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Original article

# Robinin modulates doxorubicin-induced cardiac apoptosis by TGF-β1 signaling pathway in Sprague Dawley rats

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ABSTRACT

The study focussed on the cardioprotective effect of robinin on doxorubicin-induced cardio-toxicity in Sprague Dawley rats. After the experimental period, animals were sacrificed and the various parameters such as cardiac markers, toxicity parameters, antioxidant status, ROS generation, lipid peroxidation status and inflammatory parameters were assessed. Gene expression study by RT-PCR analysis and proteins expression study by western blotting were done. Doxorubicin causes significant increase in the levels of cardiac marker enzymes, namely lactate dehydrogenase (LDH), creatine phospokinase (CPK), toxicity parameters like serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT). Antioxidant enzyme levels were decreased; lipid peroxidation products in heart tissue and inflammatory markers, namely cyclooxygenase (COX2) and lipooxygenase (LOX15) were significantly increased. Gene expression study by RT-PCR analysis of transforming growth factor-β1 (TGF-β1), Smad2, murine double minute (Mdm2), Smad3, cyclin-dependent kinase inhibitor 2A (CDKN2A), Smad4 and Smad7 were significantly altered. The western blotting study of p53, Bcl-2 and Bax also showed altered expression. The supplementation of the Robinin along with DOX caused normalised level of all the above parameters and cardio-toxicity. This study revealed the cardioprotective nature of Robinin on doxorubicin-induced cardiac toxicity by modulating TGF-β1 signaling pathway in Sprague Dawley rats.



## 1. Introduction

Doxorubicin (DOX) is a potent chemotherapeutic drug that is used extensively for the treatment of haematological malignancies and solid tumours [1]. However, long-term clinical usefulness is limited by a cumulative dose-dependent irreversible chronic cardiotoxicity which manifests itself as congestive heart failure [2–4]. DOX induced cardiotoxicity is thought to be a complex multifactorial process, which includes oxidative stress [5,6]. These reactive oxygen species (ROS) damages the heart by exceeding the oxygen radical detoxifying capacity of cardiac mitochondria [7].

The use of cardioprotective agents together with doxorubicin is a possible therapeutic approach. Several pharmacological agents have been shown to reduce the cardiotoxicity of doxorubicin, including antioxidants, iron chelating agents and haematopoietic cytokines [8–10]. Moreover, the selective toxicity for doxorubicin towards cardiac tissue rather than other tissues such as liver is due to low levels of antioxidant defences [11].

Therefore, the antioxidant therapy may be useful in the management of cardiotoxicity. Only few agents have been proven to be applicable in clinical practice and there is a search for a cardioprotective drug to suppress doxorubicin-induced complications [12]. Natural antioxidants obtained from medicinal plants are becoming popular due to their low cost, easy accessibility to everyone and perceived fewer side effects [13].

*Vigna unguiculata* leaves are edible and used as a leafy vegetable in cuisine from traditional times in India. The anti-inflammatory property of Robinin, the flavonoid from *Vigna unguiculata* in hPBMCs has been already published by our research group [14,15]. The study revealed that Robinin modulates TLR/NF-κB signaling pathway in oxidized LDL challenged human peripheral blood mononuclear cells. Robinin is one of the active principles unexploited for cardiac activity. Robinin (kaempferol 3-O-robinoside-7-O-rhamnoside) is one of the derivatives of kaempferol; it is present in flavanoid fraction of *Vigna unguiculata* leaf [16].

Transforming growth factor-β1 (TGF-β1) is formed mainly by cardiac myofibroblast and fibroblast and contributed to cardiac

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fibrosis development, hypertrophy and apoptosis [17]. The initiation of TGF-β/Smad signaling pathway started by the formation of heteromeric receptor complexes of specific type I and II serine/threonine kinases [18,19]. After binding of TGF-β to receptor type II, the receptor of type I is phosphorylated leading to phosphorylation of Smad-2 and Smad -3 then formed a complex with Smad4. This complex then entered the nucleus and binds to TGF-β responsive gene promoter that regulates their expression [20]. In addition, the expression of TGF-β1 and Smad-2, -3, and -4 is upregulated during the chronic phase of myocardial infarct scar healing [21]. Furthermore, Smad proteins are mediators for the TGF-β-induced apoptosis [22]. There is no previous report on Robinin inhibition of TGF-β1/Smad signaling pathway in cardiac toxicity.

Hence, present study was focussed on the cardioprotective role of Robinin on doxorubicin-induced cardiotoxicity in Sprague Dawley rats through TGF-β1 signaling pathway.

## 2. Materials and methods

All biochemicals and reagents used for the experimental study were purchased from Sigma Aldrich chemical company, USA. Other chemicals and solvents of analytical grade were from Sisco research laboratories chemicals and Spectrochem India respectively. RNA was isolated from the heart tissue by means of trizol reagent from Medox Biotech India PVT, Ltd. RT-PCR and PCR amplification were carried out using the RT-PCR kit from QIAGEN, India.

### 2.1. Animal experiments

Twenty-four male Sprague Dawley rats were obtained from the Department of Biochemistry, University of Kerala, with body weight of 180–200 g They were housed in cages under a day-night cycle of 12 h, at 25 ± 2 °C at room temperature. Rats were fed with the standard diet and water ad libitum throughout the experimental period.

All ethical guidelines were followed for the conduct of animal experiments in strict compliance with the institutional animal ethical committee and committee for the purpose of control and supervision of experiments on animals (CPCSEA), Government of India and ethical sanction nº IAEU-KC-24/2011-12-BC.AA (22) for the conduction of animal experiment. The rats were anesthetized by pentobarbital sodium (65 mg/kg, i.p.). Blood samples were collected for biochemical analysis. The rats were sacrificed with hearts promptly removed for biochemical and histological analysis.

### 2.2. Experimental design

The animals were randomly divided into 4 groups of 6 animals each and the experimental period was 10 days.

Group I: control; Group II: Robinin (50 mg/kg); Group III: DOX (10 mg/kg) and Group IV: Robinin plus DOX. Robinin (50 mg/kg) were orally administered everyday for 10 continuous days. DOX (10 mg/kg) were i.v. injected at day 7. The selected dose of Robinin (50 mg/kg) body weight was based on our preliminary study.

The experimental protocol is based on previously described protocol [23] with some modification.

### 2.3. Estimation of heart marker enzymes and toxicity markers

The activity of lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) in serum was measured according to a previous method [24]. The activity of serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) was measured by a previous method [25].

### 2.4. Cardiac antioxidant status

The antioxidants of heart tissue were studied; activity of superoxide dismutase (SOD) was determined by a previous method [26], catalase (CAT) activity in the samples was measured by a previous method [27], glutathione peroxidase (GPx) activity and glutathione reductase (GRd) activity was determined using a known procedure [28].

### 2.5. Level of inflammatory markers

The activity of cyclooxygenase (COX-2) was determined by a previous method [29], the activity of lipooxygenase (LOX-15) was also estimated by a known method [30].

### 2.6. Estimation of lipid peroxidation and ROS level in heart tissue

The level of thiobarbituric acid reactive substance (TBARS) in heart tissue was estimated as per the method [31] and ROS in heart tissue were estimated by a previous method [32].

### 2.7. RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis transforming growth factor-β1 (TGF-β1), Smad2, murine double minute (Mdm2) and, Smad7, Smad3, cyclin-dependent kinase inhibitor 2A (CDKN2A) and Smad4 gene from heart tissue of rat

RNA was isolated from heart tissue of rabbit by means of trizol reagent from Medox biotech India PVT, LTD. RT-PCR and PCR amplification were carried out using the RT-PCR kit from QIAGEN, India. Initial PCR activation step for 17 min for 96 °C, followed by 3 steps of cycling. Each cycle consists of denaturation for 1 min at 95 °C, annealing for 1 min at 67 °C, extension for 1 min at 74 °C, repeated for 36 cycles and final extension for 15 min at 73 °C. The PCR products were run on 0.8–1.2% Agarose gels, stained with ethidium bromide and visualized with a UV-transilluminator.

### 2.8. SDS–PAGE and western blotting studies of p53, Bcl-2 and Bax

The western blotting of p53, Bcl-2 and Bax were done by a previous method [33]. The protein was isolated from the heart tissue. Lysates were boiled for 5 min in nonreducing sample buffer (10 mmoL/L Tris [pH 6.8], 2% SDS, 20% glycerol, and 0.001% [wt/vol] bromophenol blue) and resolved by 8% SDS–polyacrylamide gel electrophoresis (PAGE) with protein lysate from an equal number of cells loaded per lane. Separated proteins were transferred to nitrocellulose membranes and blocked for 1 hour at room temperature in PBS plus 3% (wt/vol) powdered milk and 0.1% Tween 20. Primary antibody of p53, Bcl-2 and Bax (Sigma, India) was added at the indicated dilution in blocking buffer and incubated on a rocking platform for 1 hour at room temperature. Binding was detected by incubation with peroxidase-conjugated secondary antibody (Sigma), diluted 1:1000 in blocking buffer, and visualized by chemiluminescense.

### 2.9. Estimation of total protein

Total proteins of the samples were determined as described previously by the method [34] using bovine serum albumin as a standard.