 ± 0.0	14.0 ± 1.7	16.0 ± 1.0	28.5 ± 0.5
JOL 423	*S. gallinarum* ATCC 9184	7.6 ± 0.5	10.0 ± 0.0	13.3 ± 1.1	ND

Data shown represent the mean ± SE of three experiments that consisted of three replicates. ND, No activity detected.
[a]Positive control.

assay using a previously described method [33]. At Day 4 post-infection, the mice were sacrificed, and tissue specimens of the kidney, liver, intestine and spleen organs were transferred to 10% buffered neutral formalin for histopathologic examinations and then processed using standard procedures. Sections of paraffin-embedded tissues were then stained with hematoxylin and eosin.

3. Results

3.1. Determination of Antibacterial Activity by the Disc Diffusion Method. The antimicrobial efficacy of PGPE against the 16 *Salmonella* strains was evaluated by the disc diffusion method via determination of the surrounding zones of inhibition, as well as by evaluating the MIC using the agar dilution method. Table 2 shows the antimicrobial activity of *P. granatum* peel extract as determined by the disc diffusion method. The mean values of the zones of inhibition produced against the tested bacteria ranged from 13.3 to 18.6 mm, with the growth of each of the tested strains being inhibited at 500 μg per disc and the zone of inhibition increasing in a dose-dependant manner.

3.2. Determination of MICs. The MICs of the PGPE against the 16 strains of *Salmonella* are shown in Table 3. The MICs determined using the broth dilution method confirmed the results obtained using the disc diffusion method. PGPE showed antimicrobial activity against each of the tested strains, and these values ranged from 62.5 to 1000 μg mL^{-1}. The *in vivo* experiment was therefore conducted with the EtOH extract.

Table 3: Antimicrobial activity of PGPE and ampicillin (APCL) against 16 strains of *Salmonella*.

Strain	Serotypes	MIC (μg mL^{-1})	
		PGPE	APCL[a]
JOL 380	*S. typhi* ATCC 19943	250	0.97
JOL 381	*S. paratyphi* A	62.5	1.95
JOL 386	*S. enteritidis*	1000	1.95
JOL 387	*S. typhimurium*	1000	1.95
JOL 388	*S. typhimurium*	500	0.97
JOL 389	*S. typhimurium*	250	>250
JOL 407	*S. enteritidis*	250	1.95
JOL 408	*S. typhimurium*	500	1.95
JOL 409	*S. dublin* ATCC 39184	500	0.97
JOL 410	*S. derby* ATCC 6960	500	1.95
JOL 411	*S. choleraesuis* ATCC 7001	62.5	>250
JOL 419	*S. gallinarum*	62.5	1.95
JOL 420	*S. gallinarum*	62.5	1.95
JOL 421	*S. gallinarum*	125	1.95
JOL 422	*S. gallinarum*	250	1.95
JOL 423	*S. gallinarum* ATCC 9184	1000	>250

[a] Positive control.

3.3. Antibacterial Efficacy of PGPE in Mice. The *in vivo* antibacterial activity of PGPE was examined using a mouse *S. typhimurium* infection model. Briefly, mice were infected with 1×10^5 CFU of *S. typhimurium* (SI). One-hour later,

Table 4: Effects of treatment with PGPE on fecal shedding of *S. typhimurium* (CFU g^{-1}) by mice.

Group	Day of post-feeding						
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
SI-1	0	3×10^3	2×10^3	1×10^4	2×10^4	1×10^4	Death
SI-2	0	2×10^4	5×10^4	7×10^5	Death	Death	Death
SI-3	0	3×10^4	1×10^5	2×10^5	Death	Death	Death
SI-4	0	7×10^4	3×10^4	2×10^4	1×10^6	Death	Death
SI-5	0	4×10^5	2×10^5	1×10^5	3×10^6	Death	Death
SIPG-1	0	1×10^3	1×10^3	1×10^3	6×10^3	2×10^3	0
SIPG-2	0	2×10^3	1×10^3	2×10^3	3×10^3	2×10^3	2×10^3
SIPG-3	0	2×10^2	2×10^3	8×10^3	3×10^3	3×10^2	2×10^2
SIPG-4	0	1×10^3	3×10^2	2×10^2	1×10^2	3×10^2	2×10^2
SIPG-5	0	4×10^2	8×10^2	1×10^3	2×10^2	3×10^2	1×10^2

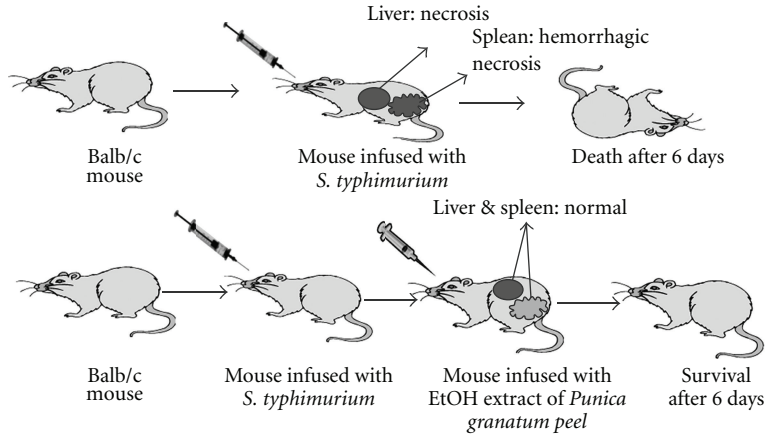

Figure 3: Importance of PGPE against *S. typhimurium* infection.

the mice were orally administered PGPE (SIPG). As shown in Table 4, treatment with the extract of *P. granatum* peel was found to have marked effects on mortality and on the number of viable *S. typhimurium* recovered from feces. At Day 1 post-infection, ten mice in the SI and SIPG group shed viable *S. typhimurium* in feces, with the feces of mice in the SI group being found to contain bacteria at a concentration of 3×10^3 to 4×10^5 CFU g^{-1} and feces of mice in the SIPG group being found to contain bacteria at a concentration of 2×10^2 to 2×10^3 CFU g^{-1}. In addition, at Day 6 post-injection, none of the mice in the SIPG group had died, whereas all five mice in the SI group had succumbed as illustrated in Figure 3.

3.4. Organ Histopathologic Changes. *Salmonella typhimurium*-infected mice that did not receive the PGPE were lethargic and showed signs of histological damage in the liver and spleen. In addition, the central and portal veins of the liver showed congestion with focal necrotic emboli-like materials (Figure 4). There were multiple small necrotizing nodular lesions in the liver parenchyma with Kuffer cell hyperplasia and inflammatory cell infiltrate. The spleen showed extensive hemorrhagic necrosis in the red pulp with multiple apoptotic bodies in the white pulp (Figure 4). No specific abnormal findings were observed in the kidney or the small intestine. Conversely, clinical signs and histological damage were rarely observed in *S. typhimurium*-infected mice fed with the PGPE.

4. Discussion

Recently, a number of antibiotics have lost their effectiveness due to the development of resistant strains of bacteria, which has primarily occurred through the expression of resistance genes [34, 35]. In addition to inducing resistance, antibiotics are sometimes associated with opposing effects such as hypersensitivity, immune-suppression and allergic reactions [36]. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases [37, 38].

In the present study, the PGPE exhibited antibacterial activity against all 16 strains of eight different *Salmonella* serotypes tested. In addition, the results of the MIC assays also confirmed its antibacterial effects against all tested *salmonella* strains. The PGPE also exhibited antibacterial activities against *Salmonella* strains JOL 389, JOL 411, JOL

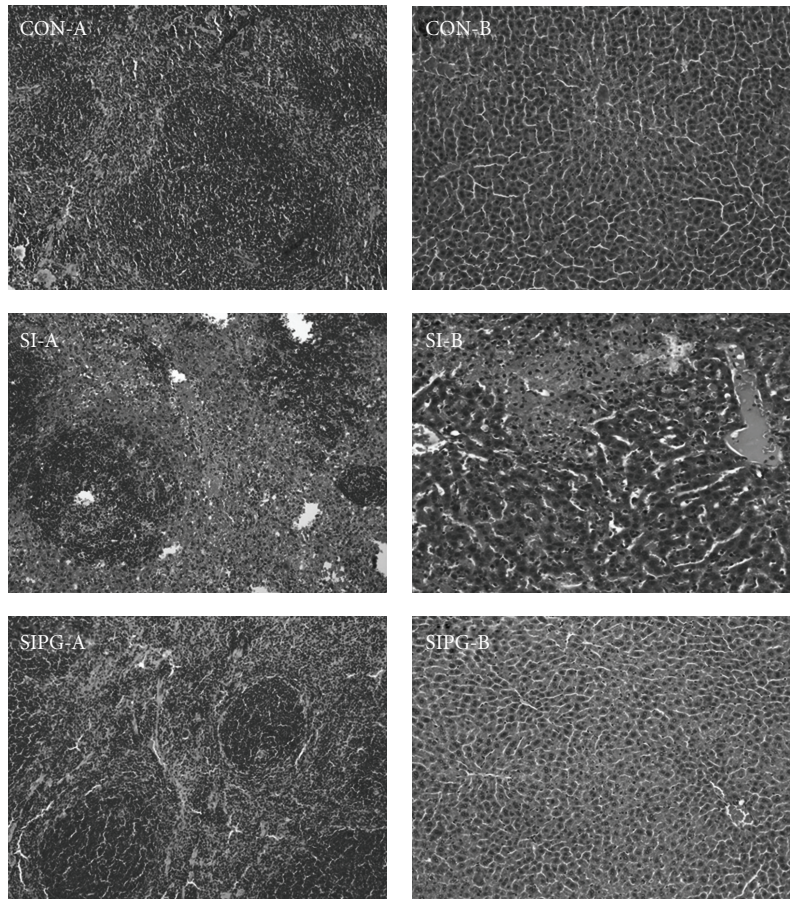

Figure 4: Histopathological changes in organs in CON, SI and SIPG. (a) spleen (×200) and (b) liver (×200).

419, JOL 420, JOL 421 and JOL 423, all of which have been shown to be resistant to two to five antibiotics (Table 1). The *in vivo* antibacterial assay also revealed that the extract effectively inhibited the growth of *S. typhimurium* and significantly reduced mouse mortality (Table 4). Furthermore, clinical signs of infection and histological damage were rarely observed in test mice, whereas untreated SI mice showed severe clinical signs and histological damage in the tested organs. This is the first study describing the antibacterial activity of *P. granatum* peel extract against *Salmonella*. Based on these promising *in vitro* and *in vivo* assay findings, we believe that *P. granatum* peel extract is likely to become a novel antimicrobial treatment for salmonellosis. It has been reported that *P. granatum* peel extracts have shown antibacterial activity against *Escherichia coli* O157 and methicillin-resistant *Staphylococcus aureus* bacteria [14, 39]. This antibacterial activity may be indicative of the presence of several metabolic toxins or broad-spectrum antibiotics. Several metabolites from herb species, including alkaloids, tannins and sterols, have previously been associated with antimicrobial activity [40].

In order to investigate components from the PGPE, the HPLC analysis was performed as shown in Figures 2(a) and 2(b). This HPLC analysis among some other minor constituents mainly shows some major phenolic compounds [26]; gallic acid and ellagic acids in addition to punicalagin as a major ellagitannin. The retention time shows it to be gallic acid and ellagic acid [41, 42] and also the presence of punicalagin isomers (Figure 1) could be deduced to be one of the major components from the results of literature reported previously [27]. Gallic acid was reported to have antibacterial activity against some intestinal bacteria [43], ellagic acid has anti-microbial activity [44] and punicalagin was reported to show anti-food-borne pathogens [45]. The site and the number of hydroxyl groups on the phenol components may increase the toxicity against the microorganisms [46]. However, it has been reported that gallic acid, ellagic acid and punicalagin have weak antibacterial activity against *Salmonella*.

The antibacterial activity of *P. granatum* peel extract might be related to the action of its antibiotic compounds or to the presence of metabolic toxins. This suggests that these components may also provide antibacterial activity against *Salmonella* and provide a plausible explanation for the higher antibacterial activity of the EtOH extract. On the other hand, the unknown minor components present have not been elucidated in terms of their activity. Further studies then need to be done. In the future, thorough investigation is needed to better ascertain the antibacterial effect of this herb extract.

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