Archives of Razi Institute, Vol. 78, No. 1 (2023) 233-239

DOI: 10.22092/ARI.2022.358579.2253

Original Article

Marrubium persicum İmproved the Biological Parameters Associated with Angiogenesis and İnflammation in Mice

Fekri, K[1], Mortezaie, M. Sh[2, 3, 4], Maleki-Dizaji, N[3], Fathiazad, F[4], Hamedeyazdan, S[4, 5 *]

1. Department of Paramedicine, Amol School of Paramedical Sciences, Mazandaran University of Medical Sciences, Sari, Iran
2. Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran
3. Department of Pharmacology and Toxicology, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran
4. Department of Pharmacognosy, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran
5. Research Center for Integrative Medicine in Aging, Aging Research Institute, Tabriz University of Medical Sciences, Tabriz, Iran

Received 4 April 2022; Accepted 7 May 2022
Corresponding Author: yazdans@tbzmed.ac.ir

Abstract

The genus *Marrubium*, belonging to the family Lamiaceae, is highly praised in herbal medicine of different countries for having renowned healing properties. Herein, the anti-inflammatory and anti-angiogenesis potential of *Marrubium persicum* methanol extract was evaluated in a mouse air pouch model of inflammation. Aerial parts of *M. persicum* were solvent extracted using the Soxhlet apparatus. Subsequently, air injections were performed (for 3 days) into the mice's backs to bring about an air pouch, while carrageenan was used to induce inflammation. The mice were divided into four groups, including; negative control (normal saline into the pouch), control (carrageenan), treatment and positive control (dexamethasone). The inflammatory markers were analyzed 48h after injecting carrageenan, and a haemoglobin assay kit assessed the quantification of angiogenesis in granulation tissue. *M. persicum* methanol extract at doses of 3.5, 5, 7.5 and 10 mg/kg represented significant decreases in inflammatory parameters. Compared to the control group, the optimum dose (3.5 mg/kg) lessened the myeloperoxidase (MPO) and angiogenesis activity, as well as haemoglobin levels. In sum, the methanol extract of *M. persicum* exhibited anti-inflammatory effects against carrageenan-induced inflammation, which could be related to its antioxidant and inhibitory effects on neutrophils' infiltration.

Keywords: Lamiaceae, methanol extract, myeloperoxidase, haemoglobin, neutrophils' infiltration

1. Introduction

As a biological reaction, inflammation occurs when the body's tissues are exposed to a harmful risk. It is a process that consists of two major phases. The acute phase is characterized by high blood flow and increased vascular porousness, which together lead to the arrival of cytokines and other inflammatory mediators in inflamed areas. During the subacute/chronic phase, exposure to inflammation-inducing agents triggers cellular and humoral immune responses around damaged tissues (1). Whether the inflammation is in the acute or chronic phase, various soluble factors have been known to be involved in leukocyte enrolment via increased development of cellular linkage molecules and chemoattraction (2). Mast cells, tissue macrophages, endothelial cells and fibroblasts are the local cells; their initiation would be modulated by a significant number of the mentioned soluble intermediaries, followed by the activation of inflammatory cells such as eosinophils, neutrophils, lymphocytes and monocytes. Taken together, these events determine the type and severity of inflammation

consequences, *e.g*. hypotension, leukocytosis, fever, cachexia or synthesis of proteins (3). Chronic inflammation, for which numerous underlying mechanisms have been suggested, is normally of low grade and a persistent stage resulting in comebacks of tissue collapse (4).

On the other hand, angiogenesis which can be a physiological response in the body, would be defined as organizing new blood vessels throughout the body (5). In addition to embryogenesis and menstrual cycle, which are physiological procedures, it is involved in the pathogenesis of some conditions such as rheumatoid arthritis, neovascular glaucoma and tumour growth (5, 6). Generally, angiogenesis leads to leukocyte recruitment and inflammation in the synovium, both known as sustaining elements for cancers and inflammatory diseases. Hence, studying the anti-angiogenic agents would be a rational approach to controlling angiogenesis-dependent diseases (7).

On the other hand, Lamiaceae, the plant family which includes a variety of species, has received significant attention for presenting valuable pharmacological properties. Among its various genera, *Marrubium* contains many important species with remarkable therapeutic effects, including the potential for treating gastrointestinal disorders (8, 9). For example, the gastroprotective effects of *M. vulgare* (one of the species of *Marrubium*) have already been reported (10). Studies have shown the presence of various flavonoids, diterpenes, alkaloids and polyphenols (as well as other classes of phytochemical compounds) in different species of *Marrubium* (11). Although plants of the genus *Marrubium* are generally considered oil-poor species (especially in terms of yielded oil quantities), essential oils of this genus would be characterized mainly by sesquiterpenes such as germacrene D, β-caryophyllene, (E)-β-farnesene, δ-cadinene and α-humulene (12).

Due to the limited studies on the phytochemistry and bioactivity of *Marrubium persicum*, native to Armenia, Azerbaijan, Turkey and Iran (13), we decided to study the anti-inflammatory and anti-angiogenesis activity of this plant in mouse air pouch model of inflammation.

2. Materials and Methods

2.1. Plant Material and Extraction

After collecting the aerial parts of *M. persicum* C. A. Mey. from its native place in Varzeqan (East Azarbaijan province, Iran), they were shade-dried and grounded by a blender. Then 100g of the powdered plant material was subjected to solvent extraction using a Soxhlet apparatus with 2L n-hexane, 2L chloroform and 2L methanol (MeOH), correspondingly. All the obtained extracts were dried by a rotary vacuum evaporator (Heidolph, Germany) at 45°C under vacuum.

2.2. Preliminary Phytochemical Assessment

Qualitative phytochemical analysis of *M. persicum* methanol extract was performed, pursuing the standard and common methods previously described (14). Tests for qualitatively detecting alkaloids, anthraquinones, coumarins, phenols, flavonoids, saponins, cardiac glycosides and tannins within the plant extract were accomplished.

2.3. *in vitro* Antioxidant Activity Assessment

scavenging capacity of all *M. persicum* crude extracts (n-hexane, chloroform and methanol) against the free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH), was evaluated by measuring the bleaching absorbance of DPPH solution which was purple (15). The stock solutions of each extract were prepared, and the sequential two-fold dilutions were also prepared. Next, the attained concentrations were mixed with similar volumes of DPPH (16-18), and absorbance of the solutions was measured against blank at 517nm after 30 minutes of incubation. This assay was conducted in triplicate using quercetin as the positive standard, and the reduction percentage of DPPH free radicals was determined for each mixture using the following formula:

R (%) = [(Blank's absorbance – Sample's absorbance) / Blank's absorbance] × 100

RC_{50} was considered the concentration of each *M. persicum* extract reducing DPPH free radicals up to 50%. The value could be calculated using graphs presenting inhibition percentages versus extract concentrations.

2.4. Laboratory Animals

Swiss mice weighing 25-30 g were held at environmentally controlled settings (22±2 ℃ temperature, 50–60% humidity and alternating 12h light and 12h dark cycles) with free access to standard laboratory diet and water.

2.5. Experimental Protocol

The standard air pouch model was induced by injecting 2.5 mL sterile air into the mice's backs for three consecutive days. Later on the 6th day, the mice were treated with a 1% solution of carrageenan subcutaneously, and after 48h, a frequently utilized technique (using pentobarbital overdose) was employed for euthanasia.

Not surprisingly, the pouch supplies a repository of cells and inflammatory mediators that manifestly could be assessed in the fluid. Endpoints such as leukocyte influx, the volume of exudate, granulomatous tissue weight and myeloperoxidase (MPO) activities together with angiogenesis could be evaluated to determine the degree of inflammation and the anti-inflammatory activity of different samples in the study. Accordingly, 48h after injecting carrageenan into each mouse, perforation of the air pouch was achieved through an initial small dorsal skin incision, and the cavity was washed with 1.0 mL of sterile phosphate buffer saline (PBS, pH 7.6) prior to the assessment of the relative inflammatory parameters. In addition, the levels of angiogenesis were also measured to reveal the potential of *M. persicum* methanol extract as an anti-angiogenic agent. As regards, 0.5 mg/kg of dexamethasone, a steroidal anti-inflammatory drug, was administered intraperitoneally (i.p.) as the standard in the positive control group to compare the obtained results with samples as well as the other control groups of normal saline (NS; pH=7.6; negative control) and vehicle-treated (control). At the outset, 30 mice were divided into five groups (n= 6) which received 2.5, 3.5, 5, 7.5, and 10 mg/kg of *M. persicum* methanol extract half an hour before carrageenan injection. Then, 48h after the carrageenan injection, the relative tissue weight, leukocyte influx and exudate volume were quantified. Concerning the acquired data compared to the control groups, the optimal dose of *M. persicum* methanol extract (3.5mg/kg) was chosen for further analysis of inflammatory parameters (n=6 in each group, a total number of 24 animals). Conclusively, the optimum dose of the methanol extract was used to determine the effect of *M. persicum* on angiogenesis and MPO activity.

2.6. Quantification of the Influx of Leukocyte and Exudate Volume

Following the euthanasia (via pentobarbital overdose), the exudates were collected, their volume was determined, and the total number of leukocytes was estimated.

2.7. Quantification of Activity of MPO

MPO activity was measured in the acquired exudates through colourimetry. Briefly, 20 µL of exudate was mixed with 180 µL of MPO test buffer, and then the mixture was exposed to multiple freeze-thaw cycles after being homogenized for 10 seconds using a sonicator. MPO activity was measured in the supernatant of the samples (centrifuged at 13,000 g in 15 min at 4ºC) by colourimetry, and the results were expressed as activity percentages.

2.8. Quantification of Angiogenesis

Two days after injecting the carrageenan solution and the formation of granulation tissue, it was dissected and weighed. PBS (pH 7.4) was used for washing the dissected granulation tissue. After that, it was slashed into tiny pieces by scissors before being homogenized in about 2.0 ml of Drabkin reagent using a homogenizer for 4min on an ice bed. The tissue homogenate was centrifuged at 10,000g and 4°C for 30min. The supernatants were filtered through a 0.22µm filter (Millipore). The haemoglobin

concentration in the supernatant was then determined at a wavelength of 540nm using a haemoglobin assay kit (Hemoglobin Colorimetric-method, Ziestchem Diagnostics). The amount of haemoglobin in the granulation tissue was calculated as mg haemoglobin/100g wet tissue.

2.9. Data Analysis

The data were presented as mean ± SEM and analyzed by one-way ANOVA. To ensure that carrageenan could induce inflammation, the first two groups were compared using independent samples T-test. Differences between the groups were considered statistically significant if $P<0.05$.

3. Results

3.1. Phytochemical Analysis

Findings of the preliminary phytochemical screenings are shown in table 1. According to table 1, *M. persicum* methanol extract contained diverse classes of phytochemicals. The table 1 also presents the RC_{50} values (which indicate in vitro antioxidant activities) for quercetin and the extract.

Table 1. Phytochemical analysis and DPPH radical scavenging capacity of *Marrubium persicum* methanol extract

Alkaloids	-
Anthraquinones	-
Phenols	+++
Flavonoids	++
Saponins	-
Tannins	+
Terpenes	+
DPPH(RC_{50})	62.1 µg/ml (Standard Quercetin: 4.5 µg/ml)

3.2. Effects of *M. persicum* on Inflammatory Parameters

Inflammatory parameters such as volume of exudate, granulomatous tissue weight and the number of leukocytes were evaluated 48h after administering the doses of *M. persicum* methanol extract. Figure 1 indicates significant decreases in inflammatory parameters following the administration. According to the figure 1, with increasing the dose, the volumes of exudate, the total number of leukocytes and the weight of granuloma tissue markedly decreased ($P<0.05$). As expected, administering dexamethasone (0.5mg/kg i.p), which was the positive control in this study, decreased the inflammatory parameters more clearly ($P<0.001$). Extract at the dose of 3.5 mg/kg i.p, 30 min before carrageenan injection (the lowest effective dose), was chosen to analyze the effects of the extract on angiogenesis and myeloperoxidase (MPO) activity.

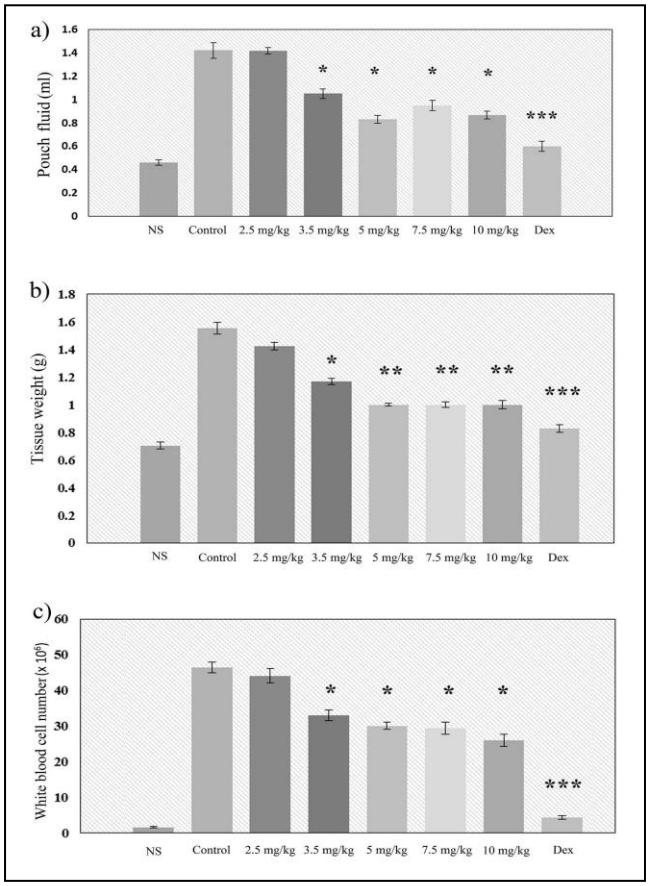

Figure 1. Effects of *Marrubium persicum* extract (in different doses) on the pouch fluid volume (**a**), granulation tissue weight (**b**) and total number of leukocytes (**c**), 48h after carrageenan injection. Values were expressed as mean±SEM presented by the vertical bars. ∗ $P<0.050$, ∗∗ $P<0.010$, and ∗∗∗$P<0.001$ based on ANOVA

3.3. Effect of *M. persicum* on MPO Activity

M. persicum methanol extract at the dose of 3.5 mg/kg i.p; caused a significant decrease in MPO activity ($P<0.05$). The reference drug, dexamethasone, also decreased this inflammatory factor ($P<0.001$). Values are expressed as a percentage of activity (Figure 2).

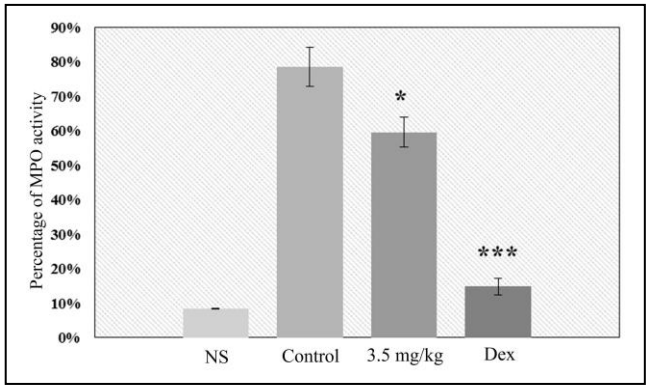

Figure 2. The effects of *Marrubium persicum* methanol extract on myeloperoxidase activity 48h after carrageenan injection. Values were expressed as mean±SEM shown by vertical bars. ∗ P<0.050, and ∗∗∗ P<0.001 based on ANOVA

3.4. Effects of *M. persicum* on Angiogenesis

As shown in figure 3, haemoglobin content which is a marker for angiogenesis, decreased following the extract administration to such an extent that the changes were statistically significant (P<0.05) compared to the control (Figure 3).

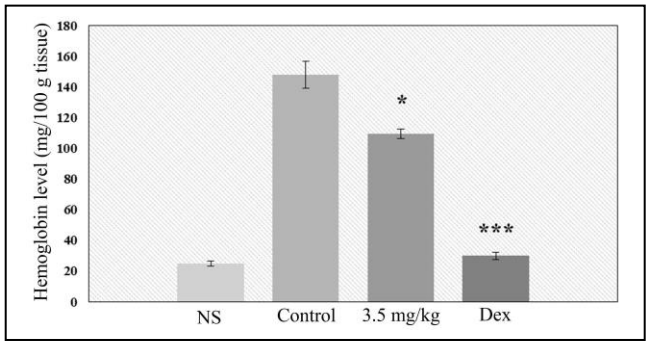

Figure 3. The effects of *Marrubium persicum* methanol extract on hemoglobin level 48h after carrageenan injection. Values were expressed as mean±SEM shown by vertical bars. ∗ P<0.050, and ∗∗∗ P<0.001 based on ANOVA

4. Discussion

According to the results, *M. persicum* methanol extract contained diverse classes of phytochemicals, including phenols, flavonoids, tannins, and terpenes among the assessed classes of compounds, although alkaloids, anthraquinones and saponins were absent in the extract. Previously, researchers had recognized some classes of these compounds in other *Marrubium* species (11).

The application of herbal products to treat different illnesses has a long history in the traditional medicine of many countries (19). In this regard, we aimed to evaluate the angiogenic and anti-inflammatory potentials of *M. persicum*, which is one of the worthy plant species from the genus *Marrubium*. In the present research, the air pouch model was utilized so that carrageenan was administered into the mouse air pouch cavity to promote an inflammatory response characterized by a significant increase in the exudates concentration and cellular proliferation similar to that observed in the synovial fluid of inflammatory diseases.

To investigate the consequences of inflammation (including the alterations in the inflammatory biomarkers), the air-pouch model has been found very applicable and reliable in the relevant pharmacological studies (20). Previously, the model was widely used to evaluate the anti-inflammatory and anti-angiogenic effects of natural compounds such as bee pollen (21) and Ficus carica Linn. Leaves (22).

Concerning the present results, *M. persicum* methanol extract considerably decreased the inflammatory parameters in a dose-dependent manner compared to the control group. Our findings strongly indicated a significant reduction in inflammatory parameters such as the influx of leukocytes, granulomatous tissue weight and exudate volume, and MPO activity and angiogenesis. It is important to note that reports have found various herbs' free radical scavenging potential responsible for their anti-inflammatory effect (23, 24). On the other hand, various reports have repeatedly mentioned antioxidant and anti-inflammatory effects for Marrubium species (11). Therefore, based on these facts, the observed effects of the extract in this study can be relevant to its antioxidant potential. Furthermore, since angiogenesis is a leading cause of cancer and chronic inflammatory conditions, the revealed anti-angiogenic property of *M. persicum* methanol extract could be helpful in the relative diseases.

Undoubtedly, the findings of this study provide evidence that the *M. persicum* extract would attenuate inflammatory responses, primarily reducing the influx of leukocytes and exudate volume. These inhibitory effects could probably be associated with inhibiting the pro-inflammatory enzyme (MPO) and proteins like TNF-α, IL-1β and VEGF-α. Notably, in a study by Ghosh et al., it was reported that COX-2-derived PGE2 underlies angiogenesis in carrageenan-induced granulation tissue through VEGF formation (25). Accordingly, we suggest that *M. persicum* extract might also inhibit COX-2 or deactivate the inflammatory cells expressing COX-2.

In this study, a mouse air pouch model was employed to investigate the possible effects of *Marrubium persicum* methanol extract on inflammation and angiogenesis. Although the exact underlying mechanisms remained unclear, this preclinical research elucidated the plant's significant anti-inflammatory and anti-angiogenic potentials. However, further studies must clarify the involved mechanisms and possible side effects before evaluating the extract in the clinic.

Authors' Contribution

Study concept and design: S. H. and N. M.
Acquisition of data: M. Sh. M.
Analysis and interpretation of data: N. M. and S. H.
Drafting of the manuscript: K. F.
Critical revision of the manuscript for important intellectual content: S. H.
Statistical analysis: M. Sh. M. and K. F.
Administrative, technical, and material support: F. F. and S. H.
Study supervision: S. H. and N. M.
It is important to note that, Apart from the assigned tasks, all authors were involved throughout the procedures.

Ethics

The procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the ethics committee of Tabriz University of Medical Sciences, Tabriz, Iran (approval code: IR.TBZMED.REC.1395.1210).

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

This article was written based on a data set of Pharm. D thesis registered in Tabriz University of Medical Sciences, Iran, with the number 3991.

References

1. Mridha AR, Wree A, Robertson AA, Yeh MM, Johnson CD, Van Rooyen DM, et al. NLRP3 inflammasome blockade reduces liver inflammation and fibrosis in experimental NASH in mice. J Hepatol. 2017;66(5):1037-46.
2. Li X, Mazaleuskaya LL, Ballantyne LL, Meng H, FitzGerald GA, Funk CD. Genomic and lipidomic analyses differentiate the compensatory roles of two COX isoforms during systemic inflammation in mice. J Lipid Res. 2018;59(1):102-12.
3. Blake DR, Allen R. Inflammation: basic principles and clinical correlates. Ann Rheum Dis. 1988;47(9):792.
4. Franceschi C, Campisi J. Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. J Gerontol A Biol Sci Med Sci. 2014;69(Suppl_1):S4-S9.
5. Colville-Nash P, Scott D. Angiogenesis and rheumatoid arthritis: pathogenic and therapeutic implications. Ann Rheum Dis. 1992;51(7):919-25.
6. Carmeliet P. Angiogenesis in health and disease. Nat Med. 2003;9(6):653-60.
7. Nyberg P, Xie L, Kalluri R. Endogenous inhibitors of angiogenesis. Cancer Res. 2005;65(10):3967-79.
8. Stankovic MS. Total phenolic content, flavonoid concentration and antioxidant activity of Marrubium peregrinum L. extracts. Kragujevac J Sci. 2011;33:63-72.
9. Yalçin FN, Kaya D. Ethnobotany, pharmacology and phytochemistry of the genus Lamium (Lamiaceae). FABAD J Pharm Sci. 2006;31(1):43-52.
10. Paula de Oliveira A, Santin JR, Lemos M, Klein Junior LC, Couto AG, Meyre da Silva Bittencourt C, et al. Gastroprotective activity of methanol extract and marrubiin

obtained from leaves of Marrubium vulgare L.(Lamiaceae). J Pharm Pharmacol. 2011;63(9):1230-7.
11. Meyre-Silva C, Cechinel-Filho V. A review of the chemical and pharmacological aspects of the genus marrubium. Curr Pharm Des. 2010;16(31):3503-18.
12. Abadi A, Hassani A. Essential oil composition and antioxidant activity of Marrubium vulgare L. growing wild in Eastern Algeria. Int Lett Chem. 2013;14:17-24.
13. Hamedeyazdan S, Fathiazad F, Sharifi S, Nazemiyeh H. Antiproliferative activity of Marrubium persicum extract in the MCF-7 human breast cancer cell line. Asian Pac J Cancer Prev. 2012;13(11):5843-8.
14. Soraya H, Moloudizargari M, Aghajanshakeri S, Javaherypour S, Mokarizadeh A, Hamedeyazdan S, et al. Angiogenic effect of the aqueous extract of Cynodon dactylon on human umbilical vein endothelial cells and granulation tissue in rat. DARU J Pharm Sci. 2015;23(1):10.
15. Mishra K, Ojha H, Chaudhury NK. Estimation of antiradical properties of antioxidants using DPPH assay: A critical review and results. Food Chem. 2012;130(4):1036-43.
16. Fathiazad F, Matlobi A, Khorrami A, Hamedeyazdan S, Soraya H, Hammami M, et al. Phytochemical screening and evaluation of cardioprotective activity of ethanolic extract of Ocimum basilicum L.(basil) against isoproterenol induced myocardial infarction in rats. DARU J Pharm Sci. 2012;20(1):87.
17. Hamedeyazdan S, Sharifi S, Nazemiyeh H, Fathiazad F. Evaluating antiproliferative and antioxidant activity of Marrubium crassidens. Adv Pharm Bull. 2014;4(Suppl 1):459-64.
18. Nahar L, Russell WR, Middleton M, Shoeb M, Sarker SD. Antioxidant phenylacetic acid derivatives from the seeds of Ilex aquifolium. Acta Pharm. 2005;55(2):187-93.
19. Baharvand-Ahmadi B, Bahmani M, Rafieian-kopaei M. A summary on the prominent herbal medicine effective for beauty, skin hygiene and wound healing in Iran. J Chem Pharm Sci. 2016;9(1):28-33.
20. Charitidis FT, Damlund DSM, Koch J. Psoriasis-like Inflammation Induced in an Air-pouch Mouse Model. In vivo (Athens, Greece). 2021;35(4):1985-97.
21. Eteraf-Oskouei T, Shafiee-Khamneh A, Heshmati-Afshar F, Delazar A. Anti-inflammatory and anti-angiogenesis effect of bee pollen methanolic extract using air pouch model of inflammation. Res Pharm Sci. 2020;15(1):66-75.
22. Eteraf-Oskouei T, Allahyari S, Akbarzadeh-Atashkhosrow A, Delazar A, Pashaii M, Gan SH, et al. Methanolic Extract of Ficus carica Linn. Leaves Exerts Antiangiogenesis Effects Based on the Rat Air Pouch Model of Inflammation. Evidence-based complementary and alternative medicine : eCAM. 2015;2015:760405.
23. Cuzzocrea S, Riley DP, Caputi AP, Salvemini D. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. Pharmacol Rev. 2001;53(1):135-59.
24. Konyalıoğlu S, Sağlam H, Kıvçak B. α-tocopherol, flavonoid, and phenol contents and antioxidant activity of Ficus carica. leaves. Pharm Biol. 2005;43(8):683-6.
25. Ghosh AK, Hirasawa N, Niki H, Ohuchi K. Cyclooxygenase-2-mediated angiogenesis in carrageenin-induced granulation tissue in rats. J Pharmacol Exp Ther. 2000;295(2):802-9.