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ORIGINAL ARTICLE

Xylocarpus moluccensis Fruit Fraction Rescues Cardiac Hypertrophy by Improving Angiogenesis and Regulating NF-κB-Mediated Inflammation

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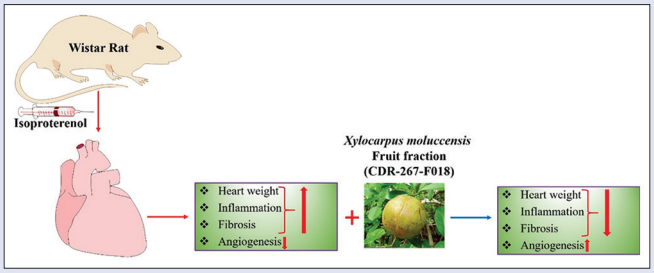
ABSTRACT

Background and Aim: The present study was undertaken to investigate the potential of ethyl acetate fraction obtained from the fruits of *Xylocarpus moluccensis* alcoholic extract (CDR-267-F018) against cardiac hypertrophy in rats. **Experimental Procedure***:* Cardiac hypertrophy was achieved in Wistar rats through isoproterenol and treated either with propranolol or CDR-267-F018 for 14 days. **Results and Conclusion:** CDR-267-F018 treatment reduced isoproterenol induced cardiac hypertrophy as assessed by 2D-echocardiography and supported by reduction in *ANP, BNP,* β*-MHC, NPP-A* and increased expression of vascular endothelial growth factor receptor 1. CDR-267-F018 treatment tightly regulated inflammation by controlling the plasma proinflammatory cytokines tumor necrosis factor-α and IFN-γ, plasma EMP level and NF-κB, Akt and ERK. Further, CDR-267-F018 treatment reduced fibrosis by regulating *Col18a1, FGF-21* and *MMP2*. In total, CDR-267-F018 protected rat heart against isoproterenol induced cardiac hypertrophy by acting on inflammation, fibrosis and by improving angiogenesis.

Key words: Angiogenesis, cardiac hypertrophy, endothelial microparticles, fibrosis, inflammation

SUMMARY

- CDR-267-F018 had shown protection against isoproterenol-induced cardiac hypertrophy. CDR-267-F018 had reduced the inflammatory load in the circulation by decreasing the levels of cytokines like tumor necrosis factor-α, and IFN-γ, but had also decreased the expression of e-selectin and reduced the circulating plasma level of endothelial microparticles and thus shown a beneficial effect in pathological condition of hypertrophy. CDR-267-F018 attenuated cardiac hypertrophy through pronounced control over NF-κB and Col18a1 and increased the vascular endothelial growth factor receptor 1 expression.
- In both, therapeutic and curative approaches, CDR-267-F018 had shown a pronounced rescue from cardiac hypertrophy condition.



Abbreviations used: Akt: Protein kinase B; ANP: Atrial natriuretic peptide; ApoE-/-: Apolipoprotein E knockout; BNP: Brain natriuretic peptide; CD31: Cluster of differentiation 31 (Platelet endothelial cell adhesion molecule); CD62E: E-selectin (endothelial-leukocyte adhesion molecule 1); COL18a1: Collagen Type XVIII Alpha 1 Chain; DBP: Diastolic blood pressure; ECM: Extracellular matrix; EMP: Endothelial microparticle; eNOS: Endothelial nitric oxide synthase 3; ERK: Extracellular signal-regulated kinases; FGF-21: Fibroblast growth factor-21; HR: Heart rate; IFN-γ: Interferon gamma; IVS; s: Intra ventricular Septum, systole; IVST: Intra Ventricular Septum Thickness; LVID; s: Left ventricular internal diameter, systole; LVLA: Left Ventricular Lumen Area; LVPW; S: Left ventricular posterior wall, systole; LVWT: Left Ventricular Wall Thickness; MAP: Mean arterial pressure; MMP-2: Matrix metalloproteinase-2; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; NPPA: Natriuretic Peptide A; p65: Nuclear factor NF-kappa-B p65 subunit; RVLA: Right Ventricular Lumen Area; RVWT: Right Ventricular Wall Thickness; SBP: Systolic blood pressure; TNF-α: Tumor necrosis factor alpha; VEGF-R1: Vascular endothelial growth factor receptor 1; VSMC: Vascular smooth muscle; vWF: Von Willebrand factor.

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INTRODUCTION

Myocardial infarction, valvular disease, cardiomyopathy, or hypertension produces excess stress on the heart, in order to compensate this excess stress, hypertrophy is developed in the heart. With persistent stress, the heart finally fails to compensate and decompensation occurs impairing cardiac ejection effecting tissue perfusion considerably. This eventually leads to heart failure. During the development of hypertrophy, severe cardiac modulations such as increased cell size, high protein content, increased myocardial fibrosis, altered metabolic reprogramming,

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mitochondrial dysfunction, impaired calcium handling, and decreased angiogenesis/capillary density take place. Major characteristics feature of cardiac hypertrophy includes an increase in ventricular mass, decreased lumen area and decreased pumping efficiency of the heart.[1]

Although pharmacological interventions like the use of Ca^{+2} channel blockers, β_2 blockers, diuretics, angiotensin antagonist are currently prescribed to reverse cardiac hypertrophy these interventions provide only symptomatic relief along with severe side-effects. Many studies have also shown a significant improvement in cardiac hypertrophy by the use of natural products like *Magnolia officinalis*,[2] *Desmodium gangeticum*,[3] and *Camellia senesis*.[4] These natural products are effective against cardiovascular complications and moreover have lesser side effects and are even cost-effective.[5]

Xylocarpus moluccensis (Lamk.) M. Roem. (Syn: *Carapa moluccensis*) (Fam: Meliaceae), a traditionally used plant, have shown the utility of its various parts such as bark, leaves, fruits, and seeds to treat various ailments related with severe infection and inflammation. The traditional use of the plant has been reported from coastal regions of India and throughout the regions south-east Asian countries including Bangladesh, Malaya, Fiji, and the Philippines.[6,7] The bark is used to treat fever, malaria, dysentery, diarrhea[8] and as astringent. The leaves are used to treat bacterial infection, cancer, and inflammation. The fruits are used for treat elephantiasis and also has aphrodisiac and bactericidal properties.[9,10] The major active constituents of Xylocarpus are limonoids or tetranortriterpenoids, alkaloids, phenolics, and steroids.[11] The fruits are rich in limonoids and have been reported for their anti-inflammatory and antioxidant.

X. molluccensis (CDR-267) from Indian mangroves possessed antihyperglycemic and antidyslipidemic activity was discovered in the alcoholic extract for the first time. Subsequent fractionation of alcoholic extract led to identify the antihyperglycemic and antidyslipidemic activity in the ethyl acetate fraction (CDR-267-F018), which is rich in limonoids (Pharmaceutical composition for treatment of diabetes and dyslipidemia, United States Patent 7959954).[5,12] Further studies on CDR-267 F018 showed a beneficial effect to improve vascular relaxation and plaque stability in dyslipidemic models of atherosclerosis.[13] The bioactive fraction CDR-267 F018 was also found safe in essential safety pharmacological studies.[14]

Considering the salubrious property of CDR-267-F018 (ethyl acetate fraction) obtained from the fruits of *X. molluccensis* alcoholic extract, we hypothesized that CDR-267-F018 could be also beneficial in cardiac hypertrophy. In this study, we have used Isoproterenol, a well-known β1, β2,-adrenergic agonist to induce cardiac hypertrophy and propranolol, which specifically acts as nonselective β-blocker and a choice of drug for treating left ventricular cardiac hypertrophy.[15] Our findings revealed that CDR-267F018 significantly reduced the cardiac hypertrophy with improved angiogenesis and reduced fibrosis and inflammation in the heart.

MATERIALS AND METHODS

Cell culture

EA. hy926 endothelial cells generated by C. J. S. Edgell (was generously provided as a gift from the University of North Carolina, Lineberger Comprehensive Cancer Centre) were cultured in DMEM (Himedia, India) supplemented with 10% FBS and 1% Penicillin/Streptomycin.[16]

Animals

Male Wistar Rats (220–250 g; 8–10 weeks old) were procured from National Animal Laboratory Centre of Council of Scientific and Industrial Research-Central Drug Research Institute (CSIR-CDRI), Lucknow, India. All the animal experiments were conducted in accordance with the guidelines of the Institutional Animal Ethics Committee of Central Drug Research Institute, Lucknow, India (Approval number, Wistar rats; IAEC number: IAEC/2014/96). All the experimental protocols were approved by the Institutional committee of Central Drug Research Institute, Lucknow, India. Animals were housed in a group of 3-5 animals per cage under natural 12 h light-12 h dark cycle at 24°C ± 0.5°C and received humane care in compliance with the guidelines for the care and use of laboratory animals.

Plant material

The fruits of *X. moluccensis* were collected from the Andaman region of India and were identified in the Botany Division of the Institute. The collection details and representative voucher specimens of the plants have been documented in the herbarium (voucher accession number: CDRI-24295) of the Botany Department, CSIR-CDRI, Lucknow for future reference.

Plant materials (1.0 kg) were dried in shade and grounded mechanically. The fine powder was extracted four times with 95% ethanol. The pooled extract was concentrated under a high vacuum on a Buchi rotary evaporator. The ethanolic extract (110 g) thus obtained was fractionated with hexane at room temperature, yielding 11 g (10%) of hexane fraction and the hexane insoluble fraction was further fractionated with ethyl acetate. Ethyl acetate soluble fraction was concentrated on Buchi rotavapor at 50°C to give a viscous mass which was finally dried under a high vacuum to obtain 27 g (24.5%) brownish powder. Ethyl acetate fraction (CDR-267-F018) was used for further biological studies.[13]

Tubulogenesis

Under serum-free condition 1×10^4 EA. hy926 endothelial cells were plated onto the matrigel coated surface in 96-well plate and treated either with control, VEGF-50 ng/mL or CDR-267-F018-10 μg/mL. After 6 h of incubation, all the respective treatments were observed for tubule formation, the pictures were captured and analyzed for tubule length using Leica Qwin V 3.0 software.[16]

Induction of cardiac hypertrophy and treatment schedule

Male Wistar rats were injected with Isoproterenol (5 mg/kg; sc) once daily for 14 days to induce cardiac hypertrophy.[17] Group 1: Control group, administered with 0.25% CMC; po + 0.9% saline; sc, while Group 2: Received isoproterenol injection 5 mg/kg; sc, Group 3: Received propranolol 5 mg/kg; po + isoproterenol injection 5 mg/kg; sc and Group 4: Received CDR-267-F018-25 mg/kg; po + isoproterenol injection 5 mg/kg; sc. All the drugs were administered once daily for a period of 14 days continuously [Figure 1a]. To rule out the possibility of age-related hypertrophy, heart weight was normalized to tibia length and expressed as heart weight to tibia length ratio (g/cm).[18,19]

For curative studies, male Wistar rats were divided into different groups as mentioned above. For 14 days, all the groups except group 1 were injected with isoproterenol (5 mg/kg; sc) once daily for 14 days to induce cardiac hypertrophy, then on 14th day onwards Group 2, 3, and 4 received isoproterenol (5 mg/kg; sc) on alternate days, while group 3 and 4 received the propranolol 5 mg/kg; po and CDR-267-F018-25 mg/kg; po daily for next 14 days. The detailed experimental plan is shown in [Figure 6a].

Echocardiography examination

The animals were anesthetized with the ketamine-xylazine mixture and were placed in supine position on thermostatically controlled pad. Electrode gel was applied on shaved chest and echocardiography

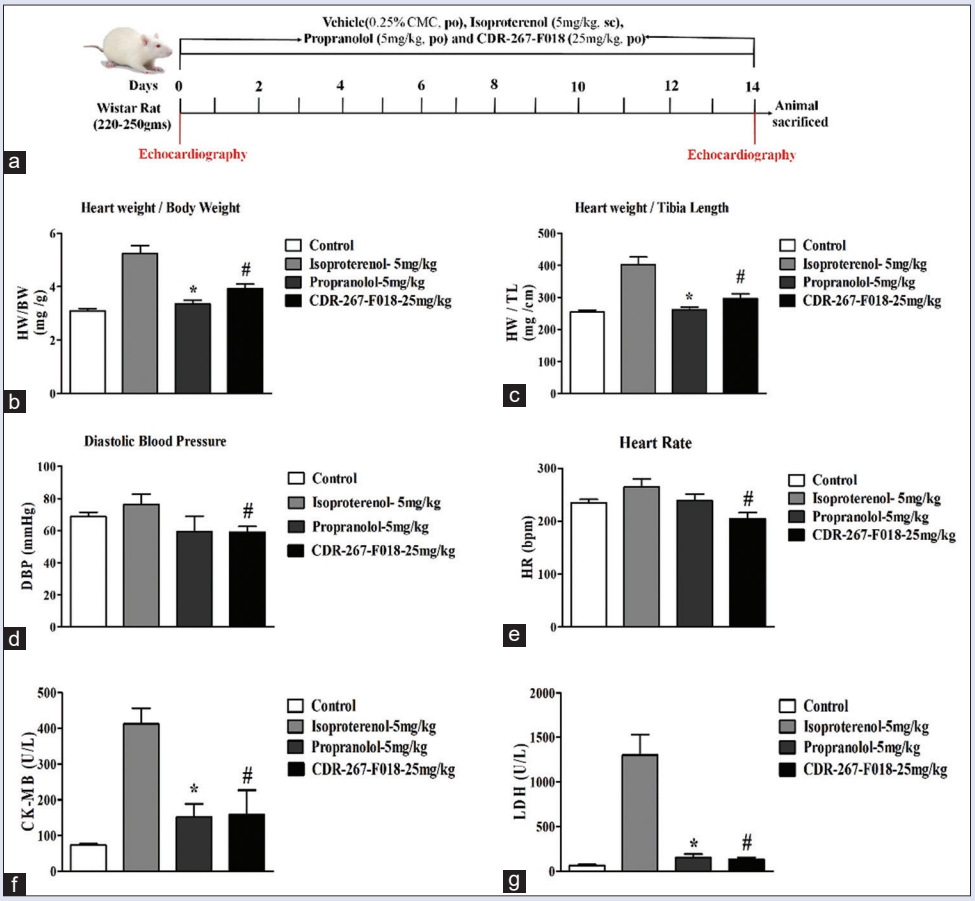

Figure 1: CDR-267-F018 exerts protection against cardiac hypertrophy. (a) Treatment schedule for rats undergoing cardiac hypertrophy. (b) Heart weight normalised to body weight, *P = 0.0006, #P = 0.0017 versus isoproterenol treated rats. (c) Heart weight normalised to tibia length, *P = 0.0010, #P = 0.0023 versus isoproterenol treated rats. (d) Diastolic blood pressure, #P = 0.0242 vs. isoproterenol treated rats. (e) Heart Rate, #P = 0.0100 vs. isoproterenol treated rats. (f) Plasma Creatinine Kinase-MB levels, *P = 0.0008 vs. isoproterenol treated rats, #P = 0.0038 versus isoproterenol treated rats. (g) Plasma lactate dehydrogenase levels, *P = 0.0310 versus isoproterenol treated rats, #P < 0.0001 versus isoproterenol treated rats. Data were expressed as mean ± Standard error of mean

was performed by using VEVO 1100 (FUJIFILM, VisualSonics, Toronto, Canada), the standard two-dimensional echocardiography acquired two views i.e., Para sternal long axis view short-axis view using MS250 (18-24 MHz) transducer for rat. Respective cine loops of all the imaged animals were analyzed with the help of Vevo LAB (version 1.7.1).[16]

Hemodynamic analysis

After 14th day the animals were anesthetized with urethane (1.5 g/kg; i. p.) and accessed for hemodynamic analysis. To avoid day-to-day variation, the instrument was calibrated before use. Briefly, after successful anesthesia, the rat was placed in supine position on thermostatically controlled pad and the exposed carotid artery was cannulated and connected to the pressure transducer to record systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate (HR) using Power Lab data acquisition system (AD Instruments, Bella Vista, Sydney, Australia).[20]

Enzyme-linked immunosorbent assay

Blood was obtained from all the animals by cardiac puncture in a tube containing 3.8% tri-sodium citrate. Blood samples were then centrifuged at 4000 rpm for 10 min at 4°C for plasma separation. All the plasma samples were stored in-80°C till next use. Cytokines Tumor necrosis factor α (TNF-α) and IFN-γ were analyzed in all the samples as per manufacturer protocol.[21]

Estimation of creatinine kinase-MB

Creatinine Kinase-MB (CK-MB) is a well-reported maker for myocardial injury. CK-MB activity was measured in plasma as per the manufacturer's protocol (CK1296, RANDOX, United Kingdom). The change in absorbance (ΔA) per minute was measured spectrophotometrically (PowerWave HT Microplate Spectrophotometer, BioTek, USA) at the wavelength of 340 nm and enzyme activity was expressed as CK-MB activity (U/L) = ΔA/min x 1651.[21]

Estimation of lactate dehydrogenase

Lactate dehydrogenase (LDH) has already been reported as a marker of injury to myocardium. Plasma LDH was spectrophotometrically determined as per manufacturer protocol (#61909500011730, Innoline, Merck, India). The change in enzyme activity was determined and expressed as a LDH (U/L) = ΔA/min x 11496 at a wave length of 340 nm.

Plasma endothelial microparticles estimation

Plasma Endothelial microparticles (EMPs) were estimated as described previously. Briefly, plasma samples were centrifuged at

1931 g for 15 min and subsequently ultracentrifuged at 100,000 g for 90 min at 4°C. EMPs were labeled with anti-CD146-PE (Abcam, Ab78488) for 30 min and analyzed using flow cytometry. Flow cytometer was calibrated with predetermined size beads and plotted FSC-SSC dot plot, number events lying within this EMP gating was measured.[21]

Western blot

At the end of treatment, hearts were collected and processed for protein extraction followed by SDS-PAGE. The membranes were immunoblotted with AKT, ERK, NF-kB, and GAPDH followed by the secondary antibody conjugated with horseradish peroxidase. Proteins were detected using Immobilon Western kit (Millipore).[16]

Gelatin zymography

Gelatin zymography was used to assess MMP-2 activity. Briefly, lysate was resolved by loading 40 µg of protein on 8.75% SDS polyacrylamide gel having 40 mg/mL gelatin. The gel was stained with 0.5% Coomassie Blue G-250 for 2 h at room temperature followed by destaining with a methanol/acetic acid solution until the bands of lysis become clear.[22]

Real time reverse transcription-polymerase chain reaction

Total RNA was isolated using TRIZOL method and processed for cDNA synthesis using H-minus Revert synthesis kit (Thermo Fischer) and amplified for *ANP, BNP, vWF, ICAM-1, VEGF-R1, VEGF-R3, β-MHC, NPP-A, Col18a1, FGF-21, p65, GAPDH, 18sRNA,* etc., (primer sequences will be provided on request) and polymerase chain reaction was performed with SYBR green.[23]

Histological analysis

The isolated heart tissues were fixed with 10% neutral buffer formalin and were processed for histological evaluation. Briefly, paraffin wax-embedded hearts were sectioned of 5–7 µm thickness (Microm HM 350, rotary microtome, Heidelberg, Germany) and mounted on Poly-L-lysine-coated slides. Subsequently, the sections were deparafinized and stained as per recommended protocol. To evaluate the general architecture of heart H and E stain was used. The sections were hydrated, stained with Hematoxylin and Eosin, then again dehydrated serially with graded alcohol and xylene and finally mounted with DPX. Myocyte size was analyzed using Leica Qwin V3.0 and complete heart picture was obtained by stitching the images using Leica Application Suite Version 4.1.0. Stitched images were further evaluated for Left Ventricular wall thickness (LVWT); Intra Ventricular septum thickness (IVST), Right Ventricular wall thickness (RVWT), Left Ventricular lumen area (LVLA) and right ventricle lumen area (RVLA). Paraffin-embedded sections were stained with CD31, vWF and E-selectin. Immunoreactivity was analyzed by Novacastra Novolink Polymer detection system (Leica Microsystems, USA) and counterstained with hematoxylin.[16]

Picro-sirius red staining for collagen

Picro-sirus red staining was used to analyze the type of collagen and fibrosis in the rat heart. Briefly, sections were deplastified, hydrated, stained with hematoxylin and picro Sirius red, then again dehydrated and finally mounted with DPX. Collagen fibers were identified under polarized light (Type I: Yellow/Orange and Type III: Green). Stitched images were further processed for analysis of fibrosis and collagen deposition.[16]

Statistics

All the groups were evaluated for the level of significance by using unpaired *t*-test with the help of GraphPad PRISM software (Version 5.01). Data were expressed as mean ± Standard error of mean a P of ≤ 0.05 was considered statistically significant.

RESULTS

CDR-267-F018 treatment reduced cardiac hypertrophy

The administration of isoproterenol for 14 days lead to cardiac hypertrophy majorly affecting the left ventricle as compared to the right ventricle, leading to increased afterload on the left ventricle with increased contractility and/or preload to maintain the same stroke volume resulting cardiac muscle hypertrophy. Upon treatment either with CDR-267-F018 or propranolol treatment, heart weight was significantly reduced as compared to the isoproterenol treated rats [Figure 1b]. To rule out the possibility of increased heart weight due to age, we normalized the heart weight to tibia length ratio, CDR-267-F018 and propranolol significantly reduced hypertrophy [Figure 1c]. As a consequence of cardiac hypertrophy, we expected a change in hemodynamic parameters after CDR-267-F018 and propranolol treatment, although we observed a non-significant change in SBP but CDR-267-F018 treatment significantly reduced the DBP, HR, and MAP as compared to isoproterenol treated rats [Figures 1c-e]. CK-MB and LDH are known markers for the cardiac injury, hence we determined their levels in plasma. CDR-267-F018 had shown a significant reduction in the levels of CK-MB and LDH level in a dose dependent manner in plasma [Figures 1f and g]. This suggests that CDR-267-F018 might be protecting the heart from cardiac hypertrophy by reducing its workload and showing its negative chronotrophic effect on the heart.

CDR-267-F018 improved the functionality of hypertrophic heart by reducing wall thickness

Further to assess the various cardiac parameters, we performed ultrasound scanning using echocardiography. Treatment with CDR-267-F018 for 14 days at 25 mg/kg and 50 mg/kg had significantly decreased the LV mass IVS, LVID, and LVPW as compared to the isoproterenol treated rats, while there was no significant difference between propranolol and CDR-267-F018 treated rats. However, we did not observe any significant reduction in LV mass IVS, LVID, and LVPW with CDR-267-F018-12.5 mg/kg [Figure 2a-e and Table 1] compared to control. Hence, our further all the rat experiments were limited to only CDR-267-F018-25 mg/kg dose only. This result shows that treatment with CDR-267-F018 reduced the cardiac hypertrophy. Further we evaluated the gross morphology of the heart as left ventricular wall thickness (LVWT), right ventricular wall thickness (RVWT), intraventricular septum thickness (IVST), left ventricular lumen area (LVLA) and right ventricular lumen area (RVLA) using H and E staining [Figure 3a]. CDR-267-F018 and propranolol treated rats had shown a significant reduction in LVWT, RVWT and IVST as compared the isoproterenol treated rats, while the lumen area is inversely proportional to the ventricular wall thickness, we found an increased left and right lumen area equal in CDR-267-F018 and in propranolol treated rats hearts as compared to the isoproterenol treated rats, suggesting CDR-267-F018 reversed cardiac hypertrophy similar to propranolol. Next, to determine the molecular effect of CDR-267-F018, we analyzed the expression of specific markers such as *ANP, NPP-A, BNP,* and *β-MHC* at mRNA level [Figure 3b-e]. Our findings revealed that CDR-267-F018 had significantly reduced the mRNA expression of *ANP* [Figure 3b], *NPP-A* [Figure 3c], *BNP* [Figure 3d] and

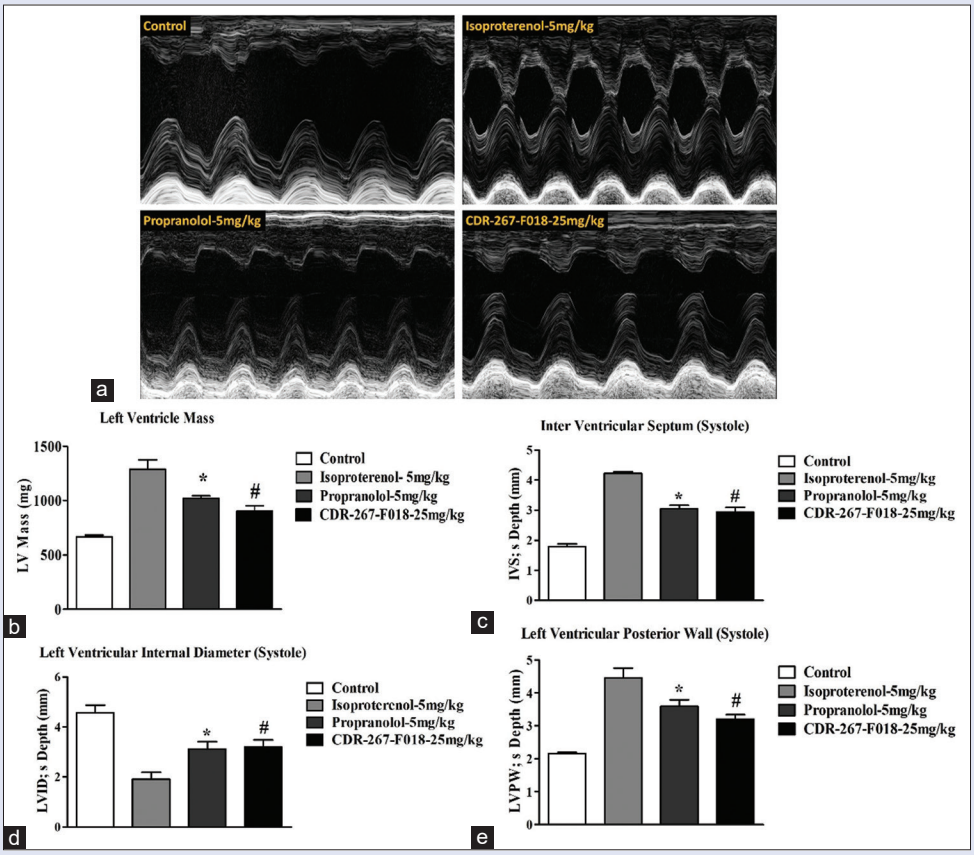

Figure 2: CDR-267-F018 improved functional parameter of hypertrophic heart. (a) Representative images of M-Mode echocardiography of left ventricle using 2D echocardiography VEVO-1100. (b) Left ventricle mass, *P = 0.0175, #P = 0.011 versus isoproterenol treated rats. (c) Inter ventricular Septum (Systole), *P < 0.0001, #P < 0.0001 versus isoproterenol treated rats. (d) Left ventricular internal diameter, (Systole) *P = 0.0144, #P = 0.0117 versus isoproterenol treated rats. (e) Left ventricular posterior wall, (Systole) *P = 0.0343, #P = 0.0054 versus isoproterenol treated rats. Data were expressed as mean ± Scanning electron microscope

β-MHC [Figure 3e]. These results confirm the hypertrophy sparing property of CDR-267-F018.

CDR-267-F018 reduced inflammation and fibrosis induced during cardiac hypertrophy

We next assessed the consequence of hypertrophic conditions resulting in inflammation, fibrosis, and angiogenesis. Treatment with CDR-267-F018 and propranolol had decreased the expression of vWF, CD62E, and CD31 as compared to the isoproterenol-treated rats suggesting that CDR-267-F018 reduced inflammation and protected the heart from cardiac hypertrophy induced inflammation. However, we also observed reduced CD31 expression with CDR-267-F018 treatment [Figure 4a]. We further assessed the effect of CDR-267-F018 on fibrosis. CDR-267-F018 treatment decreased the expression of Pro-MMP-2 and collagen deposition similar to that of propranolol treated rats as compared to the isoproterenol treated rats [Figure 4b and c]. Further to confirm this finding, we evaluated the *Col18a1* gene. CDR-267-F018 and propranolol had significantly decreased the expression of *Col18a1* at mRNA level [Figure 4d]. Hypertrophic condition leads to release of various pro-inflammatory cytokines like TNF-α and INF-γ. Treatment with CDR-267-F018 and propranolol had significantly decreased the circulating levels of TNF-α [Figure 4e] and INF-γ [Figure 4f]. These results suggest that CDR-267-F018 has potent anti-inflammatory, anti-fibrotic and anti-angiogenic property similar to that of propranolol providing a beneficial effect against cardiac hypertrophy.

CDR-267-F018 increased angiogenesis *in vitro* and reduced the expression of AKT, ERK and NF-kB during hypertrophic condition

Next, we assessed the CDR-267-F018 molecular mechanisms. Hence, we treated the EA. hy926 endothelial cells with CDR-267-F018-10 μg/mL under serum-free condition. EA. hy926 endothelial cells had increased tubule formation as compared to the VEGF treated cells [Figure 5a and b]. Further, there was a significant increase in the expression of *VEGF-R1* as compared to control [Figure 5c]. While ICAM-1, p65 and vWF were not able to show any significant change. Further, we observed a significant reduction at mRNA level of *FGF-21,* while the levels of *VEGF-R1* were up regulated under *in vivo* condition as compared to the isoproterenol treated rats [Figure 5d and e]. CDR-267-F018 treatment significantly reduced the protein expression of NFkB, Akt and ERK as well [Figure 5f]. EMPs had shown a remarkable potential in endothelial functionality, in context to this we found a significant decrease in the plasma EMPs level with CDR-267-F018 treatment in rats [Figure 5g]. Overall our results show a pronounced anti-hypertrophic effect of CDR-267-F018.

Curative treatment of CDR-267-F018 reversed hypertrophy

CDR-267-F018 being a potent anti-hypertrophic, anti-inflammatory, and anti-fibrotic. We thought whether administering CDR-267-F018 curatively might reduce hypertrophy. Hence, we treated rats for 14 days

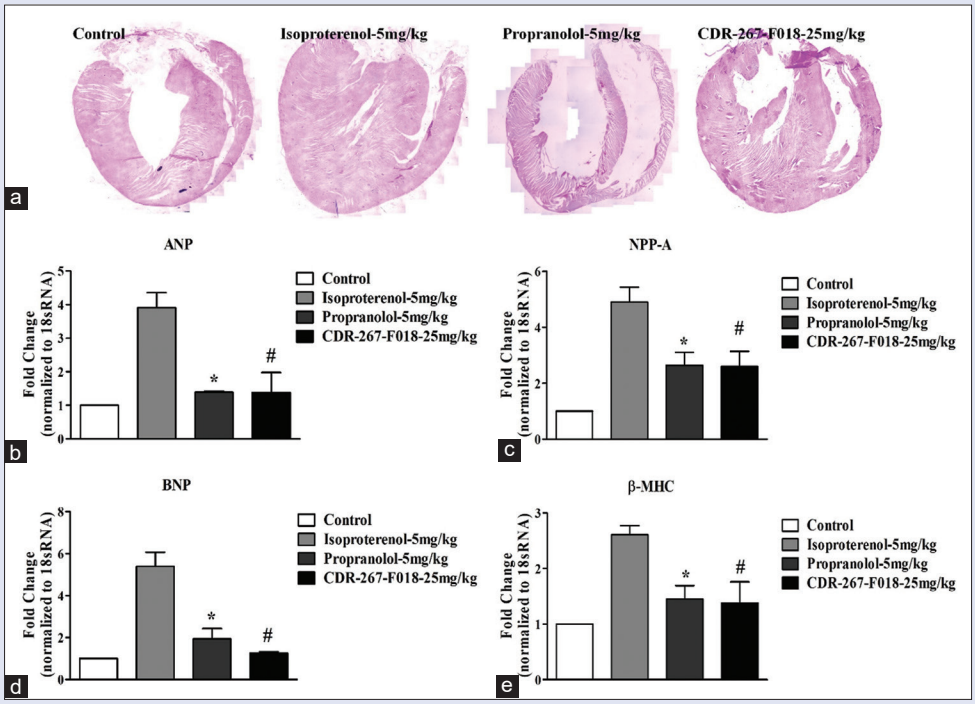

Figure 3: CDR-267-F018 improved functional parameter of hypertrophic heart. (a) Representative gross images of heart sagittal sections by haematoxylin and eosin stain. Cardiac mRNA expression of, (b) ANP, *P = 0.0048, #P = 0.0271 versus isoproterenol treated rats. (c) NPP-A, *P = 0.0327, #P = 0.0385 vs. isoproterenol treated rats. (d) BNP, *P = 0.0146, #P = 0.0038 versus isoproterenol treated rats. (e) β-MHC *P = 0.0175, #P = 0.0423 versus isoproterenol treated rats. Data were expressed as mean ± Standard error of mean

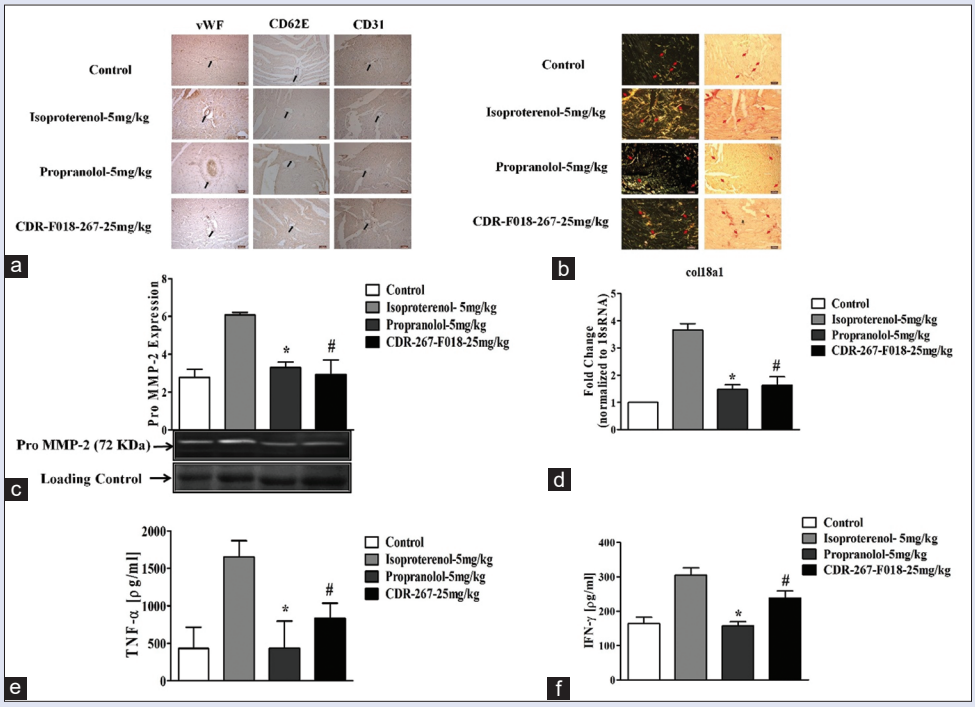

Figure 4: CDR-267-F018 decreased fibrosis and inflammation in heart. (a) Representative images of heart sections stained with vWF, CD62E and CD31. (b) Representative photomicrograph of Picro Sirius red stained sections in heart. (c) Pro-MMP2 level in heart, *P = 0.0132, #P < 0.05 versus isoproterenol treated rats. (d) mRNA level of *col18a1*, *P = 0.0017, #P = 0.0068 versus isoproterenol treated rats. Plasma cytokine level of (e) Tumor necrosis factor-α, *P = 0.0270, #P = 0.0409 and (f) IFN-γ, *P = 0.0045, #P = 0.0438 versus isoproterenol treated rats. Data were expressed as mean ± Standard error of mean

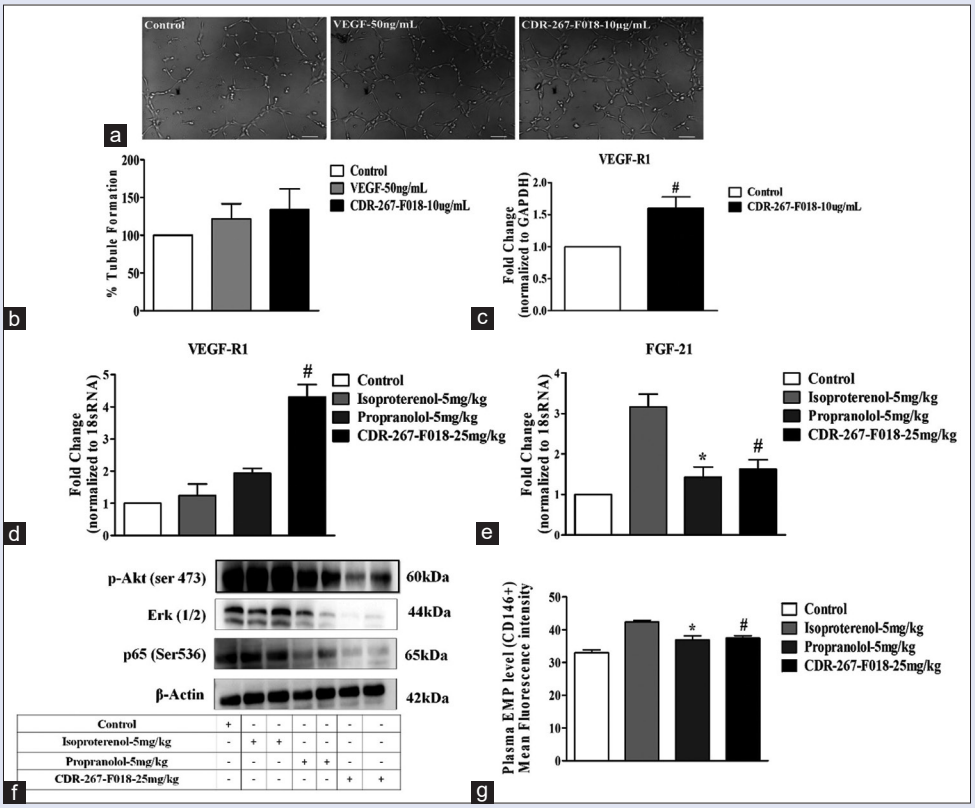

Figure 5: CDR-267-F018 increased angiogenesis and decreased inflammation in heart. (a) Representative photomicrographs of phase contrast tubule formation. (b) Bar diagram represents percent tubule formation after 6 h of CDR-267-F018 treatment. mRNA expression of (c) vascular endothelial growth factor receptor 1 in EA.hy926 endothelial cells treated with CDR-267-F018, #$P = 0.0071$ versus control, (d) vascular endothelial growth factor receptor 1 in rat heart, #$P = 0.0043$ versus isoproterenol treated rats, (e) *FGF-21* in rat heart, *$P = 0.0127$, #$P = 0.0171$ versus isoproterenol treated rats. (f) Western blot analysis of p-AKT (ser473), Erk (1/2), p65 (Ser536) and β-actin. (g) Plasma Endothelial microparticles level, *$P = 0.0010$, #$P < 0.0001$ versus isoproterenol treated rats. Data were expressed as mean ± Standard error of mean

continuously with isoproterenol followed by CDR-267-F018 for the next 14 days with alternating doses with isoproterenol [Figure 6a]. CDR-267-F018 treatment had significantly reduced the heart weight/tibia length ration as compared to the isoproterenol treated rats (data not shown). Further, ultrasound scanning of the heart showed that CDR-267-F018 has significantly reduced the LV mass as compared to the isoproterenol treated rats [Figure 6b and Table 1]. Moreover, mRNA analysis of various hypertrophic and cardiac dysfunction markers like *ANP, BNP, NPP-A, FGF-21,* Fibrotic marker *COL18a1* were found to be reduced. While in accordance with the previous finding, we observed a significant up regulation of *VEGF-R1* as compared to the isoproterenol treated rats [Figure 6c]. All these findings revealed cardio-protective benefits of CDR-267-F018 against isoproterenol induced cardiac hypertrophy.

DISCUSSION

Cardiac hypertrophy is a multifactorial disease having a poor prognostic sign, resulting in cardiac failure. Uncontrolled inflammation[24] increased fibrosis and decreased angiogenesis[25] are the major hallmarks of cardiac hypertrophy contributing to CVD-induced deaths. Our present study made a number of significant new observation in terms of plant-based therapy for controlling cardiac hypertrophy. Specifically, our study demonstrates the following findings. (1) CDR-267-F018 had reduced the cardiac hypertrophy, (2) CDR-267-F018 had shown a pronounced control over inflammation, fibrosis and angiogenesis, (3) Further treatment with CDR-267-F018 in the curative Standard error of mean approach had proven its beneficial effect in cardiac hypertrophy.

Previous studies have shown CDR-267-F018 fraction has an antidiabetic, antidyslipidemic, and endothelial protective role,[13] but its direct cardioprotective potential remained totally unexplored yet. β-adrenergic receptor agonist has been known to induce cardiac changes followed by rise in blood pressure. Activation of G protein, i.e., G (s) by β adrenergic receptor leads to the direct as well as indirect activation of ERK (1/2) followed by nuclear accumulation of ERK and resulted in increased wall thickness.[26] Like CDR-267-F018, different plant extract-based studies have shown a lowering in blood pressure could be beneficial in cardiac complications like hypertension.[27] However, under clinical condition reverting cardiac hypertrophy on the morphological basis could be a positive sign in terms of disease improvement.

Long-term stimulation of β-adrenergic receptor had been known for its adverse effect on a cardiovascular system like altering hemodynamic changes which, with the passage of time had a potential to aggravate inflammatory load by releasing TNF-α and IFN-γ in the circulation. Increased inflammatory cytokines in circulation can activate e-selectin on endothelial cell, which can further mediate the inflammatory response by regulating the leukocyte transmigration and activation of platelet adhesion factor in cardiac disease.[28] Further, overtly stressed endothelial cells generate EMPs in the circulation, can further activate the selectin mediated inflammatory effect on the heart.[16,21] Here, we

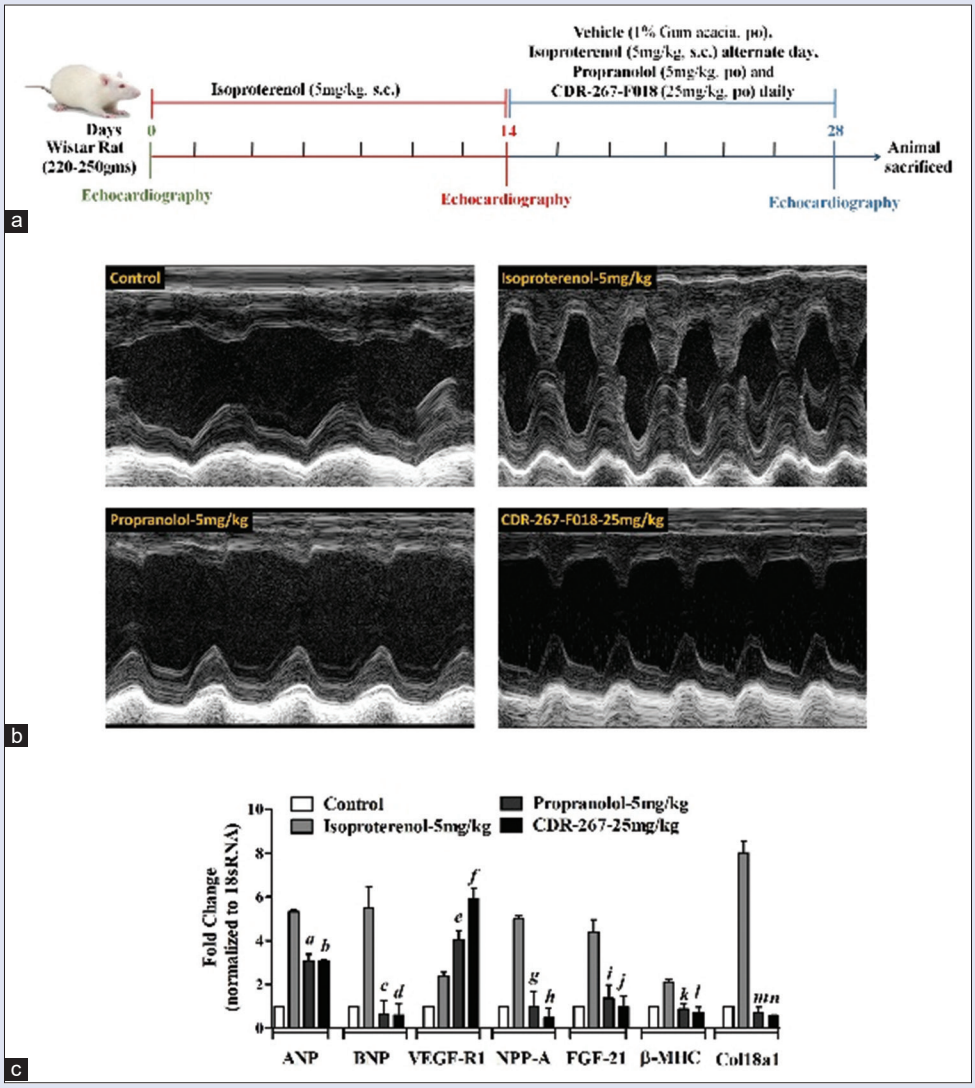

Figure 6: CDR-267-F018 reversed hypertrophy of the heart. (a) Treatment schedule for curative approach used for rats undergoing cardiac hypertrophy. (b) Representative images of M-Mode echocardiography of left ventricle using two-dimensional echocardiography VEVO-1100 after 28 days. mRNA expression of (c) ANP, $^{a}P = 0.0025$, $^{b}P < 0.0001$ vs. isoproterenol treated rats., BNP, $^{c}P = 0.0125$, $^{d}P = 0.0105$ versus isoproterenol treated rats., VEGF-R1, $^{e}P = 0.0258$, $^{f}P = 0.0027$ vs. isoproterenol treated rats., NPP-A, $^{g}P = 0.0050$, $^{h}P = 0.0006$ versus isoproterenol treated rats., FGF-21, $^{i}P = 0.0210$, $^{j}P = 0.0102$ versus isoproterenol treated rats., β-MHC, $^{k}P = 0.0116$, $^{l}P = 0.0088$ versus isoproterenol treated rats., and COL18a1, $^{m}P = 0.0003$, $^{n}P = 0.0002$ vs. isoproterenol treated rats. Data were expressed as mean ± Standard error of mean

showed that CDR-267-F018 had shown a tight control over inflammation by decreasing TNF-α levels, expression of e-selectin, and circulating EMPs level, eventually protecting the heart. Leukocyte transmigration and platelet adhesion requires adhesion factors and has been shown to aggravate disease.[29]

Cardiac fibroblast cells are well known for their dynamic behavior during heart development, disease, and aging. Under pathological hypertrophy, inappropriate fibroblast proliferation contributes toward the secretion of collagen which ultimately led to the accumulation of extracellular matrix (ECM) in the heart and contributes toward the heart failure.[30] Excessive activation of MMP's concurs with left ventricular hypertrophy and moreover lack of MMP-2 in mice had shown a beneficial effect in modulating cardiac hypertrophy.[31] A remarkable reduction in pro-MMP2 activity followed by reduction in collagen deposition accounted for the initiation of ECM remodeling process.[32]

Endothelial cells and fibroblasts contribute as a major nonmyocyte population in the adult human heart around 60%[33] which act in paracrine manner to facilitate the cardiac growth. Prolonged activation of β2 adrenergic receptor lead toward the alteration of vascular contractility because of Ca^{2+}-K^{+} activation in VSMC which could further translate the signaling to endothelial cells led toward the oxidative imbalance by uncoupling eNOS through β2 adrenergic receptor/Giα signaling pathway[34] resulting in decreased angiogenesis.[35] Increased angiogenesis and capillary density are one of the major regulators for improving myocardial vascularization and exerting a beneficial effect on cardiac hypertrophy by regulating myocardial hypoxia and growth.[36] Our findings revealed that CDR-267-F018 had shown an increased tubulogenesis which further supported by the upregulation of *VEGF-R1* at mRNA level under *in vitro* and *in vivo* condition. We hypothesize that decreased CD31 staining in tissue confirms that the surviving endothelial cells might increase the *VEGF-R1* expression

Table 1: *Xylocarpus molluccensis* alcoholic extract improved functional parameter of hypertrophic heart

For CDR-267-F018-12.5mg/kg treated rat group				
	Control	Isoproterenol- 5mg/kg	Propranolol-5mg/kg	CDR-267-F018-12.5mg/kg
Left Ventricle Mass (mg)	639.71±16.86	1239.43±77.99	988.58±47.70*	1007.88±41.87
Left Ventricular Internal Diameter (Diastole, mm)	6.70±0.155	5.43±0.132	6.84±0.226*	6.03±0.221
Left Ventricular Internal Diameter (Systole, mm)	4.01±0.172	1.19±0.253	3.32±0.297*	2.10±0.248
For CDR-267-F018-25mg/kg treated rat group				
	Control	Isoproterenol- 5mg/kg	Propranolol-5mg/kg	CDR-267-F018-25mg/kg
Left Ventricle Mass (mg)	668.84±13.69	1288.89±85.24	1077.87±64.39*	904.15±46.61#
Left Ventricular Internal Diameter (Diastole, mm)	7.10±0.470	5.14±0.089	7.06±0.326*	6.71±0.354#
Left Ventricular Internal Diameter (Systole, mm)	4.57±0.301	1.90±0.285	3.12±0.294*	3.19±0.284#
For CDR-267-F018-50mg/kg treated rat group				
	Control	Isoproterenol- 5mg/kg	Propranolol-5mg/kg	CDR-267-F018-25mg/kg
Left Ventricle Mass (mg)	736.11±13.328	1483.45±60.003	1050.25±18.186*	1227.04±52.461#
Left Ventricular Internal Diameter (Diastole, mm)	7.27±0.562	5.19±0.157	7.39±0.103*	6.22±0.321#
Left Ventricular Internal Diameter (Systole, mm)	4.76±0.307	1.90±0.287	3.11±0.297*	3.33±0.283#
For curative CDR-267-F018-25mg/kg treated rat group				
	Control	Isoproterenol- 5mg/kg	Propranolol-5mg/kg	CDR-267-F018-25mg/kg
Left Ventricle Mass (mg)	517.91±14.27	1261.81±1.72	854.67±59.39*	946.00±36.42#
Left Ventricular Internal Diameter (Diastole, mm)	7.33±0.532	5.93±0.86	6.78±0.32	6.23±0.40
Left Ventricular Internal Diameter (Systole, mm)	4.66±0.371	1.65±0.43	3.61±0.63*	3.07±0.27#

*Indicate significance between isoproterenol - 5 mg/kg and propranolol - 5mg/kg treated groups, #Indicate significance between isoproterenol - 5 mg/kg and CDR-267-F018 - 25 mg/kg and 50 mg/kg treated groups. Summarized data for rats that underwent treatment with CDR-267-F018 - 12.5 mg/kg, 25 mg/kg, and 50 mg/kg and CDR-267-F018 - 25 mg/kg curative approach. Data were expressed as mean±SEM. SEM: Standard error of mean; CDR-267-F018: Xylocarpus molluccensis alcoholic extract

for increased angiogenesis to vascularize the region. It could also be that reduced fibrosis might improve the vascularization.[37] Although mild inflammation could be beneficial in improving the angiogenesis and clearing of dead cells during pathological conditions and normal processes.

During hypertrophy, it is well-established that there is a significant increase and prolonged inflammatory load leading to the activation of master transcription regulator, i.e., NFκB, Akt, and ERK.[38] Isoproterenol (β-adrenergic receptor agonist) stimulates the production of NFκB, Akt and ERK in cardiac fibers leading to increased inflammation and cardiac hypertrophy.[39] Similar to our findings, *X. moluccensis* seed limonoids had shown a tight control over inflammation by regulation of NFκB and MAPK pathway.[40] However, further studies are required to understand how CDR-267-F018 specifically arrests inflammation and increase angiogenesis. Overall, we have shown that CDR-267-F018 exhibits protection against cardiac hypertrophy similar to propranol through reduced cytokine levels, increased angiogenesis, decreased EMP generation and improved cardiac output in different models of cardiac hypertrophy.

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Conflicts of interest

There are no conflicts of interest.

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