

An investigation on cardioprotective potential of *Marrubium vulgare* aqueous fraction against ischaemia-reperfusion injury in isolated rat heart

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Background: The aim of this study was to evaluate the cardioprotective effects of aqueous fraction of *Marrubium vulgare* hydroalcoholic extract on cardiac parameters in ischaemic-reperfused isolated rat hearts.

Materials and methods: The aerial parts of the plant were extracted with methanol 70% by maceration. The water-soluble portion of the total hydroalcoholic extract was prepared with liquid-liquid extraction (LLE). Afterwards, the antioxidant activity, total phenolic and flavonoids content of the aqueous fraction were determined. In order to evaluate the effects of the aqueous fraction on cardiac parameters and ischaemia-reperfusion (I/R) injury, the Langendroff method was used on male Wistar rats. Harvested hearts were cannulated immediately to the Langendroff apparatus and subjected into 30 min regional ischaemia and 2 h reperfusion, either by a modified Krebs-Henseleit buffer (KHB) solution or enriched KHB solution with plant extract (10, 20, 40 µg/mL).

Results: The aqueous fraction was found to be a scavenger of DPPH radical with RC50 value of 47 µg/mL. The total phenolic and flavonoids content of the fraction was 6.05 g gallic acid equivalent and 36.13 mg quercetin equivalent per 100 g of dry plant material. In addition, 40 µg/mL of *Marrubium vulgare* aqueous fraction significantly decreased infarct size in comparison to control group. All doses considerably reduced the total ventricular ectopic beats during 30 min of ischaemia. The extract at dose of 40 µg/mL noticeably decreased the arrhythmias during the first 30 min of reperfusion.

Conclusions: The results of the study indicated aqueous fraction of *Marrubium vulgare* possesses a protective effect against I/R injuries in isolated rat hearts. (Folia Morphol 2017; 76, 3: 361–371)

Key words: *Marrubium vulgare*, ischaemia-reperfusion (I/R) injury, Langendroff, ventricular ectopic beats, arrhythmias

INTRODUCTION

Ischaemia-reperfusion (I/R) injury is one of the important life-threatening medical problems worldwide. In recent years, various experiments and clinical

studies have been conducted to introduce effective natural compounds which can improve the function of the affected tissues or decrease the detrimental consequences of I/R injury [18, 29]. In ischaemia by

circulatory arrest, the aerobic metabolism turns into anaerobic due to the imbalance between oxygen demand and supply. The anaerobic metabolism leads to the reduction of intracellular pH which results in ionic homeostasis disturbances. Although a well-timed reperfusion is fundamental for recovery of a dying myocardium but by sudden restoration of blood flow to the ischaemic myocardium, a burst of reactive oxygen species (ROS) will be produced as a reaction to hyperoxia which can make the situation worse. This phenomenon is known as myocardial I/R injury [1].

The overproduction of ROS not only influences the process of injury directly but also exacerbates the circumstance indirectly. ROS can initiate the oxidation of lipids in cell membrane, oxidation of proteins and cause DNA fragmentation (directly) whereas these species can intensify the ionic derangement i.e. Ca^{2+} overload in cytosol and induce the inflammatory factors (indirectly). As a result, the myocytes death occurs and myocardium loses its ability to function well [6, 26].

Marrubium vulgare L., known as horehound, is a medicinal herb from Lamiaceae family which mainly grows in North Africa, Central and Western Asia and also in some parts of Europe [19]. This perennial plant is widely distributed in northern and eastern provinces of Iran [10, 27]. *M. vulgare* is a popular medicinal herb and in traditional medicine has been used as a neurosedative, hypotensive, antispasmodic, antinociceptive, antidiabetic and anti-inflammatory agent. Studies dealing with the biological activities on the plants of *Marrubium* genus, revealed anti-coagulant, anti-inflammatory, immunomodulating, cytotoxicity, vasorelaxant, hepatoprotective, hypolipidaemic, hypoglycaemic and antioxidant properties [4, 5]. The merit of the traditional use of *M. vulgare* along with its reported biological activities have been supported by the isolation and identification of a group of biologically active phytochemicals such as diterpenes, flavonoids, phenylpropanoid esters, tannins and sterols. Detailed phytochemical screening of *M. vulgare* showed that the plant is rich in marrubiin diterpene, flavonoid compounds such as luteolin, apigenin, quercetin, isoquercitrin, chrysoeriol, vitexin and polyphenols such as proanthocyanidins (Condensed tannins) [2, 20, 21, 24, 25]. Phenylpropanoids, a group of phenolics, were mostly detected in *M. vulgare* including acteoside, forsythoside B, arenarioside, ballotretoside, marruboside, and acethyl marruboside. Among them, acteoside, also known

verbascoside, is the principle phenylpropanoid that is believed to be one of the key bioactive compound and responsible for many health benefits of the plant. Proanthocyanidins, the water-soluble subclass of the phenolic group, are composed of flavanol (catechins) units and rich in green tea, red wine, grape seeds and fruits like blueberries, strawberries, red apple and in nuts such as hazelnut and pistachio [1, 12, 22]. Catechin and epicatechin are the two most studied compounds from proanthocyanidins. Many studies have been done in order to prove that catechins play an important role in cure of myocardial ischaemia and reduce the mortality risk of cancer, myoglobinuric acute renal failure and congestive heart failure due to antioxidant, anti-inflammatory, and vasodilatory effects [12, 16]. These components give the plant superior antioxidant content. Hence, *M. vulgare* has potential in promotion of health and prevention of conditions associated with inflammation, cardiovascular disease, dyslipidaemia, cancer, hyperglycaemia or increased oxidative stress. In this paper, concerning the polyphenols role in scavenging free radicals and with inspiration from Yousefi et al. [27] study which showed the cardioprotective effect of methanolic extract of *M. vulgare*, it has been decided to evaluate whether the aqueous extract of *M. vulgare* has the cardioprotective effect against myocardial I/R injury.

MATERIALS AND METHODS

Solvents and chemicals

Methanol, ethyl acetate, chloroform, petroleum ether and the Krebs-Henseleit buffer (KHB) solution ingredients (potassium dihydrogen phosphate, potassium chloride, magnesium chloride, sodium chloride, D-glucose, calcium chloride and sodium bicarbonate) all from Merck company (Germany) were used. Ketamine and xylazine were from Alfasan, Netherlands. Evans blue from Sigma, Japan and the triphenyl tetrazolium chloride (TTC) from Baker (England) were used. 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent and aluminium chloride all from Sigma Aldrich chemical company (USA) were used. All other reagents and chemicals were of analytical grade.

Animals

Healthy male albino Wistar rats (270–300 g) were used in this study. They were housed in the standard cages, under a 12 h light/dark cycle. The animals were given food and water freely. The present study was done regarding the Guide for the Care and Use

of Laboratory Animals of Tabriz University of Medical Sciences, Tabriz, Iran.

Extract preparation

The aerial parts of *M. vulgare* were collected during flowering stage from Kiasar, Mazandaran province, Iran. The air-dried plant materials (500 g) were grounded and powdered finely and then defatted by petroleum ether (2 L × 2) at 25°C. Afterwards, the plant materials were extracted with methanol 70% by maceration (2 L × 4) at room temperature and the solvent was removed at 40°C using rotary evaporator. Subsequently, the water-soluble portion of the total hydroalcoholic extract was prepared after removing the non-polar fractions through liquid-liquid extraction (LLE) with chloroform and ethyl acetate, respectively. The water of resultant aqueous extract containing polar compounds was dried under reduced pressure, and the dried extract was kept in the refrigerator until the phytochemical analyses and pharmacological procedure.

DPPH radical scavenging assay

This assay was performed using the method proposed by Hamedeyazdan et al. [10]. 2, 2-diphenyl-1-picrylhydrazyl, known as DPPH, is a reagent which is used in laboratory assays in order to determine the antioxidant capacity of different samples, especially the herbal extracts. It composes of free radical molecules that make the colour of a solution purple and by neutralisation the colour turns into pale yellow. The stock concentration 1000 µg/mL of the aqueous fraction of the *M. vulgare* extract was prepared and then diluted with methanol to the concentrations below: 500, 250, 125, 62.5, 31.25 and 15.62 µg/mL.

All these concentrations were prepared in the volume of 2 mL. The same volume (2 mL) of 0.004% of DPPH solution was added to each tube. After 30 min incubation in 25°C, the absorbance was read against blank at 517 nm. Tests were carried out in triplicate where the average absorption was reported for each concentration. This test was done with quercetin as the positive control. The free radical scavenging of the extract was calculated in percent by the equation below:

$$R\% = [(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}] \times 100$$

where A_{blank} is the absorbance of the solvent (containing all reagents except the plant extract) and A_{sample} is the absorbance of the sample. RC50 is the concen-

tration of the plant extract reducing 50% of the free radicals which was calculated from the graph of R% against aqueous extract concentrations in µg/mL.

Total phenolic content assay

This assay was done through Folin-Ciocalteu method in order to determine the total phenol values [10]. 0.5 mL of aqueous fraction of *M. vulgare* solution were mixed with 5 mL Folin-Ciocalteu reagent (diluted in distilled water 1:10). After 5 min, 4 mL of Na_2CO_3 1 M was added to the mixture and shaken well. Following the incubation of the mixture at room temperature for about 15 min, the absorbance was measured by UV-VIS spectrophotometer at 765 nm. The standard curve was prepared by 50–250 µg/mL solutions of gallic acid in methanol: water (50:50) against the absorbance. The total phenolic content of the aqueous fraction was expressed as gallic acid equivalents, in mg/100 g of dry plant material.

Total flavonoids content assay

In order to determine the total flavonoids content in *M. vulgare* aqueous fraction, the colorimetric aluminium chloride method was performed [10]. 0.5 mL of the aqueous fraction with 1.5 mL methanol, 0.1 mL AlCl_3 10%, 0.1 mL potassium acetate 1 M and 2.8 mL distilled water were mixed and shaken well. The mixture was allowed to stand at room temperature for 30 min, later the absorbance of the mixture was measured by UV-VIS spectrophotometer at 415 nm.

The calibration curve was drawn by 31.25–250 µg/mL of quercetin solutions in methanol. The value of total flavonoids was reported in mg per 100 g of dry plant material which was equivalent to quercetin.

Nitric oxide radical inhibition assay

This assay was done according to Griess Illosvoy reaction. The reaction mixtures (3 mL) containing sodium nitroprusside (10 mM, 2 mL), phosphate buffer (pH: 7.4, 0.5 mL) and different concentrations of *M. vulgare* aqueous fraction (0.5 mL) were incubated at room temperature for 150 min, then 1 mL of sulphanilic acid reagent (0.33% in glacial acetic acid 20%) was added to 0.5 mL of each reaction mixture. After being left for 5 min, 1 mL of naphthyl ethylene diamine dihydrochloride as a Griess reagent was added and they were allowed to stand for 30 min at 25°C. Afterward, absorbances of the mixtures were

measured by UV–VIS spectrophotometer at 540 nm. Quercetin was used as the positive control [23]. The nitric oxide radical scavenging of the aqueous fraction was calculated in percent by the equation below:

$$R\% = [(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}] \times 100$$

where A_{blank} is the absorbance of the solvent (containing all reagents except the plant extract), A_{sample} is the absorbance of the sample and R% represents the reduction percent. RC50 is the concentration of the plant extract reducing 50% of nitric oxide radicals which was calculated from the graph of R% against different concentrations of *M. vulgare* aqueous extract in $\mu\text{g/mL}$.

TLC analysis

To determine the presence of acteoside, the main phenylpropanoid, and bioactive compound in *M. vulgare* aqueous extract, the extract was subjected to qualitative thin layer chromatography (TLC) using pure acteoside as standard, solvent system chloroform: methanol: water (60:40:8) and stationary phase silicagel GF254 (Merck). The spot of acteoside was visualised by irradiation the TLC plate with ultraviolet light and spraying AlCl_3 5% reagent.

Qualitative Stiasny assay

The aqueous extract (10 mL) was mixed with mixture of hydrochloric acid and formaldehyde (1:2, 5 mL) and allowed to be heated under reflux for 30 min. The red-brown precipitation is a sign of catechins presence [8].

The pharmacological procedure

Male Wistar rats (270–300 g) were subjected into four groups ($n = 6$). Group number one is the control group that was perfused by KHB solution. The second, the third and the fourth groups were received KHB solution with different concentrations of aqueous fraction (10, 20 and 40 $\mu\text{g/mL}$, respectively) 5 min before ischaemia induction.

The rats were heparinised and anaesthetised with the mixture of ketamine and xylazine. From the moment that the hearts were cannulated to the constant-pressure Langendorff system (within the KHB solution circulation which was oxygenated by 95% O_2 /5% CO_2 and with the 80 mm Hg pressure and the temperature of 37°C) until the ischaemia induction time, was a period of 20 min which is called stabilisation phase. During this period, the hearts which were not follow-

ing a stable steady-state pattern were rejected. In the treated groups, 5 min before the end of the stabilisation phase, the hearts were perfused by *M. vulgare* aqueous fraction until the end of the reperfusion phase. Ischaemic phase started right after the stabilisation period. It was induced by occluding of left anterior descending coronary artery (LAD) via two plastic pipette tips which is known as regional ischaemia and lasted for 30 min. By removing these plastic tips after 30 min of ischaemia, the reperfusion phase began. This phase continued for 120 min and after that the suture around the LAD was tied (knot) and then the hearts were ready for Evans blue staining which was perfused via the aortic cannula [3].

Hemodynamic changes record

After mounting the heart on the Langendorff apparatus, a latex balloon connected to pressure transducer was inserted into the left ventricle in order to measure the left ventricular systolic and diastolic pressure (LVSP and LVDP, respectively), develop pressure (DP) and heart rate (HR) [3, 23]. Left ventricular developed pressure = LVSP – LVDP. The output volumes of KHB solution from the hearts were measured at special time intervals (flow rate):

- end of stabilisation phase;
- 5, 10, 15, 20, 25 and 30 min of ischaemia phase;
- 5, 10, 15, 20, 25, 30, 45, 60, 90 and 120 min of reperfusion phase.

Lactate levels measurement

The perfused KHB solutions from the hearts were collected in microtubes at the end of stabilisation period, 30 min of ischaemia phase and the first 30 min of reperfusion period. The lactate levels of these samples (the marker of the anaerobic glycolysis) were measured with lab assays [15, 20].

Infarct size and staining protocol

In regional ischaemia double staining strategy (Evans blue and TTC, respectively) is performed.

After tying secure knots and occluding LAD coronary arteries, the hearts were separated from the Langendorff apparatus and Evans blue (0.25% in saline) was infused into the hearts via cannula. The non-risk zone areas became dark blue and the risk zone areas stayed pale pink. Following the freezing of the hearts, TTC staining was applied which the viable myocytes absorbed the dye and became brick-red but the non-viable myocardium cells got the pale

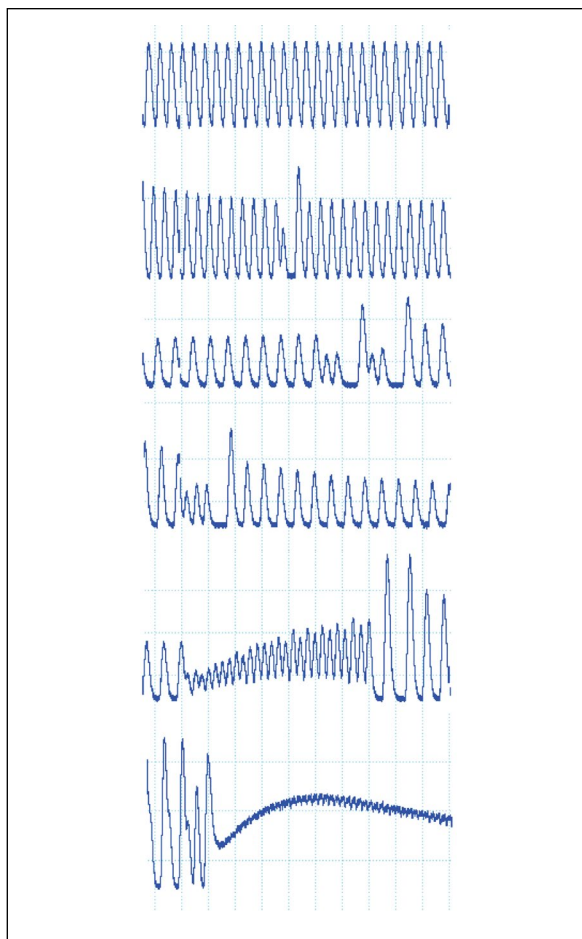


Figure 1. Arrhythmia's rhythm. From the top to the bottom: normal rhythm, Single ectopic beat, salvos ectopic beats, triplet ectopic beat, ventricular tachycardia and ventricular fibrillation.

appearances [3]. The risk zone areas and infarct sizes were measured by equations below:

- Risk zone area (%) = (non-perfused area) / (total area of the heart) \times 100;
- Infarct size (%) = (dead cells area) / (non-perfused area) \times 100.

Enumeration of arrhythmias

By a latex balloon connected to pressure transducer in the left ventricle, the normal rhythm of the hearts and the irregular heartbeats (single, salvo, triplet, ventricular tachycardia [VT] and ventricular fibrillation [VF]) were recorded. The arrhythmias were studied by the Lambeth Law. The irregular heartbeats are shown in Figure 1.

Statistical analysis

The results of infarct sizes, haemodynamic parameters and lactate concentrations in different groups were analysed by one-way ANOVA and presented

as mean \pm standard error of the mean (SEM). The results of total ventricular ectopic beats (VEBs) which is the sum of (single, salvo, triplet and VT) and the duration of VT and reversible VF in ischaemia and the first 30 min of reperfusion phase were analysed by non-parametric Mann-Whitney U-test and expressed in mean \pm SEM in different groups.

For statistical comparison, the incidence of VT and VF results in different groups are analysed by Fisher's exact test and expressed in percent.

The analysis of all data was done by SPSS Statistics 17.0 software.

RESULTS

DPPH radical scavenging assay

The radical scavenging activity of aqueous fraction of *M. vulgare* extract was determined by DPPH assay. According to the results, the RC50 value for aqueous fraction and quercetin were determined as 47 μ g/mL and 3.2 μ g/mL, respectively.

Total phenolic content assay

The total phenolic content was determined from the standard curve via the equation below:

- Absorbance = 0.0063 \times gallic acid (μ g) + 0.0372, ($R^2 = 0.999$);
- The total phenolic content of the plant aqueous extract was equivalent to 6.05 g of gallic acid per 100 g dry plant material.

Total flavonoids content assay

The assay was done by colorimetric aluminium chloride method and the absorbance of the plant extract was calculated from the equation of the standard curve which is shown below:

- Absorbance = 0.0088 \times quercetin (μ g) - 0.0625, ($R^2 = 0.9965$);
- The total flavonoids content of the plant aqueous extract was equivalent to 36.13 mg of quercetin per 100 g dry plant material.

Nitric oxide radical inhibition assay

According to the obtained data, no inhibitory activity for nitric oxide radicals by *M. vulgare* aqueous fraction was detected.

TLC analysis

Following the TLC of the extract the R_f value of acteoside was calculated as 0.62 which was consistent with R_f of standard acteoside.

Table 1. Effects of *Marrubium vulgare* aqueous fraction (10, 20 and 40 µg/mL) on ischaemia-reperfusion (I/R)-induced cardiac arrhythmias during 30 of min ischaemic phase

Ischaemic phase	Number					Duration [s]		Incidence [%]	
	Single	Salvo	Triplet	VT	VEBs	VT	VF	VT	VF
Control	458.3 ± 26.9	77.5 ± 11.9	25.3 ± 5.3	398.3 ± 141.7	959.3 ± 161	56.6 ± 21.9	5.3	100	25
<i>M. vulgare</i> ext. 10 µg/mL	231 ± 7.6*	14.8 ± 3.7*	2.4 ± 1.1*	125.3 ± 19.4	350.2 ± 25.8*	24.5 ± 4	0	100	0
<i>M. vulgare</i> ext. 20 µg/mL	83.7 ± 6.4*	7.3 ± 1.9*	2.5 ± 0.8*	46.5 ± 11*	140 ± 8.8*	9 ± 2.2*	0	100	0
<i>M. vulgare</i> ext. 40 µg/mL	79.5 ± 9.1*	7 ± 2.1*	3.3 ± 1.3*	53.3 ± 4.5*	143 ± 14.1*	9.9 ± 0.9*	0	100	0

*p < 0.05 vs. the control group; n = 6 in each group; VEBs — ventricular ectopic beats (single + salvo + triplet + VT); VT — ventricular tachycardia; VF — ventricular fibrillation

Table 2. Effects of *Marrubium vulgare* aqueous fraction (10, 20 and 40 µg/mL) on ischaemia-reperfusion (I/R)-induced cardiac arrhythmias during the first 30 min of reperfusion phase

Ischaemic phase	Number					Duration [s]		Incidence [%]	
	Single	Salvo	Triplet	VT	VEBs	VT	VF	VT	VF
Control	64 ± 9.7	6.2 ± 1.4	0.6 ± 0.4	2 ± 2	72.8 ± 10.4	0.4 ± 0.4	5 ± 5	0	25
<i>M. vulgare</i> ext. 10 µg/mL	37.6 ± 10.1	1.4 ± 0.7*	0.4 ± 0.4	0	39.2 ± 9.9	0	0	0	0
<i>M. vulgare</i> ext. 20 µg/mL	47.4 ± 7.5	1.6 ± 1.2*	0.4 ± 0.2	2 ± 2	51.4 ± 8.9	0.3 ± 0.3	0	20	0
<i>M. vulgare</i> ext. 40 µg/mL	36.7 ± 4.8*	2.5 ± 0.5*	0.3 ± 0.3	0	39.5 ± 4.8*	0	0	0	0

*p < 0.05 vs. the control group; n = 6 in each group; VEBs — ventricular ectopic beats (single + salvo + triplet + VT); VT — ventricular tachycardia; VF — ventricular fibrillation

Table 3. The effects of *Marrubium vulgare* aqueous extract (10, 20 and 40 µg/mL) on the ischaemia-reperfusion (I/R)-induced infarct size in isolated rat hearts

	Control	<i>M. vulgare</i> extract 10 µg/mL	<i>M. vulgare</i> extract 20 µg/mL	<i>M. vulgare</i> extract 40 µg/mL
Infarct size (%)	50.58 ± 1.23	54.21 ± 5.81	44.57 ± 3.23	15.61 ± 1.29***

***p < 0.001 vs. the control group; n = 6 in each group

Qualitative Stiasny assay

Following this assay, a brick-red colour precipitation revealed the presence of catechins in the aqueous fraction of *M. vulgare* hydroalcoholic extract.

The effects of aqueous extract of *M. vulgare* on the ischaemic and the first 30 min of reperfusion phase arrhythmias

The effects of aqueous extract of *M. vulgare* on the ischaemic and the first 30 min of reperfusion phase arrhythmias are shown in Table 1.

As shown in Table 1, the aqueous extract significantly decreased the total VEBs during ischaemic phase at all three doses (10, 20 and 40 µg/mL) and

also considerably reduced the VT duration at the doses of 20 µg/mL and 40 µg/mL. No VF was shown in the ischaemic period in treated groups.

The effects of the extract on arrhythmias that appeared during the first 30 min of reperfusion phase are shown in Table 2.

According to the table, the extract decreased the total VEBs with 40 µg/mL significantly. Although the doses of 10 µg/mL and 20 µg/mL decreased the total VEBs but the effects were not significant. No VT was seen in reperfusion phase in the groups which treated by 10 µg/mL and 40 µg/mL and there was also no occurrence of VF in all three treated groups.

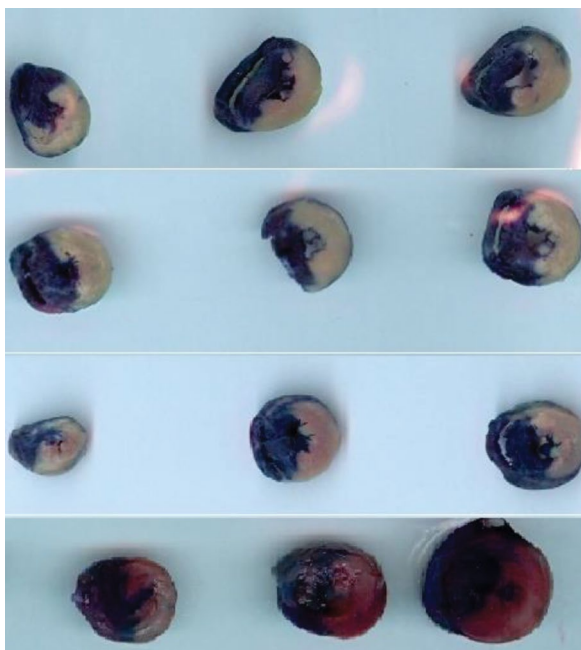


Figure 2. The samples of sliced hearts after double staining. From the top to the bottom: Control group, ext. 10 µg/mL, ext. 20 µg/mL and ext. 40 µg/mL of *Marrubium vulgare* aqueous extract. Non-risk zone areas are dark blue. Viable myocytes are brick-red. Non-viable myocardium cells are pale.

The effect of aqueous extract of *M. vulgare* on infarct size

Regarding Table 3 and Figure 2, the *M. vulgare* aqueous extract decreased infarct size at the doses of 20 µg/mL and 40 µg/mL. The reduction with dose of 40 µg/mL was striking and significant (**p < 0.001).

The effect of aqueous extract of *M. vulgare* on flow rate parameter during I/R

Figure 3 is representing the result of flow rate during the whole period of experiment. By ligation of coronary artery at the end of stabilisation phase, the flow rate decreased from 100% to 70 ± 0.05% during 30 min of ischaemia in control group. By reperfusion, the flow rate elevated again to 86 ± 0.06% and then gradually decreased to 37 ± 0.05% during 120 min of reperfusion phase. The extract at all three doses (especially 40 µg/mL) caused a great decline in flow rate in 30 min of ischaemia as compared to control group. In 120 min of reperfusion period, the flow rates in all treated groups were higher than control group, but no significant changes were occurred.

The considerable changes of flow rate in control and treated groups are shown in Figure 3 by p values (**p < 0.01, *p < 0.05).

The effect of aqueous extract of *M. vulgare* on heart rate parameter during I/R

Figure 4 is showing the result of HR during the whole period of experiment. By the ischaemia induction, the HR decreased from 97 ± 0.01% to 76 ± 0.06% in control group. By reperfusion, the HR increased up to 84 ± 0.05% and gradually decreased to 67 ± 0.04% during 120 min of reperfusion phase. Among all three doses of aqueous extract of *M. vulgare*, only the dose of 40 µg/mL decreased the HR during the both ischaemia and the reperfusion phases as compared to control group. Hence the aqueous extract induced bradycardia but not significantly.

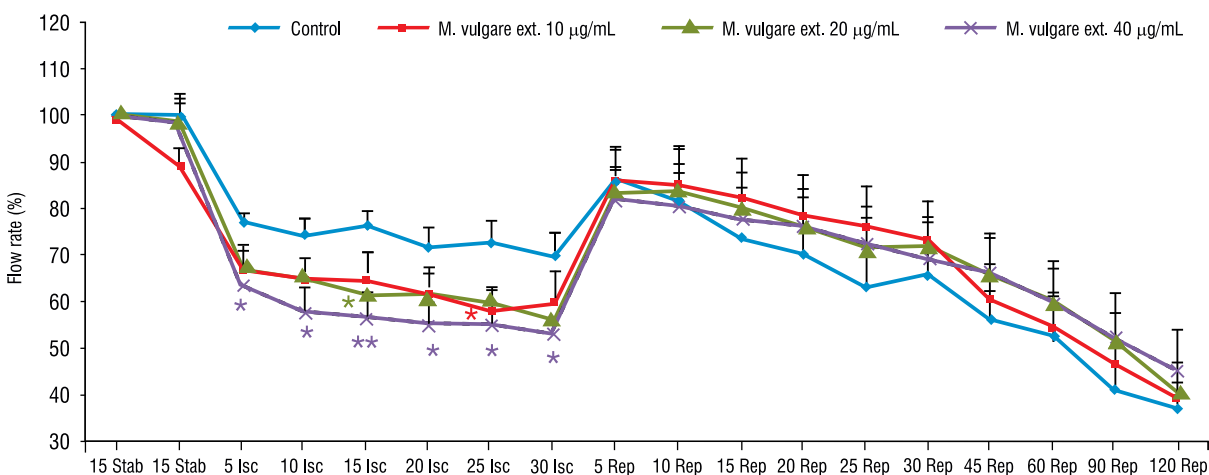


Figure 3. Flow rate in the control and treated groups receiving 10, 20 and 40 µg/mL of *Marrubium vulgare* aqueous fraction during whole experiment period. Data are normalised to 15 Stab and represented as mean ± standard error of the mean (SEM); Stab — stabilisation; Isc — ischaemia; Rep — reperfusion; Ext — extract; n = 6 rats in each group; **p < 0.01, *p < 0.05.

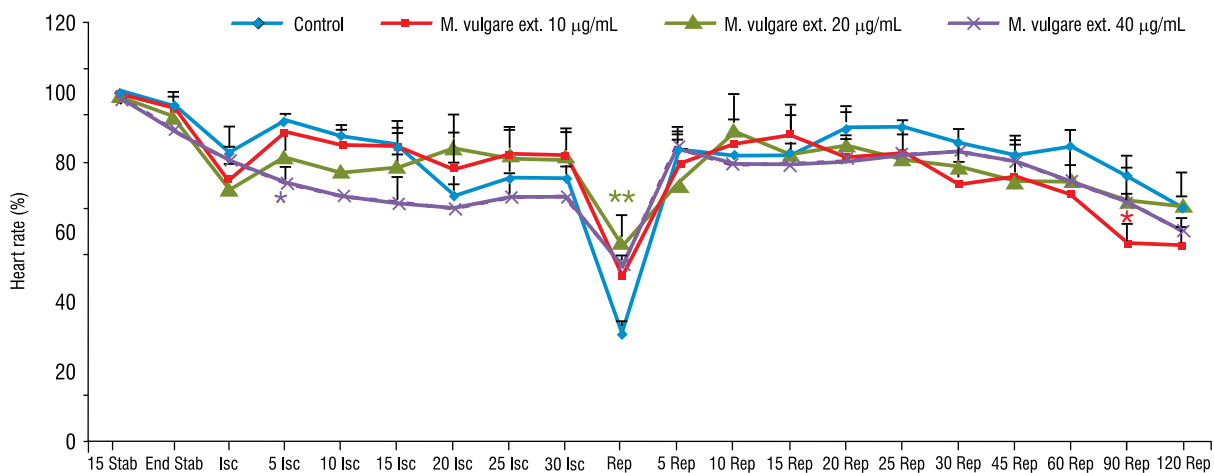


Figure 4. Heart rate in the control and treated groups receiving 10, 20 and 40 µg/mL of *Marrubium vulgare* aqueous fraction during whole experiment period. Data are normalised to 15 Stab and represented as mean \pm standard error of the mean (SEM); Stab — stabilisation; Isc — ischaemia; Rep — reperfusion; Ext — extract; n = 6 rats in each group; **p < 0.01, *p < 0.05.

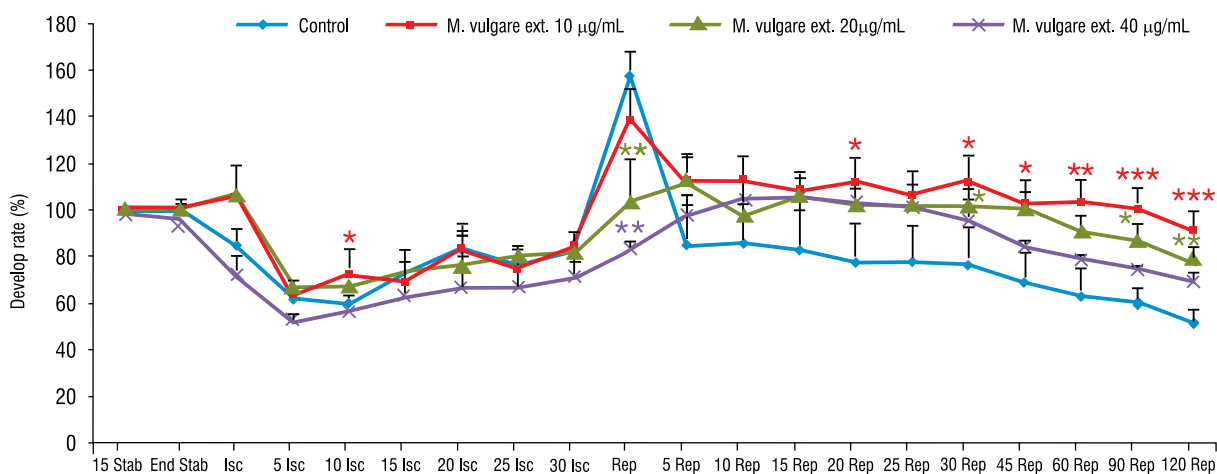


Figure 5. Develop pressure in the control and treated groups receiving 10, 20 and 40 µg/mL of *Marrubium vulgare* aqueous fraction during whole experiment period. Data are normalised to 15 Stab and represented as mean \pm standard error of the mean (SEM). Stab — stabilisation; Isc — ischaemia; Rep — reperfusion; Ext — extract; n = 6 rats in each group; *p < 0.05, **p < 0.01, ***p < 0.001.

The considerable changes of HR in control and treated groups are shown in Figure 4 by p values (**p < 0.01, *p < 0.05).

The effect of aqueous extract of *M. vulgare* on develop pressure parameter during I/R

Figure 5 is demonstrating the result of DP during the whole period of experiment. By commencing the ischaemic phase, the DP started decreasing from 100% to $82 \pm 0.08\%$ during 30 min of ischaemia in control group. By reperfusion, the DP increased up to $85 \pm 0.17\%$ and then continuously decreased to

$52 \pm 0.05\%$ during 120 min of reperfusion phase. The Figure 5 clearly show that the treated group with 40 µg/mL indicated the lower DP than control group in 30 min of ischaemia. On the contrary, the DP increased much more than control group in 120 min of reperfusion period.

The DP rhythm at doses of 10 µg/mL and 20 µg/mL in ischaemic phase were not regular but after reperfusion the DP of these both treated groups increased much more than control group especially with 10 µg/mL of the extract (p < 0.001). The considerable changes of DP in control and treated groups are

Table 4. Lactate concentration (mg/dL) in perfusate at the end of the 30 min of regional ischaemia and during the first 30 min of reperfusion phase in the control and treated groups receiving 10, 20 and 40 μ g/ml of *Marrubium vulgare* aqueous extract

Groups	Lactate concentration [mg/dL] At the end of ischaemia	Lactate concentration [mg/dL] 30 min after reperfusion
Control + I/R	2.37 \pm 0.4375	3.0220 \pm 0.28537
Normal control	0.7050 \pm 0.15842	0.7050 \pm 0.15842
<i>M. vulgare</i> extract (10 μ g/mL)	2.18 \pm 0.3414	1.9750 \pm 0.2335**
<i>M. vulgare</i> extract (20 μ g/mL)	2.0216 \pm 0.2099	1.6420 \pm 0.20346**
<i>M. vulgare</i> extract (40 μ g/mL)	2.4433 \pm 0.3960	2.608 \pm 0.2929

**p < 0.001 vs. control group; IR — ischaemia-reperfusion

shown in Figure 5 by p values (*p < 0.05, **p < 0.01, ***p < 0.001).

Concentration of lactate in perfusate

Aqueous extract of *M. vulgare* did not exert considerable positive effect on lactate (a marker of anaerobic metabolism) concentration in perfusate during ischaemia phase (Table 4). According to Table 4 the extract significantly decreased lactate level in perfusate at the dose of 10 μ g/mL and 20 μ g/mL in the first 30 min of reperfusion period in comparison to the control + I/R group (**p < 0.01).

DISCUSSION

Since cardiovascular disorders are still one of the leading cause of death all over the world especially in industrial countries, in recent years, using herbal medicines has drawn attention of many researches. The important and definite roles of ROS in the initiate and progress of heart disorders have been reported in several investigations [28]. In ischaemia by cessation of blood flow, the aerobic metabolism stops and the anaerobic metabolism begins. This kind of metabolism makes a decline in intracellular pH which leads to ionic derangement. By sudden restoration of blood flow to the ischaemic myocardium, the situation becomes worse due to the ROS production in the great amounts. ROS is reactive oxygen species that can directly injure the heart tissue by oxidation of lipids, proteins and DNA, on the other hand they can induce the inflammatory factors [1, 6, 26].

It has been formerly ascertained that there is a positive and linear relationship between plants phenolic contents and their antioxidant activity, in which higher phenolic content of a plant materials increases

the antioxidant activity of the plant. According to our results, the aqueous fraction of *M. vulgare* extract was found to accumulate the phenolic compounds including phenylpropanoids (acteoside) and proanthocyanins with a distinguished antioxidant activity. As far as we know, proanthocyanidins (condensed tannins), the water-soluble subclass of the phenolic components, are composed of flavanol (Catechin and epicatechin) units that are rich in fruits like berries [9, 22]. Catechin and epicatechin, the two most studied compounds from proanthocyanidins, have been reported to have an impressive protective role on cardiovascular system. It is believed that their effectiveness in reducing the risk of myocardial ischaemia, and congestive heart failure have apparently been associated with their antioxidant, anti-inflammatory, and vasodilatory activities [12, 14, 16]. Owing to the fact that condensed tannins scavenge the free hydroxyl and superoxide radicals, they could prevent cardiomyocytes and myocardium against oxidative stress, reduce reperfusion-induced VF and VT, and increase mitochondrial and respiratory chain enzymes (aerobic metabolism induction) as well [7, 11, 17, 22, 29]. In addition, Jing et al. [13] showed the protective effect of acteoside on lipopolysaccharide-induced inflammation in acute lung injury. They demonstrated that acteoside increased superoxide dismutase (SOD) and inhibited malondialdehyde (MDA) content and levels of proinflammatory cytokines in LPS-stimulated mice. Previously, we reported the cardioprotective effect of methanolic extract of *M. vulgare* in isoproterenol-induced myocardial infarction in rats. In that study it was found that the extract was rich in non-polar secondary metabolites of the plant and marrubiin identified as the main active component [27].

In current study, we evaluated the protective activity of aqueous extract of *M. vulgare* on I/R injuries in

the isolated rat heart along with its antioxidant activity. The phytochemical screening of the aqueous extract showed the presence of acteoside and proanthocyanidins. Based on our findings, the aqueous extract of *M. vulgare* markedly lowered infarct size, and the total VEBs in ischaemia period. Also arrhythmias were decreased in reperfusion phase. These observations point to the protective role of the *M. vulgare* aqueous extract in I/R injury. Considering the fact that ROS bursts caused by I/R injury result in further destructive reactions and defective cell functions, it seems the protective activity of the *M. vulgare* extract to be partly related to the free radical scavenging activity of the phenolics content of the extract. Moreover, the simultaneous presence of proanthocyanidins and acteoside may exerts an additive or synergistic effect in protection activity of the extract.

CONCLUSIONS

Overall, *Marrubium vulgare* aqueous extract signifying to have medicinally valuable secondary metabolites and exhibited remarkable effects on different cardiac parameters. In general, the present study showed this plant has the potential to be protective against cardiac complications such as I/R injuries. However, further rigorous research is necessary to support the beneficial impact of *M. vulgare* in preventing and/or improving I/R injuries and related risk factors. In addition, assessment of the possible toxicity of the plant extract is requisite to recommend the plant for clinical experiments.

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