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Protective Effect of Pomegranate Fruit Juice Against *Aeromonas hydrophila*-induced Intestinal Histopathological Changes in Mice

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Abstract: *Aeromonas hydrophila* is a very prevalent species. It can cause both intestinal and nonintestinal infections in humans and can often be fatal. Moreover, in the recent years, naturally occurring antioxidant compounds have gained considerable attention as antibacterial agents. The present study aimed to investigate the possible protective effect of pomegranate fruit juice (PJ) against *Aeromonas hydrophila*- induced intestinal histopathological alterations as one of the edible safe natural products. Seventy-two adult MF1 male mice were used and divided into three groups; first group, control group, was injected with phosphate-buffered saline PBS at dose and intervals parallel to the treated groups. Second group was injected i.p. with purified lipopolysaccharides (LPS) extracted from *Aeromonas hydrophila* once a week for four weeks. Third group was injected with LPS suspension as second group and synergistically received drinking water supplemented with pomegranate fruit extract. The bacteria infected group showed severe deterioration in the intestinal mucosa in the form of villar atrophy, necrosis of apical enterocytes and wide destruction of crypt cells. At the ultrastructural level, many enterocytes showed completely damaged organelles and dissolved cytoskeletal. The treatment with pomegranate juice significantly and substantially prevented the intestinal histopathological changes and normalized its morphometric parameters. It was concluded that pomegranate juice holds great promise as antimicrobial and anti-inflammatory new therapeutic. Also, we advise use of pomegranate in human nutrition as table fruit or juice for its antioxidant qualities.

Key words: Pomegranate · Aeromonas · Intestine · Endotoxemia · Morphometry

INTRODUCTION

Aeromonas hydrophila is a species of anaerobic Gram-negative bacteria. It can be found in food as well as in aquatic environments worldwide. It is a member of the family *Aeromonadaceae* and is only one of six species, *Aeromonas* species, that are known to be pathogenic in humans as well as animals. It can cause both intestinal and nonintestinal infections in humans and can often be fatal [1, 2]. Because it is so prevalent in aquatic environments, *A. hydrophila* can cause serious pathology in fish [3, 4].

Aeromonas spp. produce an array of virulence factors that include endotoxins: lipopolysaccharides (LPS). LPS is a major constituent of the outer membrane of Gram negative bacteria and serves as a potent proinflammatory stimulus by interacting with humoral and cellular mediator systems [5]. In addition, LPS harbor binding sites for antibodies and non-immunoglobulin serum factors and are, therefore, involved in the specific recognition and elimination of bacteria by the host organism's defense system [6-8].

A. hydrophila can cause some diseases including: septicemia, meningitis, pneumonia and gastroenteritis [9-13]. Septicemia with its complications is still a major challenge in contemporary medicine. Despite the new therapeutic approaches, incidence of septicemia and number of sepsis-related deaths are rising [14, 15].

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There are three major wound infections caused by *A. hydrophila*: cellulitis, myonecrosis and ecthyma gangrenosum. Cellulitis is the most common [16-18]. *A. hydrophila* is resistant to many common antibiotics such as penicillin and ampicillin [19, 20]. In the same time, this micro organism is a very prevalent species and is capable of affecting immunocompetent as well as immunocompromised individuals [10]. It was believed this species did not pose a threat to healthy individuals, but the work of Chopra and Houston [10] indicated that *A. hydrophila* is more harmful than was previously believed.

Pomegranate (*Punica granatum, Punicaceae*), is an edible fruit cultivated in Mediterranean countries, Asian countries and some parts of the United States. Pomegranate has been extensively used as a folk medicine by many cultures [21]. Edible parts of pomegranate fruit are comprised of 80% juice and 20% seed. Fresh juice contains 85% water, 10% total sugars and 1.5% pectin, ascorbic acid and polyphenolic flavonoids [22]. Pomegranate seeds are a rich source of crude fibers, pectin and sugars. Dried pomegranate seeds contain the steroid estrogen estrone [23, 24]. Pomegranate fruit is a rich source of two types of polyphenolic compounds: anthocyanins (such as delphinidin, cyanidin and pelargonidin) and hydrolyzable tannins (such as punicalin, pedunculagin, punicalagin, gallagic and ellagic acid esters of glucose), which account for 92% of the antioxidant activity of the whole fruit [25]. The soluble polyphenol content in pomegranate juice varies between 0.2 and 1.0% depending on variety [26].

Pomegranate extract is used for prevention and treatment of many major health problems. It possesses potent antioxidant activity and free radical scavenging capability [22, 25, 27, 28]. Pomegranate extract inhibits lipid peroxidation at lower concentrations than vitamin E [29]. Pomegranate also seems to have antibacterial, anti-inflammatory [30-33] and antimutagenic activity [34]. It has been proven to cause apoptosis (programed natural cell death) in cancer cells without harming healthy cells as chemotherapy dose [35, 36]. Pomegranate extract has no side effects and no known drug interactions and may be the most potent way to prevent cancer [37], strengthen the immune system [38] prevent heart disease [39], liver fibrosis [40], promote wound healing and strengthen connective tissue (which may keep cancer cells from spreading) [41].

The purpose of the present study was to examine, for the first time, the possible preventive effects of pomegranate fruit extract on the *Aeromonas hydrophila* intestinal toxic effects, as one of the edible natural products that may have more potent and safe preventive and therapeutic effects.

MATERIALS AND METHODS

Animals and Treatment: Seventy-two (30-35 g.) adult MF1 male mice (Biological Supply Center, King Fahd Centre for Medical Researches, College of Medicine, King Abdul-Aziz University, Jeddah, KSA) were used. Mice were housed in stainless-steel cages under strict hygienic conditions at 25-28°C, (8-20 hr) light and free access to feed and water. Animals were divided into three groups as following: first group remained as a control group that injected intraperitoneally (i.p.) with phosphate-buffered saline PBS (pH 7.4) 0.2 ml/mice at intervals parallel to the treated groups. The second group was injected i.p. with purified lipopolysaccharides (LPS) extracted from *A. hydrophila* (as shown later), at a dose of 20 mg/kg in 0.2 ml PBS once a week for four weeks. The third group was injected with LPS suspension as second group and synergistically received drinking water supplemented with 0.2% pomegranate fruit extract (wt/vol) as described by Malik, *et al.* [36]. At the end of the treatment period, the mice were sacrificed under mild anesthesia and small intestine samples were removed and prepared for histopathological and cytological investigations.

Preparation of *Aeromonas hydrophila* Endotoxin (LPS): The strain of bacteria used in the present study is a subculture slant identified as *A. hydrophila* (A-47) from that purchased from American Type Cell Culture, a Global Biosource Center, USA (ATCC; Cat. # 7966). The bacterial suspension of *A. hydrophila* was prepared according to Schill *et al.* [42] and modified by Austin and Austin [43]. Extraction of LPS was performed using the phenol-water method according to Westphal and Jann [44]. The LPS was purified from nucleic acids by ultracentrifugation.

Preparation of Pomegranate Fruit Extracts (PFE): PFE was prepared as described by Afaq *et al.* [46] and Malik, *et al.* [36]. This extract, PFE, was analyzed by MALDI-TOF MS and was found to contain six anthocyanins and various ellagitannins and hydrolyzable tannins [46].

Histopathological Studies: Small portions of the duodenum, jejunum and ileum were fixed and sectioned using a standard procedure. Sections of 5 μm. thickness were stained with hematoxyline and eosin.

Histomorphometrical Studies: Specimens from the middle part of the ileum of all groups were chosen for the histomorphometrical studies. Images of hematoxyline and eosin stained slides were captured as tag image format. The measurements were done by using *Leica Qwin* 500 image analyzer computer system in Image Analyzer Unit, College Medicine and Medical Sciences, Taif University. The morphometric analysis was carried out using the software of the system. Ten fields were chosen in each specimen and the mean values were obtained. The following data were measured from each specimen:

Villus Height (µm): was measured from the tip of the villus to the villus-crypt junction at nearly 130 times magnification.

Crypt Depth (µm): was measured from the villus-crypt junction to the base at nearly 500 times magnification.

The Enterocyte Height (µm): was measured from the enterocyte tip to its base at nearly 500 times magnification (measurements applied on the middle portion of the villi).

The inflammatory cells' number/ mm²

***Villus Inflammatory Cells*:** represent the number of inflammatory cells in the villi (50 small grid squares was counted and expressed as the number of cells per square millimeter of the villi, mm²).

***Crypt Associated Inflammatory Cells*:** represent the number of inflammatory cells lying between the muscularis mucosae and the villus-crypt junction (50 small grid squares was counted and expressed as the number of cells per square millimeter of the examined area (mm²).

The goblet cells' number

***Villus Goblet Cells*:** represent the number of goblet cells lying with the villus. Total goblet cells of 30 villi were counted and results were expressed as goblet cells' number/ 10 villi.

Crypt Goblet Cells: represent the number of goblet cells that lie in the crypt. Results were expressed as number of goblet cells/ 10 crypts.

Tunel Analysis: Serial sections 5 µm in thickness were prepared from ileum samples for the labeling of apoptotic cells using the TUNEL assay [47]. TUNEL staining of sections was performed using an in situ apoptosis detection kit from *Roche, Mannheim,* Germany, in accordance with the manufacturer's instructions and examined under light microscopy. The apoptotic index (the percentage of golden or dark brown stained cells) was determined at 20-random locations within the mucosa of ileum for each animal from the villi and the crypt (10 each) and for six animals from each group using a *Leica Qwin* 500 image analyzer.

Ultrastructural Studies: Specimens of 3 to 4 mm. in length were harvested from the ileum. Ultrathin sections were cut and stained with uranyl acetate and lead citrate. The ultrastructure of the tissues was examined under a transmission electron microscope.

Statistical Analyses: All morphometric data were analyzed using the PC-STAT analysis of variance program, PC-STAT, University of Georgia, [48] followed by LSD analysis. Results are expressed as mean±standard error (SE). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Histopathological Studies: The microscopic examination of the intestinal sections of control animals revealed their characteristic layers with normal architecture of villi, crypts and enterocytes (Fig. 1 A,B).

Investigation of the bacteria infected sections showed villar atrophy, necrosis and desquamation of the lining epithelium especially at the tip of villi and wide destruction of the crypt cells. There is a diffuse infiltration with inflammatory cells in the lamina propria and muscularis mucosa (Fig. 1C). The blood vessels showed severe congestion and intravascular hemolysis. Sections of the ileum showed also, lymphoid hyperplasia (Fig. 1D).

Investigation of the pomegranate-treated group revealed marked regenerative effect on the intestinal histopathological features with mucus hyper-secretion (Fig.1E). Proliferations of enterocytes were observed. (Fig.1E). Generally, the intestine essentially restored their normal histological architecture except some lymphocyte nodules were still noticed and the serosa and musculosa were still somewhat thickened.

Histomorphometrical Studies: Table (1) shows the main values of villus height, crypt depth and enterocyte height ± standard error and the percentage of changes of these values that occur in the bacteria infected group and the pomegranate treated group in relation to the control one. The results indicate a significant decrease ($P < 0.05$)

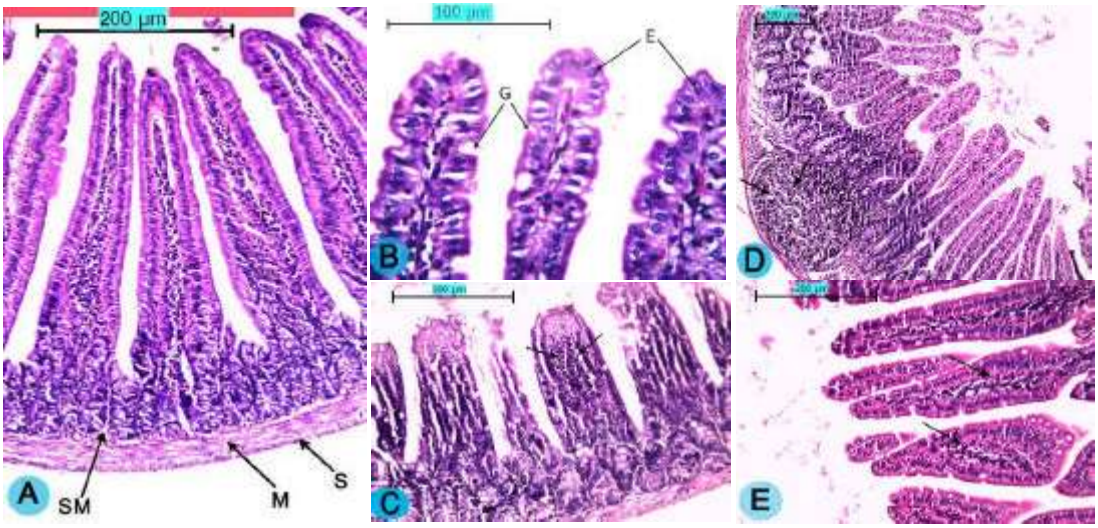

Fig 1. A: a photomicrograph of transverse section of ileum of control animals showing its characteristic layers; mucosa, submucosa (SM), muscolosa (M) and serosa (S). The villi are showing its normal architecture. B: a transverse section of ileum of control animals showing normal enterocytes (E) and goblet cells (G). C: a transverse section of ileum of bacteria infected animals showing complete destruction of mucosal layer with villar fusion and diffuse infiltration of inflammatory cells in the villar stroma (arrows). D: a transverse section of ileum of bacteria infected animals showing lymphoid hyperplasia (arrows). E: a transverse section of ileum of juice- treated animals showing normal architecture of villi with regenerated enterocytes. There were some lymphocytes still noticed in the villar stroma (arrows) H and E.

Table 1: Analysis of variance of changes in height (µm) of villus and enterocyte and depth of crypt in different groups at the end of the experimental period (4 weeks)

	Control group	Bacteria-infected group		Juice-treated group	
Experimental Parameter	Main Value ± SE	Main Value ± SE	Change %	Main value± SE	Change %
Villus height (µm)	629.01± 10.902[a]	451.87± 14.997[b]	- 28.16	628.65± 6.982[a]	- 0.001
Crypt depth (µm)	8.62±0.592[a]	7.06±0.301[b]	- 18.09	8.80± 1.099[a]	+ 0.021
Enterocyte height (µm)	40.32 ± 2.133[a]	21.13± 1.145[b]	- 47.59	39.72 ± 1.097[a]	- 0.014

(For each parameter, the values shared the same symbol (s) are not statistically significant P< 0.05)

Table 2: Analysis of variance of changes in inflammatory cells' number (number / mm2) in villar and crypt-associated areas of different groups at the end of the experimental period (4 weeks)

	Control group	Bacteria-infected group		Juice-treated group	
Inflammatory cells' number (number / mm^2.)	Main Value ± SE	Main Value ± SE	Change %	Main value± SE	Change%
Villar area	12.3 ± 0.692[c]	71.8 ± 0.318[a]	483.74	47.1 ± 1.789[b]	282.92
Crypt-associated area	12.9 ± 0.953[c]	62.2 ± 1.879[a]	382.17	45.6 ± 0.573[b]	253.48

(For each parameter, the values shared the same symbol (s) are not statistically significant P< 0.05)

in the villus height and crypt depth in the bacteria infected group and nearly complete recovery occurred after treatment with pomegranate juice for 4 weeks. There was a great decrease in the enterocyte height (47.59 %) in the bacteria infected group compared with the control one. These cells nearly restored their normal height and architecture after 4 weeks treatment with pomegranate juice.

Table (2) shows the inflammatory cells' number (number / mm^2) in the villar and crypt-associated areas of different groups at the end of the experimental period. The bacteria infected specimens show diffuse infiltration of the inflammatory cells in the lamina properia and muscular layer in both villi and crypt-associated areas. The juice treated group shows significant decrease (P< 0.05) in the number of the inflammatory cells,

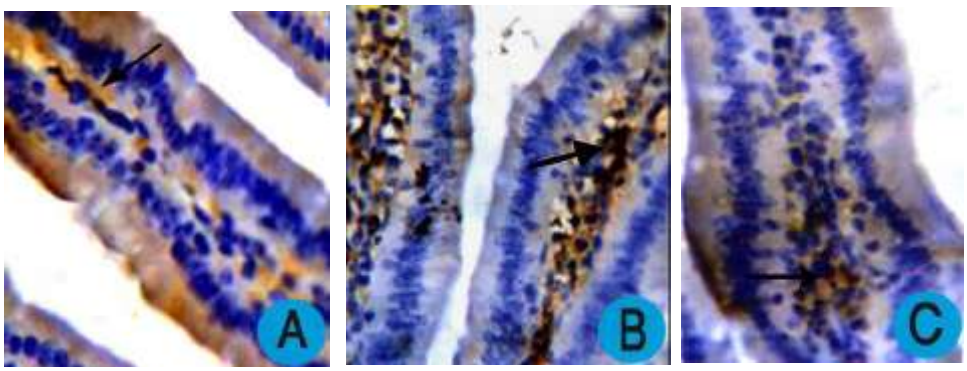

Fig. 2: Sections of rat ileum in control group stained using the TUNEL technique show dark labeled nuclear material within an apoptotic cells (arrows); A: control group. B: bacteria infected group. C: juice treated group (TUNEL X 512).

Table 3: Analysis of variance of the changes in the goblet cells' number (number / 10 villi or 10 crypts) in different groups at the end of the experimental period (4 weeks)

	Control group	Bacteria-infected group		Juice-treated group	
goblet cells' number (number / 10 villi or crypts)	Main Value ± SE	Main Value ± SE	Change %	Main value± SE	Change %
Villi	262 ± 13.624[b]	96 ± 6.823[c]	-63.35	352 ± 10.628[a]	34.35
Crypts	32 ± 2.041[b]	15 ± 1.702[c]	-53.12	68 ± 2.371[a]	112.50

(For each parameter, the values shared the same symbol (s) are not statistically significant P< 0.05)

Table 4: Analysis of variance of the changes in the apoptotic index (number of apoptotic cells / 100 cells) in different groups at the end of the experimental period (4 weeks)

	Control group	Bacteria-infected group		Juice-treated group	
Apoptotic index (number / 100 cells)	Main Value ± SE	Main Value ± SE	Change %	Main value± SE	Change %
Villi	11.2 ± 1.105[b]	39.2 ± 3.217[a]	250.00	9.8 ± 0.523[b]	- 12.50
Crypts	3.5 ± 0.528[b]	9.3 ± 1.056[a]	165.71	3.9 ± 1.278[b]	11.42

(For each parameter, the values shared the same symbol (s) are not statistically significant P< 0.05)

but the number still reveals marked increase than that of control models.

Table (3) shows the goblet cells' number (number / 10 villi or 10 crypts) in different groups at the end of the experimental period. The bacteria infected group showed significant decrease (P< 0.05) in the goblet cells' number. After 4 weeks of treatment with the pomegranate juice the number of goblet cells showed a significant increase (P< 0.05) compared with both control and bacteria infected groups.

Apoptotic Index: The percentage change of the apoptotic index showed a significant increase (P< 0.05) in the bacteria infected group in the villi and crypts compared with the control. Treatment with pomegranate juice diminished the mean apoptotic index in the villi and crypts but the values in the crypts were still higher than that of the control model (Table 4, Fig. 2).

Ultrastructural Studies: Ultrastructural investigation of the intestinal cells of control animals revealed their normal architecture; these cells were united at their uppermost lateral membrane (Fig. 3A) by a well developed tight junctions. The supranuclear region of the enterocytes had several spherical mitochondria and numerous cisternae of rough endoplasmic reticulum (Fig. 3A). The luminal surface of the enterocytes possessed many closely packed parallel finger-like microvilli (Fig. 3A).

The bacteria infected group had numerous enterocytes with completely deteriorated organelles, several lysosomes or vesicles and fused clumps of intact microvilli with dissolved cytoskeletal structures especially at the cell base or totally degenerated ones (Fig. 3B). Some of the enterocytes lost their microvilli and the remaining intact microvilli became shorter (Fig. 3B). Most enterocytes had detached lateral plasma membrane in several foci.

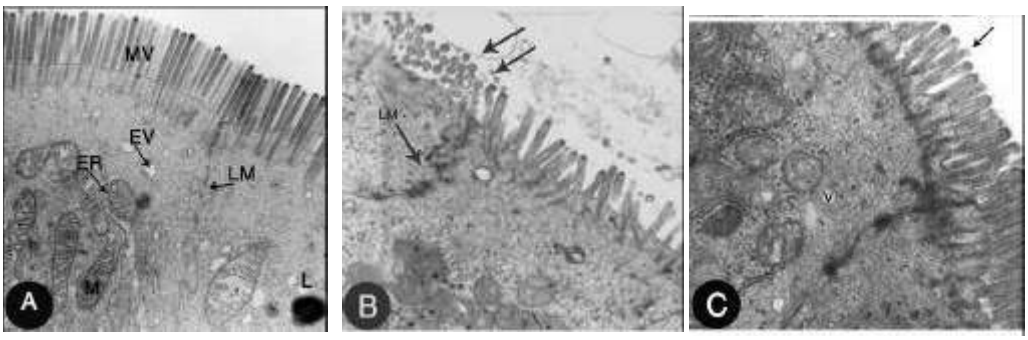

Fig. 3 A: TEM image of enterocytes in control rat small intestine. In this image, there are two enterocytes and lateral membrane (LM) between them. The microvilli (MV) are intact and tightly packed on the surface of the enterocytes. Scattered throughout the enterocytes are endocytosis vacuoles (EV), lysosomes (L), mitochondria (M) and rough endoplasmic reticulum (ER). 3700X. B: Enterocytes in bacteria infected group showing deteriorated cell organelles; several lysosomes, deteriorated microvilli (arrows) and detached lateral membrane (LM) 12000X. C: Enterocytes of juice treated group showing normal cell organelles, few numbers of vacuoles (V) and long intact microvilli (arrow) 15000X.

Investigation of the pomegranate-treated group revealed that the great majority of the enterocytes were with normal cell organelles and organized cytoskeletal elements. They had few numbers of vacuoles, normal nuclei, nearly normal mitochondria with short cristae, intact long microvilli and goblet cells with homogenously electron-lucent granules (Fig. 3C). Nevertheless, some enterocytes appeared with destructed mitochondria and degenerated microvilli at certain areas.

DISCUSSION

In recent years, naturally occurring antioxidant compounds present in the diet consumed by humans have gained considerable attention as antiviral and antibacterial agents [30-33]. The present study was designed to examine, for the first time, the possible preventive effects of pomegranate fruit extract on the *A. hydrophila*-induced intestinal structural changes, as one of the edible safe natural products and the results were wonderful.

According to our present results, the *A. hydrophila* endotoxin; lipopolysaccharide (LPS) induced severe deterioration in the intestinal mucosa in the form of villar atrophy, necrosis of apical enterocytes, which showed significant increase in the apoptotic index and wide destruction of crypt cells. There, also, were diffuse infiltrations of inflammatory cells. It is commonly known that integrity of the gastrointestinal tract acts as a critical determinant of clinical outcome in microbial septic patients where it acts as a protective barrier against bacteria and endotoxins [49, 50].

Villar atrophy and crypt destruction were indicated by significant decrease in the mean villus height, mean crypt depth and mean enterocytes height. These alterations were, mainly, related to cell necrosis and apoptosis and presumably due to reduction of available stem cells that can proliferate. Necrosis, is established as a crucial agent in sepsis-related organ failure resulted from reactive oxygen species-induced ischemia [50-53]. Moreover, the apical enterocytes of bacteria infected group showed significant increase in the apoptotic index, this finding is in agreement with many studies in recent years [53-57], that demonstrated a correlation between the increased apoptotic cell death and septic challenge. Apoptosis has, also, been suggested to be involved in sepsis-related organ failure [54]. Whatevere, cells at the villar tips have been reported to be positive for DNA strand breaks with the use of (terminal deoxy-nucleotidyl transferase) technique [58, 59]. Therefore, the small intestine has, usually, a high rate of apoptosis to counterbalance the increased cell division [60].

Lipopolysaccharide (LPS) serves as a potent proinflammatory stimulus by interacting with humoral and cellular mediator systems [5]. Many studies documented stimulation of systemic inflammatory response after endotoxin administration [61-63]. The interaction of LPS with the cellular components may be the cause of the inflammatory cells infiltration [64], while the increase in the inflammatory cells count may reflect their increased demand in the inflammatory areas to clear infection.

Moreover, the bacteria treated animals showed significant decrease in the goblet cells' number and subsequent decrease in mucoid substance. The LPS

destructive effect on crypts and villi may explain the great decrease in the goblet cells' number compared with that of control animals. Mucins allow maintenance of the normal intestinal flora by protecting mucosa from bacteria over-growth and/or penetration [65] and simultaneously providing attachment sites for intestinal flora and pathogenic bacteria [66]. This mechanism may be attenuated *via* LPS reactions.

At the ultrastructural level, the more affected cell organelle due to the bacteria endotoxin (LPS) was the brush border microvilli. Most enterocytes of intestinal epithelium lost their microvilli after LPS-treatment. Many enterocytes showed completely damaged organelles and dissolved cytoskeletal structures. These findings indicate the cytolytic action of LPS and may *per se* explain the reduction in the enterocytes' height. The extensive structural disruption of the enterocytes may exert deleterious effects through reduction of intestinal transport of nutrients because of an altered position of the carriers in the intestinal mucosa. Consistent with our results, Lorenzson and Olsen [67] reported that *A. hydrophila* could lead to shedding of the brush border membranes and decreased villous length with consequent reduction in the surface area absorption in the small intestine. Also, Arai and Nakazawa [68] explained the reduction of the actin microfilaments in the microvilli on the basis of the rearrangement of cytoskeleton filaments. Moreover, Crouser *et al.* [69] reported different degrees of mitochondrial injury in LPS-treated animals.

In pomegranate juice (PJ) co-treated animals, we noticed a wonderful result; PJ treatment significantly and substantially prevented the intestinal histopathological changes and normalized its morphometric parameters. Many recent studies showed the antimicrobial and anti-inflammatory effects of pomegranate components [30-33, 70, 71]. PJ has ability to inhibit virulence factors, in addition to being able to inhibit or kill bacteria through interference with bacterial protein production and secretion [33]. It has been shown that PJ has great efficiency in inhibiting microbial adherence [70]. The anti-inflammatory activity of PJ was explained by suppressing mast cells/basophils activation [71]. The ameliorative effects of PJ upon the histipathological alterations could be explained by the role of PJ in regulating vital cellular functions, including cell proliferation and differentiation and its potent antioxidant activity and free radical scavenging capability [22, 25, 27, 28]. Also, PJ has potent antioxidative capacity against lipid peroxidation [22, 29]. Moreover, the anticarcinogenic effect of PJ through cell cycle regulation-mediated apoptosis may play a role in amelioration of histopathological picture [36].

In conclusion, based on the present findings, it is tempting to suggest that the fruit pomegranate may posses a strong potential for development as antimicrobial and anti-inflammatory new therapeutic that may be exceedingly effective at controlling resistant organisms. The use of pomegranate as table fruit or juice can, so, be recommended in human nutrition for its antioxidant qualities.

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