



Cardioprotective effect of Butanol Fraction of *Rivea ornata* against H₂O₂ Induced Oxidative Stress in H9c2 Cells: Role of its Phenolic Content and Antioxidant Effect

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Authors' contributions

This work was carried out in collaboration between all authors. Author AS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MVJ and ND managed the analyses of the study. Author AS managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2021/v33i29A31569

Editor(s):

(1) Dr. Mohamed Fathy, Assiut University, Egypt.

Reviewers:

(1) Seema Rai, Guru Ghasidas Vishwavidyalaya, India.

(2) N. L. Dashputre, Savitribai Phule Pune University, India.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/68481>

Original Research Article

Received 05 March 2021

Accepted 11 May 2021

Published 18 May 2021

ABSTRACT

Rivea ornata used traditionally for various disease of heart. However, its protective effect against oxidative stress induced injury is unclear and unproved. So, we attempted to investigate the protective role of butanol fraction of *Rivea ornata* on H9c2 cardiomyocytes against oxidative stress induced by H₂O₂. Ethanol extract and its fractions such as n-hexane, ethyl acetate and butanol fractions were assessed for its phytochemical composition, total phenolic and flavonoid content and assessed *in vitro* antioxidant activity by using established assays such as DPPH, H₂O₂ neutralization activity, ferric reduction assays and total antioxidant capacity. Among all the extracts/fractions butanol fraction found with high phenolic (82.4 ± 4.61 mg of GAE/g) and flavonoid

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(105 ± 4.09 mg of QE/g) content as well as possess strong antioxidant abilities evident by comparative lowest IC₅₀ values for DPPH (41.80 µg/mL), H₂O₂ neutralization assay (35.39 µg/m), highest ferric reduction at 120µg/mL and high antioxidant capacity equivalent to quercetin (72.5±2.98 µg/mL). By considering these results, only butanol fraction assessed for its protective effect H₂O₂ induced oxidative stress on H9c2 cardiac cell lines was assessed by MTT, LDH assay and ROS expression studies. Butanol fraction at 25µg/mL maintain H9c2 cell lines viability (in MTT assay), cell integrity (decreased LDH release) and decreased ROS expression (decrease in percentage mean fluorescence). These findings are proved antioxidant rich polyphenolic butanol fraction protective effect against oxidative stress injury observed with H₂O₂ by maintain cell viability, cell integrity and ROS expression.

Keywords: *Rivea ornata*; total phenolic and flavonoid content; antioxidant activity; H9C2 mouse cardiac cell lines; in vitro cardioprotective effect.

1. INTRODUCTION

Cardiovascular diseases (CVDs) are the remains leading cause of mortality and morbidity throughout the world among non-communicable diseases [1]. International bodies such as WHO, European cardiovascular disease statistics 2017 and American heart association current statistics also highlight the death rates associated with cardiovascular diseases [2]. Despite patients taking preventive medicine one in three patients get another stroke or die from heart disease. Among CVDs ischemic heart disease or coronary heart disease are put highest health burden among the population. Ischemic heart disease is generally originated from blocking of coronary artery partially or completely by intraluminal accumulation of fatty plaques and associated thrombi [3]. Establishment of coronary reperfusion by surgery or use of thrombolytics are the main strategy to preserve myocardial function. However, reperfusion induced high oxygen levels to ischemic tissue generate reactive oxygen species (ROS) at a high level resulting in myocardial injury. This phenomenon is called ischemic reperfusion injury [4]. Molecular events associated with this injury is reactive oxygen species highly reactive, unstable attacks components of cell such as lipid membrane, proteins and DNA, initiate apoptosis and finally causes necrosis [5]. To overcome these challenges, administration of antioxidant rich products or medicinal substance before or after reperfusion phase is may be effective strategy to limit the extent of myocardial damage and therefore protect the heart from effects of oxidative stress [6]. So, various researchers from scientific community have been trying to find the effective antioxidant from natural sources, mainly from medicinal plants to combat detrimental effect of reactive oxygen species in ischemic reperfusion injury [7]. The early reports (clinical

and preclinical studies) were mainly focus on antioxidant rich plant extract or its compounds particularly phenolic compounds and flavonoids to overcome this issue [8]. In the search of new bioactive compounds from natural sources we tried to explore the possible role of *Rivea ornata* extract against H₂O₂ induced oxidative stress in H9c2 mouse cardiac cell lines, is an *invitro* model widely used to study efficacy in oxidative stress conditions [9]. Plant is traditionally used in disorders of the gallbladder, a disease of heart, bronchitis, and fatigue. In the Konkan, juice of the plant is used to treat piles [10]. A recent study reported methanolic extract *Rivea ornata* contains bergenin, is a polyphenolic compound as an active compound [11]. Bergenin has found diverse pharmacological actions such as antioxidant activity, anti-inflammatory activity, hypolipidemic activity, platelet aggregation inhibition activity etc. [12]. As result of broad pharmacological actions and its reported phenolic compounds we tried to explore the its protective effect on H₂O₂ induced oxidative stress in H9c2 cardiac cell lines.

2. MATERIALS AND METHODS

2.1 Introduction about Plant

Rivea ornata plant is erect herb with 1-2 meters stem. Leaves are orbicular to reniform with upper side glabrous and densely whitish tomentose underside. Flowers are oblong to elliptic oblong. Fruits are glossy brown color and seed are brown in color embedded in crumbly crust.

2.2 Collection and Identification of the Plant Material

Fresh leaves collected from flowering plant of *Rivea ornata* Aitch (Convolvulaceae family) were collected from seshachalam Tirumala hills during

between December to January and dried under shade for two weeks. The plant was authenticated by Prof. K. Madhava Chetty, Taxonomist and a voucher specimen (voucher number:0913) has been stored at department of Botany, S.V. University, Tirupati, Andhra Pradesh (India).

2.3 Preparation of Plant Extract

Dried leaves of *Rivea ornata* was ground into a coarse particle in the mechanical grinder and 1 kg of powdered plant material extracted with ethanol (99.99v/v) for the period of one week with occasional mechanism stirring. The obtained filtrate concentrated under reduced pressure by using rotary evaporator. The resulting solvent free extract residue stored at refrigerator at 2-8 °C until further use [13].

2.4 Fractionation of Ethanol Extract

Hot water dissolved ethanol extract was fractionated by solvents from nonpolar to polar (n-hexane (non-polar), ethyl acetate (medium polar) and n-butanol (low polar)) respectively. After that, each fraction was concentrated using rotary evaporator. Finally, they tested for presence of flavonoids and other phytochemicals using coloration reactions reported in standard reference books and assessed the flavonoid and phenolic content [14].

2.5 Phytochemical Screening

The solvent free extracts/ fractions (ethanol extracts, n-hexane fraction, ethyl acetate, butanol fraction and water fraction) were dissolved in their mother solvents and is subjected to the following preliminary qualitative phytochemical screening as per the literature reported [15].

2.6 Test for Carbohydrates

2.6.1 Molisch's test

To 1 mL of extracts/ fractions, pour few drops of α -naphthol in alcohol, shake and from the sides of the test tube add conc. H_2SO_4 that results in the formation of the violet ring in between two liquids indicate the presence of carbohydrates.

2.7 Test for Proteins

2.7.1 Biuret test

To 1mL of extracts/ fractions, add a strong base NaOH (4%w/v) in equal volume and add a few

drops of copper sulphate (1%w/v). Presence of protein is indicated by appearance of purple color.

2.8 Test for Steroids/Triterpenoids

2.8.1 Salkowski test

To 1 mL of extracts/ fractions, add 1 mL of chloroform and 1mL of conc. H_2SO_4 . Shake well for few minutes to get the red color.

2.10 Test for Glycosides

2.10.1 Anthraquinone glycosides

Bortrager's test: In a test tube boil the 1mL of extracts/ fractions with conc. H_2SO_4 for 5 min and filter it. To cold filtrate adds dichloromethane or $CHCl_3$ in equal volume after that separate the $CHCl_3$ layer, and to the residue add dil. Ammonia. Ammoniacal layer appears rose pink to red color indicate the presence of anthraquinone glycosides.

2.10.2 Cyanogenic glycosides

Sodium Picrate test: Few drops of $CHCl_3$ are added to the 1mL of extracts/ fractions along with conc. H_2SO_4 . Picrate paper is kept on the test tube and keeps on water bath turns yellow picrate paper into the red is considered the presence of cyanogenic glycosides.

2.10.3 Coumarin glycosides

To the 1 mL of extract of extracts/ fractions, add 1 mL of base (1N NaOH) that results in the formation of blue or green fluorescence. Indicate the presence of coumarin glycosides.

2.11 Test for Flavonoids

2.11.1 Shinoda test

To 1mL of extracts/ fractions, add few drops of conc. HCl. After that add 0.5g magnesium turnings, presence of flavonoids indicated by appearance of pink color.

2.11.2 $FeCl_3$ test

Few drops of $FeCl_3$ added to the test tube containing a 1mL of extracts/ fractions, gives intense green color gives proof of flavonoids presence.

2.11.3 Alkaline reagent test

NaOH solution is added to the 1mL of extracts/ fractions, the yellow color turns to colorless after addition of dilute acid, indicated its presence with flavonoids.

2.11.4 Lead acetate test

Lead acetate solution is added to the 1mL of extracts/ fractions that results in a yellow precipitate, indicated its presence with flavonoids.

2.12 Test for Saponins

2.12.1 Foam test

When the 1 mL of extract of extracts/ fractions is mixed with water and shake it vigorously gives foam that is stable for 15 min.

2.13 Test for Alkaloids

2.13.1 Dragendorff's test

To 1 mL of extract of extracts/ fractions add a few drops of Dragendorff's reagent. Appearance of orange-brown precipitate indicate the presence of alkaloids.

2.13.2 Mayer's test

Few drops of Mayer's reagent with 1mL of extracts/ fractions give a creamy white precipitate indicate the presence of alkaloids.

2.14 Test for Tannins and Phenolic Compounds

To 1mL of extracts/ fractions, add few drops of the following reagents and presence of tannins and phenolic compounds indicated by following color reactions.

FeCl₃ (5% w/v) solution: Deep blue-black color (Tannins)

Dil. Iodine solution: Transient red color (Tannins)

Dil. HNO₃: Reddish yellow color

Acetic acid: Red color solution

Lead acetate solution: White precipitate (Tannins)

2.15 Estimation of Total Phenolic Content

Phenolic contents of crude ethanol extracts and its fraction (n-hexane, ethyl acetate, butanol and

aqueous fraction) was estimated by Folin-Ciocalteu reagent. The (0.5 mL) diluted extracts and its fractions at concentration of 1mg/mL and varying concentration of gallic acid (20,40 ,60,80 and 100 µg /mL) mixed with 5 mL of Folin-Ciocalteu's reagent (10% v/v) and shaken it well. Add 4 mL of Na₂CO₃ (7% w/v) solution after 5 min to the above solution. Incubate the solution for 90 min at room temperature and absorbance was determined at 750 nm using a UV-Visible spectrophotometer. The total phenolic content was expressed in units as milligrams of gallic acid equivalents (GAE) per gram of dry weight (mg GAE/g DW). All samples were analyzed in triplicate [16].

2.16 Estimation of Flavonoid Content

The 1 mL of crude ethanol extracts and its fraction (n-hexane, ethyl acetate, butanol and aqueous fraction) and standard solutions of quercetin at concentration of 20, 40, 60, 80 and 100 µg /mL were mixed with 2.5 mL of freshly prepared AlCl₃(10%w/v) after 5 min and add 1M NaOH of about 2 mL and total volume make up to 10 mL with DW. The whole solution was mixed up and absorbance was measured against blank at 510 nm in UV-Visible spectrophotometer after 30 minutes of incubation period at room temperature. The total flavonoid content was expressed as milligrams of Quercetin equivalents (QE) per g dry weight (mg QE/g DW). Analysis of each sample in triplicate manner [17].

2.17 In vitro Antioxidant Activity

2.17.1 DPPH free radical scavenging activity

Aliquots of 3 mL of ethanol extract and its fractions (20 – 120 µg/ mL) and methanol solubilized vitamin C (20 – 120 µg/ mL) were added to 0.04% w/v DPPH solution (1 mL) separately. Methanol solubilized DPPH was considered as a control. Incubated test and standard solutions for 30 min at a dark place at 25°C. The colored solution absorbencies were recorded at 517 nm. The IC₅₀ was determined from the percentage scavenging versus concentration plot [18].

$$\% \text{ inhibition: } \frac{Ab \text{ of control} - Ab \text{ of sample}}{Ab \text{ of control}} \times 100$$

2.18 Phosphomolybdate Assay (Total Antioxidant Capacity)

The total antioxidant capacity was determined by phosphomolybdate method. An aliquot of 1 mL (1mg/mL) of extracts/ fractions (crude ethanol extracts and its fractions-n-hexane, ethyl acetate, butanol and aqueous) were mixed with 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 765 nm against a blank. A typical blank contained 1 mL of the reagent solution and the appropriate volume of the solvent and incubated under the same conditions. The antioxidant capacity calculated from quercetin curve (concentration versus absorbance) and expressed as mg quercetin equivalent per gram of dry weight [19].

2.19 Hydrogen Peroxide Scavenging Assay

0.1mL of the extract (1mg/mL) with varying concentration of ethanol extract and its fractions (20 – 120 µg/ mL) made up to 0.4mL with phosphate buffer (50mmol, pH 7.4) and 0.6mL of hydrogen peroxide (2mmol in phosphate buffer, pH 7.4) was added. The absorbance of the solution was measured after 10 min against a blank (only phosphate buffer) at 230 nm [20].

$$\% \text{ inhibition} = \frac{\text{Ab of control} - \text{Ab of sample}}{\text{Ab of control}} \times 100$$

2.19.1 Ferric reducing power

Reducing power assay, the reducing power of the crude ethanol extracts and its fraction (n-hexane, ethyl acetate, butanol and aqueous fraction) was determined by a method described by Oyaizu. Different concentrations of extracts/fractions in 1 mL of distilled water were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferrocyanide (1%). The mixtures were incubated at 50°C for 20 min. Aliquots 2.5 mL of trichloroacetic acid (10%) were added to the mixtures and centrifuged at 3000 rpm for 10 min. The supernatant of the solution (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of FeCl₃ (0.1%w/v). The absorbance was measured at 700 nm [21].

2.20 In vitro Cardio Protective Effect of Butanol Fraction

2.20.1 Cell viability assay by MTT assay

The viability of H9c2 cardiac cells were estimated by measuring the absorbance of formazan product from MTT by EZ count MTT cell assay kit (spectrophotometric method). 200µL of H9c2 cardiac cell suspension was incubated in a 96-well plate (20,000 cells per well) with butanol fraction (5,10,15,20,25 µg/mL) for 12 hours and later further incubated for 24 hours with 200µM H₂O₂. After washing with 100 µL of DMSO, the absorbance of dissolved formazan crystals measured on an ELISA reader at 570nm and 630nm used as reference wavelength. The IC₅₀ value was determined by using linear regression equation i.e., Y =Mx + C. Here, Y = 50, M and C values were derived from the viability graph [22].

2.20.2 LDH Release study ON Cell lines

Quantification of LDH released from damaged H9c2 cardiac cells was estimated by Lactate Dehydrogenase Assay Kit (#102526, Abcam). Cultured H9c2 cardiac cells incubated in a 6-well plate (density of 3 x 10⁵ cells/ 2 mL) initially with butanol fraction (5,10,15,20,25 µg/mL) for 12 hours followed by further 24 hours incubation with 200 µM of H₂O₂ as a toxic agent. After this, add 50 µL of the reaction reagent (Iodonitrotetrazolium Chloride) and allow to incubate at 37°C for 30 minutes. Read the absorbance of cell medium on an ELISA reader at 450nm wavelength. One-unit LDH is the amount of enzyme that catalyzes the conversion of lactate to pyruvate to generate 1.0 µmol to NADH per minute at 37°C [23].

2.20.3 ROS expression by flow cytometry

Reactive oxygen species (ROS) expression is estimated by flow cytometry assay kits. Non-fluorescent 2', 7'-dichlorodihydrofluorescein diacetate (H2DCFDA) is converted into fluorescent 2', 7'- dichlorofluorescein (DCF) in the presence of ROS. Intensity of fluorescein of DCF is proportional to ROS levels and is measured by flow cytometry. Culture cells in a 6-well plate (3 x 10⁵ cells/2 mL) are incubated with butanol fraction (5,10,15,20,25 µg/mL) for 12 hours. After washing with 1mL of phosphate buffer, treat the cells with 100 µL of H₂O₂ (200 µM) and incubate the cells for 12 hours. Dulbecco's phosphate-buffered H2DCFDA (4 mM) solution is added to wells and incubated at

37 °C for 30 min protected from light. Analyze the plate by flow cytometry using the 488 nm laser for excitation and detection at 535 nm [24].

3. RESULTS

3.1 Percentage Yield of *Rivea ornata* Extracts/Fractions

The percentage yield of ethanol and its fractions (n-hexane, ethyl acetate, butanol and aqueous fraction) was found as follows, ethanol extract (18.6g), n-hexane (6.42 g), ethyl acetate (3.58g), butanol fraction (4.28 g) and water fraction (1.24 g).

3.2 Phytochemical Composition of *Rivea ornata* Extracts

Observations of preliminary qualitative phytochemical analysis of crude ethanol extract and its fractions such as hexane, ethyl acetate and remaining aqueous portion revealed presence of secondary metabolites such as alkaloids, cardiac glycosides, steroids, flavonoids and tannins. But butanol fraction had rich sources of flavonoids and phenolic compounds than other extract/ fractions. Hence butanol fraction could be the possible sources of cardio

protective principles in our plant. Results are shown in Table 1.

3.3 Total Phenolic and Flavonoid Content

Phenolic and flavonoid content were calculated from calibration curve of gallic acid ($y = 0.0083x - 0.0214$, $R^2 = 0.9746$) and quercetin ($y = 0.0059x + 0.0857$, $R^2 = 0.9814$) respectively. Regression line of gallic acid and quercetin shown in Fig. 1. The result of the present study revealed that butanol fraction has highest contents (82.4 ± 4.61 mg of GAE/g, 105 ± 4.09 mg of quercetin/g) compared to crude ethanol and its other fractions such as n-hexane, ethyl acetate and aqueous fraction. Results are shown in Table 2.

3.4 *In vitro* Antioxidant Activity

3.4.1 DPPH radical neutralizing capacity

Butanol fraction of *Rivea ornata* exhibit higher DPPH radical neutralizing capacity. The percentage scavenging ability at higher concentration (120 μ g/mL) was 84.8 ± 0.622 %. This percentage inhibition was more than crude ethanol extract and other fractions include n-hexane, ethyl acetate and aqueous fraction. The potency of extract is expressed by its IC_{50} value. Lesser the IC_{50} greater the potency. Based on

Table 1. Physical characteristics of extract of different solvents

S. No	Test for	Crude ethanol extract	Hexane fraction	Ethyl acetate fraction	Butanol fraction	Aqueous fraction
1.	Carbohydrates	+	-	-	+	+
2.	Steroids	+	+	+	+	+
3.	Cardiac glycosides	+	+	+	-	-
4.	Saponin glycosides	-	-	-	-	-
5.	Coumarins	-	-	-	-	-
6.	Flavonoids	+	-	+	++	.
7.	Alkaloids	+	-	+	+	+
8.	Tannins	+	-	-	+	-
9.	Terpenoids	-	+	-	-	-
10.	Anthraquinone glycosides	-	-	-	-	-

Table 2. Total phenolic and flavonoid contents of *Rivea ornata* extracts and its fraction

Extract	TPC (mg of gallic acid equivalent/g)	TFC (mg of quercetin equivalent/g)
Crude ethanol extract	56.9 ± 4.30	55.1 ± 2.79
n-Hexane fraction	$6.39 \pm 1.38^{***}$	$16.7 \pm 0.351^{***}$
Ethyl acetate fraction	64.2 ± 1.56	$77.5 \pm 6.20^*$
Butanol fraction	$82.4 \pm 4.61^{**}$	$105 \pm 4.09^{***}$
Aqueous fraction	$18.1 \pm 3.52^{***}$	$27.7 \pm 4.10^{**}$

All values are expressed as the mean \pm SD of three replicate. TPC= total phenolic content, GAE= gallic acid equivalent, TFC= total flavonoid content

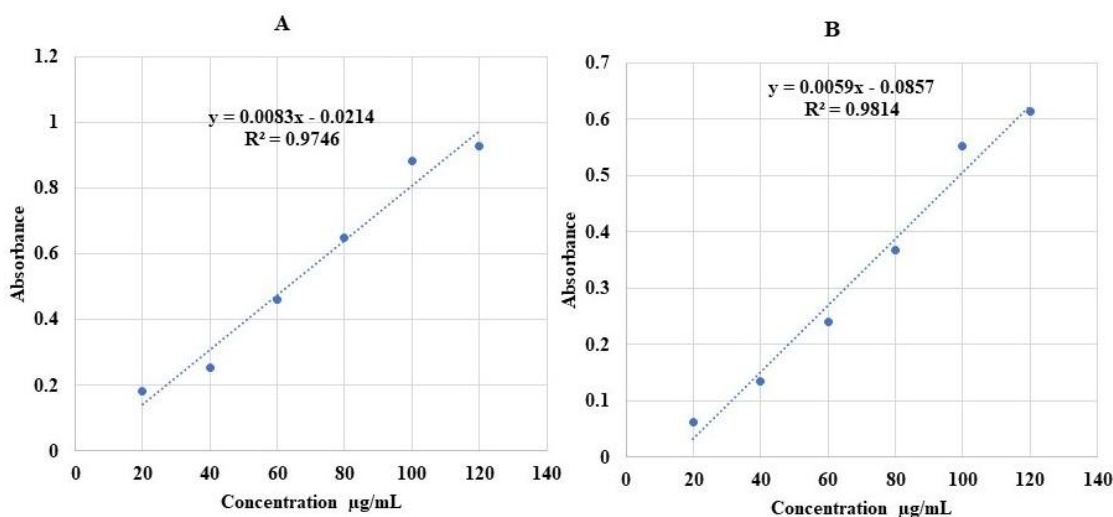


Fig. 1. Regression line of gallic acid (A) and quercetin (B)

IC₅₀ potency order of extract/ fraction was found as follows: Butanol fraction (41.80 µg/mL) > EA extract (69.13 µg/mL) > Ethanol extract (96.35 µg/mL) > Aqueous fraction (136.62 µg/mL) > NX extract (174.57 µg/mL). Results are shown in Table 3 and Fig.2.

3.4.2 H₂O₂ molecule neutralizing capacity

Butanol fraction showed highest percentage of hydrogen peroxide scavenging activity as 89.7±0.718% compared to other extract (ethanol extract, hexane, ethyl acetate and aqueous fraction). IC₅₀ value of butanol fraction was as

35.39µg/mL (lower than any other extract or fractions). Results are shown in Table 3 and Fig.2.

3.4.3 Ferric reduction power

Ferric reducing power of butanol extract was increased dose dependently as indicated by increase in absorbance at 700nm. Highest concentration (120µg/mL) showed absorbance (0.752± 0.012 nm) than any other extract or fractions. Highest absorbance indication of higher antioxidant capacity of the extract. Results are shown in Fig.2.

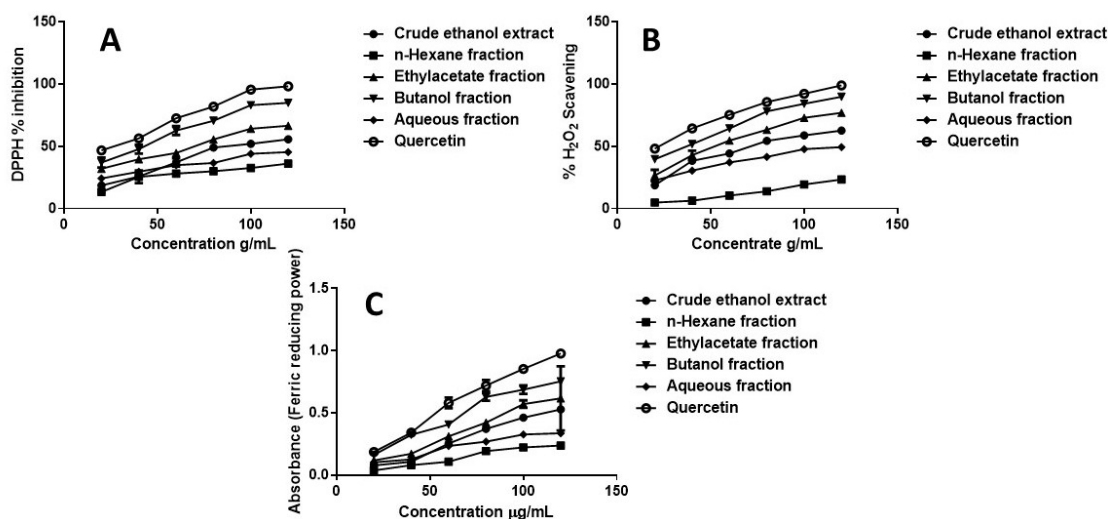


Fig. 2. Effect of crude ethanol extract and its fraction of *Rivea ornata* on various *in vitro* antioxidant assay. A. DPPH % inhibition, B. % H₂O₂ scavenges, C. Ferric reducing power assay

3.4.4 Total antioxidant activity (Phosphomolybdic acid method)

The reduction of molybdate (VI) by ethanol extract or its fractions produce green color complex, measured the absorbance at 695 nm. The absorption of reduced molybdate (V) in the presence of ethanol extract or its fractions was increased with increasing concentration. The total antioxidant capacity was calculated from standard curve of quercetin (shown in Fig.3) and expressed as mg equivalent of quercetin per gram of dried weight. Based on total anti-oxidant capacity, extract/fractions arranged descending order as follows butanol fraction (72.5±2.98 mg QUE/g of DW), ethyl acetate fraction (52.0±0.217 mg QUE/g of DW), ethanol extract (33.3±1.08 mg QUE/g of DW), Aqueous fraction (16.7±2.39 mg QUE/g of DW) and n-hexane (11.1±0.451mg

QUE/g of DW) respectively. Results were shown in Table 3.

3.5 Correlation between Phenolic Content and its Antioxidant Activity

As per observation of the Fig. 4 we found that there is a strong correlation between phenolic content and its IC₅₀ value of DPPH assay. Which is being represented with liner regression equation $Y = -1.6227x + 177.69$. Which indicate that higher phenolic content, lower is the IC₅₀ value. This correlation clearly understands the high phenolic content of the butanol fraction with lower IC₅₀ value is responsible for its high antioxidant activity compared to ethanol and other fractions, which could be the basis for taking butanol extract only for further *in vitro* cardio protective study.

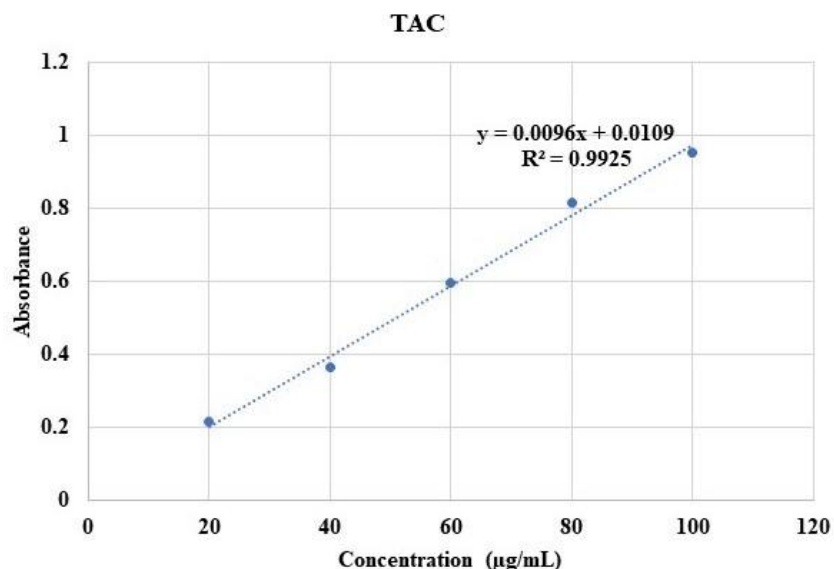


Fig. 3. Calibration curve of quercetin

Table 3. IC₅₀ value and total antioxidant capacity of different extract of *Rivea ornata*

Type of extract/ standard	IC ₅₀ / µg mL ⁻¹		Total antioxidant capacity (mg of quercetin/g)
	DPPH Radical	H ₂ O ₂	
Ethanol extract	96.35	79.36	33.3±1.08
n-hexane fraction	174.57	263.85	11.1±0.451
Ethyl acetate fraction	69.13	57.77	52.0±0.217
Butanol fraction	41.80	35.39	72.5±2.98
Aqueous fraction	136.62	113.92	16.7±2.39
Quercetin	24.17	14.908	

All values are expressed as mean ± SD of three replicate. IC₅₀= inhibitory concentration 50

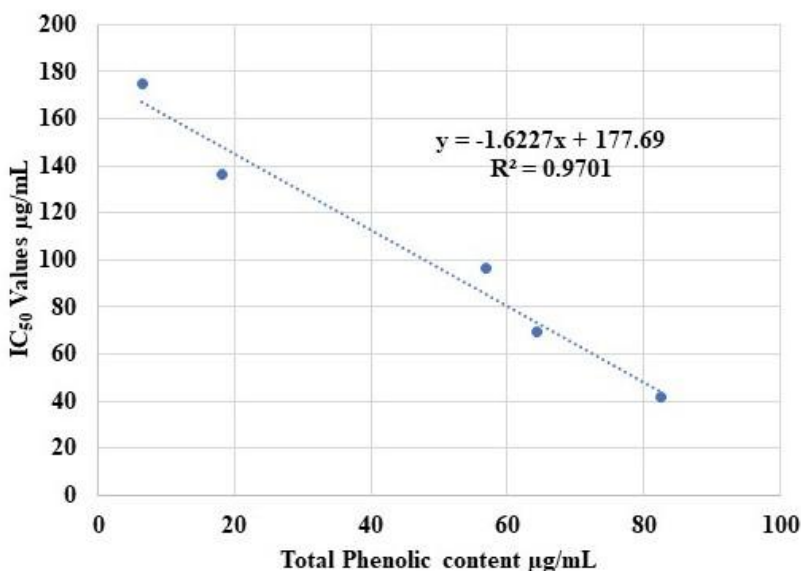


Fig. 4. Correlation between phenolic content and IC₅₀ value of DPPH assay

3.6 *In vitro* Cardio Protective Effect

3.6.1 MTT assay to measure the cell viability

H₂O₂ at concentration of 200 µg/mL drastically reduces the viability of H9c2 cardiac cell lines to 12.6% compared to control (100%). butanol fraction found to be significantly ($p < 0.001$) increased the viability of cardiac cells with increase in concentration (10, 15, 20 and 25 µg/mL). Highest concentration (25 µg/mL)

observed highest percentage viability with 79.9%. These observations also reflected in microscopic changes as follows *i.e* Untreated cell lines observed full confluent and spindle shaped cells but in the in the H₂O₂ exposed cells observed less confluent and appeared as round shaped non-viable cells. Whereas butanol fraction of *Rivea ornata* treated H9c2 cell lines observed with dose dependent increase in viable cells. Results are shown in Fig.5 and Fig.6.

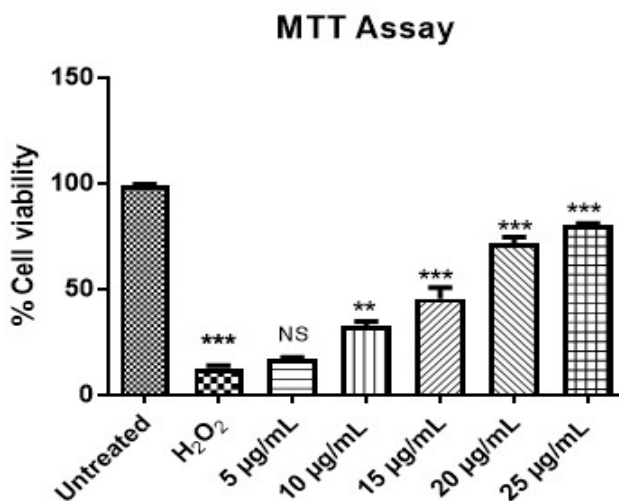


Fig. 5. Effect of butanol fraction of *Rivea ornata* on H₂O₂ induced cell viability. Each value is mean of three replicates ± SEM. $p < 0.05$, $p < 0.01$ and $p < 0.001$ represented by *, **, *** subsequently. NS means non-significance

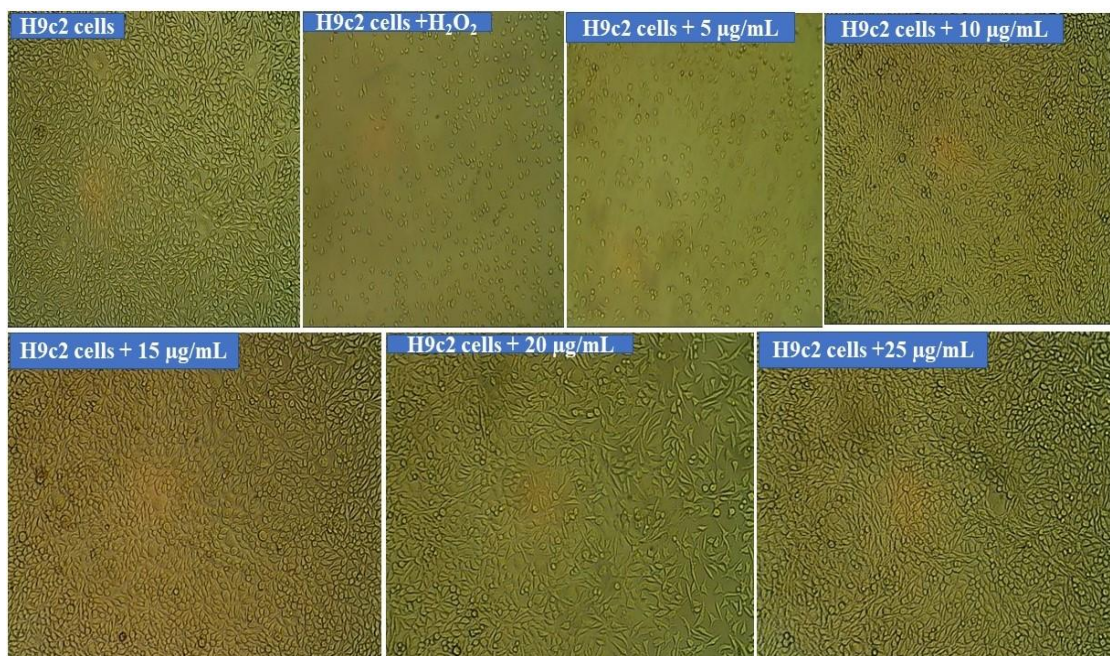


Fig. 6. Pictures of representation of H9c2 cell lines viability in different groups. Untreated H9c2 cell lines: Maximum cell viability; H₂O₂ treated H9c2 cell lines: Observed with low viable cells; 5,10,15,20 and 25 µg/mL of butanol fraction of *Rivea ornata* treated H9c2 cell lines: observed with dose dependent increase in viable cells

3.6.2 LDH release assay

Supernatant of H9c2 cell suspension in H₂O₂ exposure observed with higher level of LDH. Pre-treatment of H9c2 cells with butanol fraction

prevent the H₂O₂ induced oxidative damage which is reflected by dose dependent (15,20 and 25 µg/mL) decrease in LDH release with significant value of P<0.001. Results are shown in Fig. 7.

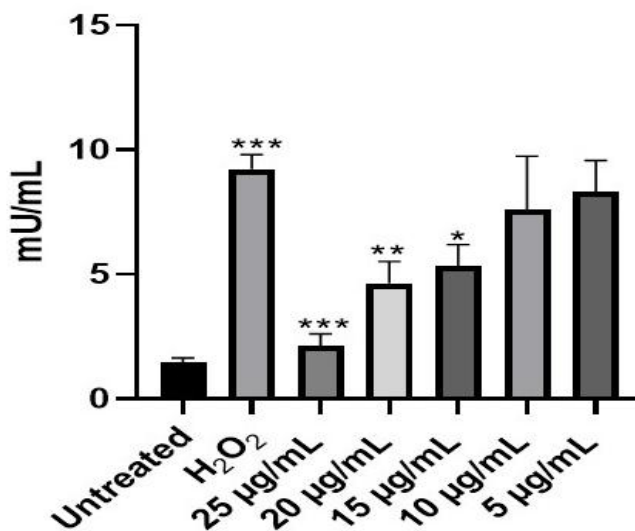


Fig. 7. Effect of butanol fraction of *Rivea ornata* on LDH release from H9c2 cardiac cells which again indicative of cell viability. Each value is mean of three replicates ± SEM. p<0.05, p<0.01 and p<0.001 represented by *, **, * subsequently. NS means non-significance**

3.6.3 ROS expression studies

Significant ROS expression in H₂O₂ exposure H9c2 cells was indicated by increased in fluorescent DCFDA positive cells with significant increase in mean fluorescence intensity. butanol fraction at the concentration of 25, 20 and 15 µg/mL observed significant ($p < 0.001$) decrease the mean fluorescence intensity compared to control. The concentration of 25 and 20 µg/mL showed highest decrease in ROS expression. Results are shown in Fig. 8.

4. DISCUSSION

Ischemic and reperfusion injury to myocardium is a common pathological event has been observed after reestablishment of coronary blood flow. There is a sufficient evidence that free radicals formed during reperfusion phase impose structural and functional damage leading to cardiac apoptosis and heart failure [25]. Intense reactive oxygen species formed during reperfusion phase and inherent lower levels of antioxidant defensive enzymes are the basis for failure of handling of oxidative storm by cardiac tissue, progress to irreversible myocardial injury. Strategic plan may follow to bring the balance between the levels of free radicals and defensive enzymes and thereby we preserve the functional capacity of heart [26]. This strategy is had been workout by introduce the oxidative stress alleviating substance before or after reperfusion phase. Even though we practiced it since long time but still we don't get appropriate results to in order get in to clinical practice [27]. Hence, searching for effective leads from natural compounds with antioxidant capacity is still considered as viable option to prevention of reperfusion injury [28]. Diversified pharmacological actions of polyphenols includes antioxidant, anti-inflammatory, antiapoptotic effect etc., can considered the polyphenolic compounds as potential candidates for oxidative stress induced cardiovascular diseases like atherosclerosis, hypertrophy, endothelial dysfunction and MI. Hence, prevention of this pathological phenomenon by giving polyphenolic rich extracts or fractions is gaining interest in the recent years [29]. Leaves of *Rivea ornata* were extracted with ethanol and further fractionated by different solvents from nonpolar to polar such as n-hexane, ethyl acetate and butanol to get the polyphenol rich fraction. Polyphenol richness was found in the butanol fraction than n-hexane and ethyl acetate fractions, which was confirmed qualitatively by the presence of flavonoids and

tannins as well as its high contents of phenols and flavonoids in butanol fraction compared to other extracts [30]. Maximum total phenolic content, total flavonoid content of butanol fraction was 34.67 ± 2.03 (mg of GAE/g) and 23.80 ± 2.36 (mg of Quercetin/g) compared to other solvent extracts/fractions (ethanol-hexane and ethyl acetate). Being a polar solvent butanol, capable to extract polar polyphenols from plant materials. With this we came to know the that butanol fraction was consider the potential sources of polyphenols. Many studies have been reported that polyphenol rich extract or fraction keeps the oxidative stress under control by virtue of its ability to capturing free radicals. Polyphenols have free hydroxyl groups especially -OH on third carbon interact with highly reactive, unstable free radicals and form stable, non-reactive, nontoxic complexes which protect the biomolecules of healthy cells [31]. So, in order to confirm the antioxidant potential we performed various *in vitro* antioxidants assays such as DPPH free radical scavenging, hydrogen peroxide scavenging, ferric reduction assay and total antioxidant activity (phosphomolybdate assay) [32]. Purple colored stable DPPH radical undergo decolor in the presence of electron donating polyphenols rich extract/fraction. The degree of discoloration is dependence up on antioxidant potency of extract. Large decrease in absorbance of DPPH indicates higher the scavenging ability of the extracts/fractions. Hydrogen peroxide is itself not a highly reactive but it plays a role in cellular oxidative stress by converting to other cytotoxic free radicals in the presence of iron such as hydroxyl radical (. OH), singlet oxygen and superoxide anion. These radicals can cause lipid peroxidation and DNA damage. Butanol fraction exhibit highest percentage neutralization ($89.7 \pm 0.718\%$) at 120 µg/mL. Polyphenols of butanol fraction may donate the electrons to H₂O₂ and thereby neutralize in to water. So, it is protecting from oxidative damage [33]. From the linear regression plot IC₅₀ were calculated to express the antioxidant potency of ethanol extract and its fraction (n-hexane, ethyl acetate and butanol fractions) for the DPPH free radical scavenging, hydrogen peroxide scavenging activity. Lower the IC₅₀ higher the potency and vice versa. From statistical comparison IC₅₀ values, we found that butanol fraction more potent than other extracts/fractions, is indicated by lowest IC₅₀ values for DPPH (251.91 ± 25.40 µg/mL) and H₂O₂ (316.94 ± 12.13 µg/mL) molecules. In the presence of butanol fraction, Fe³⁺/ferricyanide complex reduced to ferrous form which show

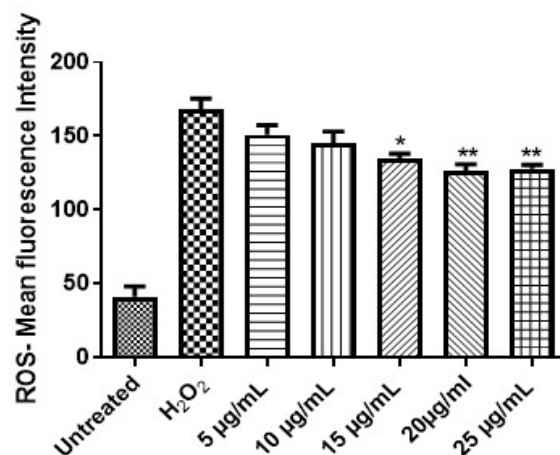


Fig. 8. Effect of butanol fraction of *Rivea ornata* on H₂O₂ induced ROS- Mean fluorescence intensity. Each value is mean of three replicates \pm SEM. $p < 0.05$, $p < 0.01$ and $p < 0.001$ represented by *, **, * subsequently. NS means non-significance**

absorbance at 700nm. Ferric reducing abilities of butanol fraction was increased with increasing the concentration from 20 to 120 $\mu\text{g/mL}$, is indicated by increasing the absorbance of green colored ferric-ferrous complex. Antioxidative reductive components of polyphenolic butanol fraction donate its hydrogen atom may responsible for its significant reducing capacities, it is observed by highest absorbance at 120 $\mu\text{g/mL}$. Therefore, which break the free radicals chain reaction, is necessitated in oxidative stress associated cardiac diseases [34]. Higher antioxidant potential abilities of butanol fraction than other extract/fractions may be because of higher phenolic contents neutralize the toxic free radicals by donating its hydrogen ions resulting in protect the cellular contents such as DNA, proteins and lipid membrane and further prevent the development of ischemic injury in cardiac tissue. Estimation of total antioxidant capacity of extracts gives an idea of its antioxidant potential, is expressed as mg of quercetin equivalent per g of extract. In our finding's butanol fraction of *Rivea ornata* demonstrated more antioxidant capacity (97.35 ± 22.14 mg of Quercetin equivalent/g of extract) than other extracts/fractions (ethanol extract, n-hexane and ethyl acetate fractions). This is attributed to presence of electron donating antioxidant principles such as polyphenols and flavonoids. These results were matched with earlier studies [35] Since butanol fraction was observed good activity profile respect to phytochemical, phenolic and flavonoid content and *in vitro* antioxidant assays, we choose butanol fraction alone to

screen *in vitro* protective effect against H₂O₂ induced oxidative stress on H9c2 cell lines, general model to study cardio protective effect of bioactive extracts and their phytochemicals before translation to animal studies [36]. H₂O₂ is one the major toxic products of molecular oxygen and causes sustained damage to our cells by initiate chain of oxidative stress by producing toxic radicals like superoxide and hydroxyl radicals [37]. By using H₂O₂ we can mimic the conditions associated with ischemic reperfusion injury induced apoptosis and reactive oxygen expression and its was assessed by cell viability assay include MTT assay, LDH release and ROS expression. MTT assay is used asses the viability of cells and it is evaluated by colorimetric measurement of purple color formazan from viable H9c2 cell lines. The intensity of formazan color is directly related to cell viability. H9C2 cell lines incubated with H₂O₂, observed showed marked reduction in percentage cell viability to 12.6 % compared to control with 99.9% cell viability [38]. Pre incubation with *Rivea ornata* butanol fraction increase the percentage cell viability dose dependently with highest percentage cell viability (79.9%) was observed with at 25 $\mu\text{g/mL}$. Cellular viability can also observe morphologically in our experiment that, H₂O₂ alone incubated cells observed lowest viability as indicated by very low cell confluent as well as loss of cell shape to oval shape compared to control cells. As the concentration of butanol fraction was increases, the more cell confluent and attainment of normal shape (spindle) were observed. Highest cell viability

observed clearly at 25 µg/mL. As the concentration of butanol increase with increase in phenolic content might be responsible for protection of oxidative effect of H₂O₂. In another viability test, cardiac specific LDH released increased up to 9.24 µg/mL with the cytotoxic effect of H₂O₂ compared to control cell lines with 1.44 µg/mL, indicate the marked reduction of cell viability in H₂O₂ incubated cells [39]. Similarly, *Rivea ornata* butanol fraction pre incubated H9c2 cells exhibit dose dependent reduction of release of LDH. Lowest release of LDH (2.14 µg/mL) at 25 µg/mL indicate that the maximum protection from oxidative cell damage with H₂O₂. Same conclusion applied as that of MTT assay that phenolic content in the butanol fraction might be responsible for cardiac cellular damages, results are in line with earlier reports [40]. Measurement of reactive oxygen species in H9c2 cells is best predictor of status of oxidative stress in healthy and diseased cells. Attenuation of excessive ROS expression by polyphenolic rich extract could be the ideal way to combat from oxidative stress [41]. In the presence of H₂O₂ (ROS family like molecule) non fluorescent DCF-DA probe converted in to fluorescent dichlorofluorescence (DCF). In the present study H₂O₂ incubated H9c2 cells has higher oxidative stress which is shown by highest percentage (168 %) increase in mean fluorescent of dichlorofluorescence (DCF) compared to lowest in control cells (41.1%) [42]. However, suppression of ROS expression associated with *Rivea ornata* polyphenol butanol fraction was evident by dose dependent reduction of percentage mean fluorescent intensity of dichlorofluorescence (DCF) positive cells [43]. Butanol fraction at concentrations of 15, 20 and 25 µg/mL able to reduced significantly mean percentage fluorescence intensity but 25 µg/mL attain maximum reduction of fluorescence intensity (128%) was observed. Consideration of above results of reduction of oxidative stress associated with H₂O₂ gives us idea about cardio protective effect of folklore medicinal *Rivea ornata* plant.

5. CONCLUSION

From the result of the study, we concluded that butanol fraction contained considerable amount of phenolic content and thus responsible for strong antioxidant abilities as confirmed by various *invitro* models. We found butanol fraction better DPPH scavenging capacity, H₂O₂ neutralizing ability, ferric reducing capacity and higher antioxidant capacity. These effects also reflected by attenuation of H₂O₂ induced

oxidative stress on H9c2 cardiac cell lines. Which is observed as preservation of viability of H9c2 cells noticed by MTT assay and LDH release assay and ROS expression. These results are attributed to high phenolic and flavonoid content in the butanol fraction scavenges free radicals and thus prevented associated cell damage because of poly hydroxy groups. Further *in vivo* study is supposed to do to confirm the cardioprotective activity.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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