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RESEARCH ARTICLE

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Metal Chelating Activity of *Glycine max* Seed Extract on Ferrous + Doxorubicin-Induced Cardiotoxicity in Rats.

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ABSTRACT

The study evaluated metal chelating activity of Glycine max seed *extract on Doxorubicin (DOX) induced cardiotoxicity in rats. In this study twenty four male Albino Wistar rats weighing between 200-250g were used. The animals were treated as follows. Group I: Animals served as vehicle control and received 0.5% tween 80 (10 ml/kg, p.o). Group II: Animal received* Ammonium Ferric Citrate (*0.6 mg/kg/day, p.o* for 14 days) *followed by doxorubicin (10mg/kg, i.v; once 48 hr before sacrifice), Group III: Animal received Deferoxamine 20 mg/kg, p.o. for 14 days followed by ferrous + doxorubicin, Group IV: Animal received Glycine max alcoholic extract 200 mg/kg, p.o for 14 days followed by ferrous + doxorubicin. In each group, body wt of rats were taken before and after doxorubicin administration. After 48 hrs of doxorubicin administration blood was collected for serum CK-MB and LDH estimation. Isolated hearts were dried and weighed. In heart tissues superoxide dismutase (SOD) & catalase (CAT), glutathione (GSH) and malondialdehyde (MDA) were estimated. Results showed that mean heart weight/body weight (HW/BW) ratio in group 2 was significantly ($p<0.001$) decreased, CKMB ($p<0.01$), LDH ($p<0.05$), MDA ($p<0.01$) increased, GSH ($p<0.01$), SOD and CAT ($p<0.01$) were decreased as compared to group 1. Group 3 & 4 has shown significant ($p<0.01$) increase in (HW/BW) ratio, decrease in CKMB ($p<0.01$), LDH ($p<0.05$), MDA ($p<0.01$), GSH ($p<0.05$), SOD ($p<0.01$) and CAT ($p<0.01$) increased as compared to group 2. It may be concluded that Glycine max alcoholic extract posseses cardioprotective and metal chelating activity in Doxorubicin induced cardiotoxicity in rats.*

Key Words: *Glycine max* seed, Cardiotoxicity, Metal chelating agent, Phytic acid.

INTRODUCTION

Cardiotoxicity is a well-known side effect of several cytotoxic drugs, especially of the anthracyclines and can lead to long term morbidity. The clinical use of Doxorubicin (DOX) is limited by dose dependence cardiotoxicity which may lead to severe and irreversible form of cardiomyopathy with congestive heart failure and high mortality is one of the factors that limit its use [1]. The cardiomyopathies represent a variety of diseases affecting the myocardium in either a diffuse or multifocal manner that frequently results in heart failure. The term congestive heart failure is used for the chronic form of heart failure in which the patient has evidence of congestion of peripheral circulation and of lungs. It is end result of various forms of serious heart diseases [2]. Doxorubicin is an anthracycline antibiotic having antitumour action and produced by the fungus *Streptococcus peucetius var. caesius*.

Doxorubicin produces clinically useful responses in a variety of human cancers. It has been shown by many investigators that the cellular damage induced by DOX is mediated by the formation of an iron-anthracycline complex that generate free radicals [3], which in turn cause severe damage of plasma membrane and interfere with cytoskeleton assembly [4,5]. The possible involvement of iron in DOX-induced cardiotoxicity became evident from studies in which iron chelators were shown to be cardioprotective [6]. Conversely, iron loading was found to potentiate anthracycline toxicity [7, 8]. DOX can form a stable complex with ferric iron [9] and the formed complex undergoes self-reduction to ferrous iron, resulting in the generation of a semiquinone free radical of DOX [10]. The semiquinone reacts with oxygen to form a superoxide anion radical that, in the presence of iron, catalyzes conversion to a hydroxyl radical. In turn, this potent radical causes lipid peroxidation and DNA damage [11].

Iron chelation reduces the generation of free radicals and therefore has been studied in many experimental systems, and in some human clinical trials, for its ability to reduce anthracycline-induced cardiotoxicity. Small clinical trials have shown that iron chelation allowed for the administration of greater doses of anthracycline and reduced the number of patients developing heart failure [12, 13]. Phytic acid (IP6) present in *Glycine max* is the most potent natural iron chelator and has strong antibiotic and antioxidant action. IP6 has been found to have similar iron-chelating properties as desferrioxamine, a drug commonly used to kill germs, tumor cells or to remove undesirable minerals from the body. The ability of myo-inositol polyphosphates to inhibit iron-catalysed hydroxyl radical formation was studied in a hypoxanthine/xanthine oxidase system.

The use of natural drug for metal chelation has been reported [14]. The soybean (US) or soya bean (UK) (*Glycine max*) is a species of legume native to East Asia, widely grown for its edible bean which has numerous uses. Together, oil and protein content account for about 60% of dry soybeans by weight; protein at 40% and oil at 20%. The remainder consists of 35% carbohydrate and about 5% ash. Soybeans contain a high level of phytic acid, which has many effects including acting as an antioxidant and a chelating agent. The beneficial claims for phytic acid [15, 16] include reducing cancer, minimizing diabetes, and reducing inflammation. However, phytic acid is also criticized for reducing vital minerals due to its chelating effect, especially for diets already low in minerals. The present study was aimed at evaluating the Cardio protective and metal chelating effect of *Glycine max* seed extracts containing phytic acid on doxorubicin-induced cardiotoxicity in rats.

MATERIALS AND METHODS

Drugs and chemicals used

Doxorubicin powder injection was gifted as doxorubicin hydrochloride by Serum Institute of India Ltd, Pune. Ammonium Ferric Citrate was procured from local medicine shop. All the solvents, chemicals used were of analytical grade and chemicals required for sensitive biochemical assays were purchased from Merck. CK-MB kit was purchased from Aspen Laboratories, Rapid Diagnostic Pvt Ltd and LDH kit from Crest Biosystems Goa, India. All drug solutions were freshly prepared before each experiment. *Glycine max* extract was suspended in 0.5% tween 80 and administered orally.

Collection of plant material

Seeds of *Glycine max* was purchased locally and authenticated by Dr. Dhabe, Dept. of Botany, Dr. Babasaheb Ambedkar Marathwada University (BAMU) Aurangabad. A voucher specimen no. 0770 has been deposited in the same department.

Preparation of extract

Glycine max (Soybean) were collected and dried under shade and coarsely powdered. The powder obtained was soxhelated using Petroleum ether at 60-80^{0}C and absolute ethanol for each drug in 1:5 proportions with solvents up to 65-72hrs. The marc was again used in same fresh solvents as second cycle for 65-72hrs. The respective filtrates were air dried and concentrated to obtain sticky dried extract. Appropriate concentrations of the extracts were made in distilled water.

Animals

Twenty four (24) Male Albino Wistar rats weighing between 200-250 gm, were procured from Wockhardt Ltd, Aurangabad. Animals were housed under standard laboratory conditions of temperature 25 ± 1ºC with free access to food (Amrut rat and mice feed, Sangli, India.) and water. The experiments were performed during the light cycle (12-12 h). The experiments were carried out according to the guidelines of the Committee for the Purpose of Control

and Supervision of Experiments on Animals (CPCSEA), New Delhi, India, and approved by the Institutional Animal Ethics Committee, Y.B. Chavan College of Pharmacy, Aurangabad.

Experimental protocol
The animals were divided in following experimental groups, each group comprising of six animals.

Group I: Animals served as vehicle control and received 0.5% tween 80 (10 ml/kg, p.o).
Group II: Animal received Ammonium Ferric Citrate (*0.6 mg/kg/day, p.o* for 14 days) + doxorubicin (10mg/kg, i.v; once 48 hr before sacrifice).
Group III: Animal served as standard drug treated and received deferoxamine 20 mg/kg, p.o. for 14 days followed by Ammonium Ferric Citrate (*0.6 mg/kg/day, p.o* for 14 days) + doxorubicin (10mg/kg, i.v; once 48 hr before sacrifice).
Group IV: Animal received *Glycine max* seed alcoholic extract 200 mg/kg, p.o. for 14 days followed by Ammonium Ferric Citrate (*0.6 mg/kg/day, p.o* for 14 days) + doxorubicin (10mg/kg, i.v; once 48 hr before sacrifice).

Cardiotoxicity Assessment Parameters
Preparation of serum:
Blood was collected from retro-orbital plexus from the inner canthus of the eye (under light ether anesthesia) using glass capillary tubes. Serum was separated using R-24 research centrifuge (Remi Instruments Ltd., Mumbai) at 3000 rpm for 15 min. and used for estimation of CK-MB [17], LDH [18].

The animals were sacrificed under ether anaesthesia by cutting the carotid artery. The hearts were quickly removed, rinsed in ice cold saline, dried on a filter paper, and weighed. A 10 % homogenate was prepared in 0.15 M KCl for the estimation of tissue malondialdehyde according to Ohkawa et al [19]. The homogenate for tissue glutathione was prepared in 0.02 M EDTA and measured according to Lindsay & Sedlak [20], Ellman [21].

Preparation of Post Mitochondrial Supernatant (PMS)
The tissues were homogenized in chilled potassium phosphate buffer (50mM, pH 7.4) using a Remi homogenizer. The homogenate was centrifuged in a refrigerated centrifuge at (10,500 rpm) for 20 minutes at 4°C to obtain the PMS, which was used for various biochemical analyses. The post mitochondrial supernatant (PMS) was used for the estimation of enzymes such as Catalase according to Clairborne [22] and Superoxide Dismutase according to Marklund [23]. Protein estimation in the PMS was done according to Lowry et al [24].

Statistics
The mean ± SEM values were calculated for each group. One-way ANOVA followed by Dunnett's multiple comparison tests were used for statistical analysis. Values of $p<0.05$ was considered statistically significant. The entire statistical analysis was performed using statistical package, GraphPad Instat Version 3 (GraphPad Software Inc., USA)

RESULTS

The present study revealed that the mean heart weight/body weight ratio in toxic control group i.e Ferrous + Doxorubicin treated group was significantly ($p<0.001$) decreased as compared to control group 1 (Tween 80, 0.5%). Deferoxamine and *Glycine max* alcoholic extract (200 mg/kg/day, p.o) treated groups showed significant ($p<0.001$) increased heart weight/body weight ratio as compared to toxic control group 2. Results in toxic control group 2 showed a significant increase in the levels of cardiac markers i.e CK-MB ($p<0.01$) and LDH ($p<0.05$) levels as compared to control group 1. The CK-MB levels in deferoxamine and *Glycine max* alcoholic extract (200 mg/kg/day, p.o) treated groups showed significant ($p<0.01$ and $p<0.05$) decrease respectively as compared to group 2, whereas LDH levels in toxic control group 2 was found to significantly increased ($p<0.05$) as compared to control group 1. The *Glycine max* alcoholic extract showed significant decrease ($p<0.05$) in LDH levels as compared to toxic control group. In toxic control group a significant decrease ($p<0.01$) in the levels of GSH was observed as compared to control group 1. *Glycine max* alcoholic extract treated group showed significant ($p<0.05$) increase in GSH levels compared to toxic control group 2 (Table 1).

In toxic control group a significant increase ($p<0.01$) in the levels of MDA as compared to control group was found. Deferoxamine and *Glycine max* alcoholic extract treated group showed significant decrease ($p<0.01$) and ($p<0.05$) respectively in MDA levels compared to toxic control group 2. The antioxidant levels of SOD enzymes were decreased in heart tissue of toxic control group 2 as compared to control group 1, where the levels of catalase enzyme was significantly ($p<0.01$) decreased in heart tissue of toxic control group 2 as compared to control group 1.

Catalase levels were significantly ($p<0.01$) increased in deferoxamine and *Glycine max* alcoholic extract treated group. The levels of superoxide dismutase enzyme was significantly ($p<0.01$) decreased in heart tissue of toxic control group 2 as compared to control group 1. Superoxide dismutase enzyme levels were significantly increased in deferoxamine ($p<0.01$) and *Glycine max* alcoholic extract ($p<0.05$) treated group. (Table 2)

Table. 1 Effect of *Glycine max* alcoholic seed extract on Heart wt. /body wt. ratio, Creatine kinase, Lactate dehydrogenase and tissue glutathione in Doxorubicin induced Cardiotoxicity.

Treatment given	Heart wt./body wt. ratio (X 10^{-3})	CK-MB (U/L)	LDH (U/L)	Tissue GSH (µmol/gm tissue)
Control (Tween 80, 0.5%)	5.376 ± 0.22	2535.192 ± 302.35	3534.22±197.53	3.838 ± 0.329
Ferrous + DOX	2.658 ± 0.145**	5040.132 ± 232.93**	5163.95±973.22*	0.297± 0.063**
Deferoxamine + (Ferrous + DOX)	4.53 ± 0.426**	2490.978 ± 169.13**	1090.08±116.17*	1.5752±0.368*
Glycine max alcoholic extract + (Ferrous + DOX)	4.67 ±0.49**	2704.04 ± 915.9*	6728.74±1447.5	1.602±0.35*

*No. of samples (n) =5. The observations are Mean ± SEM and ** p<0.01 and *(p<0.05) as compared to Ferrous + DOX induced cardiotoxicity. (One Way - ANOVA followed by Dunnett's test.)*

Table.2: Effect of *Glycine max* alcoholic seed extract on tissue MDA, Superoxide Dismutase and Catalase in Doxorubicin induced Cardiotoxicity

Treatment given	Tissue MDA (µmol/gm tissue)	SOD (Units/mg Protein)	Catalase (nmoles of H_2O_2 consumed/min/mg protein)
Control (Tween 80, 0.5%)	2.025±0.27	77.462±8.146	48.19± 3.13
Ferrous + DOX	4.99±0.6216**	37.242±3.541**	15.95±1.923**
Deferoxamine + (Ferrous + DOX)	2.74±0.5284**	72.244±3.931**	41.87±5.88**
Glycine max alcoholic extract + (Ferrous + DOX)	3.042±0.3578*	64.698±9.212*	40.35±5.35**

*No. of samples (n) =5. The observations are Mean ± SEM and ** p<0.01 and *(p<0.05) as compared to Ferrous + DOX induced cardiotoxicity. (One Way - ANOVA followed by Dunnett's test.)*

DISCUSSION

The risk of cardiotoxicity is the most serious drawback to the clinical usefulness of anthracycline antineoplastic antibiotics, which include doxorubicin (adriamycin), daunorubicin or epirubicin. Anthracyclines may promote the formation of ROS through redox cycling of their aglycones as well as their anthracycline-iron complexes. This proposed mechanism has become particularly popular in light of the high cardioprotective efficacy of dexrazoxane (ICRF-187). The mechanism of action of this drug has been attributed to its hydrolytic transformation into the iron-chelating metabolite ADR-925, which may act by displacing iron from anthracycline-iron complexes or by chelating free or loosely bound cellular iron, thus preventing site-specific iron-catalyzed ROS damage [25]**.** Iron chelation reduces the generation of free radicals and therefore has been studied in many experimental systems, and in some human clinical trials, for its ability to reduce anthracycline-induced cardiotoxicity. Small clinical trials have shown that iron chelation allowed for the administration of greater doses of anthracycline and reduced the number of patients developing heart failure. In response to acute and chronic coronary artery occlusion in dog model, myocardium showed twofold to threefold increase in CK-MB activity in both the ischemic and non-ischemic myocardium. In contrast, individual with normal cardiac tissue had low percentage of CK-MB.

Our findings are consistent with above mentioned studies and *Glycine max* alcoholic extract was found to inhibit the ferrous and DOX-induced CK-MB release in serum of rats. Our study shows that and *Glycine max* alcoholic extract led to decrease in CKMB release in dose used. There was a near complete decrease in *Glycine max* alcoholic extract treated group. Also the administration of ferrous and DOX induced cardotoxicity manifested by significant increase in serum LDH levels. The results are consistent with previous studies [26, 27]. The LDH levels are significantly decreased in deferoxamine treated group whereas *Glycine max* alcoholic extract fails to show a decrease in LDH levels. GSH may play an important role in protecting the heart from peroxidative attack [28]. DOX significantly decreased the level of tissue GSH in accordance with the previous studies [29]. Decrease in the levels of GSH represents its increased utilisation by myocardial cells due to oxidative stress. Treatment with deferoxamine and *Glycine max* alcoholic extract has significantly restored the GSH levels, this effect could be attributed either to increased biogenesis of GSH, reduction in oxidative stress levels or chelation of ferrous by phytic acid present in *Glycine max* alcoholic extract leading to decreased generation of toxic free-radical species. In our study, a significant decrease in concentration of SOD and CAT levels in Ferrous + DOX treated group was observed.

Deferoxamine and *Glycine max* alcoholic extract treatment significantly reversed the changes in antioxidant levels induced by ferrous + DOX treatment. A decrease in the activity of SOD can result in the decreased removal of superoxide ion, which can be harmful to the organs. Moreover, the enhanced SOD activity in the deferoxamine and *Glycine max* alcoholic extract treated group might be involved in scavenging of O_2^- generated from DOX. Increased levels of MDA and decreased levels of GSH, SOD and CAT were observed in heart tissue in ferrous + DOX treated animals. Deferoxamine and *Glycine max* alcoholic extract efficiently counteracted the ferrous + DOX induced cardiac tissue damage by significantly decreasing the MDA levels and increasing the GSH, SOD and CAT activities. The observed elevated CAT levels in ferrous + DOX treated animals support the above hypothesis that this increase is possibly required to overcome excessive oxidative stress [30, 31]. In our study, the average heart weight to body weight ratio in ferrous + DOX treated group was significantly decreased when compared with control group and deferoxamine and *Glycine max* alcoholic extract treated groups have shown an increase in heart weight to body weight ratio as compared with toxic control group.

CONCLUSION

Thus from above observations and results it was concluded that the *Glycine max* seed alcoholic extract possesses metal chelating and cardioprotective activity which might be due to presence of phytic acid in the *Glycine max* seed alcoholic extract and further studies showing isolation and characterization of active phytoconstituents may be needed.

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