Dose-dependent profile of ethanolic extracts of Iranian propolis on radiation-induced mucositis in rats

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Radiotherapy is a method commonly used in the treatment of head and neck malignancies. One of the most common side-effects of radiotherapy is oral mucositis, a toxic and dose and treatment limiting complication of radiotherapy, and the most significant cause of morbidity in patients undergoing chemoradiation for head and neck cancers.[1] Studies have shown that Iranian propolis contains a significant amount of flavonoids and phenolic compounds.[2] In a recent study, Iranian propolis, used to treat radiation-induced mucositis, was found to postpone the appearance of lesions and substantially reduce the severity of mucositis.[3] The aim of this study was to evaluate different doses of propolis on radiation induced mucositis in rats and investigate the effective dose of Iranian propolis for reducing radiation-induced mucositis.

This study was conducted in the Faculty of Dentistry, Shahid Rajaee Hospital, Babol University of Medical Sciences, Babol, Iran. Thirty-five male Wistar rats, aged 7-11 weeks and weighing 160 ± 20 g were included in this study. This experiment was carried out according to the International Guidelines for the Care and Use of Laboratory Animals and its design was approved by the Research and Ethics Committee of Babol University of Medical Sciences.

Fresh propolis was acquired from the Agriculture Faculty, Mazandaran University and was stored at 4°C. A fresh ethanolic extract of propolis (EEP) was made weekly using the magnetic stirring of 25 g of propolis in 100 ml of 10% (V/V) ethanol in a 250-ml closed-cap glass bottle at 42^{0}C for 2 hours. Then, the supernatant was paper-filtered at room temperature for 24 hours, and after EEP concentration measurement following centrifugation, different concentrations of propolis were prepared using 10% ethanol. The extracts were kept in a light-proof, closed containers in the refrigerator (2-8^{0}C) and placed in room to be warmed up at room temperature before injection. Rats were kept in metal cages under standard conditions (temperature, 22 ± 2^{0}C and dark/light cycle, 12/12 hours) with unlimited access to food and water. They were randomly divided into 5 groups: Group I received 10% (V/V) ethanol (control), Group II received 50 mg/kg propolis, Group III received 100 mg/kg propolis, Group IV received 200 mg/kg propolis, and Group V received 400 mg/kg propolis. The solutions were injected intraperitoneally (i.p.) into the rats 2 hours prior to radiotherapy and for the next 10 consecutive days. The rats were anesthetized with ketamine (100 mg/kg i.p.) before x-ray radiation and were immobilized on a metal shield. Then, they were irradiated by an x-ray apparatus (Siemens Co, Munich, Germany) at a 250 kV peak with a current of 12 mA and a dose of Gy15 for 9 minutes and 39 seconds.[3]

The radiation tube was 3 × 3 cm^2, and the rat's nose and jaw were in the field. After irradiation, the lips and tongues of the rats were examined daily over 10 days for signs of mucositis, according to the Parkin's scale.[4] The person responsible for the rats' daily examination was not aware of the groups' distribution (single blind), and the first evaluation was performed 24 hours after irradiation. The injection and examination continued up to 10 days (based on previous research).[3] For the histopathological study, specimens of lips and tongues were obtained on the tenth day after euthanizing the rats by CO_2. Samples were separated, coded and fixed in 10% formaldehyde for 24 hours, and after routine procedures, they were embedded in paraffin. Four micrometer-thick slices were prepared and stained with hematoxylin and eosin for light microscopic examination. An expert oral pathologist evaluated the microscopic findings. The affected areas included 1) degeneration and vacuolar alteration of the basal layer, 2) congestion and inflammatory infiltrate in the submucosa, and 3) cell changes in the stratified squamous epithelium, such as hyperchromasia, pleomorphism, necrosis and binucleation. These areas were classified into 5 grades in terms of the percentage of involved cells, according to Ertekin's scale.[5]

The severity of mucositis, the determination of the maximum effective dose, and the pathologic findings were all analyzed using the Kruskal-Wallis test, and a comparative analysis between the histological grades of each of the 2 groups was performed using the Mann-Whitney test. The results are described below. P values more than 0.05 were considered significant.

Mucositis was detected in the group receiving 400 mg/kg propolis after 7.14 ± 0.9 days (p=0.003), and the lesions were observed earlier with decreasing propolis doses (200 mg/kg [5.57 ± 1.4] (p<0.0025), 100 mg/kg [4.43 ± 1.5] (p<0.0012), 50 mg/kg [2.86 ± 0.9] (p=1) and control [2.43 ± 0.5]). There was a significant difference (p<0.05) between the control and all groups, except for the 50 mg/kg propolis group (**Table 1**). Differences between the mucositis scales of all

Table 1 - Mean scores of mucositis (Parkin's scale) recorded over 10 days.

Day	Control 10% ethanol	EEP 50 mg/kg	EEP 100 mg/kg	EEP 200 mg/kg	EEP 400 mg/kg	*P*-value
1	0.0	0	0	0	0	NS*
2	0.4	0.2	0	0	0	0.01
3	1.3	0.4	0.2	0	0	<0.0001
4	1.1	0.6	0.3	4.1	0	0.001
5	1.9	1.1	0.4	0.2	0	<0.0001
6	3.1	2.5	1.7	1.1	0.2	<0.0001
7	4.1	3.3	2.9	1.6	0.6	<0.0001
8	4.4	4.0	3.4	2.1	2.0	0.001
9	4.3	3.7	3.7	2.6	3.0	NS
10	3.0	2.6	2.4	1.6	2.0	NS

NS - not significant (p>0.05), EEP - Ethanolic Extract of Propolis

groups were significant for all days of the experiment except for the first, the ninth and the tenth days (Kruskal-Wallis test) (**Table 1**). No significant weight change was found between the groups with increasing propolis doses (p=0.51).

Degeneration and vacuolar alteration of the basal layer showed a remarkable reduction with augmentation of the propolis doses (p=0.000). In addition, minimum congestion and inflammatory infiltrates in the submucosa and maximum alteration were observed in the 400 mg/kg propolis group and the control group, respectively (p=0.000). Fewer cellular changes were found in the stratified squamous epithelial layers with enhanced doses of propolis because there were no cases of pleomorphism or severe necrosis in our control group; in contrast, marked cellular changes were observed in a few sections in the 400 mg/kg propolis group (p<0.000). The Propolis used in our study had no side effects.

In this study, we examined different doses of propolis to identify the most effective dose for reducing the severity of the lesions and for postponing the development of mucositis. The results of the present study showed delays in the lesion incidence and reductions in the lesion severity with increasing doses of propolis. The late onset of mucositis observed with higher doses of propolis indicated its effectiveness; this may be due to its anti-inflammatory properties, which plausibly lead to a delay in initiation of this phase by influencing the early (inflammatory) phase of mucositis. Histopathological findings showed reduced congestion and inflammatory infiltrate with increasing doses of propolis. This dose-dependent change was observed among different groups and is consistent with a previous study.[3] Furthermore, alterations in basal and epithelial layers diminish with dosage increase, which could be attributed to the impact of propolis on the second (epithelial) phase of mucositis. Due to the presence of phenolic compounds, propolis possesses antioxidant properties. The following 2 factors are used to assess the antioxidant characteristic of propolis: DPPH (2, 2 diphenyl-1-picrylhydrazyl) and the reducing power of iron (III).[6]

Based on the present findings, propolis caused increases in the latency of radiotherapy-induced mucositis in a dose-dependent manner. In addition, increasing doses of propolis lowered the severity of mucositis. Likewise, the histopathological effects of propolis were observed in a dose-dependent manner. According to these results, we suggest that higher propolis doses should be used in future studies in order to establish the most effective dose of propolis in human studies and evaluation of the mechanisms of its action

Lack of groups receiving doses higher than 400 mg/kg of EEP which could provide the most effective dose, was the limitation of this study.

In conclusion, according to the results of this study, increasing dose of propolis will reduce the severity of mucositis, and it is suggested that higher propolis doses should be used in detecting the most effective dose in future studies.

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