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Effect of intraperitoneal injection of *Nigella sativa* oil on 5-fluorouracil-induced oral mucositis in rats

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Abstract

Although many studies have shown that administration of *Nigella sativa* oil can decrease inflammation and facilitate tissue regeneration, the efficacy of its systemic administration for treatment of chemotherapy-induced oral mucositis has not been investigated. This study aimed to assess the effect of intraperitoneal injection of N. sativa oil on 5-fluorouracil (5-FU)-induced oral mucositis in rats. This study evaluated 72 healthy Wistar rats, weighing 250-300 g. The rats were randomly divided into three groups of control, placebo, and treatment (n=24). The rats received intraperitoneal injection of 5-FU on days 1 and 3. The rats' cheek mucosa was then wounded with a linear scratch by an 18-gauge needle on day 3. The placebo and N. sativa oil were administered in groups B and C, respectively during the study period. Histological changes in oral mucosa were assessed on days 4, 6, and 8. Data were statistically analyzed using SPSS via the ANOVA, and the Kruskal-Wallis test, followed by the Mann Whitney multiple comparisons test. The mucositis score and inflammation score significantly decreased in the treatment group compared with the control and placebo groups ($P<0.05$). But there was no significant different between the groups regarding the connective tissue changes ($P>0.05$). Our findings suggest that N. sativa oil can have a notable efficacy for improvement of oral mucositis and can decrease the inflammation score in rats undergoing chemotherapy. Our results suggest

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that *Nigella sativa* can be used as a valuable remedial agent and can be a possible candidate for treatment of chemotherapy-induced oral mucositis.

Keywords: Nigella sativa, Mucositis, Chemotherapy, 5-fluorouracil, Inflammation

1 Introduction

Oral mucositis is a common side effect of antineoplastic treatments, which increases the medical costs and decreases the quality of life and survival rate of patients undergoing chemotherapy (Scully et al., 2006; Shankar et al., 2017). Despite the advances made in cancer treatment and anti-cancer medications, chemotherapy with its disabling side effects is still among the most common treatments for cancer. Oral mucositis is a common side effect of chemotherapy with adverse clinical, social, emotional, and economical effects on patients. It decreases the quality of life, prolongs the hospital stay, and increases the costs of treatment and risk of opportunistic infections, as well as patient morbidity and mortality (Aghamohamamdi and Hosseinimehr, 2016; Peterson et al., 2008; Elting et al., 2003; Lalla et al., 2008). Although oral mucositis is a serious, painful and debilitating complication of anticancer treatments and imposes limitations on the dosage of drugs, there is currently no established treatment for it. Further investigations regarding the treatment of mucositis are therefore necessary to improve the quality of life and well-being of patients undergoing chemotherapy. The emerging concerns regarding the potentially undesirable side effects of chemical drugs have increased the tendency towards the use of medicinal herbs as an alternative to conventional medical treatments in the recent decades.

Medicinal herbs have been used for thousands of years around the world to cure various diseases including digestive, reproductive, respiratory, and inflammatory conditions (Rakotoarivelo et al., 2015; Tulunay et al., 2015; Boadu and Asase, 2017; Moslemi et al., 2016). The effectiveness of the organic compounds isolated from the medicinal plants to control inflammation has been well documented (Nworu and Akah, 2015; Verma, 2016). Various studies have indicated that medicinal plants can be used for treatment of inflammatory diseases of the oral cavity such as aphthous stomatitis (Rezvaninejad et al., 2017; Al-Attas et al., 2016; AL-Douri and Al-kazaz, 2010). Plants with anti-inflammatory and antioxidant properties have also been effectively used for the palliative care of chemotherapy-induced mucositis (Tanideh et al., 2013; Dos Santos Filho et al., 2016).

Recently, the Ranunculaceae family was suggested as an anti-inflammatory and anti-oxidative agent that may be useful for management of inflammatory diseases (Malik et al., 2017). *Nigella sativa* (N. sativa) is reported to be able to control inflammation by inhibiting the inflammatory cytokines such as interleukin-6, interleukin-1, NF-kappa B, and transcription factors (Ahmad et al., 2013). The medicinal properties of N. sativa are attributed to thymoquinone, which is the main bioactive component of N. sativa (Ermumcu and Snalier, 2017). Experimental models have shown that thymoquinone has a slight inhibitory effect on cyclooxygenase-1 expression and prostaglandin-E2 production (Al-Attas et al., 2016). In

addition, it has been shown that N. sativa can resolve inflammation and decrease the oxidative stress and subsequently help in treatment of some forms of inflammatory diseases such as rheumatoid arthritis, diabetes mellitus, bronchitis, asthma, and immune disorders (Ahmad et al., 2013; Ermumcu and Snalier, 2017). A previous study on the efficacy of N. sativa for treatment of oral inflammatory diseases such as gingivitis, periodontitis, pulpitis, and oral ulcers showed that flavonoids and other components of N. sativa make this herb an effective wound-healing accelerator (Al-Attas et al., 2016). Because of its anti-inflammatory and antioxidant properties, N. sativa is known to have beneficial effects on periodontal disease (Al-Attas et al., 2016). It has been demonstrated that the aqueous extract of N. sativa seeds has the potential to accelerate wound healing by increasing the proliferation of gingival fibroblasts (Ab Rahman et al., 2014). An animal study on a rat model evaluated the efficacy of N. sativa extract to decrease the severity of chemotherapy-induced mucositis. The results showed that gavage and topical use of N. sativa decreased the histological damage to the mucosa in an experimental oral and nasal mucositis model and suggested N. sativa as a promising agent to decrease the severity of induced oral mucositis in patients receiving anticancer treatments (Çanakci et al., 2018; Lotfy and Zayed, 2009).

This study investigated the effect of intraperitoneal injection of N. sativa oil on 5-fluorouracil (5-FU)-induced oral mucositis in Wistar rats.

2 Materials and Methods

2. 1. Drugs & chemicals

The N. sativa oil (Barij Essence Pharmaceutical Co., Iran), 5-FU (EBEWE pharma, Austria), xylazine hydrochloride, and ketamine hydrochloride (Merck, Germany), and other chemicals used in this study were purchased from local commercial sources.

2. 2. Animals

In this experimental animal study, 74 Wistar albino rats weighing 250-300 g were obtained from the animal house of Shahid Beheshti University of Medical Sciences. All animals were kept in polycarbonate cages for 7 days before the commencement of the experiment to allow for acclimatization to laboratory conditions. A 12-h light-dark cycle was maintained. The animals were kept under standard laboratory conditions and had free access to food and water ad libitum. The Animal Ethics Committee of Shahid Beheshti University of Medical Sciences approved the experimental procedure (Ethics code: IR.SBMU.RETECH.REC.1397.607).

2. 3. Treatment

At the beginning of the experiment, two of the rats were sacrificed to obtain excisional biopsies of normal buccal mucosa. Oral mucositis was induced in the rats using 5-FU (Ebewe Pharma Ges.m.b.H.Nfg.KG Unterach, Austria). All rats were injected intraperitoneally with 100 and 65 mg/kg 5-FU on days 1 and 3, respectively (Aras et al., 2013; Vanhoecke et al., 2015). On day 4, an area approximately 0.5 cm2 on the mucosa of the left cheek pouch was scratched with an 18-guage needle tip (Avapezeshk, Iran), dragging twice in a linear movement with constant force and equal depth. The common method of inducing mucositis is to create a scratch on the

3rd day after chemotherapy and repeat it on the 5th day (Peterson et al., 2008). In this study, the cheek was only scratched on the 3rd day and it was not repeated (Fig. 1). This was done to reduce the mortality and infection in rates throughout the study (based on pilot observations) and was sufficient to induce ulcerative mucositis based on histological observations. Prior to performing these procedures, the animals were anesthetized by subcutaneous injection of xylazine hydrochloride (XylazineBio; 3 mg/kg) and ketamine hydrochloride (Ketasol; 90 mg/kg). The rats were randomly divided into three groups (n=24) of control, placebo, and treatment. The control rats did not receive any other intervention. The placebo group was injected with a daily dose of 400 μL/kg placebo (Barij Essence Pharmaceutical Co., Iran), and the treatment group was injected with a daily dose of 400 μL/kg N. sativa oil intraperitoneally.

Figure 1. A slight scratch on the buccal mucous made by using a 18-gauge needle

2. 4. Tissue collection and histological staining

In each group, the animals were euthanized on days 4 (n = 8), 6 (n = 8) and 8 (n = 8), and the left cheek pouch was removed for histopathological analysis. All animals were sacrificed by an overdose of a combination of ketamine and xylazine (the anesthetic dose was administered intraperitoneally 4 times). All animals were monitored until no respiration was noted for ≥ 1 min before the biopsy. The cheek pouch areas were excised and prepared for histological examination (Fig. 2). The biopsy samples were fixed in 10% buffered formalin for 24 h, embedded in paraffin, and sliced with 5 μm thickness (Kazemi et al., 2017). The tissue sections were stained with hematoxylin and eosin (H&E), after which the degree of inflammatory cell infiltration was assessed. The sections were then observed under a light microscope (Zeiss, Germany).

Figure 2. Punch biopsy of buccal mucosa

Histopathological analysis was performed to evaluate the severity of oral mucosal damage caused by 5-FU. To do this, three different scales were defined, namely the "inflammatory scale", the "connective tissue changes scale", and the "mucositis scale". Blinded observers were then asked to grade the histological samples. For the "inflammatory scale", the samples were graded 0, 1, 2, or 3 indicating no inflammation, mild inflammation (10%-30%), moderate inflammation (20%-50%) and severe inflammation (more than 50%), respectively. For the "connective tissue changes scale", the samples were graded 0, 1, 2, and 3 indicating normal connective tissue, granulation tissue formation, fibrosis, and necrosis, respectively. A previously established grading system was used for the "mucositis scale" (Sunnyvale-Dossabhoy et al., 2015) as follows:

Grade 0: No injury, normal mucosa; grade 1: focal or diffuse alteration of basal cell layer with nuclear atypia and ≤ two dyskeratotic squamous cells; grade 2: epithelial thinning (2-4 cell layers) or ≥ 3 dyskeratotic squamous cells in the epithelium; grade 3a: loss of epithelium without a break in keratinization or presence of atrophied eosinophilic epithelium; grade 3b: subepithelial vesicle or bullous formation; grade 4: complete loss of epithelial and keratinized cell layers, ulceration (Table 1).

Table 1- Histopathologic criteria for staging of oral mucositis

Grade	Histopathologic manifestation
0	Normal mucosa
1	Focal or diffuse alteration of basal cell layer with nuclear atypia and ≤2 dyskeratotic squamous cells
2	2 Epithelial thinning (2–4 cell layer) and/or ≥3 dyskeratotic squamous cells in the epithelium
3A	Loss of epithelium without a break in keratinization or presence of atrophied eosinophilic epithelium
3B	Subepithelial vesicle or bullous formation
4	Complete loss of epithelial and keratinized cell layers; ulceration

2. 5. Statistical analysis

Data were expressed as mean ± standard error, or median, as appropriate. ANOVA, and the

Kruskal-Wallis test, followed by Mann Whitney multiple comparisons test, were used to compare the data between the three groups. The data were analyzed using SPSS version 24 (2016). The level of significance was set at 5% ($p<0.05$).

3 Results and Discussions

Table 2 represents the inflammatory reaction, connective tissue changes, and mucositis scores. Data were analyzed at the three time points to find out whether there was a significant effect of group on the three dependent variables of connective tissue changes, inflammation and mucositis scores. The Kruskal-Wallis test showed the significant effects of group on the mucositis score ($\chi 2 = 11.868b$, $p=0.003$) and inflammation score ($\chi 2 =9.480b$, $p=0.009$) but there was no significant difference between the groups regarding the connective tissue changes ($\chi 2 =0.553b$, $p=0.758$). As shown in figure 3.A, the mucositis score decreased in the N. sativa treatment group. Figure 3.B shows significant differences in the inflammation scores of the three groups. Figure 3.C shows no difference between the three groups regarding the connective tissue changes.

Table 2. Grading of mucositis in connective tissue

Grade	Histopathological manifestation
0	Normal connective tissue
1	Granulation tissue formation
2	Fibrosis
3	Necrosis

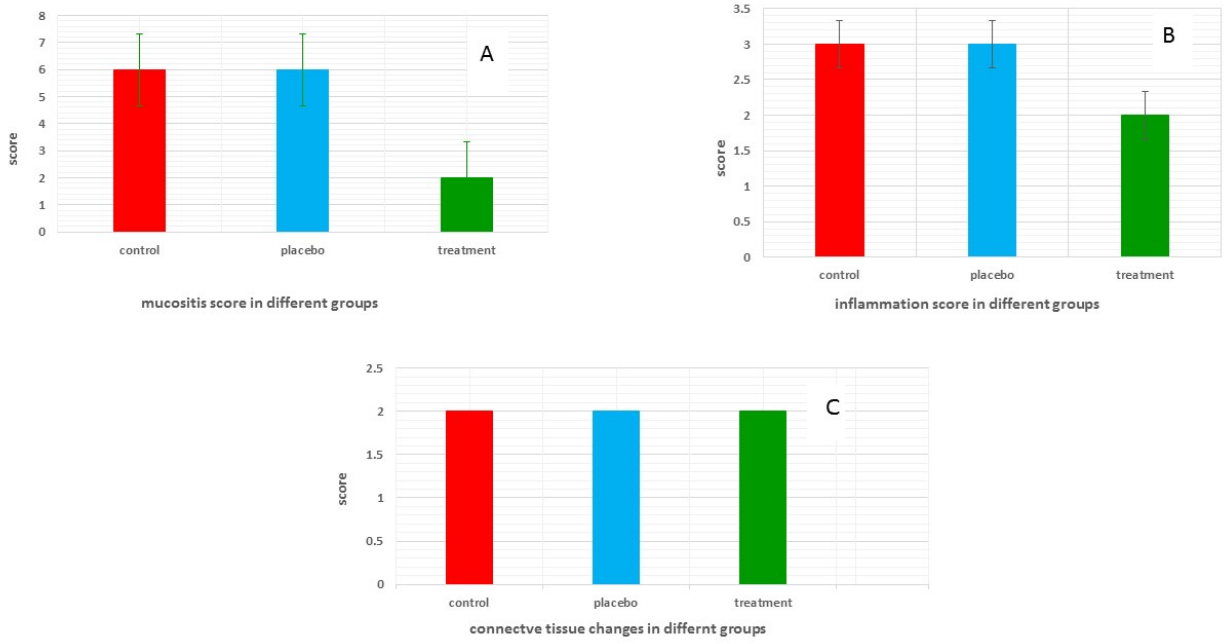

Figure 3. A. Mucositis, B. Inflammation score, and C. Connective tissue changes in the groups.

Mann-Whitney test showed significantly higher mucositis score in the control group compared with the treatment group (u=138, p=0.001). There was also a significantly higher inflammation score in the control group compared with the treatment group (u=150, p=0.003). There was no significant difference regarding the connective tissue changes between the control and treatment groups (u=260, p=0.530). No significant difference was found between the control and the placebo groups in either the mucositis score (u=250, p=0.337), the inflammation score (u=284.5, p=0.940) or the connective tissue changes (u=258, p=0.496). The results also showed a significantly higher mucositis score (u=184, p=0.02) and inflammation score (u=178, p=0.018) in the placebo group than the treatment group. There was no significant difference regarding the connective tissue changes between the placebo and the treatment groups (u=286, p=0.966).

Statistical analysis revealed that the severity of mucositis was significantly different between the groups on day 4 after chemotherapy ($\chi 2$ =9. 823b, p=0.007); whereas, there were no significant differences between the three groups on days 6 ($\chi 2$ =1.640b, p=0.440) and 8 ($\chi 2$ =4.112b, p=0.128) after chemotherapy. Statistical analysis also revealed a significant difference in the inflammation score between three groups at 4 days after chemotherapy ($\chi 2$ =7.872b, p=0.020); while, there was no significant difference between the three groups at 6 ($\chi 2$ =5.099b, p=0.078) and 8 days ($\chi 2$ =4.369b, p=0.113) after chemotherapy. There was no significant difference in connective tissue changes between the three groups at any time point (Fig. 4).

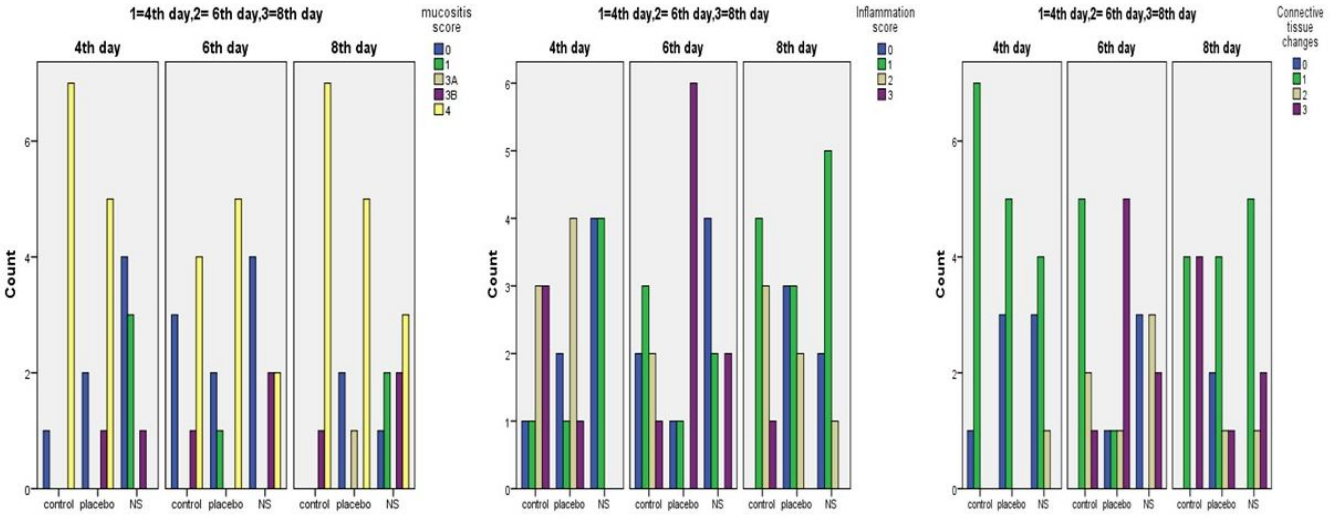

Figure 4. Mucositis and inflammation scores and connective tissue changes in different groups of study on the days 4, 6 and 8 after treatment.

On day 4, the control and the placebo groups showed severe inflammation and complete loss of epithelium. In these specimens, subepithelial vesicle formation was also noted, but the mucosa was quite normal in the treatment group, and the subjacent connective tissue exhibited moderate fibrosis. A few blood vessels and some degrees of inflammation were also visible in the treatment group (Fig 5. A.1, A.2, A.3). On day 6, the control and the placebo groups showed complete loss of epithelium with some degrees of angiogenesis and infiltration of polymorphonuclear cells in the ulcerative mucosa, but the treatment group showed normal mucosa with mild angiogenesis and moderate fibrosis (Fig 6. B.1, B.2, B.3).

On day 8, the mucositis score increased in all groups. A complete loss of epithelium was observed in the control and the placebo groups. Furthermore, severe coagulation necrosis and angiogenesis were observed in the submucosa. The treatment group showed complete loss of epithelium. The differences in the mucositis and inflammation scores were not significant between the three groups on days 6 and 8 (Fig. 7. C.1, C.2, C.3).

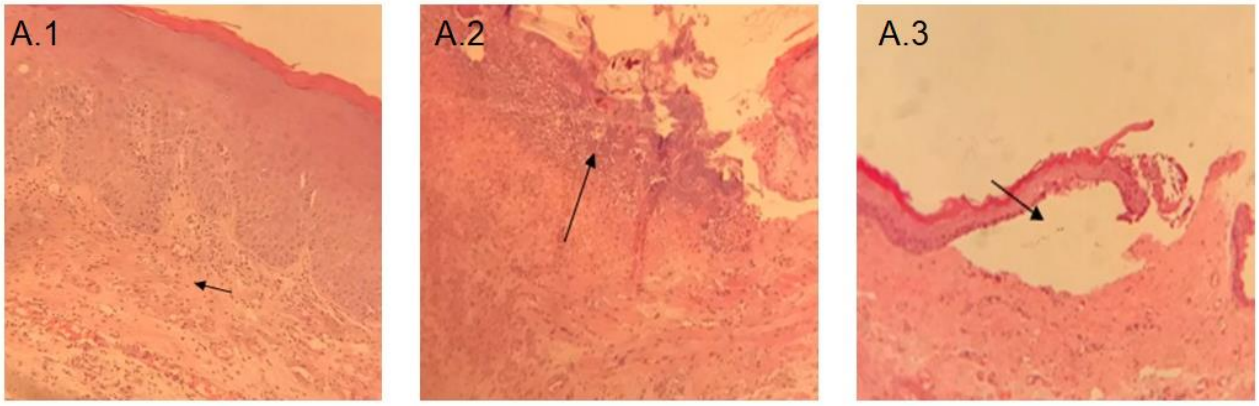

Figure 5. Histopathologic views in different groups on 4th after chemotherapy. Figure A.1 shows normal mucosa and moderate inflammation with submucosal fibrosis in treated group. Figure A.2 shows complete loss of epithelium with severe inflammation in placebo group and figure A.3 shows subepithelial vesicle formation in the control group.

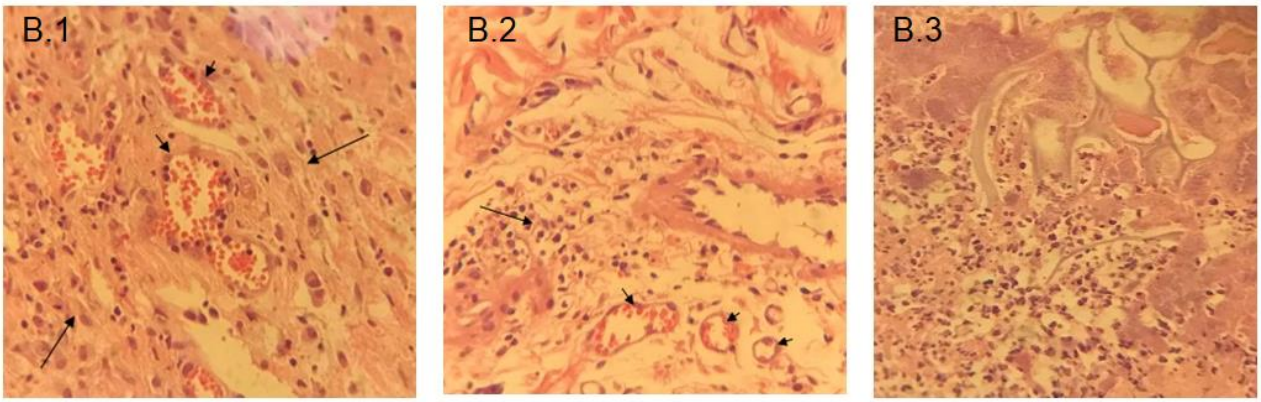

Figure 6. Histopathologic views in different groups on 6th after chemotherapy. Figure B.1 shows submucosal mild angiogenesis (short arrows) and moderate fibrosis (long arrows) in treated group. Figure B.2 shows mild angiogenesis (short arrows) and moderate fibrosis (long arrows) in placebo group. Figure B.3 shows complete loss of epithelium and severe infiltration of polymorph nuclear (PMN) cells in control group.

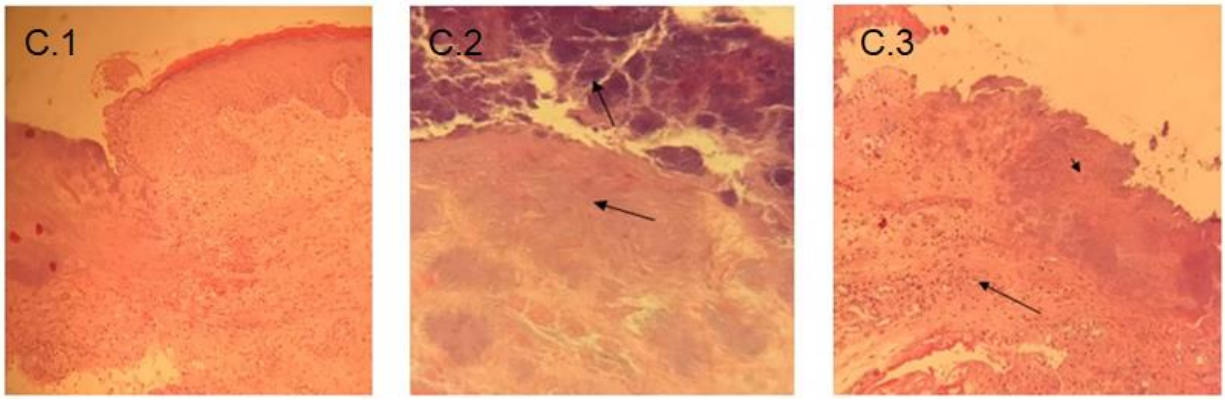

Figure 7. Histopathologic views in different groups on 8th after chemotherapy. Figure C.1 shows complete loss of epithelium in treated group. Figure C.2 shows severe coagulation necrosis of mucosa (shirt arrow) and submucosa (long arrow) in placebo group. Figure C.3 shows severe necrosis of mucosa (short arrow) and mild angiogenesis of submucosa (long arrow) in control group.

The present results reveal that the intraperitoneal administration of N. sativa oil decreased the severity of oral mucositis. The results obtained in the current study are in good agreement with the previously reported results showing that N. sativa could partially decrease the severity of oral mucositis by promoting mucosal healing (Lofty and Zayed, 2009) and that N. sativa oil can facilitate the healing of mucosal epithelium (AL-Douri and Al-kazaz, 2010). The present results are also in line with those of an in vitro study that demonstrated that the aqueous extract of N. sativa enhanced wound healing by reduce inflammation (Sari et al., 2013). Another study also reported that N. sativa accelerated the process of wound healing in burn-related skin injuries (Yaman et al., 2010). In contrast to the present findings, another study demonstrated that N. sativa increased granulation tissue formation in diabetic wounds of rats (Yaman et al., 2010). In line with our findings, another study demonstrated no significant effect of N. sativa oil on the Behcet's disease as a connective tissue disorder (Kavandi et al., 2018). The indices that we assessed to analyze the connective tissue changes have not been evaluated previously in treatment of oral mucositis. The insignificant effect of N. sativa oil on the connective tissue may be related to the insufficient dose of N. sativa, and further research is needed to give us an insight into the connective tissues changes.

Since the antioxidant effect of N. sativa oil is even higher than that of thymoquinone, we used N. sativa oil in this study (Ali and Blunden, 2003). While previous studies looked for the topical effects using gavage or topical gel, this study is unique in systemic administration of N. sativa oil for treatment of chemotherapy-induced oral mucositis. Furthermore, the method by which mucositis was induced in this study is slightly different from the previous studies because our pilot study revealed higher mortality rate in case of repeating the scratch. Also, the method adopted in this study was easier and safer.

The present study also shows that administration of N. sativa oil significantly decreased the amount of inflammatory infiltration. A large body of experimental research has confirmed the anti-inflammatory activity of N. sativa (Ahmad et al., 2013; Yildiz and Balikci, 2016; Rahmani and Aly, 2015). This anti-inflammatory effect may be appropriate to inhibit the release of mediators by thymoquinone and other constituents of N. sativa which have anti-inflammatory,

anti-parasitic, and antimicrobial properties (Yaman et al., 2010).

One possible mechanism by which N. sativa exerts its anti-inflammatory effect is through the inhibition of both cyclooxygenase and lipoxygenase pathways (Ali and Blunden, 2003). Also, the anti-inflammatory and antioxidant effects of N. sativa oil can enhance its phagocytic activity; thus, it can play a significant therapeutic role in fighting against the microorganisms (Yildiz and Balikci, 2016). The anti-inflammatory and antioxidant effects of N. sativa oil have been found to be higher than those of thymoquinone, which is known to be the active component of N. sativa oil (Ali and Blunden, 2003). In addition, N. sativa oil has been reported to possess a broad-spectrum antibiotic effect against many microorganisms; thus, it can help prevent bacterial colonization and wound infection (Al-Attas et al., 2016; Ermumcu and Şanlıer, 2017, Ali and Blunden, 2003; Yildiz and Balikci, 2016). Previous evidence indicates that the over-generation of several reactive oxygen species induced by anti-cancer treatments not only may directly damage the tissue but also can lead to a series of cellular destructive events and cascades that may indirectly lead to the destruction of the epithelial barrier (Yildiz and Balikci, 2016). Moreover, many studies have shown that inhibition of reactive oxygen species may successfully ameliorate mucositis. On the other hand, a broad range of antioxidant properties of N. sativa have been documented in the literature (Ermumcu and Şanlıer, 2017; Ali and Blunden, 2003; Yildiz and Balikci, 2016). The antioxidant properties of this medicinal plant have been mostly linked to the presence of thymoquinone and carvacrol in its composition. However, more detailed studies are needed to uncover the exact mechanism of N. sativa activity and its therapeutic effects on chemotherapy-induced oral mucositis.

Regardless of the underlying mechanism, our findings suggest that N. sativa could be a useful therapeutic agent and a safe candidate for treatment of chemotherapy-induced oral mucositis. Given the significant benefits associated with this drug, further studies are required to evaluate its therapeutic potential in treating oral mucositis and potentially other debilitating inflammatory conditions caused by chemotherapy.

4 Conclusion

This study showed that N. sativa improved the 5-FU-induced oral mucositis in rats. Our data clearly revealed the therapeutic potential of N. sativa oil in resolution of inflammation and reduction of mucositis score in rats undergoing chemotherapy.

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Conflict of interests

The authors declare that there are no competing interests.

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