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Original article

Antioxidant Activity and Cardioprotective Effect of *Potentilla reptans* L. via Ischemic Preconditioning (IPC)

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Abstract

Background and objectives: *Potentilla reptans* L. from Rosaceae family is used as traditional medicine in Iran and other countries. Previous investigations on *Potentilla* species have reported strong antioxidant activity and cardioprotective effect. In this study, antioxidant activity of aerial parts and root of *Potentilla reptans*, and the cardio protective role of its root on preconditioning ischemia reperfusion injury have been investigated. **Methods:** Antioxidant activity of aerial parts and root of this plant were measured by DPPH and FRAP methods and its total phenolics content was estimated by Folin-Ciocalteu assay. Catechin was isolated from ethyl acetate fraction by Paper chromatography. Cardioprotective role of *P. reptans* root were evaluated by thirty five rats in five groups. The hearts were subjected to 30 minutes of ischemia and 100 minutes of reperfusion. The ischemic preconditioning (IPC) protocol was applied before the main ischemia. The myocardial infarct size was estimated by triphenyltetrazolium chloride (TTC) staining. The hemodynamic parameters, arrhythmia scoring and coronary flow were measured during reperfusion. **Results:** *Potentilla reptans* root showed stronger antioxidant activity and total phenolics content compared to the aerial parts. Total extract of root significantly decreased the infarct size and increased coronary flow in a concentration-dependent manner. **Conclusion:** Our results showed that the protective effects of *Potentilla reptans* root appeared by its phenolic compounds and reactive oxygen species (ROS) inhibition mechanism.

Keyword: antioxidant; ischemic preconditioning; ischemia-reperfusion; *Potentilla reptans* L.

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Introduction

The genus *Potentilla* L. with 58 species in Iran is a major agent of Rosaceae family [1]. The species of *Potentilla* are herbaceous and woody perennials [2] that grow in Irano-Turanian, Hyrcanian (Caspian) and Zagros floristic provinces [3]. *Potentilla reptans* (creeping cinquefoil) is a perennial plant with a stout rhizome, five heart-shaped yellow petals and 5 or 7 leaves are borne on stolon stalks [4]. The aerial parts have been used to treat dental pain, ulcers and inflammation of the throat in European countries [5,6]. Previous studies have shown antioxidant activity and antiulcerogenic of the leaves [7,8] and anti-inflammatory activity of aerial part and rhizome of *P. reptans*. It has been reported that the roots of this plant have shown higher antioxidant activity than the aerial parts [9]. Investigation of phytochemical constituents of *Potentilla* species indicated that flavon-3-ols or proanthocyanidins are their major components [6,10]. On the other hand, proanthocyanidins (condensed tannin) have shown high antioxidant properties and scavengers of ROS [11,12].

There are not many studies about *P. reptans*

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spatially on its root. In a previous study, quantitative phytochemical analysis showed that total phenolics and procyanidin in aqueous extract of rhizome were more than the aerial part of *P. reptans* [9]. Likewise, phytochemical studies demonstrated eight compounds for *P. reptans* aerial parts while most of them were flavonoids [9,12]; however, for *P. reptans* rhizome 7 compounds have been reported which flavon-3-ols were the major combinations [9].

Considering cardiovascular diseases, cardiac ischemia is one of the most common cardiac malady due to coronary artery disorders and is the most causes of cardiovascular mortality worldwide [13]. On the other hand, IPC is one of the most effective ways to reduce ischemic-reperfusion injuries [14,15]. Flavon-3-ols (proanthocyanidins) are natural compounds which have high antioxidant activity [9,10] so may lead to suppress damage of ROS like in ischemia/reperfusion injury [11,12]. In this study, we have reported the antioxidant activity of *P. reptans* aerial parts and its rhizome as well as cardioprotective effect of *P. reptans* rhizome via IPC.

Material and Methods

Ethical considerations

Animal studies were approved by Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran and Golestan University of Medical Sciences, Gorgan, Iran with ethical code of (IR.TUMS.REC.1394.918, 94-02-33-29400) and (IR.GOUMS.REC.1394.149, 94.61.138) respectively.

Plant material

The aerial parts and root of *P. reptans* were gathered in July 2014 from Tangrah Village, Golestan Province, in North of Iran. A voucher specimen was identified and deposited (No. 45815 TUH) by Professor Farideh Attar in the Central Herbarium of Tehran University, Tehran, Iran.

Chemicals

Evans blue (Product No. 034115, CDH, India), Ferric chloride (103943), Folin-Ciocalteu reagent (1.09001.500), Vanillin (8.18718.0100) and 2, 3, 5-triphenyl-tetrazolium chloride (TTC, 1.08380.0010) were purchased from Merck company, Germany. BHA (B1253), DPPH (D9132), Vitamin E (25, 802-4) were procured from Sigma-Aldrich, Germany and HPLC grade and column solvents were prepared from Duksan pure chemicals South Korea.

Extract preparation

The shade-dried aerial parts (2.25 kg) and rhizome (0.75 kg) were macerated successively with methanol several times (6 L×5) at room temperature separately. A rotary evaporator at 40 °C was used for concentration of the solvents. The dried extracts of aerial parts and rhizome were fractionated separately with enough volumes of petroleum ether, chloroform, ethyl acetate and methanol, successively. The solvents were evaporated under vacuum at 40 ºC to achieve total extract and different fractions.

Determination of total phenolics content

Total phenolic content (TPCs) was measured by Folin-Ciocalteu colorimetric method [16]. In summary, Folin-Ciocalteu reagent (Merck, Germany) (1.5 mL, 1:10 diluted with distilled water) was added to the methanol solution of each sample (200 µL, 100 µg/mL) and samples were kept 5 min at the room temperature. Then, sodium bicarbonate solution (1.5 mL, 60g/L distilled water) was added to the mixture and stored 90 min at 22 °C. The absorption of the solutions was recorded at 725 nm using a spectrophotometer. The TPCs procedure was repeated for gallic acid solutions (0–100 mg/mL) to obtain standard calibration curve. The total phenolics results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dried extracts.

DPPH free radical-scavenging activity assay

Plant fractions and total extract were tested for free radical-scavenging potentials by the DPPH assay [17]. The sample concentrations ranging (5 to 50 µg/mL in methanol) and DPPH (Sigma, Germany) in the concentration of 40 µg/mL in methanol were prepared. Following, 1 mL of each diluted solutions were added to 2 mL of DPPH solution after that 30 min at 25 °C in dark environment; absorptions of the solutions were measured at 517 nm. As the positive control we used Butylated hydroxyanisole (BHA) and vitamin E in our study. Experiments were performed three times and IC_{50} values were reported as Mean±SD.

FRAP determination

The FRAP assay (ferric reducing ability of

plasma), which depends on the reduction of the ferric tripyridyltriazine [Fe(III)–TPTZ] complex to the ferrous tripyridyltriazine [Fe(II)–TPTZ] at low pH, was used to measure the antioxidant power of *P .reptans* extracts. The Fe (II)–TPTZ complex gives a blue color with an absorbance maximum at 593 nm. BHA and vitamin E were used as the positive standards [18].

Determination of catechin and proanthocyanidins

The presence of hydrolysable tannins and proanthocyanidins in total extract and ethyl acetate fraction were visualized by blue and green colors at Iron (III)-tannin interaction. Also, catechin was identified by using catechin test [19] and the dipping red color. On the other hand, they were established by TLC following an appearance of a strong red color when sprayed by vanillin/hydrochloric acid reagent. To understand the presence of flavan-3-ols in root of *P. reptans*, HPLC/PDA chromatogram also showed the (+)-catechin as marker of flavan-3-ol compounds in its extracts (figure 1). Isolated and identified catechin from this plant was used as control. HPLC was carried out on an analytical C_{18} column (Eurospher 100-C18 (5µm, 250×4.6 mm)) using an Azura LPG P6.1L pump. The sample injection volume was 20 µL. The mobile phase was used as the gradient elution with water-acetonitrile solvents (93:7, v/v, 3% acetic acid) at a flow rate of 1 mL/min [20]. The PDA UV/Vis detector (Azura DAD 2.1L) was set at 280 nm.

Isolation procedure

Because of the high antioxidant power (FRAP, DPPH) of the ethyl acetate fraction (94 g), a part of this fraction was submitted to paper chromatography. The chromatogram was developed in Whatmman No. 3 chromatographic papers (20×20 cm) using water as eluent to separate substances. Main spotlight was appeared with R_f = 0.68 (79 mg). This substance was detected at 254 nm as a dark spot and with vanillin / hydrochloric acid reagent in red color on chromatogram.

Animal care

Experiments were performed on 8-9 week old male wistar rats with a body weight of 250-280 g. Animal procedures used in this study conformed to the rules and principles of the institutional animal care and use committee of Golestan University of Medical Sciences, Golestan, Iran and Tehran University of Medical Sciences, Tehran, Iran. The rats were housed in a 12-hour light-dark cycle at 22–24˚C and 55±10% humidity with free access to water and food.

Experimental protocol and langendorff preparation

Thirty five rats were randomly divided into 5 groups (n=7 per group). The hearts were perfused for 30 min as stabilization period then followed by 30 min of regional ischemia induced by left anterior descending artery occlusion using silk string (6-0 mm). A period of 100 min of reperfusion occurred subsequently. The following protocols were performed; 1: rats received no treatment (IR); 2: ischemic preconditioning (IPC), it was achieved by 4 episodes of 5 min ischemia and 5 min reperfusion at the onset of the regional ischemia (30 min); 3-5: total extract in three concentrations (0.5, 1.0, 2.0 µg/mL) added from 40 min before global ischemia (figure 2).

Infarct size determination

At the end of the reperfusion, as described previously by Polshekan M, *et al*. briefly the LAD was stained by Evans blue 1% and TTC (2, 3, 5-tripheny tetrazolium chloride) 1% at 37 °C for 20 min. The infarct size was calculated by a computerized planimetry technique using Photoshop ME7 software. The total area at risk was expressed as a percentage of the left ventricle (AAR/LV %). Infarct size was then expressed as a percentage of the area at risk (IS/AAR %) [21].

Hemodynamic parameters determination

To measure hemodynamic parameters, left ventricular developed pressure (LVDP), the difference between the left ventricular systolic and diastolic pressures, (LVDP= LVSP–LVEDP), were measured as a valid and reliable quantitative indicator of contractile function. We also recorded the maximal rates of pressure development (+dP/dt max) as indexes of contraction and relaxation, the rate-pressure product (RPP) as an indicator of cardiac function (RPP = HR×LVDP ÷ 1,000), and the heart rate (as a hemodynamic parameter). These variables were all monitored by Power Lab software (Power Lab 8/30 AD Instruments, Australia) [21]. Coronary flow (CF) was measured at the end of the reperfusion period.

Assessment of ventricular arrhythmias

Ventricular arrhythmias were evaluated in accordance with the Lambeth Conventions [22]. In
this case, ventricular arrhythmias were analyzed as follows: 1) ventricular premature beats (VPBs) were identified by counting the premature ventricular complex (PVC), bigeminy; 2) ventricular tachycardia (VT) was defined as four or more serial ectopic beats; 3) ventricular fibrillation (VF) was characterized as an undetectable QRS complex. Arrhythmia severity was calculated according to the following scoring system [21,22].

Statistical assessment

Data were expressed as means ± SEM and analyzed by Graph Pad-Prism 5 software (San Diego, USA). The differences between groups were evaluated by using student t-test or one-way ANOVA and significant interactions by the Tukey's test. Kruskal-Wallis and Fisher's exact test were applied for analyzing arrhythmia scores and VF incidence, respectively. P values < 0.05 were considered significant level.

Results and Discussion

Antioxidant activities and total phenolics content of different fractions of *P. reptans* aerial parts and root compared to vitamin E and BHA as natural and synthetic antioxidants have been shown in table 1. In DPPH test, the root total extract exhibited a significant free radical-scavenging activity (IC_{50}=4.2 µg/mL) compared to the aerial parts total extract with IC_{50}=29.2 µg/mL (table 1). The difference between the total phenolic content and the antioxidant activity of the *P. reptans* was probably due to the difference in the type of composition in the two parts of this plant. Phytochemical studies of the aerial parts showed flavonoids as major compounds [6,9] but in root of *P. reptans* flavan-3-ols were reported as the major combinations [9]. In this genus, proanthocyanidine compounds have been reported as antioxidant active ingredients [6]. On the other hand, many studies have widely shown the cardioprotective effect of *Potentilla* genus [23-25].

The isolated compound with paper chromatography was elucidated as catechin by ^{1}H-NMR and ^{13}C-NMR (Bruker Avance 500, MHz for ^{1}H and 125 MHz for ^{13}C) as well as by comparing with published data [26].

Table1. Antioxidant activities and total phenolics of *Potentilla reptans* L.

Samples	DPPH IC_{50} (µg/mL)	FRAP (mmole Fe^{2+}/100 g)	Total phenolics (mg GAE/g of sample)
Ethyl acetate fraction[a]	-	174.6±5.5	109.00±1.39
Methanol fraction[a]	21.57	572.5±7.6	215.62±0.43
Total extract[a]	29.20	810.0±12.5	243.26±0.67
Ethyl acetate fraction[b]	8.10	2570.0±19.4	484.60±1.50
Methanol fraction[b]	11.58	1045.0±14.5	428.40±1.40
Total extract[b]	4.20	5057.5±31.7	526.40±0.83
Vitamin E	14.10	313.7±2.2	-
BHA	7.80	880.3±6.4	-

a: aerial part of *P. reptans* fractions; b: root of P. *reptans* fractions; - not determined.

Comparing the aerial parts and root, the root extract showed the higher free radical-scavenging capacity and total phenolic content (table 1); therefore, we investigated the cardiovascular protection of root part which showed a strong anti-stunning (anti-infarct) effect in the preconditioning mechanism of total root extract.

For the first time, we have used an isolated perfused rat heart model to determine the *P. reptans* root cardioprotective effect by IPC mechanism (figure 2). One of the most effective methods for reducing IR injury is the preconditioning phenomenon, while the most important IPC signaling is upregulation of ROS [27,28]. The results of our study showed that total extract of the root reduced infarct size in the IR model of isolated rat heart. The extract at the three concentrations significantly decreased IS (infarct size) compared to the IR (ischemic reperfusion) compared to the IR group by EC_{50}=1.5µg/mL (figure 3, table 2). The effect of cardiac protection on root fractions of *P. reptans* can be explained by direct effect of the antioxidant activity of the polyphenolic compounds. Previous study has shown that phenolic compounds interact directly with ROS and depress oxidative stress and protect ROS target damages [29]. In this study, total extract decreased IR injury and infarct size by high antioxidant capacity which may act via catechin type compounds through the activation of some pathways such as endogenous antioxidant enzymes or nitric oxide (NO) release [29].

More studies are needed to establish the exact cardioprotective mechanism of *P. reptans* root. This study indicated that total extract of *P. reptans* has different EC_{50} for individual cardiac parameters.

Table 2. Concentration dependent (total extract) of *Potentilla reptans* root preconditioning on infarct size and functional parameters of the isolated rat heart

Samples	% IS	Max-Min P (mmHg)	Max P (mmHg)	Min P (mmHg)	RPP (mmHg).(bpm)	Heart Rate (bpm)	Contractility Index (1/s)	+dP/dtmax (mmHg/s)
IR (Con.)	30.53±1.4	58.21±25.5	107.23±16.7	49.02±9.5	13.42±5.8	317.82±52.6	20.49±8.5	17.01±7.4
IPC (Con.)	12.06± 1.6*	93.89 ± 4.9*	119.8 ± 5.4*	20.40 ± 3.3*	21.38 ± 1.2*	226.85±12.8*	37.99±4.3*	25.28±1.8*
R (2µg/ml)	18.79 0.41*	65.5±7.9	105.4±11.2	39.9±4.5	13.79±1.6	210.47±11.9	26.03±5.3	15.64± 3.8
R (1µg/ml)	6.15± 0.66*	85.09± 7.58*	113.8± 8.2	28.73± 5.4*	17.25± 1.31	205.22± 15.8*	31.28± 3.63*	15.82±4.9
R(0.5µg/ml)	21.44±1.49*	56.35± 5.2	97.01±4.8	40.66±2.73	10.95±1.75	215.72±16.4*	20.77±2.1	15.46±1.5
EC_{50}(µg/ml)	1.50	-	-	-	-	-	-	-

IR: ischemic reperfusion; IPC: ischemic preconditioning; %IS: infarct size; Con: control; R: total extract of *P. reptans* root. R in different concentrations were applied before major ischemia; Data are means ± SEM. *P < 0.05 vs. IR.

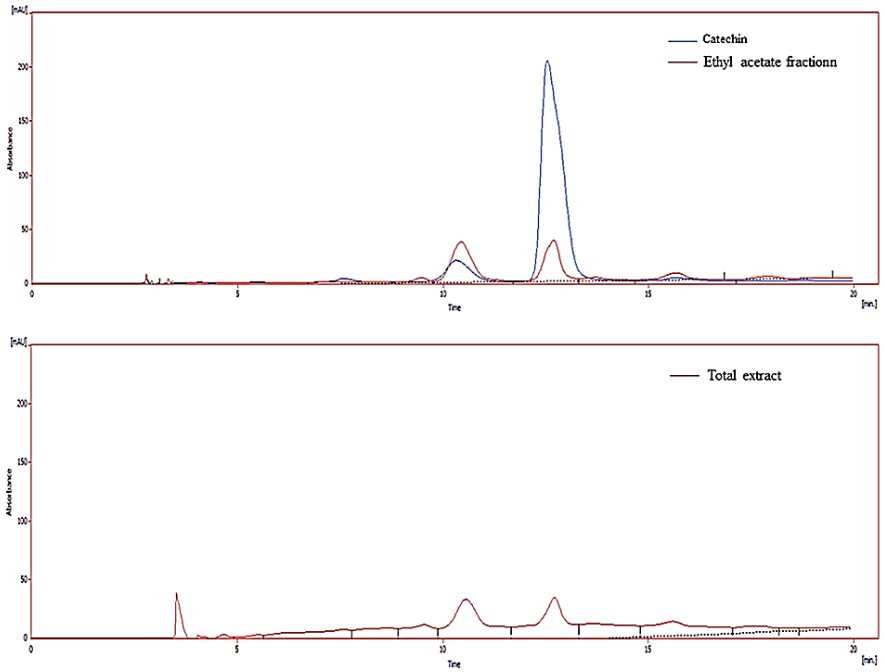

Figure 1. Reversed-phase HPLC-PDA analyses of the total extract and ethyl acetate fraction from the root of *Potentilla reptans* using a 20 min 7-15% gradient of acetonitrile in 3% aqueous acetic acid; chromatographic profiles were detected at 280 nm.

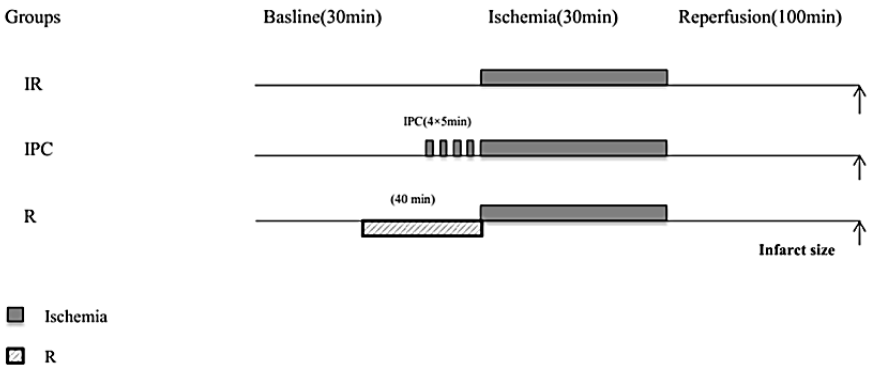

Figure 2. Experimental protocols: all experimental groups were first perfused for 30 min on langendorff apparatus to allow the isolated hearts to stabilize. The hearts were then divided into different groups. All groups were subjected to 30 min of regional ischemia followed by 100 min reperfusion. IR; Ischemia reperfusion, IPC; Ischemic preconditioning (4×5min ischemia and reperfusion before major ischemia)

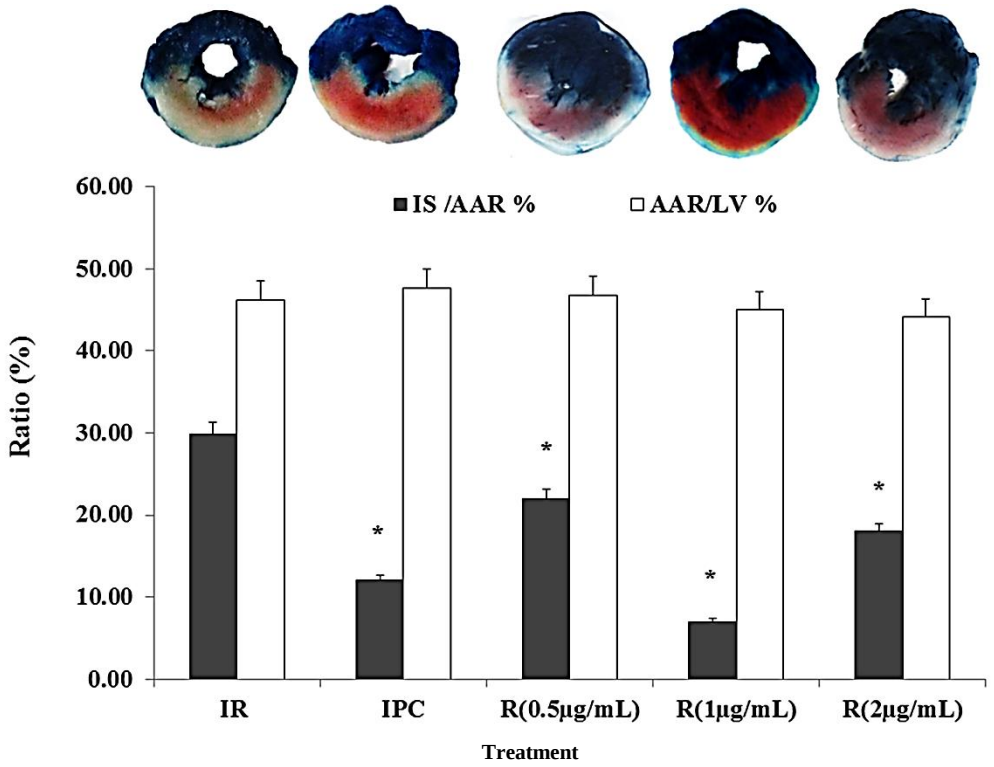

Figure 3. Cardio protective effect of *Potentilla reptans* root total extract. Representative photographs of TTC (2, 3, 5-triphenyl-tetrazolium chloride) stained rat heart sections and statistical data of myocardial infarct size (IS/AAR%) and area at risk (AAR/LV%) in isolated perfused rat. Data are presented as means ± SEM and expressed in percentage. * P < 0.05 vs. IR; LV: Left ventricular; R: total extract

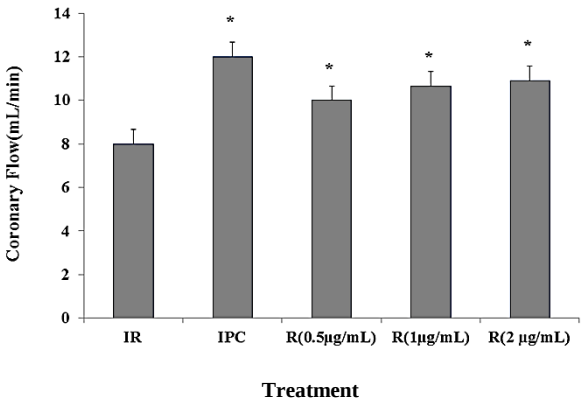

Figure 4. Changes in coronary flow in isolated rat heart. Effects of IR, IPC and R (total extract) groups on the coronary flow (CF) recovery at the end of reperfusion. Data are presented as means ± SEM and expressed in percentage of baseline values. *P < 0.05 vs. IR.

Polshekan *et al.*, have confirmed that EC_{50} of oxytocin is different for electrophysiological, biochemical and infarct size parameters of isolated rat heart IR model [30]. For example the EC_{50} of oxytocin for infarct size was 0.01µM, whereas, it's EC_{50} for LVDP, heart rate and +dP/dt max was 0.53 µM, 2.1 µM and 0.84 µM respectively. To explain this finding several previous studies have shown that different mechanism of action or different natural ingredients in herbal extracts are responsible for their variable responses to cardiac sensitive parameters [31-33]. There is a remarkable relationship among multiple active pharmacological properties of plant extract and modulation of cardiac parameters. For example, infarct size decreases by drugs which have antioxidant, mPTP blocker, and PKC activation properties. In the present study, decreased infarct size by total extract of *Potentilla reptans* was mediated with several established intrinsic activities of this plant such as antioxidant and ROS scavenger properties [9,34]. Therefore, we concluded that direct and indirect effect of total extract of *P. reptans* mediated its cardioprotective effect via several active components which showed multiple cardiac affinities in isolated rat heart IR model.

The beneficial *P. reptans* preconditioning effect increased coronary flow (figure 4).

In our study, the root extract with 0.5-2.0µg/mL concentrations significantly improved the coronary flow at the end of reperfusion (figure 4).

Increasing coronary flow by extract may be associated with NO release by flavan-3-ols compounds [35,36]. Besides, some studies have shown that NO is an important base for endogenous vasodilation [37,38]. It seems that *P. reptans* exposed this effect via the induction of NO release but the precise mechanism remained to be determined.

LV hemodynamic properties did not change by the root total extract of *P. reptans* (table 2). *Potentilla reptans* root total extract could not change ventricular arrhythmia score and ventricular fibrillation incidence (table 3). Importantly, the anti-arrhythmic and hemodynamic effects in root total extract were not significant (table 2, 3).

Table 3. The concentration-dependent effects of total extract from *Potentilla reptans* root on arrhythmia score, VT, and VF incidence during the reperfusion phase of the IR model

Samples	Arrhythmia score	VT Incidence (%)	VF Incidence (%)
IR (Con.)	3.2±0.75	53.8	30.8
IPC (Con.)	1.9±0.67*	40.0*	25.0*
R (2µg/mL)	6.0±0.75	50.0	50.0
R (1µg/mL)	4.0±0.56	80.0	60.0
R (0.5µg/mL)	6.5±0.88	100	100

IR: ischemic reperfusion; IPC: ischemic preconditioning; VT: ventricular tachycardia; VF: ventricular fibrillation; R: total extract of *P. reptans* root.

The chemical profile of the plant is an initial step in assessing the quality of its biological effects. In fact, the activity of effective compounds in the total extract of *P. reptans* root may be decreased in the presence of other root compounds; therefore, isolation and purification of the active compounds are required. Further studies are needed on active fractions and isolated compounds of *P. reptans* root to improve its cardio protective effects.

In summary, this study clearly indicated that the total extract of *P. reptans* root showed strong antioxidant activity compared to the total aerial parts. It effectively decreased infarct size via preconditioning mechanism through inhibiting ROS and high antioxidant potential; therefore, we suggest *P. reptans* root as a natural preconditioning drug for treatment of myocardial infarction. It is worth designing future studies on other potent fractions of root about the cardio protective effect signaling pathway of this plant.

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Author contributions

Aysheh Enayati performed plant preparation, extraction, isolation and identification of plant substances and pharmacological tests and drafted the manuscript; Vahid Khori planed and advised the pharmacological tests and edited the manuscript; Yousef Saeedi helped in some pharmacological tests. Narguess Yassa conceived the study, advised separation and identification of the plant substances and edited the manuscript.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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Abbreviations

AAR: area at risk; IPC: ischemic preconditioning; IR: ischemia/reperfusion; IS: infarct size; LAD: ligating of the left anterior descending branch; LVDP: left ventricular diastolic pressure; VF: ventricular fibrillation; LV: left ventricular; + dp/dtmax: maximal rate of left ventricular systolic pressure; MI/R: myocardial ischemia/reperfusion; ROS: reactive oxygen species; RPP: rate-pressure product; R: total extract of *Potentilla reptans* root; TTC: 2,3,5-triphenyl tetrazolium chloride; DPPH: 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl; FRAP: ferric reducing antioxidant power; PDA: photo-diode-array-detector, DAD: diode array detector; BHA: butylated hydroxyanisol; IC_{50}: concentration providing 50% inhibition; QRS: combination of three of the graphical deflections on a typical electrocardiogram