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Research Article

Phytochemical Screening and Anti-Inflammatory Effect of *Marrubium vulgare* L. Methanol Extract on Carrageenan-Induced Paw Inflammation in Rats

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ABSTRACT

Background: The upward desire in using traditional medicine as a remedy for treatment of different diseases has led the scientists to be thoughtful on plants as alternative sources of conventional drugs.
Methods: Herein, anti-inflammatory effects of *Marrubium vulgare* methanolic extract was evaluated in carrageenan- induced paw edema in rats through examining paw thickness, histological studies and myeloperoxidase activity (MPO). The antioxidant activity of *M. vulgare* extract and its phenolic and flavonoids content were evaluated by folin-Ciocalteau, and aluminum chloride colorimetric assay, separately.
Results: The results showed that *M. vulgare* alleviated paw inflammation as indexed by reduction paw thickness ($p<0.001$) as well as MPO activity ($p<0.001$), which was associated with a marked decrease in tissue edema. Moreover, the extract RC_{50} value for DPPH and nitric oxide antioxidant activity was 177 µg/mL and 370.5 µg/ml, besides, the total phenolic and flavonoids were as 625 mg gallic acid equivalent and 1.62 g quercetin equivalent per 100 g of dried plant material.
Conclusion: In the main, the observed anti-inflammatory and antioxidant properties of *M. vulgare* could be attributed to the high amounts of phenolic and flavonoid content identified in the extract.

Introduction

It is clear that there is a great scope for drug discovery from traditional medicines. In fact, these days plants have an important role in western medicine as alternative sources of drugs for some diseases.[1]

The genus *Marrubium*, known as horehound or hoarhound, with more than 30 species belongs to the Lamiaceae family. This plant grows in temperate regions of Europe, northern Africa and Asia.[2] *Marrubium* plants with a valuable location in folk medicine are used traditionally in different diseases such as pulmonary infections, asthma, hypotension, inflammation, cholagogues and also as a pain reliever or sedative agent.[3] Externally, they have been used in wounds and ulcers healing.[2] Nowadays, according to various studies, there is not any debate about the distinct effects of *Marrubium* genus such as cardioprotective effects,[4,5] anti-oedematogenic action,[6] gastroprotective role,[7] anti-hypertensive effect in diabetic rats,[8] analgesic effect in painful diabetic neuropathy,[9] and antispasmodic effects.[10] Also, it has been verified that administration of *Marrubium* species, including *Marrubium vulgare*, causes clear hypoglycemia,[11] reduces serum cholesterol and triglyceride and has a favorable effect on carbohydrate and lipid metabolism.[12,13] Actually, anti-proliferative property[14] and vasorelaxant action[15] of the plant has been proved. The methanolic extract of *M. vulgare* has shown incredible *in vitro* antioxidant activity and free radical scavenging property due to the presence of diterpenes, flavonoids, and phenols.[16]

Reactive nitrogen species (RNS)[17] in addition to reactive oxygen species (ROS)[18] have important role in oxidative stress conditions, especially inflammation. In recent years, the protective effects of antioxidants have been proved in inflammation[19] and provide a new insight in order to reduce

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oxidative stress conditions.

Inflammatory responses are a body defense mechanism to many harmful stimuli. It is a very important survival mechanism, but it can be dangerous in situations such as overwhelming or chronic state.[20] Carrageenan- induced rat paw edema, a fully characterized model from past, is a routine test to determine the anti-inflammatory activity and its underling mechanisms.[21]

Taking into account the former findings on superior anti-inflammatory and immunomodulatory of antioxidants and diterpenes, it was decided to research on anti-inflammatory activity of *M. vulgare* on carrageenan- induced paw edema in rats. So, herein, the effect of *M. vulgare* methanolic extract together with its antioxidant activity in different *in vitro* models was evaluated, with an approach to introduce a medicinal herb with anti-inflammatory properties.

Materials and Methods

Plant and extract preparation

The aerial parts of *M. vulgare* were gathered from Kiasar, in Mazandaran province, Iran during flowering stage (June, 2013) and the plant was identified and authenticated by Dr. Fathiazad. The voucher specimen of the plant (no: 712-TBZ-Fph) has been kept in the herbarium of the faculty of pharmacy, Tabriz, Iran. The dried aerial parts were extracted with methanol (2L×4) by maceration at room temperature and the solvent was removed at 40°C using a rotary evaporator. Finally, a greenish extract weighing 15% was obtained and kept in air tight bottle in a refrigerator for further study.

Determination of total phenolic content

Folin-Ciocalteau reagent was used to determine the total content of phenolic compounds of the *M. vulgare* methanolic extract.[22] For the experiment, 0.5 ml of the plant extract was mixed with 5 ml Folin-Ciocalteu reagent (1:10 diluted with distilled water), and incubated for 5 min, then 4 ml of aqueous Na_2CO_3 (1M) were added to the solution. Subsequent to the incubation time of 15 min at room temperature the absorbance of the produced blue color was measured at 765 nm, spectrophotometrically. Eventually, the total phenolic content was expressed as gallic acid equivalent in milligrams per 100 gram of the fresh plant material, using a standard curve generated with gallic acid. Whereas, the standard curve was prepared with different concentrations of gallic acid in methanol: water (50:50, v/v) through repeating the mentioned procedure.

Determination of total flavonoids content

The content of flavonoids was determined using aluminum chloride colorimetric assay.[22] Briefly, 0.5 ml of the extract solution were mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. Subsequently, the mixture was allowed to stand at room temperature for 30 min. Later, the absorbance of the solution was measured against a blank at 415 nm, spectrophotometrically. Finally, the total flavonoid content was calculated from a quercetin standard curve prepared through the absorption of standard solution in different concentrations under the same conditions.

Assays for in vitro antioxidant activity

DPPH radical scavenging assay

The free radical scavenging capacity of *M. vulgare* methanolic extract was measured in terms of hydrogen donating or radical scavenging ability using the stable 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radicals from the bleaching of the purple-colored DPPH solution via.[23] The methanolic extract (2 ml) at various concentrations (final concentration 1000–31.25 µg/ml) were added to 2 ml of 0.04% DPPH solution. After 30 min of incubation at 25 ºC, the resulting decrease in absorbance at 517 nm was recorded using a UV–Vis spectrophotometer (Shimadzu UV–Vis 2100), and the mean values were obtained from triplicate experiments. Besides, the same procedure was performed for quercetin as a positive control. The remaining concentration of DPPH in the reaction medium was calculated from a calibration curve, as follows:

$$I\ (\%) = 100 \times [(A\ blank - A\ sample) / A\ blank]$$

Where A sample and A blank are the absorbance values of the reaction mixture with and without plant extract, correspondingly. In addition, the IC_{50} values were established as the concentration of the sample scavenging 50% of free radical concentration.

Nitric oxide radical inhibition assay

Generally, nitric oxide generated from aqueous sodium nitroprusside solution at physiological pH interacts with oxygen to produce nitrite ions that could be measured through the Griess reagent reaction. It is known that scavengers of nitric oxide compete with oxygen bringing about diminished nitric oxide.[24] The reaction mixture contained 10 mM sodium nitroprusside, phosphate buffered saline (pH 7.4) and different concentrations of *M. vulgare* methanolic extract in a final volume of 3 ml. Later than incubation for 150 min at room temperature, 1 ml of Griess reagent (1% sulfanilamide in 20% glacial acetic acid and 0.1% naphthylethylenediamine dihydrochloride) was added to the 0.5 ml of the incubated solution. Following the production of the pink chromophore during diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine, the absorbance of

chromophore formed was read at 548 nm against a blank sample. Additionally, IC_{50} values for the concentration of the extract and quercetin, which was used as a standard antioxidant, compulsory to inhibit 50% of nitric oxide radicals were calculated and compared.

Reducing power assay

The reductive potential of *M. vulgare* methanolic extract was determined according to the method of Yen and Chen (1995).[25,26] The extract and quercetin as the standard at various concentrations were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1% w/v). The mixture was incubated at 50ºC for 20 min. After that, a portion (2.5 ml) of trichloroacetic acid (10%w/v) was added to the mixture and allowed to stand for 10 min at room temperature. Next, 2.5 mL of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride ($FeCl_3$) solution (0.1%, w/v). After all, the absorbance of the reaction mixture was measured at 700 nm spectrophotometrically, seeing that increased absorbance of the reaction mixture indicated increased the reducing power of the sample.

Animals

Male Wistar rats (180 to 200 g) were used. The animals were housed in the Animal House of the Tabriz University of Medical Sciences at a controlled ambient temperature of 25 ± 2°C and a 12 h light/12 h dark cycle with free access to food and water. The present study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of Tabriz University of Medical Sciences, Tabriz-Iran.

Carrageenan- induced paw edema

The dried extract was kept at 4°C until usage. At the experiment day, it was dissolved in the solvent (30% DMSO in normal saline) and passed through a weighed paper filter. The filtered solution was used for intraperitoneal injection. After filtration, to gain the real concentration of the extract, the filter was dried and weighed again in order to calculate the unfiltered particles.

Experimental protocol

The animals were randomly allocated into four groups which each has consisted ten rats. Rats in group 1 (control) received an i.p injection of extract solvent (500 µl) and in group 2 to 4 (treatment groups) treated with i.p injection of extract (2.5, 5 & 10 mg/kg; 500 µl) one hour before S.C injection of 100 µl of carrageenan 1% (w/v) in the left hind paw.[27] The paw thickness was measured by a dial caliper from the ventral to the dorsal surfaces[28] immediately prior to carrageenan injection and then at hourly intervals from 1 to 4 h afterward. Data were expressed as a percentage of increase in the paw thickness and were compared with those of pre-injection values. After fourth hour measurement, the rats were sacrificed by an overdose of diethyl ether or pentobarbital and the inflamed paws were excised with a guillotine and kept at -20 °C.

Myeloperoxidase (MPO) activity

Myeloperoxidase (MPO) activity as a marker of tissue neutrophils activity was measured, as previously described.[5] Briefly, the chopped tissue (n=6) was homogenized in a cold solution containing 0.5% hexa-decyltrimethyl ammonium bromide (HTAB) dissolved in 50 mM potassium phosphate buffer (pH 6). After centrifuging, at 3000 rpm for 30 min at 4°C, supernatant (0.1 ml) or standard (Sigma, Germany) was added to 2.9 ml solution of 50 mM potassium phosphate buffer at pH 6 containing 0.167 mg/ml of O-dianisidine hydrochloride and 0.0005% H_2O_2. After 5 min, the reaction was stopped with 0.1 ml of 1.2 M hydrochloric acid. The rate of change in absorbance was measured by a spectrophotometer at 400 nm. Myeloperoxidase activity was expressed in milliunits (mU) per 100 mg weight of wet tissue.

Histological examination

After four hours, the inflamed paw tissues from the control, and extract treated rats were excised under anesthesia and fixed in 10% buffered formalin then the rats were sacrificed. The tissue samples stained with haematoxylin and eosin for distinguishing tissue damages.

Statistic

All results are expressed as mean±SEM. One way ANOVA was used to make comparisons between the groups, and the significant differences were examined by the LSD *post-hoc* test. Differences between groups were considered significant at a level of $p<0.05$.

Results

Total phenols content of M. vulgare extract

The content for *M. vulgare* total phenolics showed the value 625 mg of gallic acid equivalent in 100 g of the dried plant. The following equation obtained from the gallic acid standard curve was applied in the calculation of the phenolics content (Table 1).

Sample absorbance = 0.0058 x concentration (µg/ml) + 0.0156; R^2= 0.999.

Total flavonoids content

Quantification of the total flavonoids content in *M. vulgare* methanolic extract via aluminum chloride as the shift reagent has revealed that the value for the total flavonoid content was calculated as 1.62 g quercetin equivalent in 100 g of *M. vulgare* dried

plant with reference to the relative standard curve obtained from various solutions of quercetin (Table 1).

In vitro antioxidant activity of M. vulgare extract

Three different methods were accomplished to evaluate antioxidant activity of the *M. vulgare* extract. In this regards, *M. vulgare* extract exhibited moderate antioxidant activity in DPPH radical scavenging assay with IC_{50} values of 177µg/mL for the extract and 3.8 µg/mL for the quercetin (Table 1). Also, for the nitric oxide radical inhibition assay, it was established that incubation of aqueous sodium nitroprusside solutions at physiological pH resulted in nitrite production, which was reduced by the *M. vulgare* methanolic extract. The results indicated that the extract had moderate nitric oxide scavenging activity in a dose-dependent manner with the IC_{50} value of 370.5 µg/ml as compared to the quercetin with 55.35 µg/mL value (Table 1).

As shown in Figure 1, a higher absorbance value indicates a stronger reducing power of the quercetin. However, *M. vulgare* extract showed weak reducing power.

Effects of M. vulgare on carrageenan- induced paw edema

As expected, carrageenan injection induced acute inflammation in control rats with a sharp increase in paw thickness, began 1 h after injection that reached a peak of inflammation after 4 h (Figure 2). In comparison with the pre-carrageenan control value, the paw thickness in the control group increased by 91.78 ± 3.17%, at the 4th hour. As shown in Figure 2, intraperitoneal injection of animals with the methanolic extract of aerial parts of *M. vulgare* caused a potent inhibition of the carrageenan- induced inflammation. The doses of 2.5, 5, 10 mg/kg of extract induced a significant ($p<0.001$) anti-inflammatory effect after carrageenan injection at all hours. As demonstrated in Figure 3, pretreatment by doses of 2.5, 5, and 10 mg/kg significantly decreased ($p<0.001$) the total inflammatory response measured as area under the curve (AUC) from 240.21±7.97 in control to 159.93±9.62, 144.15±7.85, and 101.58±10.85, respectively.

Effect of M. vulgare on myeloperoxidase activity

A characteristic feature of inflammation (swelling and erythema) is an accumulation of neutrophils in target tissues. As shown in Figure 4 a significant ($p<0.001$) increase in myeloperoxidase (MPO) activity, a marker of neutrophil infiltration into paw tissue, was seen 4th hour after injection of the carrageenan. The treatment of rats with an intraperitoneal injection of the single dose of *M. vulgare* (2.5, 5, 10 mg/kg) decreased MPO activity from 1889.95±145 mU/100 mg wet tissue in control to 732.88±175.98, 694.98±126.82 and 283±48.09 ($p<0.001$); respectively in treated groups.

Table 1. Total phenolic and flavonoids content of *M. vulgare* as well as its antioxidant activity *in vitro*.

	Total Phenolics *	**Total Flavonoids** **	**IC_{50} (µg/ml) DPPH**	**IC_{50} (µg/ml) Nitric oxide**
M. vulgare	625	1.62	177	370.5
Quercetin	-	-	3.8	55.3

* Values are reported as mg galic acid equivalent in 100g of dried plant material.
** Values are reported as g quercetin equivalent in 100g of dried plant material.

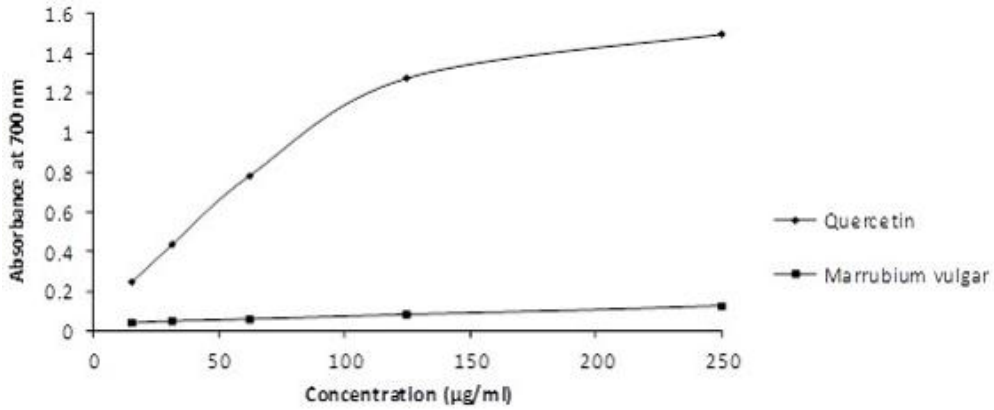

Figure 1. The reductive potential of different concentrations of Quercetin and *M. vulgare* methanolic extract using spectrophotometric detection of the Fe^{3+}–Fe^{2+} transformations.

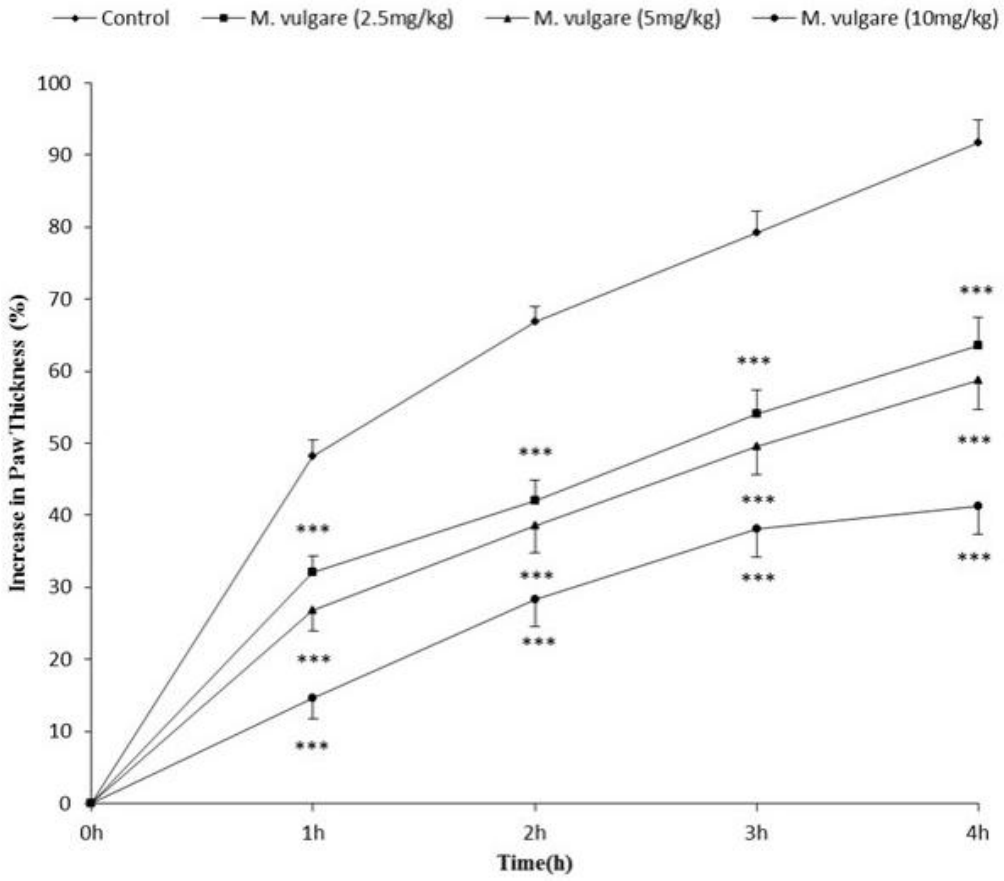

Figure 2. The effect of intraperitoneal injection of *M. vulgare* (2.5, 5, 10 mg/kg) on carrageenan- induced paw edema in rats compared to control group. *M. vulgare* was injected 1 hour before induction of inflammation by carrageenan; Results are expressed as the percentage of increase in paw thickness from values. Each point represents mean±SEM of nine rats per group. Asterisks indicate significant changes from the control value (***p<0.001).

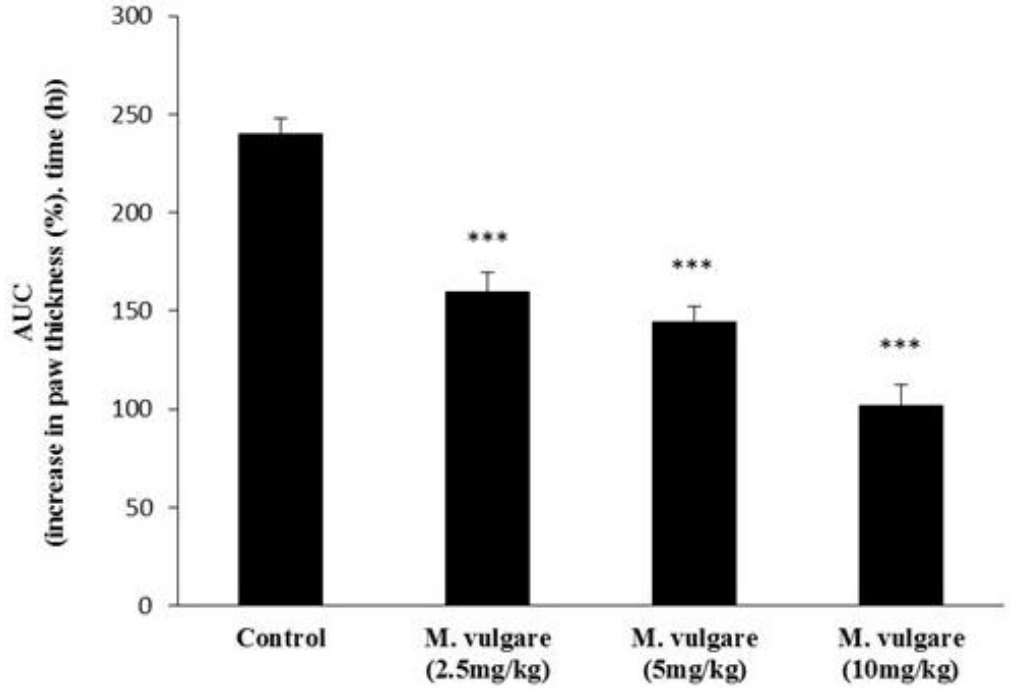

Figure 3. Total edema responses measured as area under the curve (AUC) of the *M. vulgare* treated rats compared to control. Each column represents mean±SEM of nine rats per group. Asterisks indicate significant changes from the control value (***p<0.001).

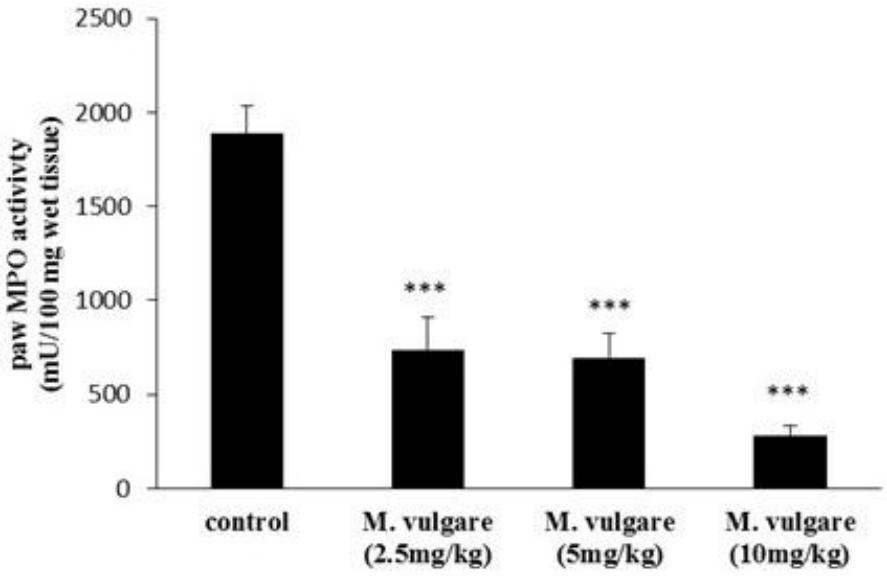

Figure 4. The effect of intraperitoneal injection of *M. vulgare* (2.5, 5, 10 mg/kg) on myeloperoxidase (MPO) activity in carrageenan- induced paw edema in rats compared to control group. *M. vulgare* was injected 1 hour before induction of inflammation by carrageenan; Results are expressed as milliunit MPO activity in 100 mg wet tissue. Each point represents mean±SEM of 6 rats per group. Asterisks indicate significant changes from the control value (***p<0.001).

Effect of M. vulgare on tissue damage

According to histopathological examination there was a sharp oedematasis in the dermis with spongy like appearance in epidermis after carrageenan administration (Figure 5A). However, all three doses of *M. vulgare* extract attenuated histological changes dose dependently. As demonstrated in Figures 5B- D there was a low oedematasis in extract treated samples without any sponge like appearance in high dose extract treated groups.

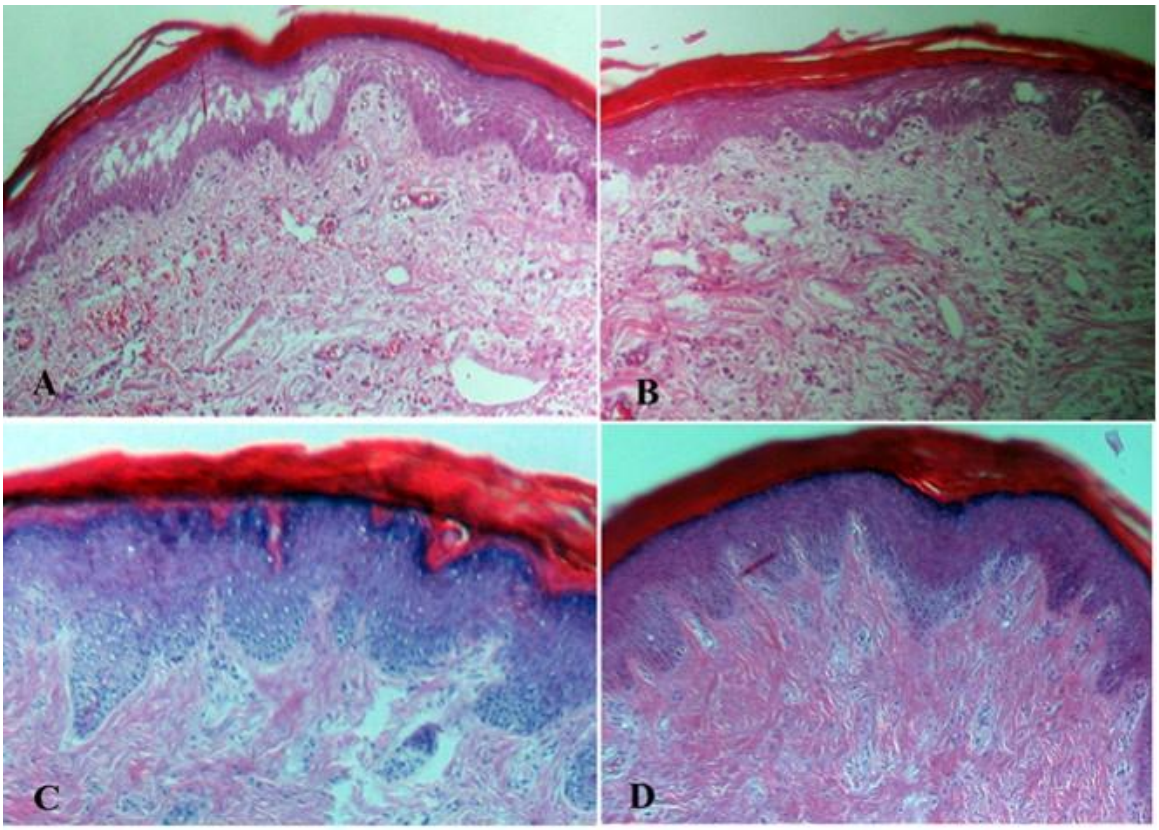

Figure 5. Photomicrographs of rat inflamed paw (N=3). A) Carrageenan control. B) Carrageenan and *M. vulgare* (2.5 mg/kg). C) Carrageenan and *M. vulgare* (5 mg/kg). D) Carrageenan and *M. vulgare* (10 mg/kg). Paw tissue of a rat subcutaneously injected with carrageenan shows intensive inflammation with oedematasis in the dermis and spongy like appearance in the epidermis. Treatment with *M. vulgare* extract considerably attenuated inflammation. Haematoxylin and Eosin (10 M).

Discussion

In the present study, we investigated the effects of methanolic extract of aerial parts of *M. vulgare* on carrageenan- induced paw edema in rats, as well as its antioxidant activity in different *in vitro* models.

It has been proved that there are two inflammatory phases in carrageenan- induced rat paw edema. During the first phase (0-1 h), histamine, 5-hydroxytryptamine (5-HT), bradykinin, and prostanoids have a key role in vascular permeability, while arachidonic acid metabolites and free radicals are more important in the second phase (1-6 h).[29,30]

The methanolic extract of aerial parts of *M. vulgare* was capable of attenuating both early and delayed phases of carrageenan- induced inflammation. Considering the main role of arachidonic metabolites in the both phases of the carrageenan-induced inflammation, it could be explained that the anti-inflammatory activity of the *M. vulgare* methanolic extract is related to the inhibition of the release and/or synthesis of cyclooxygenase and/or lipoxygenase products. In this regard, Sahpaz *et al.* (2002) showed that glycosidic phenylpropanoid esters from *M. vulgare* have COX inhibitory potential, especially on COX-2 subtype.[31] Therefore, the anti-inflammatory action of *M. vulgare* extract in our study could be attributed, at least in part, to phenylpropanoid esters.

Also, PMN accumulation has a prominent role in the second phase of the carrageenan- induced inflammation.[21] For evaluating the extract effect on PMN activity, the neutrophil infiltration into the paw tissue was assessed by myeloperoxidase activity. Myeloperoxidase (MPO) is a peroxidase enzyme which is most abundantly expressed in neutrophil granulocytes.MPO activity as a marker of neutrophil content is directly related to neutrophil number.[32] Intraperitoneal administration of *M. vulgare* extract prevented the accumulation of neutrophils in the target tissue as characterized by significant reduction in MPO activity with all doses of the extract. Production of oxygen-derived free radicals from neutrophils has remarkable effects on tissue injury.[21] Therefore, paw MPO evaluation confirmed histopathological observation in which the extract noticeably decreased morphological injury in paw tissue.

The outburst of histamine, serotonin, bradykinin, prostaglandins, and NO after carrageenan administration contributes to increase vascular permeability and recruitment of neutrophils from vessels to inflammatory area supports the inflammation by producing oxygen-derived free radicals such as superoxide anion and hydroxyl radicals.[21] Considering our results, *M. vulgare* extract acts as an effective antioxidant. Thus, the anti-inflammatory effect of *M. vulgare* could be due to its ability to efficiently scavenge free radicals and inhibit lipid peroxidation as well as COX-2 activity.

These findings are in consistent with our previous study which has been showed the protective effects of *M. vulgare* L. methanolic extract against isoproterenol induced acute myocardial infarction (MI), inflammation and fibrosis in cardiomyocyte.[5] For example, treatment with 40 mg/kg extract attenuated myocardial interstitial fibrosis in compared with MI group. Also, myocardial MPO activity, serum TNF-α as well as peripheral neutrophil count which was markedly increased after the isoproterenol injection was lowered in the treatment groups.[5]

Considering the prior phytochemical reports from genus *Marrubium*, it has been revealed that it mainly produces diterpenes as well as marrubiin which have the variety of activities.[2] Actually, immunomodulating effects of a series of diterpenes from *Marrubium* genus were declared by Karitoti *et al.* in 2007.[33]

Conclusion

In this study, we indicated that methanolic extract of *Marrubium vulgare* considerably ameliorated carrageenan- induced inflammation on the early and late phase in rats. Besides, the present study showed the antioxidant and free-radical scavenging activity of *M. vulgare* which could be related to its phenolic and flavonoids contents. These results confirm the traditional use of *M. vulgare* in inflammation. We conclude that the anti-inflammatory activity of *M. vulgare* can be explained by multiple effects, including lipooxygenase, cyclooxygenase and myeloperoxidase enzyme inhibition and free-radical scavenging activity. Future work should focus on unraveling the exact mechanism(s) responsible for its anti-inflammatory effect and determining the pharmacologically active compound(s).

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Conflict of interests

The authors claim that there is no conflict of interest.

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