



## The Beneficial Effects of Pomegranate Juice on Immunity and Intestinal Integrity in Healthy Albino Rats

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### ABSTRACT

Pomegranate has long been recognized as a "healing food," with significant health benefits in a variety of disorders. It has potent anti-inflammatory, antioxidant, anti-obesity, and anticancer effects, thus there has been lately a virtual outbreak of interest in the pomegranate as a functional food. Pomegranate may have physiological effects on the intestines, and the gut microbiota plays an essential part in its therapeutic effects. Therefore, we believe that the beneficial effects of pomegranate are intimately tied to the intestine. Hence, we evaluated the immunomodulatory effect and the intestinal health benefits of the administration of pomegranate juice (PJ). Rats received 1 ml of PJ for consecutive 28 days. The present data revealed that PJ administration did not affect the production of TNF- $\alpha$ , INF- $\gamma$ , and IL-8, but it enhanced the production of IL-4 exerting an anti-inflammatory effect. It increased villus heights and goblet cell numbers. Moreover, it improved mucosal immunity by enhancing the development of germinal center (GC) in the gut-associated lymphoid tissue (GALT) that is related to the production of efficient IgA. Therefore, pomegranate juice can increase mucosal integrity, improve intestinal morphology, which beneficially alters digestion in the small intestine, and enhance mucosal immunity against pathogens.

**Keywords:** Pomegranate, cytokines, anti-inflammation, mucosal immunity, GALT.

### 1. Introduction

The importance that nutrition plays in the prevention and treatment of many illnesses has become widely recognized during the last decades (Viuda-Martos *et al.*, 2010a), and many researchers have shown a growing interest in how food products may assist in preserving health. Recently, there is a lot of emphasis on functional foods, which, aside from their fundamental nutritional capabilities, provide physiological advantages and play a key role in illness prevention or slowing the progression of chronic diseases.

Pomegranate (*Punica granatum*), which belongs to the family Punicaceae, is an ancient fruit that has been consumed for thousands of years in numerous cultures. Pomegranate has long been recognized as a "healing food," with significant health benefits in a variety of disorders (Vidal *et al.*, 2003). Indeed, in folk medicine, the pomegranate was often used to eliminate parasites, and to treat and cure ulcers, diarrhea, acidosis, dysentery, bleeding, microbiological infections, and respiratory diseases. It was also utilized as an antipyretic (Larrosa *et al.*, 2010). Because of its multi-functionality and high usefulness in the human diet, there has been lately a virtual outbreak of interest in the pomegranate as a medical and nutritional product.

The importance of pomegranate as a functional food may be attributed to the fact that it contains a variety of compounds in various regions of the fruit with favorable physiological activities that are effective in illness risk reduction, especially with anti-oxidative (Schubert *et al.*, 1999 and Gil *et al.*, 2000) and anti-inflammation (Shukla *et al.*, 2008; Larrosa *et al.*, 2010) properties. Consumption of this fruit or its juice protects against and may even improve the course of various prevalent disorders, including obesity, diabetes, cardiovascular disease, inflammatory diseases, and even some types of

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cancer (Reviewed in Viuda-Martos *et al.*, 2010b). It has been also inferred that pomegranate may have physiological effects on the intestines (Mosele *et al.*, 2015). It is worth noting that the gut microbiota plays an essential part in the therapeutic effects of pomegranate since their metabolites boost the pomegranate's health benefits. Accordingly, the mechanism of pomegranate's anti-inflammatory activity is closely linked to microbiota that lives in the gastrointestinal tract (Zhao *et al.*, 2019).

Taking these points into consideration, we believe that the beneficial effect of pomegranate is intimately tied to the intestine. Therefore, the objective of this study was to assess the immunomodulatory effect of pomegranate juice (PJ) on the gut by measuring particular immunological parameters such as pro-inflammatory cytokines and chemokines (TNF  $\alpha$ , IFN $\gamma$ , and IL-8), anti-inflammatory cytokines (IL-4), and immunoglobulin-A. (IgA). We also investigated its effect on the ileum by performing histological and histometrical analysis.

## 2. Materials and Methods

### 2.1. Materials

Adult male albino rats (*Rattus norvegicus*) weighing between 195 and 250 g were obtained from the breeding colony of the Ministry of Health (Helwan-Egypt). Animals were housed under normal laboratory conditions and provided food and water *ad libitum*. All animals used in this experiment were cared for in accordance with institutional guidelines and followed the Guide for Care and Use of Laboratory Animals. Pomegranate fruits were purchased from a well-known market (Cairo, Egypt.). Pomegranates were washed; the seeds were manually removed then crushed and squeezed by a special juice-making machine to yield concentrated fresh juice of pomegranate daily (Amal *et al.*, 2012).

### I. Experimental design

After the adaptation period, animals were randomly divided into two groups of 9 rats each: control, and Pomegranate group (PJ).

Group 1 (control): The animals fed with a normal diet.

Group 2 (PJ): pomegranate juice at a dose of 1 ml/day was administered orally once daily for 4 weeks (Türk *et al.*, 2008).

### II. Collection of samples

At the end of the experimental period, animals were sacrificed. Blood samples were collected from the jugular vein of each rat. After incubation at room temperature, sera were collected followed by centrifugation at 4000g for 30 min and kept at -80 for immunological measurements. Samples were removed from the ileum for histological preparation.

### III. Measurement of Pro-inflammatory cytokines and Chemokines

TNF $\alpha$ , IFN $\gamma$ , and IL-8 were assayed in sera using commercial ELISA assay kits (BOSTER.; Cat. No. EK0526, BOSTER; Cat. No. EK0374, and Kamiya Biomedical; Cat. No. KT-60204, respectively) according to the manufacturer's instructions and quantified as picograms per milliliter (pg/ml).

### IV. Measurement of Anti-inflammatory cytokine

A commercial kit (BOSTER Cat. No. EK0406) was used for determining IL-4 in sera as picograms per milliliter (pg/ml) according to the manufacturer's instructions.

### V. Measurement of immunoglobulin-A (IgA)

IgA serum levels were quantified as nanograms per milliliter (ng/ml) using an ELISA assay kit (Abcam ab157735) according to the manufacturer's instructions.

### VI. Histopathological and Histometry studies

Small portions from the ileum were fixed, sectioned at 5  $\mu$ m, and stained using standard procedures. Explicitly, some sections were stained with Hematoxylin and Eosin (HE) for the evaluation of the general histological architecture, and other sections were stained with Periodic Acid Schiff reagent (PAS) for the determination of goblet cell count.

For histometry, the villus height, crypts depth, and the ratio of villi and crypts values (V/C ratio) were measured and calculated in HE-stained ileum sections (Yang *et al.*, 2013). The mean values were obtained from 10 microscopic fields in each specimen. All the histometric measurements were performed using ImageJ software (ImageJ Java software, version 1.52 v, U. S. NIH, Bethesda, MD, USA).

GALT (gut-associated lymphatic tissue) was also investigated in ileum sections, with a special focus dedicated to the lymphatic region of individual lymphatic follicles (LF) and their various components. The lymphatic follicles were divided into their specified compartments (cortical area, germinal center, coronal region, and dome region) according to Cesta (2006). These compartment areas ( $\mu\text{m}^2$ ) were measured in five LFs from each tissue sample in randomly selected fields.

## 2.2. Statistical analysis

SPSS 16.0 software (SPSS Inc. Chicago, IL, USA) was used for the statistical analysis. Statistical assessment was assessed by one-way ANOVA followed by Tukey test. The data were stated as mean  $\pm$  SE (standard error). P-values less than 0.05 were considered statistically significant.

## 3. Results

### Effect of PJ on pro-inflammatory cytokines and Chemokine (TNF- $\alpha$ , IFN- $\gamma$ , and IL-8)

As presented in Fig. 1.A., the serum levels of TNF- $\alpha$ , IFN- $\gamma$ , and IL-8 displayed a trend toward increase after PJ administration ( $P < 0.05$ ).

### Effect of PJ on Anti-inflammatory cytokine (IL-4)

PJ caused a significant increase ( $P < 0.05$ ) in IL-4 serum concentrations as compared to those of the control group (Fig. 1.B.).

### Effect of PJ on Immunoglobulin-A (IgA)

Data shown in Fig. 1.C. revealed that oral administration of PJ resulted in a significant increase ( $P < 0.05$ ) in the serum levels of IgA compared to their corresponding controls.

## Histology and Histometry studies

### Ileum section

Investigation of ileum sections from control rats (Con) showed normal architecture of crypts and villi (Fig. 2A-B). The effects of oral administration of PJ on the morphology of the ileum mucosal layer are presented in Fig. 2C-D. Specifically, administration of PJ was associated with a marked improvement in the mucosal architecture compared to the control group.

### Villus height

Table 1 shows the effects of PJ administration on villus height after 4 weeks. The villus height was significantly increased ( $P < 0.05$ ) in the PJ group compared to the control group.

### Crypt depth

As shown in Table 1, there was a slight decrease in the mean crypt depths between the PJ group and the control group ( $P < 0.05$ ).

### Villus/crypt Ratio

After 4 weeks of PJ administration, villus/crypt ratios were elevated compared to the control group (Table 1;  $P < 0.05$ ).

### Goblet cell count

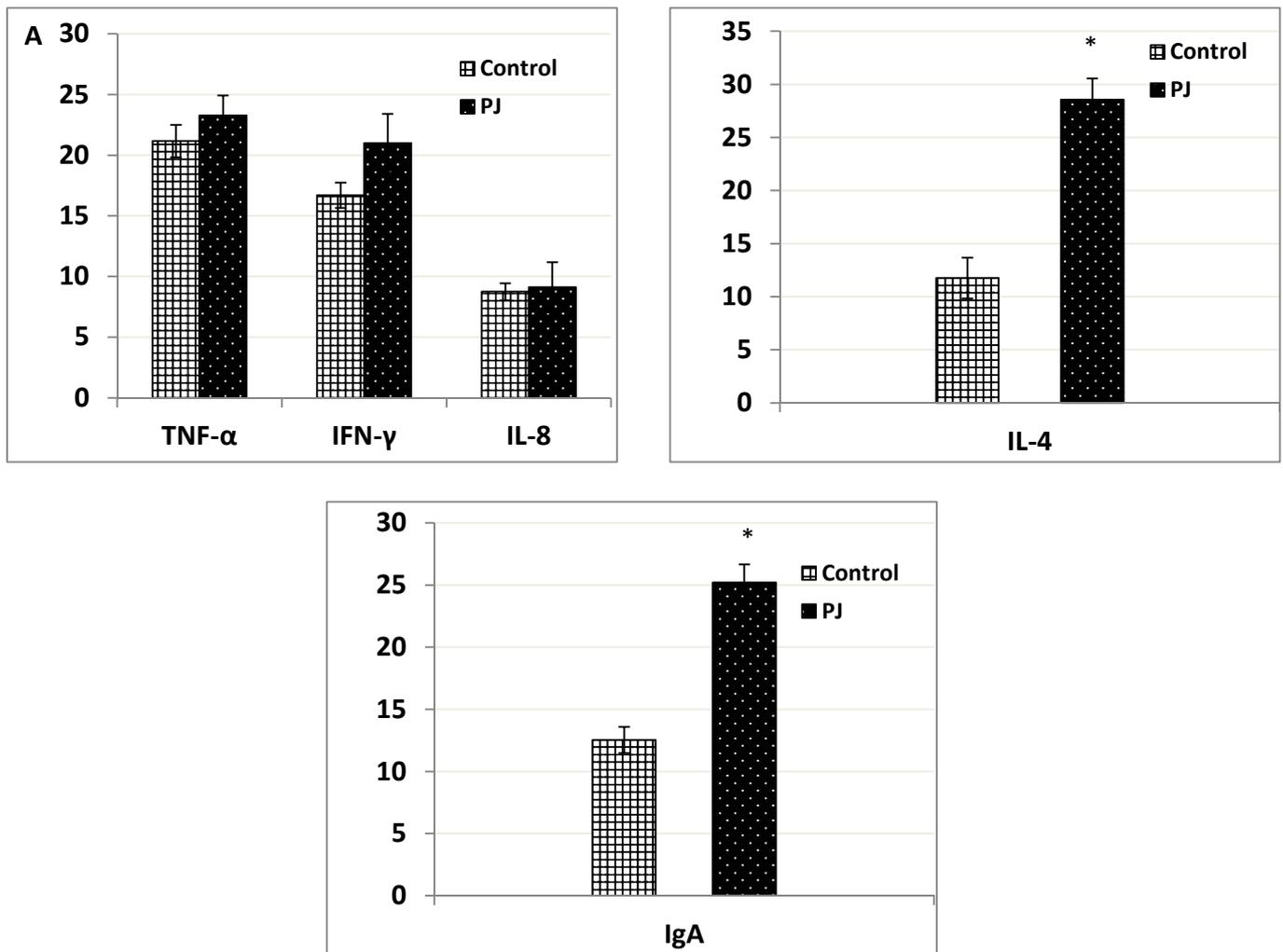
The number of goblet cells increased significantly after the administration of PJ compared to those of the control group (Fig. 2E-F; Table 1;  $P < 0.05$ ).

**Table 1:** Effect of PJ on histometrical analysis related to rat ileum

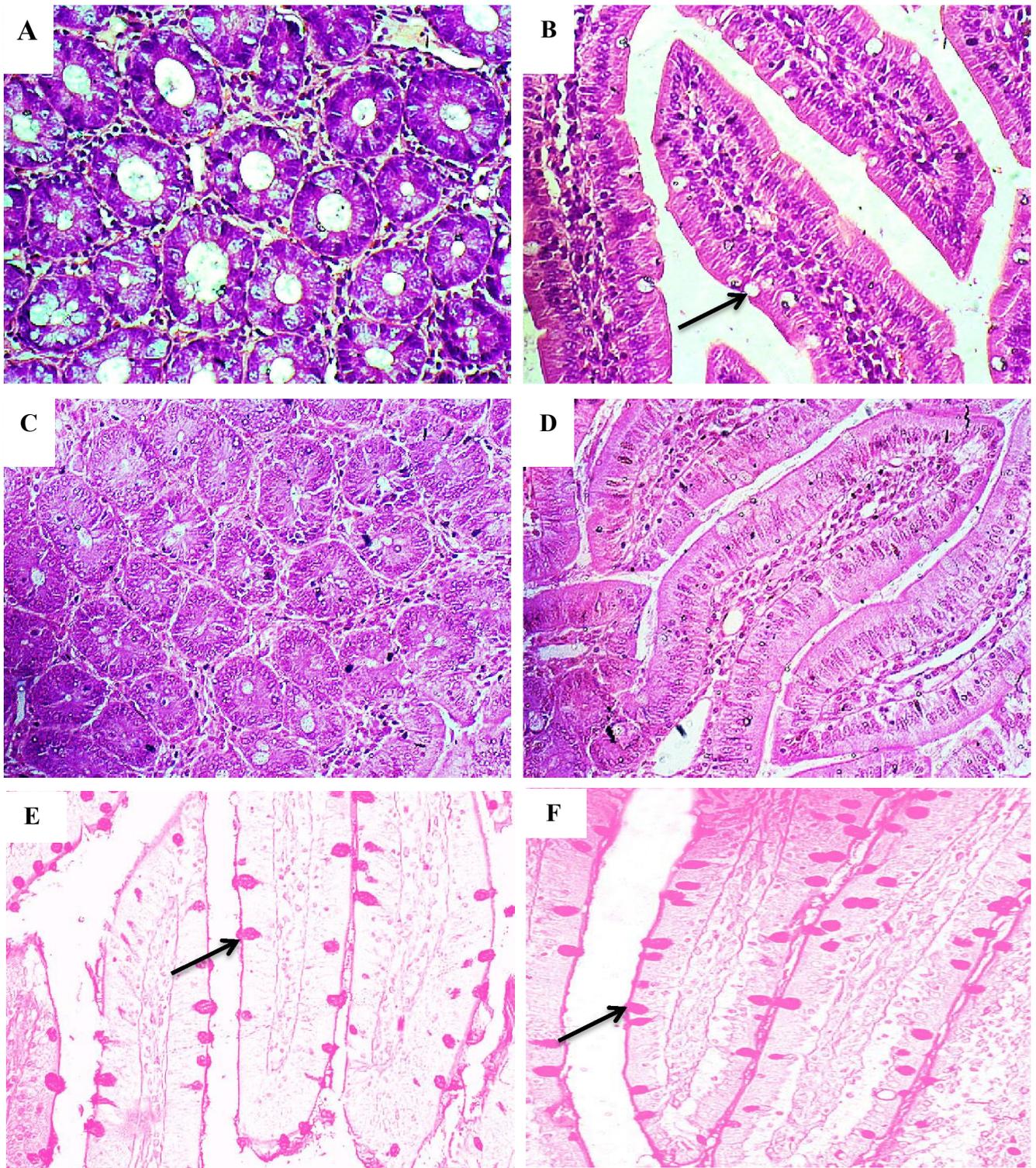
	Control	PJ
Goblet cell count	166.54 ± 13.07	228.48 ± 10.98*
Villi height, ileum, µm	140.51 ± 4.21	202.92 ± 9.23 *
crypts depth,ileum, µm	89.72 ± 4.29	76.13 ± 4.89
V/C ratio	1.56 ± 0.98	2.67 ± 1.89*

Data are expressed as means ± SEM. PJ= pomegranate juice \*=  $P < 0.05$  vs. control rats.

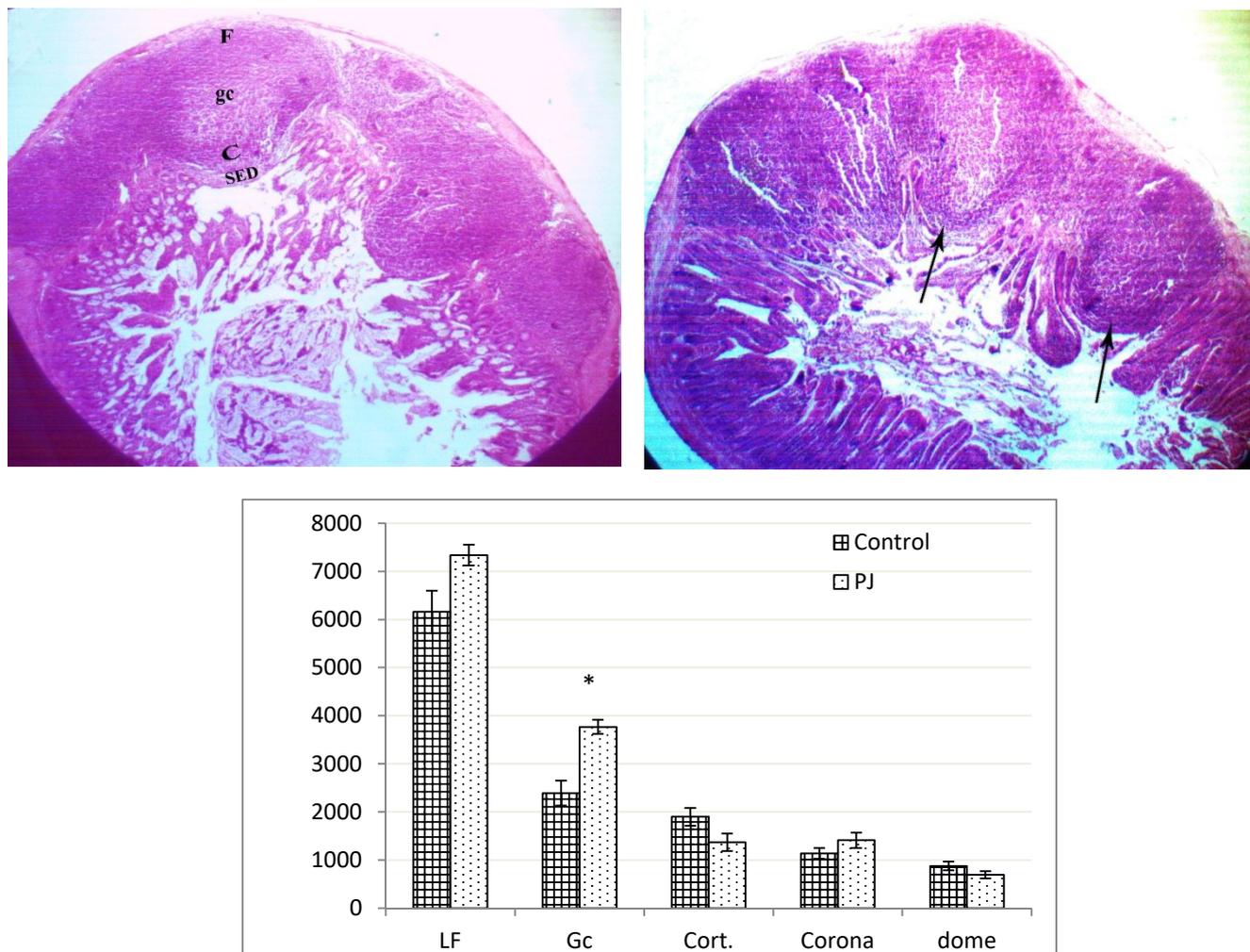
GALT appeared very well-organized in both control rats and rats administered PJ. Defined region measurements of Lymphoid Follicle (LF) compartments within the payer's patches were presented in Fig. 3A-B. Rats administered PJ exhibited a significant increase in the area of the germinal centers (Fig. 3 C;  $p < 0.05$ ) compared to control animals. Nevertheless, no difference was seen in the area measurement of other defined compartments.



**Fig. 1.** Effect of pomegranate administration on the cytokine and immunoglobulin-A concentrations A. serum levels of TNF-α, IFN-γ, and IL-8. B. serum levels of IL-4. C. serum levels of IgA; data are expressed as means ± SEM. PJ= pomegranate juice \*=  $p < 0.05$  vs. control.



**Fig. 2:** A-B ileum sections from control adult rat; A. showing normal crypts of Lieberkühn; B. showing intact villi with the presence of goblet cells in between (arrow). C-D Ileum sections from a rat administered PJ showing improvement in the architecture; C. crypts of Lieberkühn; D. villi (H&E, x400). E-F PAS-stained ileum sections (x400) obtained from: E. a healthy control rat revealing a PAS-positive brush border of columnar absorptive cells, as well as many goblet cells that appeared magenta red (arrow); F. a rat administered PJ presenting apparent increase in the numbers of goblet cells, expressed by increased intensity of PAS reaction (arrow).



**Fig. 3:** Histological sections of Peyer's patches of ileum **A.** from control rat showing centrally located follicle (f) containing a Germinal Center (gc) with Corona (C) and sub epithelial dome region (SED) (H&E, x32). **B.** from orally administered pomegranate rat with multiple lymphoid follicles (arrow) with apparent increase in the germinal center (H&E, 32). **C.** histometrical analysis of the lymphoid follicles of rat ileum, data are expressed in  $\mu\text{m}^2$  as Mean ( $\pm$ SE). PJ=pomegranate \* =  $P < 0.05$  vs. control rats.

#### 4. Discussion

The immune system is considered as a biological marker that may be used to assess the potential health benefits of dietary supplements in food for human and animal species. Pomegranate juice is high in polyphenols, tannins, and anthocyanins, as well as vitamins C, E, coenzyme Q10, and lipoic acid (Vroegrijk *et al.*, 2011). Its major antioxidative ingredients are anthocyanins and ellagic acid derivatives, which are the principal elements of the fruit's juice and give it its color (Jurenka 2008). In the current study, the administration of PJ increased the levels of the anti-inflammatory cytokine (IL-4). This is in accordance with Oliveira *et al.*, (2010) who recorded a significant increase in the anti-inflammatory cytokine (IL-4) in healthy animals after pomegranate intake. Additionally, many researchers have found that pomegranate fruit has anti-inflammatory qualities (Ahmed *et al.*, 2005, Lansky and Newman 2007; Shukla *et al.*, 2008; Larrosa *et al.*, 2010). The mechanism by which the pomegranate exerts its anti-inflammatory effect is through the inhibition of cyclooxygenase (COX) and lipoxygenase (LOX), which are crucial enzymes in the conversion of arachidonic acid to prostaglandins and leukotrienes that are important inflammatory mediators (Ardekani *et al.*, 2011).

Our data indicated that pomegranate administration resulted in a marked increase in the mucosal integrity and clarity by increasing ileal villus height and the number of goblet cells and slightly decrease the crypt depth. The goblet cells secrete mucin which protects the gastrointestinal membrane from toxic

food agents and pathogenic bacteria (Kindon *et al.*, 1995). It is well known that the heights of the intestinal villi correspond with the epithelial cells; specifically greater villi indicate more mature epithelial cells and higher intestinal absorption efficiency (He, 2006). Increased villus height can be caused by an increase in crypt cell proliferation combined with a decrease in apoptosis. The quantity of crypt cells with the ability to divide represents the intestinal membrane's growth condition. Changes in crypt depth express the rate of crypt cell division and can influence digestion in the small intestine (Kato *et al.*, 1999). Pomegranate as a rich source of antioxidant polyphenols exhibits gastrointestinal protective activity through the accumulation of large amounts of polyphenol metabolites in the intestine, which exhibits prebiotic properties (Li *et al.*, 2015). The same authors reported that both pomegranate extract and pomegranate juice enhanced the growth of lactobacilli and bifidobacteria while inhibiting the growth of clostridia and Enterobacteriaceae.

The present study showed that administration of PJ increased mucosal immunity by boosting the development of germinal center (GC) of Peyer's patches and increased the production of IgA. Previous studies indicated that microbiota and/or dietary antigens affect the properties of GC in PPs via differentiation of follicular helper T cells to produce efficient IgA, which is essential in immune protection against pathogens (Macpherson *et al.*, 2000; Hara *et al.*, 2019).

Conclusively, based on the current findings, it is interesting to confer that pomegranate juice may regulate the health by improving mucosal integrity and intestinal function, which beneficially can alter digestion in the small intestine, and enhance mucosal immunity against pathogens without any adverse side effects. As a result, the use of pomegranate juice in human nutrition can be recommended due to its favorable qualities.

#### Disclosure of interests

The authors declare that there are no conflicts of interest concerning this article.

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