The effect of ethanolic extract of propolis on radiation-induced mucositis in rats

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ABSTRACT

الأهداف: تقييم فعالية مستخلص الايثانول لصمغ النحل في العلاج الإشعاعي الذي ينتج التهاب الغشاء المخاطي في الفئران.

الطريقة: أجريت هذه الدراسة في كلية بابول للطب – ومستشفى شاهيد رجاء لجامعة بابول للعلوم الطبية خلال الفترة من أغسطس 2008م حتى سبتمبر 2009م. أجريت دراسة الحالة على 21 جرذ ذكر من نوع ويستر تتراوح أعمارهم مابين 7-11 أسبوع وتقدر أوزانهم 160±20 غرام. تم تقسيمهم إلى 3 مجموعات، تلقت مجموعة (A) حقن داخل الصفاق (EEP) بجرعة مقدارها 100مغ/كلغ وتلقت مجموعة (B) حقن داخل الصفاق بجرعة مقدارها 200 مغ/كلغ من مستخلص الايثانول لصمغ النحل، وتلقت مجموعة الحالة (C) مقدار 10% من الايثانول جرعة 10 مل/كلغ داخل الصفاق فقط قبل العلاج الإشعاعي بالأشعة السينية X. تم علاج جميع الفئران بالعلاج الإشعاعي في منطقة الرأس، والرقبة باستخدام الأشعة السينية X بمعدل جرعة مقدارها 15 جراي لمدة 9 دقائق، و39 ثواني. استمر الحقن اليومي لمدة العشر الأيام القادمة، بينما تم فحص اللسان، والشفتين يومياً لتقدير شدة الآفة الناتجة بالإشعاع.

النتائج: في المجموعة (C)، ظهرت العلامات الأولية للقرح في اليوم الأول، بينما ظهرت في اليوم الرابع في المجموعة (B)، واليوم الثالث في المجموعة (A). كانت شدة القرح مرتفعة بشكل مهم في المجموعة (C)، ومنخفضة في المجموعة (B).

خاتمة: أظهرت نتائجنا أن المادة الشمعية للنحل فعالة في تقليل وتأخير الإشعاع الذي ينتج التهاب الغشاء المخاطي في نموذج الفئران نوصي بالمزيد من الدراسة والتقييم.

Objectives: To assess the efficacy of ethanolic extract of propolis in radiation-induced mucositis in rats.

Methods: This study was performed in the Dental Faculty, Shahid Rajaee Hospital of Babol University of Medical Sciences, Babol, Mazandaran, Iran from August 2008 to September 2009, It was carried out on 21 male Wistar rats, age 7-11 weeks, and weighing 160±20g. They were divided into 3 groups. Group A received intraperitoneal (ip) injections of 100 mg/kg ethanolic extract of propolis (EEP), group B received ip injections of 200 mg/kg EEP, and the control group (group C) received 10% ethanol (10ml/kg [ip]) just before x-ray irradiation. All rats were irradiated in the head and neck region by an x-ray device at a dose rate of 15 gray (Gy) for 9 minutes and 39 seconds. The daily injection continued for the next 10 days, and the lips and tongues of the rats were examined daily to assess the intensity of lesions induced by irradiation.

Results: In group C, the first signs of ulcers appeared on the first day, while they appeared on the fourth day in group B, and third day in group A. The severity of ulcers was greatest in group C, and least in group B.

Conclusion: Propolis is effective in reducing and delaying radiation-induced mucositis in an animal model, however, further study and evaluation is required.

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Oral mucositis is one of the most common complications in patients receiving radiation for head and neck malignancies. Radiation mucositis develops after cumulative radiation doses of 30 gray (Gy)

10-14 days after initiating treatment.[1] Mucositis may be extremely painful. It severely interferes with proper food intake, increases treatment costs, and the risk of infections, and indirectly affects tumor outcomes.[1,2] Many agents have been suggested to prevent and treat mucositis, or reduce its severity.[3-6] Many molecules that interfere with the pathways of mucositis are developing. Recently, palifermin (Kepivance), a human keratinocyte growth factor was approved by the Food and Drug Administration in 2004 as an agent for mucositis.[7] Propolis is a resinous substance collected by honey bees. Honey bees use it to seal holes in beehives, and protect the entrance of the hive.[8] The components of propolis include flavonoids, organic acids, and phenols, various kinds of enzymes, vitamins, and minerals.[9,10] These components have been shown to have many biological effects such as, analgesic and anti-inflammatory, antifungal, antibacterial, anti-viral, antioxidant, tissue, and wound healing, anti-tumor, and in vitro anti-proliferative effects.[11-13] Recently, many in vitro and animal studies[14-22] were published on the radioprotective properties of propolis. These properties of propolis may affect oral mucosa, which is in the field of radiation during radiotherapy for head and neck cancers. The purpose of this study was to assess the effect of an Iranian propolis ethanolic extract of propolis (EEP) on radiation-induced oral mucositis in rats.

Methods. This experimental study was performed in the Faculty of Dentistry and Shahid Rajaee Hospital of Babol University of Medical Sciences, Shahid Rajaee Hospital, Babol University of Medical Sciences, and Babol, Mazandaran, Iran from January 2008 to September 2009. It was carried out on 21 male Wistar rats, age 7-11 weeks, and weighing 160 ± 20 g. The design of this study was approved by the Research Committee and the Ethics Committee of Babol University of Medical Sciences. The experiment was performed according to the rules and guidelines of the Medical Ethics and History of Medicine Research Center of Tehran University of Medical Sciences. A fresh local batch of propolis acquired from e Mazandaran Agriculture Office Laboratory was stored in the refrigerator, and used during the experiment period. Every week, fresh EEP was made using 10% (V/V) ethanol simply by magnet stirring of 25 g propolis in 100 ml ethanol (10%) in a 250 ml closed cap glass bottle at 42°C for 2 hours. Then the supernatant was paper filtered using Watman® No 1 filter paper (OIGT Global Distribution Inc, Lawrence, Kansas, USA) at room temperature. After the EEP concentration measurement, the corresponding dilutions (W/V) for different doses were made using 10% ethanol. The extracts were kept in light-proof, closed containers in the refrigerator (2-8°C), and warmed to room temperature immediately before injection. A pilot study was carried out to established irradiation protocol, produce a murine model of irradiation mucositis, and show the time when mucositis began, and establish the endpoint of the experiment. After 2 weeks of acclimatization, the rats were housed in metal laboratory cages at standard conditions (temperature: 22 ± 2°C, dark/light cycles: 12/12 hours) with access to food and water ad libitum. Rats were randomly allocated into 3 groups. Group A received 100 mg/kg EEP, group B received 200 mg/kg EEP, and group C (control) received 10% (v/v) ethanol (10 ml/kg, intraperitoneal [ip]) 2 hours prior to x-irradiation for the next 10 consecutive days. The rats were anesthetized with ketamine (100 mg/kg, ip) before x-ray irradiation, and immobilized on a lead shield. Then, they were irradiated by an x-ray device (Siemens Co, Munich, Germany) with a beam filter one Cu". The device was operated at 250 kilovoltage peak (kVp) with a current tube of 12 mA, resulting in a dose rate of 15 Gy in 9 minutes and 39 seconds. The tube was 3x3 cm^2, and the nose and jaws were in the field. After irradiation, the lips and tongues of the rats were examined daily for 10 days for signs of mucositis based on the Parkin's scale as follows: score 0 - normal; score 0.5 - slightly pink; score 1 - slightly erythematous; score 2 - severely erythematous; score 3 - focal desquamation, score 4 - exudation, or crusting of less than one-half of the lip; score 5 - exudation, or crusting of more than one-half of the lip.[23] A calibrated examiner who was not aware of the groups (single blind) examined the rats. The first assessment was carried out 24 hours after irradiation. The injection and examination continued daily until the tenth day (according to the pilot study).

For histological study, after the animals were euthanized by CO_2, the specimens of the lips and tongues was obtained, and encoded at endpoint. Then, the tissue samples were fixed in 10% formalin for 24 hours, and after routine processing, the tissues were embedded in paraffin wax. Four µm-thick slices were prepared, and stained with Hematoxylin and Eosin for evaluation under light microscopy. Microscopic findings were assessed by an expert oral pathologist. Damaged areas included degeneration and vacuolar alteration of the basal layer, congestion, inflammatory infiltrate in the sub-mucosa, and cell changes in the stratified squamous epithelium such as, hyperchromasia, pleomorphism, binucleation, and necrosis. Damaged areas were classified by amount of damage in percentage. They were scored on a 5-point ordinal scale proposed by Ertekin:[24] grade 0 - normal; grade 1 - minimal (≤5%); grade 2 - mild (6-20%); grade 3 - moderate (21-50%); grade 4 - marked (51-75%);

and grade 5 - severe (75-100%). The semiquantitative scores represent the population examined.

The severity of mucositis (Parkin's scale), and the grades of histologic findings were analyzed by Kruskall-Wallis test. Comparative study between histologic grades of 2 groups at a time was performed utilizing the Mann-Whitney test. A $p<0.05$ was considered significant.

Table 1 - Mean scores of mucosities (Parkin's scale) on each day of the study.

Day of the study	Group A*	Group B†	Group C‡	*P*-value
		Mean ± SD		
1	0	0	0.5 ± 0.43	0.03
2	0	0	0.61 ± 0.33	0.001
3	0.07 ± 0.8	0	1.112 ± 0.58	0.001
4	0.21 ± 0.26	0.24 ± 0.14	1.3 ± 0.6	0.01
5	0.57 ± 0.73	0.42 ± 0.34	3.0 ± 1.09	0.02
6	3.7 ± 0.48	3.5 ± 1.1	4.5 ± 1.06	0.04
8	4.2 ± 0.48	3.8 ± 0.8	4.7 ± 0.66	0.02
9	4.4 ± 0.53	3.8 ± 0.8	4.9 ± 0.66	0.02
10	3.8 ± 1.06	3.2 ± 1.8	4.8 ± 0.72	0.01

*EEP 100mg/kg, †EEP 200mg/kg, ‡Control

Results. There were significant differences in the severity of mucositis among the 3 groups (**Table 1**). in all days of the experiment except on the tenth day ($p<0.05$ in days 1, 4, 5, 7, 8, 9, and $p<0.01$ in days 2, and 3) (**Figure 1**) In group C, the first signs of mucositis were seen after one day of irradiation, while they appeared after 4 days in group B, and 3 days in group A, and this difference was statistically significant ($p<0.0001$ [Friedman test]). For each day of the study, group C had the greatest severity of mucositis, while group B had the lowest severity. The scales of mucositis between group C and groups A or B were significant ($p<0.05$) (**Figure 2**). Histological results are shown in **Figure 1**. Infiltration of inflammatory cells was greatest in group A (**Figures 2a & 2b**), and lowest in group B (**Figures 2c &2 d**). Necrosis was seen in all samples of group A (**Figures 2a & 2b**), and ulcers were seen in all samples of groups C (**Figures 1e & 1f**) and A (**Figures 2a & 2b**), but they were rarely observed in group B (**Figures 2c & 2d**). Severe pleomorphism was seen in group A, but there were only small epithelial changes in groups C (**Figures 1e & 1f**) and B (**Figures 2c & 2d**), especially in group B. The grades of the tongue and lip specimens of group B were significantly lower than group C (**Table 2**). Comparative study of the lip and tongue specimens between 2 groups at a time was performed, and in all comparisons the differences were significant.

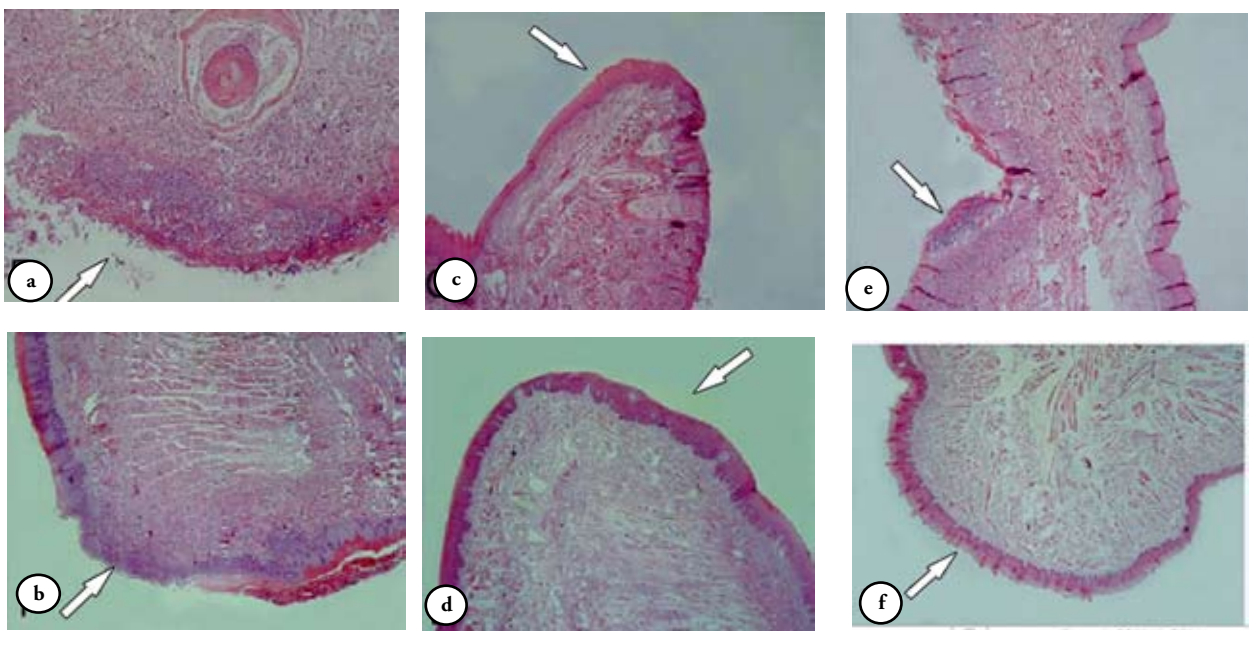

Figure 2 - Histopathologic views of the 3 groups showing in group A (ethanolic extract of propolis [EEP 100 mg/kg]), **a**) lip section: infiltration of inflammatory cells under the epithelium, **b**) tongue section: the epithelium is intact. Group B (EEP 200 mg/kg) **c**) lip section, **d**) tongue section. Group C (control) **e**) lip section ulceration, inflammation, and necrotic epithelium are seen, **f**) tongue section is covered by fibrinopulurent membrane. (Hematoxylin &Eosin staining x4)

Table 2 - Histologic grades (mean±SD) of mucositis in tongue and lip of the study groups.

Area of mucositis	Study groups (mean ± SD)			P value*
	Group C (Control)	Group A (EEP 100 mg/kg)	Group B (EEP 200 mg/kg)	
Tongue†	4.2 ± 0.8	3.28 ± 0.48	2.1 ± 0.69	<0.001
Lip‡	3.2 ± 0.66	2.28 ± 0.48	1.57 ± 0.53	<0.001

*Comparison of three groups by Kruskall-Wallis test.
†Comparison of histologic grades of tongue samples (Mann-Whitney Test): control versus propolis (100 mg/kg): p=0.01, control versus propolis (200 mg/kg): p=0.001, propolis (100 mg/kg) versus propolis (200 mg/kg): p=0.03.
‡Comparison of histologic grades of lip samples (Mann-Whitney Test): control versus propolis (100 mg/kg): p=0.02, control versus propolis (200 mg/kg): p=0.001, propolis (100 mg/kg) versus propolis (200 mg/kg): p=0.045.
EEP - ethanolic extract of propolis

Discussion. In this study, we evaluated the effects of Iranian EEP on experimental radiation mucositis in a murine model. The results showed a statistical reduction of mucositis in EEP-injected, irradiated rats. Previous studies[18,19] confirmed the radioprotective effects of propolis, or water soluble derivatives of propolis in gamma-irradiated mice.

It is believed that flavonoids are the main constituent of propolis and provides its radioprotective effects.[18,19] Iranian propolis mainly contains flavonoid, esters, and aromatic and aliphatic acids, various kinds of enzymes, vitamins, and minerals.[25] Mohammadzadeh et al[26] showed that propolis from different parts of Iran has antioxidant compounds, and may be helpful in the prevention of free radical-related diseases.

Based on antioxidant activity of flavonoids, it interact with reactive compounds of radicals, so they have the ability of direct free radical scavenging, or stabilize the ROS, which was generated in radiation mucositis.[12,27] Therefore, flavonoids can prevent the genotoxicity of radiation. This effect was also clearly demonstrated in a study by Benkovic et al,[19] which showed that propolis provided measurable protection against DNA damage from radiation. Flavonoids also have anti-inflammatory effects. They interfere with the activation of the cyclooxygenase and lipoxygenase pathways, which occur during mucositis due to the up-regulation of genes.[27,28] Additionally, the flavonoids inhibit the metabolism of arachidonic acid, and prevent the release of cytokines.[29]

Bacterial colonization occurs during the ulcerative phase of mucositis, predominantly with gram-positive, gram-negative, and anerobic organisms.[27] Propolis plays a role in this stage due to its antibacterial effects particularly against oral bacteria, and *Candida albicans*, which were described in many studies.[11,30,31] It was shown that Iranian propolis has high activity against gram positive, and some activity against gram negative bacteria, and antifungal activity as well.[25,32]

In the present study, the rats in groups A and B, which received daily doses of EEP showed lower scores of mucositis, and less histologic changes. As shown in **Figure 1**, the mucositis in group C increased from the first to the seventh day, had a peak in the eighth day, and then dropped. In groups A and B, the severity of mucositis was reduced, although the trends of mucositis in the 3 groups were not significantly different. This trend was shown in other studies as well.[23] These findings may have 2 interpretations: first, EEP may have more effective doses, which were not used in this experiment. Second, the propolis that was used in this study was not chemically analyzed, so the amount of flavonoids and other effective compounds are not clear.Even though this study showed the effect of ethanolic extract of propolis in delaying and reducing the radiation mucositis in a murine model, there are numerous questions yet to be further answered concerning effective doses, chemical composition, and the mechanism of action. It needs to be evaluated in controlled studies with different doses along with compound analysis.

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