

## HISTOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSIS OF POSSIBLE AMELIORATIVE EFFECT OF ASHWAGANDHA ON METHOTREXATE-INDUCED ORAL MUCOSITIS IN ALBINO RATS

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### ABSTRACT

**Introduction:** Oral mucositis is a common side effect of methotrexate chemotherapy; it represents a challenge for a successful cancer treatment, as it may lead to discontinuity of the chemotherapy. Ashwagandha is one of the most familiar Ayurveda herbs in India. Recently, it has gained attention due to its favorable biological effects.

**Aim:** Investigation of the possible protective effect of Ashwagandha roots extract on methotrexate-induced oral mucositis in albino rats.

**Materials and methods:** 40 rats were divided equally into 4 groups: (C): received distilled water, (ASH): received 300mg/kg ashwagandha root extract for 8 days, (MTX): received single dose of 60 mg/kg intraperitoneal injection of methotrexate on day 4, and (MTX/ASH): received 300mg/kg ashwagandha root extract daily for 8 days interrupted with 60 mg/kg i.p injection of methotrexate on day 4.

**Results:** Histopathological examination revealed normal appearance of tongue and buccal mucosa in C and ASH groups. In MTX group there was atrophy of tongue papilla, decrease of epithelial thickness, flattening of rete pegs, nuclear pyknosis, nuclear hyperchromatism, karyorrhexis, disruption of basement membrane, inflammatory cells infiltrate, blood vessels congestion, muscle atrophy in both mucosae. In MTX/ASH group, both mucosae maintained normal appearance. Immunohistochemical results revealed significant decrease of Ki67 staining in MTX group compared to C and MTX/ASH groups in both mucosae, COX2 staining revealed significant increase in MTX group compared to C and MTX/ASH groups.

**Conclusion:** Prophylactic administration of Ashwagandha root extract ameliorated the oral mucositis induced by methotrexate chemotherapy, therefore it could be a good adjuvant therapy during chemotherapeutic treatment.

**KEYWORDS:** Oral mucositis, Ashwagandha, *Withania somnifera*, Methotrexate, Chemotherapy.

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## INTRODUCTION

Oral mucositis is one of the most common and frequent adverse effects of cancer chemotherapy. Typically accompanied by symptoms as pain, discomfort, food intolerability which in consequence affects the nutritional status and patient's ability to tolerate chemotherapy. This in turn impact cancer treatment as well patient's quality of life <sup>(1)</sup>.

Methotrexate (MTX) is one of most widely used chemotherapeutic agents. It is an antimetabolite and immune modulating drug. When administered at high doses, it establishes a chemotherapeutic effect and used for treatment of cancer. On the other hand, when administered in low doses to treat rheumatic diseases <sup>(2)</sup>.

MTX is a folic acid antagonist, a crucial co-factor for DNA synthesis and cell turnover, and this is the primary reason of its chemotherapeutic effect, and oral mucositis as a side effect <sup>(3)</sup>.

*Withania somnifera*, commonly known as Ashwagandha or Indian ginseng, is one of the most familiar Ayurvedic herbs in India over 3000 years. It has been known to have many health benefits owing to its favorable biological effects<sup>(4)</sup>. It has been reported to have anti-inflammatory<sup>(5)</sup>, antioxidant<sup>(6)</sup>, immunomodulatory<sup>(7)</sup>, anticancer<sup>(8)</sup>, antidiabetic<sup>(9)</sup>, cardioprotective<sup>(10)</sup>, and anti-stress effects<sup>(11)</sup>. These beneficial effects have been credited to its withanolide content, which is the pharmacologically active steroidal lactones present in it.

In Ayurveda, the roots of Ashwagandha have been used topically to treat ulcers, heal skin sores, and reduce swelling <sup>(12)</sup>. In research, it was proven to be useful in the prevention of induced ulcers, suggesting that this effect may be attributed to its antioxidant and anti-inflammatory effects <sup>(13)</sup>.

In the light of the mentioned beneficial effects of Ashwagandha, the present study was conducted to investigate its potential protective effect on methotrexate-induced oral mucositis.

## MATERIALS AND METHODS

### Experimental animals:

40 adult male albino rats weighing ~200g were used in the present study, animals of each group were dwelled in separate stainless steel ventilated cages in the animal house at The Faculty of Medicine, Minia University. Rats were housed and handled properly, fed a stock diet, and kept in stable, appropriate environments. The experiment was conducted in accordance with the guidelines of animal care ethical committee, Faculty of Dentistry, Minia University (Ethics approval no. 875/2023)

### Drugs and chemicals:

Ashwagandha was purchased from ZETA PHARMA® under trade name (Dozova Ashwagandha KSM-66) in the form of capsules, each capsule contains 300 mg Ashwagandha root extract with 1.25% withanolides.

Methotrexate vials 50 mg/2ml under trade name of (Unitrexate), were purchased from HIKMA pharmaceuticals, Cairo, Egypt.

### Preparation of ashwagandha root extract solution

The contents of each capsule were dissolved in 10 ml of distilled water and then Shaken vigorously to assure their complete dissolution <sup>(14)</sup>.

### Induction of mucositis:

For induction of oral mucositis 60 mg/kg b.w methotrexate were injected intraperitoneally to rats <sup>(15,16)</sup>.

As ashwagandha to be tested for its protective potential, it was administered 4 days before and after methotrexate injection <sup>(15)</sup>.

### Experimental design:

Animals were randomly and equally distributed into 4 groups as follow:

- **C (Control):** rats were administered 0.6 ml of distilled water by oral gavage for 8 successive

days interrupted with injection of 0.5 ml saline on day 4 of experiment.

- **ASH:** rats were administered 300 mg/kg b.w.<sup>(17)</sup> of Ashwagandha root extract by oral gavage for 8 successive days interrupted with injection of 0.5 ml saline on day 4 of experiment.
- **MTX:** rats were administered 0.6 ml distilled water by oral gavage for 8 successive days interrupted with injection of 60 mg/kg b.w i.p methotrexate on day 4 of experiment.
- **MTX/ASH:** rats were administered 300 mg/kg b.w Ashwagandha root extract by oral gavage for 8 successive days interrupted with injection of 60 mg/kg b.w i.p methotrexate on day 4 of experiment.

At the end of 8<sup>th</sup> day, rats were sacrificed by anesthesia overdose (ketamine 150 mg/kg b.w and xylazine 15 mg/kg b.w)<sup>(18)</sup>, then the tongue and cheek mucosa were dissected.

#### **Histological procedures:**

Specimens were immediately fixed in 10% formol saline for 24 hrs, followed by water rinse then dehydrated in ascending alcohol concentrations ending in 100% absolute alcohol. The specimens were then cleared by 2 xylene exchanges, 10 minutes for each. After that, tissues were infiltrated with melted soft paraffin wax for replacement of all xylol. Then, embedded in hard paraffin wax and 4 µm sections were obtained and mounted on pre-cleaned glass slides and finally stained with hematoxylin and eosin (H&E) stain<sup>(19)</sup>.

#### **Immunohistochemical staining steps:**

After preparing 4 µm - thick sections, immunohistochemical staining was performed using the streptavidin-biotin-peroxidase technique. First, the sections were deparaffinized, rehydrated, and placed for 10 minutes in a 3% H<sub>2</sub>O<sub>2</sub> solution at room temperature, then immersed in an antigen

retrieval solution. Next, the sections were incubated in 10% normal goat serum in phosphate buffered saline solution (PBS) to prevent non-specific protein binding, followed by the addition of ki67 primary antibody (Dako, M7240, 1:100)<sup>(20)</sup> or COX2 primary antibody (cat, RB-9072-P0)<sup>(21)</sup> Lab Vision, USA and incubated in a moist chamber overnight at 4°C. Further, drops of streptavidin peroxidase were added for 30 minutes then rinsed in PBS. Freshly prepared Diaminobenzidine (DAB) solution was used as a chromogen; it was added to the sections for 5-10 minutes, followed by washing in PBS for 3 changes 2 minutes each, and finally counterstained with Mayer hematoxylin. Cover slips were then mounted using Canada balsam<sup>(22)</sup>.

#### **Image analysis procedures:**

Ki67 and COX2 immunoreactivity were measured using Image J 22 software. Area fraction was measured in a standard measuring frame per 5 different fields in each group using magnification (x200) by light microscopy transferred to the monitored screen.

Positive cells for Ki-67 were identified by brown nuclei, while COX2 expression was identified by brown nuclear and cytoplasmic staining. Brown staining, regardless of its intensity, was chosen for measurement, and these areas were masked by a red binary color and then measured by the software.

#### **Microscopic examination of stained sections:**

The anterior 2/3 of tongue dorsal surface and the buccal mucosa were examined and photomicrographed using the biological light microscope LEICA, model DM LB equipped with a digital camera (LEICA Microsystems DFC295, Germany), Faculty of Dentistry, Minia University.

#### **Statistical analysis:**

Data entry and statistical analysis were performed using the Statistical program SPSS for Windows version 20. Graphics were created using Microsoft

Office Excel 2022. Quantitative data were displayed as mean and standard deviation. The Mann Whitney U test was chosen to compare differences between 2 independent groups when the dependent variable is not normally distributed. 0.05 or less represented significant difference.

## RESULTS

### Hematoxylin and Eosin Staining Results

#### *Control group:*

Histopathological examination of the anterior 2\3 of tongue dorsal surface showed a regular cover of cone-shaped filiform papillae with thick keratin covering them and a few scattered mushroom shaped fungiform papillae with one taste bud in their superior surface and covered with a thin keratin layer. Epithelial rete pegs were long and regular. Lamina propria was formed of collagen fibers and scattered fibroblasts. Skeletal muscle bundles were found to be comprising the main bulk of the tongue (Figure 1A).

On the other hand, buccal mucosa showed normal histological appearance of the stratified squamous keratinized epithelium with deep and broad rete pegs. The underlying lamina propria appeared consisting of collagen fibers and dispersed fibroblasts. Deep to lamina propria, there was skeletal muscles formed of interlacing bundles (Figure 1B).

#### *ASH (Ashwagandha-treated) group:*

Lingual mucosa of oral tongue dorsal surface appeared as in control group with a regular cover of tongue papillae, normal appearance of lamina propria and muscle layer (Figure 2A).

The buccal mucosa also seemed as in control group; it showed normal histological appearance and thickness of surface epithelium with deep epithelial rete pegs and regular keratin layer, the underlying lamina propria appeared also normal (Figure 2B).

#### **MTX (Methotrexate- treated) group:**

Dorsal surface of the tongue showed a very thin covering epithelium with severely atrophied filiform tongue papillae, slightly atrophied fungiform papillae. Many basal cells showed nuclear hyperchromatism, pyknotic nuclei were also seen in upper epithelial layers, the keratohyalin granules were diminished. Keratin layer appeared variable, in some areas was thin and detached or even lost, other appeared thickened. Rete pegs flattening was evident in most area; however, some short and irregular epithelial rete pegs still existed. Lamina propria showed inflammatory cell infiltrate, spacing and congested blood vessels. Muscle fibers appeared atrophied and widely spaced (Figure 3A&B).

Buccal mucosa also showed thin irregular and atrophied surface epithelium with very thin keratin layer. The junction between epithelium and connective tissue found to be smooth in most area with no or very few rete pegs. Lamina propria showed spacing, inflammatory cells, and their blood vessels appeared dilated with RBCs and inflammatory cells inside their lumen. Muscle layer showed marked atrophy and spacing.

Basal cell layer of the buccal mucosa appeared irregular with some parts showed disrupted basement membrane. Some epithelial cells appeared with vacuolated cytoplasm and pyknotic nuclei, granular cells appeared swollen with diminished keratohyalin granules, some nuclei showed karyorrhexis, other were hyperchromatic. Some fibroblasts appeared also with hyperchromatic nuclei (Figure 4A&B).

#### **MTX\ASH (Methotrexate /Ashwagandha- treated) group:**

In combination group, the lingual mucosa preserved its normal thickness and appearance. It Presented a regular cover of tongue papillae, normal lamina propria and blood vessels. Muscle fibers seemed to be regularly arranged (Figure 5A).

Buccal mucosa appeared as of the control group, its epithelium maintained its thickness, rete pegs were regular with adequate length. Keratin was found to be mostly regular while in some areas it was detached. Lamina propria appeared normal and didn't show blood vessel congestion (Figure 5B).

### **Immunohistochemical Staining Results:**

#### ***Ki67 immunostaining results:***

##### ***Control group:***

Examination of ki67 stained sections of the anterior 2/3 of tongue dorsal surface and the buccal mucosa revealed moderate and intense positive immune staining in basal and para basal layers of surface epithelium. Most connective tissue cells showed weak, moderate, and intense positive immunoreactivity. Many muscle cells had weak, moderate, and intense positive nuclear immunoreactivity (Figure 1 C & 1D).

##### ***ASH (Ashwagandha-treated) group:***

Dorsal surface of tongue displayed moderate nuclear immunoreactivity in basal and parabasal layer of surface epithelium together with some connective tissue and muscle cells (Figure 2 C).

Buccal mucosa-stained sections revealed moderate and intense nuclear immune staining in basal cell layer and moderate staining in para basal layer of surface epithelium. Most connective tissue and muscle cells also showed positive immunostaining varying from weak to intense (Figure 2 D).

##### ***MTX (Methotrexate- treated) group:***

Tongue showed weak, moderate, and intense immunostaining in most but not all cells of basal layer with few stained parabasal cells. Some connective tissue cells and muscle cells showed moderate nuclear immunostaining (Figure 3 C).

In buccal mucosa, basal cell layer showed moderate and intense nuclear immunostaining with little nuclei had moderate staining in para basal layer. Some connective tissue cells and muscle were moderately stained with ki67 (Figure 4 C).

##### ***MTX\ASH (Methotrexate /Ashwagandha- treated) group:***

Tongue in this group showed deeply stained nuclei of all basal cell layer and moderated staining of nuclei in upper epithelial layers. Many connective tissue cells appeared with positively stained nuclei while some of muscle cells showed positive immunostaining (Figure 5 C).

In buccal mucosa, intense nuclear immunostaining in basal and parabasal layer, weak and moderate staining of some nuclei in upper epithelial layers. Most fibroblasts and muscle cells showed positive nuclear staining (Figure 5 D).

#### ***COX 2 immunostaining results:***

##### ***Control group:***

Tongue mucosa appeared almost negative to COX2, cheek mucosa immunostained sections showed very weak staining of some endothelial cells in the lamina propria (Figure 1 E&F).

##### ***ASH (Ashwagandha- treated) group:***

Tongue appeared almost negative with very few connective tissue cells showed positive immunostaining, cheek mucosa showed weak staining of some cells in lamina propria (Figure 2 E&F).

##### ***MTX (Methotrexate- treated) group:***

Tongue mucosa showed intense immunoreactivity in nearly all endothelial cells, many connective tissue and muscle cells, while few epithelial cells showed positive cytoplasmic reaction (Figure 3 D).

Buccal mucosa also showed strong immunoreactivity in most endothelial cells and connective tissue

cells. Also, epithelial cells showed weak cytoplasmic staining while some muscle cells showed variable weak and moderate immunostaining (Figure 4 D).

#### MTX\ASH (Methotrexate/Ashwagandha- treated) group:

Tongue and buccal mucosa immunostained sections with COX2, showed weak and moderate immunoreactivity in some endothelial and connective tissue cells (Figure 5 E&F).

#### Statistical analysis results:

On measuring area fraction of Ki67 immunoreactivity in both tongue and buccal mucosa, the MTX group recorded the least value. By comparing mean and standard deviation of Ki67 labelling index of MTX group with control or combination (MTX/ASH) groups, there was significant decrease. On the other hand, COX2 immunoreactivity results showed significant increase of mean and standard deviation of area fraction in MTX group, in comparison with control and combination groups (Table 1, fig. 6 & 7).

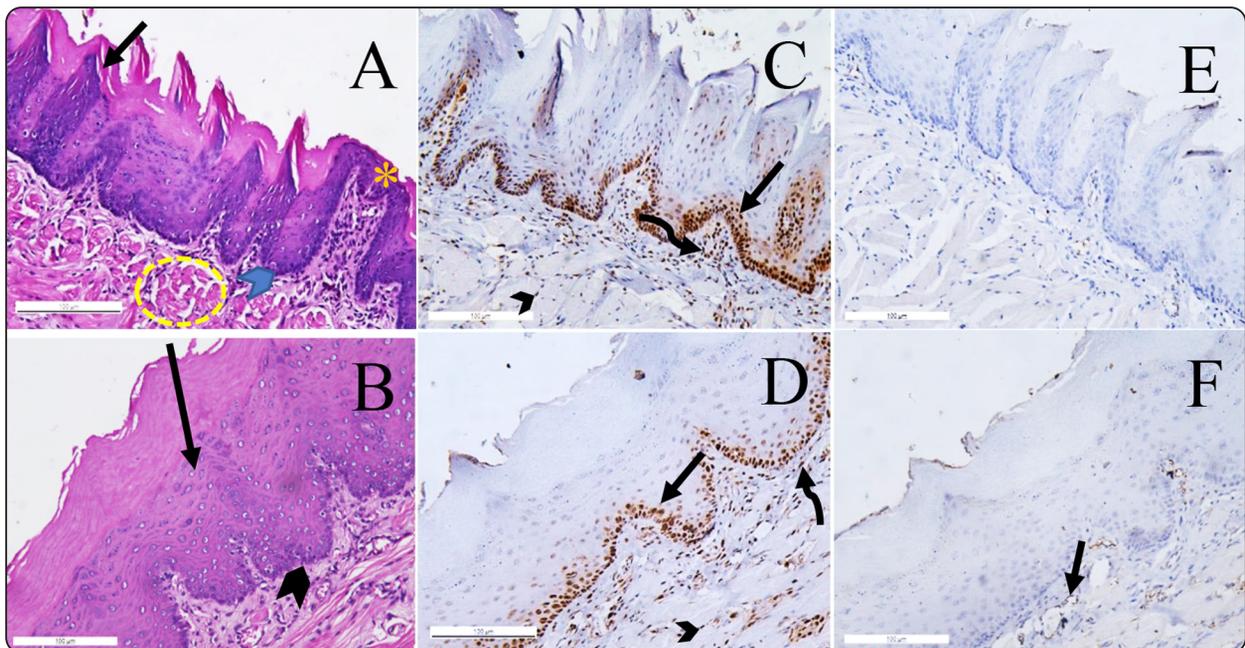


Fig. (1) Photomicrographs of tongue (A, C, E) and buccal mucosa (B, D, F) of control group showing: (A) full thickness of epithelium with regular cover of conical filiform p. (arrow), and fungiform papilla with one taste bud in its superior surface (\*), deep rete pegs (arrowhead), muscle bundles (circle) (H&E x200), (B) normal appearance and thickness of str. sq. keratinized epi. of rat buccal mucosa (arrow), deep rete pegs (arrowhead) (H&E x200). (C&D) moderate and intense nuclear staining of basal and para basal layer of epi. (arrow), nuclear staining in connective tissue cells (s arrow) and muscle cells (arrowhead) (Ki67 x200), (E) nearly negative COX2 immunostaining (COX2 x200), (F) very weak COX2 immunostaining (arrow) (COX2 x200).

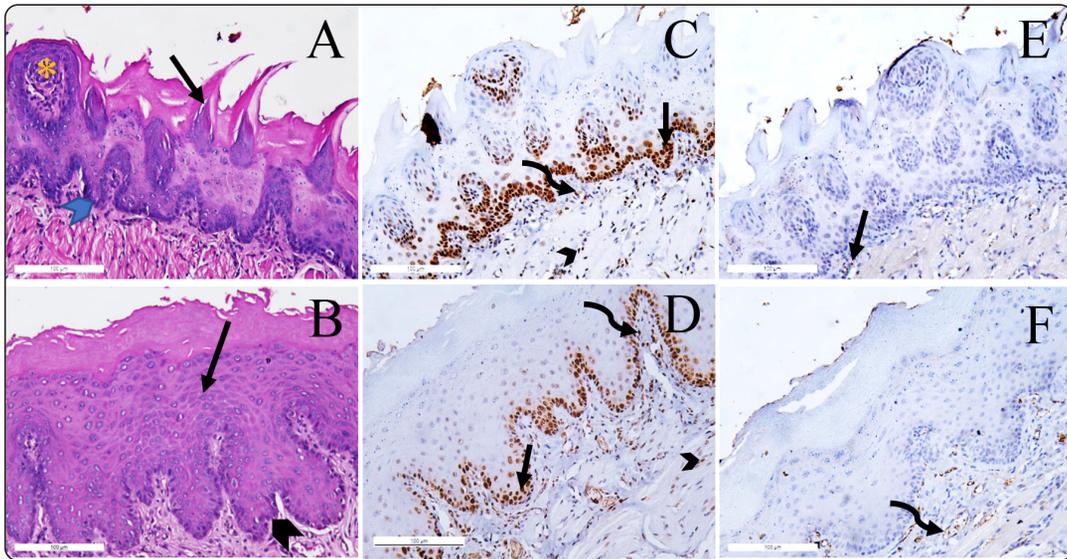


Fig. (2) Photomicrographs of tongue (A, C, E) and buccal mucosa (B, D, F) of ASH group showing: (A) full thickness of epithelium with regular cover of tongue filiform (arrow) and fungiform (\*) papilla, deep rete pegs (arrowhead) (H&E x200), (B) normal appearance and thickness of epithelium (arrow), deep rete pegs (arrowhead) (H&E x200). (C&D) moderate and intense nuclear staining of basal and para basal layer of epi. (arrow), connective tissue cells (S arrow) and muscle cells (arrowhead) (Ki67 x200). (E) Positive immunostaining of few connective tissue cells (arrow), (F) weak immunostaining of some endothelial cells (S arrow) (COX2 x200).

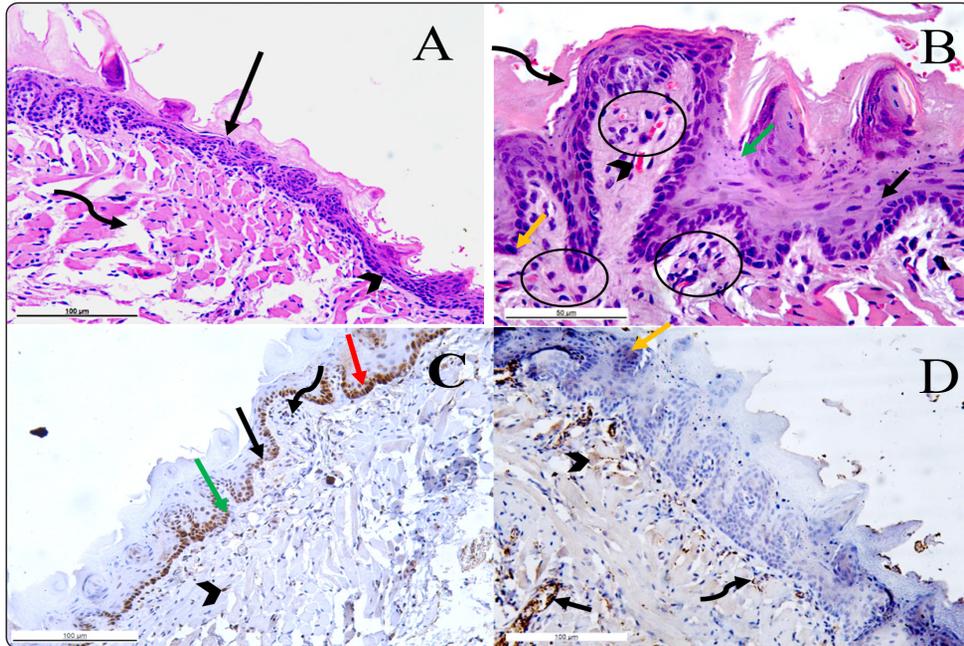


Fig. (3) Photomicrographs of tongue in MTX group showing: (A) thin epithelium with severely atrophied papilla (arrow), flattening of rete pegs (arrowhead), wide spacing between atrophied muscle fibers (S arrow) (H&E x200), (B) atrophied fungiform p. with detached keratin (S arrow), hyperchromatic nuclei (yellow arrow), pyknotic n. (black arrow), diminished keratohyalin granules (green arrow), inflammatory cells (circle), congested blood vessel (arrowhead) (H&E x400), (C) weak (↑), moderate (↑) and intense (↑) nuclear immunostaining of basal and parabasal epi. cell layers, moderate staining of some C.T cells (S arrow) and muscle cells (arrowhead) (Ki67 x200), (D) intense immunostaining in most endothelial cells (black arrow), cytoplasm of some epithelial cells (yellow arrow), connective tissue cells (S arrow), and muscle cells (arrowhead) (COX2 x200).

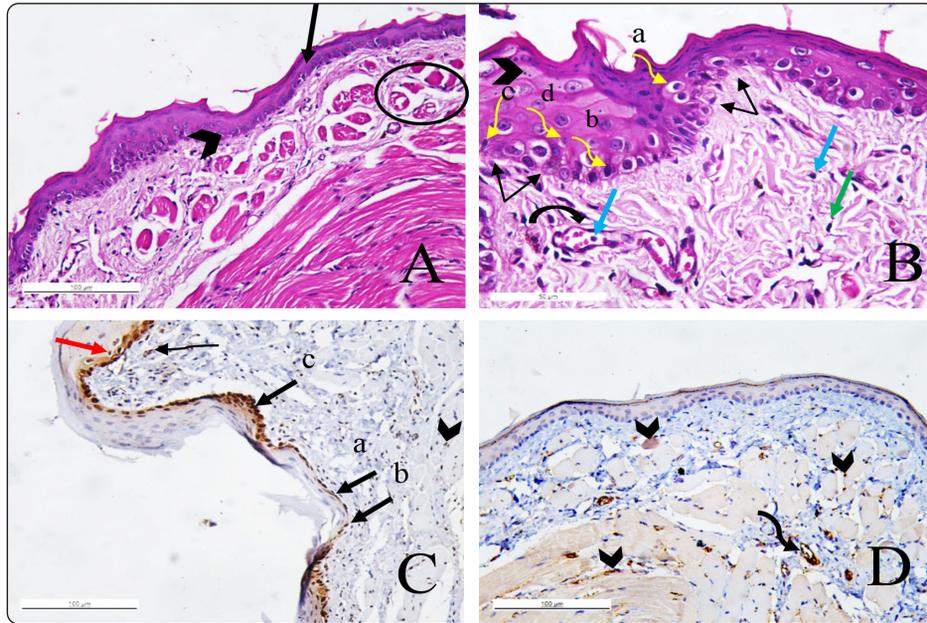


Fig. (4) Photomicrographs of buccal mucosa in MTX group showing: (A) very thin atrophied epithelium (arrow), flat rete pegs (arrowhead), wide spacing between atrophied muscle fibers (circle) (H&E x200), (B) cytoplasmic vacuolation (a), hyperchromatic nuclei (b), pyknotic n. (c), karyorrhexis (d), diminished keratohyalin granules (arrowhead), disrupted basement membrane (v arrow), dilated congested b.v. (U arrow), inflammatory cells (blue arrow), hyperchromatic n. of fibroblast (green arrow) (H&E x400), (C) weak (a), moderate (b) and intense (c) nuclear immunostaining of basal layer of epi., few stained nuclei in para basal layer (red arrow)m moderate staining of some C.T cells (Ki67 x200), (D) intense immunostaining apparent in endothelial cells (S arrow), and muscle cells (arrowhead) (COX2 x200).

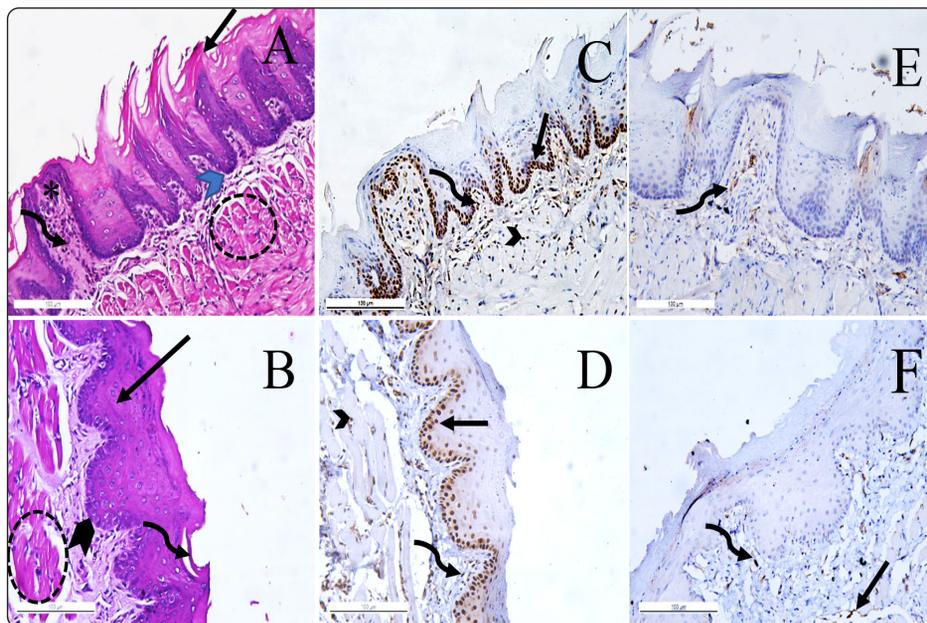


Fig. (5) Photomicrograph of tongue (A, C, E) and buccal mucosa (B, D, F) of MTX/ASH (protected) group showing: (A) full thickness of epithelium with regular cover of filiform papilla (arrow), fungiform p. (\*), regular rete pegs (arrowhead), normal c.t (S arrow) and muscle fibers (circle) (H&E x200), (B) nearly normal thickness of surface epithelium (arrow), with detached thin keratin (S arrow), regular rete pegs (arrowhead), nearly normal muscle bundles (circle) (H&E x200). (C&D) intense nuclear staining of basal and para basal layer of epi. (arrow), connective tissue (S arrow) and muscle cells (arrowhead) (Ki67 x200), (E&F) moderate immunostaining of some C.T cells (S arrow), (F) weak staining of some endothelial cells (arrow) (COX2 x200).

TABLE (1) P and mean values of Ki67 and COX2 immunostaining area fraction of control and experimental groups in both tongue and buccal mucosa:

Group	Mean ±SD (Tongue)	P value		Mean ±SD (Buccal m.)	P value	
<b>Ki67</b>						
Control	5.7±1.527			3.68±0.784		
ASH	6.98±1.163	P1	0.175	4.52±1.04	P1	0.117
MTX	2.8±0.409	P2	0.009*	1.89±0.49	P2	0.009*
MTX/ASH	6.26±1.125	P3	0.7	3.58±0.53	P3	0.75
		P4	0.009*		P4	0.009*
<b>COX2</b>						
Control	0.186±0.124			0.9±0.53		
ASH	0.146±0.589	P1	0.75	0.54±0.26	P1	0.251
MTX	4.069±0.855	P2	0.009*	4.55±0.55	P2	0.009*
MTX/ASH	0.277±0.19	P3	0.175	1.133±0.36	P3	0.175
		P4	0.009*		P4	0.009*

*P1, P2, P3 in comparison with control group, P4 in Comparison with MTX group., \* Significant*

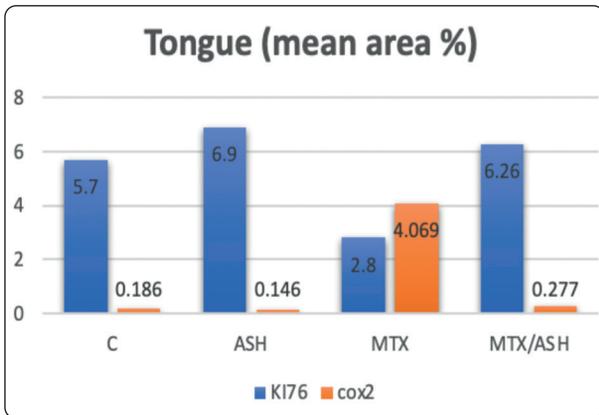


Fig. (6) A bar chart showing comparison between mean values of Ki67 and COX2 area fraction in tongue of control and experimental groups.

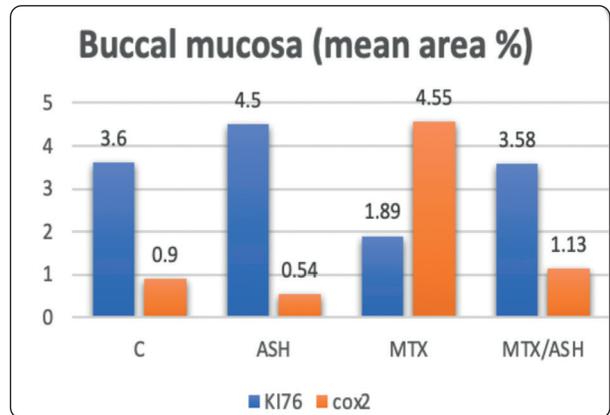


Fig. (7) A bar chart showing comparison between mean values of Ki67 and COX2 area fraction in buccal mucosa of control and experimental groups.

## DISCUSSION

Oral mucositis is an inflammatory condition that results from damage to oral mucosa following cytotoxic cancer chemotherapy including methotrexate. The severity and duration of mucositis are dependent on the dose and type of the drug used; however, in general, it could lead to compromised nutrition and general health of the patient, and as a consequence, this may compel a chemotherapy dose reduction or postponement due to the severe pain or other related symptoms<sup>(23)</sup>.

Besides being antioxidant, anti-inflammatory, anti-ulcerogenic, Ashwagandha has been reported to have selective killing ability for cancerous cells<sup>(8)</sup>, suggesting that it would be a favorable adjuvant to chemotherapeutic drugs that may help in protecting oral tissues without affecting the efficacy of the anticancer treatment. However, till now, no studies were conducted to evaluate its possible protective effect on methotrexate-induced oral mucositis. So, it was our concern in the current study.

Our results revealed that methotrexate induced atrophic and inflammatory changes in both the buccal mucosa and the examined area of anterior part of the dorsal surface of the tongue. These changes were represented in hematoxylin and eosin-stained sections by a decrease in epithelial thickness, slight atrophy of the tongue fungiform papillae and severe atrophy of filiform papillae, keratin detachment, disruption of basement membrane, cytoplasmic vacuolation, decrease of keratohyalin granules, nuclear changes as pyknosis, karyorrhexis, and hyperchromatism. Inflammatory cell infiltrate, dilated congested blood vessels, atrophy of muscle fibers, and an increase in spacing in connective tissue and between muscle fibers was also detected. Immunohistochemically, MTX induced a significant increase in COX2 and a significant decrease in Ki67 immunostaining as compared with control group.

On the other hand, in combination group of the current study, ashwagandha has been shown to mitigate the atrophic and inflammatory changes induced

by MTX administration, this was demonstrated in our histopathological and immunohistochemical staining results; histologically, by preserving the normal morphology and thickness of buccal and lingual mucosal tissues, and immunohistochemically, by a significant enhancement of Ki67 immunoreactivity concurrently with significant lowering of COX2 levels to values close to control groups, as indicated by a statistically non-significant difference in both Ki67 and COX2 labeling indices when compared to the control group and a statistically significant difference when compared with the MTX-treated group.

Ki-67 protein is an indicative marker for proliferation, it plays an important role in the cell cycle during the interphase and mitotic phase. During interphase, it is required for the normal distribution of cellular heterochromatin and the nucleolar association of heterochromatin. While in mitosis, it is essential for the formation of the perichromosomal layer (PCL), a ribonucleoprotein sheath coating the condensed chromosomes, in addition to prevention of chromosomal aggregation<sup>(24)</sup>.

COX2 is an enzyme that has been known to be a major player in inflammatory process. It is rapidly expressed in several cell types in response to growth factors, cytokines, and pro-inflammatory molecules and primarily responsible for production of prostanoic acid including Prostaglandins, which mediate the inflammatory process in acute and chronic inflammatory conditions<sup>(25)</sup>.

Chemotherapy has been reported to stimulate the production of transcription factor nuclear factor kappa B (NF- $\kappa$ B), that upregulates COX2 and in turn results in the production of PGs and inflammation<sup>(26)</sup>.

In the current study, the apparent decrease in COX2 immunoreactivity in ASH/MTX group, is supported by Jayaprakasam and Nair<sup>(27)</sup> and Ichikawa et al<sup>(28)</sup> who reported that Ashwagandha has an anti-inflammatory effect with a selective COX2 enzyme inhibitory effect. It has been suggested that the anti-

inflammatory effect of Ashwagandha is attributed to its bioactive components withanolides specifically withaferin A, which has the ability to inhibit the activation of Nuclear Factor Kappa B (NF- $\kappa$ B), and alpha-2 macroglobulin <sup>(29)</sup>.

The anti-inflammatory effect of ASH demonstrated in our study comes in co-ordinance with Azab et al <sup>(17)</sup> who reported that ashwagandha produced a significant reduction in level of inflammatory markers (IL-17, IL-10, and  $\alpha$ 7-nAChR) and oxidative stress markers (MDA, GSH, and ROS) in spleen and liver tissues of rats treated with ASH and exposed to  $\gamma$ -radiation, and Elhadidy et al <sup>(30)</sup> who proved that ashwagandha extract produced anti-inflammatory and anti-oxidant effects against aluminum chloride (AlCl<sub>3</sub>)-induced neurotoxicity in rats, the pretreatment with ASH extract counteracted the significant rise in TNF- $\alpha$ , MDA and NO levels induced by AlCl<sub>3</sub> to non-significant changes as compared with the control group.

Regarding the preservation of lingual and buccal mucosal tissues seen in our combination group, this might be attributed to lowering of COX2 elevated by MTX. As in oral mucositis, the elevated COX2 has been reported to activate matrix metalloproteinases (MMPs), resulting in the breakdown of the subepithelial collagenous matrix, destruction of the basement membrane <sup>(31)</sup>, and mucosal tissues by mediating apoptosis through disruption of normal cell kinetics <sup>(32)</sup>. Thus, lowering COX2 by ASH constricts tissue damage and apoptosis, which also illustrates the significant increase of Ki67 expression seen in MTX\ASH group in comparison to MTX group.

The increase in cellular proliferation shown in MTX/ASH in comparison to MTX-treated rats, is supported by Sanap et al <sup>(33)</sup> who reported that preconditioning culture medium with *Withania somnifera* root extract, was found to increase proliferation and inhibit senescence of Wharton's Jelly Mesenchymal Stem Cells.

In the light of the previously discussed, our study demonstrated that ashwagandha has anti-ulcerative and anti-mucositis effects on buccal and lingual mucosa of MTX-treated rats, this result is supported by sing et al <sup>(34)</sup> who investigated the anti-ulcerogenic effect of it for the first time, they proclaimed that ashwagandha reduced the incidence of gastric ulcers induced by aspirin and physical stress, significantly reduced milk-induced leukocytosis, and prevented the decrease in ascorbic acid and cortisol content. Authors argued that ashwagandha enhanced the adaptability of rats and mice to physical as well as chemical stress and it might induce a state of non-specifically increased resistance (SNIR) during stress.

Our result is also supported by Sanjay et al <sup>(35)</sup> who reported that 20 days of treatment with 250 mg/kg, 500 mg/kg alcoholic extract, and 1000 mg/kg powder of ashwagandha roots, after induction of gastric ulcer by aspirin, produced a significant reduction in ulcer index that is comparable to a standard anti-ulcer drug, famotidine. Histopathologically, re-epithelialization of ulcer and restoration of morphology were to a lesser extent than in the famotidine-treated rats. Authors suggested that the antiulcer effect of ashwagandha may be due to inhibition of histamine and serotonin activity in the central nervous system.

Moreover, corroborating our findings is Bhatnagar et al <sup>(36)</sup> who compared the anti-ulcer effect of ashwagandha with a standard drug, ranitidine, in NSAIDs and stress-induced gastric ulcers in adult rats. Authors found that 15 days of pretreatment with 100 mg/kg orally administered ashwagandha root extract presented healing activity in the histopathological observation, alongside a significant reduction in the ulcer index, an increase in antioxidant enzymes and ascorbic acid and a concomitant significant decrease in lipid peroxidation. These results were more pronounced in stress-induced gastric ulcer, authors proposed that this was due to the antistress activity of ashwagandha that could be useful in reducing the

changes in stress-induced ulceration besides its anti-secretory and cytoprotective effect.

This anti-ulcerogenic properties of ashwagandha have been linked to its content of sitoindoside VII and VIII compounds, as declared by Bhattacharya et al.<sup>(37)</sup> who proved that the two compounds extracted from ashwagandha possessed anti-ulcerative activity and produced a significant decrease in the incidence and severity of stress-induced gastric ulcers in rats. Additionally, ashwagandha has been shown to be rich in flavonoid content, which was reported to have an anti-ulcerogenic effect, as presumed by Parmar and Parmar<sup>(38)</sup>

## CONCLUSION

From the present study, we concluded that prophylactic administration of Ashwagandha root extract ameliorated the oral mucositis induced by methotrexate chemotherapy, and therefore, it could be a good adjuvant therapy during chemotherapeutic treatment.

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