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Cardioprotective, hypotensive and toxicological studies of *Populus ciliata* (Wall. ex Royle)

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ABSTRACT

Populus ciliata Wall ex. Royle has folkloric repute to treat various cardiovascular ailments and related disorders. The current study was designed to evaluate the toxic profile, cardioprotective and hypotensive effects of *Populus ciliata* (Wall. ex Royle). *Populus ciliata* crude ethanolic extract (Pc. Cr) and its aqueous (Pc. Aq) & organic (Pc. Dcm) fractions were tested on isolated aorta of rat and rabbit having intact and non-intact endothelium respectively. Pc. Cr & Pc. Aq relaxed the contractions induced by PE (1 μM)-induced and K^+ (80 mM)-induced on aorta, possibly by mediating endothelium derived relaxing factor (EDRF) in intact endothelium and voltage dependent L-type calcium channels blocking (CCB) mechanism in non-intact endothelium. Pc. Cr showed anti-hypertensive & cardioprotective activity by decreasing force of contraction & heart rate on isolated rabbit paired atria and reduced blood pressure in anesthetized rat. Cardioprotective effect of Pc. Cr was assessed in isoproterenol induced acute myocardial infarction (AMI) and left ventricular hypertrophy (LVH) in Sprague Dawley rats. In LVH, Pc. Cr exerted positive effects by decreasing angiotensin II & renin and increasing cGMP & nitric oxide (NO) with reduced cardiac fibrosis, necrosis and cardiac cell size. In AMI, Pc. Cr responded effectively by decreasing cardiac markers creatinine kinase (CK), creatinine kinase myocardial band (CK-MB) and lactate dehydrogenase (LD) in blood associated with less edema and necrosis. Presence of catechin, vinallic acid, P-coumeric acid and quercitin identified through HPLC support the effectiveness of Pc. Cr in hypertension, AMI and LVH. Pc. Cr showed no significant adverse effects in Sprague Dawley albino rats after acute & sub-acute treatment in histopathological investigation. Extract of *Populus ciliata* showed vasorelaxant, hypotensive and cardioprotective effect in Sprague Dawley albino rats and white albino rabbit by mediating EDRF and voltage dependent L-type CCB mechanism respectively.

1. Introduction

Cardiovascular diseases (CVDs) are the leading cause of death in all regions of the world and a prime barrier to human sustainable development [1]. Non-communicable diseases, including CVDs, are a great concern for global health as recognized by UN in 2011 [2]. Hypertension is responsible for 51% of cerebrovascular disease and 45% of ischemic heart disease [3]. Due to chronic hypertension, number of physiological changes occurs at cellular level which leads to the growth of heart called hypertrophy [4]. Globally myocardial infarction (MI) and left ventricular hypertrophy (LVH) are the most prevailing cause of death [5].

Health benefits of alternative medicine and natural products have led

Abbreviations: AgII, angiotensin II; ALT, alanine transaminase; AMI, acute myocardial infarction; ANF, atrial natriuretic factor; BNF, brain natriuretic factor; CCB, calcium channels blocking; CK, creatinine kinase; CK-MB, creatinine kinase myocardial band; CVDs, Cardiovascular diseases; DBP, diastolic blood pressure; EDRF, endothelium derived relaxing factor; H&E, hematoxylin and eosin; ISO, Isoproterenol; LD, lactate dehydrogenase; LVH, left ventricular hypertrophy; MABP, mean arterial blood pressure; NO, nitric oxide; Pc.Aq, Populus ciliata aqueous extract; Pc. Cr, Populus ciliata crude ethanolic extract; Pc. Dcm, Populus ciliata dimethanolic extract; PE, phenylephrine; PP, pulse pressure; SBP, systolic blood pressure.

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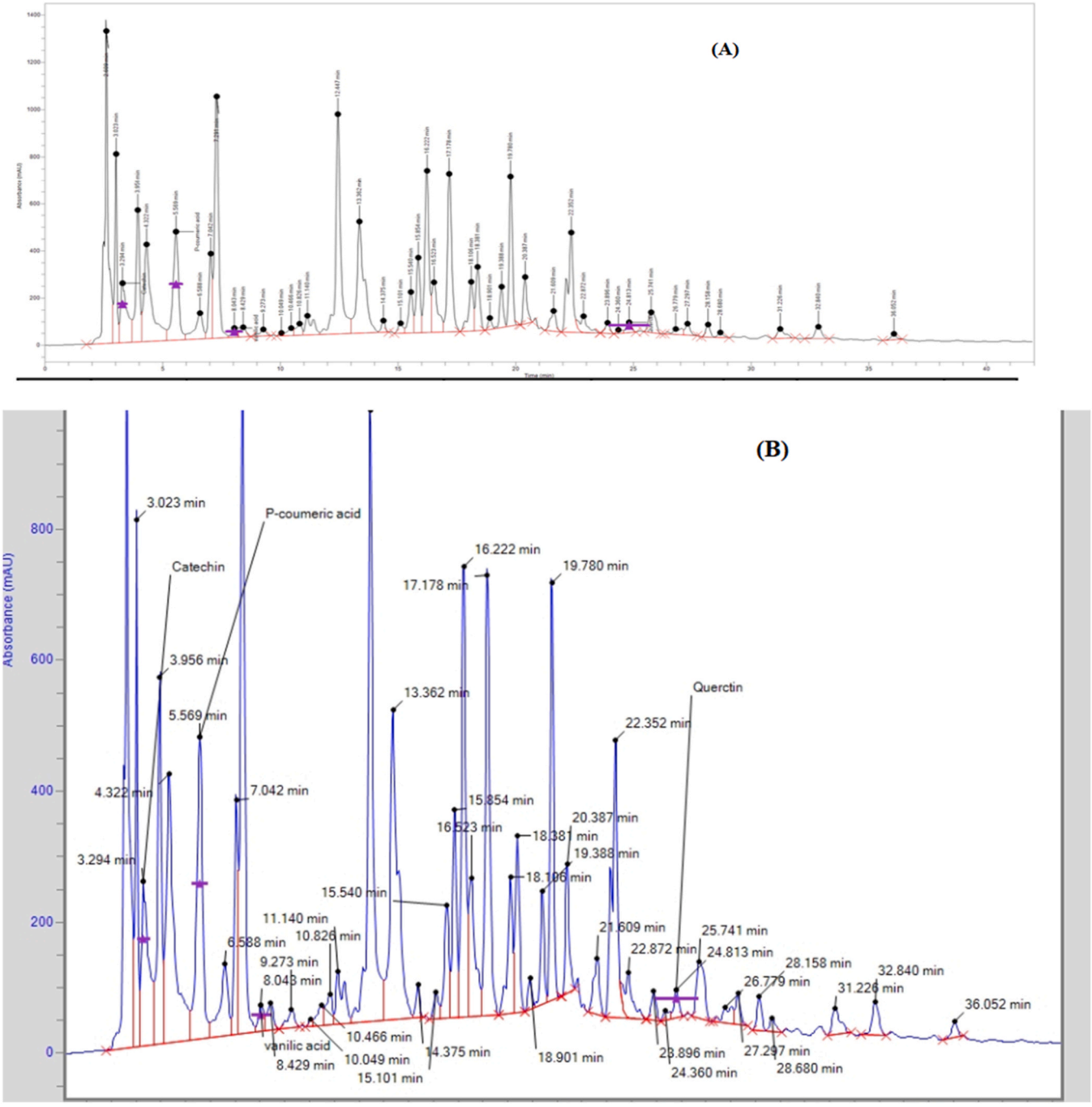

Fig. 1. (A) HPLC chromatogram of Pc.Cr (B) Standard chromatogram of HPLC.

to increased interest in traditional remedies used for the treatment of cardiovascular disorders and allied ailments [6–9]. For example leaves of *Syzygium guineense, Passiflora neplensis wall, Ginko biloba, Stephania tetrandra* and *Uncaria rhynchopylla* are used for hypertension [10,11], *Allium sativum* for atherosclerosis, *Crataegus oxyacantha* and *Crataegus monogyna* for angina and cardiac hypertrophy [12–14]. Similarly *Viola tricolor Lin.* and *Anogeissus acuminate* are used to treat acute myocardial infarction and left ventricular hypertrophy [15,16]. *Populus ciliata* is used for the treatment of various cardiovascular disorders and hypercoagulability [17,18]. *Populus tremuloides, Populus balsamifera and Populus nigra* are used against cardiac disorders [19,20].

Populus ciliata (*Salicaceae*) is native to Subcontinent and North America, Central and North Asia. It is spread out in Pakistan in Mirpur Azad Kashmir, Murree Hilly areas, Gilgit, Swat, Hazara, Tirah, Chitral and Dir. The leaves and stem are used as a tonic, stimulant and cleanser. Plant leaves possess anti-bacterial activity [21]. Bark is commonly used for the treatment of muscular swelling, temperature, pain and thinning blood. It is also used as tonic or blood purifier especially for menstrual ache and in rheumatism [22]. Despite many therapeutic advantages against cardiovascular ailments and related disorders, there is no reported study of *Populus ciliata* Wall ex. Royle as hypotensive, cardiac relaxant, vasorelaxant and cardio-protective agent. So the present study was designed to investigate the cardio-protective and hypotensive effects and toxic potential of extract of *Populus ciliata* Wall ex. Royle.

Table 1
Retention times of standards and detected compounds of Pc.Cr.

Standard used		Pc.Cr			
Compound used	Retention time (min)	Compound detected	Peak area	Retention time (min)	Peak conc. detected (μg/g)
Gallic Acid	2.806	–	–	–	–
Ferulic Acid	12.967	–	–	–	–
Butylatedhydroxytoluene	7.041	–	–	–	–
P-coumaric Acid	5.596	P-coumaric acid	7,784,528.8	5.569	2335.35
Chlorogenic Acid	5.227	–	–	–	–
Vanillic Acid	8.096	Vanillic acid	634,675.2	8.043	27.92
Caffeic Acid	7.842	–	–	–	–
Catechin	3.386	Catechin	5,017,644.7	3.294	1881.61
Sinapic Acid	12.679	–	–	–	–
Quercetin	24.893	Quercetin	598,008.8	24.813	56.81

2. Materials and methods

2.1. Plant material and preparation of crude extract

Populus ciliata Wall. ex Royle was collected in August 2020 from Mirpur Azad Kashmir, Pakistan. Plant (Voucher # TPL1.1/record/tro-28300081) was verified by Dr. Zafar Ullah Zafar, a Botanist at Bahauddin Zakariya University, Multan, Pakistan. Stem and leaves were dried under shade. The plant material was triturated by mortar and pestle. Coarse plant powder (1 kg) was macerated for 7 days in solution of aqueous ethanolic solution (70%*w*/*w*) with occasional shaking. It was filtered with muslin clothe and remaining filtrate was filtered with Whatmann-1 filter paper. Filtrate was evaporated through rotavapour (Buchi, Swiss model# 9230) and chiller at 37 °C under low pressure created by vacuum pump (V-550, Labotech). The yield of the crude extract was 32.16% w/w. The semisolid extract (Pc.Cr) obtained was stored in amber glass bottle. Pc.Cr (10 mg) was dissolved in distilled water (50 mL) for fractionation in separating funnel along with dichloromethane (50 mL) for 48 hrs. Aqueous layer was freeze dried whereas DCM layer was dried by rotary evaporator [23].

2.2. Chemicals and reagent

Sodium dihydrogen phosphate, Sodium bicarbonate, Potassium dihydrogen phosphate, Methanol, Magnesium sulphate, $CaCl_2$, NaCl, Ketamine, Ethylenediaminetetraacetic acid, KCl, Phenylephrine, Isoproterenol, Ketamine, Carbamyl choline HCl, Acetylcholine and dilutions freshly prepared at once. All above mentioned chemicals were bought from Jiangyin Lanyu chemical Co., China.

2.3. Experimental animals

Animals involved in studies were white albino rabbits of either sex (1–2 kg; 8–9 months old) and Sprague Dawley albino rats of either sex (200–250 g; 2–3 months old) about 10 rabbits and 108 rats were used in invivo and invitro activities, each group consisted of 6 randomly selected animals. They were kept in cages floored with sawdust (changed after 4 hrs.) at room temperature exposed to dark and light cycles in animal house of department of pharmacy, BZU Multan. Animals were fasted overnight with water ad libitum, treated with great care while experimentation and executed according to drafted rules of life sciences of animal resources commissioned by Laboratory of Bahauddin Zakariya University as authorization committee to ethical institution of Multan (EC/06 PhL-2019–2021). Animals were given a standard diet consisting of (g/kg): flour 380, choker 380, NaCl 5.8, molases 12, nutivet L 2.5, potassium metabisulphate 1.2, vegetable oil 38,fish meal 170 and powdered milk 150.

2.4. HPLC analysis

A binary gradient solvent system comprising of C-18 column (250 × 4.6 mm, 5 μm), run time of 36 min with flow rate of 0.8 mL per minute was used for the elucidation of Pc.Cr. chromatogram. Catechin, gallic acid, P-coumaric acid, ferulic acid, hydroxy benzoic acid, caffeic acid, querctin, BHT, sinapic acid and chlorogenic acid were used as reference samples in this reverse phase HPLC procedure [13]. The mobile phase comprised of two solvents A & B. Solvent A was acetonitrile and methanol (70:30) while solvent B was 0.5% glacial acetic acid in double distilled water. Spectra were recorded at 275 nm UV. Sample peaks were identified by comparing with the absorbance spectrum and retaining time of reference compounds.

2.5. In vitro experiments

2.5.1. Aortic tissue preparation

Rabbit and rat aorta (2–3 mm) from thoracic region was cut and immersed in Kreb's solution tissue bath (20 mL) at 37 °C having supply of carbogen. Isolated tissue was mounted for 1 h for stabilization with preloaded tension (2 g). Mounted aortic rings contraction data was recorded with power lab (Model KCYL100) connected to computer having displacement force transducer [23].

2.5.2. Paired atrium tissue preparation

Rabbit paired atrium was isolated and dipped in tissue bath (20 mL) having Krebs (pH: 7.4) solution along with carbogen at 32 C. Isolated tissue was mounted for 20 min with 1 g of basal tension. Mounted tissue contraction data was recorded with power lab connected to computer having displacement force transducer, contractions were due to pacemaker cells over atrium [24].

2.6. In vivo experiments

2.6.1. Intra-arterial blood pressure measurement (invasive method)

Anesthesia (diazepam 5 mg/kg; ketamine 80 mg/kg) was given intraperitoneally with 5 min gap to Sprague Dawley albino rats. After anesthetization, right carotid artery was cannulated to insert polyethylene tube (PE-50) for normal saline solution as well as heparin (1000 Usp/mL) 0.1 mL to avoid from clotting of blood. BP transducers were attached to measure blood pressure. When animal was stabilized, it was instilled with plant doses along with 0.1 NS through jugular vein and regression in BP was recorded and observed over computer screen [25].

2.6.2. ISO induced left ventricular hypertrophy

Rats (30) of 200–250 g were taken in 6 groups @6 rats/group. All rats were given ISO (5 mg/kg/day) for initiation of hypertrophy.

Group I: Control group (@ 0.9% normal saline).

Group II (Intoxicated group): ISO (subcutaneously; 5 mg/kg/day) for

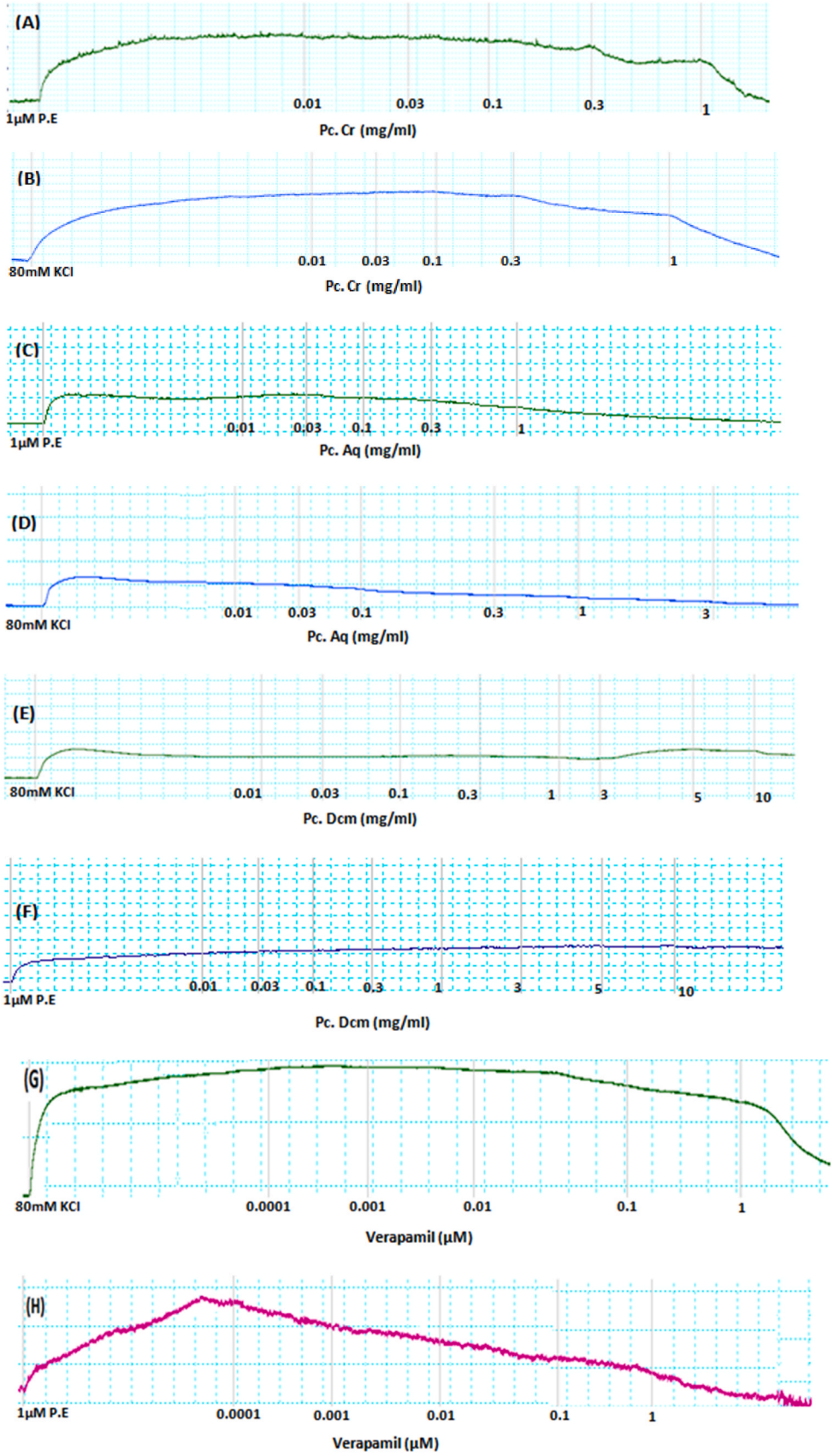

Fig. 2. Tracings representing effects of: Pc. Cr (A and B), Pc. Aq (C and D), Pc. Dcm (E and F), and verapamil (G and H) on contractions via PE (1 μM)-induced and K^+ (80 mM)-induced, respectively, on separated rat aorta (n = 5).

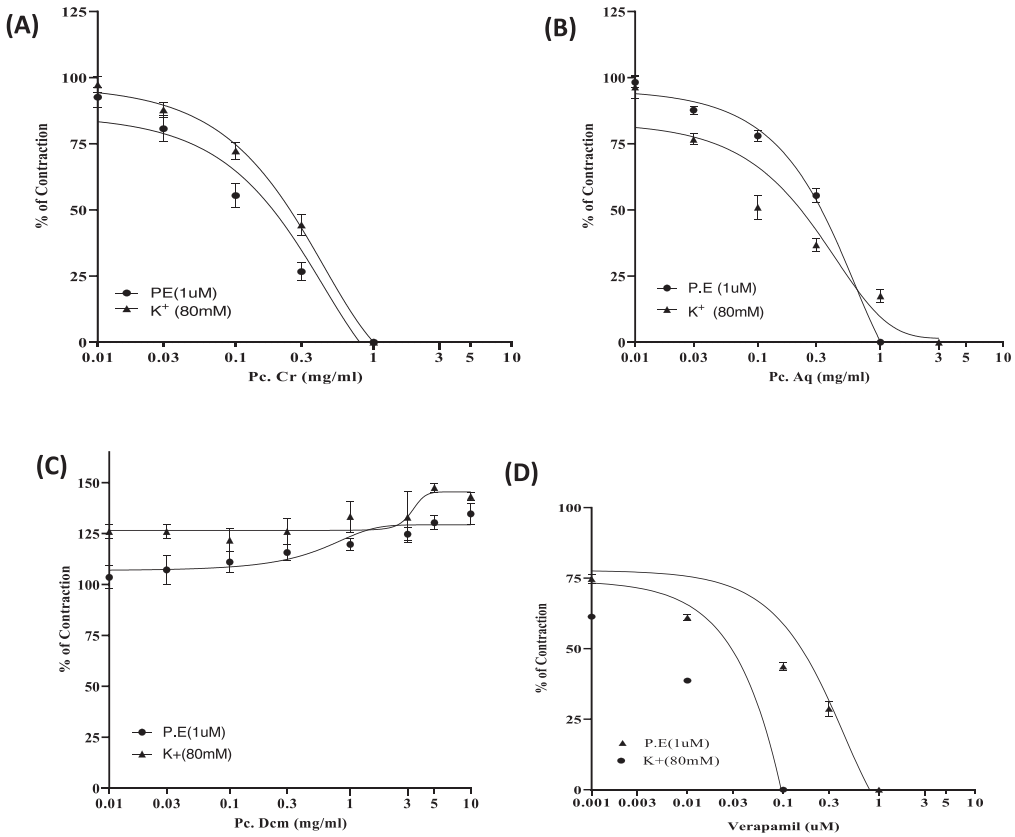

Fig. 3. Effect of: (A) Pc. Cr, (B) Pc. Aq, (C) Pc. Dcm, and (D) Verapamil on contractions induced through PE (1 μM)-induced and K^+ (80 mM)-induced respectively, on rat aorta. All values are shown as mean ± SEM, (n = 5).

10 days for inducing LVH and for 14 days normal saline was given orally.

Group III (carvedilol+ ISO): Standard drug carvedilol (orally, 10 mg/kg/day) for 14 days after cardiac injury initiation.

Group VI (100 mg/kg + ISO): Pc.Cr @14 days after buildup of hypertrophy.

Group V (200 mg/kg + ISO): Pc.Cr @14 days after LVH intermission.

Group VI (300 mg/kg + ISO): Pc.Cr @14 days after hypertrophy of heart.

Calculations of heart wt. to final body wt., tail length to heart wt. and tibial length to heart wt. ratios were measured.

After experiment, final body weights of all rats were measured. After dissection, cardiac tissue was removed and weighed by balance. Tail length and tibial length were measured. Then ratios were calculated.

2.6.2.1. Estimation of hemodynamic markers. Plasma renin and cyclic GMP (cGMP) were assayed by enzyme immunoassay kit (catalog #: A03E5546, Ray Biotech, USA), plasma nitrate/nitrite by nitrate/nitrite assay kit (catalog#: 7680201), and angiotensin (Ag II) levels by renin ELISA kit, (catalog number: 581021) manufactured by Shaigan chemicals, Pakistan respectively. All assays were done according to instructions given by manufacturer [26].

2.6.2.2. Estimation of heart to body weight, weight of heart to tail length, and weight of heart to tibia length ratio. Heart weight index was assessed by division of heart weight over body weight but for tail and tibia indexes, length of these parts was divided over heart weight [27].

2.6.2.3. Histopathological Examination. After collection of blood for biochemical analysis, heart was isolated and immersed in 10% solution of formalin and transferred into paraffin blocks, 5 μm thick tissue was stained with hematoxylin and eosin (H&E) dye. An experienced histopathologist read all the slides under compound microscope.

2.6.3. ISO Induced Myocardial Infarction

Sprague Dawley albino rats were exposed to ISO (85 mg/kg) at last two days of 14 days study after interval of 24 h. Myocardial infarction was identified by serum level of CK-MB which was elevated in infracted tissues. Biochemical analysis of blood collected from rat sinocular cut was performed. The assay kit used was electrochemiluminescence immunoassay kit by Highnoon, Germany. Rats were divided into 6 groups@ 6 rats/group.

Group I: Control group (@0.9% normal saline).

Group II: ISO treatment group (subcutaneously; 85 mg/kg) continuously for just 2 days of 24-h interval at 13th and 14th day.

Group III: Standard group taking carvedilol (10 mg/kg) for 14 days orally and ISO (85 mg/kg) was administering on 13th and 14th day.

Group IV: Pc.Cr (100 mg/kg) for 14 days and on 13th and 14th day ISO (85 mg/kg) was also introduced.

Group V: Pc.Cr (200 mg/kg) for 14 days and on last 2 days ISO (85 mg/kg) was given.

Group VI: Pc.Cr (300 mg/kg) for 14 days and at 13th and 14th day ISO (85 mg/kg) was also used.

All rats were anaesthetized with chloroform and sample of blood was taken in tubes from vein of eye and tail of rats for biochemical test and cervical beheading was done to kill all rats. Heart organs were removed, weighed, soaked in cold saline for cleaning and preserved all organ in 10% formalin and then histopathological lab findings was done [28].

2.6.3.1. Cardiac heart markers quantification. Cardiac serum markers mainly such as CK, CK-MB and LDH were assessed by immunoassay kit (Highnoon, Germany).

2.6.3.2. Histopathological Examination. After collection of blood for biochemical analysis, heart was isolated and immersed in 10% solution of formalin and transferred into paraffin blocks then 5 μm thick tissue

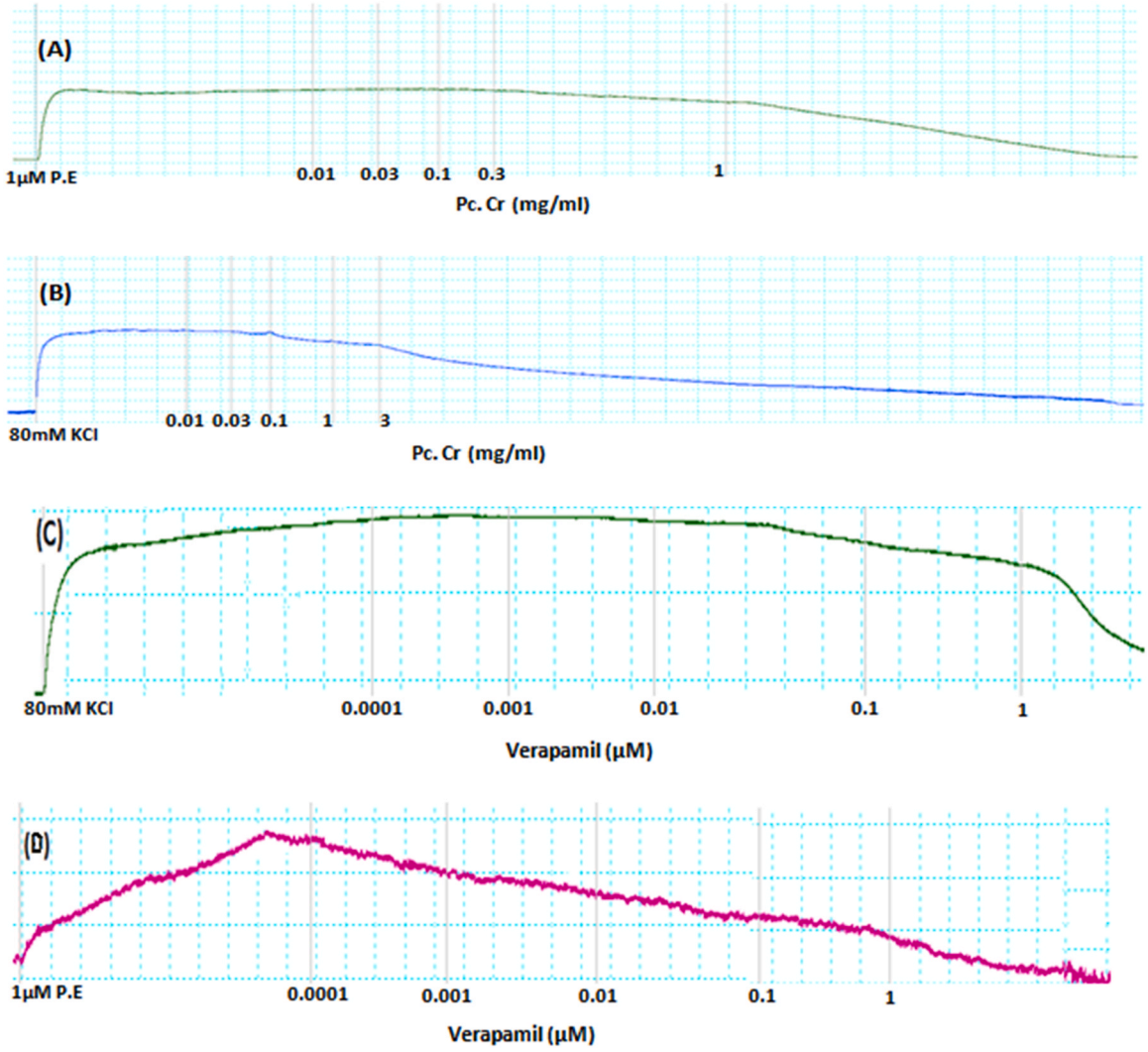

Fig. 4. Tracings representing effects of: Pc. Cr (A and B) and Verapamil (C and D) on contractions via PE (1 μM)-induced and K^+ (80 mM)-induced, respectively, on separated rabbit aora (n = 5).

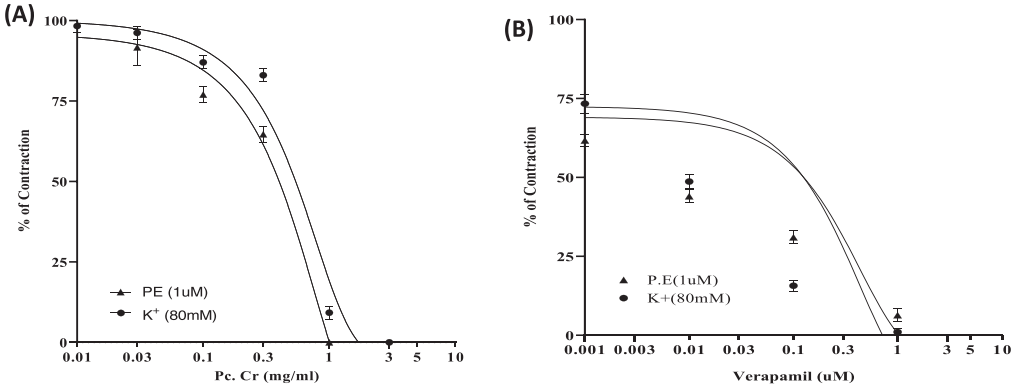

Fig. 5. Effect of: (A) Pc. Cr and (B) Standard drug (verapamil) on contractions induced through PE (1 μM)-induced and K^+ (80 mM)-induced on isolated rabbit aorta, respectively. All values are shown as mean ± SEM, (n = 5).

was stained with hematoxylin and eosin (H&E) dye. An experienced histopathologist read all the slides under compound microscope [29].

2.7. Toxicity study

2.7.1. Determination of acute toxicity

The toxic profile of Pc. Cr was tested in Sprague Dawley albino rats (150–200 g) of either sex under maintained conditions. The single

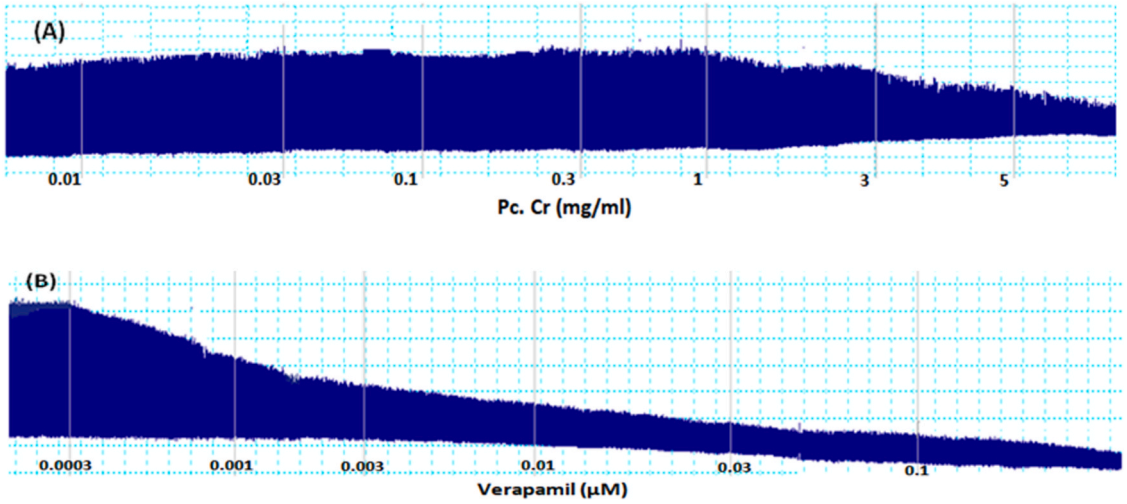

Fig. 6. Tracings showing of the effects of (A) Pc. Cr and (B) verapamil on isolated rabbit paired atrial preparation.

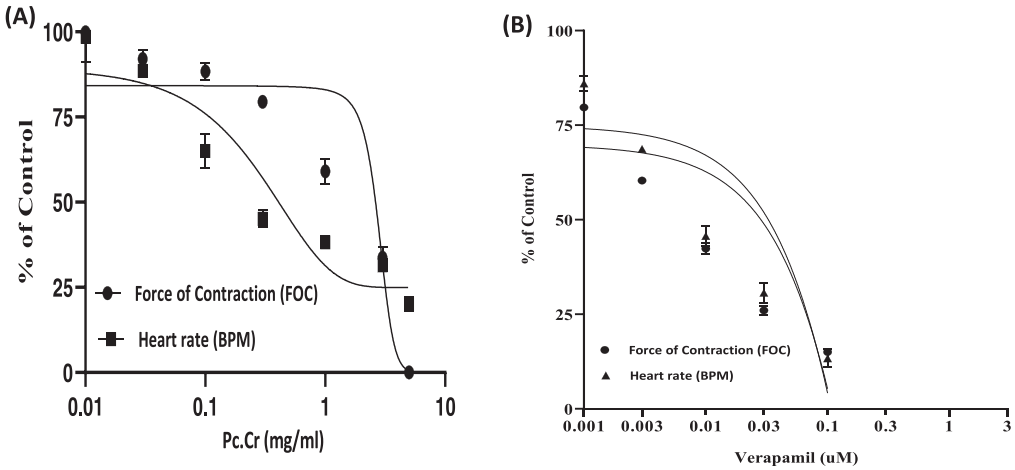

Fig. 7. Effects of (A) Pc. Cr and (B) verapamil on isolated rabbit paired atrial preparations. All values are shown as mean ± SEM, (n = 5).

intraperitonial dose of 1500 mg/kg and 3000 mg/kg was given to rats and observed for 4 h for any mortality or change in behavior so study was conducted for 14 days and every week their weight was observed. On last day beheading was done to take organs for histopathology of organs [30].

2.7.2. Determination of chronic toxicity

Sprague Dawley albino rats were divided into 2groups@6rats/ groups, acclimatized for 7 days according to lab environment and baseline values were taken. Blood samples of normal group were taken to establish normal biochemical data. Rest of the groups was treated with Pc.Cr @400 mg/kg/day and 800 mg/kg/day for 28 days. They were observed for their behavioral and physical changes every day but weights and food intake was observed weekly. At the end day of study animals were killed to get blood samples and organs were also dissected for histopathological data [11].

2.8. Statistical analysis

The results of in vitro cardio relaxant and vasorelaxant activities were demonstrated as mean ± SEM. Effective concentration EC50 with 95% confidence interval were assessed with the help of Graph Pad Prism version 8.0 software program (Graph Pad Software, San Diego, CA, USA). Dose response curves were assessed by non-linear regression sigmoidal response curve (variable slope). Likewise invivo (invasive method) results; statistical significance was assessed by one-way and two-way ANOVA. $^{*}p < 0.01$, $^{**}p < 0.001$, $^{***}p = 0.0001$, $^{****}p < 0.0001$ was observed to be statistically significant.

3. Results

3.1. HPLC studies

Analysis of Pc.Cr through HPLC confirmed that the catechin, P-coumeric acid, vanillic acid and quercitin were present (Fig. 1A, B and Table 1).

3.2. Results of in vitro studies

3.2.1. Effect on isolated rat aorta

The Pc. Cr relaxed the contractions induced through PE (1 μM)-induced and K^+ (80 mM)-induced @1 mg/mL, having EC_{50} = 0.17 mg/mL (95% CI: 0.12–0.22 mg/mL; n = 5) & EC_{50} = 0.49 mg/mL (95% Cl: 0.37–0.68 mg/mL; n = 5) respectively. Pc. Aq relaxed effect @1 mg/mL & 3 mg/mL, having EC_{50} = 1.2 mg/mL (95% CI: 0.78–2.143 mg/mL; n = 5) & EC_{50} = 0.11 mg/mL (95% Cl: 0.07–0.20 mg/mL; n = 5) respectively. Verapamil relaxed the contractions induced through PE (1 μM)-induced and K^+ (80 mM)-induced @1 μM and 0.1 μM, having

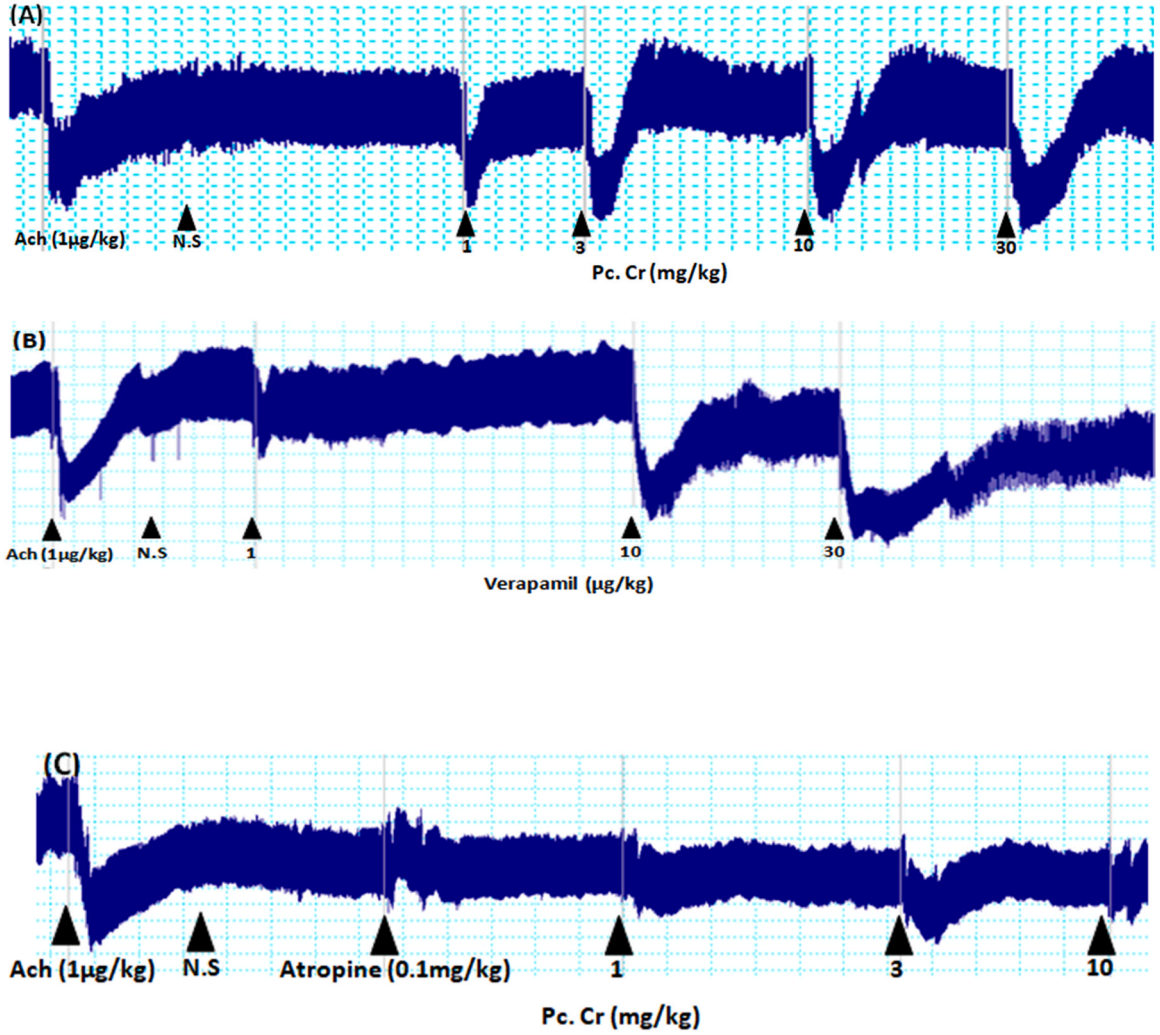

Fig. 8. Tracings showing effect of blood pressure lowering effect of (A) Pc. Cr compared with the effect of (B) verapamil (C) after atropine @0.1 mg/kg in anesthetized rats.

EC_{50} = 0.12 mg/mL (95% CI: 0.02–0.35 mg/mL; n = 5) & EC_{50} = 0.005 mg/mL (95% Cl: 0.001–0.014 mg/mL; n = 5) respectively (Figs. 2 and 3).

3.2.2. Effect on isolated rabbit aortic preparation

Pc. Cr relaxed contractions induced by PE (1 µM)-induced and K^+ (80 mM)-induced @1 mg/mL, having EC_{50} = 1.53 mg/mL (95% CI: 0.54–3.81 mg/mL; n = 5) & EC_{50} = 1.75 mg/mL (95% Cl: 0.87–3.01 mg/mL; n = 5) respectively. Pc. Cr induced contractions on isolated rabbit aorta, blocked by doxazosin (1 µM). Verapamil produced similar response on both induced contractions @1 µM and 0.1 µM, having EC_{50} = 0.11 µM (95% CI: 0.03–0.18 µM; n = 5) & EC_{50} = 0.03 µM (95% Cl: 0.01–0.16 µM; n = 5) respectively (Figs. 4 and 5).

3.2.3. Effect on isolated rabbit paired atria preparations

Pc. Cr decreased FOC (force of contractions) and BPM (beats per minute) of isolated rabbit paired atria @10 mg/mL, having EC_{50} = 4.93 mg/mL (95% CI: 2.27–17.92 mg/mL; n = 5) & EC_{50} = 0.10 mg/mL (95% Cl: 0.07–0.15 mg/mL; n = 5) respectively. Verapamil decreased the FOC and BPM of isolated rabbit paired @0.1 µM, having EC_{50} = 0.02 µM (95% CI: 0.01–0.04 µM; n = 5) (Figs. 6 and 7).

3.2.4. Effect on BP measurement through invasive technique

In anesthetized rats, Pc. Cr @1,3,10 and 30 mg/kg, exhibited hypotensive effect and decreased the blood pressure of 28 ± 4, 29 ± 5, 32 ± 3 and 47 ± 2 mmHg in MSBP (n = 3). Similarly verapamil (µg/kg) reduced blood pressure in range of 16 ± 5, 37 ± 6 and 55 ± 4 mmHg in MSBP (n = 3). Pc. Cr reduced the BP in a dose dependent way just like verapamil. An anticholinergic drug atropine administered to the rat and assessed the blood pressure of Pc. Cr in the presence of antagonist (Figs. 8 and 9).

3.3. Results of toxicity studies

3.3.1. Acute toxicity study

3.3.1.1. Changes in behavior, body weight and organ due to PC.CR. Effect of Pc. Cr @1500 mg/kg and 3000 mg/kg on rats was normal. There were no toxicity, no mortality and behavior was mostly normal. It means LD_{50} was more than 3000 mg/kg. The animal weight and weight of separating organs (liver & kidney) were slightly more than that of normal group (Tables 2 and 3).

3.3.1.2. Histopathological results. The slides of treated groups were almost similar to normal groups. No change of color, texture, shape and morphological alterations occurred. The results are similar to normal groups (Fig. 10).

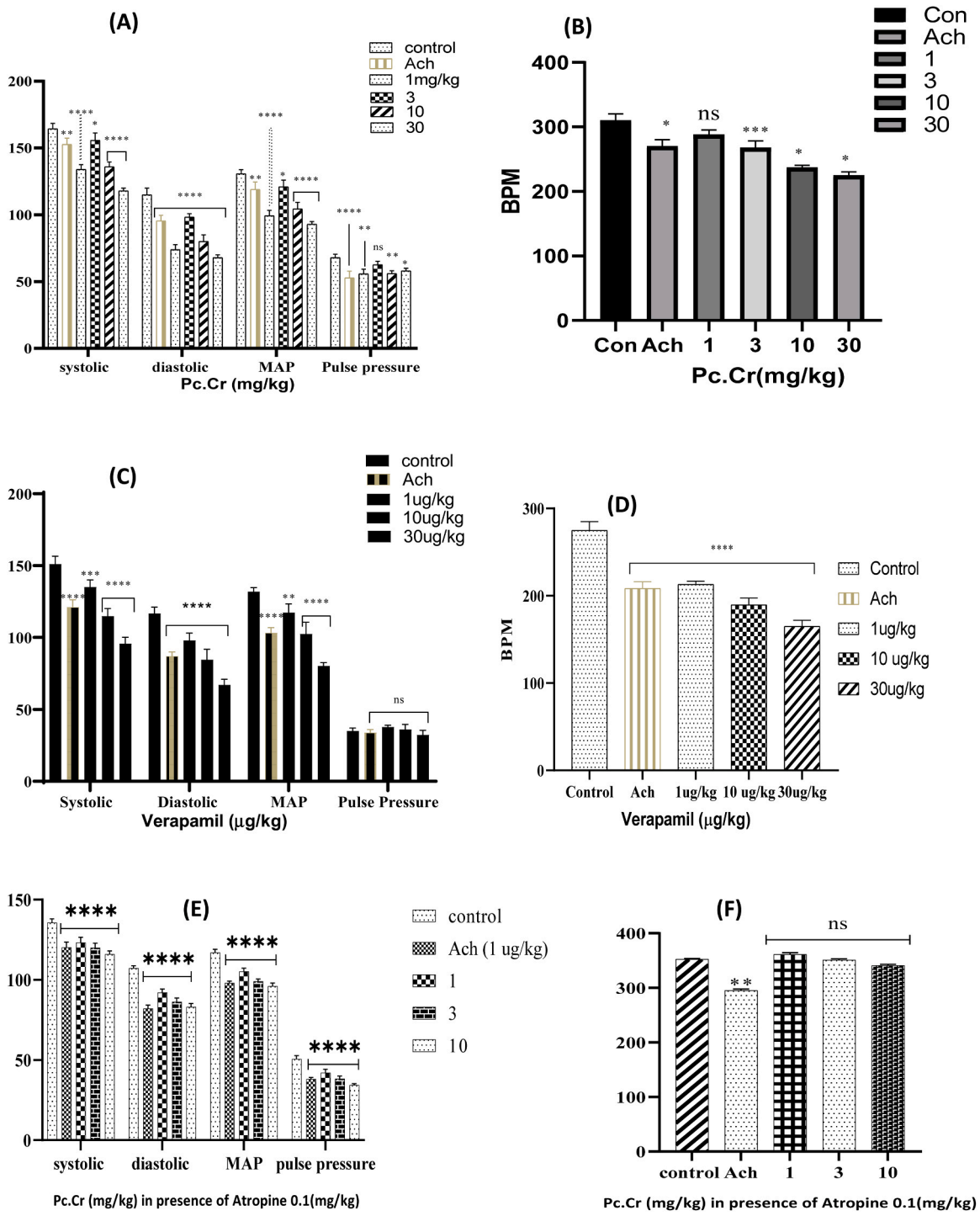

Fig. 9. The effect of (A) Pc. Cr, (D) Verapamil and (E) Pc. Cr in the presence of atropine on MAP, SBP, DBP and PP by using Two-way ANOVA. The effect of (B) Pc. Cr, (C) Verapamil (F) and Pc. Cr in the presence of atropine on BPM (Heart rate), applying One-way ANOVA (****P < 0.0001, ***P = 0.0001, **P = 0.001 and ns = not significant data).

Table 2
Effect of Pc.Cr on weight of the organ (rats).

Observational Parameters	Doses (mg/kg)		
	Control	1500	3000
Final body weight(g)	260 ± 5	250 ± 8	245 ± 10
Kidney (g)	1.1 ± 0.03	0.97 ± 0.02	0.95 ± 0.01
Liver (g)	10.2 ± 1	9.8 ± 0.5	9.9 ± 0.8

3.3.2. Results of chronic toxicity study

3.3.2.1. Changes in behavior, body weight and organ due to Pc. Cr. The effects of 400 mg/kg and 800 mg/kg dose of Pc. Cr on rats were normal. As in acute study, there was no toxicity, no mortality and almost seen with normal behavior. The animal weight and weight of separating organs were almost close to normal control group (Table 4).

3.3.2.2. Effect of Pc. Cr on hematological and biochemical markers. The

Table 3
Effect of Pc.Cr on behavior of rats.

Observations	Normal	Pc.Cr (mg/kg)	
		1500	3000
Vigilant	☑	☑	☑
Touch response	☑	☑	☑
Motion response	☑	☑	☑
Sounds reaction	☑	☑	☑
Food consumption	☑	☑	☑
Clutch response	☑	☑	☑
Loose motions	☒	☒	☒
Over activity	☒	☒	☒
Trembling	☒	☒	☒
Light response	☑	☑	☑
Mortality	☒	☒	☒
Fits	☒	☒	☒
Change in skin color	☒	☒	☒
Inertia /slowness	☒	☒	☒
Water intake	☑	☑	☑
Saliva secretion	☑	☑	☑

☒ = Absent, ☑ = Normal

hematological markers of treated groups were seen within reference range and close to normal group (Table 5). The biochemical markers of treated groups were seen within reference range and close to normal group. Values of LDH and triglycerides were slightly increased (Table 6).

3.3.2.3. Histopathological results. The slides of treated groups were almost similar to normal groups. No change of color, texture, shape and morphological alterations occur. The results are similar to normal

Table 4
Effect of Pc. Cr on weight of rat organs.

Observation	Control	Pc. Cr mg/kg	
		400	800
Final Body Weight(g)	260 ± 5	240 ± 8	245 ± 5
Liver (g)	10.8 ± 1	9.5 ± 0.8	9.8 ± 0.5
Spleen (g)	0.61 ± 0.2	0.54 ± 0.03	0.56 ± 0.03
Kidney (g)	1.1 ± 0.03	0.97 ± 0.01	0.98 ± 0.02
Paired Lungs (g)	2.8 ± 0.1	2.6 ± 0.2	2.7 ± 0.1
Heart (g)	0.81 ± 0.02	0.76 ± 0.05	0.78 ± 0.02

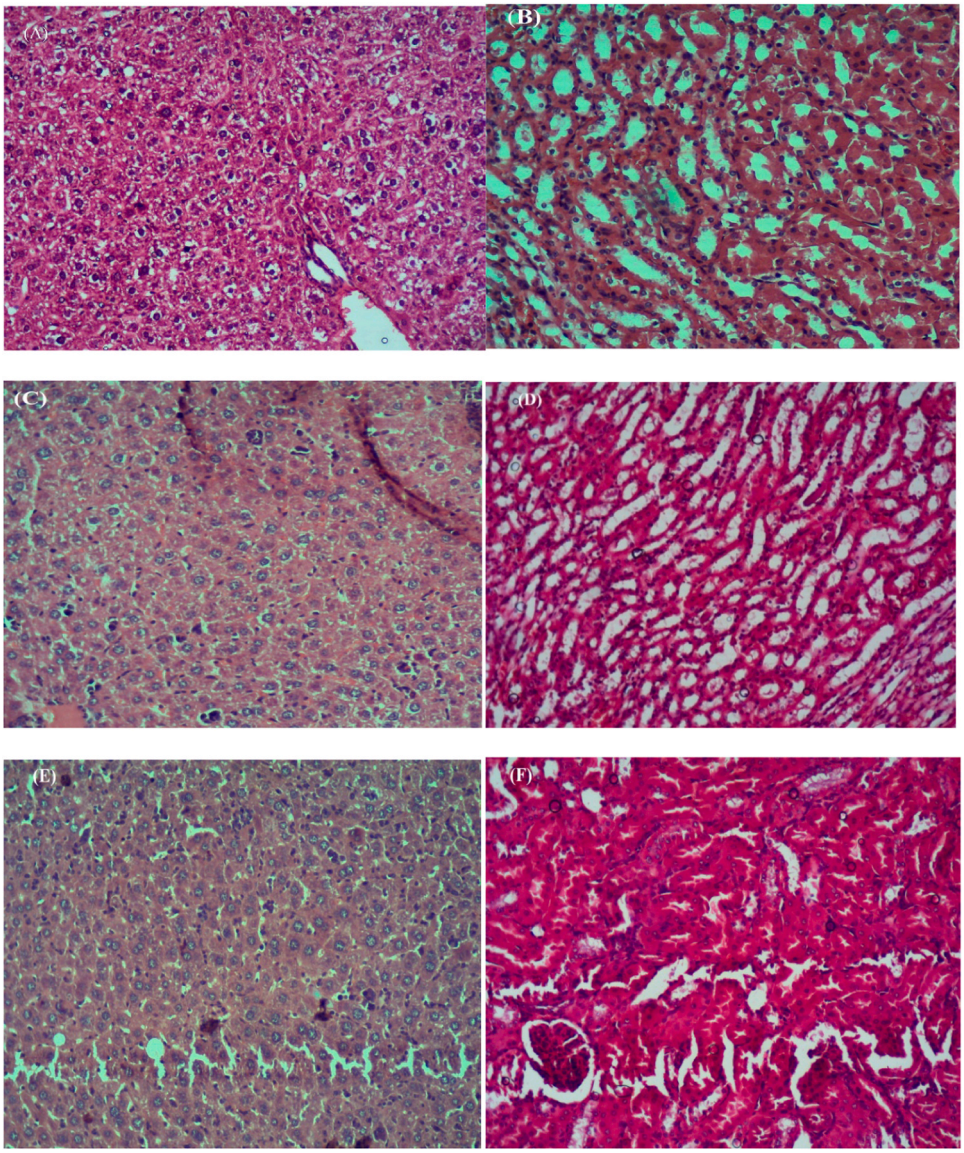

Fig. 10. Histological analysis of liver and kidney tissues in rats during acute toxicity study as observed by hematoxylin-eosin staining (10 ×). No change of color, texture, shape and morphological alterations occur due to Pc. Cr (A): Normal liver; (B): Normal kidney; (C): Pc. Cr (1500 mg/kg) liver; (D): Pc. Cr (1500 mg/kg) kidney; (E): Pc. Cr (3000 mg/kg) liver; (F): Pc. Cr (3000 mg/kg) kidney.

Table 5
Hematological values obtained from rats treated with Pc. Cr.

Hematological Parameters	Normal	Pc. Cr mg/kg	
		400	800
WBC's (10^5/μL)	2.5 ± 0.1	2.9 ± 0.03	2.7 ± 0.02
Neutrophils (%)	40 ± 15	47 ± 7	58 ± 2
Lymphocytes (%)	45 ± 18	65.3 ± 5	70.4 ± 6
Eosinophills (%)	1 ± 0.5	0.9 ± 0.03	0.8 ± 0.01
RBC's (10^6/μL)	8.6 ± 2	8.9 ± 0.7	9.1 ± 0.3
Hemoglobin (g/dl)	13 ± 2	14 ± 1	15 ± 1
Hematocrite (%)	45 ± 1	47.3 ± 3	44.7 ± 4
MCV (f/L)	55.8 ± 10	54.1 ± 5	51.6 ± 5
MCH (pg)	17.7 ± 2	17.1 ± 1	16.8 ± 1
MCHC (%)	31.6 ± 3	29 ± 2	31 ± 2
Platelets (10^5/μL)	5.8 ± 1	6.1 ± 0.2	5.9 ± 0.3

Table 6
Biological serum values obtained from rats treated with Pc. Cr.

Parameters of Biological Serum	Control	Pc.Cr mg/kg	
		400	800
Albumin (g/dl)	1.8 ± 0.5	2.4 ± 0.1	2.38 ± 0.3
T. Protein (g/dl)	3.8 ± 2	3.9 ± 0.4	4.7 ± 0.3
Albumin/Globulin	2.1 ± 0.01	2.39 ± 0.02	2.5 ± 0.03
Alanine Transaminase (U/L)	25.5 ± 8	38.9 ± 5	30.8 ± 4
Lactate Dehydrogenase (U/L)	1546 ± 350	2249 ± 200	2415 ± 235
Asparate Transaminase (U/L)	98.2 ± 12	127.3 ± 16	112.3 ± 13
Total Cholesterol(mg/dl)	57 ± 22	71 ± 12	79 ± 5
T. bilirubin (mg/dl)	0.19 ± 0.02	0.4 ± 0.01	0.2 ± 0.01
Uric Acid (mg/dl)	0.7 ± 0.03	0.8 ± 0.01	0.9 ± 0.01
Creatinine (mg/dl)	1.5 ± 0.1	1.8 ± 0.5	1.0 ± 0.39
Alkaline Phosphatase(U/L)	280 ± 60	354 ± 20	398 ± 18
Sodium (mmol/L)	107 ± 20	155 ± 10	179 ± 12
Triglycerides (mg/dl)	121 ± 50	147 ± 15	161 ± 12
Potassium (mmol/L)	2.8 ± 0.1	3.9 ± 0.3	5.5 ± 0.5

groups (Fig. 11).

3.4. Results of AMI Study

3.4.1. Pc. Cr effect on hemodynamic markers

The cardiac markers were increased in ISO treated group as compared to normal. Pc. Cr treated group decreased the amount of cardiac markers as compare to ISO treated group. Other hemodynamic markers were seen in normal range as compare to intoxicated group. Remarkable progress seen in the standard groups as its decrease the parameters as contrast to intoxicated group (Table 7).

3.4.2. Histopathological results

Myocardial fibrosis along with the increase number of cardiac cells with enlarged inflammatory and necrotic cells were seen in intoxicated group as compare to normal. Morphological alterations were seen as compare to normal controlled group. All the treated groups of Pc crude were normal in texture, morphology. Necrotic and inflammatory cells were less in numbers. Standard groups showed remarkable progress as contrast to group treated with ISO (Fig. 12).

3.5. Results of LVH Study

3.5.1. Effect of Pc. Cr on tail, heart and tibia indexes

The heart index, tibia index and tail index ratio increased in a group treated with ISO as compared to normal. Groups treated with Pc. Cr showed reduced tail, heart and tibia index. Standard groups also reduced the above indexes as compared to ISO treated group (Table 8).

3.5.2. Effect of Pc. Cr on hematological markers

Group treated with ISO, increased the plasma concentration of renin and angiotensin II as compared to normal, and while Pc. Cr treated groups reduced these markers significantly. Nitric oxide and cGMP level decreased in groups treated with ISO, while these markers increased in Pc. Cr treated groups. Groups treated with standard drugs exhibited great progress by decreasing the renin and angiotensin II and increasing NO and cGMP in plasma as compared to ISO group (Table 10).

3.5.3. Histopathological results

Myocardial fibrosis along with the increased number of cardiac cells with enlarged inflammatory and necrotic cells were seen in intoxicated group as compared to normal. Morphological alterations were seen as compared to normal control group. All treated groups of Pc. Cr were normal in texture and morphology. Necrotic and inflammatory cells were less in numbers. Standard groups showed remarkable progress as compared to groups treated with ISO. Pc. Cr increased cell count along with decreased surface area and cell diameter as contrast to intoxicated group. Standard groups showed great progress as contrast to group treated with ISO (Fig. 13 and Table 9).

4. Discussion

Currently many plants are used for the treatment of cardiovascular diseases like AMI, LVH and hypertension [31]. Present study was designed to evaluate the toxicity profile, cardioprotective and hypotensive effect of Pc. Cr. HPLC confirmed the presence of four major compounds in Pc. Cr, i.e. P-coumeric acid, vanillic acid, catechin and quercetin. These compounds play a significant role in the treatment of hypertension, AMI and LVH. Catechin possess significant effects in cardiac ischemic diseases especially which occur due to dysfunction of endothelial cells. Catechin helps body against hypertension and other cardiovascular events [32]. P-coumeric acid prevents cardiovascular events such as hypertension, LVH and infarctions [29–33]. Vanillic acid (VA) has therapeutic potential to treat hypertension [34,35]. Quercetin plays a vital role in acute myocardial infarction. It acts positively to control hypertension [36] and used as cardioprotective agent especially in AMI [37] and LVH [38].

Pc. Cr showed negative ionotropic and chronotropic effect in a dose dependent way on isolated rabbit paired atria. Decreased availability of cytosolic Ca^{++} leads to ionotropic and chronotropic effects [24]. Verapamil reduced the force of contraction and heart rate just like Pc. Cr. So Pc. Cr possibly acts as voltage dependent L-type CCB [20]. The Pc. Cr showed vasorelaxant effect on isolated rabbit aorta (non-intact endothelium) as it relaxed the contractions stimulated through PE (1 μM)-induced and K^+ (80 mM)-induced. As Ca^{++} quantity toward or within the cell increases, the cell membrane depolarizes [39]. Calcium channel blocker inhibits the effect of K^+ (80 mM)-induced through voltage dependent L-type CC and considered as vasorelaxant. PE (1 μM)-induced contractions are because of the activation of α-adrenergic receptors which enhanced the influx of calcium and control Ca^{++} channels [40]. Pc. Cr relaxed K^+ (80 mM)-induced and P.E (1 μM)-induced contractions similar to verapamil possibly indicating CCB effect on rabbit aorta. Pc. Cr showed contractions on isolated rabbit aorta baseline which blocked with the help of adrenergic antagonist doxazosin (1 μM). There is a possibility that Pc.Cr has some effect on adrenergic receptors because it relaxed contractions induced due to phenylephrine [20]. The contractions which was induce by PE (1 μM)-induced and K^+ (80 mM)-induced on isolated rat aorta (intact endothelium) relaxed by Pc. Cr, Pc. Aq and Pc. Dcm. The possible mechanism of this relaxation is due to the activation of endothelial derived relaxing factor (EDRF) which is a vasoactive agent secreted from endothelial cells. In smooth muscles it stimulates soluble guanylate cyclase which increase cGMP level and cause vasorelaxation [41]. This EDRF is also known as nitric oxide (NO), synthesized by enzyme nitric oxide synthase (NOS). So it is considered that Pc. Cr and its fractions either by mediating EDRF or by CCB effect relaxed the rat isolated aorta [42]. The antihypertensive effect of Pc. Cr was assessed on anesthetized

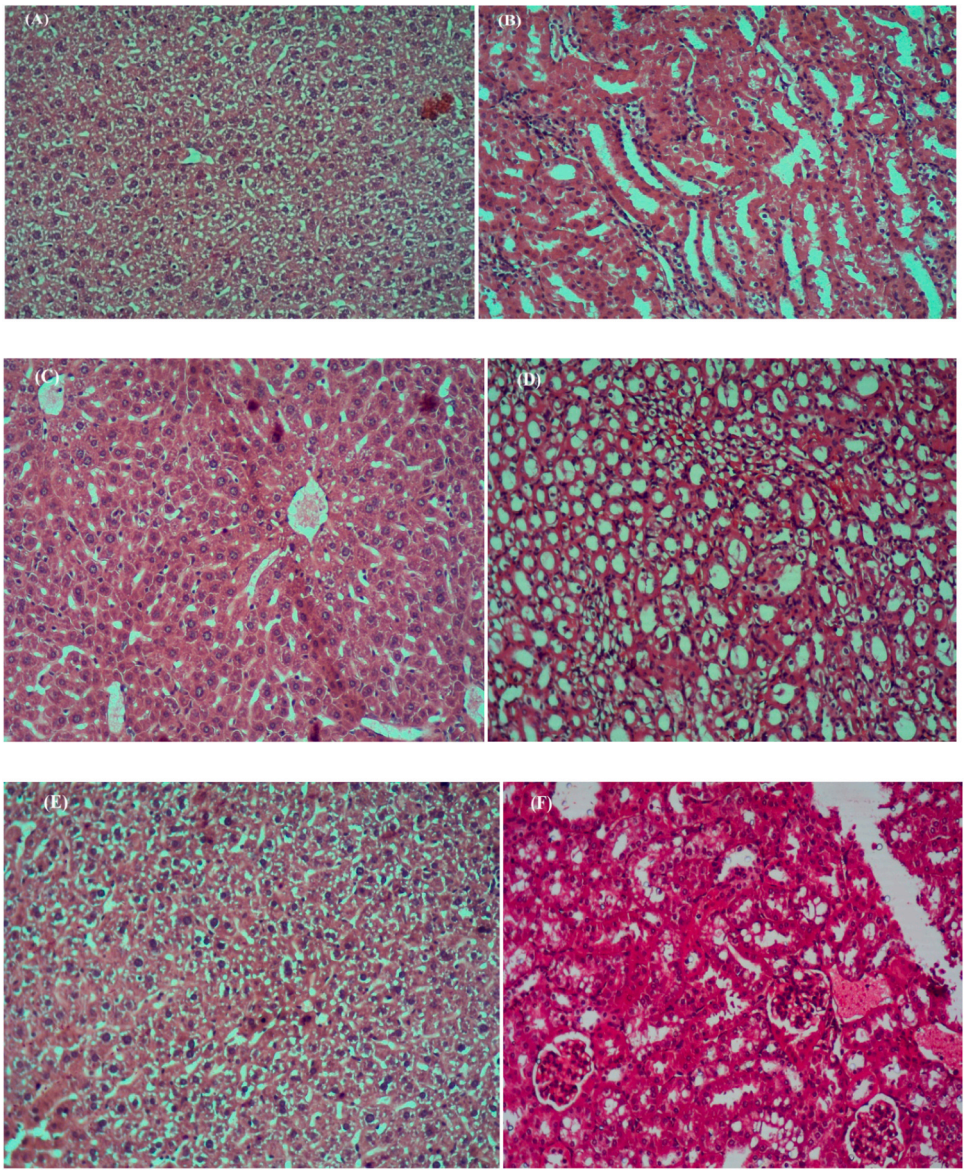

Fig. 11. Histological analysis of liver and kidney tissues in rats during chronic toxicity study as observed by hematoxylin-eosin staining (10 ×). No change of color, texture, shape and morphological alterations occur due to Pc.Cr. (A): Normal liver; (B): Normal kidney; (C): Pc.Cr (400 mg/kg) liver; (D): Pc.Cr (400 mg/kg) kidney; (E): Pc.Cr (800 mg/kg) liver; (F): Pc.Cr (800 mg/kg) kidney.

Table 7
Effect of Pc. Cr on biological markers in ISO-induced AMI rats.

Biological Markers	Groups						
	Control	Intoxicate	Dose 100	Dose 200	Dose 300	Carvedilol	Verapamil
CPK (U/L)	125 ± 12	398.2 ± 22	289.1 ± 4	229.3 ± 3	160.1 ± 2	141.2 ± 4	132.2 ± 2
Ag II Conc. (pg/mL)	92.7 ± 6	248.4 ± 2	154.3 ± 2	122.3 ± 2	110 ± 2	102.9 ± 1	99.1 ± 2
Renin Conc.(pg/mL)	24.9 ± 2	310.5 ± 20	220 ± 3	135.3 ± 5	77.2 ± 1	42.3 ± 5	31.3 ± 2
cGMP (pmol/mL)	15.5 ± 5	8.2 ± 1	9.8 ± 0.5	11.3 ± 1	12.8 ± 2	16.2 ± 1	16.9 ± 1
NO (μmol/l)	47.2 ± 5	22.7 ± 3	28.1 ± 0.3	34 ± 0.1	38 ± 0.5	41.2 ± 1	43.1 ± 2
ANF (pg/mL)	249 ± 10	189.2 ± 40	218.8 ± 5	224.2 ± 1	239.3 ± 1	243.6 ± 5	244 ± 2
BNF (pg/mL)	11.2 ± 12	2.2 ± 2	4.5 ± 0.3	6.4 ± 0.3	9.3 ± 0.5	14.1 ± 1	15.1 ± 1
ACE (μg/mL)	40.6 ± 4	148.6 ± 8	73.1 ± 0.5	62 ± 0.2	51.2 ± 0.3	45.3 ± 0.3	4.3 ± 0.1
CKMB (U/L)	75.4 ± 7	472.7 ± 30	323.2 ± 8	231.1 ± 5	120.3 ± 3	89.3 ± 3	83.1 ± 1
LDH (U/L)	82.3 ± 8	369.2 ± 33	231.3 ± 7	196.5 ± 2	135.3 ± 5	100.4 ± 2	94.2 ± 2
AST (U/L)	127 ± 14	466.3 ± 48	221.3 ± 3	186.1 ± 1	171.2 ± 3	154.6 ± 3	143.3 ± 2
ALT (U/L)	38.3 ± 4	167.3 ± 1	105.3 ± 1	93.1 ± 0.3	59 ± 0.5	47.1 ± 1	44.1 ± 1

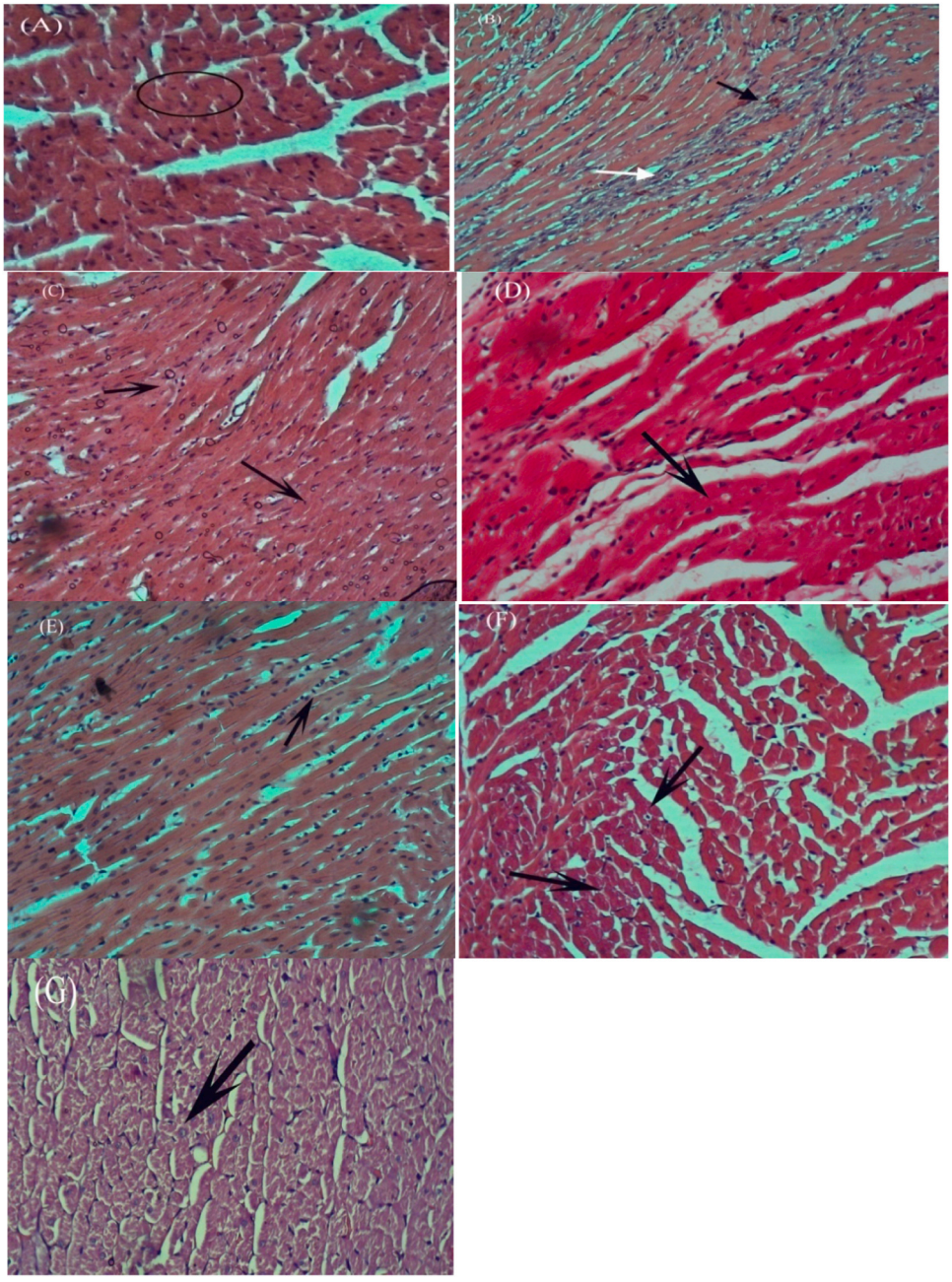

Fig. 12. Histological analysis of heart tissue in rats during ISO induced acute myocardial infarction (AMI) study as observed by hematoxylin-eosin staining (10×). The effect of Pc. Cr (A) control (B) intoxicated ISO (C) 100 mg/kg (D) 200 mg/kg and (E) 300 mg/kg (F and G) carvedilol and verapamil in rats. Oval circle represents normal cells. The single filled arrow black in color show edema while white show necrosis and notched arrow showed normal cluster of cardiac cells.

Table 8
Effect of Pc. Cr and standards on (a) heart weight index (b) tail length index and (c) tibia length index in ISO induced LVH in rats.

Groups	Animal Final Weight (g)	Tail Length Index (g/cm)	Surface Area (mm)	Tibia length Index (g/cm)	Heart Weight Index (g/cm)
Control	188 ± 12	0.044 ± 0.02	0.73 ± 05	0.17 ± 0.01	0.002 ± 0.03
Intoxicated	240 ± 20	0.05 ± 0.01	2.5 ± 0.3	0.27 ± 0.03	0.004 ± 0.5
Dose 100	181 ± 14	0.04 ± 0.7	1.81 ± 0.01	0.19 ± 0.1	0.003 ± 0.4
Dose 200	195 ± 20	0.04 ± 0.1	1.63 ± 0.02	0.21 ± 0.01	0.003 ± 0.1
Dose 300	210 ± 14	0.04 ± 0.3	1.20 ± 0.03	0.20 ± 0.01	0.003 ± 0.3
Verapamil	210 ± 15	0.04 ± 0.1	1.10 ± 0.01	0.19 ± 0.3	0.003 ± 0.5
Carvedilol	190 ± 18	0.04 ± 0.3	1.09 ± 0.03	0.19 ± 0.02	0.003 ± 0.1

Table 9
Effect of Pc. Cr and standards on (A): cell diameter; (B): cell count and (C): cross sectional surface area of the heart tissues observed by hematoxylin and eosin (H and E) stain in ISO-induced LVH.

# of Groups	Cell Count n = 6	Diameter of Cell (μm) n = 6	Surface Area of Cell (μm) n = 6
Control	1295 ± 105	7.2 ± 3	98.6 ± 5
Intoxicated	700 ± 90	25 ± 1	201.2 ± 3
Dose 100	905 ± 18	13.2 ± 1	166.1 ± 2
Dose 200	945 ± 10	11.2 ± 2	149.1 ± 5
Dose 300	972 ± 14	10 ± 1	141.3 ± 3
Verapamil	1001 ± 8	7.8 ± 0.1	118 ± 7
Carvedilol	1021 ± 6	7.5 ± 0.2	110 ± 4

rat through invasive method. Pc. Cr showed hypotensive effect in a dose dependent way @1, 3, 5 and 10 mg/kg likewise standard drug verpamil. Parameters of BP such as pulse pressure (PP), systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial blood pressure (MABP) were assessed and compared with verapamil [43]. Atropine which is a muscarinic antagonist given to the animal. After that Pc. Cr, showed less effect as compared to without atropine. It means muscarinic receptors are blocked by atropine. It confirms the hypotensive mechanism of action of Pc. Cr.

The cardioprotective activity of Pc. Cr was tested on AMI and LVH model. ISO @5 mg/kg administered for 10 days, induced LVH while ISO @85 mg/kg for consecutive days induced AMI. ISO is a non-selective β adrenergic agonist widely used in cardioprotective research studies [27]. Alteration in the cardiac markers such as creatinine kinase (CK), creatinine kinase myocardial band (CK-MB), lactate dehydrogenase (LDH), atrial natriuretic factor (ANF), brain natriuretic factor (BNF), angiotensin II (AgII), renin, cyclic guanosine monophosphate (cGMP), aspartate aminotransferase (AST) and alanine transaminase (ALT) occurred due to two days consecutive administration of ISO @85 mg/kg during induction of AMI [44]. Different doses of Pc. Cr were assessed; compared with both ISO treated group and standard group (verapamil and carvedilol). Level of cGMP and NO decreased while that of LDH, CK, CK-MB, Ag II and renin increased. Pc. Cr @100, 200 and 300 mg/kg groups was not affected by the dose of ISO on repeated days. It's a possibility that receptors which are already occupied by Pc. Cr are not replaced by ISO and less changes in cardiac markers occurred because the presence of Pc. Cr in the blood as compared to ISO group. It is supposed that Pc. Cr normalizes the cardiac markers due to their 13 days administration and showed its cardioprotective effect and protect heart from infraction [20]. In ISO treated group, increased myocardial fibrosis, edema, necrotic cells with no normal texture and alteration in morphology were seen during histopathological examination. Less inflammation, necrosis and edema with normal texture and morphology were seen in Pc. Cr treated groups as compared to ISO group [19]. ISO @5 mg/kg was administered to the rats for 10 days repeated for the induction of LVH. Plasma level of AgII and renin increased while that of cGMP and NO decreased in ISO treated group. Vasoconstriction increased the stress on heart and induces hypertrophy [45]. In Pc. Cr treated groups, the elevated markers level decreased but increased the reduce ones and contrast their results with standard groups.

In LVH treated group, increased myocardial fibrosis, edema, necrotic cells with no normal texture and alteration in morphology were seen during histopathological examination. While less inflammation, necrosis and edema with normal texture and morphology were seen in Pc. Cr treated groups in contrast to ISO group [46]. Pc. Cr extract reduced the cardiac cell surface area, diameter and increased cell count.

Pc.Cr was evaluated for 14 days and 28 days for acute and sub-acute study respectively. Rats treated with 1500 and 3000 mg/kg for acute toxicity were seen with normal behavior, with no mortality and toxicity. It means LD_{50} was above 3000 mg/kg [47]. Animals and their organs weight were slightly increased and close to control group. The shape, texture and color of the slides of liver and kidney are normal with no morphological alterations were seen during histopathoogical studies [11].

Rats treated with 400 and 800 mg/kg for sub-acute toxicity were seen with normal behavior, no mortality and toxicity [47]. Animals and their organs weight such as liver, spleen, paired lungs, kidney and heart were slightly increased and close to control group [48]. Biological and hematological parameters of treating groups were almost in normal range [49]. The shape, texture and color of the slides of liver and kidney are normal with no morphological alterations were seen during histopathoogical studies [11].

5. Conclusion

The Pc. Cr showed hypotensive, vasorelaxant and cardioprotective effect during in vitro and in vivo studies. In ISO induced AMI, Pc. Cr positively reduced the cardiac markers with decreased edema and necrosis during hemodynamic and histopathological examination. Similarly in ISO induced LVH, Pc. Cr positively increased the potent vasodilator enzymes, and reduced the vasoconstrictors along with decreased cardiac cell size and lack of inflammatory cells as shown in hemodynamic and histopathological examination. HPLC screening of Pc. Cr confirmed the presence of catechin, P-coumeric acid, vanillic acid and quercetin which have vital role in cardiovascular diseases especially hypertension induced AMI and LVH. *In vivo* and in vitro studies of *Populus ciliata* showed therapeutic potential against cardiovascular diseases. In Future, *Populus ciliata* Wall ex. Royle can be used as antihypertensive, cardioprotective and vasorelaxant after separation of active constituents.

5.1. Studies in animals

All animal experiments complied with the ARRIVE guidelines and carried out in accordance with the U.K. Animals (Scientific Procedures)

Table 10
Effect of Pc. Cr on biological markers in ISO-induced LVH rats.

Biological Data	Groups (n = 6 Rats)						
	Control	Intoxicated	Dose 100	Dose 200	Dose 300	Carvedilol	Verapamil
ACE (μg/mL)	40.6 ± 4	58.6 ± 2	53.2 ± 0.3	51 ± 0.1	48 ± 0.5	47.3 ± 1	45.3 ± 1
Renin Conc.(pg/mL)	24.9 ± 2	149.1 ± 12	120 ± 3	105 ± 5	60 ± 1	41.3 ± 4	33.3 ± 1
ANF (pg/mL)	249 ± 10	148.4 ± 14	158 ± 2	169.5 ± 1	203.2 ± 1	212.6 ± 5	221.4 ± 2
BNF (pg/mL)	11.2 ± 12	5.3 ± 1	6.5 ± 0.3	8.8 ± 0.3	14.1 ± 0.5	17.1 ± 1	19.1 ± 1
LDH (U/L)	82.3 ± 8	160.7 ± 13	135 ± 2	125 ± 2	115 ± 3	102.4 ± 2	98.2 ± 2
Ag II Conc. (pg/mL)	92.7 ± 6	118.6 ± 2	114.3 ± 1	112 ± 1	107 ± 2	103.9 ± 1	100.1 ± 2
NO (μmol/l)	47.2 ± 5	32.5 ± 1	33 ± 0.3	35 ± 0.1	37 ± 0.5	40.2 ± 1	42.2 ± 2
CPK (U/L)	125 ± 12	286.4 ± 26	248 ± 4	202 ± 3	170 ± 2	149.2 ± 4	141.2 ± 2
CKMB (U/L)	75.4 ± 7	189.3 ± 17	152 ± 3	127 ± 5	110 ± 3	92.3 ± 3	87.1 ± 1
cGMP (pmol/mL)	15.5 ± 5	4.6 ± 4	5.7 ± 0.5	7.2 ± 1	9.1 ± 0.3	13.2 ± 1	17.1 ± 1
ALT (U/L)	38.3 ± 4	57.3 ± 1	55 ± 1	53 ± 0.3	49 ± 0.5	46.1 ± 2	44.1 ± 1
AST (U/L)	127 ± 14	210.5 ± 20	181 ± 3	176 ± 1	169 ± 3	157.6 ± 5	149.3 ± 2

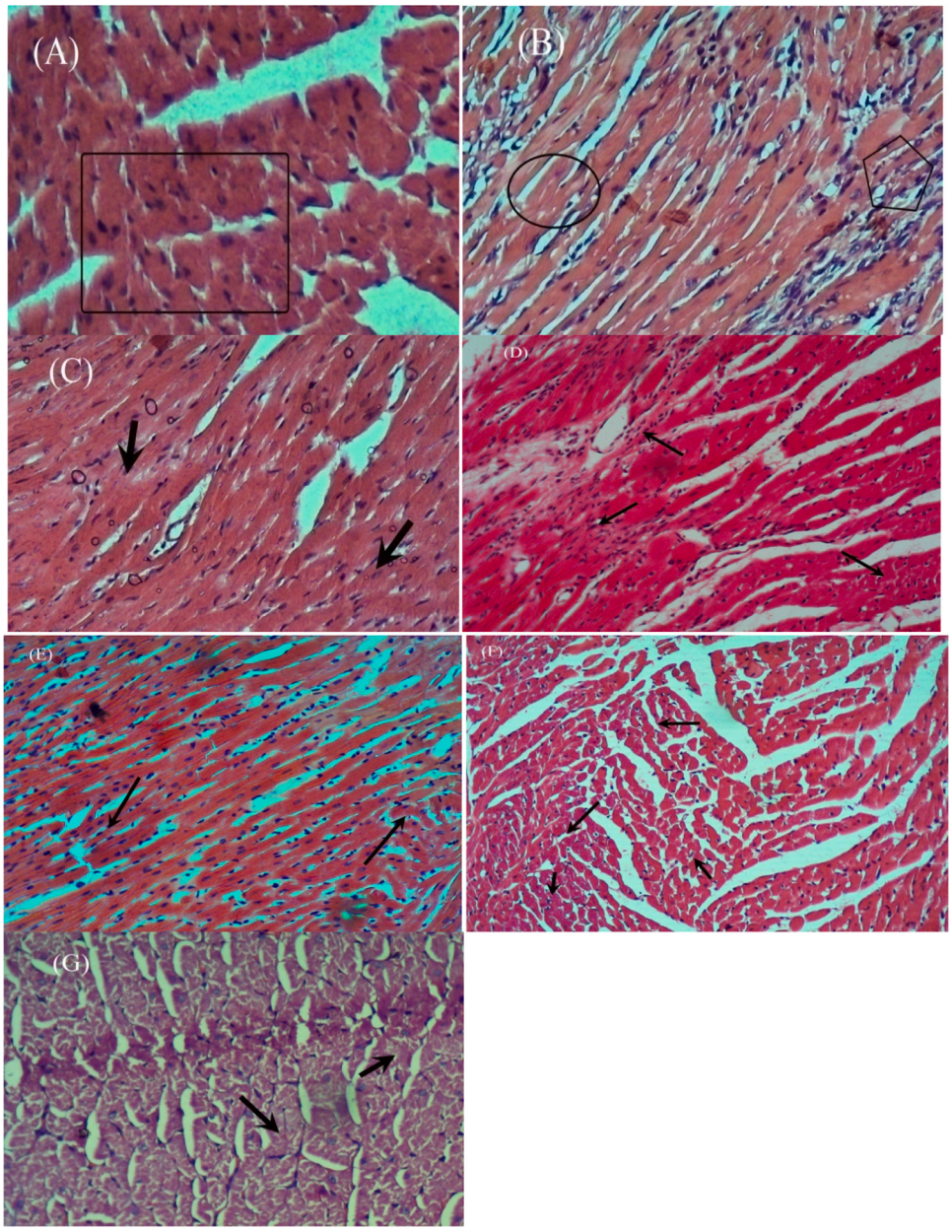

Fig. 13. Histological analysis of heart tissue in rats during ISO induced LVH study as observed by hematoxylin-eosin staining (10×). Representing the effect of Pc. Cr (A): Control; (B): Intoxicated ISO; (C): 100 mg/kg; (D); 200 mg/kg; (E): 300 mg/kg; (F) and (G) Standard carvedilol and verapamil in rats. The rectangle represents normal cells, oval shaped represent enlarged cardiomyocytes, polygon represent cellular infiltration and notched arrow showed normal cluster of cardiac cells.

Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

CRediT author contribution statement

F. Saqib, **A. Ali** and **H.T. Ahmedah** designed the study and performed experiments. **F. Saqib**, **A. Ali**, **C.A. Irimie**, **S.I. Toma**, **B.E. Popovici**, **M. Moga**, and **M. Irimie** collected data and wrote the manuscript. All authors revised the manuscript.

Declaration of Competing Interest

All authors declared that there is no conflict of interest.

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