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Research Article

Effect of Methanolic Extract of *Scrophularia subuphylla* on Ischemia and Reperfusion-Induced Myocardial Injury

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ABSTRACT

Background: Coronary artery disease is a leading cause of death worldwide. The present study has been designed to investigate efficacy of methanolic extract of *Scrophularia subuphylla* (*S. subuphylla*) on ischemia and reperfusion-induced myocardial injury in isolated rat heart.

Methods: The isolated male Wistar rat hearts (n= 5) were perfused by Krebs-Henseleit solution enriched with the extract (0, 1, 5, and 10 µg/ml), using the langendorff method. After 15 minutes stabilization, the hearts were subjected to 30 minutes regional ischemia and then 120 minutes reperfusion.

Results: Administration of the extract did not improve any of cardiac markers of flow rate, heart rate and developed pressure. Number, percentage and duration of arrhythmias were not affected by any concentrations of the extract. However, the concentration of 1 and 5 µg/ml increased the VT duration compared to control group ($P<0.05$). Furthermore, there was not considerable infarct size difference between none of the groups. Methanolic extract did not show any cardioprotective effect, while it had high anti-oxidant activity as well as high amounts of total phenol and flavonoid contents.

Conclusion: Generally, the methanolic extract of *S. subuphylla* at the doses which studied exhibited no protective effects against I/R-induced injures, which might be due to the high amount of cardiac glycosides with low therapeutic index.

Introduction

Cardiovascular diseases (CVDs) is the leading global cause of death, accounting for 17.3 million deaths per year.[1] Among various types of CVDs, coronary heart disease or ischemic heart disease is the most common mortal type in most countries.[2] Myocardial injury due to ischemia-reperfusion can result in cardiac contractile dysfunction, microvascular damage, arrhythmias, reversible mechanical dysfunction and irreversible myocyte damage.[3] The blood restoration and the introduction of oxygen into the transiently ischemic tissue induce oxygen radicals production and oxidative stress.[4] Despite huge advances in modern medicine, traditional medicine is still used in all over the world and this points to the importance of research on natural compounds used in folk medicine. Plants which have some active secondary metabolites with anti-oxidant properties like flavonoids,[5,6] phenyl ethanoides,[7] saponins,[8,9] phenyl propanoides, phenolic acids[10] and iridoids[11] might be the candidate for investigations on this field. Among these compounds flavonoids and phenylethnaoids as polyphenols have important impact on decreasing cardiovascular diseases.[12] One of the plants which is rich in above mentioned metabolites is *Scrophularia*,[6] so it can be considered as a cardioprotective agent.

The genus *Scrophularia* (commonly known as figwort) is one of the largest genera of the Scrophulariaceae family which is distributed widely in Asia, North America and central Europe, especially in the Mediterranean region. *Scrophularia subaphylla* (*S. subaphylla*) is one of the endemic species growing in East Azerbaijan province of Iran.[13] It is a square stems with woody rhizomes perennial herb, opposite leaves and open two-lipped flowers.[14,15] Since ancient times, in folk remedies some species of this genus were used in treatment of skin disorders, inflammatory conditions and fever.[9,16-18]

S. ningpoensis from this family has been used in treatment of upper respiratory disorders, GI difficulties and fever in Chinese medicine.[19] Furthermore, this species was used in restraining ventricular remodeling in order to ameliorate heart failure with subsequent event of MI.[20] Based on our published paper, *S. frigida* has been shown

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cardioprotective effect on ischemia and reperfusion-induced myocardial injury.[21] Hence, it has been proposed to evaluate cardioprotective effect of *S. subaphylla.* Accordingly, the aim of our study was to evaluate the effects of methanolic extract of *S. subaphylla* on Ischemia/Reperfusion induced injuries in the isolated rat heart with an approach to introduce an herb with protective effects against ischemic heart diseases.

Materials and Method

Animals

Adult male Wistar rats weighing 270-300 g were supplied by the Laboratory Animal Center, Medical Sciences University of Tabriz, Iran. 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 maintained with free access to standard rat food and tap water. The experiments reported were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No 85-23, revised 1985).

Chemical reagents

The following chemicals were purchased: NaCl, $NaHCO_3$, KCl, KH_2PO_4, $MgSO_4$, $CaCl_2$, D-glucose (Merck Company), Sodium pentobarbital (Kela Company, Belgium) and Heparin (Daru-pakhsh Company, Iran). Triphenyltetrazolium chloride (TTC) and Evan's blue were purchased from Baker Analyzed (USA). Ferric chloride, Gallic acid, Folin- Ciocalteau reagent, Na_2CO_3, HCl, $AlCl_3$ and sodium acetate were all prepared from Merck, Germany. Furthermore, magnesium ribbon and DPPH reagent both of them (Sigma-Aldrich, Germany) were used.

Plant material

Aerial parts of *S. subaphylla* were collected during flowering stage from Mishodagh mountain, Eastern Azerbaijan province, Iran, in July 2013. Voucher specimen (TBZ-fph-747) of the plant was identified then has been deposited in the Herbarium of the Faculty of Pharmacy, Tabriz University of Medical Sciences.

Extraction

The air-dried and powdered aerial parts of *S. subaphylla* (100 g) were Soxhlet extracted successively with n-Hexane, DCM and MeOH (500 mL each). The obtained extracts were concentrated using a rotary evaporator at 45 °C.

Phytochemical analysis

Extracts were investigated to identify the phytochemicals constituents such as tannins, iridoids and flavonoids following standard procedures.[22-24]

Test for Tannins and Phenolic Compounds (Ferric Chloride Test)

The extract solution gives blue green color with few drops of ferric chloride. [22]

Total phenol content (TPC)

Total phenolic constituents was determined using Folin-Ciocalteau assay method. Gallic acid (GAE; Merck, Germany) was used as a standard. Briefly, one mL of samples with concentration of 5 mg/mL in acetone: water (60:40) was mixed with 0.2 mL Folin- Ciocalteau reagent (Merck, Germany) and 1 mL 2% Na_2CO_3. The samples were incubated in room temperature for 30 minutes and the absorbance was measured at 750 nm (Pharmacia biotech ultrospec 2000, UV/Visible spectrophotometer, England). The calibration curve was drawn with different concentrations of gallic acid and the same method. All measurements were performed in triplicates.[21]

Test for iridoids

1ml of Trim-Hill reagent was added to the concentrated extract and then was heated for a few minutes. A blue-green or red color indicated the presence of iridoids.[23]

Test for flavonoids

Evaluation of flavonoids content was done through Shinoda test (Magnesium Hydrochloride reduction test. Concisely, this test was investigated by adding few pieces of magnesium ribbon (Sigma-Aldrich, Germany) and also adding concentrated HCl (Merck, Germany) drops to methanolic extract. The pink scarlet, crimson red or occasionally green to blue color appears after few minutes.[22]

Total flavonoid content (TFC)

Total flavonoids were estimated using $AlCl_3$ (Merck, Germany) method. Sample solutions were prepared in 80% methanol. To prepare $AlCl_3$ reagent, 133 mg crystalline aluminum chloride and 400 mg crystalline sodium acetate (Merck, Germany) were dissolved in 100 ml of 80% methanol. For estimating the flavonoid content 2 ml of sample, 400 µL of water and 1 mL of $AlCl_3$ reagent were added. Absorbance was recorded at 430 nm against blank containing no $AlCl_3$ reagent. Stock solution of quercetin (1 mg/mL) was prepared in 80% methanol. Various dilutions of quercetin (5-25 µg/mL) were prepared in methanol and a standard curve was plotted. The amount of flavonoids was calculated as quercetin equivalent from the calibration curve of quercetin (5-25 µg/mL).[24]

Free radical scavenging activity test

Antioxidant activity of the extracts was performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent (Sigma-Aldrich, Germany).[21] DPPH solutions were prepared (0.08 mg/mL) in methanol for evaluating the antioxidant activity of MeOH extract.

The extract were dissolved in MeOH to obtain the stock concentration of 1 mg/mL. Serial dilutions were made to obtain the range of concentrations. Furthermore, diluted solutions (5 mL each) were mixed with DPPH solution (5 mL) then remained for 30 min for occurring any reaction. The UV/Visible absorbance was recorded at 517 nm. The percentage of reduction capacity was calculated as:

$R\% = \left((A_{blank} - A_{sample})/A_{blank}\right) * 100$ Eq. (1)

A_{blank} and A_{sample} were the absorbance of the control and the absorbance of the extract respectively. Extract concentration which is providing 50% inhibition (IC_{50}) was calculated. The experiment was done in triplicate and the same manner was followed for the positive control, quercetin.

Preparation and isolated heart perfusion

Preparation and isolated heart perfusion was performed as previously described[25] with minor modifications. Rats were heparinized (1000 IU/kg, intraperitoneally) and then anesthetized with ketamine/xylasin (60 / 10 mg/kg; intraperitoneally). When the rats didn't responded to external stimuli, the surgery for harvesting the heart was done. The rat hearts were immediately excised and kept in ice cold oxygenated modified Krebs–Henseleit buffer (K/H) (pH=7.4), before the aorta was cannulated on Langendorff apparatus (ML176-V Langendorff Apparatus, ADInstruments, Australia). The hearts were perfused at a constant pressure (80 mmHg) with a K/H containing NaCl 125, KCl 4.3, KH_2PO_4 1.1, MgCl2. $6H_2O$ 1.3, CaCl2. 2 H_2O 2.4, $NaHCO_3$ 25, and glucose 13.32(in mmol/l). The perfusate was gassed with carbogen (5% CO2/95% O2) to set the pH in 7.38-7.56 at 37°C.

When the harvested heart was mounted to the apparatus, the suture by 6.0 silk surgical 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. To measure left ventricular contractility, a water-filled latex balloon attached to a pressure transducer (MLT844 physiological pressure, ADInstruments, Australia) was inserted into the left ventricle (LV) through an incision in the left atrium.

The hearts were allowed to equilibrate for 15 min (stabilization) with infusion by K/H, and then time was set to zero and K/H without or with extract (1, 5, and 10 µg/cc in separate groups) was infused 5 min before occlusion and maintained for duration of the experiment.

Infarct size and staining protocol

In regional ischemia double staining strategy (Evans blue and TTC respectively) is performed. After re-ligating the LAD in the same location, the hearts were separated from the langendorff apparatus and Evans blue (0.25% in saline) was directly infused into the hearts via cannula for delineation of the ischemic zone from the nonischemic zone. 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 infarct area is demarcated as a white area while viable tissue stains red [3]. The risk zone areas and infarct sizes were measured by equations below:

$Risk\ zone\ area(\%) = ((None - perfused\ area))\ /\ ((total\ area\ of\ the\ heart) * 100)$ Eq. (2)

$Infarctsize(\%) = ((Dead\ cells\ area))\ /\ ((None - perfused\ area) * 100)$ Eq. (3)

Statistical analysis

Results expressed as mean± SEM. To compare the hemodynamic factors in different groups the Mann-Whitney non-parametric U-test were employed. Differences were considered significant at a level of P< 0.05.

Results

Phytochemical results

Phytochemical tests of MeOH extract of S. *subaphylla* indicated the existence of iridoids, phenolic compounds (tannins) and flavonoids.

TPC and TFC results

The total amount of phenolic contents was 63.46 ± 0.14 mg GAE/g of extract and the constituent of flavonoids was equal to 141.56 ± 20.31 mg rutoside equivalent in 1 g of powdered plant material.

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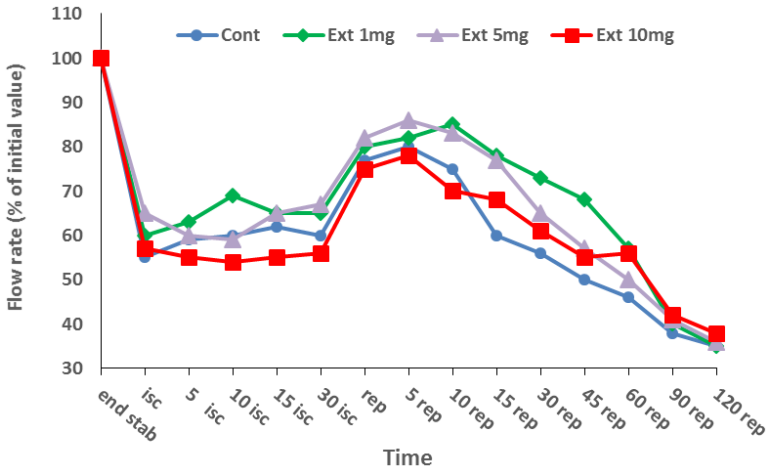

Figure 1. Flow rate in the control and isolated rat hearts receiving methanol extract of S. *subaphylla* (1, 5, 10 µg/ml) during 30 min ischemia followed by 120 min reperfusion. Data are represented as Mean±SEM. Stab: stabilization, isc: ischemia, rep: reperfusion, cont: control, Ext: extract. . N average = 5 rats in each group.

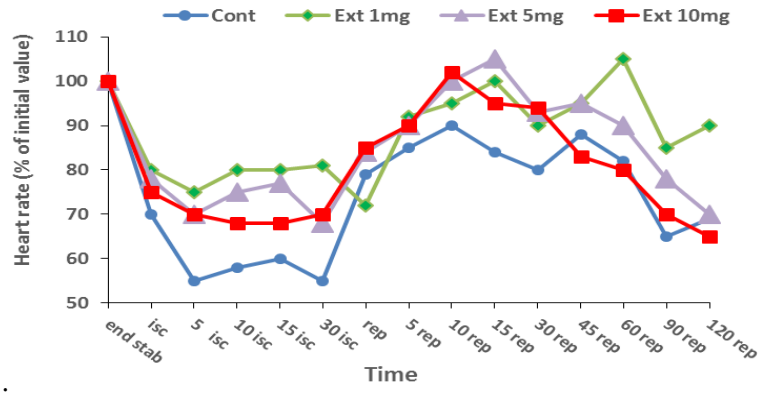

Figure 2. Heart rate in the control and isolated rat hearts receiving methanol extract of S. *subaphylla* (1, 5, 10 µg/ml) during 30 min ischemia followed by 120 min reperfusion. Data are represented as Mean±SEM. Stab: stabilization, isc: ischemia, rep: reperfusion, cont: control, Ext: extract. N average = 5 rats in each group.

Free-Radical-Scavenging Activity

Free radical scavenging activity of the MeOH extract was based on the reduction of DPPH. The potency of antioxidant activity of the extract (IC_{50}) was (0.28 ± 0.08mg/ml) in comparison to the value of quercetin (0.003 ± 0.00 mg/ml) as a positive control.

Effects of MeOH extract of S. subaphylla on flow rate of isolated rat heart

Figure 1 represents the change of flow rate (% of initial value) during whole period of experiment. Ligation of coronary artery at the end of stabilization led a marked decline in perfusion flow rate from 100% to 55%. Reperfusion of the ischemic area caused an again increase in the flow rate to 78% that gradually decreased to 35% at the end of the reperfusion time. The perfusion of extract in all concentrations of 1, 5 and 10 µg/ml did not induce any significant change in flow rate

Effects of MeOH extract of S. subaphylla on heart rate of isolated rat heart

Figure 2 represents the change of heart rate (% of initial value) during whole period of experiment. Ligation of coronary artery at the end of stabilization led a continious decline in heart rate from 100% to 56% at the end of ischemic phase. With reperfusion of the ischemic area an increase occurred in the heart rate to 78% that gradualy decreased to 68% at the end of the reperfusion time. The heart rate changes under perfusion of extracts at 1 and 5 and 10 µg/ml was similar to that of the control group with no significant difference

Effects of MeOH extract of S. subaphylla on developed pressure of isolated rat heart

Figure 3 represents the developed pressure changes (% of the initial value) during whole period of experiment which is determined as the difference of systolic and diastolic pressure. Ligation of coronary artery at the end of stabilization led a mark rise in developed pressure from 100% to 130% which followed by a decrease pattern to the amount of 117 at the end of ischemic phase. Reperfusion of the ischemic area caused an again rise in the developed pressure to 130% of the initial value that gradually decreased to 110% at the end of the reperfusion time. The extract at the concentrations of 1, 5 and 10 mg/l had similar rhythm with no significant effect on the developed pressure during whole period of perfusion time.

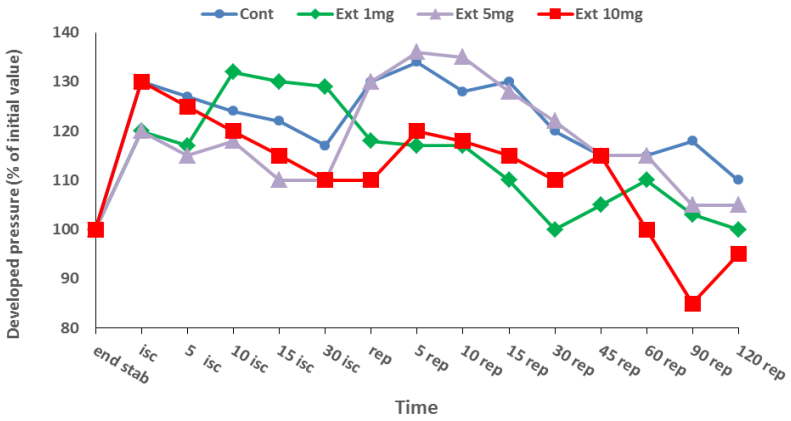

Figure 3. Developed pressure in the control and isolated rat hearts receiving methanol extract of S. *subaphylla* (1, 5, 10 µg/ml) during 30 min ischemia followed by 120 min reperfusion. Data are represented as Mean±SEM. Stab: stabilization, isc: ischemia, rep: reperfusion, cont: control, Ext: extract. N average = 5 rats in each group.

Table 1. Effects of MeOH extract of *S. subaphylla* on I/R-induced cardiac arrhythmias after 30 min regional ischemia followed by reperfusion in isolated rat heart.

Ischemic phase	Number				Duration (sec)		Frequency (%)	
	Single	Salvous	Triplet	VT	VT	VF	VT	VF
Control	195 ± 34	75 ± 6	14 ±1	264 ± 87	98 ± 23	12 ± 10	100	45
Ext. 1mg/l	203 ± 23	64 ± 4	11± 4	305 ±100	84 ± 5	15 ± 11	100	56
Ext. 5mg/l	215 ± 54	89 ± 7	18 ±8	296 ± 87	168 ±3*	8 ± 4	100	61
Ext. 10mg/l	188 ± 45	90 ± 9	26± 2	312 ±102	146 ±17*	23 ± 10	100	64
Reperfusion phase	Number				Duration (sec)		Frequency (%)	
	Single	Salvous	Triplet	VT	VT	VF	VT	VF
Control	168 ± 12	35 ± 2.9	5 ± 0.4	79 ± 23	16 ± 10	1.6 ±0.4	75	20
Ext. 1mg/l	149 ± 3	25 ± 1.4	2.8 ± 1	51 ± 7.5	11 ±2	2.4 ±0.2	33.3	22
Ext. 5mg/l	134 ± 22	34 ± 2	3.4 ± 0.2	47 ±12	17 ±0.5	2.5	28.6	32
Ext. 10mg/l	185 ± 13	18 ±1.7	4.7 ± 0.5	85 ±7.2	22 ± 2.5	3 ± 3.5	16.7	35

Isolated hearts in control groups receiving methanol extract of *S. subaphylla* (1, 5, 10 µg/cc) were studied during 30 min ischemia followed by 120 min reperfusion. Data are represented as mean±SEM. N average = 5 rats in each group.*p<0.05 versus the control group. Cont: control, Ext: extract, VT: Ventricular Tachycardia, VF: Ventricular Fibrillation, Total arrhythmias: Single+ Salvos+ Triplet+ VT.

Effects of MeOH extract of S. subaphylla on ischemia/reperfusion-induced arrhythmia in isolated rat heart

The effects of administration of extract on reperfusion-induced cardiac arrhythmias after 30 min regional ischemia are summarized in Table 1. The obtained results from arrhythmia table indicate that, in any of ischemia nor reperfusion phase were not observed a significant change in numbers, duration and as well as frequency of VT and VF compared to the control group. The only significant change was in duration of VT arrhythmia which was increased in the doses of 5 and 10 µg /ml compared to control group (P<0.05).

Effects of MeOH extract of S. subaphylla on infarct size in the isolated rat heart

As demonstrated in Figure 4, the infarct size was 70±7 % in the control group while the perfusion of the MeOH extract of S. *subaphylla* by 1, 5 and 10 µg/ml changed it to 80±9 %, 85±13 % and 75±12% respectively, which none of them were significantly different from control group.

Discussion

According to the obtained results of this study, significant changes in the infarct size of different groups were not observed compared to the control group. Furthermore, the results of the arrhythmias studies showed that none of the used doses of the extract could reduce the number of arrhythmia in ischemia nor reperfusion phases. However, the doses of 5 and 10 µg/ml induced a significant increase in ventricular tachycardia duration in comparison to control group (P<0.05). Other arrhythmic factors such as the percentage of VT and VF showed no significant change compared to the control group. In addition, markers of cardiac function such as heart rate, flow rate and developed pressure were not affected by extract of *S. subaphylla.*

Meanwhile, further isolation of active pure composition of the extract, which was carried out in a separate study resulted in isolation of high levels of iridoid glycosides and phenolic compounds like phenylethanoid (unpublished paper). Additionally, high amount of total phenol and total flavonoids which was reported in this study indicated the potent antioxidant ability of the plant. Despite the existence of such phenolic compounds with high antioxidant activity in S. *subaphylla*, the result of our study demonstrated no cardioprotective effects. These evidences may propose a high potency of this herbal extract which can be related to high amounts of cardiac glycosides and phenylethanoids.

Consequently, these compositions especially in high value with synergistic effect on cardiac function not only induced no improvement in factors under our investigation, but also developed adverse effects such as arrhythmia.

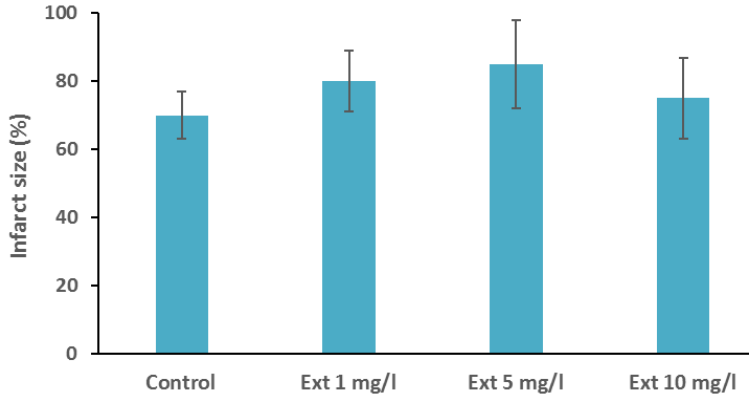

Figure 4. Myocardial infarct size in the control and isolated rat hearts receiving methanol extract of S. *subaphylla* (1, 5, 10 µg/ml) during 30 min ischemia followed by 120 min reperfusion. Data are represented as mean±SEM. N average = 5 rats in each group.

Whereas this plant and other species of this plant contain phenylethanoid and irioid[26-28] and cardiac effects of these compounds have already been demonstrated.[7,11,12,28,29] Besides, our previous study showed remarkable cardiovascular effect of *S. frigida* on isolated rat heart.[21] Even though, *S .frigida* showed significant cardioprotective effects, *S. subaphylla* indicated no efficacy. On the other hand, both plants were gathered from the same place (Mishodagh mountain) with different altitude, whereas they showed different activity on isolated heart rat. It is proposed that, different heights of growing and climatic conditions may cause variations in the chemical composition contents which is responsible for the different biological effects. [9]

Conclusion

It is supposed that as like as narrow therapeutic index of scrophulariaceae family (*Digitalis lanata*: digoxin), the high amount of cardiac glycosides in this plant also is the responsible for such unfavorable effects on cardiac function. Accordingly, due to arrhythmogenic effect (which have been recorded in doses of 5 and 10 µg/ml) and no other protective effect of *S. subaphylla*, it is recommended to investigate the effects of lower concentration of this extract and also the other species of this family. Moreover, some extensive work in this direction could also lead to explore the possible other mechanism of *S. subaphylla* in cardiovascular system.

Conflict of interests

The authors claim that there is no conflict of interest.

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