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SALVADORA PERSICA AQUEOUS EXTRACT PROMOTES HEALING OF 5-FLUOROURACIL INDUCED ORAL MUCOSITIS IN RATS: A POSSIBLE ROLE OF KGF

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ABSTRACT

Oral hygiene decreases severity of chemotherapy induced oral mucositis. Miswak (*Salvadora persica*) is the most widely used chewing stick for oral hygiene. This study examines the preventive action of aqueous *Salvadora persica* (*S. persica*) extract against 5-FU induced mucositis. Aqueous *S. persica* (500 mg/kg) were introduced orally two times daily from day 1 to day 8. Mucositis was induced using single IP injection of 5-FU (150 mg/kg) at 5th day of study. According to WHO grading system 67 % of rats treated with 5-FU showed mucositis while 0% of *S.persica* treated rats expressed severe mucositis ($P \leq 0.05$). *S.persica* showed no significant changes in measured antioxidant markers. Also myeloperoxidase didn't decrease significantly by *S.persica*. *S.persica* induces significant ($P \leq 0.05$) increase in keratinocyte growth factor (KGF) in mucosa tissues compared to 5-FU. Oral care using aqueous *S.persica* extract promotes healing of 5-FU induced mucositis in part due to induction of KGF in mucosa tissue.

KEYWORDS

Oral mucositis, *Salvadora persica*, Keratinocyte growth factor and Reactive oxygen species.

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INTRODUCTION

Oral mucositis is one of complications induced by radiation and administration of anti-cancer drugs, such as 5-Fluorouracil (5-FU)^{1,2}. The prevalence of mucositis in chemotherapy treated patients is approximately 40%, raised to more than 50% in high-dose chemotherapy protocols³.

5-FU is a pyrimidine analog which used as chemotherapeutic agent for different type of

tumors⁴. Including anal, breast, colorectal, esophageal, stomach, pancreatic and skin cancers⁵. Incorporation of 5-FU in chemotherapy protocols is associated with oral mucositis³.

Oral mucositis is an inflammatory change in oral mucosa. It is a painful condition that significantly affects patients' quality of life⁶. Anti-neoplastic drugs induce direct epithelial cell injury starting with DNA strand breaks concurrently with production of reactive oxygen species (ROS)⁷. Amplification of tissues injury is done through production of proinflammatory cytokines such as tumor necrosis factor α (TNF- α), Interleukin 1 β (IL-1 β), and Interleukin 6 (IL-6)^{8,9}.

Although clinical features of oral mucositis are mainly result from oral epithelial injury, endothelial cells may have an important role in this injury. Endothelial cells produce growth factors which trigger epithelial cells to grow and differentiate. Keratinocyte growth factor (KGF) was identified as a central molecule in communication between endothelial and epithelial cells¹⁰.

Oral hygiene is critical for patients receiving chemotherapy to remove any source of infection that may be life threatening in those patients¹¹. Preventing infection plays important role oral mucositis severity. Bacteria, fungi and viruses can superimpose secondary infections on the damaged mucosa. In severe stages micro-organisms may entrapped into the circulation resulting in life-threatening septicemia, especially in myelosuppressed patients¹².

Different antimicrobial agents were investigated for their efficacy in preventing and/or reducing mucositis severity¹³. Topical antimicrobial lozenges containing polymyxin, tobramycin, and amphotericin B reduce oral mucositis induced by radiation therapy^{14,15}. Similarly, oral rinse containing antimicrobial agents such as chlorhexidine reduce the severity of oral mucositis.

Oral hygiene has a potential role in preventing or decreasing severity of mucositis induced by chemotherapy¹⁶. Miswak (*Salvadora persica* L.) is the most widely used chewing stick for oral hygiene

in middle -eastern and eastern African cultures, which is prepared from the roots or stems of *Salvadora persica* L. (*S. persica*)¹⁷. World health organization¹⁸ reported that *Salvadora persica* L plays a role in the promotion of oral hygiene.

The aqueous extracts of *S. persica* contains important phytoconstituents such as vitamin C, salvadorine, salvadorene, alkaloids, trimethylamine, cyanogenic glycosides, tannins, saponins and salts mostly as chlorides^{19,22}. Moreover, it has been reported that *S. persica* aqueous extract contains potential antimicrobial anionic compound such as CL, SO₄ and SCN¹⁷.

Different studies reported immediate antimicrobial effect of *S. persica* aqueous extract on cariogenic bacteria in vitro and in patients^{23,24}. *S. persica* antibacterial activity were extensively studied in different studies. These studies documented that *S. persica* is effective against most pathogen found in oral cavity including *Porphyromonas gingivalis*²⁵, *Staphylococcus aureus*, *Streptococcus mutans* and *Candida albicans*²⁶. In addition, 10% aqueous extract of *S. persica* is an effective antimicrobial agent when utilized clinically as an irrigant in the endodontic treatment of teeth with necrotic pulps²⁷. This study investigate the use of *S. persica* as a potential agent for oral hygiene in preventing or decreasing the incidence of oral mucositis induced by 5-fluorouracil. Potential targets of possible healing effect will also be evaluated.

MATERIAL AND METHODS

Animals

Adult male Wistar albino rats weighing 100-150 gm were obtained from animal house of National Research Center (Dokki, Giza, Egypt) and housed in a pathogen-free facility in 6 wire mesh plastic cages with Sawdust bedding. The facilities were maintained at 25 \pm 2 °C, relative humidity of approximately 50% and a 12-hr light: dark cycle. All rats were fed standard diet and water *ad libitum*. The experiment was performed in accordance with ethical guidelines of internationally accepted principals for laboratory use and care in animal research (Health research extension act of 1985).

Also the study protocol followed the Damanhour University (Egypt) guideline for the use and care of animals.

Plant material and extract preparation

The stems of *S.persica*. (Meswak) were purchased from the local market of makka, Saudi Arabia. The plant was re-identified by a department of pharmacognosy, faculty of pharmacy. Damanhour University. The plant name has been checked with www.plant list.com.

S. persica extract was prepared according to the method of¹⁷ briefly. The sticks were grinded. 10 grams of the powdered stem were transferred to sterile wide-mouthed screw-capped bottles. Sterile de-ionized distilled water was added to the powdered stems until the volume reached to 100 ml. The mixture were allowed to soak for 24 h at 4°C then centrifuged at 2000 rpm for 10 min at 4°C. The supernatants were filtered freeze dried and reconstituted upon use. Extract is then injected orally at dose 500 mg/kg²⁸ at volume of 0.5 ml/100 g b.w.

Drugs and chemicals

5-FU ampoules (Utoral) was obtained from EMC united pharmaceutical (Cairo, Egypt). All other chemicals used were of the highest quality and analytical grade.

Induction of Mucositis and treatment regimens

Thirty male Wister rats were used during the study. Rats were divided into 3 groups (10 rats each) and treated as follow: The first group (S EXTgroup): Rats were given aqueous *S persica* extract by oral gavage at a dose 500 mg/kg twice a day (from days 1–8)²⁸. Rats of this group were also injected with single I.P injection of 5-FU (150 mg/kg)²⁹ on the 5th day and right cheek pouch was scratched with a wire brush to induce mucositis. The second group (5-FUgroup): Rats were injected with single I.P injection of 5-FU (150 mg/kg)²⁹ on the 5th day and right cheek pouch was scratched with a wire brush to induce mucositis. The third group (Control group): Rats were gavaged with single I.P injection of saline on the 5th day (Figure No.2). All animals were scarified at the 9th day and blood samples immediately obtained via cardiac puncture and

collected into uncoated tubes and allowed to clot at room temperature for 60 min. The samples were then centrifuged (3000 x g, 10 min, 4°C), and the resultant serum in each supernatant was recovered and stored at -20°C until analysis.

Assessment of Mucositis damage

Mucositis was graded at the end of treatment by an independent observer who was blinded to the treatments according to World Health Organization (WHO) grading system³⁰ for mucositis as follow:

| Grade | Description |
|-----------------------|---|
| 0 (none) | None |
| I (mild) | Oral soreness, erythema |
| II (moderate) | Oral erythema, ulcers, solid diet tolerated |
| III (severe) | Oral ulcers, liquid diet only |
| IV (life-threatening) | Oral alimention impossible |

Preparation of mucosa homogenates

Mucosa tissues of the left pouch were desiccated and kept frozen under -80°C. 0.25 gm of frozen tissues was used to prepare 10% homogenate in phosphate saline buffer centrifuged at 3000 rpm for 10 min at 4°C the obtained supernatant was used for biochemical analysis.

Assay of oxidative stress in mucosa tissue

Oxidative stress in mucosa homogenate was assayed through measuring markers of oxidative stress " lipid peroxidation and superoxide dismutase". Lipid peroxidation was measured as the level of malondialdehyde (MDA) determined by the thiobarbituric acid (TBA) reaction according to method of Satoh,³¹ using kits from (Biodiagnostic, Giza, Egypt -CAT No.MD2529).

Superoxide dismutase (SOD) activity in mucosa tissues was measured based in reaction with reduced phenazine methosulfate and molecular oxygen according to the method of Nishikimi *et al.*³² using kits from (Biodiagnostic, Giza , Egypt - CAT No.SD 2521).

Assessment of leukocyte involvement

Myeloperoxidase (MPO) activity was assessed as a marker of neutrophil infiltration using enzyme linked immunoassay (ELISA) kit according to

manufacturer instruction (china). The detection limit of the kit (0.7-20 ng/ml).

Assay of keratinocyte growth factor (KGF)

Serum and mucosa level of KGF was assayed using enzyme linked immunoassay (ELISA) kit according to manufacturer instruction (wkeamed supplies, china). The detection limit of the kit (20-800 ng/L).

Histopathological analysis

Mucosa tissues of right pouch were removed for histopathological examination. Mucosa tissues were fixed in 10% formol saline for twenty four hours. The samples were embedded in Paraffin then 4 μ m sections were stained by hematoxylin and eosin³³ and examined blindly by histopathologist.

Statistical analysis

Data analysis was performed using the Graphpad Prism version 6 (Graphpad software, San Diego, CA, USA). Results were expressed as mean \pm standard error (SE). Statistical significant difference was determined by unpaired *t*-test. Categorical variable were compared using Fisher's exact test (two-sided). A probability value of $P \leq 0.05$ was considered statistically significant.

RESULTS

***S. persica* extract decreases incidence of mucositis:**

According to WHO grading system, control rats showed no evidence of mucositis (Figure No.2A) where 67% Rats treated with 5-FU showed mucositis (grade 3) and 33% showed moderate mucositis (grade 2) evidenced by erythema and ulceration (Figure No.2 B). *S.persica* decreased the mucositis severity significantly ($P \leq 0.05$) 83% of *S.persica* treated rats showed moderate mucositis (grade 2) while 17% showed mild mucositis (grade 1) (Figure No.2C) (Table No.1).

***S. persica* extract improves histological damage induced by 5-FU in rat buccal mucosa**

Control rats showed normal histological structure of the stratified squamous keratinized epithelium of the lining mucosa with the underlying lamina propria and muscular layer (Figure No.2D). Rats treated with 5-FU showed hyperkeratosis and acanthosis of the vacuolized cellular epithelial

mucosal layer with finger like projections (Figure No.2E.). Rats treated with *Salvadora persica* extract showed only edema in the lamina propria (Figure No.2F.). The severity of histopathological alteration on buccal mucosa in different treatment groups are shown in Table No.2.

Effect of *S. persica* extract on mucosa content of antioxidant markers (MDA and SOD)

Rats treated with 5-FU showed significant ($p \leq 0.05$) increase on mucosa content of MDA (241.3 \pm 6.7) and marked decrease in SOD (235.6 \pm 27.5) activity ($p \leq 0.001$) compared to control rats (188.3 \pm 8.025) and (1678 \pm 81.9) respectively. Rats treated with *S. persica* extract showed a non-significant change in MDA or SOD activity in mucosa compared to 5-FU rats (Figure No.3a and b).

Effect of *S. persica* extract on mucosa content of MPO

Rats treated with 5-FU showed more than two fold increase in MPO mucosa content compared to control rats ($P \leq 0.01$), while rats treated with *S. persica* showed a non-significant decrease in MPO mucosa content compared to 5-FU rats (Figure No.4).

***S. persica* extract induces KGF expression in mucosa tissues**

Rats treated with 5-FU group showed significant ($P \leq 0.001$) decrease in KGF expression (77.8 \pm 7.8) compared to control rats (142.4 \pm 3.8). Rats treated with *S. persica* extract showed a significant ($P \leq 0.05$) increase in KGF expression in mucosa (115.2 \pm 8.4) compared to 5-FU treated rats (Figure No. 5A).

Effect of *S. persica* extract on serum level of KGF

Rats treated with 5-FU showed significant ($P \leq 0.01$) decrease in serum KGF (46.4 \pm 2.6) compared to control rats (60.2 \pm 2.9) where rats treated *S. persica* extract showed a non-significant change in KGF mucosa level compared to 5-FU treated rats (Figure No.5B).

DISCUSSION

Management of oral mucositis includes three main arms. The first is general oral care including oral

hygiene protocols³⁴. The second is prevention using many protective pathways such as anti-inflammatory drugs^{35,36}, ROS inhibitors³⁷, infection prevention¹⁵ and growth factors such as palifermin (recombinant keratinocyte growth factor 1)³⁸. The third arm is palliative care including avoidance of alcohol, tobacco, spicy food and analgesic¹¹.

S.persica is one of the most commonly used medicinal plants for oral hygiene among global muslim community³⁹. World Health Organization¹⁸ approved *S.persica* use for oral hygiene (WHO, 2000)¹⁸. In addition, the antibacterial activity of *S.persica* had been extensively studied previously^{25,26}.

This study evaluated the use of *S.persica* aqueous extract in preventing oral mucositis induced by chemotherapy. The induction of mucositis was based on using single dose of 5-FU in the fifth day of study then scarifying animal after 3 day of 5-FU injection²⁹. 5-FU induces sever mucosal damage expressed as erythema, congestion and ulceration. Histological examination of mucosal tissues showed damage of the cellular epithelial layer. On the other hand, rats treated with *S.persica* extract express less inflammation, edema and relatively intact epithelial layer.

S.persica antiulcer activity was previously reported in other studies. It has been reported that lyophilized decoction of *S.persica* roots possesses a significant protective effect on ulceration induced by ethanol, indomethacin and cold restraint stress in rats⁴⁰. In addition, it has been reported that *S.persica* exerted antiulcer effect in different ulcer model^{41,40}. However, it is not clear if *S.persica* antiulcer effect is due to preserving oral hygiene and preventing secondary infection or other mechanism may be involved.

For clarification of anti-mucositis effect exerted by *S.persica* we evaluated the antioxidant activity of *S.persica* extract. Our results showed that aqueous extract doesn't affect oxidative stress induced by chemotherapy. It doesn't inhibit lipid peroxidation nor increase SOD activity in mucosa tissues. These results pointed out that antiulcer effect achieved by

S.persica is not mediated through antioxidant activity.

In contrast to our results, different studies clearly indicated that *S.persica* having effective antioxidant activity^{42,43}. However, it should be noted that these studies use hydroalcoholic or methanolic extracts.

MPO is an enzyme found in primary granules of polymorpho nuclear neutrophils and used as an index for inflammation severity⁴⁴. It is well-known that this enzyme is increased in 5-FU induced mucositis⁴⁵. *S.persica* treatment resulted in reduction of MPO activity in oral mucosa compared to 5-FU treated rats however this decrease didn't reach statistical significance. This result revealed that *S.persica* aqueous extract didn't have anti-inflammatory activity. This was in contrast with results of Ibrahim and his colleagues⁴⁶ who reported an ant inflammatory activity of *S.persica* ethanolic extract. There is a paucity of data detecting effect of *S.persica* on MPO activity.

The present study examined the effect of *S.persica* on keratinocyte growth factor. KGF first described in 1989 as a growth factor that stimulates the proliferation of mouse keratinocytes^[47]. Our results showed that *S.persica* extract increased KGF expression in mucosa tissues while couldn't affect KGF serum level. KGF has been reported to prevent the epithelial cells from radiation damage⁴⁸. The recombinant protein of human KGF (Palifermin) has been used to treat the acute mucositis induced by chemoradiotherapy⁴⁹. Induction of KGF in mucosal tissues may be in part responsible for healing action exerted by *S.persica*. To the best of our knowledge there is a paucity of data reporting effect of *S.persica* on KGF. Further studies using KGF receptor antagonist may help to confirm the role of KGF in *S.persica* extract induced mucositis healing.

Table No.1: Incidence and severity of oral mucositis induced by 5-FU in all rats groups

| S.No | Grade | Control | 5-FU* | S EXT [▪] |
|------|---------|---------|-------|--------------------|
| 1 | Grade 0 | 100% | 0% | 0% |
| 2 | Grade 1 | 0% | 0% | 17% |
| 3 | Grade 2 | 0% | 33% | 83% |
| 4 | Grade 3 | 0% | 67% | 0% |
| 5 | Grade 4 | 0% | 0% | 0% |

* Significant compared to control rats ($P \leq 0.05$) (fisher's exact test)

▪ Significant compared to 5-FU rats ($P \leq 0.05$) (fisher's exact test)

Table No.2: The severity of the histopathological alteration of buccal mucosa in different group

| Histopathological alteration | Group No | Control | 5-FU Group | S EXT Group |
|--|----------|---------|------------|-------------|
| Epidermis | | | | |
| • Hyperkeratosis | | -- | +++ | -- |
| • Acanthosis | | -- | +++ | -- |
| • Cellular vacuolization | | -- | ++ | -- |
| Dermis | | | | |
| • Focal inflammatory cell infiltration | | --- | ++ | -- |
| • Edema | | -- | +++ | ++ |
| • Congestion | | -- | +++ | -- |

| | |
|----------|-----|
| Severe | +++ |
| Moderate | ++ |
| Mild | + |
| Nil | -- |

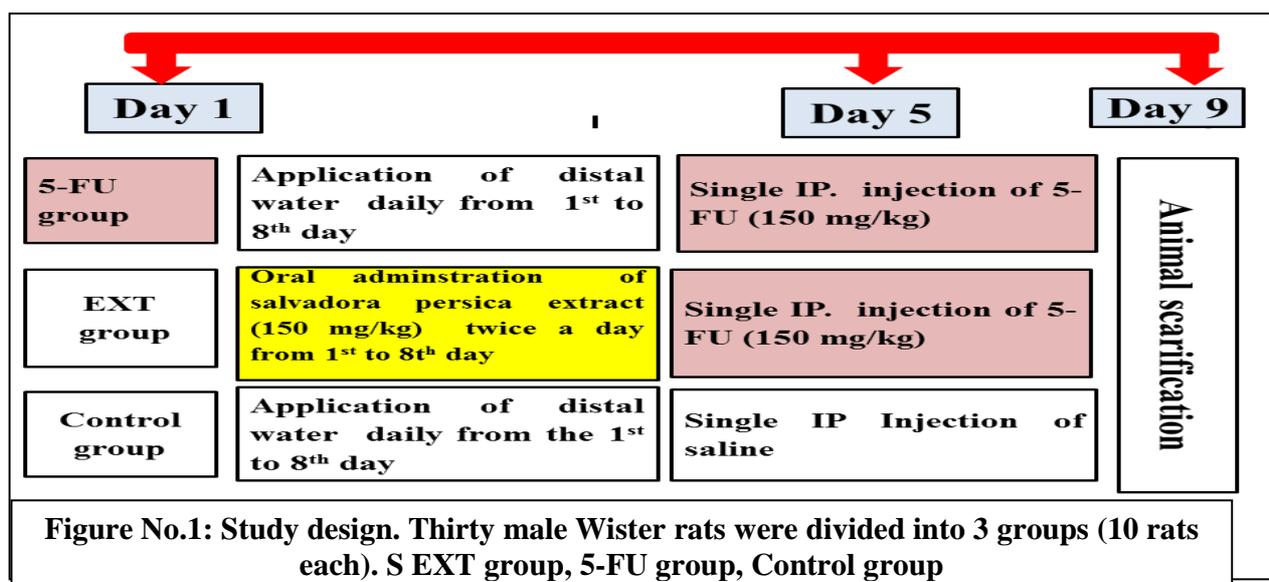


Figure No.1: Study design. Thirty male Wister rats were divided into 3 groups (10 rats each). S EXT group, 5-FU group, Control group

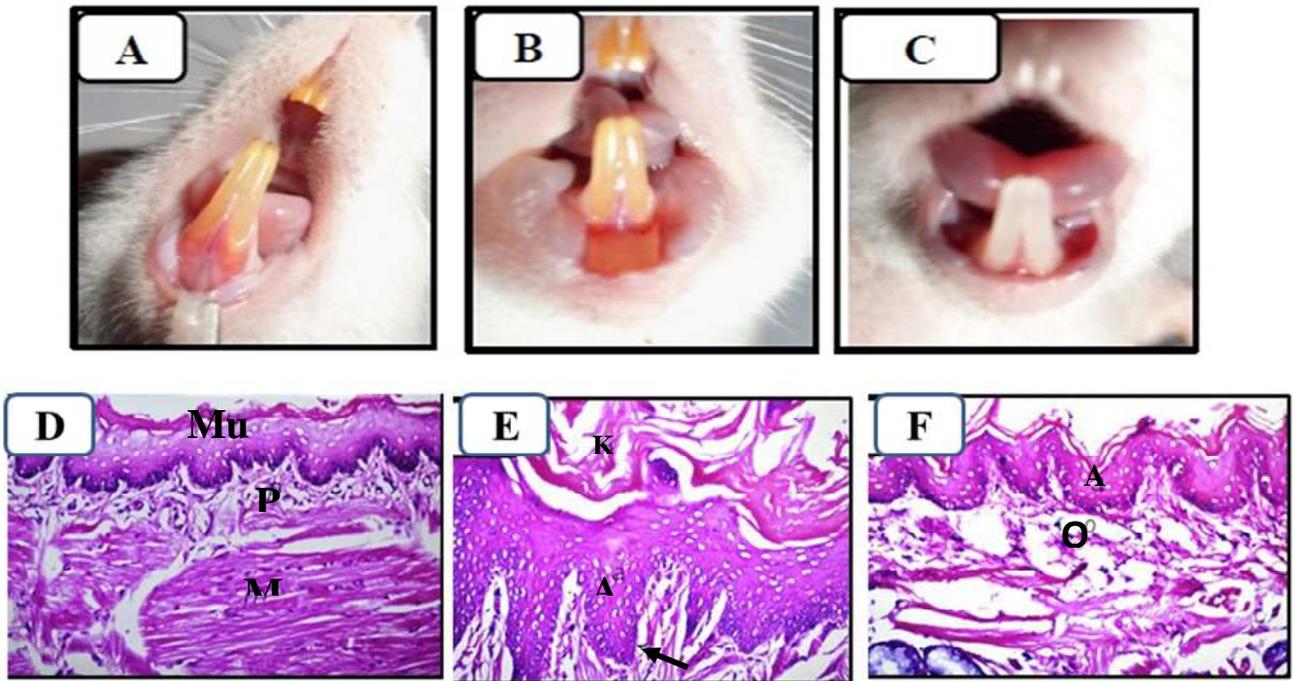


Figure No.2: Rats showing different grades of mucositis. Figure No.2A: Rat from control group showed grade 0 mucositis Figure No.2B: Rat from 5-FU group showed moderate to severe mucositis (grade 2-3). Figure No.2C: Rats from S EXT group showed mild to moderate mucositis (grade 1-2). Figure No.2D: Histological section from control rats showed normal histological structure of the stratified squamous keratinized epithelium (mu) of the lining mucosa with the underlying lamina propria (p) and muscular layer (m). Figure No.2E: Histological section from rats treated with 5-FU showed Hyperkeratosis (k) and acanthosis (a) of the vacuolized cellular epithelial mucosal layer with finger like projections (arrow) protruded in the lamina propria Figure No.2F: Rats treated with *S.persica* extract showed only edema (O) in the lamina propria

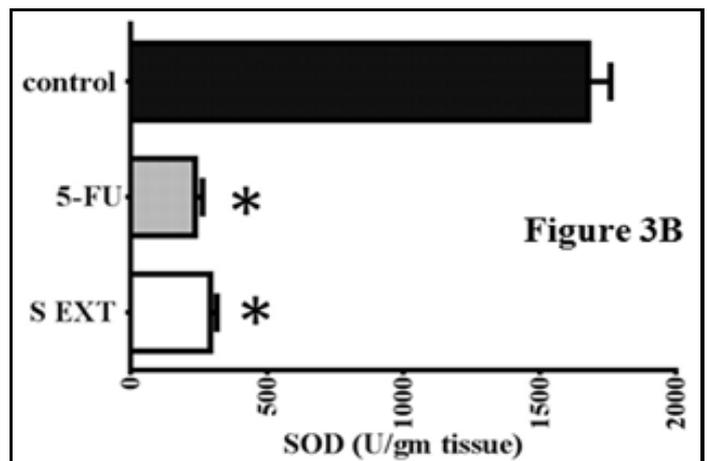
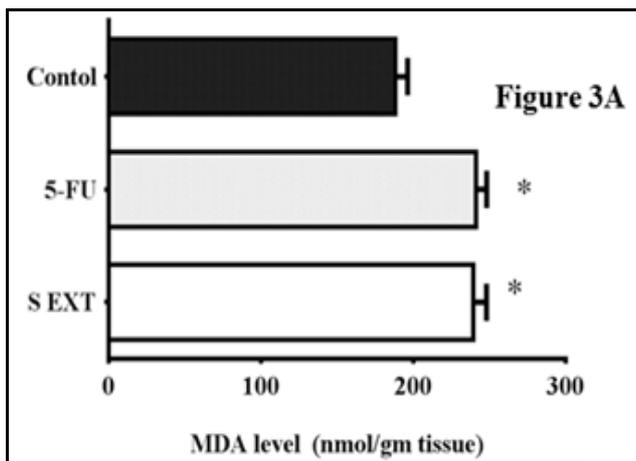


Figure No.3: Changes of MDA (Fig3A) and SOD (Fig3B) in mucosa tissues
 * Significant compared to control rats. $p \leq 0.05$

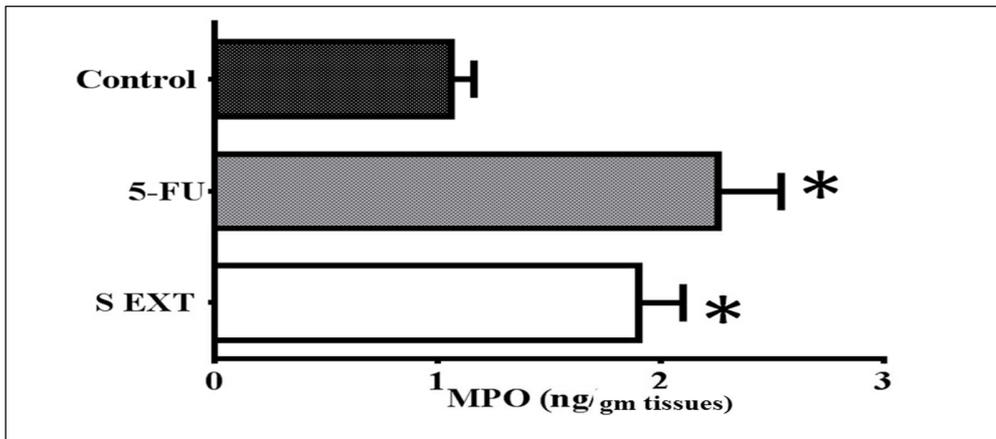


Figure No.4: Changes of MPO in mucosa tissues
* Significant compared to control rats. $p \leq 0.05$

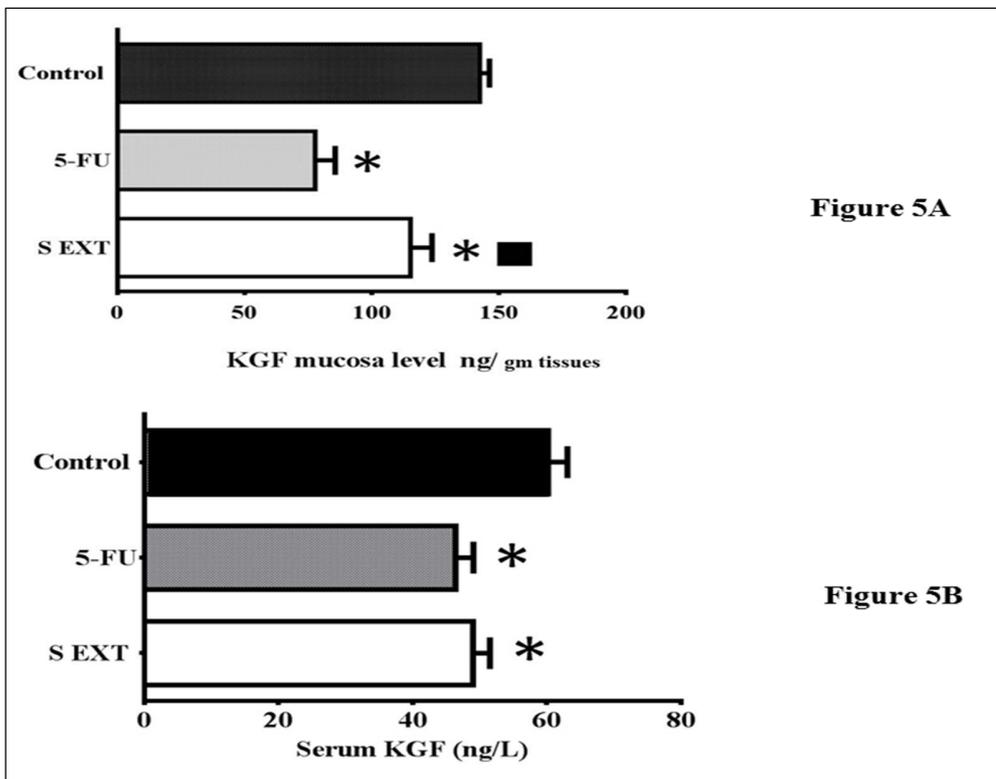


Figure No.5: Changes of KGF in mucosa tissues (Figure No.5A) and KGF in serum (Figure No.5B)
* Significant compared to control rats. $p \leq 0.05$
■ Significant compared to 5-FU rats. $p \leq 0.05$

CONCLUSION

Chemotherapy induced oral mucositis is a painful dose limiting toxicity. Oral hygiene prevents secondary infection and decrease severity of

mucositis. Oral care using aqueous extract of *S.persica* ameliorated severity of 5-FU induced mucositis. This healing effect induced by *S.persica* was not associated with antioxidant or anti-

inflammatory activity however it was correlated with induction of KGF expression in mucosa tissues. The exact role of KGF in *S.persica* healing activity needs further investigation using KGF antibody. Preserving oral hygiene using *S.persica* extract may be therapeutic alternative for decreasing severity of chemotherapy induced mucositis.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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