

## Effect of Chamomile Extract on the Tongue of Chemotherapy Treated Albino Rats (Histopathological and Immunohistochemical Study)

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### Abstract

**Background and objectives:** 5-Fluorouracil (5-FU) is a commonly used drug for the treatment of malignant cancers. The control of oral mucositis result from 5-FU use is becoming increasingly more important, and effective intervention is considered a high priority in cancer patient care. The aim of this study was to investigate the effect of chamomile extract on the pathogenesis of 5-FU induced tongue mucositis in Albino rat.

**Materials and methods:** In current study forty female Albino rats, weighing 220-280 g were used. For the induction of mucositis, 60 mg/kg of 5-FU was administered intraperitoneally to each animal in the study group on day 0, and 40 mg/kg was administered on day 2. The control animals were intraperitoneally injected by normal saline in the same manner and dose like 5-FU on day 0 and 2. Then the rats in each group were randomly divided into two groups: Distilled water treated group and chamomile extract treated group (10 animals each).

A volume of distilled water equal to chamomile extract was given by intragastric gavage tube, while the other group was gavaged with chamomile extract at a dose of (100 mg/ kg) two times daily. The treatment with distilled water or the chamomile extract was initiated on day 5 and the experiment continues for twelve days. The animals were sacrificed on day 8 and 12 (five animals each). In each experiment, the middle third of the tongue was removed for histopathological and immunohistochemical analysis using Ki-67 and Bcl-2 immunolabeling.

**Results:** Chamomile can protect the tongue from fluorouracil-induced cytotoxicity and attenuate or decrease the associated injury. The chamomile in 5-FU+chamomile group causes significant increase in Ki-67 and Bcl-2 immunoexpression in comparison with 5-FU+water group at day eight. But longer duration of taking chamomile can cause cytotoxic and damaging effect to the tongue mucosa.

**Conclusion:** Chamomile can protect the tongue mucosa from fluorouracil-induced mucositis. It attenuate the associated injury if it taken for short duration, but the reverse was occurred if it taken for longer period.

**Keywords:** Mucositis; Tongue; Chamomile; 5-FU

### Introduction

In rats, the tongue mucosa consists of an outermost keratinized stratified squamous epithelium beneath which is a dense network of connective tissue called the lamina propria. The dorsal mucosa contain different shapes and types of papillae- filiform, fungiform papillae, these papillae are mostly found in the anterior region of the tongue, and circumvallate papillae, they are few and seen in the posterior region of the tongue. The muscular core consists of a mass of skeletal muscle of longitudinal, transverse and oblique fibers [1].

In addition to moving food about in the mouth, the tongue performs sensory and secretory functions: It is equipped with chemosensitive taste buds that test food quality, and exhibit significant morphological variations that appear to represent adaptation to the current environmental conditions of each respective habitat [2].

Mucositis is the inflammation of the mucous membrane coating the digestive tract. When it involves the mucous membrane of the oral and

oropharyngeal regions, it is termed as oral mucositis [3]. Oral mucositis remains one of the most common and troubling side effects of anti neoplastic radiation and drug therapy [4]. Virtually, every patient with an oral cancer who receives chemoradiation can expect to develop the confluent painful and deep mucosal ulcerations that characterize the condition [5]. It is generally accepted that oral mucositis results from the direct inhibitory effects of chemoradiotherapy on DNA replication and mucosal cell proliferation, resulting in a reduction in the renewal capabilities of the basal epithelium. It is characterized by the atrophy and ulceration of squamous epithelial cells, vascular tissue damage and infiltration of inflammatory lymphocytes to the basement region [6,7]. This injury occurs as a consequence of chemotherapy and radiotherapy, which are targeted to eliminate rapidly dividing cancer cells [8]. The high rate of cellular replication makes the oral mucosa particularly susceptible to this cytotoxicity [7].

5-Fluorouracil (5-FU) is an anti-metabolite drug work by inhibiting essential biosynthetic processes, or by being incorporated into macromolecules, such as DNA and RNA, and inhibiting their normal

function. The mechanism of its cytotoxicity has been ascribed to the misincorporation of fluoronucleotides into RNA and DNA and to the inhibition of the nucleotide synthetic enzyme thymidylate synthase, an important enzyme in nucleotide metabolism [9]. 5-FU is widely indicated for treatment of malignant tumors, particularly of the breast, colon or rectum; and in the treatment of gastric, primary hepatic, pancreatic, uterine, ovarian and bladder carcinomas. The use of 5-FU is one of the most common causes of oral mucositis. It is a major clinical problem that has led to the development of a series of clinical trials aimed at studying methods to decrease this problem [10].

Chamomile is an herb that has been in use since ancient times due to its many advantages and properties. Chamomile is said to have antioxidant, anti-inflammatory, anti-bacterial and anti-fungal properties [11]. The phenolic compounds are commonly found in chamomile, have multiple biological effects. Flavonoids are especially common in flowers and leaves. Extracts of chamomile may exert antioxidant effects within human body. The majority of their antioxidant activity is due to flavones, falvenols, isoflavones, flavonoids, anthocyanin, cumarin, tannins acid, and isocatechins [12].

It is a well-known widely used plant for various gastro-intestinal disorders, and commonly used for many human ailments such as hay fever, inflammation, muscle spasms, menstrual disorders, insomnia, ulcers, wounds, rheumatic pain, and hemorrhoids [13,14].

Nowadays, researches are focusing on exploring the pharmacological profile of compounds from natural origin, where promising results aroused. Few interventions are of proven efficacy in reducing the severity or duration of oral mucositis. For this reason, the present study was designed to study the effects of 5-FU on rat's tongue mucosa and evaluate the effectiveness of chamomile extract as a treatment for the induced tongue cytotoxic changes. As variables to evaluate the grade of protection, we used histological and immunohistochemical investigations to clarify its effect on cell proliferation and cell apoptosis.

## Materials and Methods

### Rats and housing

In current study forty female Albino rats, weighing 220-280 g were supplied and cared in the Animal House of College of Medicine, Hawler Medical University, Erbil, Iraq. The animals were kept under a standard laboratory conditions and maintained on a 12 hour light/dark cycle at  $20 \pm 5^\circ\text{C}$ . The animals were kept in standard room conditions and fed with a standard rat chow and allowed to drink water ad libitum. The research project was approved by the Research Ethics Committee at College of Dentistry, Hawler Medical University under protocol.

### Induction of mucositis

For the induction of mucositis, 60 mg/kg of 5-FU (Kocak farma/Turky) was administered intraperitoneally to each animal in the study groups on day 0, and 40 mg/kg was administered on day 2, following the protocol proposed by Sonis et al. [15] and modified by Leitao et al. [16].

### Experimental design

The rats were randomly divided into two groups.

**Control groups (Normal saline groups):** Consist of distilled water treated group and chamomile extract treated group (10 animals each). In the distilled water treated group, a volume of distilled water equal to chamomile extract was given by intragastric gavage tube, while the chamomile extract-treated group was gavaged with chamomile extract (*Matricaria recutita* organic alcoholic extract-United States-Code HS3751002) at a dose of (100 mg/kg) two times daily [17]. The animals were intraperitoneally injected by normal saline in the same manner and dose like 5-FU on day 0 and 2, and the treatment with distilled water or the chamomile extract was initiated on day 5 and the experiment continue for twelve days.

**Study groups (5-FU groups):** After induction of mucositis, the animals in this group were also divided into two groups: the distilled water treated group and chamomile extract treated group (10 animals each) and treated in the same manner like the control group.

**Histopathological analysis:** The animals were sacrificed by over dose of anesthesia on day eight and twelve (five animals each). In each experiment, the middle third of the tongue was removed for histopathological analysis. Samples were then fixed in neutral buffered 10% formalin, processed for H&E and trichrome stains, and for immunohistochemical analysis using Bcl-2 and Ki-67 immunolabeling.

**Immunohistochemical staining and analysis using Ki-67 and Bcl-2 immunolabelling:** Cell proliferation was assessed by Ki-67 immunohistochemistry, while the anti apoptosis was assessed by Bcl-2 immunostaining and were performed using monoclonal Mouse Anti-Human Ki-67 Antigen, Clone MIB-1, Code No. M 7240 staining system, and a monoclonal Mouse Anti-Human Bcl-2 Oncoprotein Clone 124 Code No 1587 ready to use N-series primary antibody, for use with Dako EnVision™, EnVision™ double staining and LASAB™ 2 systems. The staining procedure sections of the instructions included with each detection system were followed. Positive and negative controls were run simultaneously with biopsy specimen.

Positive cells expressing Ki-67 were identified by brown nuclei, while Bcl-2 was demonstrated brown cytoplasmic staining. To ensure the objectivity of the analysis, the evaluation was carried out by two independent observers. Five sections were randomly chosen for each animal. Approximately 1000 cells from cell population were counted by two observers at a magnification of 400X and the percentages of Ki-67 and Bcl-2 positive cells were calculated. All microscopic analyses were performed using a light microscope (Olympus, Tokyo, Japan).

The level of Ki-67 and Bcl-2 expression was evaluated according to the scoring system of Seleit et al. [18]. The application of this system gives a score ranging from 0 to 3 for both degree of positivity: Percentage of positively stained cells ((absent: <1%), (mild: 1 - 10%), (moderate: 10 - 50%), (strong: >50%)).

**Statistical analysis:** Statistical analysis was performed using Bonferroni Post Hoc 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.

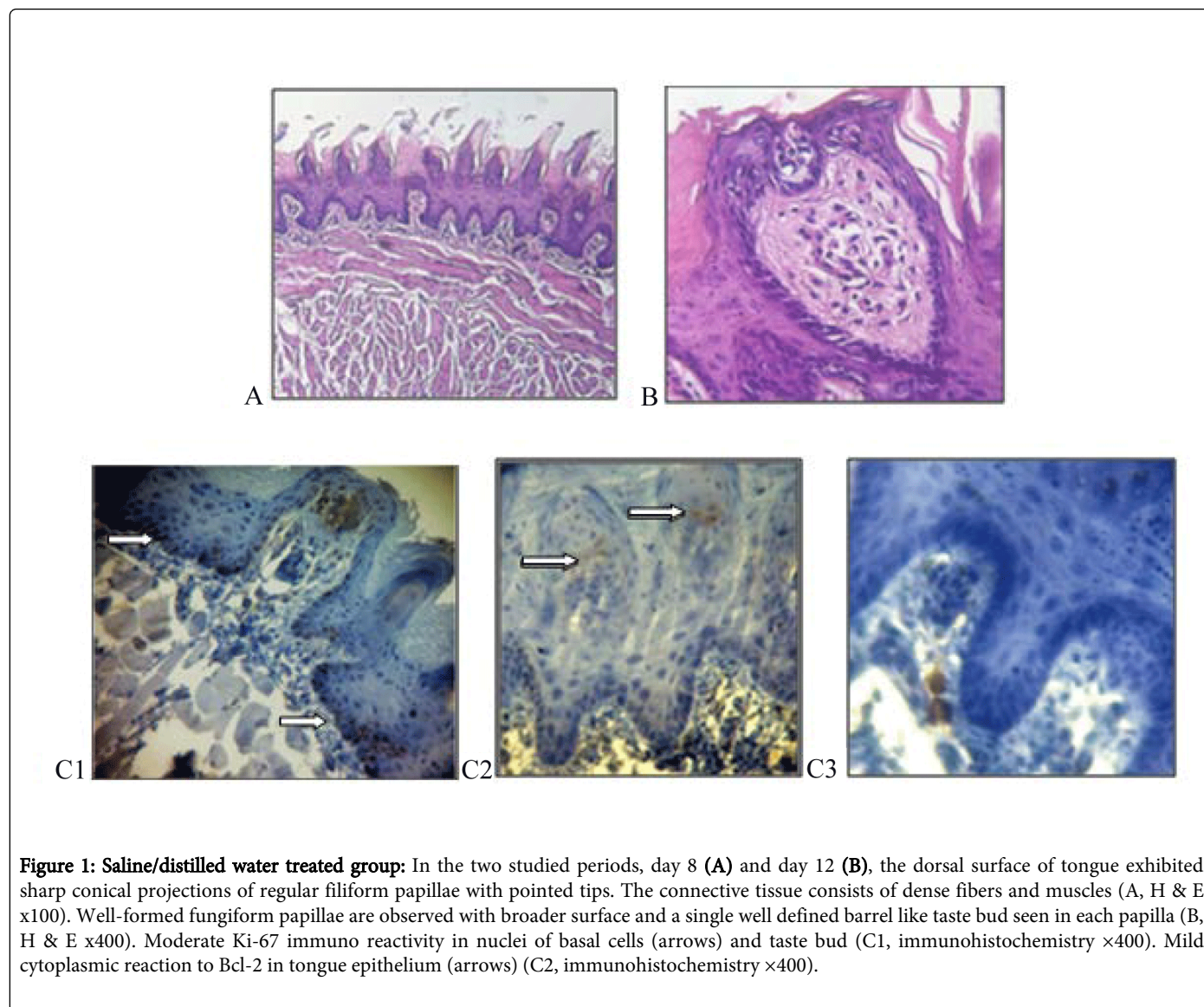
## Results

### Histological findings

**Saline/distilled water treated group:** The dorsal surface of tongue in the studied periods (day 8 and 12) exhibited sharp conical projections

of filiform papillae with pointed tips. The papillae are regular in distribution, height, shape and orientation with smooth keratinized epithelial covering. The connective tissue papillae are normal. Skeletal muscle fibers running in different directions are seen underneath the papillae (Figure 1A). Well-formed fungiform papilla are observed in

between filiform papillae with broader surface and wide vascular connective tissue and covered by normal epithelium. A single well defined taste bud with peripheral arranged cells was seen at the summit of each papilla (Figure 1B).



**Figure 1: Saline/distilled water treated group:** In the two studied periods, day 8 (A) and day 12 (B), the dorsal surface of tongue exhibited sharp conical projections of regular filiform papillae with pointed tips. The connective tissue consists of dense fibers and muscles (A, H & E x100). Well-formed fungiform papillae are observed with broader surface and a single well defined barrel like taste bud seen in each papilla (B, H & E x400). Moderate Ki-67 immuno reactivity in nuclei of basal cells (arrows) and taste bud (C1, immunohistochemistry x400). Mild cytoplasmic reaction to Bcl-2 in tongue epithelium (arrows) (C2, immunohistochemistry x400).

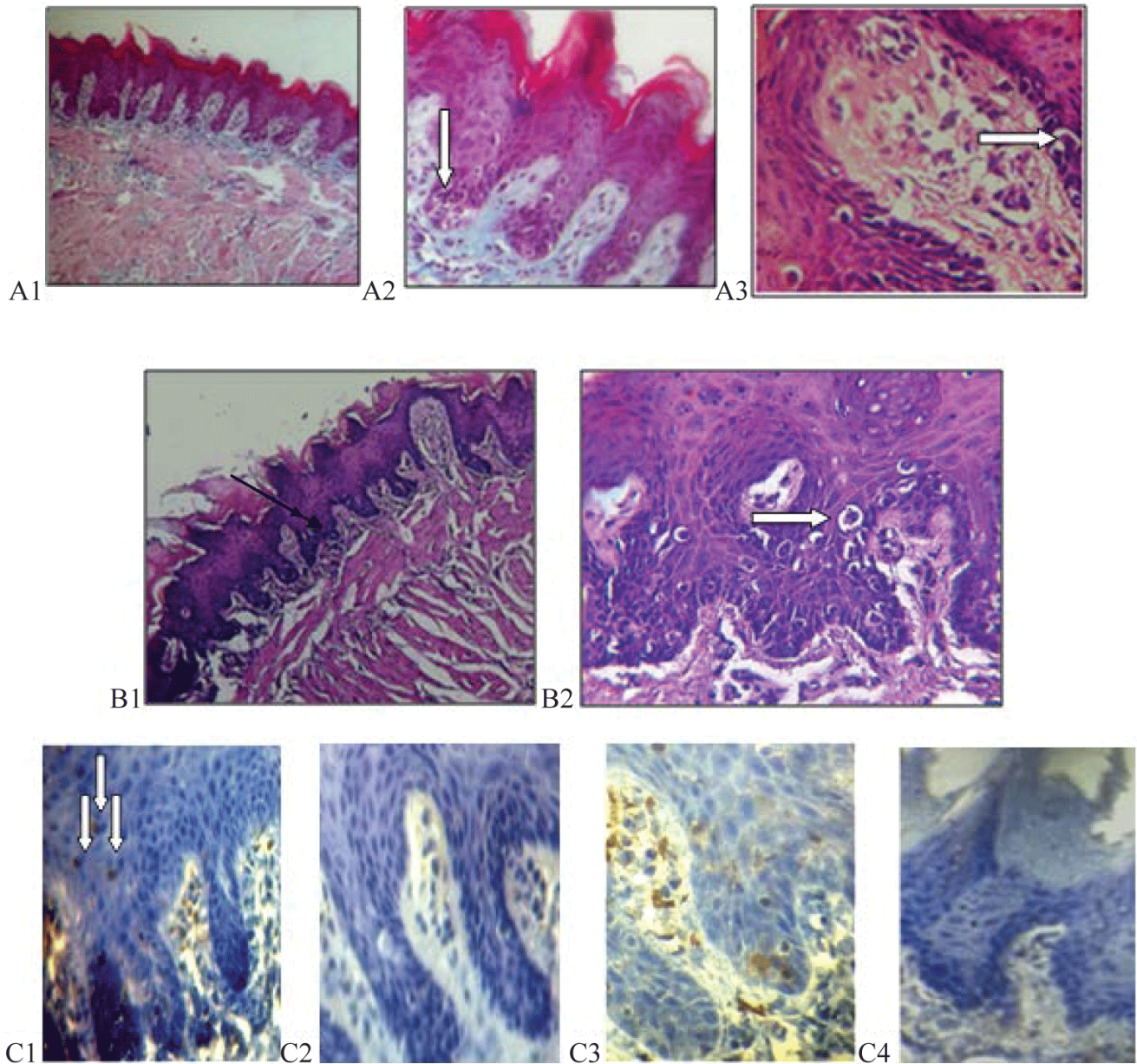
**Saline/chamomile extract treated group:** At day 8, some filiform papillae of the tongue showed loss of the normal appearance, they showed flattening of the tips with loss of their characteristic conical shape (Figure 2A1). Vacuolar degeneration of the basal cell layer was seen in some areas (Figures 2A2 and 2A3).

At day 12, more changes seen in the shape of papillae and rete ridges, with acanthosis and hyperkeratosis of the covering epithelium and separation of the keratin layer from underlying epithelial cells (Figure 2B1). Vacuolated cells are increased in number and appear in basal and supra basal cells layer (Figure 2B2).

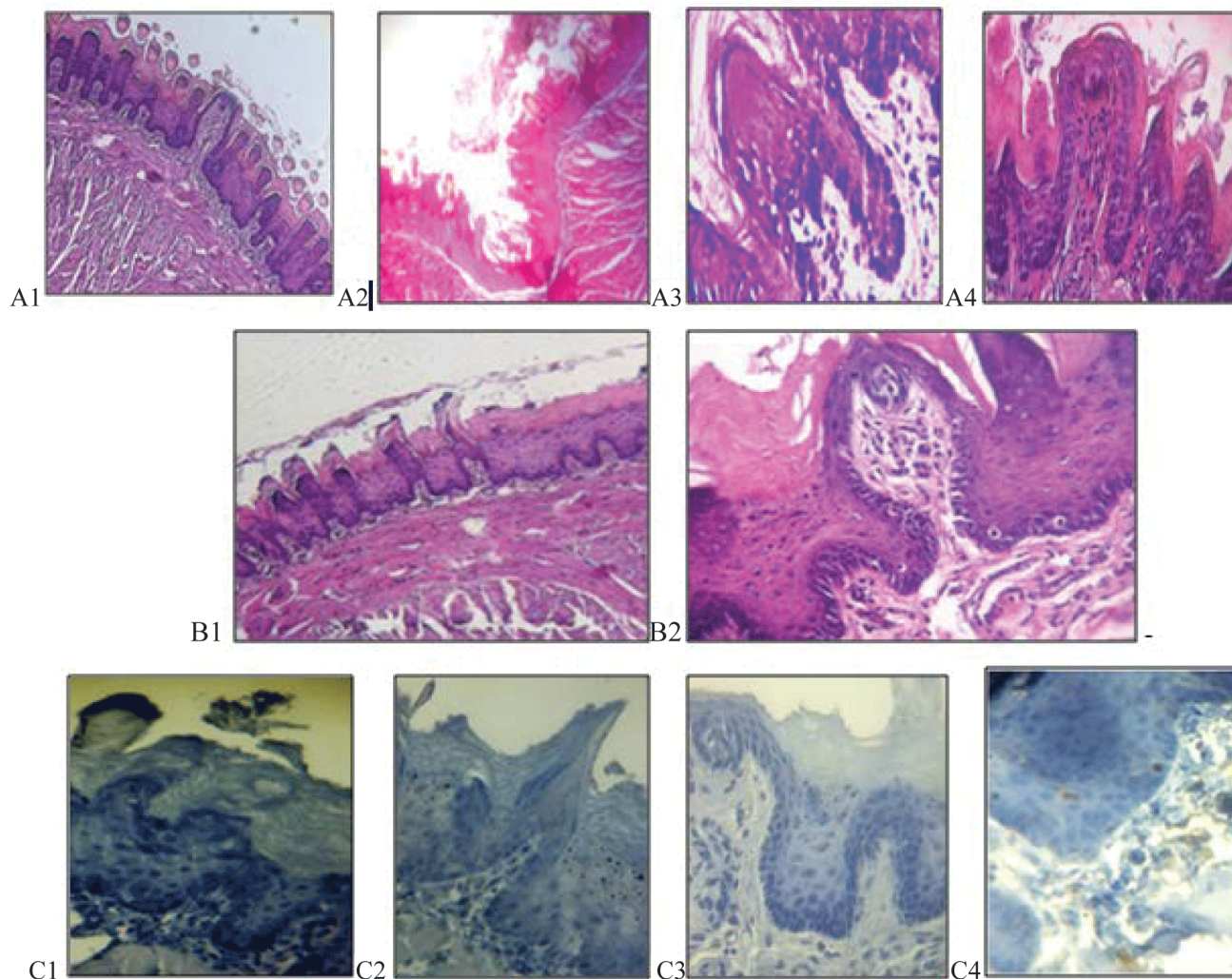
**5-FU–distilled water treated group:** At day 8, the dorsal surface of tongue revealed loss of the normal appearance of filiform papillae, and most of them showed flattening of the tips with loss of their characteristic conical shape, with few shallow epithelial ridges (Figure

3A1). The keratin layer shows separation from underlying epithelial cells and vacuolation of some epithelial cells were also seen (Figure 3A2). Fungiform papillae showed atrophic changes in the shape and orientation of the taste bud cells, the gustatory pore appear ill defined with infiltration of inflammatory cells in the connective tissue (Figure 3A3).

At day 12, some of the filiform papillae exhibited sharp conical projections and with pointed tips, but at the same time, some filiform papillae were missing. Hyperkeratosis with few shallow epithelial ridges was also seen (Figure 3B1). The shape of fungiform papillae appear similar to normal but with some cellular vacuolation. The vacuolation of the epithelial cells was decreased, and mild inflammatory cells infiltration in connective tissue was still present (Figure 3B2).



**Figure 2: Saline/chamomile extract treated group:** At day 8, the dorsal surface of tongue revealed atrophy of filiform papillae, with loss of the normal appearance and most of them showed flattening of the tips with loss of their characteristic conical shape (A1, Trichrome, x100). Vacuolar degeneration was mostly seen associated with the basal epithelial layer of filiform and fungiform papillae, arrows (A2, Trichromex400; A3, H & E x400). At day 12, more changes seen in the shape of papillae and rete ridges with separation of keratin from the surface of papillae, the deeply stained nuclei, and the vacuolated cells are increased in number and seen in basal and supra basal layer, arrows (B1, H & E 100; B2, H & E x400). Mild (arrows) and negative Ki-67 immune reactivity in nuclei of epithelial cells at day 8 and 12 respectively (C1, immunohistochemistry x400; C2 immunohistochemistry x400). Mild and negative cytoplasmic reaction to Bcl-2 in tongue epithelium at day 8 (C3, immunohistochemistry x400) and 12 (C4, immunohistochemistry x400) respectively.

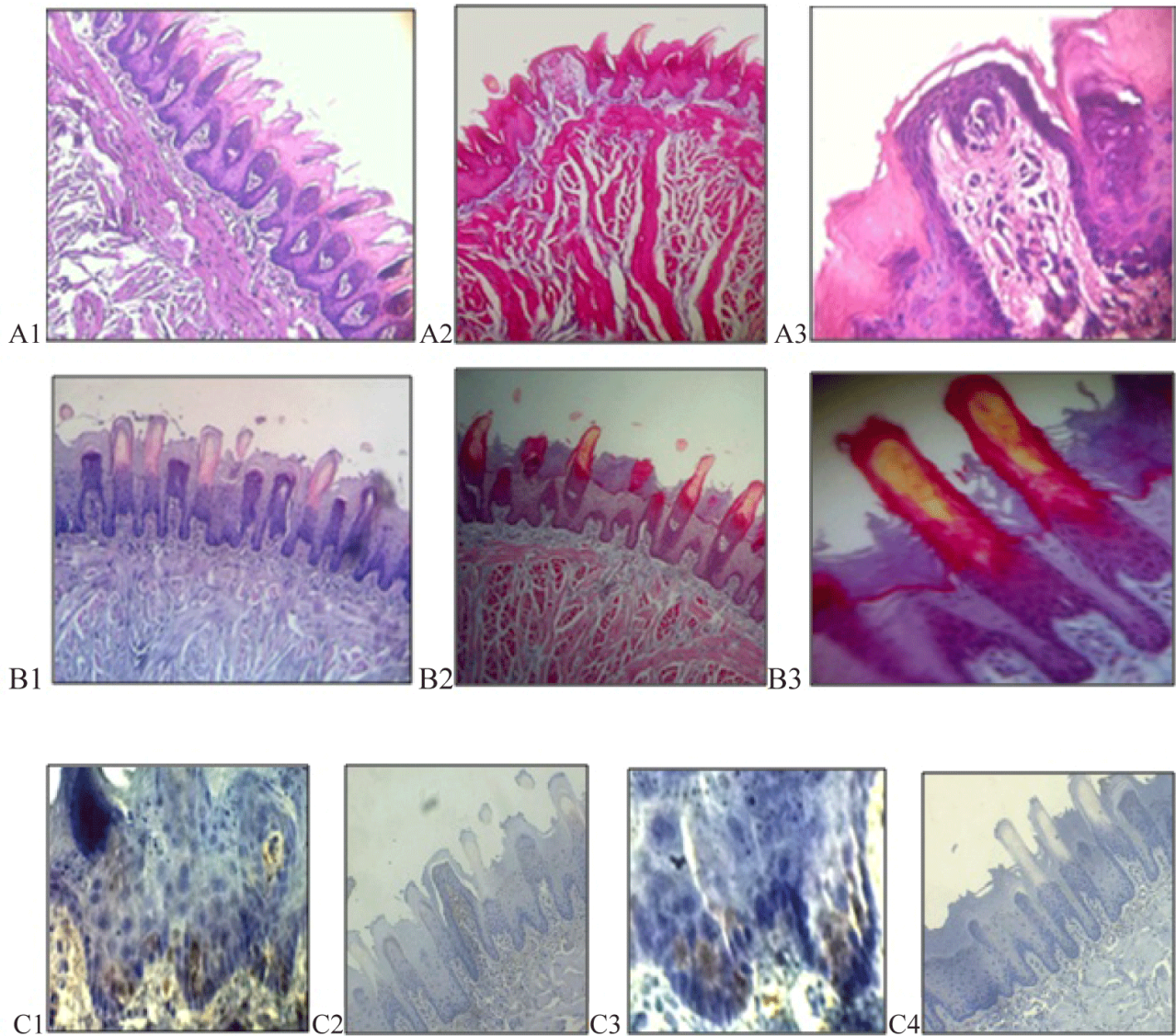


**Figure 3: 5-FU-distilled water treated group:** At day 8, the dorsal surface of tongue revealed atrophy and loss of the normal appearance of filiform papillae. Filiform papillae showed flattening of the tips with few shallow epithelial ridges (A1, H & E x100). Vacuolar degeneration of basal and supra basal epithelial layer were seen (A2, H & E x400). Atrophic changes in the shape of fungiform papillae and orientation of the taste bud cells were also seen, the gustatory pore appear ill defined with infiltration of inflammatory cells in the connective tissue (A3, H & E x400). At day 12, some filiform papillae were still missing but others restore its conical shape with hyperkeratosis with few shallow epithelial ridges, the shape of fungiform papillae appear similar to normal but with some cellular vacuolation (B1, H & E x10; B2, H & E x400). Negative immune reactivity to Ki-67 (C1,C2, immunohistochemistry x400) and Bcl-2 (C3,C4,immunohistochemistry x400) in tongue epithelium at day 8 and 12 respectively.

**5-FU/chamomile extract treated group:** At day 8, the dorsal surface of tongue showing almost restoration of normal histology of epithelium, and connective tissue. The filiform papillae appear with pointed tips, and nearly normal histology of fungiform papilla was seen. Epithelial cells vacuolation and inflammatory cells infiltration in connective tissue papillae was not seen (Figures 4A1-4A3).

At day 12, the filiform papillae showing changes in its shape and covered by a thick keratin covering giving the appearance of a birthday candle specially when stained by trichrome stain (Figures 4B1-4B3).

**Immunohistochemical findings (Table 1):** At day 8, the number of cells staining positive for Ki-67 in the 5-FU/chamomile group was significantly different in relation with the other three groups ( $p < 0.05$ ). 5-FU in the 5-FU/water group caused significant decrease in the number of Ki-67 positive cells in relation with the other three groups ( $p < 0.05$ ). Chamomile in the chamomile/water group cause significant decrease in the number of Ki-67 positive cells in relation with the saline/water group ( $p < 0.05$ ).



**Figure 4: 5- FU/Chamomile extract treated group:** At day 8, the dorsal surface of tongue showing almost restoration of normal histology of epithelium, and connective tissue. The filiform papillae appear with pointed tips (A1, H & E x100; A2, Trichrome x100), and nearly normal histology of fungiform papilla was also seen (A3, H & E x400). At day 12, the filiform papillae showing changes in its shape with thick keratin covering (B1, H&E x100) and appear like the birth day candle (B2, Trichrome x100; B3, Trichrome x400). Mild and negative Ki-67 immuno reactivity in nuclei of epithelial cells at day 8 and 12 respectively (C1, immunohistochemistry x400; C2 immunohistochemistry x100). Mild and negative cytoplasmic reaction to Bcl-2 in tongue epithelium at day 8 (C3, immunohistochemistry x400) and 12(C4,immunohistochemistry x100) respectively.

At day 12, the number of cells staining positive for Ki-67 in the 5-FU/chamomile group was significantly different in relation with the saline/water group ( $p < 0.05$ ), but this difference was non-significant in relation with the saline/chamomile and 5-FU/chamomile groups ( $p > 0.05$ ). Chamomile in the chamomile/water group cause significant decrease in positive Ki-67 immune expression in relation with the saline/water group ( $p < 0.05$ ). 5-FU in the 5-FU/water group caused significant differences in the number of Ki-67 positive cells in relation with the saline/water group

( $p < 0.05$ ), but this difference was non-significant in relation with the saline/chamomile and 5-FU/chamomile groups ( $p > 0.05$ ). Chamomile in the chamomile/water group cause significant decrease in positive Ki-67 immune expression in relation with the saline/water group ( $p < 0.05$ ).

At day 8, the number of cells staining positive for Bcl-2 in the 5-FU/chamomile group was significantly different in relation with the other three groups ( $p < 0.05$ ). 5-FU in the 5-FU/water group caused significant decrease in the number of Bcl-2 positive cells in relation with the other three groups ( $p < 0.05$ ). Chamomile in the chamomile/water group cause significant decrease in the number of Bcl-2 positive cells in relation with the saline/water group ( $p < 0.05$ ).

At day 12, the number of cells staining positive for Bcl-2 in the 5-FU/chamomile group was significantly different in relation with the

saline/water group ( $p < 0.05$ ), but this difference was non-significant in relation with the saline/chamomile and 5-FU/water groups ( $p > 0.05$ ). 5-FU in the 5-FU/water group caused significant differences in the number of Bcl-2 positive cells in relation with the saline/water group ( $p < 0.05$ ), but this difference was non-significant in relation with the saline/chamomile and 5-FU/chamomile groups ( $p > 0.05$ ). Chamomile in the chamomile/water group cause significant decrease in positive Bcl-2 immune expression in relation with the saline/water group ( $p < 0.05$ ).

Mean & SD		Saline/water (10 rats)	Saline/chamomile (10 rats)	5-FU/water (10 rats)	5-FU/chamomile (10 rats)
<b>Ki-67 antigen-positive cell rate</b>	Day 8	19.20 ± 0.45	1.01 ± 0.54	0.01 ± 0.00	2.24 ± 0.195
	Day 12	18.2 ± 0.67	0.11 ± 0.116	0.6 ± 0.154	0.14 ± 0.6
<b>Bcl-2 antigen-positive cell rate</b>	Day 8	1.1 ± 0.122	1.87 ± 0.17	0.21 ± 0.05	3.2 ± 0.51
	Day 12	1.36 ± 0.25	0.11 ± 0.06	0.21 ± 0.14	0.16 ± 0.08

**Table 1:** Immunohistochemical results of the tongue following water or chamomile extract treatment in female Albino rats post saline or 5-FU injection in the studied periods of all groups in the study.

## Discussion

The filiform papillae are widely distributed on the dorsal surface of the tongue and they undergo lengthening, loss and atrophic changes faster and earlier than other papillae. It was stated that the filiform papillae are of high metabolic activity, so any enzymatic disturbance, vascular insufficiency, nutritional deficiency, or drug toxicity may result in atrophy of these papillae. The filiform papillae were seen as a mirror that reflects the general health status [1].

The atrophy and changes of the lingual papillae in the 5-FU-water group in the present study may be due to the mechanism of inhibition of epithelial reproduction through the cytotoxic effect of 5-FU. 5-FU administration was accompanied by a significant reduction in Ki-67 and Bcl-2 positive cells. It seems that inhibition of DNA synthesis, DNA damage and the production of reactive oxygen species by chemotherapy impair the metabolism in progenitor cells and cause inhibition of mitosis and increase of apoptosis [7]. Clinically, the direct mucotoxic effects of chemotherapy on the oral mucosa begin shortly after therapy has begun, and peak in severity approximately day seven or day ten of therapy, with eventual resolution occurring within two weeks [19].

To our knowledge no investigation was done using chamomile extracts to fight the cytotoxic effect of 5-FU on rat tongue mucosa. The combined treatment of 5-FU and chamomile extract ameliorated the histological changes in the dorsal surface of the tongue induced by 5-FU alone. The Ki-67 and Bcl-2 immunostaining was significantly lowest in the 5 FU- water group than the 5-FU- chamomile group at day eight. Thereby indicating that chamomile increased epithelial proliferation and anti apoptosis. It is probable that this protective effect of chamomile may prove useful in clinical practice to prevent or decrease oral mucositis resulting from 5-FU treatment. This may be due to its antioxidant and cytoprotective properties [20], or its anti-inflammatory activity by reducing IL-6 and TNF- $\alpha$  production [21].

The chamomile plant contains many different substances with antibacterial and antifungal properties, as chamazulene, alpha bisabolol, bisabol oxides, spiroethers, and flavonides. Flavonoids act as antioxidants, enhance the effects of vitamin C, and strengthen

connective tissue around capillaries [22]. Curra et al. [23] study the effect of topical chamomile (mouthwash) on immunohistochemical levels of IL-1 $\beta$  and TNF- $\alpha$  in 5-FU induced oral mucositis in hamsters. They found the group treated with chamomile had lower scores for both pro-inflammatory cytokines. For comparison, no other studies were found use oral chamomile extract for treatment of oral mucositis.

Bhaskaran et al. [24] demonstrate the cytoprotective effects of chamomile on hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) induced cellular damage in macrophage cells. Pretreatment of cells with chamomile markedly attenuated H<sub>2</sub>O<sub>2</sub> induced cell viability loss in a dose dependent manner. The mechanisms by which chamomile protected macrophages from oxidative stress was through the induction of several antioxidant enzymes including NAD (P)H: Quinone oxidoreductase, superoxide dismutase, and catalase and increase nuclear accumulation of the transcription factor Nrf2 and its binding to antioxidant response elements. Furthermore, chamomile dose dependently reduced H<sub>2</sub>O<sub>2</sub> mediated increase in the intracellular levels of reactive oxygen species.

At the same time, chamomile exerts harmful effect to the tongue epithelium. It decreases the Ki-67 and Bcl-2 immune expression in relation with the saline-water group, in addition to the severe microscopical changes seen at day twelve. The harmful effect of chamomile may come from its constituents, like bisabolol, volatile oils, anthemic and tannic acid, and chamazulene. Cavalieri et al. [25] found that  $\alpha$ -bisabolol is a small oily sesquiterpene alcohol, it is a pro-apoptotic agent and enhance apoptosis. This study represents a reported demonstration of the anticancer effects of chamomile. Further investigations are warranted in evaluating the potential usefulness of the systemic use of chamomile extract at a concentration of (200 mg/kg /day) in the management of cancer patients.

## Conclusion

Fluorouracil chemotherapy has a deleterious effect on the rat tongue mucosa leading to marked microscopic changes. Our results, for the first time, demonstrate that systemic use of chamomile has protective effects against fluorouracil-induced cytotoxicity and might

be beneficial to provide defense against oral mucositis by significant increase in the Ki-67 and Bcl-2 immunoreactivity, it attenuates or decrease the associated injury if it taken for short period, therefore can be used as a protective natural product to prevent oral mucositis in individuals undergoing cancer therapy. Longer duration of taking chamomile can cause cytotoxic and damaging effect to the rat tongue mucosa.

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