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Cardio Protective Activity of Calophyllolide on Doxorubicin Induced Myocardial Damage

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Abstract

This study aimed to examine *in vitro* antioxidant, acute toxicity studies and *in vitro* cardioprotective activity of Calophyllolide against Doxorubicin-mediated myocardial damage on H9c2. The anti-oxidant potential of Calophyllolide was evaluated by performing DPPH free radical scavenging assay and NO free radical scavenging assay. The Doxorubicin (DOX) induced hepatotoxicity studies were performed on female rats (130-160 g) and were administered Calophyllolide doses (5, 50, 200, 2000 mg/kg) orally. The rats were monitored for two weeks for signs of toxicity, and haematological and biochemical parameters were assessed post-experiment using respective analyzers. The results suggest that the doxorubicin-treated cells exhibited 60%, 48%, and 30% viability after 24, 48, and 72 hrs, respectively, compared to the normal group. Cells treated with DOX + Calophyllolide at 50 µg/mL showed 68%, 52%, and 60% viability after the same time points. Treatment with DOX + Calophyllolide at 100 µg/mL significantly increased cell viability to 78%, 81%, and 84%, while DOX + Calophyllolide at 150 µg/mL further improved viability to 90%, 98%, and 99%, indicating that Calophyllolide protects the heart muscle from DOX-induced cardiac toxicity. Doxorubicin administration significantly increased LDH activity (162%) compared to the control group, but Calophyllolide at 100 µg/mL and 150 µg/mL significantly reduced LDH activity to 122% and 70%, respectively, suggesting protection against DOX-induced cardiac damage. Similarly, doxorubicin significantly elevated ROS activity (160%) compared to the control group, while Calophyllolide at 100 µg/mL and 150 µg/mL reduced ROS levels to 98% and 78%, respectively, indicating protection against DOX-induced oxidative cardiac damage. This study underscores the potential of Calophyllolide as a candidate for further investigation as a cardio-protective agent.

Keywords: Antioxidant, Cardioprotective, Cell Lines, *In Vitro*

1. Introduction

Cardioprotective activity refers to the ability of certain substances or interventions to protect the heart from damage and maintain its normal functioning, especially in the face of various cardiovascular stressors[1,2]. The heart is designated as a vital organ that pumps blood throughout the body, and its well-being is crucial for overall health. Conditions such as ischemic heart disease, heart attacks, and heart failure can significantly impact cardiac health. Several factors contribute to the development of cardiovascular diseases, including oxidative stress, inflammation, blood pressure, and dyslipidemia. Cardioprotective agents aim to mitigate

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these factors and safeguard the heart from potential damage. These agents may be pharmaceutical drugs, natural compounds, lifestyle modifications, or a combination of these approaches[3-6]. Research in the field of cardioprotection focuses on identifying and understanding substances or strategies that can prevent or minimize heart damage, enhance cardiac function, and improve overall cardiovascular health. Cardio-protective interventions often target pathways involved in cellular protection, antioxidant defence, anti-inflammatory responses, and the regulation of blood pressure and lipid levels[7-10]. Globally, cardiovascular diseases will cause high morbidity and mortality, and the exploration of cardioprotective measures remains a critical area of study. The development of effective cardioprotective strategies holds the potential to reduce the incidence and severity of heart-related disorders, improving the overall quality of life for individuals at risk or already affected by cardiovascular conditions[11,12].

Calophyllolide is a natural compound that has been identified in certain plant species. One notable source of calophyllolide is the Calophyllum genus, particularly Calophyllum inophyllum. Calophyllum inophyllum, also known as tamanu or kamani tree, is a tropical tree found in various regions, including Southeast Asia and the Pacific. Research suggests that calophyllolide, along with other compounds found in Calophyllum species, and may possess biological activities, including anti-inflammatory and antimicrobial properties. These properties make these compounds of interest for potential medicinal and pharmaceutical applications.

2. Materials and Methods

2.1 DPPH* Free Radical Scavenging Assay of Calophyllolide

Blois MS (1958) introduced a method for assessing DPPH* free radical scavenging activity. Calophyllolide was weighed at 1 mg/mL, and dissolved in Dimethyl sulfoxide (DMSO), and various concentrations were prepared at 10, 20, 50, 100, 150, 200, and 250 µg/mL. In this experimental procedure, aliquots of varying concentrations of the test compound were combined with 50 µg/mL concentration of methanolic DPPH solution (0.1 mM) at a volume ratio of 1:3. The resulting mixture was then allowed to react for 20 mins, to facilitate the scavenging of free radicals by the compound under investigation. Following the incubation period, solution absorbance was determined at 517 nm spectrophotometer (220 double beam, Thermo Fisher Scientific). The radical scavenging ability was evaluated by correlating reaction mixture absorbance with blank. The antioxidant activity of the compound was subsequently calculated as a percentage, reflecting the degree of inhibition of DPPH radicals by the compound relative to the control[13].

2.2 NO* Free Radical Scavenging Assay of Calophyllolide

Precisely weighed Calophyllolide samples were dissolved in DMSO to prepare a 1 mg/mL solution. From this stock solution, various concentrations of 10 to 250 µg/mL were prepared and each level solution was mixed with a similar volume of sodium nitroprusside solution at 10 mM concentration. Then it was incubated at 37°C for 3 hrs to induce Nitric Oxide (NO) production. Following the incubation period, Griess reagent (0.3 mL) was added to each aliquot of the incubation solution to facilitate the quantification of nitrite ions, a byproduct of NO production. The solution absorbance was taken at 570 nm on a UV spectrophotometer. The % inhibition of radicals by the test sample was determined using the formula:

$$\%\ inhibition\ = \frac{A0 - A1}{A0} \times 100$$

In the equation, A_0 will be the control absorbance (without the test sample), and A_1 will be the test sample absorbance. This experimental procedure enables the evaluation of the antioxidant activity of calophyllolide by assessing its ability to scavenge NO radicals, providing valuable insights into its potential therapeutic applications.

2.3 Acute Toxicity Studies

The acute oral toxicity study[14] was conducted according to the guidelines set in test no 423 by the Organization for Economic Cooperation and Development (OECD). This study employed Calophyllolide at a dose of 5 mg/kg to 2000 mg/kg. The choice of doses ranging from 5 mg/kg to 2000 mg/kg in acute toxicity studies ensures a comprehensive assessment of the compound's safety profile. The low dose (5 mg/kg) identifies potential adverse effects at minimal exposure, intermediate doses (50 mg/kg and 200 mg/kg) provide insights into dose-dependent toxicity and the therapeutic window, while the high dose

(2000 mg/kg) tests the upper limits of tolerance and identifies severe toxic effects. This range, recommended by regulatory guidelines, helps determine the safe dosage range and potential risks, providing a thorough safety profile for the compound.

Female rats within a weight range of 130-160 grams, devoid of pregnancy and porous characteristics, were carefully selected for the study. These rats were then allocated into four distinct groups, each comprising six individuals.

Group I Administered with Calophyllolide at a dose of 5 mg/kg, orally

Group II Administered with Calophyllolide at a dose of 50 mg/kg in a 0.5% CMC suspension, orally

Group III Administered with Calophyllolide at a dose of 200 mg/kg in a 0.5% CMC suspension, orally

Group IV Administered with Calophyllolide at a dose of 2000 mg/kg in a 0.5% CMC suspension, orally

All four groups underwent close monitoring for mortality and manifestation of toxicity-related clinical signs, encompassing general behaviour, respiratory patterns, cardiovascular indicators, motor activities, reflex responses, as well as alterations in skin and fur texture. Assessments were conducted at designated time intervals of 30 mins, 12 hrs, 24 hrs, 48 hrs, 1 week, and 2 weeks post-administration. This systematic approach allowed for a comprehensive evaluation of the acute oral toxicity profile of Calophyllolide across a range of doses, thereby providing valuable insights into its safety profile and potential adverse effects.

2.3.1 Haematological Assessments

Following the experimental phase, haematology analyzers (DxH 900 Analyzer, Beckman Coulter) were employed to assess blood samples gathered in heparin-coated tubes. Various haematological parameters were examined, encompassing platelet count, haemoglobin estimation, leucocyte count (WBC), haematocrit, Differential Leucocyte Count (DLC), erythrocyte count (RBC), Mean Platelet Volume (MPV), Mean Corpuscular Haemoglobin Concentration (MCHC), Mean Corpuscular Haemoglobin (MCH) and Red Cell Distribution Width (RCDW).

2.3.2 Biochemical Assessments

A biochemistry analyser (Cobas c 311 Clinical Chemistry Analyzer, oche Diagnostics) with automated functionality was utilized to assess various biochemical parameters. These included uric acid, urea, triglycerides, creatinine, glucose, BUN (blood urea nitrogen), SGOT (serum glutamic-oxaloacetic transaminase), SGPT (serum glutamate-pyruvate transaminase), ALP (alkaline phosphatase), conjugated bilirubin, total bilirubin, total cholesterol along with total protein. The plasma for analysis was derived from blood samples, which were subjected to centrifugation in uncoated tubes for ten mins at 3000 rpm and 5°C.

2.3.3 Cell Culture and Treatment

The H9c2 cell line, procured from the American Type Culture Collection (ATCC, USA), was thawed from cryogenic storage in a Cryotube and allowed to equilibrate in a water bath set at 37°C. Following dissolution, the cells were transferred to T75 cm^2 flasks for expansion. Subsequently, H9c2 cells were seeded onto a 96-well plate and maintained in a CO_2 incubator at 37°C with 5% CO_2 humidity. After 48 hrs, the cells were counted and adjusted to a density of 2×10^5 cells/well in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). Various concentrations of Calophyllolide in 50 to 150 µg/mL were applied to the cells. Experimental groups included a Control group treated with saline, a DOX group subjected to 10 µM ISO treatment, and DOX + Calophyllolide groups pre-incubated with different concentrations (50, 100, and 150 µg/mL) of Calophyllolide for 24 hrs, followed by a 24 hr treatment with 10 µM DOX[15,16].

2.4 MTT Assay

Using the MTT assay, the proliferation kinetics of H9c2 cells were evaluated throughout 24, 48, and 72 hrs. In 96-well plates, H9c2 cells were seeded at 1×10^4 cells per well density and subjected to various treatments. Absorbance readings at 570 nm were obtained thrice using a microplate reader spectrophotometer[17,18].

The inhibition of cell growth was determined by calculating the percentage survival of treated cells relative to control cells, denoted as T/C%. This was achieved using the formula:

$$\%\ Growth\ Inhibition = 100 - \left[\frac{OD(test\ sample) - OD(blank)}{OD(control) - OD(blank)}\right] \times 100$$

where OD represents optical density. This method allowed for the quantification of cell viability in response to different treatments, providing valuable insights into the potential cytotoxic or cytostatic effects of the test samples on H9c2 cells.

2.5 LDH Activity

LDH release served as an indicator for assessing the extent of cellular damage. In 96-well plates, H9c2 cells were seeded at 1×10^6 cells per well density and subjected to various treatments. Following treatment, the culture media were analyzed using an LDH assay kit and a microplate reader, adhering to the manufacturer's instructions, to quantify LDH activity[19-21].

2.6 ROS Production

ROS production in H9c2 cells was assessed using 2′7′-dichlorofluorescein diacetate (DCF-DA) dye. DCF-DA is employed due to it detects ROS within cells. Once inside the cell, DCF-DA is converted to the non-fluorescent 2′7′-dichlorofluorescein (DCFH), which is then oxidized by ROS to the fluorescent compound DCF. The fluorescence intensity of DCF, measured by a fluorescence spectrophotometer, directly correlates with ROS levels, making DCF-DA a reliable indicator of oxidative stress and the antioxidant capacity of test compounds.

The cell seeding to treat 8 hrs period with 150 µM DOX was administered. Subsequently, the cells were exposed to 10 µM DCF-DA and allowed to incubate for 30 mins at 37°C to facilitate dye uptake. Upon removal of the Dulbecco's Modified Eagle Medium (DMEM), the cells underwent two washes with phosphate-buffered saline (PBS; 0.01M, pH 7.4). Following this, cell lysis was performed for ten mins at 37°C using 1% Triton X-100 in PBS (0.01M, pH 7.4). Fluorescence measurements were conducted to detect ROS, with excitation at 490 nm and emission at 525 nm, providing quantitative insights into ROS production levels within the cells. This experimental approach enabled the elucidation of the oxidative stress response elicited by DOX treatment in H9c2 cells, offering valuable mechanistic insights into its cardiotoxic effects.

3. Results

3.1 DPPH Assay

Table 1 presents a comparative analysis of the DPPH scavenging activity between calophyllolide and the standard antioxidant, ascorbic acid, across various concentrations measured in µg/mL. At a concentration of 50 µg/mL, the DPPH scavenging activity of ascorbic acid is notably higher, at 44.63%, compared to callophyllolide, which exhibits a scavenging activity of 16.31%. This trend continues across all concentrations tested. As the concentration increases, both substances show an increase in DPPH scavenging activity, indicating a dose-dependent response. However, even at the highest concentration tested (250 µg/mL), ascorbic acid demonstrates superior antioxidant activity, scavenging 92.56% of DPPH radicals, while calophyllolide scavenges 56.33%. These findings suggest the potential antioxidant efficiency of calophyllolide compared to standard ascorbic acid.

The results are presented as Mean±SEM (n=3), where SEM stands for Standard Error of Mean. Statistical significance was determined through ANOVA, followed by Bonferroni post-tests for multiple comparisons. Statistically significant differences are denoted by '*' with a significance level of $p<0.001$, in comparison to the positive control. ANOVA refers to the Analysis of Variance.

3.2 NO Scavenging Activity

Table 2 displays a comparison of the NO scavenging activity between calophyllolide and the standard antioxidant, ascorbic acid, at various concentrations measured in µg/mL. At the lowest concentration of 50 µg/mL, calophyllolide exhibits a NO scavenging activity of 24.18%, which is notably lower than the activity of ascorbic acid at 46.21%. However, as the concentration of calophyllolide increases, its NO scavenging activity also increases significantly. At 250 µg/mL, calophyllolide demonstrates a remarkable NO scavenging activity of 58.43%. Results suggest that ascorbic acid initially exhibits higher NO scavenging activity at lower concentrations, and calophyllolide demonstrates a remarkable enhancement

Table 1. DPPH assay result

Concentration (µg/mL)	DPPH scavenging activity (%)	
	Ascorbic acid	Calophyllolide
50	44.63±0.26	16.31±0.81*
100	52.78±0.37	21.35±0.32*
150	65.92±1.21	38.87±1.01*
200	88.51±1.37	43.9±0.56*
250	92.56±0.17	56.33±0.37*

in its scavenging activity as the concentration increases, eventually surpassing that of ascorbic acid at higher concentrations. These findings suggest the potential of calophyllolide as a potent NO scavenger, particularly at higher concentrations.

The results are presented as Mean±SEM (n=3), where SEM stands for Standard Error of Mean. Statistical significance was determined through ANOVA, followed by Bonferroni post-tests for multiple comparisons. Statistically significant differences are denoted by '*' with a significance level of $p<0.001$, in comparison to the positive control. ANOVA refers to the Analysis of Variance.

3.3 Acute Toxicity Studies

The acute toxicity evaluation of calophyllolide at various doses (5 mg/kg, 50 mg/kg, 200 mg/kg, and 2000 mg/kg) provided crucial insights into its potential adverse effects and safety profile. Throughout the observation period of 30 mins to 2 weeks post-administration, no mortality was recorded across all treatment groups, indicating that calophyllolide did not induce acute lethal toxicity within the tested dose range.

In terms of clinical signs and symptoms, no significant adverse effects were observed in the parameters assessed, including skin condition, mucous membrane integrity, salivation, lethargy, convulsions, tremors, and diarrhoea. This suggests that calophyllolide did not elicit overt signs of toxicity or induce severe physiological disturbances, even at the highest dose of 2000 mg/kg (Table 3).

Interestingly, transient alterations in sleep patterns were noted at the 2-week time point in the group treated with the highest dose of calophyllolide (2000 mg/kg), where animals exhibited increased sleep duration compared to the control group. However, this observation was not indicative of toxic effects, as it did not progress to more severe manifestations such as coma or convulsions.

The absence of mortality and significant adverse effects in the acute toxicity evaluation suggests that calophyllolide has a favourable safety profile at the doses tested. However, it is essential to acknowledge the limitations of acute toxicity studies and the need for further investigations, including subacute and chronic toxicity studies, to comprehensively assess the safety of calophyllolide over extended periods and at various doses.

The haematological parameters assessed in this study were tabulated in Table 4 and results provide valuable insights into the potential systemic effects of different doses of the treatment on various blood components. Overall, the results indicate that the administration of calophyllolide at different doses did not induce significant alterations in most haematological parameters compared to the control group.

Table 2. NO scavenging activity

Concentration (µg/mL)	NO scavenging activity (%)	
	Ascorbic acid	Calophyllolide
50	46.21±0.72	24.18±0.34*
100	57.26±0.42	31.62±0.41*
150	68.18±0.93	37.62±1.21*
200	81.46±1.03	44.68±0.63*
250	90.22±0.43	58.43±0.42*

Table 3. Observational changes in rats after oral administration of Compound A

Parameter	30 m	12 hrs	24 hrs	48 hrs	1 week	2 weeks
Skin	NSC	NSC	NSC	NSC	NSC	NSC
Mucus membrane	NA	NSC	NSC	NSC	NSC	NSC
Salivation	NA	NSC	NSC	NSC	NSC	NSC
Lethargy	NA	NSC	NSC	NSC	NSC	NSC
Sleep	NSC	NSC	NSC	NSC	SC	SC
Coma	NSC	NSC	NSC	NSC	NSC	NSC
Convulsions	NSC	NSC	NSC	NSC	NSC	NSC
Tremors	NSC	NSC	NSC	NSC	NSC	NSC
Diarrhoea	NSC	NSC	NSC	NSC	NSC	NSC
Mortality	NSC	NSC	NSC	NSC	NSC	NSC

NSC indicates no significant changes observed whereas, observable changes represented with SC

Regarding RBC parameters, including RBC count, hematocrit, and haemoglobin levels, there were no substantial differences observed among the treatment groups compared to the control. This suggests that calophyllolide did not exert significant effects on erythropoiesis or red blood cell indices.

Similarly, the WBC count and differential counts of leukocytes, including neutrophils, basophils, eosinophils, monocytes, and lymphocytes, remained relatively stable across all treatment groups. These findings indicate that calophyllolide did not induce notable changes in the immune response or alter the distribution of different leukocyte subpopulations.

Platelet parameters, such as platelet count and mean platelet volume, also showed no significant differences between the treatment and control groups. This suggests that calophyllolide did not affect platelet production or platelet size. However, some minor fluctuations were observed in certain parameters, such as MCV and MCH, particularly at higher doses of calophyllolide. These variations, although statistically significant, were relatively small and may not have clinical significance.

In summary, the haematological evaluation indicates that calophyllolide administration at doses of 5 mg/kg, 50 mg/kg, 200 mg/kg, and 2000 mg/kg did not exert significant adverse effects on hematopoiesis or blood cell populations in the experimental animals. These findings suggest that calophyllolide may possess a favourable safety profile regarding haematological parameters at the doses tested. However, further studies are warranted to comprehensively assess the safety profile of calophyllolide and its potential long-term effects on haematological parameters.

Result data are expressed as Mean±SEM, n=6 (rats). The statistically significant difference has been determined by ANOVA accompanied by Dunnett's tests for multiple comparisons and is statistically significant with '*'$p<0.05$ compared with the control group.

The evaluation of serum biochemical parameters provides valuable insights into the potential systemic effects and organ toxicity induced by calophyllolide administration at different doses. These parameters serve as indicators of liver function, kidney function, and metabolic processes, shedding light on the compound's safety profile.

Among the liver function markers, SGOT and SGPT levels were moderately elevated in the treatment groups compared to the control group, particularly at higher doses of calophyllolide (2000 mg/kg). However, these increases remained within the normal range for SGOT and SGPT, suggesting mild hepatocellular injury that did not progress to severe liver dysfunction.

Table 4. Hematological studies results

Haematological parameters	Unit of expression	Treatment groups			
		5 mg/kg	50 mg/kg	200 mg/kg	200 mg/kg
RBC	$x10^{12}$/L	8.80±0.25	8.48±0.08	9.01±0.13	8.86±0.15
WBC	$x10^{9}$/L	10.77±0.23	9.88±0.29	10.27±0.72	10.23±0.60
Platelet count	$x10^{9}$/L	884.48±82.08	816.45±162.25	995.40±175.70	978.03±153.77
Mean platelet volume	fL	9.67±0.02	9.74±0.01	9.63±0.04	9.74±0.01
Hematocrit	%	48.38±0.36	47.93±0.56	48.67±0.24	49.45±0.60
RCDW	%	13.78±0.15	13.38±0.49	14.36±0.34	13.83±0.15
MCV	fL	58.89±1.76	61.59±1.48	63.52±1.64	59.47±1.47
Haemoglobin	g/dL	15.52±0.21	15.59±0.58	15.49±0.26	15.84±0.10
MCH	pg	19.70±0.24	18.91±0.54	19.88±0.36	18.87±0.12
MCHC	g/dL	31.73±0.18	31.03±0.33	31.81±0.13	32.70±0.30
Basophils	%	0.48±0.07	0.53±0.04	0.57±0.08	0.47±0.02
Eosinophils	%	0.89±0.03	0.98±0.06	0.84±0.05	0.95±0.01
Neutrophils	%	11.71±2.05	14.80±0.79	13.02±1.53	13.60±1.78
Monocytes	%	1.52±0.07	1.73±0.16	1.42±0.16	1.38±0.20
Lymphocytes	%	85.07±1.31	82.02±1.73	84.15±2.36	83.62±3.00

Conversely, ALP levels did not show significant alterations, indicating minimal impact on hepatic biliary function.

Total protein levels remained relatively stable across all treatment groups, indicating no significant disturbances in protein metabolism. Similarly, total bilirubin and conjugated bilirubin levels did not exhibit substantial deviations from the control group, indicating preserved hepatic bilirubin metabolism and excretion.

In terms of kidney function markers, urea and creatinine levels showed slight fluctuations across treatment groups, with the highest dose of calophyllolide (2000 mg/kg) demonstrating a mild decrease in urea and creatinine levels compared to the control group. This suggests a potential mild improvement in renal function at the highest dose tested. Additionally, BUN levels were significantly reduced at the highest dose, indicating potential nephroprotective effects of calophyllolide at higher concentrations.

Metabolic parameters such as glucose, triglycerides, and total cholesterol exhibited minimal alterations across treatment groups, suggesting that calophyllolide did not significantly influence glucose metabolism or lipid profiles under the tested conditions.

The serum biochemical studies as tabulated in Table 5 indicate that calophyllolide administration at the tested doses did not induce substantial systemic toxicity or organ dysfunction. The observed changes in liver enzymes and kidney function markers were within acceptable limits and may reflect mild adaptive responses rather than significant pathological effects.

The results are presented as Mean±SEM, with 'n' indicating the number of rats. Statistical significance was determined using ANOVA, followed by Dunnett's tests for multiple comparisons. Statistically significant differences are denoted by '*' with a significance level of $p<0.05$, in comparison to the control group.

3.4 Effect of CA on H9c2 Cells Viability

3.4.1 Assessment of Cell Viability by MTT Assay

In our study doxorubicin(150 uM) treated cells exhibit 60%,48% and 30% cell viability after 24,48 and 72 hr, respectively when compared to the normal group. Whereas DOX + Calophyllolide 50 µg/mL treated cells exhibited 68%, 52% and 60% cell viability after 24, 48 and 72 hr, respectively when compared to Dox treated cells. Whereas DOX + Calophyllolide 100 µg/mL treated cells significantly increase cell viability by 78%,81% and 84% after 24, 48 and 72 hr, respectively when compared to Dox treated cells. Whereas DOX + Calophyllolide 150 µg/mL treated cells significantly increase cell viability by 90%, 98% and 99% after 24, 48 and 72 hr, respectively when compared to Dox treated cells This indicates that Calophyllolide protects the heart muscle from Dox induced cardiac toxicity.

Table 5. Serum biochemical studies results

Serum biochemical parameters	Unit of expression		Treatment groups		
		5mg/kg	5 mg/kg	200 mg/kg	2000 mg/kg
SGOT	U/L	235.88±23.81	248.90±19.91	278.01±12.87	268.18±12.65
SGPT	U/L	69.77±3.12	70.76±3.23	78.46±4.02	52.44±2.67*
ALP	U/L	158.92±10.73	150.35±15.66	156.77±13.09	146.72±11.98
Total protein	g/dL	6.03±0.34	6.33±0.17	6.26±0.38	6.87±0.33
Conj. bilirubin	µmol/L	8.66±0.27	7.24±0.76	8.74±0.88	7.92±1.02
Total bilirubin	µmol/L	21.45±1.65	18.98±2.34	25.09±2.91	25.69±1.55
Creatinine	mg/dL	0.53±0.03	0.55±0.03	0.55±0.03	0.51±0.03*
Uric acid	mmol/L	0.38±0.04	0.82±0.05	0.67±0.05	0.19±0.02
Urea	mmol/L	6.99±0.67	5.64±0.38	7.09±0.46	6.71 ±0.63
BUN	mg/dL	18.07±2.04	20.10±3.90	18.98±2.05	12.82±0.66*
Triglycerides	mg/dL	12.55±2.48	11.37±3.07	17.80±4.11	14.80±2.54
Glucose	mg/dL	117.13±16.9	109.45±8.41	100.46±14.24	120.23±7.85
Total cholesterol	mg/dL	70.34±4.22	68.22±4.67	81.22±5.09	69.45±6.91

Table 6. Cell viability was assessed by MTT assay

S. No	Test	% Cell viability 24 hr Mean ±SEM	% Cell viability 48 hr Mean ±SEM	% Cell viability 72 hr Mean ±SEM
1	Control	100±0.12*	100±0.02*	100±0.87*
2	DOX (150uM)	60±0.78**	48±0.36**	30±0.69**
3	DOX + Calophyllolide 50 µg/mL	68±0.47	52±0.05	60±0.46
4	DOX + Calophyllolide 100 µg/mL	78±0.14	81±0.36	84±0.34
5	DOX + Calophyllolide 150 µg/mL	90±0.98***	98±0.98***	99±0.74***

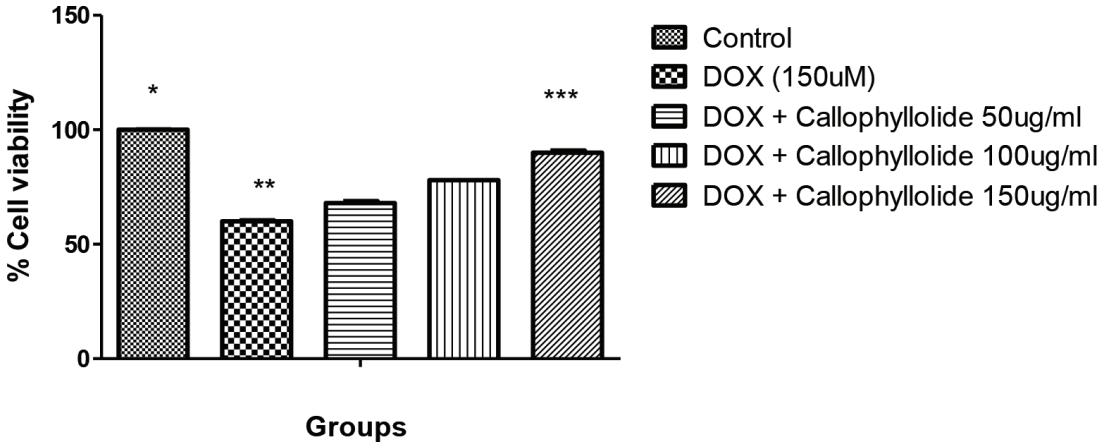


Figure 1. Effect of calophyllolide on cell viability. The values are the means ± SEM(n=6) Doxorubicin administration significantly decreased the cell viability after 24 h. (**p<0.001) when compared to control. Treatment with Calophyllolide 150 µg/mL dose of Calophyllolide significantly increased the cell viability after 24 hr. (***p<0.001) when compared to the Doxorubicin group.

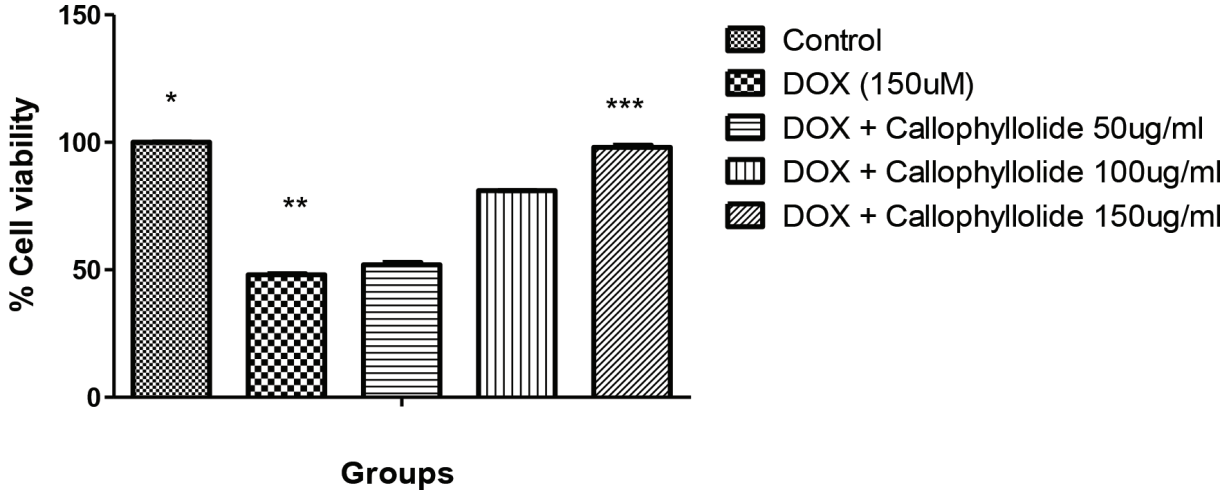


Figure 2. Effect of calophyllolide on cell viability. The values are the means ± SEM(n=6) Doxorubicin administration significantly decreased the cell viability after 48 hrs. (**p<0.001) when compared to control. Treatment with Calophyllolide 150 µg/mL dose of Calophyllolide significantly increased the cell viability after 48 hrs. (***p<0.001) when compared to the Doxorubicin group.

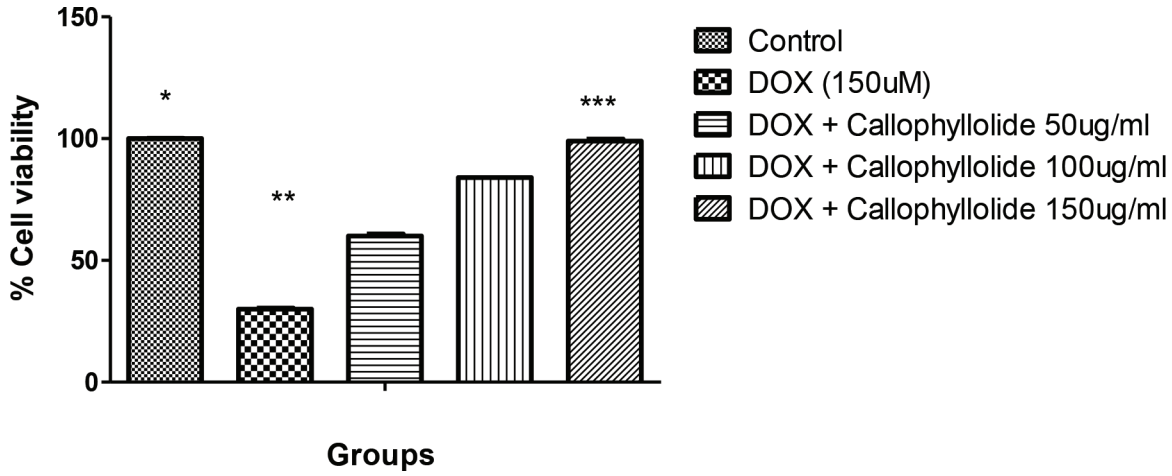


Figure 3. Effect of calophyllolide on cell viability. The values are the means ± SEM(n=6) Doxorubicin administration significantly decreased the cell viability after 72 hrs. (**p<0.001) when compared to control. Treatment with Calophyllolide 150 µg/mL dose of Calophyllolide significantly increased the cell viability after 72 hrs. (***p<0.001) when compared to the Doxorubicin group.

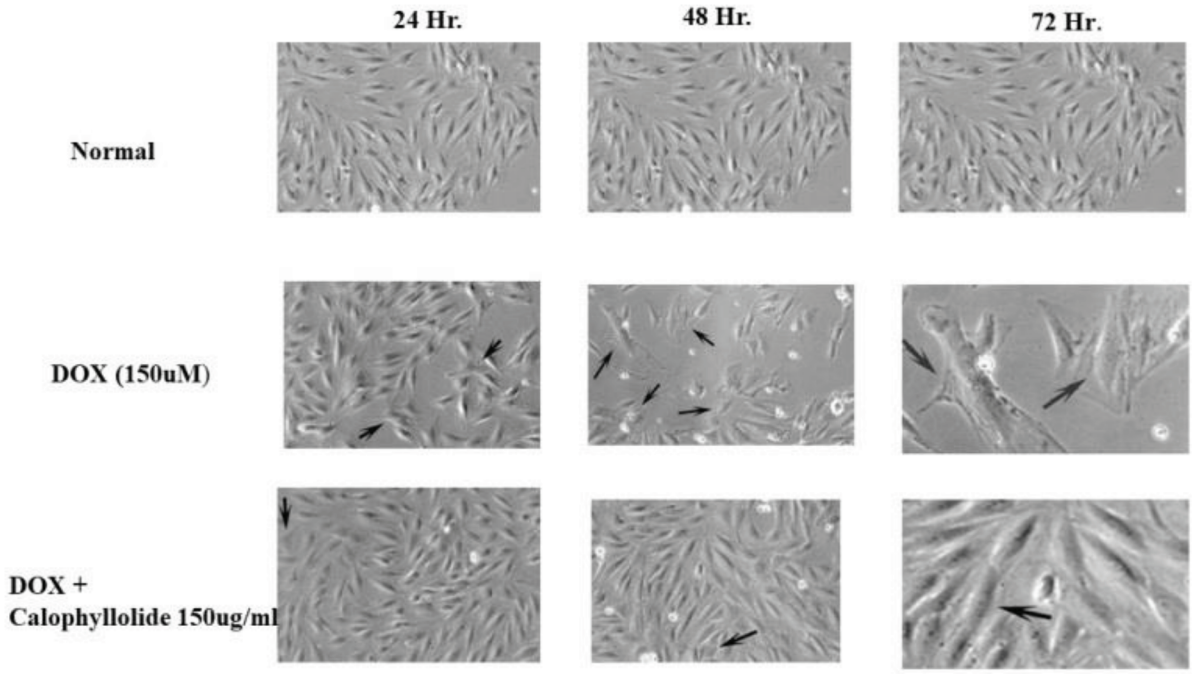


Figure 4. Effect of calophyllolide on cell viability.

3.5 Effect of CA on LDH

Doxorubicin administration significantly increased the activity of LDH (162%) when compared to the control group, which are enzymatic biomarkers of myocardial injury. Treatment with Calophyllolide 50 µg/mL showed no significant difference in LDH (150%) when compared to Dox treated group; however, 100 µg/mL and 150 µg/mL dose of Calophyllolide significantly decreased the LDH (122%; 70%). It indicates that Calophyllolide protect the heart from Doxorubicin-induced cardiac damage

3.6 Effect of CA on ROS Release

Doxorubicin administration significantly increased the activity of ROS (160%) when compared to the control group, which are enzymatic biomarkers of myocardial injury. Treatment with Calophyllolide 50 µg/mL showed

Table 7. Effect of CA on LDH

S.No	Test	LDH Release (%Control) Mean ±SEM
1	Control	100±0.24*
2	DOX (150uM)	162.8±0.14**
3	DOX + Calophyllolide 50 µg/mL	150.7±0.84
4	DOX + Calophyllolide 100 µg/mL	122.3±0.87
5	DOX + Calophyllolide 150 µg/mL	70.4±0.45***

Table 8. Effect of CA on ROS release

S.No	Test	ROS Release (%Control) Mean ±SEM
1	Control	100±0.47*
2	DOX (150uM)	160±0.25**
3	DOX +CA 50 µg/mL	122±0.47
4	DOX + Calophyllolide 100 µg/mL	98±0.89
5	DOX + Calophyllolide 150 µg/mL	78±0.74***

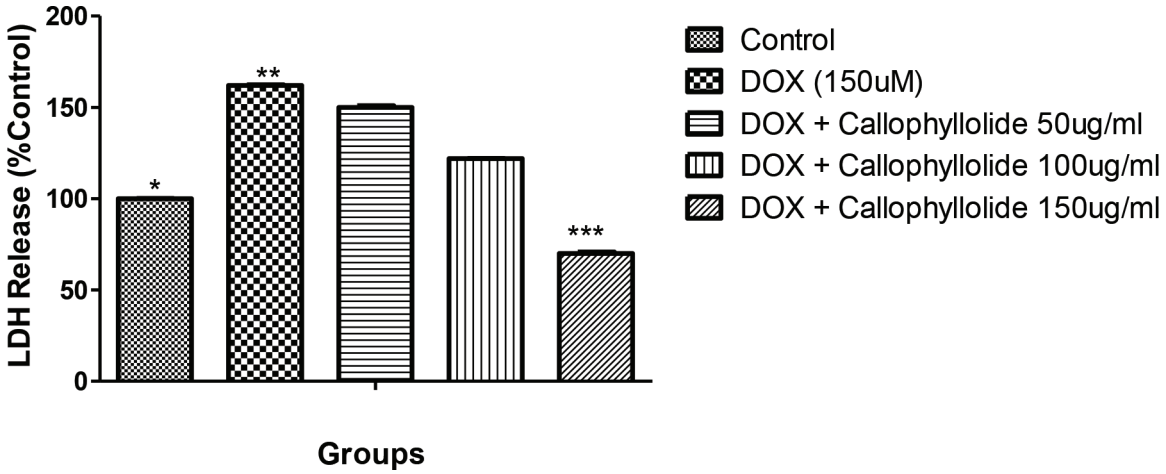


Figure 5. Effect of calophyllolide on LDH release. The values are the means ± SEM(n=6) Doxorubicin administration significantly increased the LDH level (**p<0.001) when compared to control. Treatment with Calophyllolide 150 µg/mL dose of Calophyllolide significantly decreased the LDH release (***p<0.001) when compared to the Doxorubicin group.

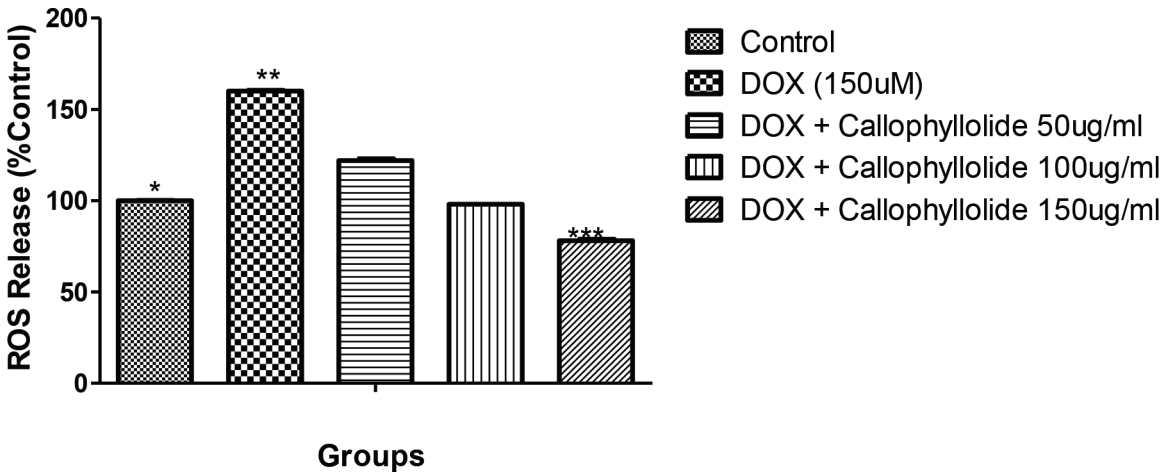


Figure 6. Effect of calophyllolide on ROS release. The values are the means ± SEM(n=6) Doxorubicin administration significantly increased the ROS level (**p<0.001) when compared to control. Treatment with Calophyllolide 150 µg/mL dose of Calophyllolide significantly decreased the ROS release (***p<0.001) when compared to the Doxorubicin group.

no significant difference in ROS (122%) when compared to Dox treated group; however, 100 µg/mL and 150 µg/mL dose of Calophyllolide significantly decreased the ROS (98%; 78%). It indicates that Calophyllolide protects the heart from Doxorubicin-induced oxidative cardiac damage.

4. Conclusion

This study highlights Calophyllolide's significant antioxidant and cardioprotective effects against doxorubicin-induced myocardial damage. The comprehensive evaluation of acute toxicity, haematological parameters, serum biochemical studies, and cytotoxicity assays provides valuable insights into the safety and pharmacological potential of calophyllolide. The acute toxicity assessment demonstrated that doxorubicin-treated cells exhibited decreasing viability of 60%, 48%, and 30% at 24, 48, and 72 hrs, respectively. In contrast, cells co-treated with doxorubicin and Calophyllolide showed improved viability, reaching 68%, 52%, and 60% at 50 µg/mL, 78%, 81%, and 84% at 100 µg/mL, and 90%, 98%, and 99% at 150 µg/mL. Additionally, Calophyllolide treatment significantly reduced LDH activity from 162% in doxorubicin-treated cells to 122% and 70% at 100 µg/mL and 150 µg/mL, respectively. ROS levels were also markedly decreased, from 160% in doxorubicin-treated cells to 98% and 78% with 100 µg/mL and 150 µg/mL Calophyllolide, respectively. Additionally, analysis of haematological parameters and serum biochemical markers indicated minimal alterations, suggesting that calophyllolide does not induce significant hematotoxin, hepatotoxic, nephrotoxic, or metabolic disturbances. Moreover, cytotoxicity assays revealed that calophyllolide attenuated the cytotoxic effects of doxorubicin (DOX) on H9c2 cells, with dose-dependent improvement in cell viability observed at 48 and 72 hrs. These findings collectively suggest that calophyllolide possesses promising safety and cytoprotective properties, highlighting its potential as a therapeutic agent.

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