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Research Article

Cardioprotective Activity of N'',N'''-Bis[5-methyl-2-oxo-1, 2-dihydro-3H-indol-3-ylidene]carbonohydrazide Derivative against Doxorubicin Induced Cardiotoxicity in Rats

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The present study was aimed at evaluating the cardioprotective effect of novel synthetic N'',N'''-bis[5-methyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene]carbonohydrazide derivative, by estimating the various biomarkers like creatine kinase-myoglobin (CK-MB), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and triglycerides (TG) in plasma and antioxidants like catalase, superoxide dismutase in heart tissue homogenate, and histopathological examination of heart tissues. The results showed the significant ($P < 0.05$) dose dependent decrease in elevated cardiotoxic biomarkers CK-MB, LDH, AST, and TG levels. The histopathological studies of heart tissues showed mild degeneration of muscle bundles and less interstitial edematous changes. The results showed the significant ($P < 0.05$) dose dependent increase in antioxidant enzymes catalase and superoxide dismutase in heart tissue homogenates. These observations enable us to conclude that N'',N'''-bis[5-methyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene]carbonohydrazide has cardioprotective activity against doxorubicin induced cardiotoxicity.

1. Introduction

Cardiotoxicity is the occurrence of heart electrophysiology dysfunction or/and muscle damage. The heart becomes weaker and is not as efficient in pumping and therefore circulating blood. Cardiotoxicity may be caused by chemotherapy, heavy metal intake, or in an incorrect administration of drug like bupivacaine [1]. Ischaemic heart disease is a leading cause of morbidity and mortality worldwide. The World Health Organization (WHO) estimates there will be about 20 million CVD deaths in 2015, accounting for 30 percent of all deaths worldwide [2]. Myocardial infarction is the acute condition of myocardial necrosis that occurs as a result of imbalance between coronary blood supply and myocardial demand. The patient may experience significant disability or die [3].

Isatin and its derivatives are used in organic synthesis and they are used in evaluating new product that possesses different biological activities. In the past few decades, isatin and its derivatives have received much attention due to their chemotherapeutic values [4]. Isatin and its analogs show considerable pharmacological actions such as antimicrobial, anticancer, antiviral, anticonvulsant, anti-inflammatory, and

analgesic [5]. From these results, ideas for future molecular modifications leading to compounds with greater favourable pharmacological properties may be derived.

Doxorubicin is broad spectrum antibiotic used as an anticancer drug which was limited by its dependent cardiotoxic effects [6].

In the present study, we have evaluated the cardioprotective effect of novel synthetic isatin derivative N'',N'''-bis[5-methyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene]carbonohydrazide against doxorubicin induced cardiotoxicity in rats by estimating various cardiotoxic biomarkers like creatine kinase-myoglobin (CK-MB), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), triglycerides (TG) in plasma, and histopathological studies of rat heart tissues and the antioxidant capacity of test compound was known by estimating catalase, superoxide dismutase in heart tissue homogenates and glutathione levels in plasma.

2. Materials and Methods

2.1. Animals. All the experiments were carried out with male wistar albino rats, (200 ± 20 g) supplied by Mahaveer enterprises, Hyderabad. They were housed individually in cages under hygienic conditions and placed in a controlled environment with a 12 : 12 h light : dark cycle at 25°C + 2°C and 45 + 10% humidity for 7 days before the experiment. The animals were allowed a commercial standard rat diet and water *ad libitum*. The animal care and experimental protocols were in accordance with CPCSEA/IAEC.

2.2. Test Compound. We have selected the following newly synthesized isatin derivative to evaluate cardio protective activity. This compound was gifted by University College of Pharmaceutical Sciences, Kakatiya University, Warangal, India. The structure and physicochemical properties of the compound are as follows.

2.3. Chemistry

N'',N'''-Bis[5-methyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene] carbonohydrazide ***(3)***. To a solution of 5-methyl isatin (0.04 mol) and carbohydrazide (0.02 mol) in the 30 mL of ethanol, a few drops of glacial acetic acid is added to the reaction mixture and was refluxed for 2-3 hours. The reaction was monitored by TLC. Then the reaction mixture was cooled overnight and the precipitate was collected by filtration. The solids were purified by column chromatography and recrystallized with ethanol.

N'',N'''-Bis[(3Z)-5-methyl-2-oxo-1,2-dihydro-3H-indol-3-ylidene]carbonohydrazide ***(3)***. % yield: 75%, M.P (°C): 250–252. IR (KBr) cm^{-1}: 3320 (N–H), 3198 (N–H), 3010 (C–H, Ar), 2980 (C–H str), 1730 (C=O), 1680 (s, C=O, lactam), 1608, 1472 (C=C, Ar), 1620 (C=N). ^{1}H-NMR (DMSO-d6) ppm: δ 10.28 (s, 2H), 9.48 (s, 2H), 6.83–8.72 (m, 6H), 2.31 (s, 6H). Mass (ESI-MS): m/z 377 (M+1, 68%), 399 (M+Na, 100%). Elemental analysis: for $C_{19}H_{16}N_6O_3$ calculated 60.63% C, 4.28% H, 22.33% N; found 60.69% C, 4.23% H, 22.38% N.

2.4. Induction of Cardiotoxicity. In the present study, cardiotoxicity was induced by a single dose of doxorubicin (15 mg/kg, i.p) treatment in rats [7].

3. Experimental Design

A total of 20 rats were randomly divided into 4 groups of five rats each.

Group I. Rats were orally administered with 0.1% CMC for 8 days as the normal control.

Group II. Rats were orally administered with 0.1% CMC for 8 days and on 7th day single dose of doxorubicin (15 mg/kg, i.p).

Group III. Rats were pretreated with test compound (50 mg/kg), orally for 8 days and on 7th day single dose of doxorubicin (15 mg/kg, i.p).

Group IV. Rats were pretreated with test compound (100 mg/kg), orally for 8 days and on 7th day single dose of doxorubicin (15 mg/kg, i.p).

4. Collection of Blood Samples and Organs

After 24 h following doxorubicin treatment, blood samples were collected into Eppendorf tubes containing sodium citrate through retroorbital puncture. After centrifugation, plasma was separated and stored at −20°C for further biochemical assay.

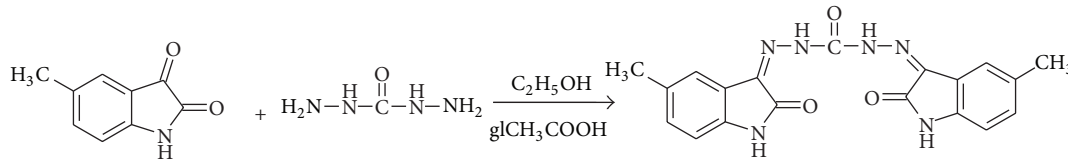
Rats were killed by overanesthesia and hearts were removed rapidly. The tissues of heart were excised and washed with prechilled physical saline, homogenized with prechilled physical saline in tissue homogenizer, and then centrifuged at 10,000 rpm for 10 min at 4°C. The supernatants were assayed immediately.

5. Assessment of Cardiotoxicity

Determinations of creatine kinase-myoglobin (CK-MB), lactate dehydrogenase (LDH), triglycerides (TG) were assayed using commercial available reagents.

5.1. Estimation of Lactate Dehydrogenase (LDH). The estimation of LDH is carried out based on the method of Andries [8]. The principle involved is that LDH specifically catalyzes the oxidation of lactate to pyruvate and transphosphorylation of ADP to ATP, with subsequent formation of NADH. The rate at which NADH was formed is proportional to enzyme activity. The increase in absorbance per minute, as a function of NADH production, is measured calorimetrically at 340 nm.

5.2. Estimation of Creatine Kinase-Myoglobin (CK-MB). The immunoinhibition method of CK-MB analysis was performed using an enzymatic kit manufactured by Coral Clinical Systems, Verna, Goa, India. The test was carried out as previously described [9].



1 5-Methylisatin **2** Carbohydrazide **3**

Scheme 1: Synthetic route for the bis-isatin derivative **3**.

5.3. Estimation of Triglycerides (TG). The levels of triglycerides were estimated by GPO-POD method [10]. Briefly, triglycerides are hydrolysed by lipase to glycerol and free fatty acids. Glycerol is phosphorylated by ATP in the presence of glycerolkinase (GK) to glycerol-3-phosphate (G-3-P) which is oxidized by the enzyme glycerol-3-phosphate oxidase (G-P-O) producing hydrogen peroxide. Hydrogen peroxide so formed reacts with 4-aminopyrine and ESPAS in the presence of the enzyme peroxidase (POD) to produce a brown color complex. The intensity of the color developed is proportional to triglycerides concentration.

5.4. Determination of Antioxidant Enzyme Activities. Biochemical estimation of catalase, superoxide dismutase, and glutathione peroxidase in plasma and/or heart tissues was measured by respective diagnosing kits and assay procedures.

Catalase was assayed according to the method of Aebi [11]. The enzyme superoxide dismutase (SOD) was determined in erythrocytes using photooxidation method as reported earlier in literature [12]. Glutathione was measured spectrophotometrically according to the method of Beutler et al., 1963 [13].

6. Histopathological Examination

One heart from each group was stored in formalin solution. Tissues were embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin (H&E). The sections were examined under light microscope, and then photomicrographs were taken.

7. Statistical Analysis

All the values were expressed as mean ± standard deviation (S.D.) ($n = 5$). Statistical comparisons between different groups were done by using one way analysis of variance (ANOVA) followed by Dunnett's test. P value less than 0.05 was considered as statistically significant.

8. Results

We report the simple and efficient method for synthesis of some new bis-5-methyl isatin carbohydrazone derivatives as planned in Scheme 1. The desired derivative is prepared by the reaction of 5-methyl isatin with carbohydrazide in the presence of glacial acetic acid in ethanol under reflux condition. The synthesized compounds were purified by column chromatography. All of the derivatives were supported by spectral data. The FT-IR, ^{1}H NMR, and mass spectra are in agreement with the proposed structures.

The IR spectra of the compounds **3** showed the absorption bands at around 3320, 1730, and 1620 cm^{-1} regions, resulting from the –NH, C=O, and C=N functions, respectively. Absence of carbonyl (C=O) peak around 1715 cm^{-1} reveals the formation of carbohydrazone derivatives. The ^{1}H NMR spectra of compound **3** which displayed broad singlets in the range of δ 11.0-13.0 are due to –NH protons. The aromatic protons are shown in multiplets around δ 7.5-8.5 ppm. All compounds have shown an excellent agreement between the calculated and experimental obtained data for elemental analysis. Mass spectra the base peak are shown as M+1 peak and also characteristic of all the remaining derivatives.

In the present study, administration of doxorubicin at a single dose of 15 mg/kg, (i.p) to rats produces significant changes in cardiotoxic biomarkers that include elevation of plasma CK-MB, LDH, TG, and AST levels. Elevation of all these parameters might be due to cardiac tissue damage, which was reported in previous studies [14]. This confirms that single dose of Doxorubicin 15 mg/kg, i.p will produce cardiotoxicity in rats.

Cardioprotective activity of test compound was evaluated by measuring CK-MB, LDH, TG, AST levels and the histopathological examination of rat hearts treated with test compound. The antioxidant capacity in rats after treatment with test compound was evaluated by estimating catalase and superoxide dismutase levels in heart tissue homogenate.

9. Estimation of Plasma Parameters

9.1. Creatine Kinase-Myoglobin (CK-MB). Creatine kinase-myoglobin (CK-MB) is an indicator of myocardial damage. The results were as shown in Figure 1.

In this study, we found that single dose of doxorubicin to rats causes significant ($P < 0.001$) elevation of plasma CK-MB levels. This elevation indicates that cardiotoxicity is produced with doxorubicin. Pretreatment with test compound at the dose of 50 mg/kg and 100 mg/kg before a single dose of doxorubicin administration showed a significant ($P < 0.01$ and $P < 0.001$) decrease in CK-MB levels compared to doxorubicin group in Figure 1.

9.2. Lactate Dehydrogenase (LDH). Lactate dehydrogenase is an enzyme that presents in heart tissues. If there is any damage to heart tissues it releases LDH into blood stream. The results were as shown in Figure 2.

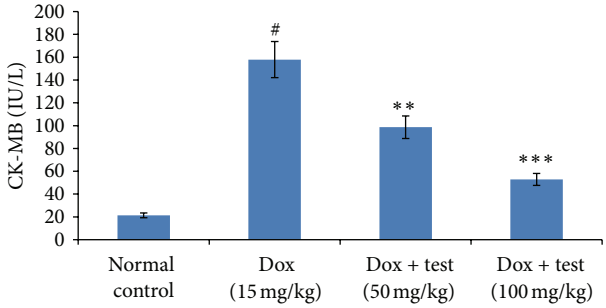


Figure 1: Effect of test compound on plasma CK-MB levels in rats treated with doxorubicin. $^{\#}P < 0.001$ versus normal control; $^{*}P < 0.01$; $^{***}P < 0.001$ versus dox.

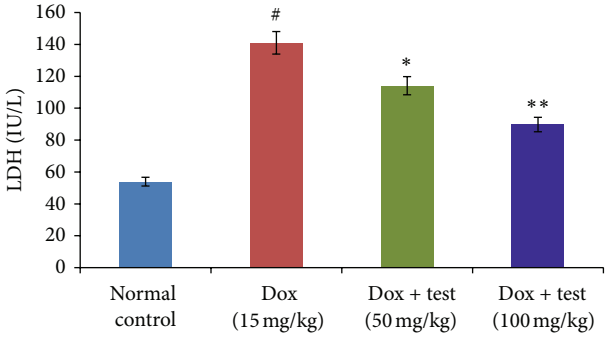


Figure 2: Effect of test compound on plasma LDH levels in rats treated with doxorubicin. $^{\#}P < 0.001$ versus normal control; $^{*}P < 0.05$; $^{**}P < 0.01$ versus dox.

In this study, we found that single dose of doxorubicin to rats causes significant ($P < 0.001$) elevation of plasma LDH levels. This elevation indicates that cardiotoxicity is produced with doxorubicin. Pretreatment with test compound at the doses of 50 mg/kg and 100 mg/kg before a single dose of doxorubicin administration showed a significant ($P < 0.05$ and $P < 0.01$) dose dependent decrease in LDH levels and these results were comparable to doxorubicin group.

9.3. Triglycerides (TG). Triglyceride is fatty substance that is composed of three fatty acids. Triglycerides levels in blood either come from the diet or the liver. High TG levels in blood indicate cardiac complications like atherosclerosis which may cause toxic events in cardiac tissues. The results were as shown in Figure 3.

In this study, we found that single dose of doxorubicin to rats causes significant ($P < 0.001$) elevation of plasma TG levels. This elevation indicates that cardiotoxicity is produced with doxorubicin. Pretreatment with test compound at the doses of 50 mg/kg and 100 mg/kg before a single dose of doxorubicin administration showed a significant ($P < 0.05$ and $P < 0.01$) dose dependent decrease in TG levels compared to doxorubicin group.

9.4. Aspartate Aminotransferase (AST). The elevation of AST levels in plasma indicates tissue damage. The results were as shown in Figure 4.

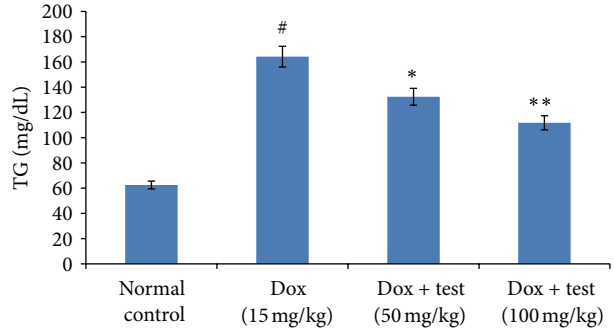


Figure 3: Effect of test compound on plasma TG levels in rats treated with doxorubicin. $^{\#}P < 0.001$ versus normal control; $^{*}P < 0.05$; $^{**}P < 0.01$ versus dox.

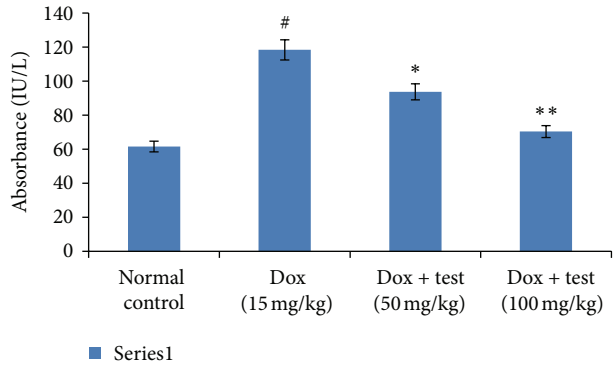


Figure 4: Effect of test compound on plasma AST levels in rats treated with doxorubicin. $^{\#}P < 0.001$ versus normal control; $^{*}P < 0.05$ versus dox, $^{**}P < 0.01$ versus dox.

In this study, we found that single dose of doxorubicin to rats causes significant ($P < 0.001$) elevation of plasma AST levels. This elevation indicates that cardiotoxicity is produced with doxorubicin. Pretreatment with test compound at the doses of 50 mg/kg and 100 mg/kg before a single dose of doxorubicin administration showed significant ($P < 0.05$ and $P < 0.01$) dose dependent decrease in AST levels compared to doxorubicin group.

10. Estimation of Antioxidants

10.1. Catalase. Catalase is a common enzyme found in nearly all living organisms that are exposed to oxygen. To prevent damage caused by hydrogen peroxide, it must be quickly converted into other less dangerous substances. To end this, catalase is frequently used by cells to rapidly catalyse the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules. Catalase activity in heart tissue homogenate represents antioxidant status in cardiac tissues.

In this study, we found that single dose of doxorubicin to rats causes significant ($P < 0.001$) decrease in catalase levels. This reduction indicates that toxicity is produced with doxorubicin. Pretreatment with test compound at the doses of 50 mg/kg and 100 mg/kg before a single dose of doxorubicin administration showed a significant ($P < 0.05$ and $P < 0.01$)

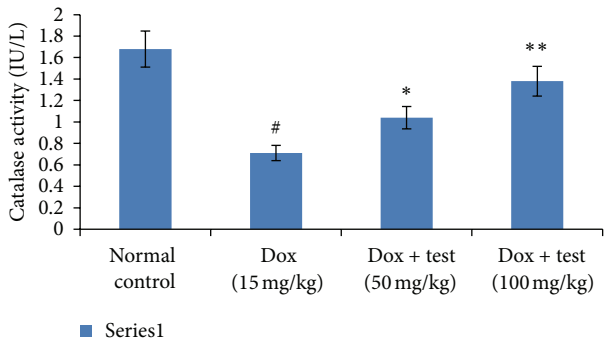


Figure 5: Effect of test compound on catalase levels in heart tissue homogenate in rats treated with doxorubicin. $^{\#}P < 0.001$ versus normal control; $^{*}P < 0.05$ versus dox; $^{**}P < 0.01$ versus dox.

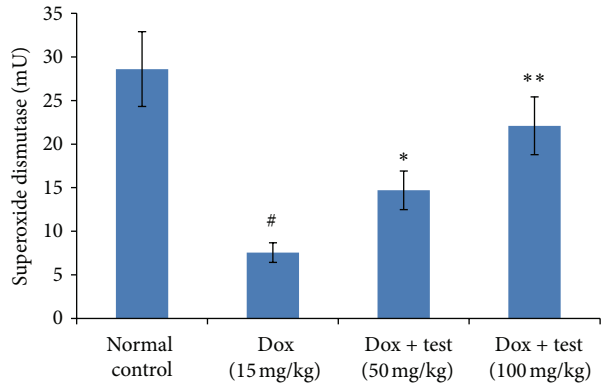


Figure 6: Effect of test compound on superoxide dismutase levels in heart tissue homogenate in rats treated with doxorubicin. $^{\#}P < 0.001$ versus normal control; $^{**}P < 0.01$ versus dox; $^{***}P < 0.001$ versus dox.

dose dependent increase in levels compared to doxorubicin group.

10.2. Superoxide Dismutase. Superoxide dismutases are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. It is an important antioxidant defense in nearly all cells exposed to oxygen. Superoxide dismutase activity was estimated in tissue homogenate with the help of pure bovine superoxide dismutase standard.

In this study, we found that single dose of doxorubicin to rats causes significant ($P < 0.001$) decrease in superoxide dismutase levels. This reduction indicates that toxicity is produced with doxorubicin. Pretreatment with test compound at the doses of 50 mg/kg and 100 mg/kg before a single dose of doxorubicin administration showed a significant ($P < 0.01$ and $P < 0.001$) dose dependent increase in levels compared to doxorubicin group.

10.3. Glutathione. Glutathione, the dominant intracellular thiol, plays an important protective role against oxidative stress caused by reactive oxygen species. It is an endogenous antioxidant. Glutathione was estimated in plasma with the help of pure glutathione standard graph. The values were shown in Figure 7.

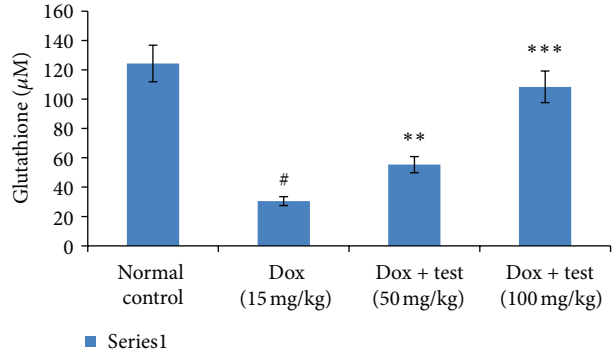


Figure 7: Effect of test compound on glutathione levels in plasma in rats treated with doxorubicin. $^{\#}P < 0.001$ versus normal control; $^{**}P < 0.01$ versus dox; $^{*}P < 0.001$ versus dox.

In this study, we found that single dose of doxorubicin to rats causes significant ($P < 0.001$) decrease in glutathione levels. This reduction indicates that toxicity is produced with doxorubicin. Pretreatment with test compound at the doses of 50 mg/kg and 100 mg/kg before a single dose of doxorubicin administration showed a significant ($P < 0.01$ and $P < 0.001$) dose dependent increase in levels compared to doxorubicin group.

11. Histopathological Examination

The heart tissue of different groups of animals was histopathologically examined and the results were as shown in Figure 8.

Histopathological observations in rat heart tissues:

Group I. (Normal control) showed no lesions of pathological significance.

Group II. (Doxorubicin 15 mg/kg i.p) showed degeneration of muscle bundles.

Group III. (Doxorubicin + test compound 50 mg/kg) showed degeneration of muscle bundles and interstitial edematous change.

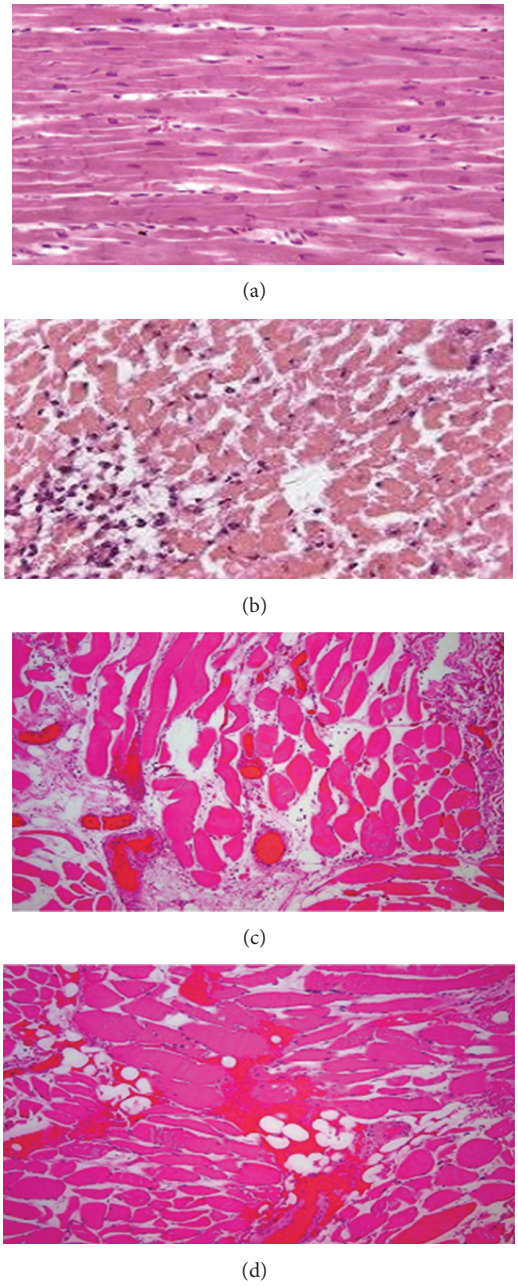
Group IV. (Doxorubicin + test compound 100 mg/kg) showed mild degeneration of muscle bundles and less interstitial edematous change.

These above results indicate that test compound showed cardioprotection against doxorubicin induced cardiotoxicity.

12. Discussion

The present study was aimed to evaluate the cardioprotective activity of test compound by measuring various cardiotoxic biomarkers, antioxidant enzymes, and histopathological examination in rats.

In the present study cardiotoxicity was induced by a single dose of doxorubicin (15 mg/kg, i.p) in rats. Doxorubicin treatment causes significant elevation in plasma CK-MB,



(a)

(b)

(c)

(d)

Figure 8: Light micrograph of heart tissue from rats treated with (a) normal control, (b) doxorubicin treated, (c) doxorubicin + test compound 50 mg/kg, and (d) doxorubicin + test compound 100 mg/kg.

LDH, TG, and AST when compared with normal control group. There is a significant reduction in catalase, superoxide dismutase levels in heart tissue, and significance depletion in glutathione levels in plasma when compared with normal group. This confirms the induction of cardiotoxicity in rats which is supported by histopathological changes observed in heart tissues [7].

Doxorubicin is broad spectrum antibiotic used as an anticancer drug which was limited by its dose dependent cardiotoxic effects [15]. The role of oxygen free radicals and oxidative stress has been well established in case of doxorubicin induced cardiac damage in rats. Doxorubicin is converted into its semiquinone form in cardiac myocytes by CYP450 and flavin monooxygenases. Then it reacts with molecular oxygen, initiates cascade of reactions, and produces ROS. ROS reacts with lipids, proteins, and other cellular constituents to cause damage to mitochondria and cell membranes of the heart muscle [16].

Pretreatment with 50 mg/kg and 100 mg/kg of test compound for 8 days shows significant protection against cardiotoxicity in rats by decreasing the cardiac biomarkers like CK-MB, LDH, TG, and AST and elevation of antioxidant enzymes like catalase, superoxide dismutase levels in heart tissue, and glutathione levels in plasma.

Histopathological observations of hearts of animals treated with a single dose of doxorubicin (15 mg/kg, i.p) showed degeneration of muscle bundles. Pretreatment with 50 mg/kg and 100 mg/kg of test compound for 8 days showed less or mild degeneration of muscle bundles and mild interstitial oedematous change was seen (Figure 8). This shows that test compound has cardioprotection against doxorubicin induced cardiotoxicity.

Catalase is a free radical scavenging enzyme which has cellular defence against oxidative stress and scavenging reactive oxygen radicals [17]. In the present study doxorubicin treatment produced notable decrease in heart tissue catalase levels when compared with normal. Pretreatment with 50 mg/kg and 100 mg/kg of test compound for 8 days showed marked elevation in catalase levels compared with doxorubicin group (Figure 5).

Superoxide dismutases are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. As such, they are an important antioxidant defense in nearly all cells exposed to oxygen [12]. In the present study doxorubicin treatment produced decrease in SOD levels when compared with normal. Pretreatment with 50 mg/kg and 100 mg/kg of test compound for 8 days showed marked elevation in SOD levels compared with doxorubicin group (Figure 6).

Glutathione is a most important antioxidant enzyme, preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides. Glutathione is found almost exclusively in its reduced form, since the enzyme that reverses it from its oxidized form, glutathione reductase, is constitutively active and inducible upon oxidative stress [13]. It exhibits antioxidant activity by reacting with superoxide radicals, peroxy radicals, and so forth [18]. In the present study doxorubicin treatment produced decrease in plasma glutathione levels when compared with normal. Pretreatment with 50 mg/kg and 100 mg/kg of test compound for 8 days showed marked elevation in glutathione levels compared with doxorubicin group (Figure 7).

All the results were supported with previous reports that N-(benzo[d]oxazol-2-yl)-2-(5-bromo-2-oxoindolin-3-ylidene)hydrazinecarboxamide has cardioprotective activity against doxorubicin induced cardiotoxicity in rats. Pretreatment with compound significantly reduced the elevated levels of cardiotoxic biomarkers in plasma [14], *N*-substituted isatin derivatives are more potent small molecules with enhanced

free radical scavenger properties and the cytoprotective effect on the apoptosis of PC12 cells induced by H_2O_2 was screened [19]. These above reports mainly support that isatin derivatives have free radical scavenger properties and cardioprotective activity. The present study results show that novel synthetic isatin derivative decreases the cardiotoxic biomarkers like CK-MB, LDH, TG, and AST and elevates the antioxidant enzymes like catalase and superoxide dismutase; glutathione which supports that novel synthetic isatin derivative has cardioprotective activity.

13. Conclusion

On the basis of our findings, it may be worthy to suggest the following.

(i) Novel synthetic isatin derivative has cardioprotective effect against doxorubicin induced cardiotoxicity in rats by decreasing the cardiotoxic biomarkers like CK-MB, LDH, TG, and AST in plasma.

(ii) Novel synthetic isatin derivative has antioxidant effect, evaluated by measuring antioxidant enzymes. There is an increase in catalase, superoxide dismutase in heart tissues, and glutathione levels in plasma in doxorubicin induced cardiotoxicity in rats.

(iii) Novel synthetic isatin derivative has cardioprotective effect against doxorubicin induced cardiotoxicity by observing the histopathological changes in rat heart tissues.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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