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ORIGINAL ARTICLE

The Synbiotic Healing Effect of Probiotics and Prebiotics on Indomethacin Induced Gastric Ulcer in Rats

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ABSTRACT

Background*: Probiotics are becoming one of the main alternative medicational solutions used worldwide nowadays. Besides to their function in alleviating the immune system, they are used for the treatment of gastric ulcer. Along with the mixing with prebiotics, this could increase the healing percentage and maybe rate as well.* ***Methodology****: In this study, the synbiotic effect of the Bifidobacteria and lactobacillus probiots with the Punica granatum, Glycyrrhiza glabra, Cynara cardunculus. Prebiots were experimented separately to identify whether they would increase the healing effect or not. We divided the Ulcer-induced rats into 13 groups and treated for 14 days.* ***Results:*** *The results showed a decrease in WBCs count in the synbiotic mixture of Lactobacillus and P. granatum followed by Bifidobacterium with P. granatum. The lactobacillus and Cynara cardunculus mixture gave the highest WBCs count. Also, a decrease in both IL6 and IFN-γ after treatment with all synbiotic mixtures. The histopathology showed decreased gastric lesions and infiltration by mononuclear cells in all mixtures where the lactobacillus, P. Granatum mixture showed the best healing effect compared to the rest of groups and Ranitidine reference group. These results suggest that using probiotics along with prebiotics could be a potential treatment of gastric ulcer via enhancing the immune system.*

INRODUCTION

Gastric ulcer is a chronic disease accompanied with unexpected complications including bleeding, stenosis and perforation, as well as a high incidence of recurrence[1]. Gastric ulcer is described as a necrotic lesion penetrating through the entire mucosal thickness of the stomach. It results from tissue necrosis induced by various etiological factors such as: aspirin, indomethacin, bile acids, stress, aging and H. pylori infection [2]. Ulcer healing is an orchestrated process of filling the mucosal defect with epithelial and connective tissue cells, including cell proliferation, migration, regeneration, active angiogenesis, leading to scar formation. So to heal gastric ulcer, and besides using medications new researches suggests the fact that we can use probiotics and/or prebiotics [3]. Probiotics are live micro-organisms, which upon ingestion provide health benefits to the host, not available in inherent basic nutrition [4]. The mechanisms of probiotic action differ, depending on the species administered, existing intestinal environment and disease setting, however, some common mechanisms include the promotion of crypt cell proliferation (via short-chain fatty acid production), reduction of proinflammatory cytokine expression, production of antimicrobial factors, and competition with pathogenic bacteria for adherence [5].

There are numerous probiotic genera and species including *lactobacilli* and *bifido-bacteria* [6]. *Lactobacillus rhamnosus* and *Bifidobacterium bifidum* have been reported to protect the gastric mucosa from indomethacin induced damage [5]. It was found that mixing some compounds with the probiotic bacteria, enhance their activity and increase their viability, these compounds are called prebiotics. Prebiotics are indigestible diet ingredient that advantageously affects the host by particularly exhilarating the progression and/or action of one or a definite number of bacteria in the bowel [7].

Prebiotics have anti-inflammatory and anti-ulcer properties when ingested alone. Studies have shown that by harnessing both the benefits of these prebiotics and probiotics into synergy, the number of good bacteria in the digestive systems increased. The consumption of a synbiotic diet cause a modification of the gut metabolic activities with a maintenance of the bowel biostructure[8]. On the basis of the above discussion, this study is based on the use of the probiotics (*Lactobacillus rhamnosus* and *Bifidobacterium bifidum*) and prebiotics (Punica granatum, Cynara cardunculus and Glycyrrhiza glabra)

synergistic curing effect on indomethacin induced gastric ulcer in rats.

METHODOLGY

1. Experimental animals

Sixty five male Sprague dawley rats (body weight 200–250 gram) were purchased from VACSERA, Egypt. The animals were maintained in separate cages 5 per group, under standardized environmental conditions. Following a 7-day acclimation period, rats were randomized into experimental and control groups for induction of gastric ulcer. They were allowed to food and water *ad libitum* and they were deprived of food but had free access to water for 24 hours before ulcer induction by indomethacin [10].

Rats were divided into 13 groups

Group 1: Normal (Negative control).

Group 2: is ulcerated without treatment (Positive control).

Group 3: is ulcerated and treated by Ranitidine drug only.

Group 4: is ulcerated and treated by *Lactobacillus rhamnosus* only.

Group 5: is ulcerated and treated by *Bifidobacterium bifidum* only.

Group 6: is ulcerated and treated with the *Punica granatum* peels only.

Group 7: is ulcerated and treated with the Cynara cardunculus only.

Group 8: is ulcerated and treated with the *Glycyrrhiza glabra* only.

Group 9: is ulcerated and treated with the *Punica granatum peels* and *Lactobacillus rhamnosus*.

Group 10: is ulcerated and treated with the *Punica granatum* peels and *Bifidobacterium bifidum*.

Group 11: is ulcerated and treated with the Cynara cardunculus and *Lactobacillus rhamnosus*.

Group 12: is ulcerated and treated with *Glycyrrhiza glabra* and *Lactobacillus rhamnosus.*

Group 13: is ulcerated and treated with *Glycyrrhiza glabra* and *Bifidobacterium bifidum.*

2. Plant material (Prebiotics) preparation

Fruits of *Punica granatum*, Cynara cardunculus and *Glycyrrhiza glabra* were collected from local market in Cairo. Pre-treatment is done to change the initial matrix structure is generally recommended, which is grinding to gain smaller particles, that can be more easily penetrated by the solvent [11].

In our study, the peels of *P. granatum* and Cynara cardunculus were manually separated from the whole fruits and lyophilized for 48 hrs to increase the surface area and to save the active ingredient from degradation. Where the *Glycyrrhiza glabra* was obtained already in the powder form. After lyophilization, we boilied the three prebiots for two hours and decanted them overnight. (VACSERA, Egypt).

3. Bacteria (Probiotics) preparation and activation

Lactobacillus rhamnosus (ATCC 7469) and *Bifidobacterium bifidum* bacterial slants (EMCC: 1334) slants were purchased from Cairo MIRCEN, Ain Shams University. The *Bifidobacterium* broth media was used for bifidobacterium and *Lactobacillis* MRS Broth for lactobacillus. MRS is based on the formulations of de Man, Rogosa and Sharpe (MRS) [12].

Bifidobacterium broth was prepared by suspending 78.65 grams in 1000 ml distilled water. Media was completely dissolved by gentle heating for a few minutes. After sterilization by autoclaving at 15l pressure for 15 minutes, Both bifidobacteria was activated by inoculating 1 ml of it in 50 ml the media and incubating for 24-48 hours.

MRS media was prepared (Acumedia, United States) by dissolving 55 g of the medium in one liter of purified water, mixing them thoroughly and autoclaving at 121ºC for 15 minutes. After that the activation of the bacteria by inoculating 1 ml of *Lactobacillus rhamnosus* in 50 ml MRS media and incubate for 24-48 hours under anaerobic conditions.

4. Preparation of Indomethacin

Non-steroidal anti-inflammatory drugs (NSAID) are widely used in the treatment of pain, fever and inflammation. However, NSAIDs have been linked with ulcer and in several animal experiments and are widely used in ulcer induction in vivo which is “indomethacin” in our study [13].

In our experiment, indomethacin was prepared by adding 0.5 gm of Gum accacia to 250 gm of indomethin powder and solubilizing them in distilled water using the morter and pistle to produce a 500 ml suspension.

5. Microdilution method

Microdilution is the use of small, disposable, plastic trays for measuring antimicrobial susceptibility and MIC. Standard trays contain 96 wells, each containing a volume of 0.1 ml [14]. In the present study, the aim of doing this test was to estimate whether the prebiotic extract is toxic to the probiotic bacteria not in order to avoid antagonism.

We used McFarland standard to calculate the bacterial concentration. We prepared a test suspension by obtaining a fresh, pure culture of the test organism and inoculating a suitable broth. Then we visually adjusted the turbidity of the log growth of the bacterial suspension to that of a known McFarland Equivalence Standard. Microdilution test was done by inoculation of 10^6 cfu [0.5 McFarland St.] of the bacteria in the 100 µl *punica granatum*, Cynara cardunculus and *Glycyrrhiza glabra* extracts separately in each well of the plates by 10 fold dilution. Following overnight incubation at 35 ºC, the tubes were examined for visible bacterial growth by spectrophotometer as evidenced by turbidity.

6. Ulcer induction

Indomethacin was used to induce ulcer as it act as an NSAID (Non-steroidal anti-inflammatory drug) of

strong anti-ulcer properties. Gastric ulceration was induced in the animals according to the procedure described by Sayanti [15]. Briefly, rats were administered with a single oral dose of indomethacin 40 mg/kg body weight in 1 ml water. They were deprived of food but had free access to water 24 hours prior to ulcer induction.

7. Animal treatment

The 13 group where were orally administered the 1 ml of the treatment for 14 constitutive days and they accessed to food and water ad libitum. The protocol conforms to the guidelines of the National Institute of Health (NIH, 1985) for laboratory animal care and use. Where the Ranitidine dose was 50 mg/kg body weight, the bacterial dose was $6x10^8$ *cfu* according to McFarland standard and 500 mg/kg from the each prebiotic extract.

8. Blood Sample collection

Blood samples were taken from the rats' eyes in EDTA-collecting tubes for making complete blood count (CBC). Serum was collected in the day 15 after sacrificing the animals in blood collecting tubes, then separation by centrifugation at 2000 rpm for 15 minutes at 4°C, aliquotted and stored at -80°C for further investigations (ELISA).

9. ELISA for IL-6 and IFN-γ

Levels of IL-6 were quantified by Rat Il-6 ELISA Kit and IFN-γ were quantified by Rat IFN-γ ELISA Kit (Sigma-Aldrish, USA). Absorbencies were measured at 650 nm using ELISA plate reader (Biotek®, USA). The ELISA reader-controlling software (Masterplex readerfit) processes the digital data of raw absorbance value into a standard curve from which cytokine concentrations of unknown samples can be derived directly. In our study we used sandwich ELISA kit. Avidin-HRP as the conjugate, ABTS as the liquid substrate solution and BSA as a blocking buffer.

10. Stomach excision and collection of gastric tissue

Tissue samples were collected after indomethacin induction and at day 14 after treatment to compare between them. The specimens for each rat were fixed in 10% neutral buffered formalin solution and dehydrated in a graded alcohol series. Each specimen were examined grossly for dissection and detection of ulcer.

RESULTS

Microdilution methods

The results showed no antagonism or toxic effect in all mixtures almost in all concentrations used in the Microdilution test, which means that it's safe to use both prebiotic and probiotic together as a mixture synbiots.

Complete blood count

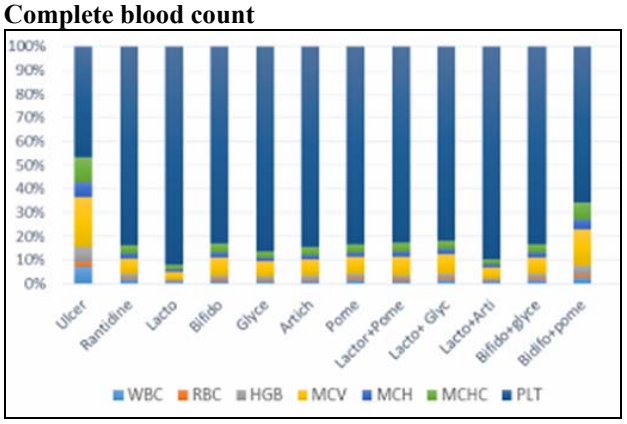

Fig. 1: Leukocytes and Lymphocytes had the highest level in control ulcer indicated the inflammation and they were decreased in probiotics with ulcer indicating decreased inflammation. They decreased the most in both Ranitidine and the mixture of *punica granatum* and lactobacillus. Neutrophils increased in Ranitidine and synbiots mixture from the normal because it plays a big role in healing process.

ELISA for IL-6 and IFN-γ cytokines

- *Interferon-γ*

The five synbiotic mixtures showed a significant decrease by different degrees in the Interferon- γ levels compared to the positive control. Ranitidine was the most effective treatment that decreased the levels of IFN-γ compared to the mixtures. This may indicate that the inflammation is decreased, thus the ulcer is decreasing and in the process of healing.

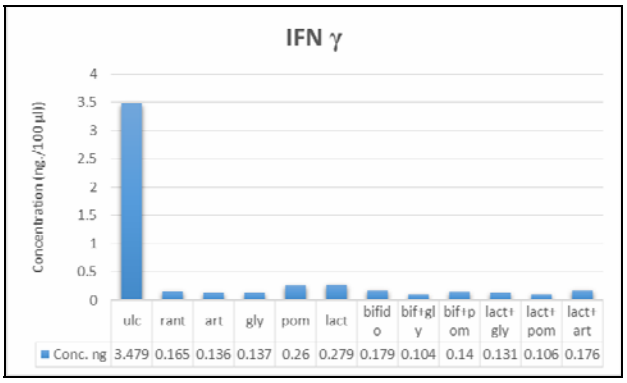

Fig. 2: Different concentrations of IFN-γ in 12 groups compared to the ulcer. A great decrease in cytokines levels is shown in both Ranitidine and the mixture (Bifidobacterium + Glycyrrhiza glabra).

- *Interleukin -6*

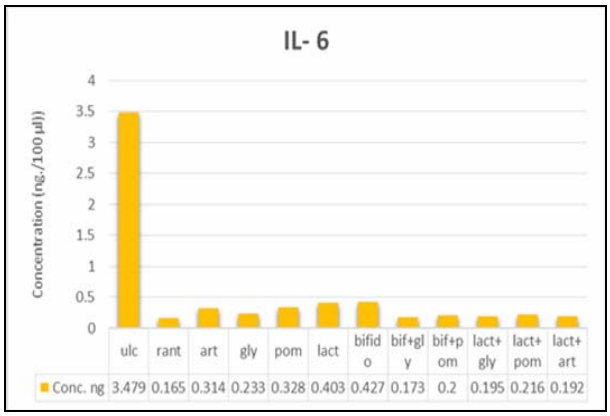

Fig. 3: Ranitidine showed the most decrease in IL-6 levels, followed by the synbiotic mixture (Bifidobacterium + Glycyrrhiza glabra) followed by (lactobacillus+ Glycyrrhiza glabra) compared to the positive control. This may indicate that the synbiotic mixture has anti-ulcer properties showed by decreasing interleukin levels.

Histopathology

In this study, histopathological examination upon Lactobacillus and Bifidobacterium treatment showed enhanced ulcer healing with mild ulcerations compared to the ulcerated control. In case of treatment by Punica granatum, it also showed mild healing with erosion of the superficial mucosal epithelium and infiltration with mononuclear cells. Surprisingly the healing effect improved in case of using both synbiotcs compared to using each one alone. As described also by [22], Ranitidine shows the most healing effect among all samples with regeneration of mucosal epithelium, infiltration with mononuclear cells in the submucosal layer as shown in Figure 4 -11.

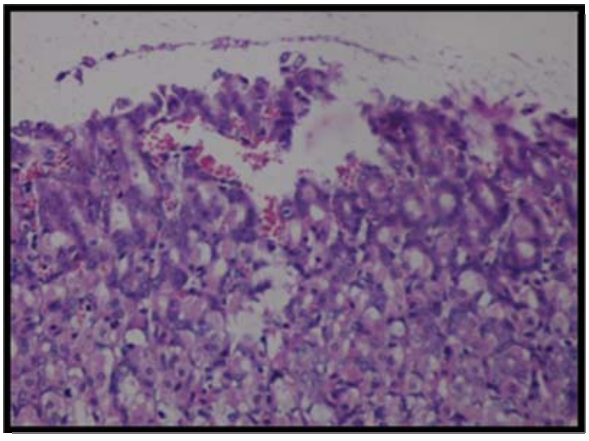

Fig. 4: Ulcer. Stomach shows gastric ulcer with sloughing of superficial mucosal epithelium and hemorrhages, gastric gland shows vacuolar degeneration and necrosis (H&E x400).

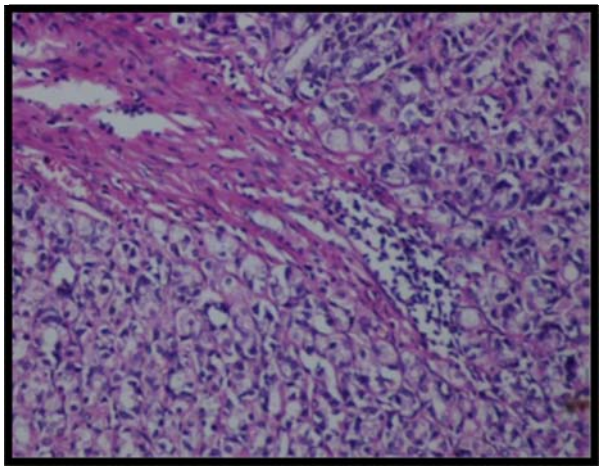

Fig. 5: Lactobacillus rhamnosus. Stomach shows infiltration by mononuclear cells and regeneration of gastric mucosa and mild necrosis of gastric gland with desquamation of its lining cells and infiltration by mononuclear cells (H&E x400). Healing capacity of this sample is estimated to be 30% and denoted by (++).

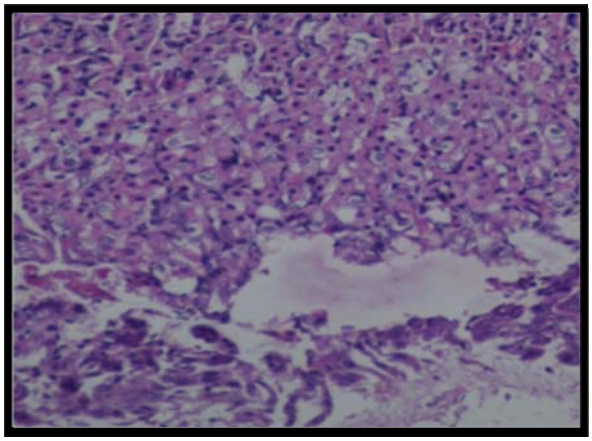

Fig. 6: Bifidobacterium bifidum. Stomach shows gastric ulcer, erosion in the mucosal epithelium, destruction and necrosis of gastric gland with infiltration by mononuclear cells and Stomach showed destruction and necrosis of the epithelial lining gastric gland with infiltration of the submucosa by mononuclear cells (H&E X 400).

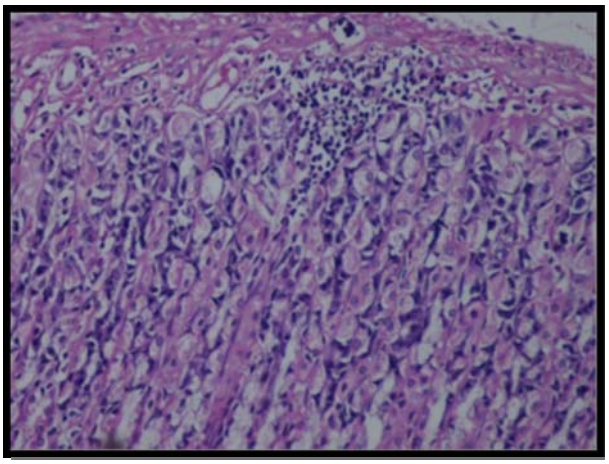

Fig. 7: Punica granatum. Stomach shows degeneration and necrosis of epithelial lining of most gastric glands with infiltration with mononuclear cells and shows erosion and sloughing of mucosal epithelium with necrosis of stomach gastric gland. Healing capacity of this sample is estimated to be less than 30% and denoted by (+). (H&E x400)

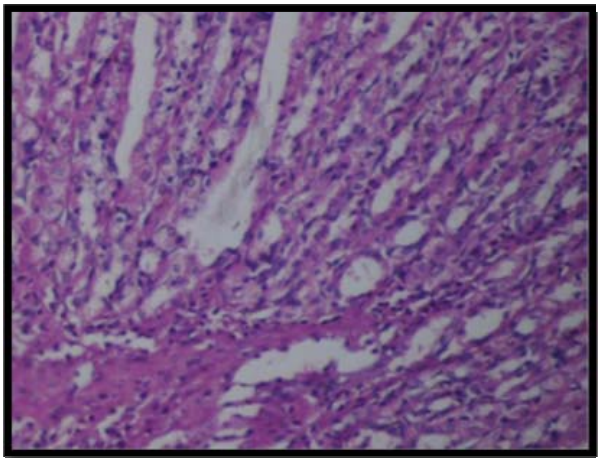

Fig. 8: Glycyrrhiza glabra. Stomach showed gastric gland with mild destruction and necrosis of its epithelium lining and mononuclear cells infiltration.

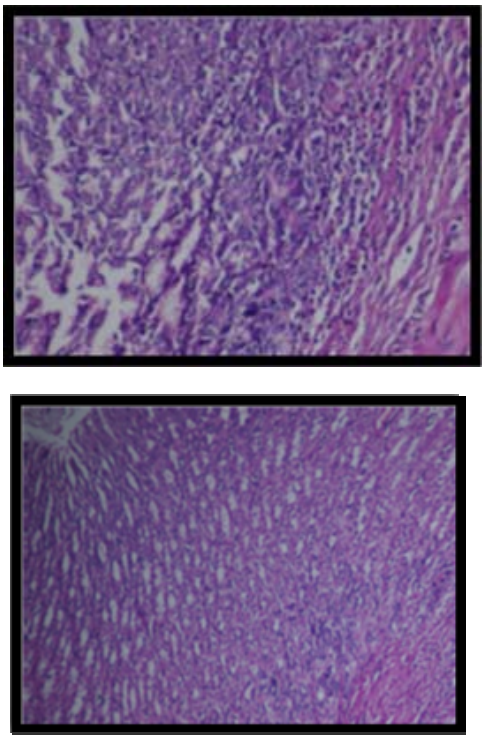

Fig. 9: Cynara cardunculus | Stomach from group received Cynara cardunculus showed superficial gastric ulcer with tissue debris and mononuclear cells infiltration (H&E x400).

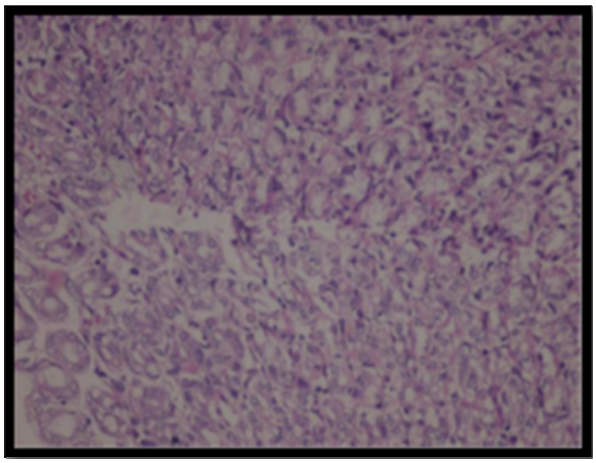

Fig. 10: Ranitidine. Stomach shows mild regeneration of gastric glands (H&E x200). Stomach shows slight degeneration and necrosis of some gastric glands with mononuclear cells infiltration in the submucosal layer (H&E x400). Healing capacity of this sample is estimated to be more than 50% and denoted by (+++).

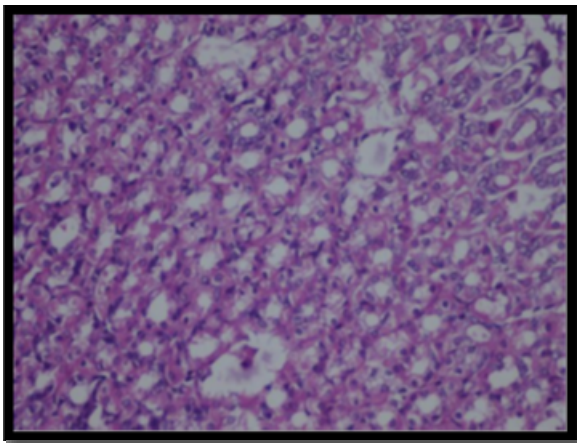

Fig. 11: Lactobacillus and punica granatum. Stomach shows mild degeneration of gastric gland and re-epithelization of mucosal lining (H&E x200). Stomach shows mild erosion of gastric mucosa and infiltration by mononuclear cells (H&E x400). Healing capacity of this sample is estimated to be more than 50% and denoted by (+++).

DISCUSSION

Microdilution method was done to estimate whether the prebiotic extracts (*Punica granatum*, *Cynara cardunculus* and *Glycyrrhiza glabra*) are toxic to the probiotics (*Lactobacillus rhamnosus* and *Bifidobacterium bifidum*) or not in order to avoid antagonism. This concern was, as explained by [16]. that Punica granatum had antimicrobial effects on some bacterial strains, but in our experiment the bacterial strain used was not affected by the antimicrobial effects of Punica granatum.

The complete blood count (CBC) is a good, sensitive indicator of the organism health. WBCs estimates the body's ability to fight infections and diseases. WBCs count said to be increased in case of gastric ulceration [17]. According to previous studies, *lactobacillus rhamnosus* enhances gastric ulcer healing shown by decreasing WBCs count if used alone [18] and the same was found in some *in vitro* studies when Bifidobacterium bifidum was used [19]. Also, *Punica granatum* peels decreases WBCs count in gastric ulcer healing if used alone [20]. In this study, our *Lactobacillus* and *Bifidobacterium* strains, *Punica granatum*, *Cynara cardunculus* and *Glycyrrhiza glabra* causes a significant decrease in WBCs count by different degrees, compared to the positive control.

It was demonstrated in a lot of researches that live probiotic strains as *Lactobacillus* and *Bifidobacterium* enhances the epithelial cell regeneration and inhibit epithelial cell apoptosis. Interleukin-6 and γ-interferon showed decreased levels when live probiotic *Lactobacillus* was used in gastric ulcer treatment [21] and the same for *Bifidobacterium* [19]. *Punica granatum* also as a prebiotic was found in a lot of studies to have antiulcer property.

Healing effect was approved by elevation of IL-6 and IFN- γ levels in treating ulcer in vivo [20]. On the other hand some studies showed decreased levels of both cytokines upon punica granatum treatment [22]. It was found that the prebiotics increase the proliferation of probiotics and enhance their healing capability [23].

It was found that *Lactobacillus rhamnosus* [24] and Bifidobacterium [25] showed to decrease cellular apoptosis and enhance re-epithelization in induced gastric ulcer rats in vivo. Histopathological examination in some researches showed a great progression and enhanced healing effect upon lactobacillus administration [26]. Researches showed that also punica granatum peels enhance gastric mucosa regeneration and increase the antioxidants levels [22]. It also enhances the immune cells recruitment to site of infection in order to accelerate the healing mechanism [23].

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