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POTENTIAL ROLE OF POMEGRANATE PEEL EXTRACT VERSUS PUMPKIN SEED OIL IN PREVENTION OF TONGUE CYTOTOXICITY INDUCED BY METHOTREXATE IN MALE ALBINO RATS: HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY

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KEYWORDS

Pomegranate peel extract, Pumpkin seed oil, Lingual mucosa, PCNA.

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ABSTRACT

Methotrexate (MTX), a chemotherapeutic drug, causes severe cytotoxic effects on lingual mucosa. Pomegranate peel extract (PPE) and pumpkin seed oil (PSO) are natural substances, that are used to decrease the toxic effect on tissues because of their anti-oxidant effects. **Aim of the study:** The current study was conducted to evaluate and compare between the preventive role of pomegranate peel extract and pumpkin seed oil against cytotoxicity induced by methotrexate on albino rats' lingual mucosa. **Methodology:** This investigation involved 32 adult male albino rats, which were evenly distributed into four groups, each containing eight rats: control, MTX, PPE+MTX, and PSO+MTX. Following a duration of 21 days, all the animals were humanely euthanized. Their tongues were then collected and subjected to staining with Hematoxylin & Eosin (H&E) and immunohistochemical analysis for PCNA. **Results:** MTX group revealed markedly atrophic degenerative changes in lingual papillae, lamina propria showed degeneration and dissociation of collagen fibers, inflammatory cell infiltrations and acinar and ductal degeneration compared to control group. However, PPE and PSO groups showed fewer damaging effects on tongue papillae comparing to MTX group only. PCNA was significantly increased in PPE and PSO groups compared to MTX group. **Conclusions:** Pomegranate peel extract showed obvious improvement against methotrexate degenerative effect than pumpkin seed oil which demonstrated poor improvement thus, PPE was outstanding anti-inflammatory and anti-oxidant than PSO.

INTRODUCTION

Chemotherapy is a family of drugs that is used in different forms these days to treat cancer. It's critical to find drugs that can mitigate the side effects of anti-cancer drugs without reducing their efficacy, increasing their toxicity or endangering the target organs [(1)]. Chemotherapeutic agents significantly affect the mouth, leading to both primary and ancillary toxicity. This arises from several reasons, notably the swift regeneration of cells in the mucous membrane of the mouth [(2)].

Methotrexate (MTX), an analog of folic acid, is commonly prescribed for several autoimmune diseases such as psoriasis, lichen planus, and rheumatoid arthritis, in addition to being used in the treatment of various cancers, including acute leukemia and osteosarcoma [(3)]. The primary mechanism of action of MTX relies on inhibiting DNA synthesis.

MTX disturbs the balance between pro-oxidants and antioxidants, resulting in oxidative stress, leading to increased reactive oxygen species and later cellular apoptosis [4].

Despite being a common component of cancer therapy, it also has adverse effects on a variety of tissues. These adverse effects caused by MTX include nephrotoxicity, hepatotoxicity, lung toxicity, and bone marrow toxicity [5]. Stomatitis, diarrhea, nausea, vomiting and alopecia are the most frequent side effects in MTX patients; these side effects are frequently brought on by the production of free radicals and lipid peroxidation [6].

Administering natural antioxidants could be a viable strategy to mitigate the negative effects associated with treatment using MTX [7]. Because antioxidants play a crucial role in preserving both general and dental health, there has been a particular focus on using them in treatment. Utilizing natural dietary additives, particularly those enriched with components like extract from pomegranate peels and oil from pumpkin seeds, can hasten recuperation from critical health conditions and lessen the adverse effects caused by treatments such as chemotherapy and radiotherapy [8].

Pomegranates (Punica granatum L.) have gained a lot of attention because of its advantageous and nutraceutical qualities in relation to a number of illnesses, including diabetes mellitus, cancer, and cardiovascular disorders[9]. Extract from pomegranate peels is rich in phytochemicals, predominantly from the family of phenolic compounds, including elements like anthocyanins, tannins, flavonoids, and specific phenolic acids (like gallic acid) [10].

Pomegranate peel extracts (PPE) exhibit notable properties such as antioxidation, anti-inflammation, infection resistance, microbial defense, liver protection, anti-atherosclerosis, diarrheal prevention, and mutation prevention capabilities [11]. Polyphenols such as ellagannins and the pomegranate-peculiar punicalagin play a crucial role as antioxidants and anti-inflammatory agents. According to the results of recent studies, PPE has a more significant and promising role in the regulation of oxidative stress due to its ability to scavenge radicals and chelate transition metals [9].

Pumpkins, with origins dating back over 4,000 years in South America, belong to the genus Cucurbita maxima and the family "Cucurbitaceae" where they were first cultivated. Rich in bioactive compounds such as proteins, peptides, polysaccharides, para-aminobenzoic acid, sterols, and fixed oils, pumpkin consumption is known to combat cancer, eye disorders, and skin conditions. Pumpkin seeds enhance the body's immune response and reduce cell damage. Their nutritional and health-maintaining qualities have attracted considerable attention[12].

Oil derived from pumpkin seeds is rich in phenolic substances such as vanillic acid, tyrosol, and vanillin. It also contains abundant selenium and lutein, alongside squalene, phytosterols, carbohydrates, minerals, and proteins. The pharmacological properties of the pumpkin seed oil include anti-inflammatory, antibacterial, antifungal, anti-diabetic, antitumor, anti-obesity and antioxidant properties [13]. Several studies have documented its therapeutic benefits including immunomodulation, anticancer and antihypertensive properties [14].

MATERIAL AND METHODS

Approval of the Ethics Committee of Scientific Research, Faculty of Dentistry, Suez Canal University, has been obtained before starting the search (approval number: 430\2021).

Materials

Methotrexate: was purchased as ampoule (50 mg/2 ml) (Sigma Chemical Company, Saint Louis, Mo, USA. Cat. No. A6770).

Pumpkin seed oil: was purchased from Nano Gate Company Cairo in form of cold-pressed PSO (liquid)

Pomegranate peel extract: was prepared at Nano Gate Company, Cairo.

PCNA for IHC stain: mouse monoclonal anti-PCNA (Cat # MS-862-P, Thermo Scientific, CA, USA at dilution 1:300) was used as primary antibodies**.**

Preparation of pomegranate peel extract:

Pomegranate peel extract prepared at Nano Gate Company, Cairo according to manufacture instructions[15], 100 g oven dried peel of pomegranate (Punica granatum L. Family: Punicaceae) were extracted by adding 1000 ml of distilled water and boiled at 80ºC for 30 min. The extract underwent filtration through filter paper, and the resulting filtrate was then subjected to evaporation using a rotary evaporator at low pressure until dry. Subsequently, 5 grams of this dried extract was reconstituted in 100 milliliters of distilled water prior to administration. The aqueous extract was then delivered orally via an oropharyngeal tube, at a dosage of 250 mg per kilogram of body weight, for a duration of three weeks.

Study design and animals grouping

Animals: This study was carried out on 32 male albino rats, weighting (200 ±15) grams. The sample size for this study was calculated according to ***Charan and Biswas,*** [16] used the following equation:

$$\mathbf{N = \frac{(Z\alpha)^2 * (SD)^2}{(d)^2}}$$

N = Total sample size

$Z\alpha$= Is Standard normal variate and its equal 1.96 at $P < 0.05$

SD = Standard deviation of variable

d = Absolute error or precision

Zα	SD	d
1.96	5.77	2

$$\textbf{Total sample size N} = \frac{(1.96)^2 \textbf{ x } (\textbf{5.77})^2}{(2)^2} \textbf{ = 31.97} \approx \textbf{32 samples}$$

The sample size in this study is in agreement with ***Ghamarzad Shishavan et al.***[17] who have published on this point.

The rats were purchased from College of Veterinary Medicine. They were housed into four groups in a well-ventilated room with controlled temperature, 50-70% humidity and 12-hour day/night cycle. All animals were given water and recommended diet adlibitum.The experiment was performed in the Animal House, Faculty of Dentistry, Suez Canal University.

Thirty-two rats were divided into four (4) groups as follows:

Group 1 (control negative): containing 8 rats, the rats did not receive any treatment.

Group 2 (MTX group): containing 8 rats, the rats were received methotrexate (MTX) 10mg\kg intramuscularly for 3 consecutive days, beginning from the 10th day [18].

Group 3 (PPE + MTX group): containing 8 rats, the rats were received 250mg\kg pomegranate peel extract (PPE) orally by oropharyngeal tube for 21 days and methotrexate (MTX) 10mg\kg intramuscularly for 3consecutive days, beginning from the 10th day [15].

Group 4 (PSO + MTX group): containing 8 rats, the rats were received pumpkin seed oil 1.5 ml/kg/day orally by oropharyngeal tube once daily for 21days and methotrexate (MTX) 10mg\kg intramuscularly for 3 consecutive days, beginning from the 10th day [19].

Following euthanasia (achieved through an excessive amount of ether vapor), the tongues were extracted for histological and immunohistochemical analysis. These tongue samples were preserved in a 10% buffered formalin solution for a period of 48 hours. After fixation, the samples were dehydrated using increasing concentrations of ethyl alcohol, cleared in xylene, and subsequently embedded in paraffin. Using a microtome, each block was sectioned to produce five serial sections, each 5µm thick, which were then prepared for Hematoxylin & Eosin (H&E) and Immunohistochemical (IHC) staining for PCNA [20].

Immunohistochemical procedures:

For the immunohistochemical examination of PCNA, sections of 5 microns thickness were sliced from each paraffin block and placed onto positively-charged glass slides. These sections were then rid of paraffin in xylene and passed through a series of ethanol solutions of decreasing concentration for rehydration. Following this, the sections were rinsed with TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.4). Subsequently, they underwent a 30-minute room temperature treatment with 0.3% H_2O_2 in distilled water to suppress endogenous peroxidase activity. Adhering to the manufacturer's guidelines, antigen retrieval was carried out. The slides were then treated with 100 μl of blocking solution from Abcam for 30 minutes at ambient temperature. This was followed by an overnight incubation at 4ºC with primary monoclonal antibodies (specifically, anti-PCNA primary antibody, Cat # MS-862-P, from Thermo Scientific, CA, USA, used at a 1:300 dilution).

The sections were first rinsed in 1X Phosphate Buffered Saline (PBS) and then treated with a biotinylated secondary antibody (anti-mouse) in a blocking buffer. This incubation took place for one hour at room temperature within a moisture-controlled chamber. To visualize peroxidase activity, the sections were soaked in an ABC solution for one hour at a consistent temperature. The development of color began with the application of DAB solution (0.5 mg/ml DAB and 0.1% H_2O) on the sections. Once the color reaction reached the intended level, it was stopped by rinsing the sections in H_2O for a duration of 5 to 10 minutes. Following a gradual dehydration process, the sections were prepared with coverslips for observation. Immunohistochemical staining was evaluated using a light microscope (Olympus BX53, Tokyo, Japan). An image analysis system (Image J / Fiji 1.46) was employed to analyze the results of immune-expression.

Statistical Analysis:

For the statistical evaluation of IHC outcomes, the Statistical Package for the Social Sciences (SPSS) software, version 26, was utilized. The intensity of the IHC staining was presented as a mean ± standard deviation. To conduct comparisons across different groups, a One-way ANOVA was employed. For specific pairwise comparisons, Bonferroni post hoc test was used. A P value of less than 0.05 was deemed to indicate statistical significance.

RESULTS

Histological evaluation

Group 1 (control group): Histological analysis of the rats' tongue mucosa revealed typical characteristics of the surface epithelium and the supporting lamina propria beneath. Various papilla types such as filiform, fungiform, and circumvallate were observed on the tongue's dorsal aspect **(Fig. 1.A,B,C)**, while the lingual glands exhibited standard histological structure **(Fig. 2.A,B).**

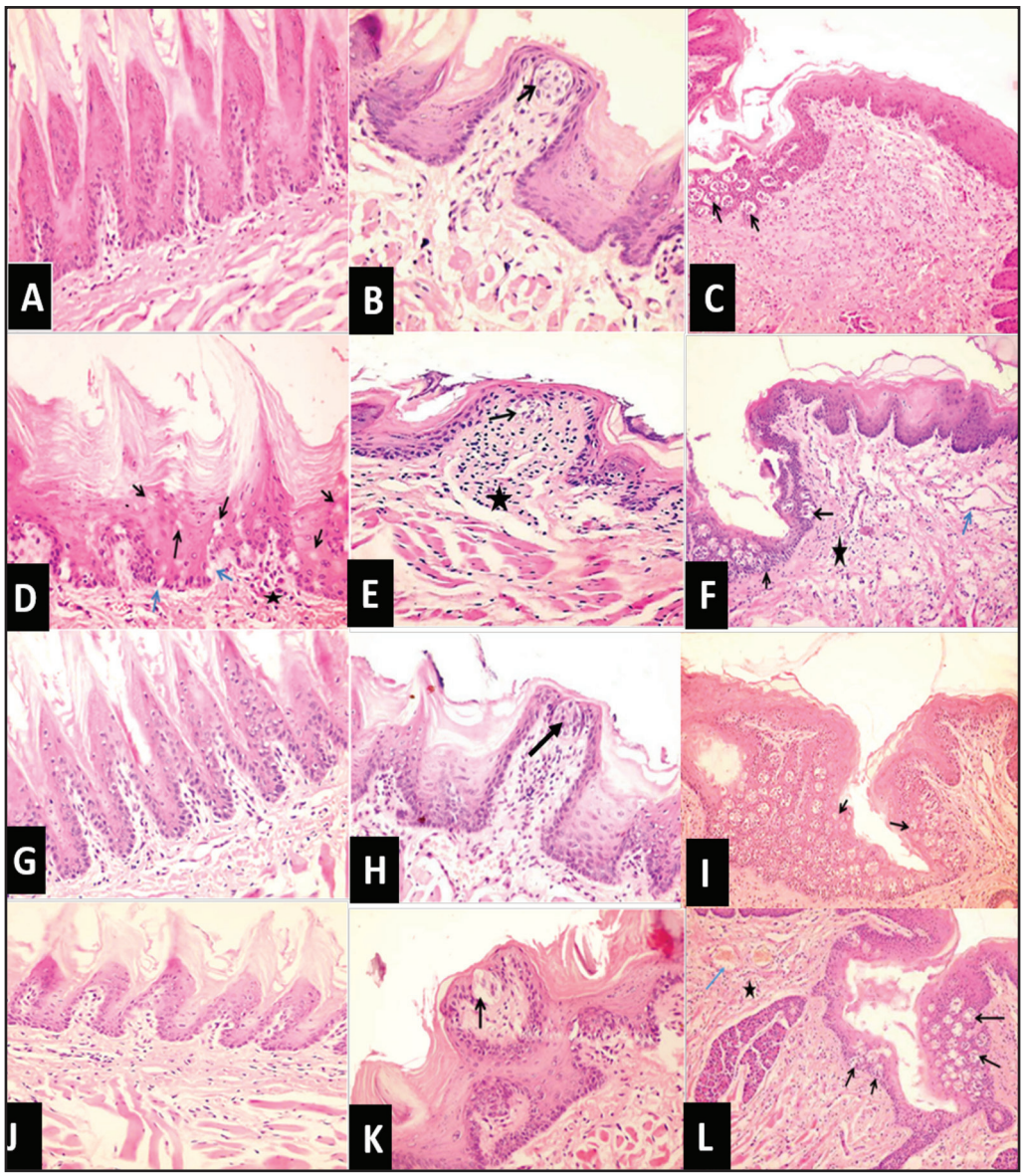

Fig. (1) Photomicrographs of dorsal surface of the tongue. **A:** Control group showing normal structure of filiform papillae with keratinized stratified squamous epithelial coverage and lamina propria **B:** Control group showing a fungiform papillae with taste bud (arrow) **C:** control group showing circumvallate papillae with normal stratified squamous epithelium, lamina propria and a lot of taste buds in its side walls (arrows) **D:** MTX group showing atrophied filliform papillae with cytoplasmic vacuolization of epithelium (black arrows), focal disruption of basment membrane (blue arrows), hyperkretanization, and inflammatory cell infilteration in the underlying lamina propria (star) **E:** MTX group showing atrophic fungiform papilla with degenerating taste bud (arrow) and inflammatory cells infilteration (star) **F:** MTX group showing degenrated taste buds (black arrows),dialtion of blood vessels engorged with RBCs (blue arrow) and disassociaton of underlying connective tissue with inflammatory cell infilteration (star) **G:** PPE+MTX group showing normal filiform papillae with normal keratinization and normal underlying lamina propria **H:** PPE+MTX group showing almost normal fungiform with cytoplasmic vacuolization in taste bud (arrow) **I:** PPE+MTX group showing circumvallate papillae with almost normal stratified squamous epithelium and taste buds with cytoplamic vacuolizations (arrows) **J:** PSO+MTX group showing atrophied filiform papillae with almost normal underlying lamina propria **K:** PSO+MTX group showing atrophied fungiform papilla with cytoplasmic vacuolization in its taste bud (arrow) **L:** PSO+MTX group showing circumvallate with atrophied epithelium, degenerated taste buds (arrows), the underlying lamina propria showed inflammatory cell infilteration (star) and blood vessels engorged with RBCs (blue arrow) (H&E, orig. mag. 400,200).

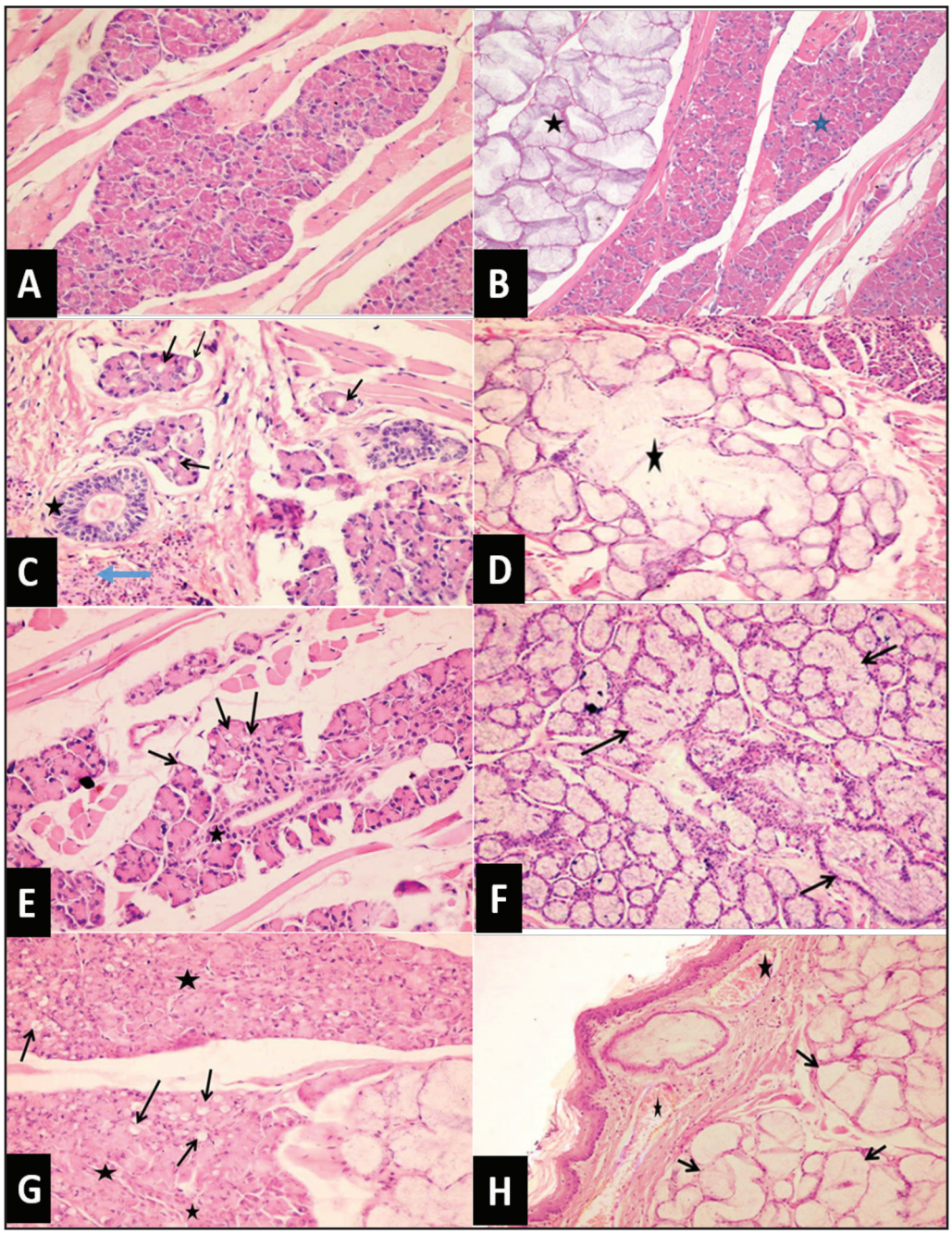

Fig. (2) Photomicrographs of immunohistochemistry-stained sections of tongues **(A):** Control group showing moderate positive nuclear PCNA immunoexpression in the basal and suprabasal cells **(B):** MTX group showing weak nuclear PCNA immunoexpression in the basal and suprabasal cells **(C):** PPE+MTX group showing strongly positive nuclear PCNA immunoexpression in the basal and suprabasal cells **(D):** PSO+MTX group showing weak nuclear PCNA immunoexpression in the basal and suprabasal cells (orig. mag. 400).

Group 2 (MTX group): showed the filiform papillae were markedly atrophic and with decreasing in their number. Atrophy and contraction were observed in the fungiform and circumvallate papillae, along with degeneration in their taste buds. In the lamina propria, there was evidence of collagen fiber degeneration and separation, infiltration by inflammatory cells, and expanded blood vessels filled with congested red blood cells **(Fig.1. D, E, F)**. The lingual salivary glands displayed vacuolization in the cytoplasm of serous acinar cells and cyst-like changes in mucous acinar cells, accompanied by infiltration of inflammatory cells in the adjacent connective tissue **(Fig. 2. C, D).**

Group 3 (PPE + MTX group): showed obvious improvement in their histological picture represented by regain of the epithelium thickness of lingual papillae and almost normal taste buds architecture with marked decreasing in cytoplasmic vacuolization. The lamina propria showed an apparent increase in cellularity and vascularity with no inflammatory cell infiltrations recording **(Fig.1. G, H, I)**. The lingual glands exhibited typical histological features, except for a few mucous acinar cells undergoing cystic changes and minor cytoplasmic vacuolization in some serous acinar cells **(Fig. 2. E, F).**

Group 4 (PSO + MTX group): The histological appearance of PSO-treated rats revealed minimal improvement in their histological image represented by little cytoplasmic vacuolization in the epithelium of dorsal surface with diminished in thickness and partial degeneration of the lingual taste buds. Lamina propria showed partially regain their cellularity and vascularity with localized areas of inflammatory cell infiltration **(Fig.1. J, K, L)**. The lingual salivary glands exhibited vacuolization in the cytoplasm of serous acinar cells and a cystic transformation in mucous acini **(Fig. 2. G, H).**

Immunohistochemical localization of proliferating cell nuclear antigen (PCNA):

Group 1 (control group): Immunohistochemical examination of the surface epithelium of the lingual mucosa of the control group showing moderate positive nuclear PCNA immunoexpression in the basal and suprabasal cells **(Fig. 3 A)**.

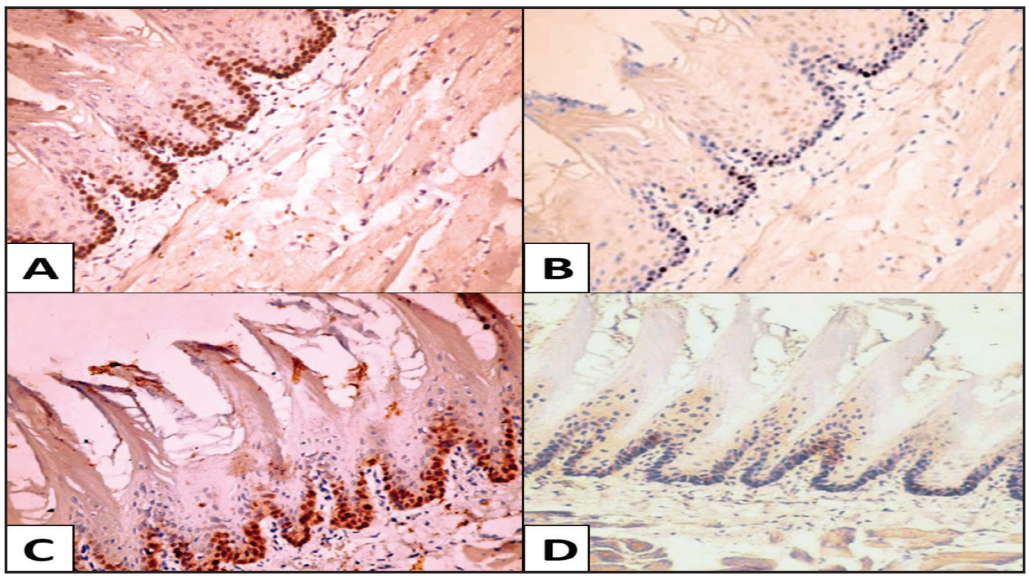

Fig. (3) Photomicrographs of immunohistochemistry-stained sections of tongues (A): Control group showing moderate positive nuclear PCNA immunoexpression in the basal and suprabasal cells (B): MTX group showing weak nuclear PCNA immunoexpression in the basal and suprabasal cells (C): PPE+MTX group showing strongly positive nuclear PCNA immunoexpression in the basal and suprabasal cells (D): PSO+MTX group showing weak nuclear PCNA immunoexpression in the basal and suprabasal cells (orig. mag. 400).

Group 2 (MTX group): In the lingual mucous membrane treated with a 10 mg/kg dose of MTX, the basal and suprabasal cells of the surface epithelium exhibited a faint response to PCNA **(Fig. 3 B)**.

Group 3 (PPE + MTX group): In group III, treated with PPE at a dosage of 250mg/kg, the surface epithelium of the tongue's mucous membrane displayed pronounced immunoreactivity to PCNA in both basal and suprabasal cells **(Fig. 3 C)**.

Group 4 (PSO + MTX group): In group IV, which was treated with PSO at a dosage of 1.5 ml/kg/day, the surface epithelium of the mucous membrane of the tongue displayed a weak immune response to PCNA **(Fig. 3 D)**.

Statistical Analysis for PCNA:

Table 1 presents the outcomes of the statistical analysis, indicating distinct and significant differences when compared to the control group, MTX, PEE+MTX and PSO+MTX groups for PCNA ($p < 0.001$) using one way ANOVAs at a significant level $P < 0.05$. The pairwise comparison showed a significant difference between each group to another. The **PEE+MTX** group had the high mean value (24.84±1.35) followed by **Control** group (21.32±0.85) then **PSO+MTX** (13.35±1.52) while MTX group was the lowest one (5.32±0.54).

Table (1) *Illustrates difference in mean of PCNA optical density between different group*

	Mean	SD	F test	P value
Control (G1)	21.32 [b]	0.85	470.17	<0.001**
MTX (G2)	5.32 [d]	0.54		
PPE+MTX (G3)	24.84 [a]	1.35		
PSO+MTX (G4)	13.35 [c]	1.52		

Multiple Comparisons using Bonferroni post-hoc				
Pair wise	Mean difference	95% Confidence Interval(CI)		P value
		Lower	Upper	
G1 Vs G2	16.01	14.39	17.62	<0.001**
G1 Vs G3	-3.52	-5.13	-1.90	<0.001**
G1 Vs G4	7.97	6.36	9.58	<0.001**
G2 Vs G3	-19.52	-21.13	-17.91	<0.001**
G2 Vs G4	-8.04	-9.65	-6.42	<0.001**
G3 Vs G4	11.49	9.87	13.10	<0.001**

Test used: ANOVAs test (F)

***; and different letters means significant at P<0.05*

DISCUSSION

Methotrexate (MTX) is a cytotoxic chemotherapy drug that is recommended for inflammatory disorders such as rheumatoid arthritis and psoriasis, as well as for treating various tumour types such as breast cancer, lung cancer, leukaemia, testicular tumours, and other malignancies [(3)]. Oral mucositis, a condition characterized by inflammation and ulceration, commonly occurs in cancer patients undergoing chemotherapy and radiotherapy. Around 40% of individuals undergoing chemotherapy and 80% of patients receiving radiotherapy for head and neck cancers suffer from oral mucositis [(21)].

The present study demonstrated the negative side effects of methotrexate on lingual mucosa and aimed to evaluate the preventive role of pomegranate peel extract and pumpkin seed oil against cytotoxicity induced by methotrexate on albino rats' lingual mucosa.

In current investigation, the tongue was tissue of choice because it is a helpful investigative tool for examining the effects of pharmacological substances on various tissues as the enormous morphological variations of the tongue which is

crucial for chewing and swallowing food, are a reflection of different lifestyles [22] additionally, it has high turnover and renewal rate of epithelial cells, thus cytotoxic medicines that target rapidly proliferating cells can have an adverse effect on it[23]

In this study, rats administered with MTX exhibited signs of atrophy and degeneration affecting the surface epithelium and lamina propria of the dorsal surface of the tongue, including the lingual salivary glands. Surface epithelium of the dorsal surface demonstrated a lot of vacuolizations in the epithelial cells, atrophy and hyperkertanization that could be due to its direct inhibitory effects on DNA replication and mucosal cellular proliferation reduce the basal epithelium's ability for regeneration. The study of ***Ahmad and Kaz*** [24] was agreed with the present study result, the authors demonstrated atrophied epithelium with flattening or shortening of rete ridge during studying the histological effect of methotrexate and folinic acid on oral epithelium of albino rats.

The lamina propria displayed signs of collagen fiber degeneration and separation, along with infiltration by inflammatory cells and the widening of blood vessels, which were filled with an abundance of red blood cells. These results are in agreement with ***Al-Refai et al.*** [25] study. In their research, the authors examined the defensive effects of honey on the dorsal side of the tongue in rats treated with methotrexate, concluding that methotrexate induced degenerative alterations in the lingual mucosa.

Marked hemorrhage, vascular congestion and inflammatory cell infiltration in lamina propria were also noticed in the present study, these results were supported by ***Hsu et al.*** [26]. The author proposed that the primary cause of the observed effects was the oxidative stress induced by MTX. It was found that MTX, during its intracellular metabolism, reduced glutathione-based antioxidants and generated oxygen free radicals. This led to lipid peroxidation, subsequently causing lysis of organelles and plasma membranes due to an imbalance between oxidants and antioxidants. Such inflammatory responses might aim to enhance blood flow to the damaged tissues. According to ***Garipardic et al.***[27] () and ***Abeer et al.*** [28] elucidated that the dilation and congestion observed were due to an inflammatory reaction to the MTX treatment, which heightened the permeability of endothelial cells.

The current findings related to MTX are consistent with the research of ***Ahmed et al.*** [29], which focused on the impact of α-Lipoic acid on oral mucositis and oxidative stress caused by methotrexate in rats. The authors deduced that methotrexate led to degenerative alterations in the surface epithelium and lamina propria of the buccal and lingual mucosa.

After MTX administration, lingual glands showed degenerative changes represented as cytoplasmic vacuolization, number of mucous acinar cells revealed cystic transformations. Some serous acinar cells presented with marked atrophy, areas of complete loss of acinar cells and inflammatory cell infilteration of surrounding connective tissue. These findings align with the research of ***Abdel-Fatah et al.*** [30], who showed that MTX treatment led to the disruption of the normal structure of parotid tissue. This was characterized by a total disappearance and deterioration of parotid acini, creating vast empty spaces, while some acini displayed a high number of intracellular vacuoles. Additionally, ducts were observed to be expanded with accumulated secretions. The presence of collagen fibers, infiltration by inflammatory cells, overloaded blood vessels, and hemorrhaging among the acini were also noted.

El-Agamy et al. [31] suggest that the vacuolization observed in the cytoplasm of certain epithelial or acinar cells could stem from cytoplasmic

deterioration that leads to the formation of voids, or from the buildup of fat droplets. This fatty degeneration, resulting in empty spaces, may be attributed to unused fatty acids, a consequence of cellular malfunction.

Methotrexate triggers the generation of reactive oxygen species, which harm the DNA of epithelial cells, initiating a cascade of biological responses. This includes the activation of nuclear factor-kappa B, culminating in the release of inflammatory cytokines that lead to tissue damage [32]. The interaction of these reactive species with biological macromolecules correlates with the creation of lipid peroxides, impairment of cellular function, membrane breakdown, and the production of deactivated proteins and mutated DNA [33]. Pro-inflammatory cytokines and reactive oxygen species both play a vital role in the onset of mucositis [34].

Some antineoplastic drugs' mode of action during cancer chemotherapy involves the production of free radicals which further causes cellular damage and necrosis in cancer cells. As a result, taking antioxidant supplements helps patients tolerate potentially greater effective chemotherapy doses, which increases the likelihood of a stronger tumour response and a higher survival rate. So that, co-administration of antioxidant during chemotherapy has been highly controversial topic [35].

Antioxidants defend the body against the damaging effects of free radicals and reduce or repair the damage through slowing down or stopping cellular damage and are essential for oxidative stress prevention [36]. As a consequent of previous researches, the present investigation suggested that the antioxidant could have preventive role during methotrexate treatment. Pomegranate peel extract and pumpkin seed oil were chosen as antioxidant agents [37] in the present study to evaluate their preventive role against the negative effect of MTX.

Pomegranate peel extract (PPE) is a valuable source of bioactive compounds such as carbohydrates, minerals, and bioactive substances like flavonoids, ellagitannins, phenolics, and proanthocyanidins. Poly-phenols and flavonoids have antioxidant properties that include scavenging reactive species, modifying enzymes to interfere with cell signalling, and oxidative stability [38].

This study found that pre-treatment with PPE prior to administering MTX (PPE+MTX) significantly enhanced the histological condition of the lingual mucosa in rats treated with MTX. Notably, there was a restoration of normal epithelial thickness of the dorsal surface of the tongue, a decreased extent of disintegration and separation of the collagen fibers in the lamina propria, and a reduction in the infiltration of inflammatory cells. Minor salivary glands showed almost normal histological picture and their ducts showed normal appearance with normal potent lumen.

The outcomes align with the findings of ***Bassiri-Jahrom et al.*** [39], who determined that pomegranate peel extract acts as an in vivo suppressor of oral candida infections, making it a promising candidate for research into novel anti-candida agents. Additionally, ***Al-Gareeb and Mohammed,***[40] investigated the liver-protective properties of pomegranate against acute liver injury caused by methotrexate. Their research indicated that pomegranate lessened ROS and inflammation, and ameliorated the histological alterations induced by MTX.

These marked improvement in the histological structures of the lingual mucosa of rats that received PPE could be explained as a plentiful source of organic antioxidants, immunostimulants, cancer-fighting, anti-atherosclerosis, anti-inflammatory and anti-microbiota agents [41]. Pomegranate peel extract reduces oxidative stress, which in turn reduces cell death and tissue fibrosis in wounded tissues.

It includes phenolic compounds that can prevent lipid peroxidation by activating antioxidant enzymes including glutathione reductase, glutathione peroxidase, and sodium oxide dismutase or by scavenging free radicals. This impact will maintain the level of both enzymatic and non-enzymatic antioxidants [42].

Pumpkin seed oil (PSO) has a variety of anti-tumor, antioxidant, antibacterial, anti-diabetic, and anti-obesity properties. They are a good source of phenolic compounds like vanillic acid, tyrosol, and vanillin, as well as high levels of selenium and lutein. They also contain, phytosterols, carbohydrates, minerals, and proteins [13].

In the present investigation, PSO-treated rats before MTX administration (PSO+MTX) showed partial improvement in their histological picture of lingual mucosa of rats treated with MTX. The epithelium of the dorsal surface of the tongue partially retain thickness comparing to MTX group, filliform showed partial regain of the their number, decreasing in inflammatory in filtration, connective tissue fibers of lamina propria regained their density and arrangement. A number of mucous acinar cells exhibited cystic changes, while some serous acinar cells displayed signs of vacuolization.

These results correspond with those of ***Kassab et al.*** [43], who found that pumpkin seed oil may play a protective role in mitigating damage to the mucosa of the tongue caused by orlistat in adult male albino rats. Furthermore, this is consistent with the research by ***Siham et al.*** [44], who explored the protective effects of pumpkin seed oil on nephrotoxicity induced by methotrexate in rats. Their study revealed that PSO administration significantly reduced nephrotoxicity caused by methotrexate in rats, attributed to its antioxidant and anti-inflammatory properties.

Pumpkin seed oil greatly reduces the severity of lipid peroxidation and increases the activity of antioxidant enzymes this due to high amount of tocopherol (vitamin E) was thought to be the main cause of antioxidant effect. It is also a strong scavenger of peroxyl radicals which can shield biological cell membranes from the negative effects of free radicals. It inhibits DNA oxidative damage by neutralizing the increased generation of reactive oxygen species [45].

PCNA plays a critical role in the regulation of the cell cycle, as well as in DNA replication and repair. The expression of PCNA has been suggested as a possible biomarker for evaluating the proliferation rate in tissues [46]. The current study's immunohistochemistry findings showed that the MTX group's PCNA immunoexpression was noticeably lower than that of the control group showing that MTX directly inhibits DNA replication and mucosal cell proliferation, resulting in a low rate of cellular proliferation.

These results are corroborated by ***Ahmed et al.*** [29], which indicated that MTX diminishes the proliferative ability of epithelial cells, consequently leading to a thinner epithelium. This reduction is due to the impaired renewal capability of the basal epithelium. MTX acts by hindering the synthesis of purine and pyrimidine through its competitive binding to dihydrofolate reductase, resulting in DNA damage and triggering apoptosis [47].

This study demonstrates that PPE enhances the proliferation of epithelial cells, as evidenced by the statistical data from the PPE+MTX group, which recorded the highest mean value of 24.84±1.35. This finding is in line with the research of ***Ricci et al.*** [48], which showed a link between the use of pomegranate peel extract and the regulation of critical cell functions such as cell growth and differentiation. Their study also highlighted the strong antioxidant

properties of pomegranate peel extract and its effectiveness in neutralizing free radicals.

On the other hand, PSO+MTX enhanced the epithelium's ability to proliferate but not enough to improve degeneration caused by MTX that indicated statistically as mean value of PSO+MTX (13.35±1.52). PSO partially increased thickness of epithelium when compared to MTX group that indicated histologically.

Pumpkin seed oil contains active ingredients such as fatty acids, tocopherols, and phytosterols. These bioactive components contribute to the oil's regenerative abilities, offering a connective tissue matrix that aids in complete re-epithelialization and the migration of fibroblasts. Moreover, vitamin E in pumpkin oil is an antioxidant and prevents cell degeneration, it helps the oil's healing properties by promoting DNA synthesis [49]. Accordingly, the present investigation revealed that PPE were potent than PSO through increasing proliferation rate of epithelium and consequently improving the degenerated lingual mucosa , which was confirmed by histological results.

CONCLUSIONS

Pomegranate peel extract has more potential preventative effect against MTX cytotoxicity than pumpkin seed oil.

RECOMMENDATIONS

- More researches utilizing combination of pomegranate peel extract and pumpkin seed oil are recommended as a trial to reinforce their preventive effects
- Further studies using different dose concentration of pomegranate peel extract and pumpkin seed oil to determine the perfect preventive effective dose.

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