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The role of *Hippophae rhamnoides* L. on 5-fluorouracil-ınduced oral mucositis in rats

Muhammet Dilber[1], Bahadir Suleyman[2], Renad Mammadov[2], Zeynep Suleyman[2], Bulent Yavuzer[2], Mine Gulaboglu[3], Ertugrul Erhan[4], Ismail Salcan[4] and Halis Suleyman[2]

[1]Dilber ENT & Aesthetic Clinic, Istanbul, Turkey
[2]Department of Pharmacology, Faculty of Medicine, Erzincan Binali Yildirim University, Erzincan, Turkey
[3]Department of Biochemistry, Faculty of Pharmacy, Ataturk University, Erzurum, Turkey
[4]Department of Otorhinolaryngology, Faculty of Medicine, Erzincan Binali Yildirim University, Erzincan, Turkey

Abstract: Current study aimed to research the effect of *Hippophae rhamnoides* (HRE) on potantial oral oxidative and inflammatory damage of 5-FU in rats. The rats were assigned to three groups; healthy (HG), 5-FU 100mg/kg (FUG) and HRE 50mg/kg +5-FU 100mg/kg (HRFU). The 5-FU was injected in the FUG group intraperitoneally. The HRFU was injected 5-FU at 100mg/kg IP one hour after the 50mg/kg HRE was given orally. Olive oil was used as a solvent for the HG. HRE was given to the rats three times a day for ten days. 5-FU was given one dose on the 1st, 3rd and 5th days. On the 10th day, the tissues removed from the animals were euthanized with high-dose anaesthesia and were macroscopically examined. The levels of the oxidant, antioxidant and proinflammatory cytokines were investigated.It was seen that HRE alleviated the symptoms of severe mucositis by antagonizing the effects of 5-FU on oxidant, antioxidant and proinflammatory cytokines such as malondialdehyde, total glutathione, superoxide dismutase, catalase, nuclear factor kappa-B and interleukin-6 in inner cheek and tongue tissue. These results recommend that HRE may be benefical in the cure of 5-FU-associated oral mucositis.

Keywords: 5-Fluorouracil, *Hippophae rhamnoides* L. oral mucositis, oxidative and inflammatory, rats.

INTRODUCTION

5-Fluorouracil (5-FU) is an antimetabolite utilized as a chemotherapy drug (Dos Santos, 2022). 5-FU's mechanism of action is related to interaction with DNA synthase and inactivation of thymidylate synthase. 5-FU inhibits DNA synthesis by limiting the availability of thymidylate during the S phase of cell cycle (Shiga & Hiraide, 2020). 5-FU is used alone or in combination with other chemotherapeutics in the cure of various cancers(Cunningham *et al*., 2017; Jahani *et al*., 2019). Although generally it is well tolerated, therapy-related toxicity remains a source of deep concern in using 5-FU (Diasio & Offer, 2022). As it is known, the 5-FU has many side effects that reduce the patient's quality of life and sometimes interrupt the treatment. The most common side effects were reported to be fever, tiredness, nausea, diarrhea, vomiting, oral and intestinal mucositise (Vodenkova *et al*., 2020). Oral mucositis is the most significant side effect of 5-FU treatment (Takeuchi, 2020) Oral mucositis is inflammation of the oral cavity with ulceration, which is unfortunately common in patients (Fonseca *et al*., 2021). The increase in the manufacture of reactive oxygen species (ROS) is liable for basicallyof 5-FU-associated oral mucositis. It is also stated to rise the expression of inflammatory factors by activating the NF-κB pathway (Cao *et al*., 2022). The literature indicates that antioxidant drugs that inhibit the increase in proinflammatory cytokine production may be benefically in the cure of 5-FU-induced oral mucositis (Sun *et al*., 2022).

In our study, the antioxidant, anti-inflammatory, antiulcer and pro-inflammatory cytokine antagonist properties of Hippophae Rhamnoides plant fruit extract (HRE), aimed to investigate the protective effect of 5-FU against possible oral damage in rats, are known. It is thought that the vitamins, carotene, triterpenoids, glycosides, flavonoids, alkaloids in the content are what give HRE these properties (Han *et al*., 2021). Kubczak et showned that vitamins, amino acids and phenolic compounds in the content of HRE show strong antioxidant activity (Kubczak *et al*., 2022). In the literature reported that HRE protects the heart tissue from oxidative damage (Gumustekin *et al*., 2010). It has also reported that HRE prevents methotrexate-related oxidative and inflammatory cheek, lower lip and tongue tissue damage in rats (Kuduban *et al*., 2016). This information suggests that HRE may be effective against possible oxidative and inflammatory oral mucosa damage (oral mucositis) of 5-FU in animals. Also, in internet browsingthere were no studies exploring the effect of HRE against 5-FU-related oral mucositis. Our study aims to investigate the effect of HRE on 5-FU's possible oral oxidative and inflammatory injury in albino Wistar rats, macroscopically and biochemically.

MATERIALS AND METHODS

Animals

In current study, eighteen rats weighing 276 and 290 grams were used, totally. All animals were obtained from

**Corresponding author:* e-mail: halis.suleyman@gmail.com

an Experimental Animals Application and Research Center. Before the experiment, animals were housed in the appropriate laboratory medium at regular room temperature (22°C) and the rats were fed ad libitum. The procedures were meet with approval by the Local Animal Experimentation Ethics Committee (Date 25.08.2022, meeting No.08/40).

Chemicals

In the experiment; thiopental sodium was obtained from IE. Ulagay (Turkiye), *Hippophae rhamnoides* L. from PhytoLab (Russia) and Fluorouracil (1000mg. 20ml, solution for i.v. injection) from Training and Research Hospital (Turkiye) dependented with the Ministry of Health.

Experimental groups

The animals were divided to three groups; a healthy group (HG), administered only 5-FU 100mg/kg group (FUG) and administered HRE 50mg/kg + 5-FU 100mg/kg (HRFU) group.

Experimental procedure

The HRE 50mg/kg was givenby gavage orally into the HRFU (n=6)group. Theolive oil was administered as a solvent into groups of the HG (n=6) and FUG (n=6) . One hour later the administration of HRE, the HRFU and FUG groups were injected 5-FU 100mg/kg IP. HRE was given three times a day for ten days. On the other hand, 5-FU was administered as one dose (3 doses in total) on the first, third and fifth days. On the tenth day, the animals were euthanized with 50mg/kg thiopental sodium and the inner cheek and tongue tissues were extracted and examined macroscopically and biochemically. All results were compared with the FUG group.

Biochemical analyzes

Preparation of samples

After the tissue samples were washed with physiological saline, they were placed in Petri dishes and the tissues were place into dust in liquid nitrogen. To determine the malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and protein levels, tissue samples were homogenized. Supernatants were used for MDA, GSH, SOD and CAT protein analyses.

Malondialdehyde, glutathione, superoxide dimutase and catalase analysis

The define of "malondialdehyde (MDA; µmol/g protein)", "total glutathione (tGSH; nmol/g protein)" and "superoxide dimutase (SOD; U/g protein)" in tissue samples taken will be evaluated with commercial enzyme-linked immunosorbent assay (ELISA) kits which were produced for rats and each analysis will be carried out according to kit instructions (Item no., respectively, 10009055, 703002 and 706002 Cayman Chemical Company). Determination of Catalase (CAT; U/g protein) will be made according to the method proposed by Goth (Goth, 1991). Determination of protein will be determined spectrophotometrically at 595 nm as per the Bradford method (Bradford, 1976).

Nuclear Factor Kappa B and Interleukin 6 analysis

The weight of models judged and at that time cut off we quickly froze them using liquid nitrogen and homogenized them via pestle and mortar; maintained samples at 2-8°C after melting. We added Phosphate-Buffered Saline (PBS) (pH 7.4), 1/10 (w/v) and then vortex for 10 seconds, centrifuge for 20 min at 10,000 xg. were collected carefully. "Nuclear factor-kappa B (NF-κB; pg/L)" was assayed using a Rat Nuclear Factor Kappa B ELISA immunoassay kits (SunRed). The levels of "interleukin 6 (IL-6; ng/L)" was analysed using a trading kit obtained by "Eastbiopharm Co. Ltd. ELISA kit, China".

STATISTICAL ANALYSIS

The results were stated as "mean value ± standard deviation" (x ± SD). The normality test was done with Shapiro-Wilk test. The One way ANOVA test was used to make comparisons between groups. Then, Fisher's LSD (least significant differences) was made as a post-hoc test. The "SPSS for Windows, 25.0 (Armonk, NY: IBM Corp.)" and "GraphPad Prism 8" were used for analysis and the $p < 0.05$ value was accepted significant.

RESULTS

Biochemical Analysis

MDA analysis

As in fig. 1, the level of MDA in the inner cheek and tongue tissues cured with 5-FU augmented seriously in comparison to HRFU and healthy groups ($p<0.001$). MDA levels difference between the HRFU and healthy group was significantly insignificant in the inner cheek ($p=0.860$) and tongue tissue ($p=0.624$).

tGSH analysis

The HRE significantly prevented the decrease of tGSH amount in the inner cheek and tongue tissues with 5-FU ($p<0.001$). The difference in the amount of tGSH between the HRFU and healthy was statistically significant in tongue tissue ($p=0.014$) and insignificant in inner cheek tissue ($p=0.692$) (fig. 2).

SOD analysis

As in fig. 3, the level of SOD in the inner cheek and tongue tissue of animals administered 5-FU was lower than the HRFU and healthy groups ($p<0.001$). The difference in the amount of SOD in HRFU and healthy groups was major in tongue tissue ($p<0.001$) and insignificant in inner cheek tissue ($p=0.367$).

Table 1: Biochemical analysis results in inner cheek and tongue tissue.

Biochemical parameters		Mean±standard deviation			p values		
		HG	FUG	HRFU	HG vs. FUG	HG vs. HRFU	FUG vs. HRFU
Inner check	MDA	1.63±0.17	3.07±0.34	1.70±0.18	<0.001	0.860	<0.001
	tGSH	4.56±0.20	2.46±0.19	4.46±0.21	<0.001	0.692	<0.001
	SOD	5.57±0.42	2.54±0.10	5.36±0.16	<0.001	0.367	<0.001
	CAT	8.89±0.07	4.24±0.08	7.72±0.14	<0.001	<0.001	<0.001
	NF-kB	2.84±0.14	5.48±0.21	3.28±0.28	<0.001	0.009	<0.001
	IL-6	2.14±0.04	4.54±0.09	2.32±0.12	<0.001	0.012	<0.001
Tongue	MDA	1.40±0.16	2.50±0.15	1.48±0.12	<0.001	0.624	<0.001
	tGSH	4.18±0.07	2.17±0.13	3.98±0.12	<0.001	0.014	<0.001
	SOD	6.19±0.09	3.27±0.15	5.80±0.09	<0.001	<0.001	<0.001
	CAT	9.18±0.12	3.63±0.12	7.61±0.21	<0.001	<0.001	<0.001
	NF-kB	3.22±0.07	5.63±0.21	3.42±0.12	<0.001	0.077	<0.001
	IL-6	2.39±0.08	4.77±0.09	2.48±0.08	<0.001	0.135	<0.001

MDA; malondialdehyde, tGSH; total glutathione, SOD; superoxide dismutase, CAT; catalase, NF-kB; nuclear factor kappa B, IL-6; interleukin 6, HG; healthy group, FUG; administered only 5-FU 100mg/kg group, HRFU; administered HRE 50mg/kg + 5-FU 100mg/kg group. Analysis was done by one-way ANOVA and then Tukey or Games-Howel was used as post-hoc.

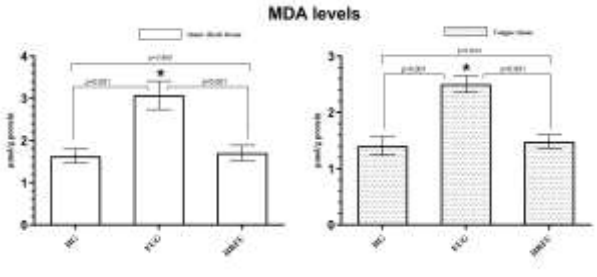

Fig. 1: MDA analysis of inner cheek and tongue tissue in study groups. *p < 0.001, according to HG and HRFU groups. MDA; malondialdehyde, HG; healthy group, FUG; only administered 5-FU group, HRFU; administered HRE 50mg/kg + 5-FU 100mg/kg group

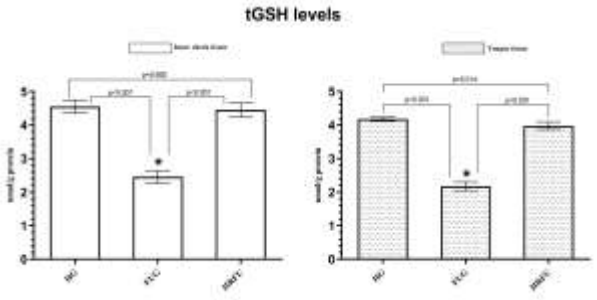

Fig. 2: tGSH analysis of inner cheek and tongue tissue in study groups. *p < 0.001, according to HG and HRFU groups. tGSH; total glutathione, HG; healthy group, FUG; only administered 5-FU group, HRFU; administered HRE 50mg/kg + 5-FU 100mg/kg group.

CAT analysis

The level of CAT in the inner cheek and tongue tissues taken with 5-FU decreased significantly compared to the HRFU and healthy groups (p<0.001). The CAT difference between the HRFU and healthy group was statistically significant (p<0.001) (fig. 4).

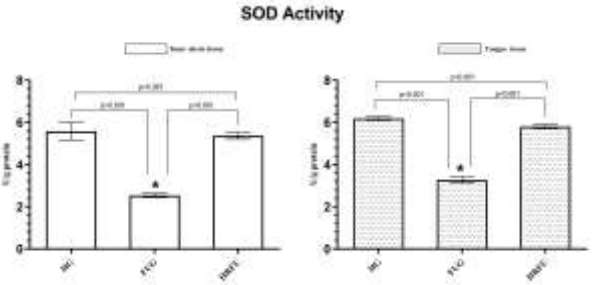

Fig. 3: SOD analysis of inner cheek and tongue tissue in study groups. *p < 0.001, according to HG and HRFU groups. SOD; superoxide dismutase, HG; healthy group, FUG; only administered 5-FU group, HRFU; administered HRE 50mg/kg + 5-FU 100mg/kg group.

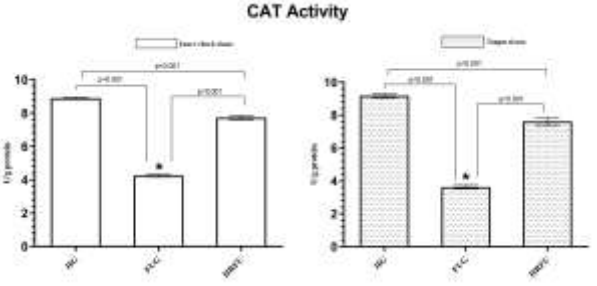

Fig. 4: CAT analysis of inner cheek and tongue tissue in study groups. *p < 0.001, according to HG and HRFU groups. CAT; catalase, HG; healthy group, FUG; only administered 5-FU group, HRFU; administered HRE 50mg/kg + 5-FU 100mg/kg group.

NF-kB analysis

The 5-FU increased the amount of NF-kB in the inner cheek and tongue tissues. However, the HRE significantly (p<0.001) inhibited the increase of NF-kB with 5-FU. The amount of NF-kB was found to be statistically significant

(p=0.009) in the inner cheek tissue and insignificant (p=0.077) in the tongue tissue between the HRFU and healthy group (fig. 5).

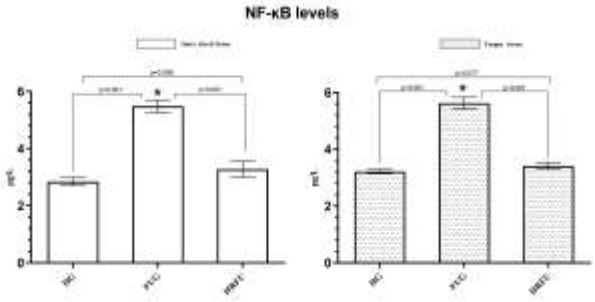

Fig. 5: NF-kB analysis of inner cheek and tongue tissue in study groups. *p < 0.001, according to HG and HRFU groups. NF-kB; Nuclear Factor Kappa B, HG; healthy group, FUG; only administered 5-FU group, HRFU; administered HRE 50mg/kg + 5-FU 100mg/kg group

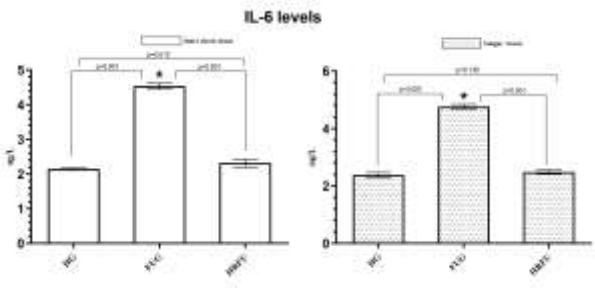

Fig. 6: IL-6 analysis of inner cheek and tongue tissue in study groups. *p < 0.001, according to HG and HRFU groups. **p>0.05, according to HG group. IL-6; interleukin 6, HG; healthy group, FUG; only administered 5-FU group, HRFU; administered HRE 50mg/kg + 5-FU 100mg/kg group.

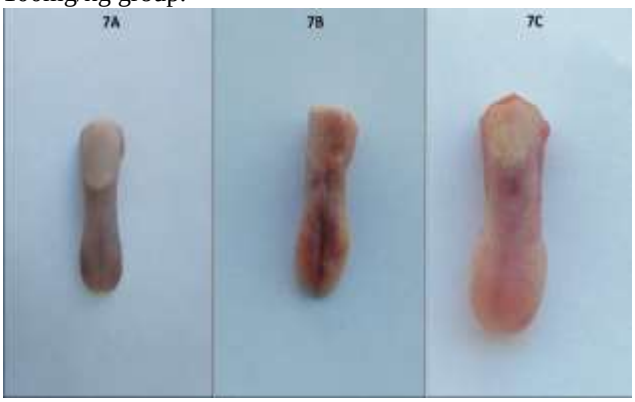

Fig. 7: Histopathological evaluation of tongue tissue in study groups. A. Normal macroscopic appearance of apex, corpus, radix, epithelial layer and papillae in tongue tissue of HG group. B. Edema and swelling in the entire tongue tissue of FUG group, a thickened epithelial layer at the apex part, peelings in the corpus part, hyperemia, hemorrhage and thickened papilla structure with loss of its feature (HxE). C. The near-normal appearance of the apex part of the tongue tissue belonging to the HRFU group, mild edema in the entire tongue tissue, mild hyperemia in the corpus part and moderate papilla loss. (HxE).

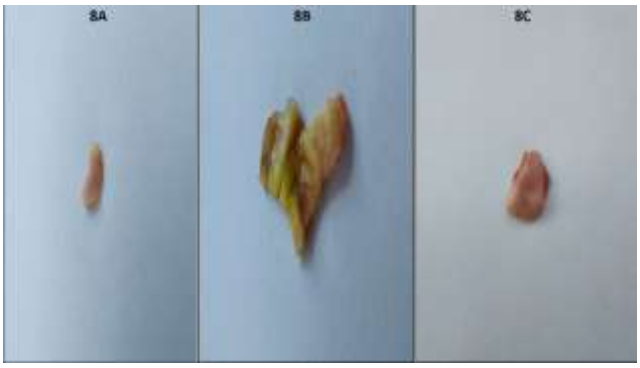

Fig. 8: Histopathological evaluation of inner tissue in study groups. A. Normal macroscopic view of the inner cheek tissue of the HG group. B. In the FUG group, findings of severe hyperemia, hemorrhage, widespread edema, swelling and significant thickening of the epithelial layer (HxE). C. Mild hyperemia and mild edema findings in the inner cheek tissue of the HRFU group.

IL-6 analysis

HRE hindered the rise of IL-6 with 5-FU in inner cheek and tongue tissue significantly (p<0.001). The amount of IL-6 was found to be significant (p=0.012) in the inner cheek tissue and insignificant (p=0.135) in the tongue tissue between the HRFU and healthy groups (fig. 6).

Histopathological Analysis

Macroscopic findings of inner cheek and tongue tissue

As in fig. 7A, no pathological results were observed macroscopically in the inner cheek tissue of the HG group. However, severe hyperemia, hemorrhage, widespread edema, swelling and marked thickening of the epithelial layer were observed in the FUG group's inner cheek tissue (fig. 7B). Except for mild hyperemia and mild edema, no pathological findings were observed in the inner cheek tissue of the HRFU group in which HRE was applied (fig. 7C).

As seen in Fig 8A, macroscopically normal apex, corpus, radix, epithelial layer and papillae are seen in the tongue tissue of HG group animals. However, the entire tongue tissue of FUG group animals is edematous and swollen, a thickened epithelial layer at the apex part, peelings in the corpus part, hyperemia, hemorrhage and a thickened papilla structure that has lost its feature are being observed (fig. 8B). While the apex part of the tongue tissue belonging to the HRFU group was in near-normal view, mild edema in the entire tongue tissue, mild hyperemia in the corpus part and moderate papilla loss were observed (fig. 8C).

DISCUSSION

In current study, the effect of HRE on 5-FU-induced oral mucositis in animals was evaluated macroscopically, also the tissues were examined biochemically. Our experimental results have shown that thickening in the epithelial layer, peeling and inflammatory symptoms developed in the cheek and tonguetissue of the group administered 5-FU alone, macroscopically. Also, in a previous study was reported that 5-FU forms macroscopically similar damage like this to the animal oral mucosa (Vilar *et al*., 2020). In the literature, basal epithelial cell death seen in chemotherapy-induced oral mucositis has been associated with the formation of the reactive oxygen species (ROSs) (Bell & Kasi, 2022). As is known, the ROSs cause lipid peroxidation (LPO) (Hua *et al*., 2017) and LPO leads to malondialdehyde (MDA) production (Capuzzi *et al.*, 2022). As can be understood from our biochemical results, the level of MDA in the 5-FU group was found to be significantly higher than in the healthy and HRE groups. In the literature, MDA has been shown as a proof of oxidative stress in the pathogenesis of mucositis (Sardaro *et al*., 2019). This points out that the 5-FU triggers oxidative stress.

Furthermore, in our study, the amount of total glutathione (tGSH) in the 5-FU group was lower than the HG and HRE groups. GSH is an endogenous antioxidant molecule that reacts with hydrogen peroxide (H_2O_2) and protects cells from ROSs damage by detoxifying H_2O_2 (Xu *et al.*, 2019). Therewithal, it has also been reported in prior studies that GSH reduces H_2O_2-induced cell death (Chen *et al*., 2022). The oxidant-antioxidant balance in healthy tissues is protected by the advantage of antioxidants. The change of this balance that way oxidants cause oxidative stress (Daenen *et al*., 2019). Our experimental results show that the balance between oxidants and antioxidants in the cheek andtongue tissues of the 5-FU group has changed in favor of oxidants.

Enzymatic antioxidant parameters such as SOD and CAT are also used to evaluate oxidative stress (Nguyen *et al*., 2022). These SODs and CATs, by neutralizing excessively produced ROSs, participate in maintaining tissue integrity and functions at normal levels (Daenen *et al*., 2019). In our study, it was found that SOD and CAT activities declined in the inner cheek and tongue tissues of the group in which we applied 5-FU. SOD is a metalloprotein that catalyzes the dismutation reaction of the superoxide anion radical and protects cells against oxidative stress (Policar *et al.*, 2022). It has been reported that SOD plays an important task in the pathogenesis of chemotherapy-associated oral mucositis (Menezes *et al*., 2021). The reason why we measure both SOD and CAT activities at the same time is that SOD converts the superoxide anion radical to H_2O_2. The complete removal of the formed H_2O_2 from the environment is carried out by CAT (Buettner *et al*, 2019). Current findings and literature indicate that oxidative damage developed in the inner cheek and tongue tissue of the administered 5-FU group.

In the inner cheek and tongue tissues of the 5-FU group, where oxidant parameters were determined to be high, proinflammatory cytokine levels such as NF-κB and IL-6 were also found to be high. It is known that oral mucositis is the inflammation of the oral mucosa (Fonseca *et al*., 2021). It is suggested that the underlying factor of oral mucositis is the ROS. It is advocated that these ROSs directly trigger inflammatory events that increase the severity of cellular damage (Bell & Kasi, 2022). It has been reported that 5-FU increases ROS production and, at the same time, activates signal transduction pathways such as NF-κB, which is an important factor in theformation of mucositis (Ribeiro *et al*., 2021). As is known, NF-κB also induces the manufacture of IL-6 and other proinflammatory cytokines (Ribeiro *et al*., 2017; Ribeiro *et al*., 2021).

As seen in our experimental results, HRE antagonized the effect of 5-FU on oxidant-antioxidant and pro-inflammatory parameters in inner cheek and tongue tissue. The antioxidant activity of HRE has also been reported in previous studies (Wei *et al*., 2022). Studies showing that the HRE inhibits both oxidant parameters and proinflammatory cytokine increase support our experimental results (Turan *et al.*, 2021). The absence of pathological findings other than mild hyperemia and mild edema in the inner cheek tissue of the HRE group may be due to its suppression of the increase in oxidative stress and proinflammatory cytokines. Hou *et al*. reported that the HRE inhibits NF-κB signaling pathways and forms an anti-inflammatory effect (Hou *et al*., 2017). It has also been shown that the HRE inhibits IL-6 increase as well as NF-κB (Gu *et al*., 2022). In the study of Erhan *et al*. the role of oxidant and proinflammatory cytokine increase in the pathogenesis of oral mucositis was expressed. It has been also emphasized that the HRE alleviated mucositis symptoms by inhibiting the increase in oxidant and proinflammatory cytokines (Erhan *et al*., 2017).

CONCLUSION

The 5-FU caused both oxidative and inflammatory injury in rats' inner cheek and tongue tissues. The HRE antagonized the effects of 5-FU on the MDA, tGSH, SOD, CAT, NF-κB and IL-6 in inner cheek and tongue tissue and alleviated the symptoms of severe mucositis. Our experimental results recommended that the HRE may bebenefical in the treatment of 5-FU-associated oral mucositis.

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