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The Effects of Solvent Fractions of Bee Pollen Crude Extract in an Animal Model of Inflammation

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ABSTRACT

BACKGROUND AND OBJECTIVE: Conventional anti-inflammatory drugs, despite their beneficial effects, have serious and irreversible side effects. Due to the remarkable anti-inflammatory properties of bee pollen crude extract, the present study was conducted to investigate the effects of 100% methanol fraction (MF) and dichloromethane fraction (DF) in an air pouch model of inflammation.

METHODS: This experimental study was performed on 42 male Wistar rats in 7 groups of six. First, the hairs on the back of the rats were shaved. Subcutaneous injection of sterile air (20 and 10 mL) was performed on the dorsum of anaesthetized rats (day 1 and day 3, respectively). On day 6, inflammation was induced by intra-pouch injection of carrageenan. Group I: Normal saline; Group II, III, and IV: the rats treated with 100, 500 and 2500 µg MF; Group V, VI, and VII: the animals treated with 100, 500 and 2500 µg DF by intra-pouch injection simultaneously with carrageenan after 24 and 48 h. Three days after carrageenan administration, the inflammatory parameters and angiogenesis were measured.

FINDINGS: Compared to the control group (5.3±0.5 g), granulation tissue weight was significantly decreased ($p<0.001$) by approximately 30% and 40% by all three doses of MF and DF, respectively. Inflammatory parameters such as exudate volume and angiogenesis were significantly reduced by DF ($p<0.001$) and MF ($p<0.01$), respectively. In both fractions, a dose of 2500 µg increased the number of leukocytes.

CONCLUSION: Although different effects were observed in the two fractions of extract due to different amounts of flavonoids, a promising inhibitory effect on granulation tissue and angiogenesis was obtained, which could be considered as a natural source of anti-rheumatoid and anti-cancer agents.

KEY WORDS: *Apitherapy*, *Air Pouch*, *Inflammation*, *Angiogenesis Inhibitors*.

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Introduction

Approximately, 20% of all cancers are started or worsened by inflammation. The onset and progression of malignancies is also closely related to angiogenesis (1, 2). Despite the importance of angiogenesis in the growth and reproduction (3), it is overproduced in the microenvironment of the tumor to maintain tumor growth and metastasis by facilitating migration of inflammatory cells to the site of inflammation in order to deliver oxygen and nutrients. Thus, inhibition of angiogenesis is an important strategy for treating chronic inflammatory diseases (4).

Many of currently available therapies for inflammatory diseases have a number of serious adverse effects. Therefore, research for finding more effective and safer anti-inflammatory agents is one of the important areas of investigation (5). Apitherapy is a method of using bee products such as honey, pollen, royal jelly, propolis, and bee venom to prevent or treat diseases (6). Bee Pollen is a highly nutritious substance made by bees from various plant sources and used in traditional medicine as a supplement (7).

Based on the previous results in suppressing of inflammation and angiogenesis by the crude extract of bee pollen (8), the present study was designed to evaluate the anti-inflammatory and anti-angiogenesis effects of methanol fraction (MF) and dichloromethane fraction (DF) of total bee pollen extract in an experimental model of air pouch. The air pouch is an *in vivo* model used to study acute or chronic inflammation. It is also an appropriate model for the study of angiogenesis and articular diseases, especially RA (9).

Methods

The present study is basic research and was performed experimentally after approval by the ethics committee of Tabriz University of Medical Sciences with the code IR.TBZMED.VCR.REC.1397.036 and IR.TBZMED.VCR.REC.1398.082. In this study, 42 male Wistar rats (200-250 g) were used. Conditions for keeping and caring for animals in accordance with the standards and ethical guidelines for research on laboratory animals were notified by the Ministry of Health and the study began after receiving ethics codes from the ethics committee of Tabriz University of Medical Sciences.

Extraction and fractionation methods: The ground bee pollen (350 g) was macerated with methanol (MeOH) for 12 h, followed by filtering. To prevent chemical changes, the extraction process was performed in a dark place. After 12 hours, the resulting extract was filtered twice through a Whatman filter to obtain a clear liquid. The solvent extraction was repeated three times and the filtrate was dried and concentrated using a rotary evaporator at a maximum temperature of 45 °C. The final extract was stored at -20 °C until consumption. Extraction Phase Solid (SPE) method was used to fractionate the whole extract. A portion of the MeOH extract (10 g) was subjected to solid-phase extraction (SPE) on Sep-Pak C18 cartridges (Waters, Ireland) eluting with a step gradient of water–methanol mixtures (10:90, 20:80, 40:60, 60:40, 80:20 & 100:0) as well as dichloromethane. The obtained fractions were dried and concentrated separately using a rotary evaporator at a maximum temperature of 45 °C (10).

Total flavonoid content: Total flavonoid content was measured using aluminum chloride reagent (Scharalau, Italy) and rutin used as a standard. 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride solution in ethanol, 0.1 mL of 1 M potassium acetate and 2.8 ml of distilled water were added to 0.5 mL of each fraction. After incubation of the samples at room temperature for 30 minutes, the absorbance of the samples was read with a spectrophotometer (UV Brucker Tensor 27, USA) at 510 nm against control sample and the total flavonoid content was expressed as rutin equivalents in mg per 100g of dried extract (11). 100% MF and DF fractions were selected to study inflammation.

Study groups: The rats received 2 ml of carrageenan 2% as a phlogistic agent to induce inflammation. The animals with induced inflammation were divided into 7 experimental groups, each consisting of six animals; Group I: Normal Saline; Group II, III, and IV: the rats treated with 100, 500 and 2500 µg MF; Group V, VI, and VII: the rats treated with 100, 500 and 2500 µg DF.

Creating experimental model of air pouch: Based on the defined protocol (12), an air pouch was created on the back of the rats by subcutaneous injection of 20 ml of sterile air on the first day after light anesthesia and disinfection of the injection site. Subsequently, the air pouches were maintained by injecting 10 ml of air into the pouch on the third day. A 2% solution of lambda carrageenan was prepared and to sterilize it, the resulting suspension was autoclaved at 151 ° C for 15 minutes. On the sixth day, inflammation was induced by injecting 2 mL of carrageenan (2% w/v) into the pouch to all groups of rats. In the control group, 1 ml of normal saline and in the experimental groups, MF and DF fractions with doses of 100, 500 and 2500 micrograms were injected into the pouch at the same time as

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carrageenan injection, 24 and 48 hours after carrageenan injection.

Evaluation of inflammatory parameters: Twenty-four hours after injecting the last dose of fractions, the rats were sacrificed, the formed pouch was surgically removed, then the exudate was extracted and its volume was measured. The number of leukocytes in the exudate was counted under the light microscope. Granulation tissue around the pouch was carefully separated and weighed (13).

Evaluation of angiogenesis: To evaluate angiogenesis, the granulation tissue was cut into small pieces after washing with PBS at pH=4.7 and drying. Drabkin solution prepared from a hemoglobin kit (Biochemical Company, Iran) was added to it and homogenized for 4 minutes at a speed of 15000 rpm and in ice bed (4 °C) by a homogenizer (Heidolph, Germany). The homogeneous tissue was then centrifuged at 10000 RCF at 4 °C for 30 minutes (Eppendroph, Germany). The supernatant was filtered through a 0.22 micron filter and the amount of hemoglobin as an indicator of angiogenesis was calculated using the standard curve of the hemoglobin kit by a spectrophotometer at a wavelength of 540 nm (14).

Statistical Analysis: All data were reported as Mean±SEM and analyzed using one way ANOVA followed by LSD post-hoc test in SPSS 24 software and $p<0.05$ was considered significant.

Results

Pollen extraction and analysis: The amount of MF and DF obtained from 10 g total extract of bee pollen was 9% (900 mg) and 8% (800 mg), respectively. The absorption of MF and DF were 1.65 and 0.85 nm, respectively, indicating the presence of high flavonoid content in MF rather than DM. The MF and DF were found to contain 119.35 and 62.12 mg rutin equivalent per 1 gram of dry sample which corresponds to 11.93% and 6.21% of total flavonoid, respectively.

Effect of pollen solvent fractions on the inflammatory parameters: The volume of exudate in the MF groups not only had not been changed in comparison with the control (12±0.76 ml) but also showed significant increasing at a dose of 2500 µg ($p<0.05$) (Fig. 1A). Unlike MF, DF at doses of 100, 500 and 2500 µg decreased the volume of exudate by 47.5%, 44.2% and 62.5%, respectively ($p<0.001$). There was a significant difference between MF and DF in terms of exudate lowering. Both fractions failed to decrease leukocyte accumulation. On the other hand, the number of leukocytes with the dose of 2500 increased significantly (Fig. 1B). Compared to the control group (5.3±0.5 g), granulation tissue weight was significantly decreased ($p<0.001$) by approximately 30% and 40% by all three doses of MF and DF, respectively (Fig. 1C). In addition, doses of 500 and 2500 µg of the two fractions showed a statistically significant difference in granulation weight loss ($p<0.05$ and $p<0.01$, respectively).

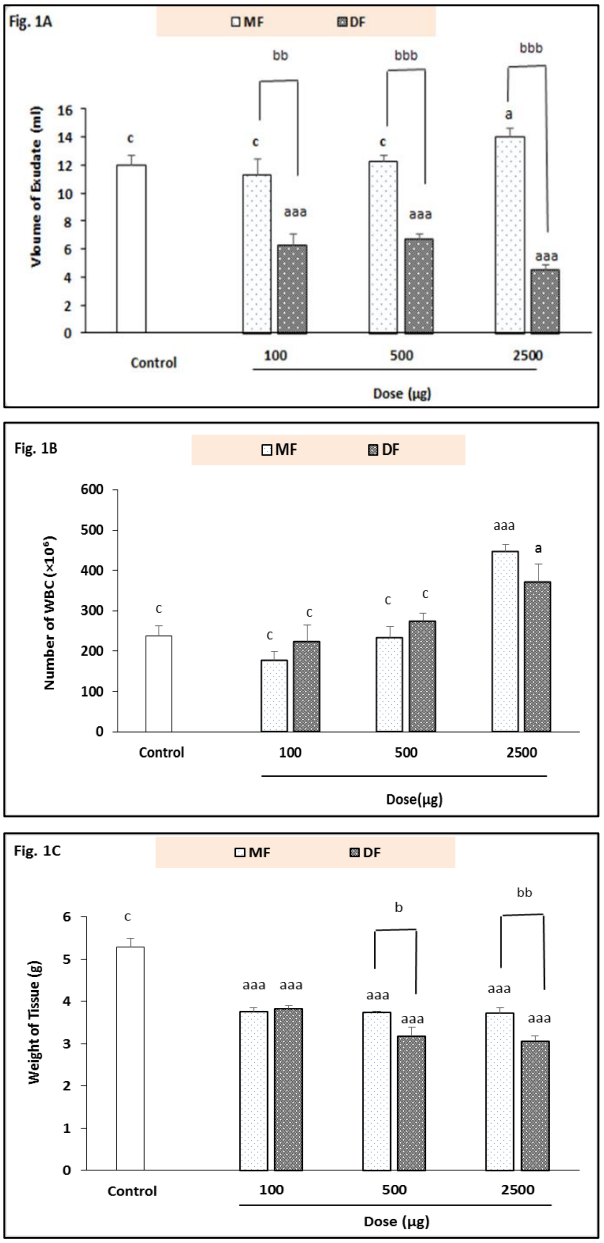

Fig. 1. Effect of MF and DF on exudate volume (A), leukocyte accumulation (B) and granuloma tissue weight (C). **a indicates a significant difference between the fractions and the control group (a: $p<0.05$, aaa: $p<0.001$), b indicates a significant difference between the groups (b: $p<0.05$, bb: $p<0.01$) and c indicates that there is no significant difference between the control group and the treatment groups.**

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Effect of solvent fractions of Bee pollen total extract on the angiogenesis: Treatment with MF at 500 and 2500 µg significantly reduced ($p<0.01$) the angiogenesis in 31.5% and 23.5% compared with the control saline group, respectively. The DF not only had no effect on angiogenesis but also showed significant difference with MF in this respect (Fig. 2).

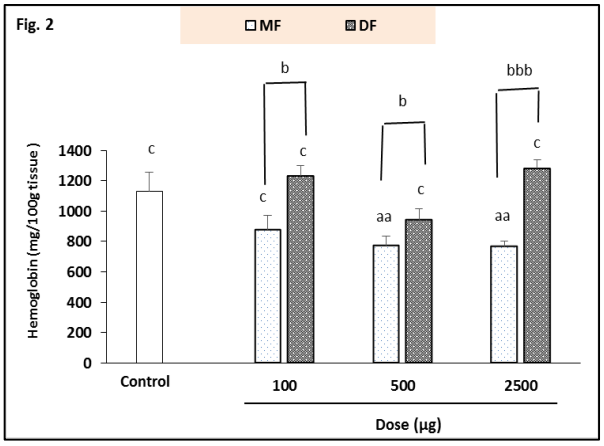

Fig. 2. Effect of 100% methanolic (MF) and dichloromethane (DF) fractions of pollen extract on angiogenesis. a indicates a significant difference between the treatment groups and the control group (aa: $p<0.01$), b indicates a significant difference between the treatment groups (b: $p<0.05$, bbb: $p<0.001$) and c indicates that there is no significant difference between the control group and the treatment groups.

Discussion

We reported here for the first time that both fractions of bee pollen extract effectively reduced some inflammatory parameters and angiogenesis in inflammatory model of air pouch in rats. These activities are probably correlated with the immunomodulatory and anti-inflammatory properties of the various flavonoids present in the MF and DF. Different types of flavonoids in pollen extract were reported, the most abundant of which were quercetin and kaempherol (15). Our results showed that DF significantly decreased the volume of exudate. According to Lopes (2019), quercetin inhibits the activity of histidine decarboxylase, reduces the level of histamine, and may also inhibit the cascade of arachidonic acid metabolism, decreasing the level of pro-inflammatory prostaglandins (16).

One of the flavonoids in pollen is kaempferol, which has been shown to inhibit hyaluronidase enzyme. By inhibiting the enzyme, kaempferol strengthens the walls of the arteries and reduces the exudation. Hyaluronic acid also protects granulation tissue from the dangers of free radicals (17). Other mechanism that may involve in the anti-inflammatory properties of fractions is the general characteristic of flavonoids in their ability to scavenge free radicals, which exacerbate inflammation (18). In this study, granulation tissue weight was significantly decreased by both fractions of bee pollen total extract. Granulation tissue formation is an important feature of chronic inflammation (19), which is affected by nitric oxide released by macrophages (20).

Results of a study revealed that ethanolic extract of bee pollen had notable inhibitory effect on inducible nitric oxide synthase (iNOS) (21). The results of a study showed that ethanolic extract of bee pollen has a significant inhibitory effect on induced nitric oxide synthase (iNOS) (21) and perhaps one of the mechanisms of granuloma tissue weight loss by pollen extract fractions is inhibition of iNOS enzyme. Based on the results of this study, the rate of angiogenesis was reduced by intra-pouch injection of MF fraction. The amount of vascular endothelial growth factor (VEGF) in the inflammatory fluid is directly related to angiogenesis. Results of the study of Eteraf-Oskouei et al. revealed that bee pollen crude extract decreased the concentration of VEGF and TNFα, and consequently reduced angiogenesis (8). Compounds that affect angiogenesis, especially substances that affect VEGF concentration, are more likely to be present in the MF portion of the total extract.

Although bee pollen extract showed an anti-inflammatory effect according to our previous study (8), allergic reactions and anaphylaxis have been reported after consuming bee pollen (22). Considering that the MF, despite reducing the weight of granulation tissue, caused a significant increase in the number of leukocytes and exudation, it seems that some compounds such as phenol amines are involvement in this condition (23).

The results of this study indicated that the most effective solvent fraction in reducing angiogenesis and exudation was MF and DF, respectively. However, both of them had a significant effect on weight loss of granulation tissue. Maybe, the flavonoid content of bee pollen fractions and also their antioxidant properties have a key role in modulating inflammation. Although bee pollen has potential therapeutic properties, future research is needed before its using in treatment of diseases. Moreover, to find the exact mechanism of action, the composition of bee pollen should be studied in more detail in future studies.

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