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The impacts of treatment with newly developed probiotic versus phaleria macrocarpa leaves extract on the histological features in immunocompromised New Zealand white rabbits

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Abstract. This research aimed to examine the possible effectiveness of treatment with a freshly formulated new probiotic versus *Phaleria macrocarpa* leaves extract indicated by the histopathological changes occurred to the colon of the immunocompromised New Zealand White (NZW) rabbits. The study included 40 rabbits, divided into 4 groups. Three of the groups were fed with the immunosuppressive medication (azathioprine, 500 mg/kg/day) to be immunocompromised, Group B was treated with *P. macrocarpa* extract, Group C treated with our developed probiotic, and the group D left untreated as a positive control, Group A regarded as control negative. The duration of immunocompromising drug induction was set as 14 days; and the period of treatment was set for another 14 days. Distinctly, the treated rabbits were showing improvements of healing processes compared to non-treated, where the signs of lack of normal mucous folds, different degrees of mucosal edema and congestion, submucosal hemorrhage, accumulative score of gross colon morphology and major severe lesions of the colon tissue of the non-treated were significantly improved in the probiotic treated, as well as, *P. macrocarpa* treated groups. As a conclusion, the results indicated that uptake of the formulated probiotic could strengthen rabbits' immune responses of the immunocompromised rabbits significantly (both the specific and non-specific responses) and retain the weakened colon tissues (caused by the immunosuppressant drug). These findings are recommended to be used in future for development of promising commercial supplements to mitigate the medical risks that comes with immunosuppressant medications currently given to animals or human patients under certain circumstances.

1. Introduction

The immune system contains certain organs, muscles, cells, powerful molecules and genes [1]. When an external antigen invades some biological structure, it stimulates the host's immunity such that all essential processes, in all structures in the body, work normally [2].

Azathioprine (Imuran®) is an immunosuppressive antimetabolite drug [3], and since 1996 there is no further reports in the literature referring to any modifications on its chemical structure [4].

Probiotics may provide the host with health benefits if delivered in sufficient quantities" [5] Recently, lactobacilli probiotics have gained more attention for their direct and indirect inhibitory effects on cells initiation and progression. Several studies indicated the anti-tumor properties of certain



Lactobacillus strains [6], through different mechanisms, such as inhibition of pathogens colonization [7], induction of immune system, direct cytotoxic effects on cancer cells [8], anti-mutagenic effects [4], and modulation of carcinogens metabolism and prevention of DNA from oxidative damage [9]. Moreover, the antifungal activities of Lactobacillus spp. strains have demonstrated previously as well [10].

Phaleria macrocarpa belongs to the family of Thymelaeaceae. Traditionally, P. macrocarpa is well known to contribute to human health and vitality, whereas, its extracts have been reported in numbers of valuable medicinal properties [11].

Several active substances have indicated in P. macrocarpa extractions, such as alkaloids, which can help in detoxification that neutralize toxins in the body. The presence of saponin acts as a source for antibacterial and antifungal activities and helps to promote and boost body immune system. Moreover, flavanoid contents could reduce the cholesterol and reduce the accumulation of fat in the blood vessel wall, it also acts as antioxidant agent and help to reduce the pain if there is bleeding or swelling [12].

This study aimed to examine the possible effectiveness of treatment with a freshly formulated new probiotic versus Phaleria macrocarpa leaves extract indicated by the histopathological changes occurred to the colon of the immunocompromised New Zealand White (NZW) rabbits.

2. Materials and methods

2.1. This study has been conducted into three main phases, as follow:

The First Phase (the pilot studies), prior to starting the experiments and grouping, several randomly selected rabbits were used for pilot studies, to determine the proper mode of induction, the accurate dosage of immunosuppressant drug to use, the number of doses required, and the number of days needed for experimental rabbits to be immunocompromised. Pilot studies were also important to obtain the safety (acute oral toxicity and skin irritation test) and the effectiveness of the treatment (probiotic and P. macrocarpa extract) used. These pilot studies were quite important to decide the best effective number of doses and the time intervals needed for both induction and treatment periods, quantitatively and qualitatively.

The Second Phase (the plant extraction), in this phase the preparation of P. macrocarpa extract was prepared, at the chosen dosage, and given as an oral supplement to the experimental rabbits to test its effectiveness on the enhancement of immune system functions and histological features of colon in immunocompromised rabbits.

The Third Phase (the histopathological studies), in this phase histology slides of all rabbit colon samples were prepared, by sectioning and staining with H&E and Alcian Blue-Periodic Acid Schiff (Alcian Blue-PAS). Histopathological alterations of rabbit's colon tissue, from all groups studied, were determined and used to estimate any cytotoxic effect and/or other side effects that may arise due to the administration of the probiotic and plant extract.

2.2. P. macrocarpa Extract Preparation

The powder of P. macrocarpa leaves (2kg) was soaked in methanol solvent 1:3 (divided into 100 g of the coarse powder in a conical flask with 300 ml of absolute methanol). The mixture kept at room temperature for 36 hours, stirred intermittently at a 4 hours interval.

Then, the mixture was filtered using filter papers (Whatman grade No.1) to recover the supernatant, and evaporated under low pressure using a rotary evaporator fitted with vacuum pump (BUCHI Switzerland) at 40°C till the concentrated methanol solvent was obtained. At the end of the drying process, the total amount of 389.20 g of plant paste obtained and stored in 500 ml normal saline (pH 7.0) to prepare the oral doses for treatment [11].

Qualitative biochemical tests were done phytochemically for analyzing the prepared extract for its active components.

2.3. Acute Oral Toxicity Test

The test was performed to assess the oral toxicity of *P. macrocarpa* extraction, as per the results obtained earlier, through the series of pilot studies to achieve the optimal effective dosage of the extract. Three pairs of animals (total of six rabbits) were fed orally (using standard feeding needle) with different dosages of the extract (300, 500, 700 mg/kg) daily for five days. Rabbits observed at a time interval of 4, 6, 12, 24, 48, 72, and 96 hours after feed. The dose of (500 mg/kg) of the extract showed best results, no death was recorded along the short and long-term outcomes, furthermore, referring to the delayed type of toxic signs, no depilation, diarrhea, or suppression of body weight was observed, compared to other doses. Thus, the dose of 500 mg/kg/day considered safe to be used to the experimental group.

2.4. Preparation of Probiotic

The bacterial colonies transferred to MRS broth (de MAN, ROGOSA and SHARPE, Merck), series of dilutions prepared to conduct selective enrichment media, and further microbial biochemical diagnostic tests were performed in order to use for preparation of the probiotic.

The concentration of two billion CFU/ml per strain was used to prepare the probiotic, depending on results of series of different concentrations tested during the pilot studies. These strains, at two billion CFU/ml in nutrient broth, were mixed with 10% skimmed milk (as carrier material), by adding 10 g of skim powder milk (SUNLAC, New Zealand) in 100g of distilled water (reduce pH to 4.6), and kept in room temperature for 2 hours [13], prior to feed orally to the rabbits using the feeding needle.

2.5. The Histopathological Study

At the end of induction period, five rabbits of each group have been sacrificed (using 30 mg/kg of Fatal-Plus® solution injected IV), and same scenario took place after treatment period. Rabbit's gut removed within 30 minutes after sacrificing the animal, two elongated samples from each proximal and distal areas of the colon immersed in 10% phosphate-buffered formalin at room temperature, and kept for overnight. This was done according to [14].

3. Results and Discussions

3.1. The Histopathological Findings

Results indicated that the Immunosuppressant used (Azathioprine) has induced severe microscopic changes (based on the severity of reaction or lesion index). The injury was mainly located in the distal colon with crypt distortion and inflammatory cell infiltration. Overall observation of colon sections of non-treated immunocompromised rabbits (group B) showed development of severe acute colitis, numerous areas showed with loss of mucosal folds, variable degrees of congestion and mucosal edema along with submucosal hemorrhage and focal ulceration. The cumulative score of colon alterations indicated an increase of substantial serious lesions in Group B versus those showed in treated groups (C and D), compared to normal (group A).

The composition of mucin secretion (of the four groups studied) of rabbits' colon has observed in sections-stained H&E and Alcian Blue-PAS. The findings indicated that the distribution of musin were identical (both in proximal and distal areas) and no differences in mucin secretion lesions shown within each group, with the two methods of staining used. However, results showed significant changes among the four groups studied.

The Cross section of normal rabbit's colon (group A), showed the cellularity of lamina propria is quite evident, capillaries were identified within the mucosa, lymphatic spaces were dilated and exist as a plexus around the muscularis mucosae, not extended higher than the bases of crypts of Lieberkuhn (Figure 1-4).

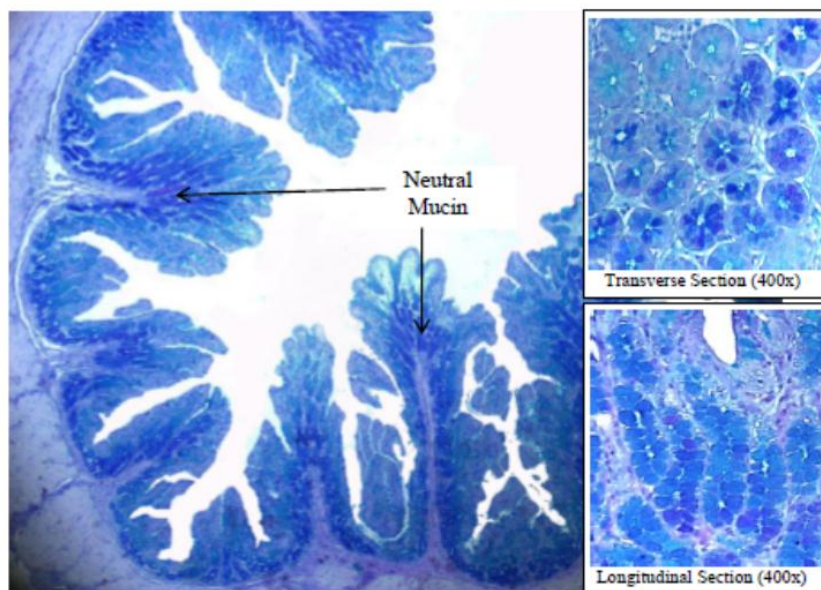


Figure 4.12 Cross section of normal rabbit's colon (group A), showing neutral mucin appeared obviously (darker colour). Alcian Blue-PAS (100x)

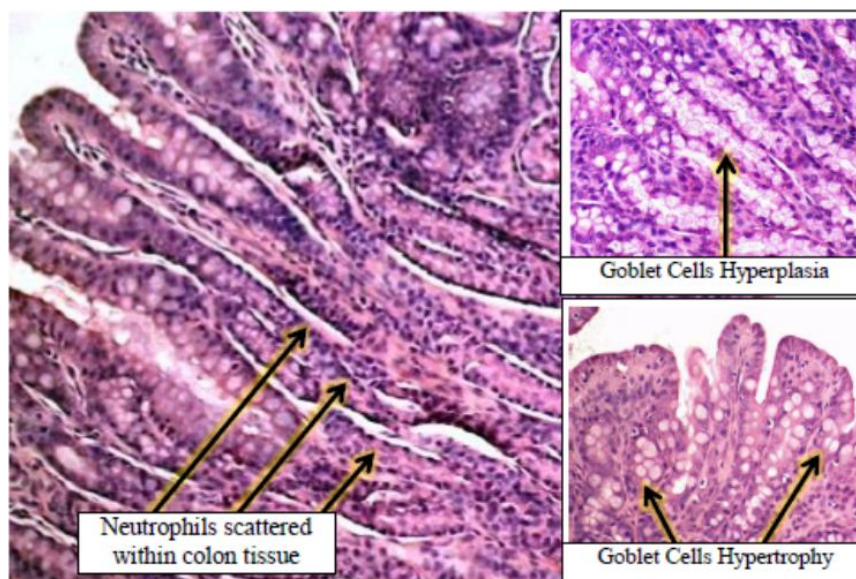


Figure 4.13 Cross section of non-treated rabbit's colon (group B), showing inflammatory cell infiltration of mixed leukocytes. H&E (200x)

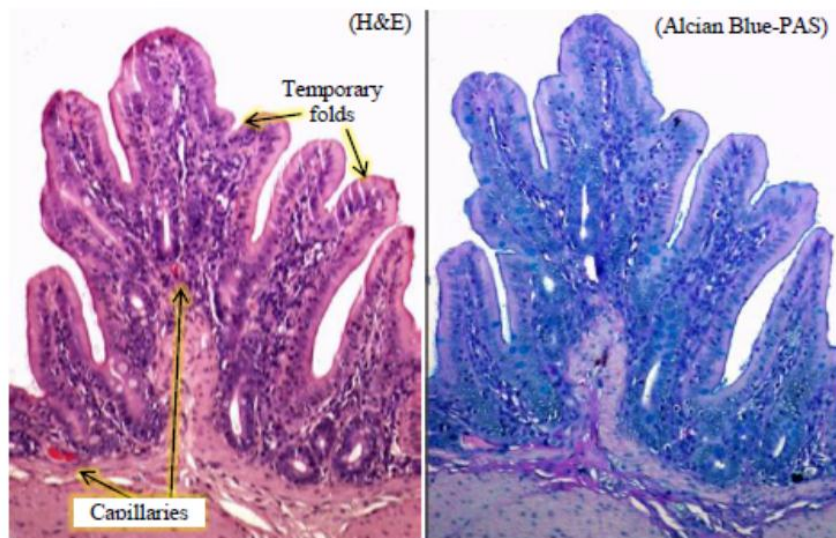


Figure 4.16 Cross section of probiotic-treated rabbit's colon (Group C), showing thinner mucosa and temporary folds (200x)

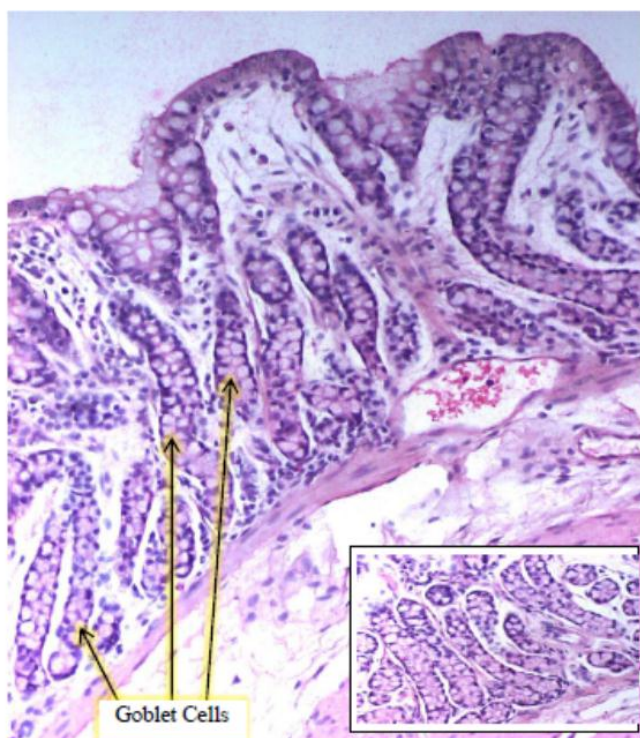


Figure 4.17 Cross section of *P. macrocarpa* treated rabbit's colon (Group D), showing goblet cells composition within colon tissue. H&E (400x)

Microscopic observation of non-treated immunocompromised colon samples (group B) showed an extreme increase of goblet cells hyperplasia and hypertrophy, an increase of mucin secretion, and increased infiltration of leukocytes (i.e. granulocytes, monocytes and lymphocytes). These changes

were common in all rabbit samples of group B, which indicating a severe inflammation occurred to the colon tissue. Although, it is not confirmed whether these histopathological changes caused by the pathogenic microbes or the normal microbiota. Further studies and tests are still needed to identify the particular cause of these histological alterations.

The immunocompromised rabbits treated with *P. macrocarpa* extract Group D showed remarkable reduction of mucin secretions, even though goblet cells hyperplasia is still seen in few sections. This might be explained as a reflection of the recovery process of impaired colon tissue (due to the immunosuppressant drug). These observations were supported by the increase of goblet cell compositions. However, it is still unclear whether these alterations were contributed to the initiation, or occur as a result of the inflammation (or both).

Furthermore, samples of immunocompromised rabbits treated with the probiotic (group C) and treated with *P. macrocarpa* extract (group D) were showed distinct decrease of mucin secretion and of goblet cells structured composition, as well as, a significant recovery of body weight gain, physical activity, reduction of pro-inflammatory immune markers, and abundant rates of neutrophils in plasma. In contrast, these signs were remarkably elevated in non-treated immunocompromised rabbits (group B). The NZW rabbit is very similar to the human in terms of (not only anatomy) but also the location of microflora within the GI tract. The upper GI tract in humans and rabbits contain relatively small numbers of bacteria, whereas large numbers of bacteria are found in the upper GI tract of mouse and rat models. Moreover, the upper intestine of both humans and rabbits also contains similar species of microflora (e.g. *Bifidobacteria*, *Bacteroides*). The addition of the live microbe population (via probiotic administration) resulted in decreased colonization of the upper GI tract. It also prevented translocation of the pathogen and seeding of other intra-abdominal organs [15]. In the modern era, there was suggestion that the consumption of yogurt containing *Lactobacilli* reduces the number of toxin-producing bacteria in the gut, thereby leading to longevity in the host. There has been an explosion of research investigating the use of probiotics over the past decade in particular, showing promise in treating or preventing a number of intestinal disorders (in both animals and humans), including antibiotic associated diarrhea, acute gastroenteritis, inflammatory bowel disease, food allergy, and colon cancer prevention [16; 15].

4. Conclusion

Treatment with *P. macrocarpa* extract showed remarkable recovery for the histopathological changes seen in the colon tissue of the immunocompromised NZW rabbits, in comparison to those treated with the formulated probiotic or non-treated groups.

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