Involvement Of *Inula Ra*cemose Hook Extract on Ischemic Preconditioning Mediated Cardioprotection in Diabetes

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Abstract: Objective: Ischemia of the heart is one of the major cause behind diabetes mediate death in peoples. PI3K/Akt phosphorylation is inhibited in IPC-induced infarcts, preventing the opening of the mitochondrial permeability transition pore (MPTP). In diabetics, the beneficial effects are lessened. IPC-mediated cardioprotection in diabetic Wistar rat hearts was observed in this study after pre-treatment with *Inula racemosa* Hook root extract (*IrA*). Ischemia reperfusion (I / R) and cycles of IPC were performed on an isolated rat heart mounted on Langendorff's assembly. MPTP is opened by atractyloside (ATR). Method: RP-HPLC and 1H and 13C NMR were used to identify the chemical components. Western blot, oxidative stress, mitochondrial function, integrity, and haemodynamic parameters are all examined in this study. Result: Pretreatment with *IrA* reduced LDH, CKMB, myocardial infarction size, ventricular fibrillation, and cardiac collagen content, as well as decreased oxidative stress levels and mitochondrial dysfunction in diabetic rat hearts in all groups, according to the data. By blocking the opening of MPTP in diabetic rats, ATR reduced the cardioprotective effect of *IrA* on IPC. Conclusion: This shows that IPC-mediated cardioprotection can be restored by pre-treatment with *IrA*, which inhibits the opening of the MPTP in diabetic rats.

Keywords: Ischemic preconditioning, Diabetes, Mitochondria, Oxidative stress

INTRODUCTION

Coronary artery disease, predominantly myocardial infarction, which is one the leading factor for morbidity and mortality in the world population [1]. During of Ischemic reperfusion (I/R) injury, blood supply to the myocardium is reduced. The repeated short phase of ischemia and reperfusion, i.e. Ischemic preconditioning (IPC), allow myocardium to get resistant against the sustained ischemia for longer duration [2]. The cardioprotection by IPC is mediated through phosphatidylinositol 3-kinase (PI3K/Akt), nitric oxide generation, phosphorylation of eNOS, decreasing the oxidative stress, apoptotic cell death and by preventing the opening of mitochondrial permeability transition pore (MPTP) [3]. The beneficial phenomenon, IPC gets significantly declining in the various conditions like aging, heart failure, hypertension, obesity, hyperlipidaemia and diabetes mellitus [4]. In diabetic patient after myocardial ischemia the mortality rate gets double than the normal diabetic patients [5]. Cardioprotective mechanism of IPC is to prevent the opening of MPTP, which gets attenuated due to the inhibition of PI3K/Akt signalling phosphorylation [6]. MPTP is pore of inner membrane of mitochondria which on opening causes reperfusion injury in myocardium [7] and phosphorylation of PI3K/Akt signal gets depleted [6]. IPC mediate its beneficial action of cardioprotection by inhibiting the opening of MPTP, mediated through phosphorylation of PI3K/Akt. However, various researches shows that the IPC mediated cardioprotection gets attenuated during the condition of Diabetes mellitus which is may be due to involvement of MPTP opening.
In last two decades the research is focused on the study of natural drugs and medicinal plants that conventionally has been involved as source of new therapeutics for long-term prevention of cardiovascular diseases [8]. The traditional plant *Inula racemosa* Hook (*IrA*) commonly known as Pushkarmool shows its beneficial effect in cardio-resp*ira*tory [9] and cardiovascular diseases [10, 11]. Plant contains various phytoconstituents sesquiterpenes (alantolactone, isoalantolactone and alloalantolactone) which is responsible for the beneficiary effect. Several researches shows that the alantolactone is responsible for the pharmacological activity [12]. Its beneficial effect has been seen as antioxidant, β-blocker, and in myocardial necrosis [13]. Ayurvedic, Chinese, and Mediterranean medicine have reported that Inula species are used in anginal pain [14]. The beneficiary potential of this medicinal plant generates the interest of investigating the effect of *Inula racemosa* Hook on cardioprotective effects of IPC in diabetic hearts.

MATERIALS AND METHODS

Rats weighing approximately 180-215 g of either sex is employed in this study. All rats were feed with standard chow diet and kept the water supply ad libitum. In the animals house the animals were acclimatised and exposed to normal day-night time cycle. All experimental works on animals were conducted in accordance with the consent of Institutional Animal Ethics Committee (KNIMT/PHAR/IAEC/18/05) according to national guidelines for animal research.

Drugs and Chemicals

All analytical chemicals and reagents used were of investigative grade and procured from Sigma-Aldrich, India. Atractyloside potassium (ATR) was dissolved in minimum amount of distilled water and transfer to Krebs Henseleit (KH) solution. The reagents used in study are freshly prepared prior to use.

Plant material, Extraction and Phytochemical Analysis

The roots of *Inula racemosa* Hook. (*IrA*) plant is available in regions of the Himalayas, Himachal Pradesh and Kashmir [15]. The air-dried roots of *Inula racemosa* was procured from local supplier and authenticated by the Council of Scientific and Industrial Research- National Institute of Science Communication and Information Resource (CSIR-NISCAIR), New Delhi (NIScPR/RHMD/Consult/2021/3881-82). The air-dried roots(500 g) are mechanically ground to fine powder. 50:50 methanol and water were used to perform a cold maceration extraction process at 25 ºC. The filtered hydro-alcoholic extract under reduced pressure evaporated to remove the solvent content using a rotary evaporator, and a viscous mass was obtained [16].

Furthermore, a Shimadzu system equipped with an LC-10ATvp pump, SPD-M 10Avp diode array detector, SIL-10ADvp auto injector, and LC-10ADvp system controller was used to analyse the identification and phytochemical components in *IrA*. Shimadzu software was used for the data gathering. Milli-Q nylon syringe filters with a 0.45 pore size are used to filter samples. The IR spectra of a pure substance were obtained using -FTIR (Bruker Optik, GmbH-Ettlingen Germany). The spectrometer's high-throughput ZnSe ATR crystal can be manipulated in a variety of ways thanks to the OPUS programme. The spectra of 1H and 13C NMR were recorded at 300 and 75 MHz, respectively, on a Bruker AV-300 Supercon NMR system, with the samples being scanned between 600 and 4000 cm-1. Deuterated chloroform (CDCl3) in Trimethyl silane (TMS) serves as the solvent and internal standard, respectively. With TMS set to 0, the scale is used to express chemical shift values.

Induction of experimental diabetes

Animals were given a single dosage of streptozotocin (65 mg/kg i.p.) to cause diabetes mellitus in an experimental setting [17]. After one week, the serum glucose level was measured using a spectrophotometer set to 505 nm and the glucose oxidase/pyruvate oxidase (GOD-POD) technique. Animals were considered hyperglycemic if their serum glucose levels were greater than 200 mg/dl[18].

Isolated rat heart preparation

Sodium pentobarbital (60mg/kg, i.m.) [19] was used to induce deep anaesthesia in the study animals, and heparinised (500 IU/l, i.p.) (Gland Pharma Ltd., Hyderabad, India) was injected intravenously to prevent excessive blood clotting. After being removed from the animal, the heart was placed in a double-walled container and immediately put on Langendorff's assembly [20]. Warm water circulation was used to keep the temperature at 37 degrees Celsius. The preparations were perfused with 95% oxygen and 5% carbon dioxide at a rate of 7-9 ml/min via the coronary arteries at a constant pressure of 80 mm Hg using Kerbs Henseleit (KH) buffer (KCl 4.7 mM, NaCl 118 mM; $MgSO_4.7H_2O$ 1.2 mM, CaCl2 2.5 mM; $KH2PO_4$ 1.2 mM, $NaHCO_3$ [21].

Experimental Protocol and Induction of IPC

There are seven different groups used in this study (n=6). Each group of animals was given a week to adjust to their new environment before being randomly assigned to different groups. Experiment group protocols are discussed in full, with Fig. 1 providing a visual aid. For the sham control, an isolated rat heart was stabilised for 10 minutes, then perfused continuously with KH buffer for 190 minutes, all while avoiding the effects of global ischemia. (2) I/R Control: After 10 minutes of stabilisation, 30 minutes of global ischemia and 120 minutes of reperfusion are administered to a rat heart preparation. After 10 minutes of stabilisation, an isolated rat heart underwent four cycles of preconditioning consisting of 5 minutes of ischemia followed by 5 minutes of reperfusion with KH buffer. The heart was then exposed to 30 minutes of global ischemia followed by 120 minutes of reperfusion. (4) I/R in diabetic rats: after 10 minutes of stabilisation, an isolated rat heart preparation was subjected to 30 minutes of global ischemia, followed by 120 minutes of reperfusion. Five minutes of occlusion, followed by five minutes of reperfusion with KH buffer, after stabilisation for ten minutes, was used to induce preconditioning (IPC) in the hearts of diabetic rat preparations. After 120 minutes of reperfusion, the animal hearts underwent another 30 minutes of global ischemia. Sixth, IrA-induced preconditioning (IPC) in the hearts of diabetic rats: an isolated heart preparation from these animals was treated with an *IrA* hydro-alcoholic extract, and then subjected to four episodes of preconditioning consisting of occlusion for five minutes, followed by reperfusion for five minutes with a KH buffer, and then satabilization for ten minutes. After 30 minutes of global ischemia, the hearts of rats were reperfused for 120 minutes. (7) Intrinsic Polymorphic Chromosomes in Atractyloside Perfused Hearts of *IrA* -Treated Diabetic Rats: Four preconditioning episodes were performed in diabetic rats, each lasting 10 minutes. Each episode began with an occlusion lasting 5 minutes and ended with a reperfusion using KH buffer lasting 5 minutes. The most recent instalment of IPC featured the implementation of atractyloside (20 M)reperfusion. After 120 minutes of reperfusion, the rat heart was deprived of oxygen for 30 minutes.

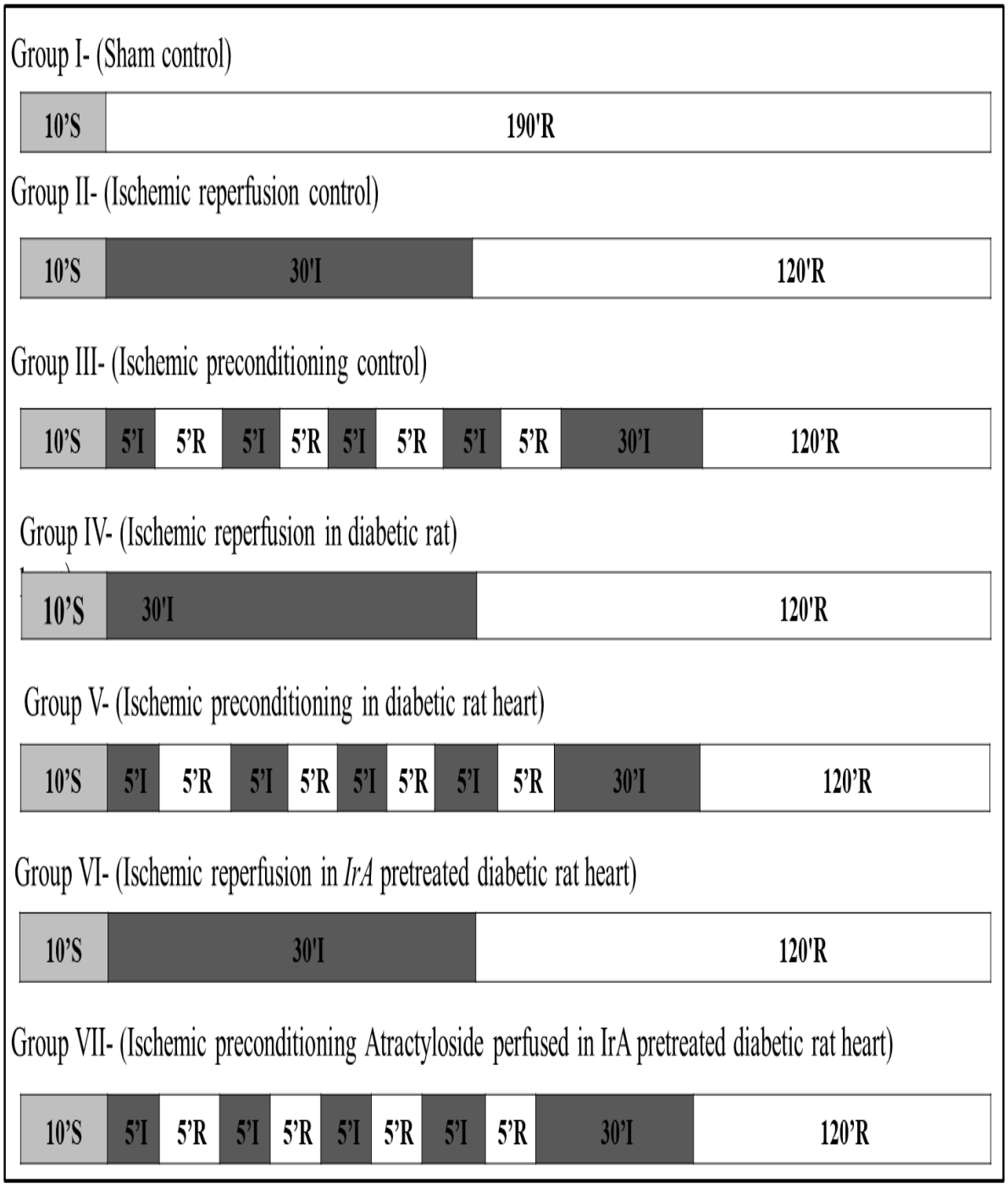


Fig.1 The representation of experimental protocol.

Assessment of myocardial infarction size

Once the cycle was complete, the isolated heart was kept at -85 degrees Celsius for half an hour. Following a top-to-bottom slicing procedure on a frozen heart, cross sections were obtained. The thickness of each slice was determined to be between two and three millimetres. The infarct area was not stained after being treated with 1% triphenyl-tetrazolium chloride (TTC), while the other live myocardial tissues were a bright red colour. ImageJ was used to calculate the proportion of myocardial infarction [21].

Assessment of myocardium cell injury

The extent of myocardial cell damage was assessed by estimating the levels of LDH and CKMB in coronary drainage by spectrophotometric analysis using commercially available kit (Coral clinical system Pvt. Ltd., India) [22].

Haemodynamic parameter

The isolated heart was placed in the Langendorff's apparatus, where the left ventricular end-diastolic pressures (LVEDPs) were maintained at a stable 5–10 mm Hg by means of an inflated LV balloon. Acquisition system software was used to capture heart rate (HR), left ventricular end-diastolic pressure (LVEDP), left ventricular developed pressures (LVDPs), and LV contractility (+ dP / dt) (PowerLab). in each experimental group, this is the related parameter. We took a sample of the heart's blood flow and stored it for further analysis. After inducing global ischemia, heart rate and the occurrence of reperfusion-mediated ventricular fibrillation (VF) were recorded at baseline, 0 (no ischemia), 30 (normal), and 120 (reperfusion) minutes [23].

Troponin-T release

In the cardiac preparation, the troponinT release was measured with a commercially available troponin T test kit **(**Coral Clinical System Pvt. Ltd., India) (Srivastav et al., 2013).

Nitrite estimation

The nitrite concentration was easily measured as compared to nitric oxide (NO). The nitrite concentration was used to identify the change in production of NO. The release of nitrite in the coronary drainage was measured by using Griess reagent and spectrophotometric analysis was performed at 550 nm. The nitrite concentration was determined andresults were expressed as micromoles per litre [18].

Assessment of oxidative stress in myocardial tissue

Unstiffened samples of myocardial tissue were homogenised in 0.1 M phosphate buffer (10% w/v, pH 7.4) at 7000 rpm for 5 minutes. Two millilitres of supernatant were collected after centrifuging the homogenate for a second time at 4 degrees Celsius and a speed of three thousand revolutions per minute. Adding an equal volume of 5% tricarboxylic acid (TCA) to the residual tissue homogenate, centrifuging at 4 °C for 10 minutes at 4000 rpm, and collecting the supernatant yielded a protein fraction that was easily isolated and purified [21].

Assessment of Superoxide dismutase (SOD) activity

Analyzing spectrophotometrically at 560nm, SOD activity in the heart is quantified. Blank, standard, and tissue samples all had their own 96-well plates made for them. Using a multichannel pipette, add 300 l Tris buffer to the blank, 290 l Tris buffer to the autoxidation, and 10 l each of tissue homogenate, Tris buffer, and pyrogallol to the sample wells. The blank does not get any Tris buffer [24].

Assessment of Catalase (CAT) activity

Hydrogen peroxide built up in the myocardium while CAT wasn't around. The sample was diluted with 50 mM phosphate buffer and placed in a 3 ml cuvette (pH 7.0). Absorbance was monitored for 30 seconds at 240 nm after 1 ml of 30 mM hydrogen peroxide was administered at 15 second intervals [24].

Assessment of glutathioneperoxidase (GSH) activity

Cumene hydroperoxide's ability to catalyse the oxidation of glutathione by glutathione peroxidase (GPX) is used to evaluate GPX activity. To measure GSH, we mixed 0.5 millilitres of 5,5 dithiobis-2-nitrobenzoic acid (DTNB) with 0.5 millilitres of supernatant, 2.0 millilitres of phosphate buffer (0.3 M, pH 8.4), 0.4 millilitres of double-distilled water, and 0.1 millilitre of supernatant. Following a 10-minute incubation period, the reaction mixture's absorbance was measured at 412 nm [18].

Isolation of mitochondria from Heart tissue

In all groups of experiments, the mitochondria were instantly isolated from the heart using the standard protocol after their perfusion in the control group. In addition, mitochondria in all groups were isolated from the heart immediately after reperfusion and then exposed to diabetic models. The amount of protein available in the mitochondria were determined [25].

Assessment of Mitochondrial function

The ability of mitochondria to break down 3-(4,5-dimethylthiazol-2-yl) -2,5- diphenyltetrazolium bromide (MTT) was evaluated by measuring the amount of formazan formed during the reduction process. Spectrophotometric analysis at 595 nm was used to calculate the amount of formazan produced. The amount of formazan produced in the experiments was given as mg/min/mg protein [26].

Histopathological examination and cardiac collagen content

10% buffered formalin solution was used to repair the heart slice. These tissues were embedded in paraffin wax and then sectioned transversely through the middle of the ventricles at a thickness of about 5 m using a microtome. Haematoxylin and eosin necrosis with myocyte size and Picrosirius red F3BA were used to evaluate fibrosis in these sections (i.e., collagen content) [27].

Western blot Analysis

The heart muscle was snap frozen in a vat of liquid nitrogen and then kept at -80 degrees Celsius for later use. Proteins were extracted from the tissue. Using a modified Bradford assay (Bio-Rad, CA, USA), protein concentration was determined in the supernatant obtained after centrifugation at 14,000 g for 20 minutes [28]. Electrophoresis on SDS-PAGE gels and transfer to polyvinylidene difluoride (PVDf) membranes were performed on aliquots of supernatants containing 35 g of protein. Primary antibodies were used to treat membranes. As a loading control, actin was employed in conjunction with the secondary antibody (1:1000) for 1 hour at room temperature in the dark. E-Gel Imager images were taken and analysed using the enhanced chemiluminescence (ECL) method (Bio-rad Laboratories, USA) to detect the band's intensity (Tanon 5200 Multi, Shanghai, China).

Statistical Analysis

Utilizing GraphPad Prism, statistical analysis was carried out. The data are presented as a mean standard deviation. One-way analysis of variance (ANOVA) with a Bonferroni post hoc test was performed to compare

the activities of the enzymes LDH and CKMB to those of the hemodynamic, coronary flow rate, and HR variables. All statistical data is analysed using one-way ANOVA and the Student-Newman-Keuls post hoc test. Statistical significance is defined as a p-value that is less than 0.05.

RESULT

Chemical analysis of *IrA* by RP-HPLC, FTIR and NMR.

The main chemical constituents in IrA were analysed by RP-HPLC. The main peaks were identified as Alantolactone/Isoalantolactone (Fig. 2a) by comparing the retention time and UV spectra and structure are shown in Fig. 2b. The content of alantolactone/isoalantolactone was found to be 1.74 ± 0.185 % (w/w).

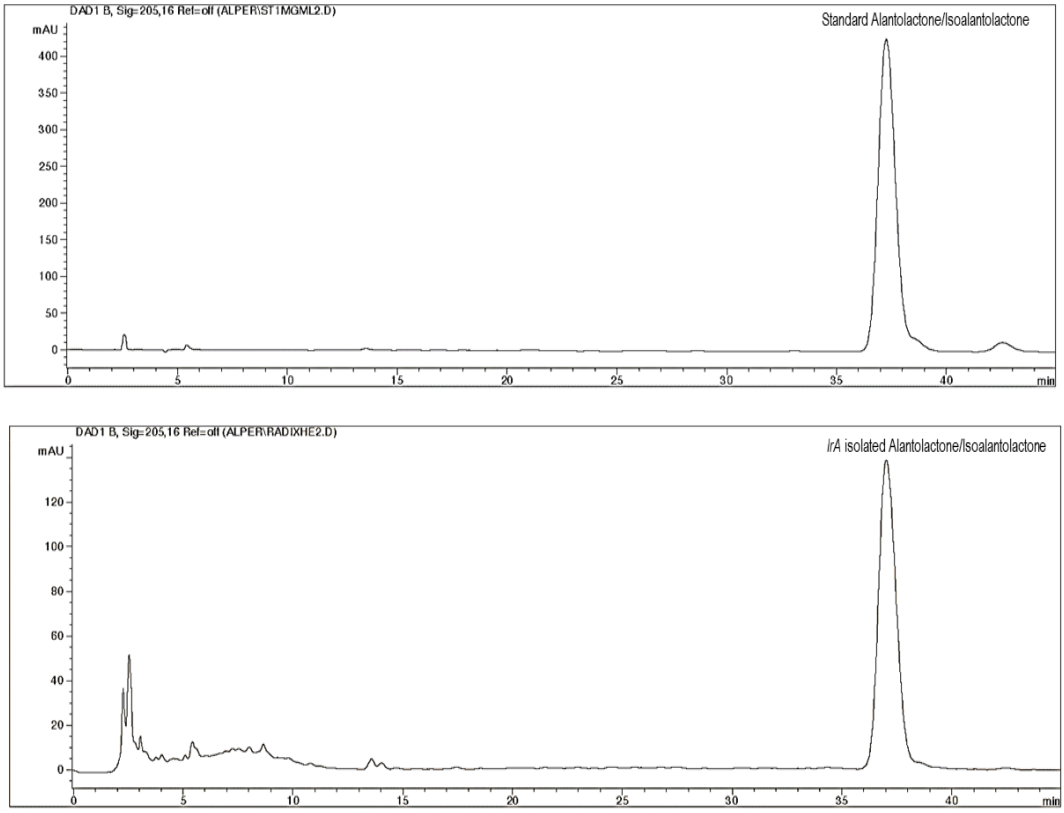


Fig.2a HPLC-UV chromatgram of IrA and standard at 205nm.

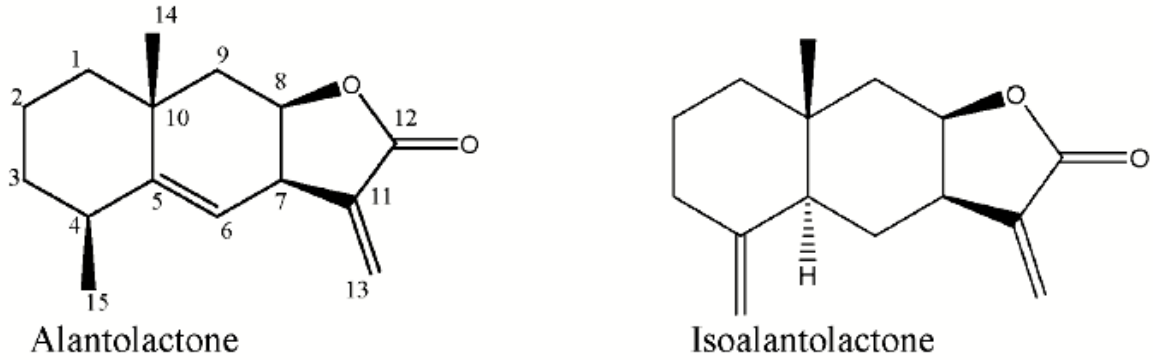
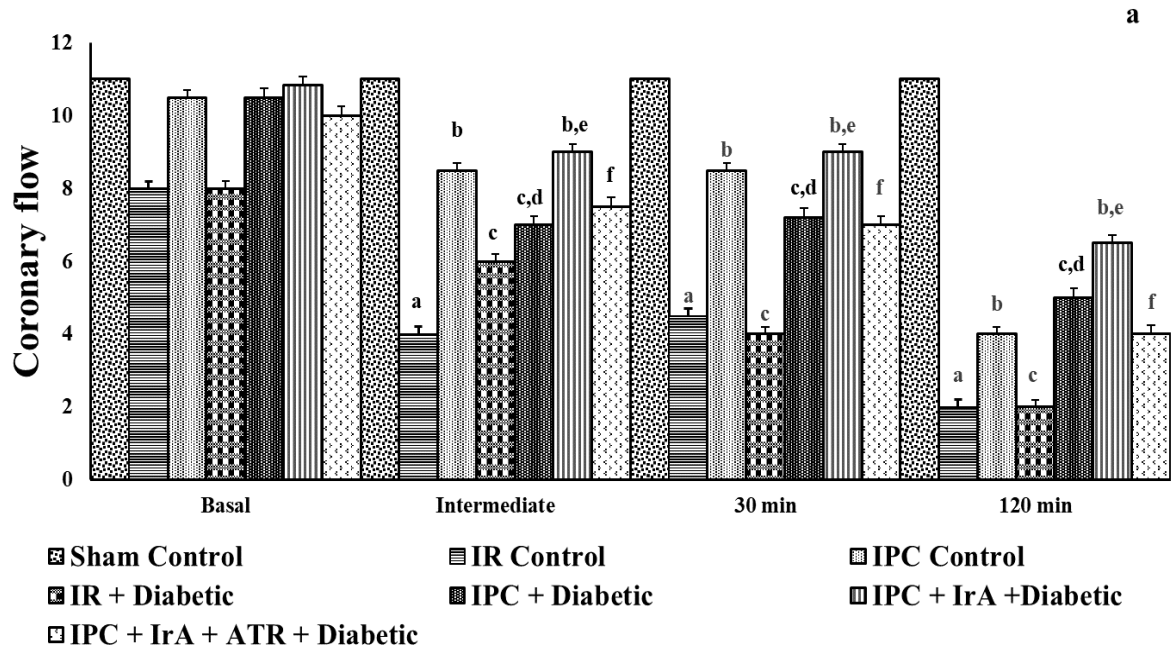


Fig.2b. Structure of main constituents of *IrA*

IrA potentiate the IPC-mediated coronary flow in Diabetic rat heart

It was revealed that IrA affected IPC-mediated coronary flow in the hearts of diabetic rats (Fig.3a). Using a post hoc test, we found no statistically significant differences in coronary effluent flow at the baseline time between any of the experimental groups. Comparing the I/R control and IPC + diabetic groups, we find that IPC mediates a decrease in coronary flow in the heart of diabetic rats, whereas IrA augments the IPC-mediated increase in coronary flow.



***IrA* potentiate the IPC-mediated enhancement in HR in diabetic heart**

Diabetic hearts are depicted in Fig. 3b, where the influence of IrA on the IPC-mediated alterations in HR is noted. Post-hoc analysis confirmed that the experimental groups did not differ significantly from one another in their baseline HR. As shown here, heart rate (HR) decreases in the immediate, 30- and 120-minute periods following the onset of ischemia in the rat heart, regardless of whether the rats were observed in the I/R control, IPC control, or IPC+ diabetic groups, while IrA increases IPC influence increase in HR of diabetic rats. This effect lasted all the way through the tests.

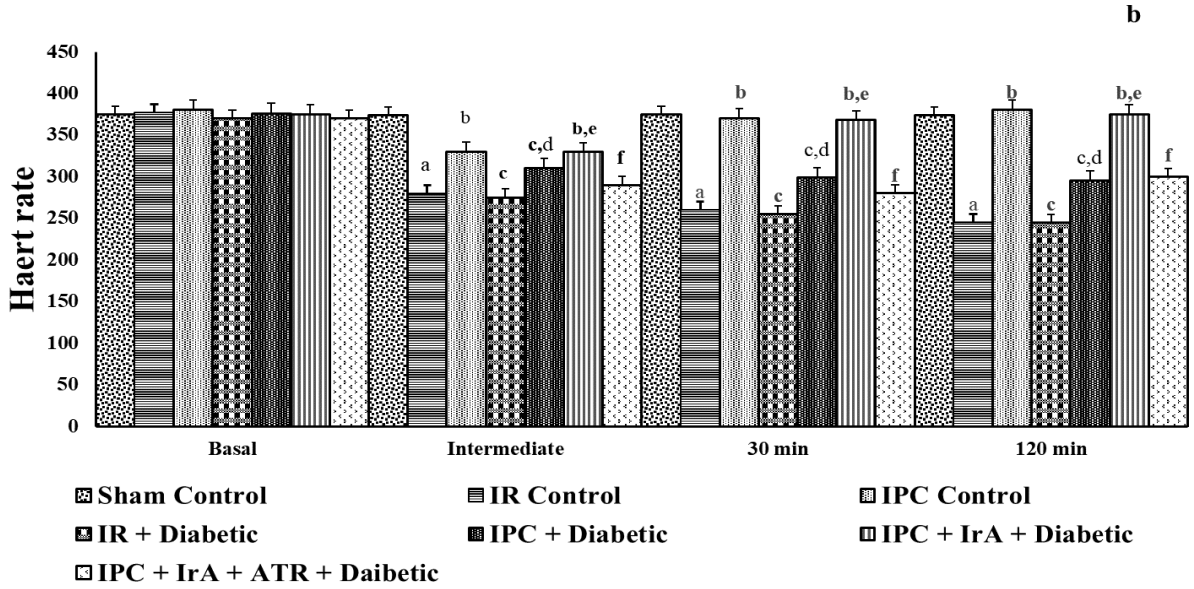


Fig. 3 *Inula racemosa* restored in IPC mediated changes in coronary flow and heart rate ofdiabetic heart. All values are stated as mean ± S.D (n=6). [a]*P*< 0.05 compared with sham control, [b]*P*< 0.05 compared with I/R control, [c]*P*< 0.05 compared with IR control, [c,d]P< 0.05 compared with I/R control and I/R +Diabetic, [b,e]*P*< 0.05 compared with I/R control and IPC + Diabetic, [f]*P*< 0.05 compared with IPC control and IPC + IrA + Diabetic(one way ANOVA followed by Bon-ferroni Post hoc).

***IrA* potentiate the IPC-mediated enhancement in LVDP, +dP/dtmax, and -dP/dtmax in diabetic rat heart**LVDP, +dP/dtmax, and -dP/dtmax were all altered by IrA's effect on the IPC, as shown in Fig. 4. There was no statistically significant difference between the experimental groups in terms of LVDP, + dP /dtmax, or - dP /dtmax during the baseline period, as determined by post hoc analysis. When comparing I/R control and IPC+ diabetic hearts, this study found that IPC-mediated reductions in LVDP, + dP /dtmax, and - dP/dtmax occurred in the latter two groups, while IrA treatment increased all three. This effect lasted all the way through the tests.

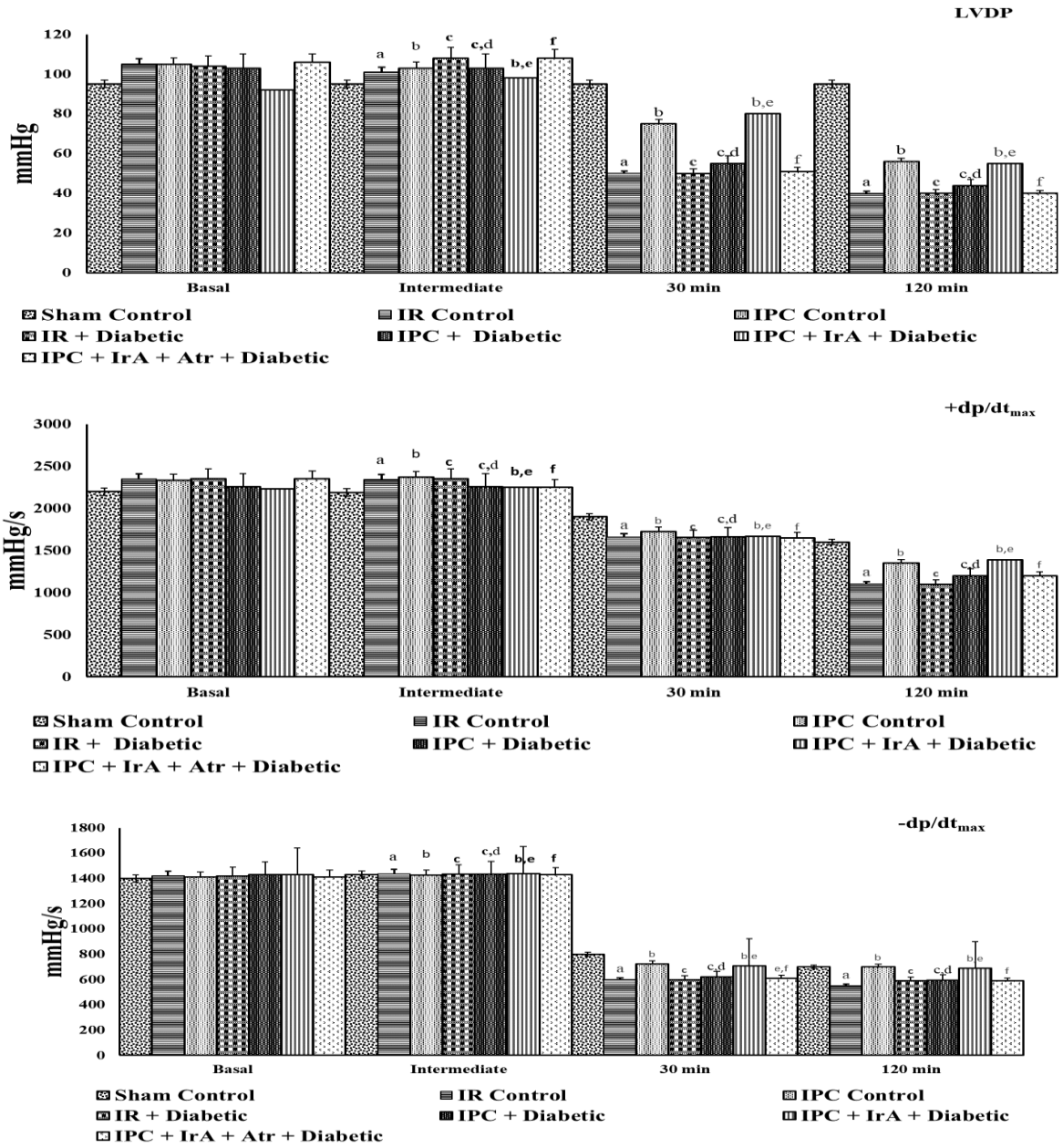
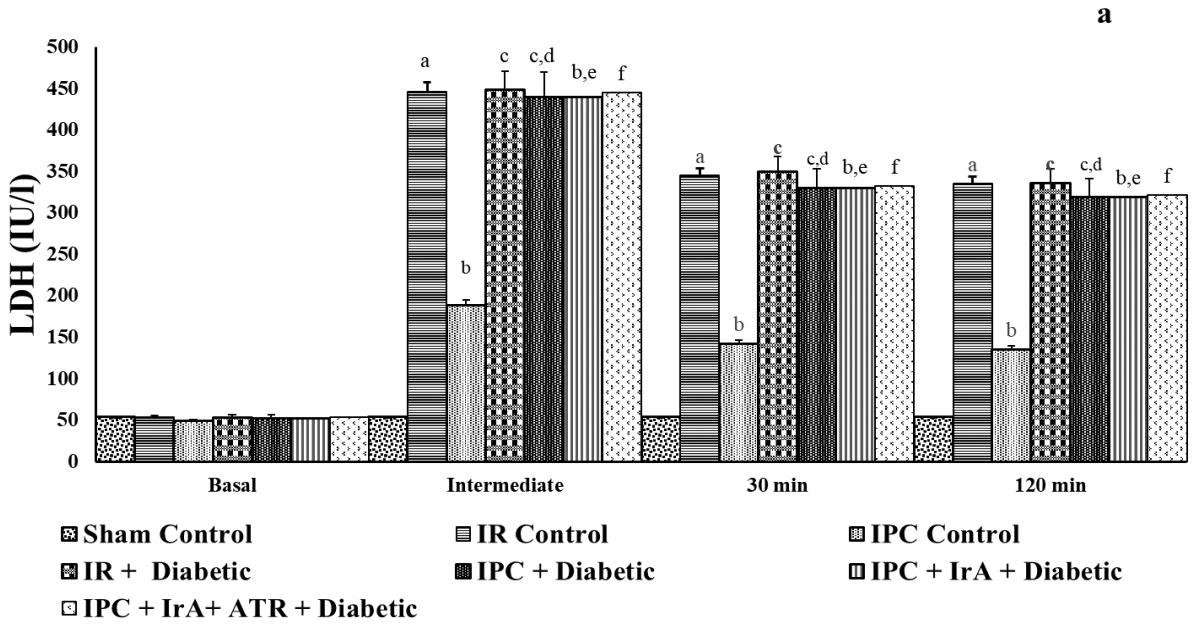


Fig. 4 *Inula racemosa* restored in IPC mediated changes in LVDP, +dp/dt$_{max,}$and-dp/dt$_{max}$ in diabetic heart. All values are stated as mean ± S.D (n=6). [a]*P*< 0.05 compared with sham control, [b]*P*< 0.05 compared with I/R control, [c]*P*< 0.05 compared with IR control, [c,d]P< 0.05 compared with I/R control and I/R + Diabetic, [b,e]*P*< 0.05 compared with I/R control and IPC control, [e,f]*P*< 0.05 compared with IPC control and IPC + Diabetic, [f]*P*< 0.05IPC + IrA + Diabetic (one way ANOVA followed by Bon-ferroni Post hoc).

***IrA* facilitates IPC-mediated attenuation in LDH level in diabetic rat heart**

In Fig.5a. the influence of *IrA* on IPC mediated change in LDH activity on diabetic hearts. Post hoc shows that there is no significant difference in LDH level at basal time among all experimental groups. In this study, an IPC-influenced decrease in LDH level in coronary effluent of *diabetic* rat heart was decreased, while *IrA* decreases the LDH level as compared to I / R control and IPC+ diabetic hearts. The effect lasted until the end of the experiments.



***IrA* facilitates IPC-mediated attenuation in CKMB level in diabetic rat heart**

In Fig. 5b. the influence of *IrA* on IPC mediated changes in CKMB activity on diabetic hearts. Post hoc shows that there is no significant difference in CKMB level at basal time among all experimental groups. In this study, an IPC- mediate decrease in CKMB level in coronary effluent of diabetic heart was decreased, while *IrA* decreases CKMB level as compared to I / R control and IPC+ diabetic hearts. The effect lasted until the end of the experiments. Also, Atractyloside along with *IrA attenuated* the IPC mediated decrease in level of CKMB in diabetic hearts.

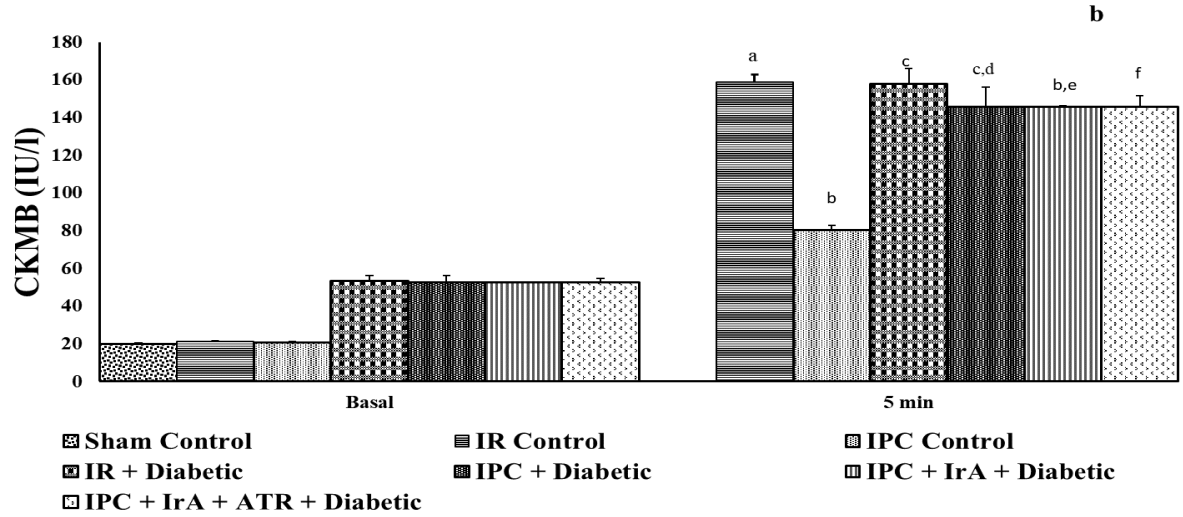


Fig. 5*Inula racemosa* restored in IPC mediated changes in LDH and CKMB in diabetic heart. All values are stated as mean ± S.D (n=6). $^{a}P< 0.05$ compared with sham control, $^{b}P< 0.05$ compared with I/R control, $^{c}P< 0.05$ compared with IR control, $^{c,d}P< 0.05$ compared with I/R control and I/R +Diabetic, $^{b,e}P< 0.05$ compared with I/R control and IPC + Diabetic, $^{f}P< 0.05$ compared with IPC control and IPC + IrA + Diabetic(one way ANOVA followed by Bon-ferroni Post hoc test).

***IrA* facilitates IPC-mediated attenuation in infarct size as well as ventricular fibrillation in diabetic rat heart**

The influence of *IrA* on IPC mediated changes on infarct size and VF in diabetic hearts shown in Fig. 6a, b. Post hoc indicates that IPC significantly lessened diabetic induced increases in myocardial infarct size and VF in all the experiment groups. *IrA* further increases IPC induced reduction of the infarct size and VF in diabetic hearts as compared to the I / R control and IPC+ diabetic hearts. Also, Atractyloside along with *IrA* diminished the IPC mediated decrease in infarct size and VF in diabetic rat hearts.

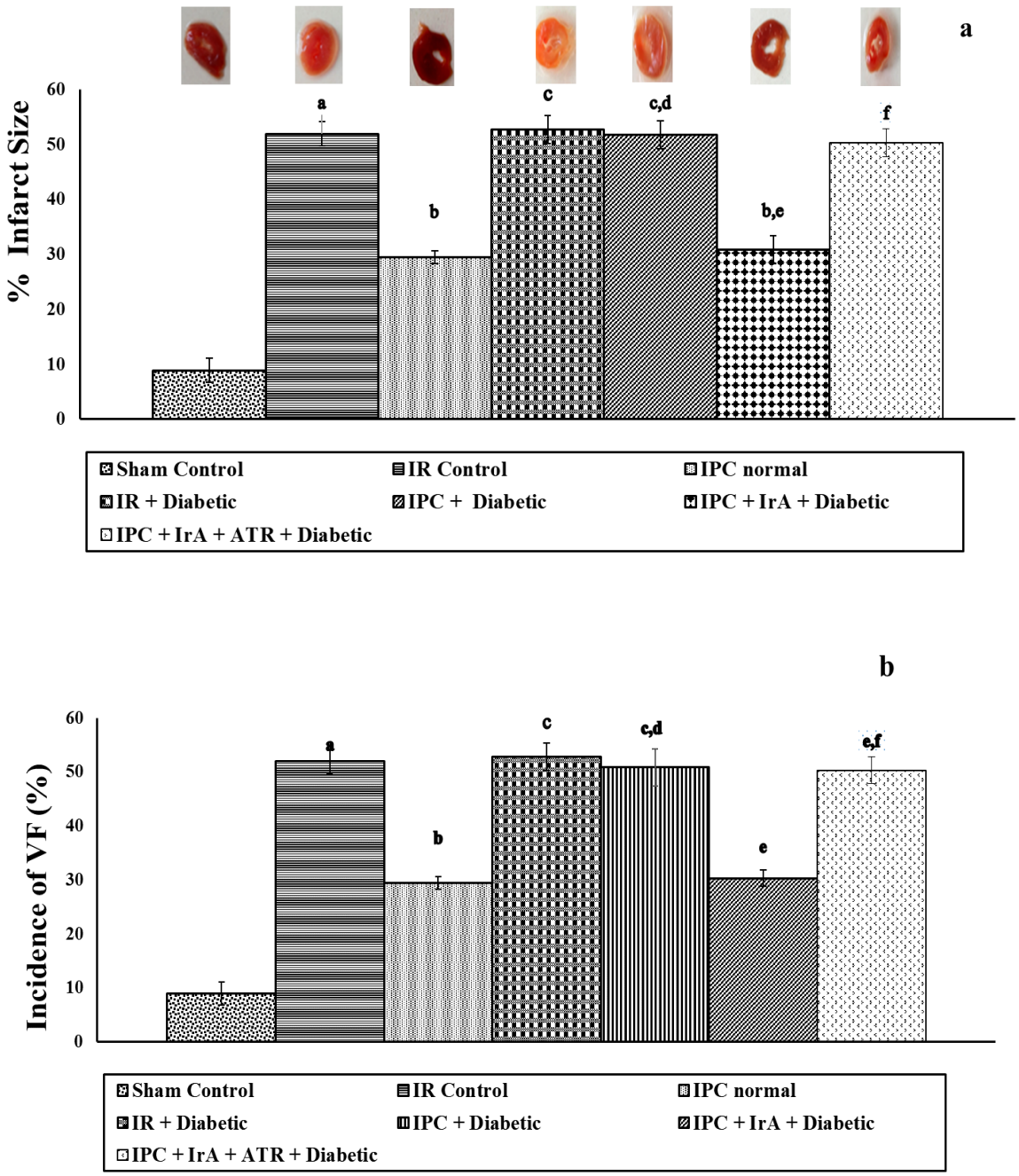


Fig. 6*Inula racemosa* restored in IPC mediated changes in heart infarct size and ventricular fibrillation indiabetic heart. All values are stated as mean ± S.D (n=6). [a]*P*< 0.05 compared with sham control, [b]*P*< 0.05 compared with I/R control, [c]*P*< 0.05 compared with IR control, [c,d]P< 0.05 compared with I/R control and I/R +Diabetic, [b,e]*P*< 0.05 compared with I/R control and IPC + Diabetic, [f]*P*< 0.05 compared with IPC control and IPC + IrA + Diabetic(one way ANOVA followed by Bon-ferroni Post hoc).

***IrA* facilitates IPC-mediated attenuation in infarct size as well as ventricular fibrillation in diabetic rat heart**

The influence of *IrA* on IPC mediated changes in mitochondrial function was measured in terms of formazan level and TMRM fluorescence intensity (integrity) in diabetic hearts are shown in Fig. 7a, b. Thepost hoc shows that mitochondrial function was noticeably reduced in diabetic hearts when comparing with sham control. *IrA* increasesIPC attenuated mitochondrial function in diabetic hearts as compared to the I / R control and IPC+ diabetic groups. Also, Atractyloside together with *IrA attenuated* the IPC mediated increase in mitochondrial function in diabetic hearts.

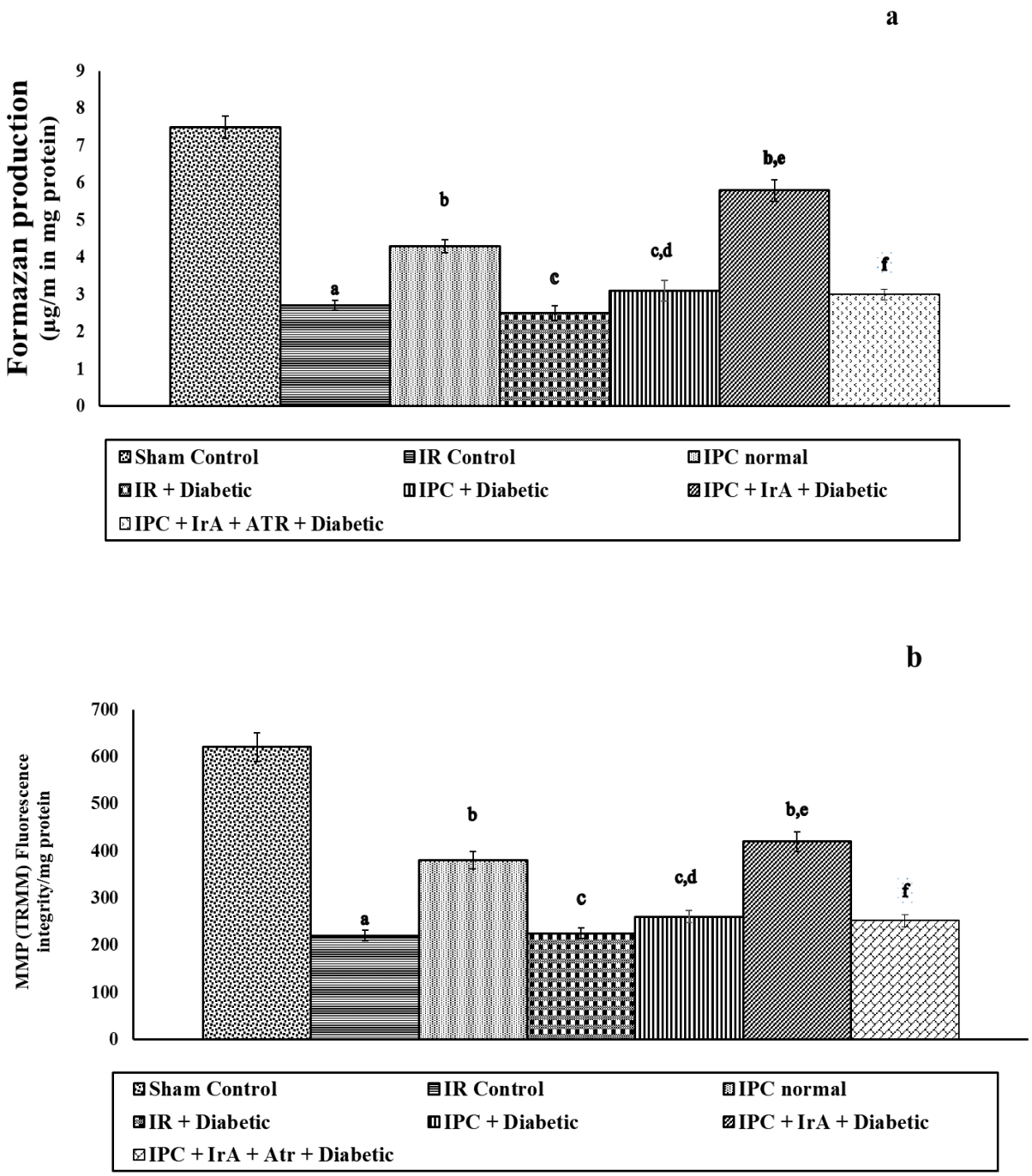


Fig. 7*Inula racemosa* restored in IPC mediated changes in mitochondrial function (formazan production) and integrity (TMRM fluorescence intensity) indiabetic heart. All values are stated as mean ± S.D (n=6). [a]*P*< 0.05 compared with sham control, [b]*P*< 0.05 compared with I/R control, [c]*P*< 0.05 compared with IR control, [c,d]P< 0.05 compared with I/R control and I/R +Diabetic, [b,e]*P*< 0.05 compared with I/R control and IPC + Diabetic, [f]*P*< 0.05 compared with IPC control and IPC + IrA + Diabetic(one way ANOVA followed by Bon-ferroni Post hoc).

***IrA* facilitates IPC-mediated attenuation in the oxidative stress in diabetic rat heart**

The influence of IrA on IPC mediated changes on GSH, CAT and SODactivities are represented in Table 1in diabetic hearts. The post hoc shows that there is no noticeable difference on GSH, CAT and SOD (antioxidant) activities in diabetic heart when compared with sham control. IPC attenuated enhanced activities in diabetic rat hearts. IrA increases the IPC mediated decrease in antioxidant level in diabetic rat hearts as compared to the I / R control and IPC+ diabetic groups. Interestingly, Atractyloside together with IrA attenuated the IPC mediated increase in oxidant stress markers in diabetic hearts.

Table.1. All values are mean ± S.D. (n=6).[a]*P*< 0.05 compared with sham control, [b]*P*< 0.05 compared with I/R control, [c]*P*< 0.05 compared with IR control, [c,d]P< 0.05 compared with I/R control and I/R +Diabetic, [b,e]*P*< 0.05 compared with I/R control and IPC + Diabetic, [f]*P*< 0.05 compared with IPC control and IPC + IrA + Diabetic (one way ANOVA followed by students Newman KeulsPost hoc test).

Oxidative Stress	GSH (units/min/mg protein)	SOD (units/min/mg protein)	CAT (units/min/mg protein)
Sham Control	2.15 ± 0.01	0.58 ± 0.09	2.93 ± 0.16
I / R Control	0.46 ± 0.09[a]	0.20 ± 0.10[a]	1.49 ± 0.25[a]
IPC Control	1.13 ± 0.073[b]	0.54 ± 0.056 [b]	2.48 ± 0.23[b]
I / R + Diabetic	0.47 ± 0.06[c]	0.20 ± 0.02[c]	1.47 ± 0.19[c]
IPC+ Diabetic	0.552 ± 0.16[c,d]	0.23 ± 0.018[c,d]	1.53 ± 0.21[c,d]
IPC + IrA + Diabetic	1.24 ± 0.09[b,e]	0.55 ± 0.02[b,e]	2.47 ± 0.21[b,e]
IPC + IrA + Atr + Diabetic	0.861 ±0.04[f]	0.22 ± 0.02 [f]	1.52 ± 0.27[f]

IrA facilitates IPC-mediated attenuation troponin-T in diabetic rat heart

In Fig. 8 we see that IrA modulates IPC-mediated variations in troponin-T levels in diabetic hearts. Post hoc analysis revealed that IrA decreases troponin-T relative to I/R control and IPC+ diabetic hearts, while IPC significantly decreased the rise in troponin-T relative to sham control. To my surprise, the elevation in troponin-T that is caused by IPC was blunted when Atractyloside was combined with IrA in diabetic hearts.

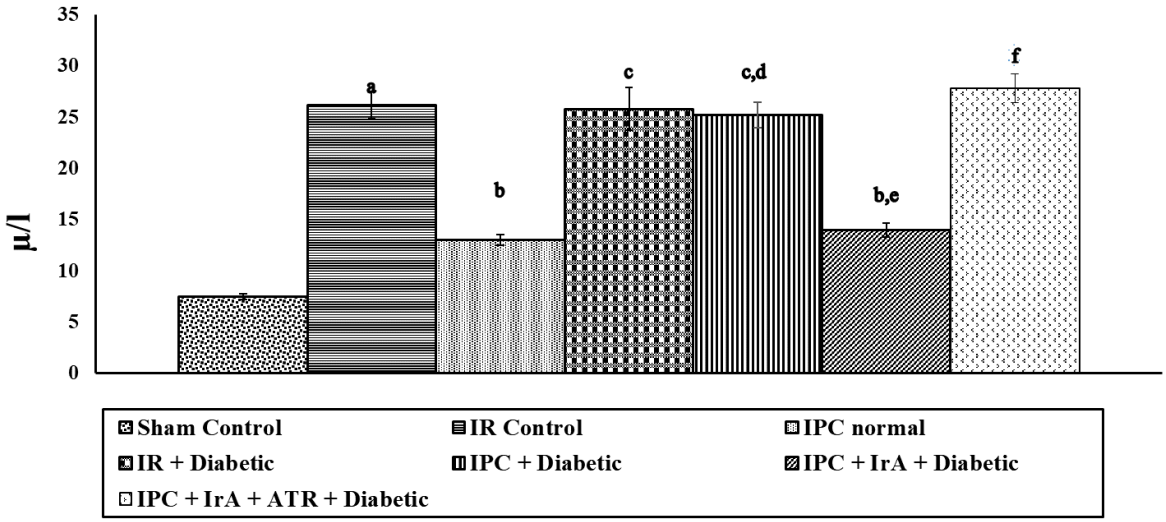


Fig. 8*Inula racemosa* restored in IPC mediated changes in Troponin-T inDiabetic heart. All values are stated as mean ± S.D (n=6). [a]*P*< 0.05 compared with sham control, [b]*P*< 0.05 compared with I/R control, [c]*P*< 0.05 compared with IR control, [c,d]P< 0.05 compared with I/R control and I/R +Diabetic, [b,e]*P*< 0.05 compared with I/R control and IPC + Diabetic, [f]*P*< 0.05 compared with IPC control and IPC + IrA + Diabetic(one way ANOVA followed by Bon-ferroni Post hoc).

IrA facilitates IPC-mediated attenuation in myocardial histological changes in diabetic rat heart

The histological changes in various group of rat heart are studied in photomicrograph are shown in Fig.9. The histological images of sham control shows that all the heart muscles (endocardium, papillary muscles, epicardiumand also vasculature are found normal. There is no cardiac damage is seen under the microscope.In I/R control treated group graph confirms the focal myonecrosis with myophagocytosis and lymphocytic infiltration. In sub-endocardium region under microscope prominent chronic inflammation in cells are visible In IPC given group shows less amount of infiltration of inflammation in cell and also decrease in the myonecrosis. However, under microscope changes in sub-endocardium and myonecrosis has been visible. In IPC+ diabetic group, some severity of myonecrosis was seen, whereas lesser infiltration of inflammation and decrease in myophagocytosis in cells as compared to IPC group has been seen. However, under microscope changes in sub-endocardium vacuolar are also seen. IPC + *IrA* group shows less amount of infiltration of inflammation in cells and decreases the grade of myonecrosis under Microscope. IPC + diabetic + *IrA*treated group were showing minor severity of myonecrosis, myophagocytosis and lesser infiltration in lymph vessels. Very less infiltration and oedema in inflammatory cells were visible. In IPC + diabetic + *IrA* + Atractyloside treated group there is visibility of myonecrosis, infiltration of inflammation and decrease in myophagocytosis in cell as compared IPC and IPC + diabetic + *IrA* group.

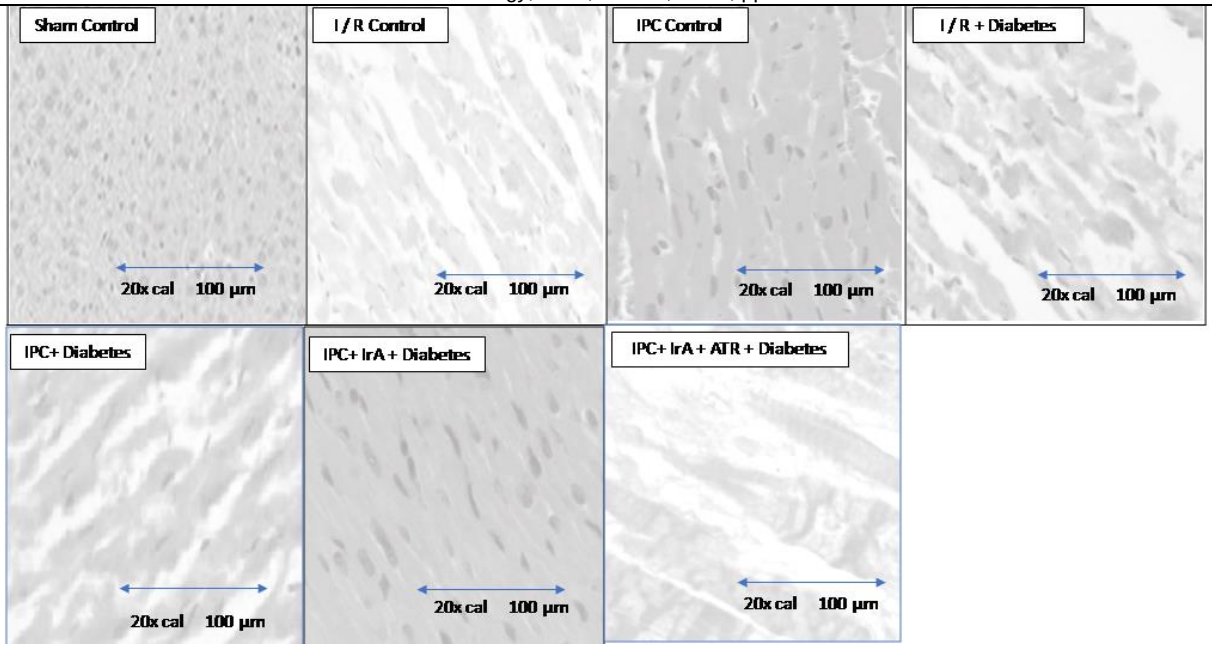


Fig. 9. Effect of I/R on histopathological change in normal, Diabetic heart, IrA pretreated diabetic heart, Effect of IPC on histopathological change in normal and diabetic hearts, Effect of IPC in pretreated IrA, Atr, pretreated IrA and Atr on histopathological report of heart effect of diabetic heart. Sham control shows normal myocardial cell architecture, I/R control shows necrotic changes in myocardial tissue, IPC control shows regenerative change in myocardial tissue, I/R + Diabetic shows necrotic change in tissue architecture, IPC + Diabetic shows lesser regenerative change in myocardial tissues, IPC + IrA + Diabetic shows more regenerative change in myocardial tissue architecture, IPC + IrA + Atr + Diabetic show necrotic changes in myocardial tissues.

***IrA* facilitates IPC-mediatedattenuation of PI3K expression in diabetic rat heart**

IrA influence the IPC mediated change in expression of PI3K, in diabetic hearts shown in Fig.10. Post hoc indicates that extent of PI3K significantly improved in diabetic rat heart tissue as compare to sham control. IPC attenuates the extent of expression of PI3K in diabetic heart. *IrA* again increases the IPC-induced reduction in expression of PI3K in diabetic heart. Interestingly, Atractyloside with *IrA* causes significant change to IPC-mediated extent of expression in diabetic rat heart.

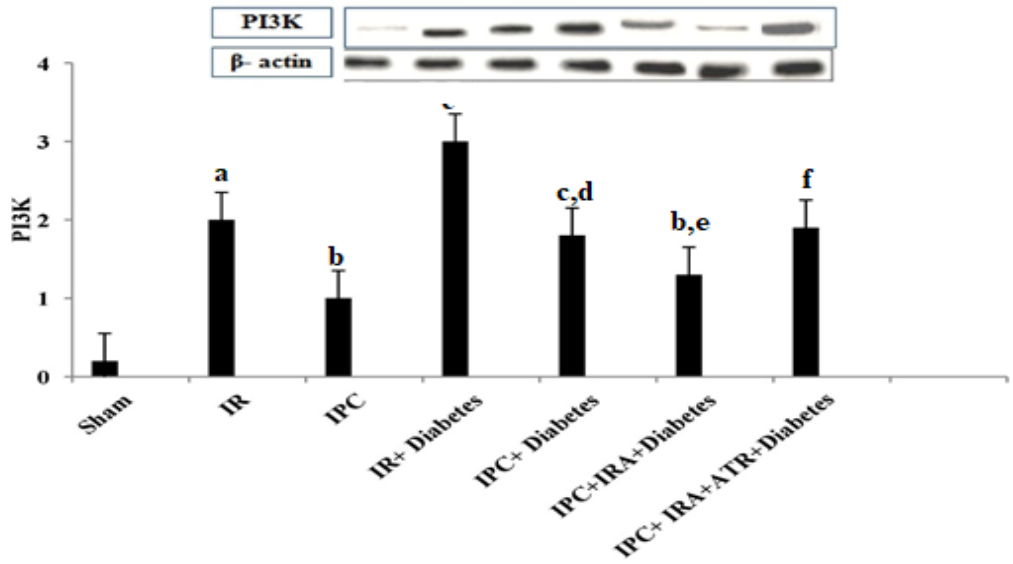


Fig. 10 *Inula racemosa* restored in IPC mediated changes in level of expression in diabetic heart tissue. The histogram of PI3K protein is expressed as the arbitrary unit of expression of PI3K to β-actin. All values are stated as mean ± S.D (n=6). $^{a}P< 0.05$ compared with sham control, $^{b}P< 0.05$ compared with I/R control, $^{c}P< 0.05$ compared with IR control, $^{c,d}P< 0.05$ compared with I/R control and I/R +Diabetic, $^{b,e}P< 0.05$ compared with I/R control and IPC + Diabetic, $^{f}P< 0.05$ compared with IPC control and IPC + IrA + Diabetic(one way ANOVA followed by Bon-ferroni Post hoc).

DISCUSSION

A comparison of the I/R control group and the 5-minute ischemia followed by KH buffer reperfusion with additional 30-minute global ischemia after 120 minutes of reperfusion in an adiabetic heart preparation mounted on Langendorff's' assembly did not reveal a significant cardioprotective phenomenon. However, a significant cardioprotective response was shown versus I/R and IPC + diabetes groups when Inula racemosa extract was administered over 30 days prior to heart isolation. It was also found that the MPTP opener Atractyloside dramatically reduced the cardioprotective effects of IPC with Inula racemosa extract in diabetic rats. This study adds to the existing body of knowledge by showing that the cardioprotection afforded by Inula racemosa, when administered to hearts with disease (such as diabetes), differs from that afforded to hearts without disease in terms of its ability to prevent I/R-induced damage via MPTP opening inhibition.

The chemical components of *Inula racemosa* extract was profiled by RP-HPLC and ^{1}H and ^{13}C NMR. The major identified chemical constituents. Alantolactone and Isoalantolactone shows the beneficial effect. During the study, the 30 days dose of the *Inula racemosa* in normal heart had not produced any significant change with IPC but noticeably in diabetic hearts it restores the IPC-mediated cardioprotective effect. The influence noticed in term of weakening of the cardiac disfunction by reducing the LVDP, $+dp/dt_{max}$ and $-dp/dt_{max}$, coronary flow and heart rate. In this study, it was observed that in diabetic hearts the haemodynamic parameter was sustained by treatment with *Inula racemosa*, compared to IR and IPC + Diabetic groups.

The damage to the heart muscles during ischemia -reperfusion correlates directly with the extent of release of LDH, CK-MB, myocardial infarct size and VF. Thepre-treatment of *Inula racemosa* extract lowers the concentration of the enzyme LDH and CK-MB in coronary effluent and also reduced the myocardial infarctsizeand VF in diabetic hearts. But decreased release of CK-MB and LDH, myocardial infarction size, VF with atractyloside perfused *Inula racemosa* pre-treated in diabetic hearts show an increased level. The primary mechanism is that IPC arbitrates the cardioprotective action by inhibiting the opening of MPTP [29, 30, 31] and further activation of Mitochondrial ATP sensitive Potassium channel (K_{ATP}) (Oldenburg et al., 2002). The decrease in the level of formazan level and decreased MMP indicate the myocardial dysfunction and the decrease in the level of markers for oxidative stress; GSH SOD and CAT are attenuated with IPC in diabetic rats. However, pre-treatment of *Inula racemosa* extract enhanced the cardioprotective therapy of IPC in diabetic rat groups. Perfusion with atractyloside with IPC pretreated *Inula racemosa* extract in diabetic rats weakened the cardioprotective effect due to the opening of MPTP. This observation strongly suggests that the *Inula racemosa* extract enhances the cardioprotective effect of IPCin diabetic hearts by inhibiting the opening of MPTP and is confirmed by the data represented in scientific data which showed that inhibition of MPTP opening is protective pathway.

Collagen maintains the construction of ventricles and then transfers the force of contractility from the myocytes to ventricular lumen. However, the cardioprotection by IPC failed in certain diseased states. Chronic fibrosis of the myocardial tissue occurs during this disease [32]. For this purpose, collagen-related data are observed for IPC in diabetic state. Myocardial injury occurs during myocardial infarction and which results in the appearance of an extracellular matrix rich scar [33], but diabetic affects cardiac collagen. In addition, when atractyloside has been co-administered with previously *Inula racemosa*, the cardioprotective effects gets attenuated in diabetic rat hearts.

During IPC activated the PI3K/Akt pathway which inhibit the MPTP opening which considered to be the major pathway in cardioprotection which is gets declining in diabetic rat heart. The effect of IPC mediated activation gets attenuated in diabetic condition. On treatment with *Inula racemosa* extract restores the attenuated PI3K/Akt phosphorylation and further inhibit the MPTP opening. This may modulate the IPC mediated cardioprotection in diabetic rat heart.

The data presented in the experimental study suggested that *Inula racemosa* can protect the heart from diabetic induced heart damage by using IPC. The result obtained thus expresses that the extract of *Inula racemosa*can be useful for patient with acute heart attack and responsible for reduction in further occurrence of ischemic attacks. Treatment of *Inula racemosa* to heart disease patients could be a potential cardioprotective adjuvant.

CONCLUSION

Based on the above discussion, it is further concluded that hydro alcoholic extract of roots *of Inula racemosa* Hook may restore the reduced cardioprotective effect of ischemic preconditioning in diabetic hearts by inhibiting the opening of MPTP. Supporting this claim is the fact that administration of atractyloside, an MPTP opener, diminished the cardioprotective effects of ischemic preconditioning in diabetic heart. Hence, *Inula racemosa* could be the potential cardioprotective adjuvant in acute myocardial infarction.

DECLARATION

Animal Ethical Approval

The Institutional Animal Ethics Committee (KNIMT/PHAR/IAEC/18/05) approved all experimental techniques in conformity with national rules for animal experimentation

Plant Authentication

NIScPR/RHMD/Consult/2021/3881-82 is the reference number for the *Inula racemosa* roots that were obtained from a local supplier and authenticated by the Council for Scientific and Industrial Research (CSIR-NISCAIR) in New Delhi.

Conflict of Interest

No conflict of Interest

CONTRIBUTION OF AUTHORS

AKT and PSG are involved in the design and conduct of the experiments. A research protocol was drafted by PSG and MP in conjugation with first author. Data processing and interpretation had done by PSG and MP. The design, evaluation and analyse the experimental research was possible with joint contribution of all authors. Also all authors are involved in writing the manuscript.

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