

Evaluation of the antiapoptotic effect of matricaria chamomilla through Bcl-2 immune reaction in rat tongue mucositis model

تقييم فعالية ماتريكاريا كاموميللا ضد موت الخلايا المبرمج من خلال دراسة التفاعل المناعي الى Bcl-2 للغشاء السفلي المخاطي في لسان الفئران.

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Abstract

Bcl-2 is specifically considered as an important anti-apoptotic protein. The present study was designed for evaluation of the anti-apoptotic activity of Matricaria chamomilla by study the Bcl-2 immune reaction in ventral rat tongue mucositis.

Albino rats (Forty in number) were used in this study. They were divided randomly into control and study groups. In order to induce this inflammation in that tissue, 60 mg/kg of 5-Fluorouracil (5-FU) was administered intraperitoneally to each experimental animal in the study group at day 0 while at day two, 40 mg/kg was administered. The control group of experimental animals was injected intraperitoneally by distilled water in the same manner and dose as the study group. Each group was subdivided into: distilled water and chamomile extract treated groups. The distilled water treated experimental animals were gavaged by distilled water, while the others were gavaged by Matricaria chamomilla at a dose of 100mg/kg, two times daily. The treatment with water or Matricaria chamomilla was started at day five. The experimental animals were sacrificed at day eight and 12 (five animals each) and biopsies from the ventral tongue mucosa were taken. At day eight, the study animals that were developed tongue mucositis and were treated with Matricaria chamomilla showed significant increase in the thickness of epithelium with significant decrease in damage score, and significant increase in Bcl-2 immune expression in comparison with the water treated group ($p < 0.05$). At day 12 the result showed that longer duration of taking Matricaria chamomilla extract can cause damaging effect to the ventral tongue mucosa. In conclusion, Matricaria chamomilla can attenuate the injury caused by 5-FU, but long duration intake gave the reverse action.

الخلاصة

يعتبر Bcl-2 على وجه التحديد بروتين مهم لمكافحة موت الخلايا المبرمج. الهدف من هذه الدراسة لتقييم فعالية ماتريكاريا كاموميللا ضد موت الخلايا المبرمج من خلال دراسة التفاعل المناعي الى Bcl-2 للغشاء السفلي المخاطي في لسان الفئران. استخدمت أربعون من الفئران البيضاء في الدراسة، وقسمت عشوائيا إلى مجموعتين للسيطرة والدراسة. لتحريض التهاب الغشاء المخاطي، تم إعطاء 60 ملغم / كغم من 5-فلورويوراسيل داخل البريتون لكل حيوان في مجموعة الدراسة في اليوم 0، و 40 ملغم / كغم في اليوم الثاني. تم حقن المجموعة الضابطة داخل البريتون بالماء المقطر بنفس الطريقة والجرعة التي اتبعتها مجموعة الدراسة. تم تقسيم كل مجموعة إلى: المجموعة المعالجة بالماء المقطر و المجموعة المعالجة بالبابونج. المجموعة المعالجة بالماء المقطر تم إعطائها الماء المقطر عن طريق المعدة في حين أن المجموعة الثانية ثم إعطائها ماتامريكاريا كاموميللا بجرعة 100mg / كغم مرتين يوميا. وقد بدأ العلاج بالماء أو ماتريكاريا كاموميللا في اليوم الخامس. تم التضحية بالحيوانات في يوم ثمانية وأثني عشر (خمسة حيوانات لكل منهما) وخزعات من الغشاء المخاطي السفلي لللسان تم اخذها. في اليوم الثامن، أظهرت حيوانات الدراسة التي تم ظهور عندها التهاب الغشاء المخاطي للسان وعولجت بماتريكاريا كاموميللا زيادة معنوية في سماكة البطانة، وانخفاض معنوي في درجة الضرر، وزيادة معنوية في التعبير المناعي الى Bcl-2 بالمقارنة مع المجموعة المعالجة بالمياه ($P < 0.05$). في يوم اثني عشر وأظهرت النتيجة أن مدة أطول من أخذ ماتريكاريا كاموميللا يمكن أن يسبب الضرر للغشاء المخاطي اللساني السفلي. في الختام، ماتريكاريا كاموميللا يمكن أن يخفف من الاصابة الناجمة عن 5-FU، ولكن إذا اتخذت لفترة طويلة عكس يحدث.

Introduction

Matricaria chamomilla is the more familiar and more commonly used type of chamomile and considered as the star among medicinal species [1]. It demonstrates anti-bacterial and fungicidal [2], antiviral [3], anti-inflammatory [4], and anti-oxidant activity [5]. It exhibited anti-proliferative and apoptotic activity in various human cancer cells[6,7], accelerate the healing of the incisional wound in the skin of rat [8], used for numerous gastrointestinal conditions[9], treat infant colic disorders [10], control of breast pain(cyclic mastalgia)[11] relieved hypertensive symptoms [12], and ameliorates hyperglycemia and diabetic complications[13].

The survival cycle for the cell is dedicated by the balance between cell death sup pressor and cell death promoter signals provided by external stimuli as well as by intracellular molecules [14]. The Bcl-2 genes family has a central role in the control of the program, they encodes proteins that inhibit apoptosis produced by damage factor withdrawal (Bcl-xL, Bcl-w, A1, Mcl-1, Bcl-2) and also proteins that can promote apoptosis (Bad Bak, Bik/Nbk, Bid, Bag-1, Bax, Bcl-xS)[15].

Apoptosis which is a programmed cell death indicated by cell shrinkage, condensation of nuclei and internucleosomal degradation of DNA. Bcl-2 is a member of a growing family of apoptosis regulators. Apoptosis is the predominant mechanism in chemotherapy by which cancer cells die [16].

A pyrimidine analogue used to treat a wide range of solid tumors is 5-Fluorouracil (5-FU) because of its capacity to induce apoptosis in malignant cells mostly through Bcl-2 which trigger the mitochondrial pathways [17,18]. The present study was designed for evaluation of the anti-apoptotic activity of Matricaria chamomilla through Bcl-2 immune reaction in ventral surface of rat tongue mucositis caused by 5-FU.

Materials and methods

Animals grouping: Albino rats (Forty in number) , weighing 280-295 g were cared in the Animal House, Medical College, Hawler Medical University, Erbil/Iraq. The experimental animals maintained on a 12 hour dark / light cycle at $25 \pm 6^{\circ}\text{C}$ and fed with a standard animal food and allowed drinking water ad libitum. The research project was approved by the Research Ethics Committee at Dental College, Hawler Medical University, Erbil/Iraq, under protocol. The experimental animals were randomly divided into control and study groups (20 animals in each).

The control group of experimental animals in this study consists of: (A) Group treated with distilled water in which a volume of it was equal to that of Matricaria chamomilla extract. This volume was given by intragastric gavage tube, and (B) Group treated with Matricaria chamomilla organic alcoholic extract (USA-Code 1, HS3751002) gavaged with dose of 100 mg / kg two times daily [19]. The experimental animals were intraperitoneally injected by normal saline in the same manner and dose like 5-FU on day 0 and two, and the treatment with distilled water or the chamomile extract was initiated at day five and the experiment continue for 12 days.

The experimental study group is also consists of: (A) Group treated with distilled water and (B) Group treated with Matricaria chamomilla and they were treated in the same manner like the control group. Mucositis was induced by intraperitoneal injection of 60 mg / kg of 5-FU (Kocak farma / Turkey) to each animal at day 0 and 40 mg/kg was administered at day two [20].

The experimental animals were sacrificed at day eight and 12 (five animals in each), and the mid-third of the tongue was removed, then fixed in neutral buffered formalin, processed by H&E and immunohistochemistry for histopathological analysis.

Morphometric analysis:

The epithelial thickness of the tissue were assessed by two independent physicians, the thicknesses of all layers in the epithelium was measured (the major epithelial thickness) in five photograph fields from each tongue mucosal epithelium section by objective micrometer at high power magnification (x400). In order to measure tongue mucositis damage scores, five regions in the epithelial area and the connective tissue were selected (linearly adjacent to each other), and these areas were evaluated for the following:

- *Cell changes in squamous epithelium such as hyperchromasia, pleomorphism, binucleation, and necrosis.
- * Degeneration and vacuolar alterations of basal and suprabasilar layer.
- * Inflammatory infiltrate in submucosa layer.
- * Blood vessels Congestion.

The changes in the tissue were assessed by one blind evaluator to the type of the sample with scores of 0 to five. This method was modified from the study proposed by Üçuncu et al [21] to assess the degree of tongue mucositis.

The anti-apoptosis in the tissue was assessed by Bcl-2 immunostaining by using monocle. Mouse Anti-Hum. Bcl-2 Oncoprot. Clone 124 Code No 1587 ready to use N-series primary antibody, with Dako EnVision™, EnVision™ double staining and LASAB™ 2 systems. Positive and negative controls were run simultaneously with biopsy specimen. Positive cells expressing Bcl-2 was demonstrated brown cytoplasmic staining. Two observers counted approximately 1000 cells from cell population for each section of the tissue at a magnification of 400X. Using a light microscope (Olympus, Tokyo, Japan), the percentages of Bcl-2 positive cells were calculated and Bcl-2 expression was evaluated according to the scoring system of Seleit et al [22]. Percentage of positively stained cells were: absent: <1%, mild: 1 - <10%, moderate: 10 - <50%, or strong: > 50%.

Statistical analysis of this study was performed using Mann -Whitney u test to assess statistical analysis for every individual pair in a group. P value less than or equal to 0.05 was considered statistically significant.

Results

A. Hematoxylin and eosin results:

At day eight, photomicrograph of the ventral tongue mucosa of animal (Figure-1), in Saline / Water treated group indicate normal keratinized squamous epithelium and connective tissue, with absence of inflammatory cells and blood vessels congestion. While the photomicrograph of the same tissue of animal in Saline / Matricaria chamomilla treated group reveal a nearly normal epithelium and connective tissue, only few epithelial cells shows vacuolation (Figure-1). The 5-FU / Water treated group shows several changes like: Severe decrease in the epithelial thickness and keratin layer, keratin separation, cytoplasmic vacuolation, loss of few basal cells layer, flattening of rete ridges, edema, inflammatory cells infiltration, and vascular dilation and congestion. Increase in the epithelial thickness in 5-FU / Matricaria chamomilla treated group in comparison with 5-FU / Water treated group at this day, less congested blood vessels seen, but cytoplasmic vacuolation of some epithelial cells is still present.

At day 12 (Figure-2), photomicrograph of the same tissue of animals in Saline / Water treated group also reveal normal keratinized squamous epithelium and connective tissue. Saline / Matricaria chamomilla treated group shows some vacuolation of epithelial cells and changes in the shape of rete ridges. The 5-FU / Water treated group shows number of changes like: Increase in the epithelial thickness, decrease in number of inflammatory cells and congested blood vessels, and less epithelial vacuolation in comparison with day eight of the same group. 5-FU / Matricaria chamomilla treated

group shows cytoplasmic vacuolation of basal epithelial cells, inflammatory cells infiltration, and congestion of connective tissue blood vessels.

At day eight (Table-1), the 5-FU cause significant decrease in epithelial thickness in comparison with group treated with the Saline / Water at this day ($p<0.05$). The mean epithelial thickness in the 5-FU / Water treated group was ($17.16 \pm 0.82 \mu\text{m}$), but the mean epithelial thickness in the 5-FU / Matricaria chamomilla treated group was ($25.08 \pm 0.87 \mu\text{m}$), the statistical analysis showed that Matricaria chamomilla can cause significant increase in epithelial thickness at this day ($p<0.05$). The mean damage score for the 5-FU/Water treated group was 3.03 ± 0.33 , but the mean damage score for the 5-FU/ Matricaria chamomilla treatment group was 1.91 ± 0.31 . Statistical analysis showed that chamomile can cause significant decrease in damage score at day eight ($p<0.05$).

In day 12 (Table-1), the mean epithelial thickness in the 5-FU/ Water treated group was ($27.59 \pm 0.66 \mu\text{m}$), but the mean epithelial thickness in the 5-FU/ Matricaria chamomilla treated group was ($23.1 \pm 0.85 \mu\text{m}$), the statistical analysis showed that Matricaria chamomilla can cause significant decrease in epithelial thickness at this day ($p<0.05$). The mean damage score for the 5-FU/Water treated group was 1.02 ± 0.18 , but the mean damage score for the 5-FU/ Matricaria chamomilla treatment group was 2.14 ± 0.74 . Statistical analysis showed that Matricaria chamomilla can cause significant increase in damage score at day twelve ($p<0.05$).

B. Immunohistochemical result:

The immunohistochemical results (Table-2) showed that the group treated with 5-FU / Water can cause significant decrease in Bcl-2 immune expression in comparison with the group treated with Saline / Water at day eight and twelve ($p<0.05$).

At day eight, the mean Bcl-2 immune expression in 5-FU / Water and 5-FU / Matricaria chamomilla treated groups was 0.132 ± 0.016 , and 0.722 ± 0.013 respectively. Significant differences statistically present between them ($p<0.05$). At day 12, the mean Bcl-2 immune expression in 5-FU / Water and 5-FU / Matricaria chamomilla treated groups was 0.738 ± 0.025 , and 0.118 ± 0.014 respectively. Significant differences statistically present between them ($p<0.05$).

DISCUSSION

Several methods was used to prevent oral mucositis development, such as oral hygiene protocols, amifostine, calcium phosphate, cryotherapy, cytoprotectors (sucralfate, oral glutamine, hyaluronic acid), growth factors, topical polyvinylpyrrolidone, and low power laser irradiation[23]. Since the biology of oral mucositis is complex, so it is difficult to establish a fully therapeutic agent. Advances in the knowledge of its immunopathology will accelerate the treatment and prevention of this clinical condition [24].

One of the most widely used management strategies in cancer is antineoplastic chemotherapy, either alone or in combination with other types of treatment. Lack of selectivity is the main side effect of chemotherapy. It acts upon both tumor cells and rapidly multiplying normal cells such as oral mucosal cells [25, 26]. The most common side effects of cancer treatment like chemotherapy is oral mucositis that giving rise to erythematous areas in combination with oral ulcerations that can reach a large size and causing severe oral complications [27].

5-FU is prescribed for treatment of cancer and remains as the most common causes of oral mucositis. Its major action is blocking DNA synthesis. Severe inflammation, ulceration and bleeding can occur in the mouth, esophagus and intestine [28, 29].

Day eight: The present results showed that 5-FU / Water treated experimental group showed the followings: Significant decrease in the epithelial thickness, significant increase in the mean microscopic damage score and significant decrease in Bcl-2 immune expression ($p<0.05$) in comparison with the Saline / Water treated group. This may be due to the increases in the release of

proinflammatory cytokines which cause tissues damage and inflammatory response [28]. Al-Refai *et al* [30] found that 5-FU administration was accompanied by the following: A significant reduction in Ki-67 and Bcl-2 positive cells. The production of reactive oxygen species by chemotherapy disturb the metabolism in progenitor cells and cause inhibition of mitosis and increase in apoptosis [31].

5-FU/Matricaria chamomilla treated group showed the followings: Significant increase in the epithelial thickness, significant decrease in the mean microscopic damage score and significant increase in Bcl-2 immune expression ($p < 0.05$) in comparison with the 5-FU / Water treated group at this day. The result of this study showed that chamomile can lessen the 5-FU induced mucositis at day eight. This may be due to the enhanced release of growth factors, increased neovascularization and collagen formation [32]. The Matricaria chamomilla plant contains many different substances with antibacterial and antifungal actions, such as chamazulene, alpha bisabolol, bisabol oxides, spiroethers, and flavonoids. Flavonoids act as antioxidants, encourage the effects of vitamin C, and strengthen connective tissue around capillaries [33]. Zadeh *et al* [1] also found that the anti-inflammatory, wound-healing, and antimicrobial effects of Matricaria chamomilla are attributed to the essential oil that contains sesquiterpene alcohol, alpha-bisabolol, chamazulene, and flavonoids.

Day 12: The 5-FU/ Water treated group showed a less 5-FU induced side effects at day 12 in comparison with day eight of the same group. The direct mucotoxic side effects of chemotherapy on the oral mucosa begin shortly after therapy has begun, and decrease gradually with eventual resolution occurring within two weeks after treatment [34].

Matricaria chamomilla caused the followings: Significant decrease in the epithelial thickness, significant increase in the mean microscopic damage score and significant decrease in Bcl-2 immune expression ($p < 0.05$) in comparison with the Saline/ Water and 5-FU / Water treated group at this day. Harmful effects of chamomile may be come from its constituents, like bisabolol, volatile oils, anthemic, chamazulene, and tannin [35]. Cavalieri *et al* found that the α -bisabolol is a proapoptotic agent and enhances apoptosis [36]. Further investigations are needed to evaluate the topical effects of Matricaria chamomilla extract at different concentrations in the management of oral mucositis.

Conclusion

Histopathological results of the experimental animals that developed mucositis and were treated with Matricaria chamomilla extract exhibited a lesser degree of mucositis and showed significantly more cells staining positive for Bcl-2 in comparison to the 5-FU/Water treated group ($p < 0.05$) at day eight, but longer duration of usage of Matricaria chamomilla can cause damaging effect to the rat tongue ventral mucosa.

Acknowledgment: Great amount of gratitude for Dr Rafil T. Yaqo, Head of Histopathology Unit at the Duhok Central Public Lab for thoughtful co-operation in processing and the staining of all samples of the study.

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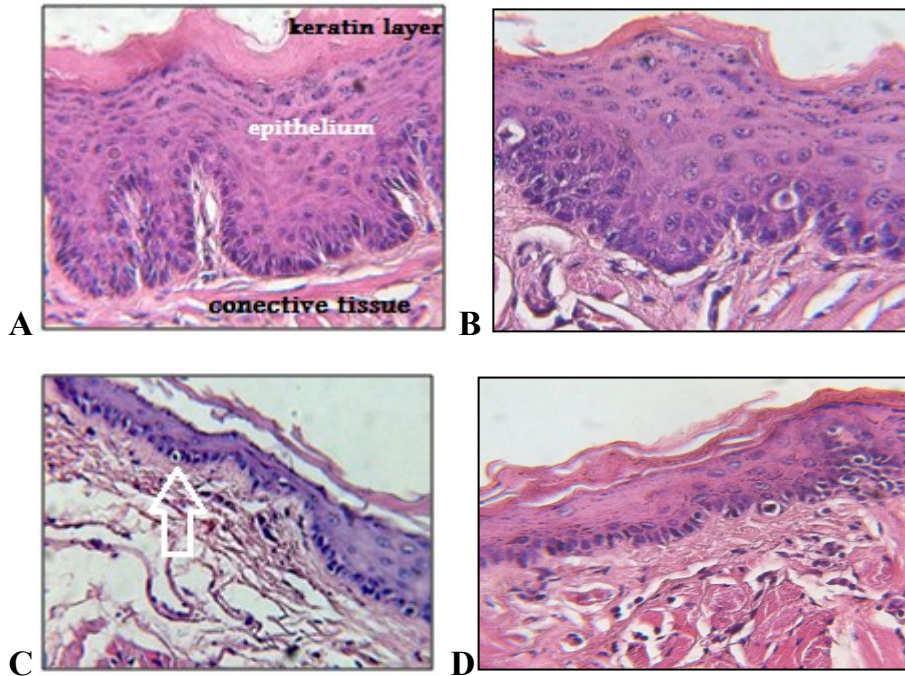
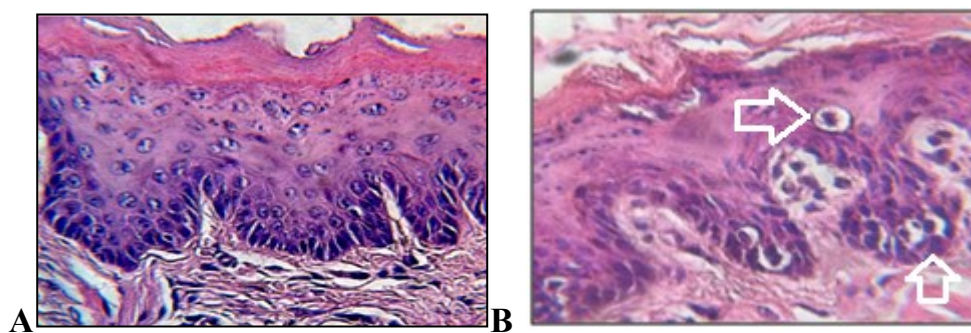


Figure 1: The ventral tongue mucosa photomicrograph of rat at day eight. (A) Saline / Water treated group reveal normal keratinized squamous epithelium and normal connective tissue. (B) Saline / Matricaria chamomilla treated group reveal a nearly normal epithelium and connective tissue, only few epithelial cells shows vacuolation. (C) 5-FU/ Water treated group shows severe decrease in the epithelial thickness and keratin layer, keratin separation, cytoplasmic vacuolation(arrow), loss of some basal cells layer, and flattening of rete ridge. (D) 5-FU / Matricaria chamomilla treated group shows increase in the epithelial thickness of the tissue comparing with group treated with 5-FU/Water at this day, cytoplasmic vacuolation of some basal epithelial are also seen (H&E, x400).



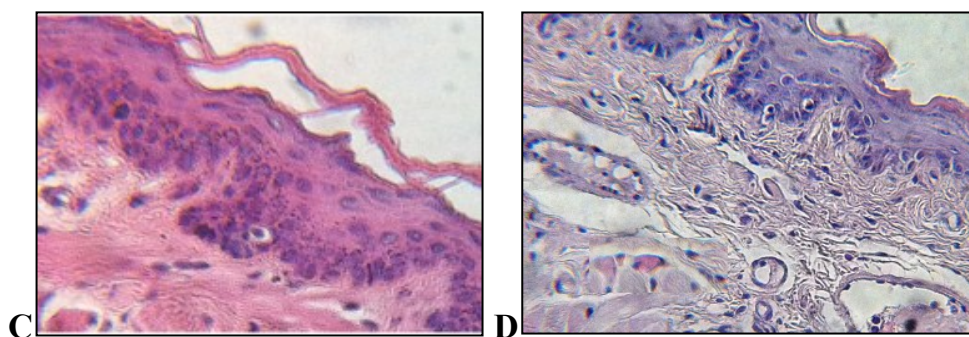


Figure 2: The ventral tongue mucosa photomicrograph of rat at day 12. (A) Saline / Water treated group reveal normal keratinized squamous epithelium and normal connective tissue. (B) Saline / Matricaria chamomilla treated group shows more vacuolation of basal and suprabasilar cells (arrows). (C) 5-FU / Water treated group shows the followings: Increase in the epithelial thickness with less vacuolar degeneration in comparison with day eight of the same group. (D) 5-FU / Matricaria chamomilla treated group shows cytoplasmic vacuolation of some basal epithelial and congestion of connective tissue blood vessels (H&E, x400).

Table 1: Means and standard deviations of epithelial thickness and damage scores of the examined tissue after water or Matricaria chamomilla treatment in Albino rats after saline or 5-FU injection in all groups in the study.

Groups	Major epithelial thickness(μm) Mean \pm SD				Damage score Mean \pm SD			
	Day 8	P-value	Day 12	P-value	Day 8	P-value	Day 12	P-value
Saline/water	48.62 \pm 0.87		50.08 \pm 0.72		0.16 \pm 0.05	0.057	0.13 \pm 0.04	
Saline/ Matricaria chamomilla	47.94 \pm 1.14	0.051	42.46 \pm 3.61	0.012	0.27 \pm 0.07		1.17 \pm 0.04	0.012
Saline/water	48.62 \pm 0.87		50.08 \pm 0.72		0.16 \pm 0.05	0.012	0.13 \pm 0.04	
5-FU/water	17.16 \pm 0.82	0.012	27.59 \pm 0.66	0.012	3.03 \pm 0.33		1.02 \pm 0.18	0.012
Saline/water	48.62 \pm 0.87		50.08 \pm 0.72		0.16 \pm 0.05	0.012	0.13 \pm 0.04	
5-FU/ Matricaria chamomilla	25.08 \pm 0.87	0.012	23.1 \pm 0.85	0.012	1.91 \pm 0.31		2.14 \pm 0.74	0.008
Saline/ Matricaria chamomilla	47.94 \pm 1.14		42.46 \pm 3.61		0.27 \pm 0.07	0.012	1.17 \pm 0.04	
5-FU/water	17.16 \pm 0.82	0.012	27.59 \pm 0.66	0.012	3.03 \pm 0.33		1.02 \pm 0.18	0.144
Saline/ Matricaria chamomilla	47.94 \pm 1.14		42.46 \pm 3.61		0.27 \pm 0.07	0.012	1.17 \pm 0.04	
5-FU/ Matricaria chamomilla	25.08 \pm 0.87	0.012	23.1 \pm 0.85	0.012	1.91 \pm 0.31		2.14 \pm 0.74	0.008
5-FU/water	17.16 \pm 0.82		27.59 \pm 0.66		3.03 \pm 0.33	0.012	1.02 \pm 0.18	0.008
5-FU/ Matricaria chamomilla	25.08 \pm 0.87	0.012	23.1 \pm 0.85	0.012	1.91 \pm 0.31		2.14 \pm 0.74	

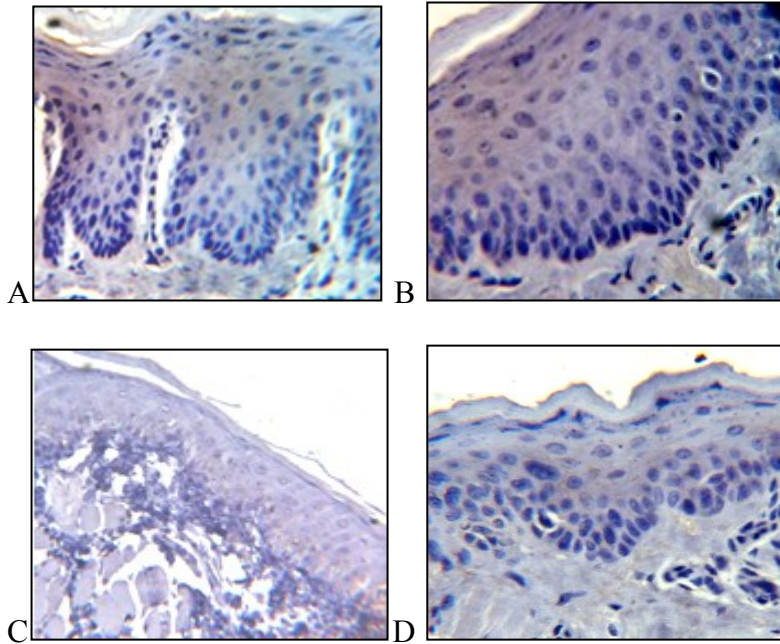


Figure 3: The ventral tongue mucosa photomicrograph of rat at day eight. (A) Saline / Water treated group reveal mild Bcl-2 immune expression. (B) Saline / Matricaria chamomilla treated group shows mild Bcl-2 immune expression. (C) 5-FU / Water treated group shows negative Bcl-2 immune expression. (D) 5-FU / Matricaria chamomilla treated group shows negative Bcl-2 immune expression (Immunohistochemistry x400).

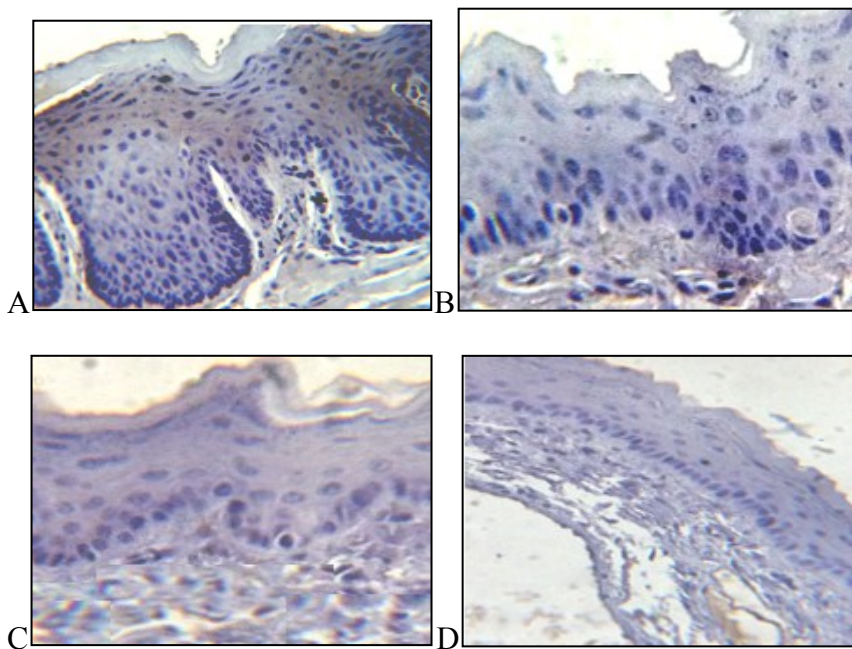


Figure 4: The ventral tongue mucosa photomicrograph of rat at day 12. (A) Saline / Water treated group reveal mild Bcl-2 immune expression. (B) Saline / Matricaria chamomilla treated group shows negative Bcl-2 immune expression. (C) 5-FU / Water treated group shows negative Bcl-2 immune expression. (D) 5-FU / Matricaria chamomilla treated group shows negative Bcl-2 immune expression (Immunohistochemistry x400).

Table 2: Means and standard deviations of Bcl-2 immune expression of the examined tissue after water or Matricaria chamomilla treatment in the experimental animals after saline or 5-FU injection in all groups in the study.

Groups	Bcl-2 immune expression(Mean ±SD)			
	Day 8	P-value	Day 12	P-value
Saline/water	1.184±0.005	0.062	1.199±0.015	0.012
Saline/ Matricaria chamomilla	1.172±0.008		0.808±0.022	
Saline/water	1.184±0.005	0.012	1.199±0.015	0.012
5-FU/water	0.112±0.016		0.738±0.025	
Saline/water	1.184±0.005	0.012	1.199±0.015	0.012
5-FU/ Matricaria chamomilla	0.722±0.013		0.118±0.014	
Saline/ Matricaria chamomilla	1.172±0.008	0.012	0.808±0.022	0.012
5-FU/water	0.112±0.016		0.738±0.025	
Saline/ Matricaria chamomilla	1.172±0.008	0.012	0.808±0.022	0.012
5-FU/ Matricaria chamomilla	0.722±0.013		0.118±0.014	
5-FU/water	0.112±0.016	0.012	0.738±0.025	0.012
5-FU/ Matricaria chamomilla	0.722±0.013		0.118±0.014	