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Effect of Methanolic Extract of *Marrubium crassidens* Boiss on Ischemia/Reperfusion Induced Arrhythmias and Infarct Size in Isolated Rat Heart

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ABSTRACT

Background: Methanolic extract of *Marrubium crassidens* Boiss has potent antioxidative effects and can have cardio-protective effects on Ischemia/Reperfusion (I/R) injuries in heart. ***Methods:*** The extract was prepared by maceration. The isolated rat hearts were perfused by Krebs-Henseleit solution enriched with the extract (0, 10, 50, and 100µg/ml), using the langendorff method. After 15 minutes stabilization, the hearts subjected to 30 minutes regional ischemia and then 120 minutes reperfusion. During the experiments hemodynamic functions were recorded and cardiac arrhythmias were determined. At the end, the infarct size was measured. ***Results:*** The extract at 100µg/ml caused a significant reduction in the number of ischemia and reperfusion induced ventricular-ectopic-beats (P<0.05). The extract at 100µg/ml also remarkably (P<0.001) reduced the number of ischemic ventricular tachycardia (VT). The incidence of ischemic VT was reduced from 100% in the control group to 20% in the group treated with 100 µg/ml (P<0.01). The infarct size was 70.74±10.35% in the control group whereas, perfusion of ischemic hearts with the extract (10, 50µg/ml) reduced the size to 19.11±6.26 (P<0.001) and 25.27±3.89% (P<0.01), respectively. ***Conclusions:*** *M. crassidens* has protective effects against I/R injuries in isolated rat hearts and the protective effects could be related to antioxidative activities of the extract.

Introduction

Cardiovascular diseases (CVDs) are the number one cause of death globally and more people die annually from CVDs than any other reasons.[1] Among various types of CVDs, coronary heart disease or ischemic heart disease is the most common mortal type in most countries, and a major cause of hospital admissions.[2] Despite huge advances in modern medicine, traditional medicine is still used in all over the world as supplementary and this points to the importance of research on natural compounds used in folk medicine. The genus of Marrubium with forty species is considered as a herbal medicine and some species are used traditionally as a beneficial agent in different disease such as asthma, pulmonary infections, inflammation, hypotension, cholagogues and also as sedative agent and pain reliever[3] Numerous studies have scientifically proven many distinct effects of Marrubium genus, such as anti-oedematogenic action [4,5], anti-hypertensive effect in diabetic rats,[6,7] analgesic effect in painful diabetic neuropathy,[8,9] antispasmodic effects,[10,11] gastro-protective role,[12] and cardio-protective effects.[13,14]

According to the evidences administration of Marrubium species causes clear hypoglycemia,[15-19] reduces serum cholesterol and triglyceride and has a beneficial effect on carbohydrate and lipid metabolism [20,21]. The plant have also vasorelaxant activity [22] and anti-proliferative property [23-25] which all of these effects are beneficial characteristics in cardiac diseases. Furthermore, Marrubium species contain high amount of polyphenols and flavonoids [26,27] which can attenuate oxidative stress and inflammatory reactions caused by oxygen free radicals involved in ischemic heart conditions. Recently our group has shown that the *M. vulgare* total extract has a strong protective effects against isoproterenol-induced myocardial infarction and it seems that the protection is due to its anti-inflammatory effects.[13,14]

Accordingly, the aim of our study was to evaluate the effects of methanol extract of *Marrubium crassidens* Boiss on Ischemia/Reperfusion induced arrhythmias and on infarct size in the isolated rat heart with an approach to introduce an herb with protective effects against ischemic heart diseases.

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Material and methods

Animals

Healthy male albino Wistar rats (290±20 g) were used in this study. They were housed in standard polypropylene cages, six per cage, under a 12 h light/dark cycle in temperature of 22±2 °C with 50±10% relative humidity. The animals were given food and water freely. 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 (National Institutes of Health Publication No. 85-23, revised 1985).

Chemical reagents

Triphenyltetrazolium chloride (TTC) and Evan's blue were purchased from Baker Analyzed (USA). The other reagents were of a commercial analytical grade.

Plant extraction and preparation

M. crassidens was collected during the flowering stage from Chichaklou in East Azarbaijan province, Iran, in June 2011. A voucher specimen of the plant (Tbz-Fph-719) representing this collection has been deposited at the Herbarium of the Faculty of Pharmacy, Tabriz University of Medical science, Iran. The air-dried and well-grounded aerial parts of *M. crassidens* (0.5 kg) were extracted with solvents of increasing polarity, petroleum ether (40–60°C), dichloromethane and methanol (5L of each solvent, thrice every 48h) by maceration at room temperature.

Preparation and isolated heart perfusion

Preparation and isolated heart perfusion was performed as previously described [28] with minor modifications. Male Wistar rats were heparinized (1000 IU/kg ip) and then anesthetized with kethamin/xylasin (60 / 10 mg/kg; ip). The pedal pain withdrawal reflex to the external stimuli was evaluated and when no response was observed, an incision was performed along the xyphoid-sternum to the lateral ends of costal margins. The ribs at the right and left anterior axillary lines were cut to create a clamshell thoracotomy. The surgery for harvesting of the heart was possible after the anterior chest wall had been deflected upwards. The harvested heart was placed in cold KHBS and extra tissues were removed. After that the heart was cannulated to the langendorff apparatus and perfused retrogradely by the buffer immediately within less than 5 min from opening the thorax for harvesting the heart till the heart being completely perfused with buffer to avoid damage or preconditioning.[28]

The ML176-V Langendorff Apparatus, ADInstruments, Australia, was used. The hearts were retrogradely perfused at a constant flow (10 mL/min/ g (heart weight)) with a KHBS containing (in mmol/L) NaCl 125, KCl 4.3, KH_2PO_4 1.1, $MgCl_2$. $6H_2O$ 1.3, $CaCl_2$. 2 H_2O 2.4, $NaHCO_3$ 25, and glucose 13.32. The perfusate was gassed with carbogen (5% CO_2/95% O_2). The pH was 7.38-7.56 at 37°C. After stabilization period (15 min), time was set to zero and KHBS enriched with the extract (10, 50, 100 µg/ml in separate groups, n=10) was infused commencing 5 min before occlusion and maintained for the whole period of the experiment.

As soon as the harvested heart was attached to langendorff setting, the suture is put in place around the left anterior descending artery (LAD) and formed to make a snare. During ischemia, the snare is tightened around LAD and loosed during reperfusion.

For monitoring coronary perfusion pressure (CPP), the aortic cannula was connected to a pressure transducer (MLT844 physiological pressure, ADInstruments, Australia). To measure left ventricular contractility, a latex balloon attached to a second pressure transducer was inserted into the left ventricular cavity via the mitral valve after removing the atrial appendage. Left ventricular developed pressure (LVDP) was calculated as the difference between peak-systolic and end-diastolic pressure, the maximum and minimum rate of left ventricular pressure (dP/dt_{max}, dp/dt_{min}) and heart rate (HR) as a marker of left ventricular contractility were continuously recorded by PowerLab 8/35, ADInstruments, Australia. Cardiac arrhythmias were determined based on the Lambeth conventions.[29]

Measurement of myocardial infarct size

According to Bell *et al.* study [28] with some modification, to determine the infarct size double staining strategy is necessary. After120 min reperfusion period, the ligature around the LAD artery re-tied. To distinguish the perfused area from non-perfused area (area at risk), the cannulated heart was detached and perfused slowly by 1 cc Evan's blue dye (0.25% w/v) via aortic cannula. Then the heart was stored at -20 °C. For second staining the frozen heart was sliced from apex to base (into 1-2 mm sliced). The slices incubated with 1% (w/v) triphenyltetrazolium chloride (TTC) solution in phosphate buffer for 15 min at 37 °C to dye the non–infarcted region. At the end the slices were fixed in 10% formalin overnight. This procedure resulted in the normally perfused tissue being stained blue, non-infarcted and non-perfused tissue stained brick red, infarcted tissue remaining unstained and appeared pale. Digitally photographed sliced were imported to Image J software (Wayne Rasband, National Institute of Health, USA) and infarct size was computed.

Statistical analysis

Except for the incidence of VT and ventricular fibrillation (VF) that indicated as percentage, all results expressed as mean±SEM. To compare the number of VT, ventricular ectopic beats (VEBs) and duration of VT and VF, between groups, the Mann-Whitney non-parametric U-test was employed. Analyzing the incidence of VT and VF was accomplished by Fisher test.

Hemodynamic changes were analyzed between and

within groups, using one-way ANOVA followed by LSD and Paired-Samples T-Test, respectively. The percentage of infarct size was analyzed by Mann-Whitney non-parametric U-test. Differences were considered significant at a level of $P<0.05$.

Table 1. Effects of methanol extract of *M. crassidens* (10- 50- 100 µg/ml) on hemodynamic parameters during 5 minute before ischemia. Left ventricular developed pressure (LVDP), heart rate (HR), coronary perfusion pressure (CPP), left ventricular dp/dt$_{max}$ (dp/dt $_{max}$), and left ventricular dp/dt$_{min}$ (dp/dt $_{min}$), bpm (beat per minute). Data are represented as Mean±SEM for 7 rats in each group.

	M. crassidens 10 µg/ml		*M. crassidens* 50 µg/ml		*M. crassidens* 100 µg/ml	
	Before extract	**5 min after extract**	**Before extract**	**5 min after extract**	**Before extract**	**5 min after extract**
LVDP (mmHg)	87.9±4.97	94.5±5.37	116.25±14.03	100.25±16.63	97.25±9.23	127.5±11.72*
HR (bpm)	259±7.78	216±26.68	274.75±11.02	227±13.32**	243±14.84	224±7.06
CPP (mmHg)	119.4±11.73	112.4±11 *	58.5±6.74	54.75±10.45	50.25±6.7	61.25±4.37
dp/dt$_{max}$ (mmHg)	3045.2±209.28	3124.1±204.17	3496±360.67	2664.25±414.34	2966.5±331.6	3741.75±444.97
dp/dt$_{min}$ (mmHg)	-1906.1±174.2	-1810±95.1	-2196±263.7	-1790±265.9	-1931.5±160.01	-2397.25±168.88*

$^{*}P<0.05$ and $^{**}P<0.01$ compared to the baseline value (Before extract).

Results

Effects of methanolic extract of Marrubium crassidens Boiss on hemodynamic parameters in isolated rat heart

Perfusion of heart with KHBS enriched with *M. crassidens* methanolic extract was started 5 min before ischemia. The effects of application of the extract for 5 min before ischemia on hemodynamic changes were evaluated within groups (Table 1). Perfusion of total extract of the *M. crassidens* with 10 µg/ml caused significant reduction in coronary perfusion pressure (CPP) ($P<0.05$). Heart rate (HR) was decreased significantly from 274.75±11.02 to 227±13.32 by 50 µg/ml of the extract ($P<0.01$). Meanwhile, *M. crassidens* with concentration of 100 µg/ml significantly increased LVDP from 97.25±9.23 to 127.5±11.72 mmHg ($P<0.05$) and decreased LVdp/dt $_{min}$ from -1931.5±160.01 to -2397.25±168.88 mmHg ($P<0.05$). A slight but not significant increase in LVdp/dt $_{max}$ was seen by 10 and 100 µg/ml. However, LVdp/dt $_{max}$ was decreased by 50 µg/ml with no significant differences.

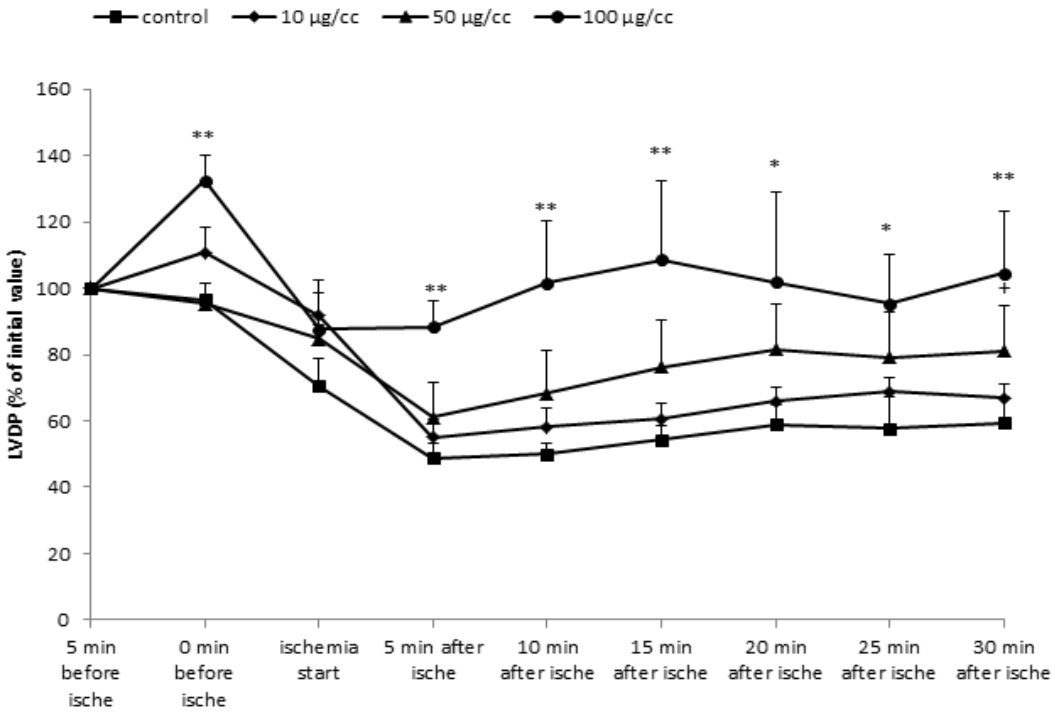

Figure 1. The effects of methanolic extract of *M. crassidens* (10, 50, 100 µg/ml) on LVDP during ischemia. Data are presented as Mean±SEM. N=7. $^{+}P<0.05$ showing the significant differences of group 50 µg/ml *vs.* control and $^{*}P<0.05$ and $^{**}P<0.01$ showing the significant differences of group 100 µg/ml *vs.* control. LVDP: left ventricular developed pressure; ische: ischemia.

In addition, the hemodynamic changes from the beginning of the extract perfusion to the end of regional ischemia, within 5 min intervals, were evaluated and compared to ischemic control group. The extract at 100 µg/ml remarkably (P<0.01) increased LVDP during stabilization period. Further, LVDP was significantly improved by 100 µg/ml of the extract during ischemia (P<0.01-0.05). A slight but not significant increase in LVDP was seen by 10 and 50 µg/ml of the extract during the period of ischemia (Fig. 1).

When compared to the untreated control group, both LV dP/dt_{max} and LV dP/dt_{min} were significantly elevated by 100 µg/ml (P<0.01) at the end of stabilization period when there is no ischemai. In addition to increase of myocardial contractility, the high dose of the extract at 100 µg/ml also significantly improved LV dP/dt_{max} and LV dP/dt_{min} during the period of ischemia (P<0.001; P<0.01 and P<0.05). Besides, LV dP/dt_{max} and LV dP/dt_{min} were significantly enhanced at 25 (P<0.05), and 30 min (P<0.01, P<0.05, respectively) after ischemia by 50 µg/ml. The concentration of 50 µg/ml of the extract improved (P<0.05) LV dP/dt_{max} at 20 min of ischemia. The concentration of 10 µg/ml of the extract produced no significant changes in the heart contractilty (Fig. 2).

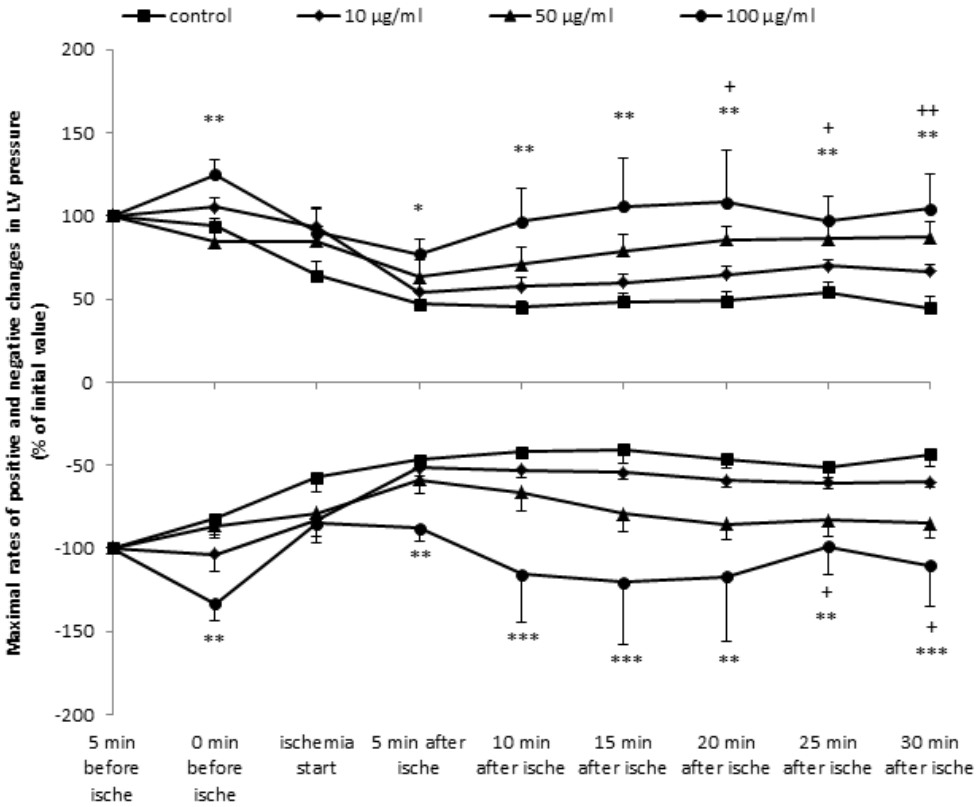

Figure 2. The effects of methanolic extract of *M. crassidens* (10, 50, 100 µg/ml) on maximal rates of positive and negative changes in left ventricular pressure (LV dp/dt_{max} ; LV dp/dt_{min}) during ischemia. Data are represented as Mean±SEM. N=7. ^{+}P<0.05, ^{++}P<0.01 showing the significant difference of group 50 µg/ml *vs.* control. $^{*}P$<0.05, $^{**}P$<0.01, and $^{***}P$<0.001 represents the significant difference of group 100 µg/ml in comparison with control. ische: ischemia.

Table 2. Effects of methanol extract of *M. crassidens* (10, 50, and 100 µg/ml) on duration of VT and VF and VT incidence during ischemia and in the first 30 minutes of the reperfusion period.

Groups	Ischemia time			Reperfusion time		
	VF Duration (sec)	**VT Duration (sec)**	**VT incidence (%)**	**VF Duration (sec)**	**VT Duration (sec)**	**VT incidence (%)**
Control	7.875±7.87	24.9±10.28	100	0	5.1±1.77	87.5
M.crassidens (10 µg/ml)	0.74±0.65	5.98±2.25*	80	1±0.93	3.79±1.53	73.3
M.crassidens (50 µg/ml)	4.94±3.87	24.85±7.5	100	0.98±0.9	16.74±3.88*	100
M.crassidens (100 µg/ml)	0	0.9±0*	20**	20.33±20.33	62.8±39.48*	100

Data are represented as Mean±SEM. $^{*}P$<0.05 and $^{**}P$<0.01 versus control group, respectively. n=7 rats in each group. VT: Ventricular tachycardia, VF: ventricular fibrillation.

Only a significant decrease in the heart rate and CPP were seen by 10 and 50 µg/ml (P<0.05) in the beginning of ischemia (Figs. 3 and 4). Methanolic extract of *M. crassidens* had no significant effects on the hemodynamic parameters during reperfusion time (data are not shown). The extract at 10 (P<0.05) and 100 µg/ml (P<0.001) decreased the VT number and only 100 µg/ml of the extract significantly decreased the number of single, triplet and total VEBs during the ischemic period (P<0.05). At ischemic period, only 100 µg/ml of the extract significantly reduced the number of total VEBs occurring as single and salvoes (P<0.05). At reperfusion time, VT duration was increased by 50 and 100 µg/ml (P<0.05) and there is not any significant different for VF duration and VT incidence when the treated groups were compared to control group (Table 2).

As shown in Figure 2, only the extract at 100 µg/ml significantly decreased the number of single, triplet and total VEBs during reperfusion period (P<0.05). VT number was increased by 50 µg/ml (P<0.05)

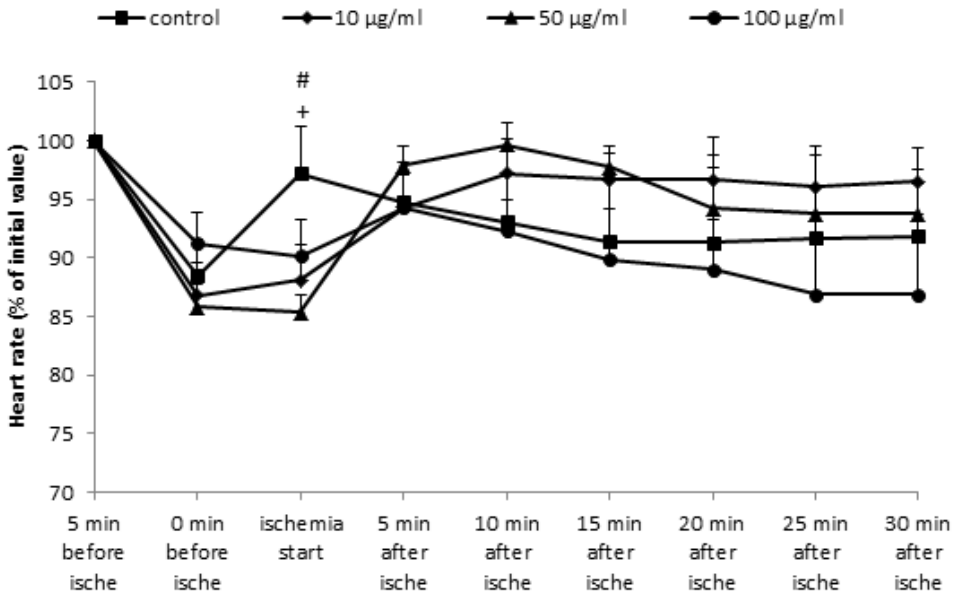

Figure 3. The effects of methanolic extract of *M. crassidens* (10, 50, 100 µg/ml) on heart rate during ischemia. Data are represented as Mean±SEM. N=7. $^{\#}P$<0.05 showing the significant difference of group 10µg/ml *vs.* control and ^{+}P<0.05 represents the significant difference of group 50 µg/ml in comparison with control. ische: ischemia.

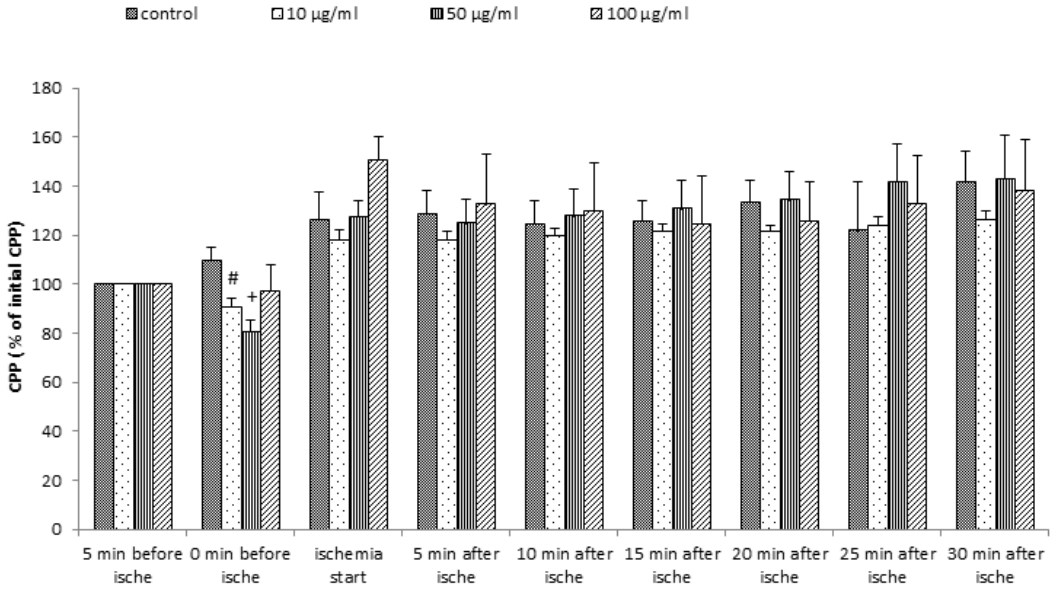

Figure 4. The effects of methanolic extract of *M. crassidens* (10, 50, 100 µg/ml) on CPP during ischemia. Data are represented as Mean±SEM. N=7. $^{\#}P$<0.05 showing the significant difference of group 10µg/ml *vs.* control and ^{+}P<0.05 represents the significant difference of group 50 µg/ml in comparison with control. CPP: coronary perfusion pressure; ische: ischemia.

Effects of methanolic extract of Marrubium crassidens Boiss on infarct size in the isolated rat heart

As demonstrated in Figure 7, the infarct size was 70.74±10.35% in the control group while the perfusion of the total extract of *M. crassidens* at 10 and 50 µg/ml remarkably decreased it to 19.11±6.26 (P<0.001) and 25.27±3.89 % (P<0.01), respectively.

In addition, the extract at the concentration of 100 µg/ml had no significant effect on the infarct size following 30 min ischemia and 2 h reperfusion. To show that the places of ligation at coronary artery are same in all experiments the ischemic risk zone was calculated. As shown in Figure 8, there is no significant difference between areas at risk in all groups.

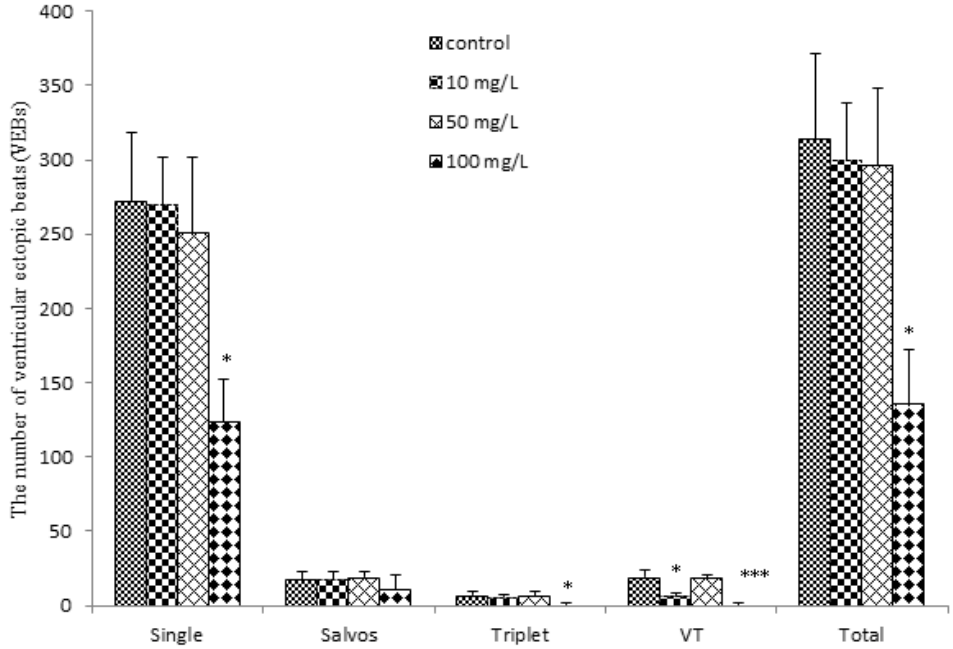

Figure 5. The total number of VEBs in the control and treated groups receiving 10, 50 and 100 µg/ml methanol extract of *M. crassidens* during ischemia. Data are represented as Mean±SEM. $^{*}P$<0.05, and $^{***}P$<0.001 *vs.* the control group respectively. N=7. VEBs: ventricular ectopic beats; ische: ischemia.

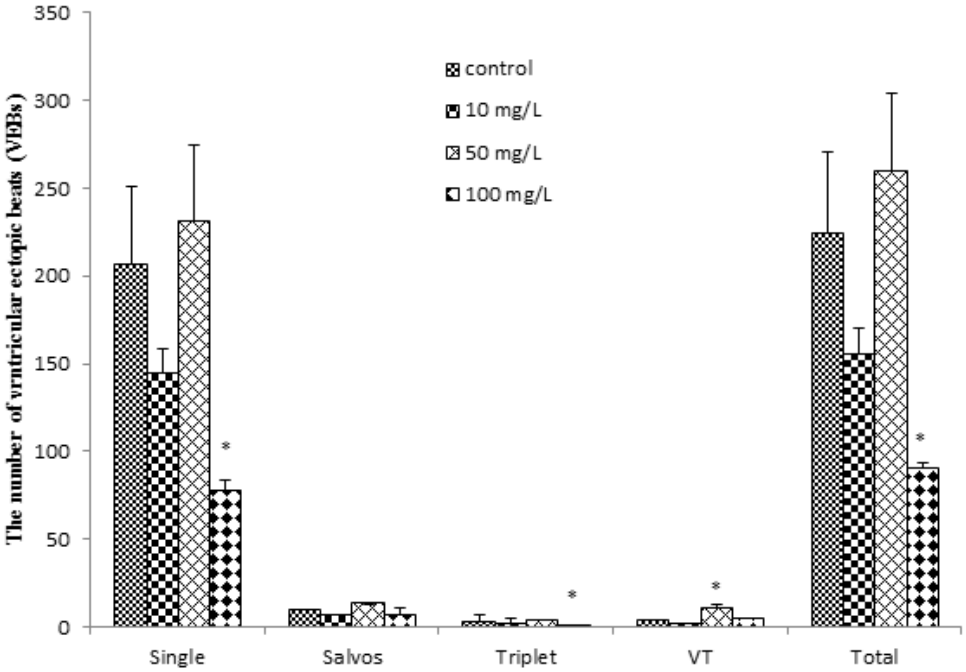

Figure 6. The total number of VEBs in the control and treated groups receiving 10, 50 and 100 µg/ml methanol extract of *M. crassidens* in the first 30 min of reperfusion period. Data are represented as Mean±SEM. $^{*}P$<0.05 *vs.* the control group. N=7. VEBs: ventricular ectopic beats; ische: ischemia.

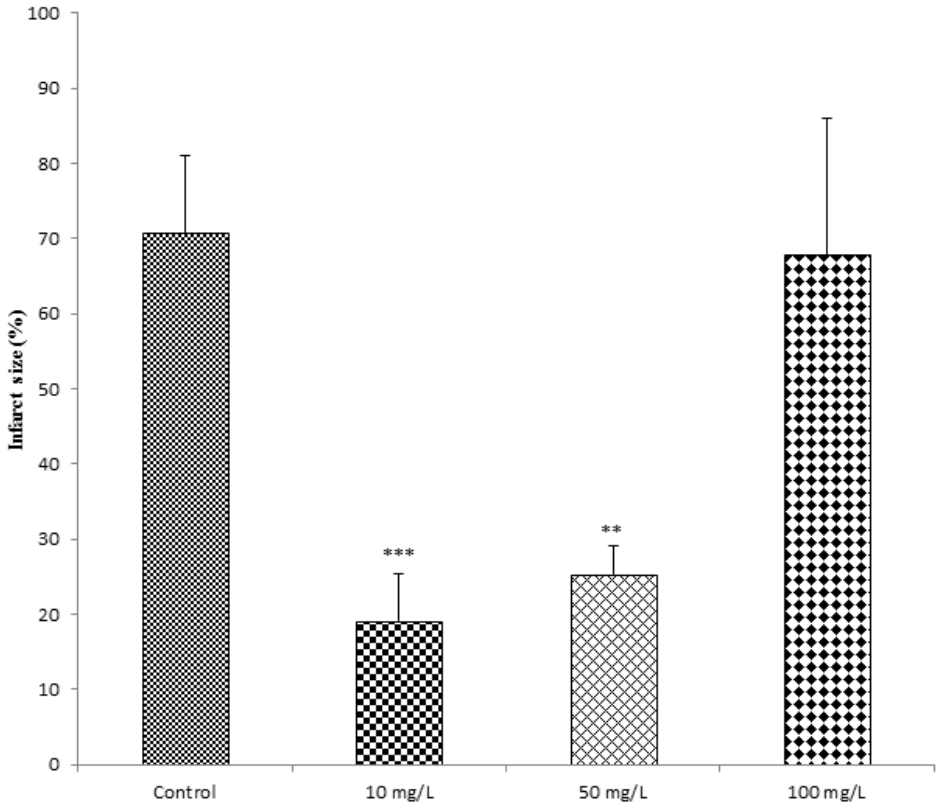

Figure 7. Myocardial infarct size in the control and isolated rat hearts receiving methanol extract of *M.crassidens* (10, 50, 100 µg/ml) during 30 min ischemia followed by 120 min reperfusion. Data are represented as mean±SEM. $^{**}P<0.01$, $^{***}P<0.001$ *vs.* control group respectively. N=7.

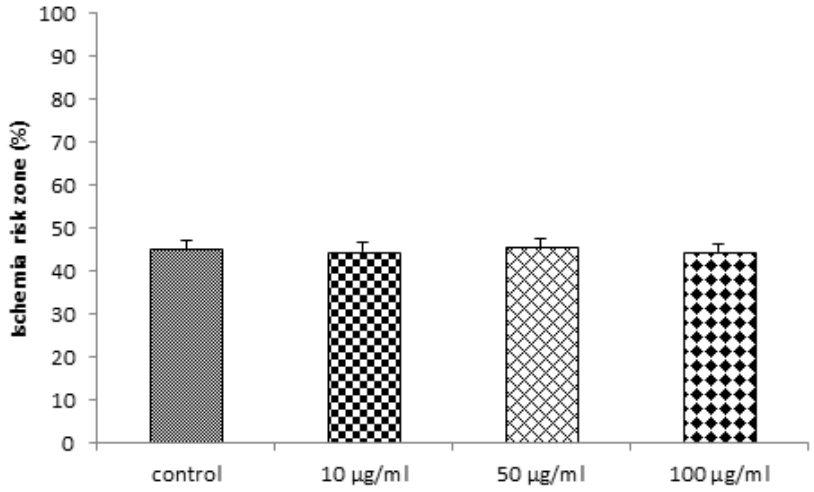

Figure 8. Myocardial ischemia risk zone in the control and isolated rat hearts receiving methanol extract of *M.crassidens* (10, 50, 100 µg/ml) during 30 min ischemia followed by 120 min reperfusion. Data are represented as mean±SEM. N=7.

Discussion

Ischemic heart disease is a major cause of morbidity and mortality worldwide. During acute coronary events, ischemia followed by reperfusion causes pivotal damages to myocardium and so decreases the heart performance. Obviously, suppression of the ischemia/reperfusion injuries lead to improve the heart performance [30]. Ventricular arrhythmias are the most important causes of mortality in the way of cardiac surgery and myocardial infarction. [31]

Genus Marrubiom which is contained high amount of polyphenols and flavenoids [26,27] is being used traditionally in the treatment of inflammatory and respiratory disorders. [3] However, little is known about cardioprotective actions of *Marrubom* species in cardiovascular diseases. In the present study, we

investigated the therapeutic efficacy of the methanolic extract of the aerial parts of the *M. crassidens* on I/R induced arrhythmias and on infarct size in the isolated rat heart.

High concentration of the extract at 100 µg/ml had suppressive effects against the arrhythmias. For the first time, the results of the present study showed that the high concentration of methanolic extract of *M. crassidens* produces anti-arrhythmic effects against ischemia/reperfusion (I/R)-induced arrhythmias such as the number of single, triplet, VEBs when it is used during 30 min ischemia and 30 min reperfusion so its effects are dose dependent. The significant effect of the extract on the reduction of number, incidence and duration of VT just was observed only in the ischemic period.

Surprisingly, the higher concentrations of the extract increased the duration and number of VT during reperfusion time (Table 2, Figure 1 & 2). This discrepancy is explicable since the anti-arrhythmic drugs not only help to control arrhythmias but also can cause some type of them. [32]

The results also revealed that the methanolic extract of *M. crassidens* caused distinct and strong protective activity against I/R injuries by reducing infarct size. The most striking result to emerge from the data is the inverse relation between the extract concentration and reduction of infarct size, since the infarct size in high concentration of extract was nearly similar to the control group. Taking all together, the results of the present study show that methanolic extract of *M. crassidens* creates anti-arrhythmic effects against I/R induced arrhythmias especially during ischemia and decreases infarct size. However, the effect of the extract on the infarct size is not correlated positively with its effects on the ischemic arrhythmias. So that, the high dose of the extract has the most protective effects against arrhythmias while the infarct size is limited more effectively by the lower concentration of the extract. This discrepancy sometimes is expected since the ischemic/reperfusion induced arrhythmias and infarction is the endpoints that different and probably separate mechanisms are involved in their developments.

Concerning a recently published study by our group [25] the content for *M. crassidens* total phenolics compound showed the value of 512.64 mg of gallic acid equivalent in 100 g of dry plant material. Besides, the amounts of flavonoid contents was calculated as 212.73 mg quercetin equivalent in 100 g of powdered plant material, comparing the absorbance values for methanolic extract solution with the standard solutions of quercetin. Regarding the results of the same study significant antioxidant activity related to the flavonoids and other phenolic compounds was detected in the methanolic extract of *M. crassidens.*

There is an inverse association between flavonoid intakes and coronary heart disease mortality.[33] A clinical study showed that flavonoids may reduce mortality from coronary heart disease [34]. The most important property of the flavonoids is their antioxidative activity that could be due to scavenging of free radicals, interfering with inducible nitric-oxide synthase activity and inhibition of xantin oxidase.[33] So, the antioxidative properties and of course flavonoid contents of the extract may have important roles in preventing I/R induced injuries such as arrhythmia and infarction.[31,35]

In addition, at the stabilization time, application of different concentrations of the extract for 5 min before ischemia also significantly decreased CPP (with 10 µg/ml, $P<0.05$), HR (with 50 µg/ml, $P<0.01$), and increased LVDP (100 µg/ml, $P<0.05$). Besides, LV dp/dt_{min} decreased significantly with 100 µg/ml extract ($P<0.05$) as a marker of contractility promotion. It was mentioned previously that marrubenol and phenypropanoid esters from a spices of Marrubium exhibited L-type calcium channel blocking activity. The results of our study show that the *M. crassidens* extract has a depressive effect on the normal and healthy hearts and this effect as mentioned above could be due the calcium antagonistic action of some components of the extract. Generally, the results suggest that the methanolic extract of *M. crassidens* may have calcium channel blocking and vasodilator effects [22]. So, anti-arrhythmic effects of the extract could be due to blocking the cardiac calcium currents, slowing the conduction and increasing the refractory period in calcium-dependent tissues such as the AV node.[32] In summary, regarding the total phenols and flavonoids content of *M. crassidens* methanol extract [25], it seems that the extract may protect the isolated hearts against I/R induced injuries such as arrhythmia and infarction via anti-oxidant activity and calcium channel blocking properties.

Conclusion

The present study demonstrated that perfusion of methanolic extract of *M. crassidens* decreased the number of ventricular ectopic beats during ischemia and reperfusion in the isolated rat hearts. In addition, the lower concentration of the extract also profoundly diminished the I/R induced infarct size. It may be concluded that the methanolic extract of *M. crassidens* could protect I/R hearts by suppressing arrhythmia and reduction of infarction injuries. Future studies are required to determine the effective component(s) and the cardio protective mechanism(s) of *Marrubium crassidens* Boiss.

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