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Research Article

Antioxidant and Cardiac Enzyme Marker Studies of *Thevetia peruviana* Seed Hydro-Methanol Extract in Wistar Male Albino Rats

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ABSTRACT

Thevetia peruviana seed kernels are used for suicide attempts in many countries centuries back. The aim of the present study was to evaluate the level of toxicity exposure of seed kernels by acute and subacute studies on male Wistar rats taking antioxidant enzyme levels in the vital organs, like liver, kidney, heart, and brain tissues myocardial marker enzyme levels in serum. Results revealed that antioxidant enzyme (SOD, GPX, GSH) levels were normal in the lower groups (25, 50 mg/kg), but drastic hike was observed in CKMB and LDH cardiac biomarker enzyme levels in 100 mg/kg groups. In the liver tissues of group IV animals, a significant dose-dependent increase was observed in the activities of SOD (3.15 ± 0.58), GPx (46.55 ± 4.79), and GSH activity (18.20 ± 0.56). In kidney homogenates, SOD and GSH level showed a statistically insignificant ($p < 0.05$) elevation, but the increase in GPx level shown by group IV animals (41.50 ± 7.04) was significant ($p < 0.05$). The activities of SOD in brain homogenates were increased significantly in group III (2.17 ± 0.24) and group IV (2.51 ± 0.27) animals. The GPx enzyme level also increased dose-dependently ($p < 0.01$), but the level of GSH was found an insignificant hike. The heart, supposed to be the most adversely affected organ on cardiac glycoside administration, showed undisturbed values of enzyme levels. A noticeable elevation was observed in the serum CKMB and LDH enzyme levels in a dose-dependent manner, but the extract did affect only the higher dosed animals (100 mg/kg) significantly ($p < 0.05$). In contrary to that, tissue homogenates of subacute animals under study showed a markedly significant hike in both CKMB and LDH levels. In conclusion, the level of toxicity and safety margin is very narrow, and the seeds really take the lives of organisms, whose intake is accidentally or deliberately.

Introduction

Although herbal preparations have vital roles in health and disease management system, the safety of herbal medicines have to be evaluated before recommending as a source of natural remedy. Most members of Apocynaceae have reported medicinal as well as toxic properties. The taxon *T. peruviana*, a garden evergreen ornamental, is well known for its toxicity. All plant parts, especially the seeds were reported as a rich treasure of many valuable cardiac glycosides.[1] Even though the plant has much therapeutic properties,[2] it is enlisted as a toxic plant because of the narrow margin of safety between therapeutic and toxic doses.

Deliberate seed intake of yellow oleander with suicidal and homicidal purpose and as an abortifacient is in usual practice in many countries, like India and Sri Lanka.[3,4] Also, the exposure of yellow oleander poisoning cases in humans with various clinical complications, like vomiting, diarrhea, arrhythmias, and palpitation were reported elsewhere.[5,6] Similarly, *Nerium oleander*, a close relative of *Thevetia*, also reported to have many toxicological compounds.[7] The typical symptoms of *Digitalis purpurea*

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intoxication include extreme fatigue, visual disturbances, weakness, nausea, headaches, and abdominal pain.[8]

The toxic nature of *Thevetia* seeds was earlier reported in many experimental animals like mice, albino rats, and rabbits with severe clinical malfunctioning.[9-12] Preceding studies in Wistar rats treated with a single dose of 70% methanol extract (500 mg/kg) caused 100% mortality and LD_{50} is calculated as 375 mg/kg, revealed the narrow margin of safety lies in between 250 and 500 mg/kg, on seed intake. In continuation with this, the present work was conducted to evaluate the effect of seed extract exposure on vital organs of Wistar male albino rats by analyzing the antioxidant and myocardial enzyme levels present in the serum and tissue homogenates, after oral administration of 14 days.

Materials and Methods

Animals

Male Wistar albino rats procured from the Small Animal Breeding Station (SABS), Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India, were maintained under standard environmental conditions in the animal house of Amala Cancer Research Centre, Trichur. Animals were divided into four groups of five each for acute and sub-acute studies. All animal experiments were conducted as per Organization for Economic Co-operation and Development (OECD) guidelines (423) and with the prior permission of the Institutional Animal Ethics Committee (IAEC) of the research center, approved by government of India.

Preparation of Drug Samples

Approximately 60 grams of coarsely powdered seed kernels were de-fatted with petroleum ether and extracted using 70% methanol in a soxhlet extractor for 20 to 22 hours. The residue was collected, evaporated off the solvent and stored in airtight bottles at 4°C for toxicity studies.

Experimental Design

For acute studies, all the three animal groups (II, III, and IV) were given orally a single dose of the drug at 100, 250, and 500 mg/kg body weight and a dosage of 25, 50, and 100 mg/kg body weight were given to subacute groups for a consecutive 14 days. Group I is treated as control in both cases. On 15th day, animals were anesthetized, and blood samples were collected for serum biochemical studies. Vital internal organs like liver, kidney, heart, and brain were dissected out, observed visually for any necrotic lesions, washed in chilled phosphate buffered saline (PBS), blotted dry, and weighed. All tissue and plasma samples were stored at –80°C for further analyses.

Tissue Homogenization

Tissue samples of liver, kidney, heart, and brain (1-gram) were homogenized using ice-cold 0.1 M Tris HCl buffer (pH 7.4) in a homogenizer (Fork Scientific Industries, Mumbai), and 25 or 10% (w/v) homogenates were prepared. Centrifuged the samples in a cooling centrifuge (Remi Instruments Ltd.) at -4°C at 10,000 rpm, for 60 minutes. The supernatants were stored at -20°C for performing antioxidant enzyme assays, *viz*., superoxide dismutase (SOD), glutathione (GSH), and glutathione peroxidase (GPx), protein estimation, creatinine kinase (CKMB), and lactate dehydrogenase (LDH) cardiac biomarker enzyme assays.

Antioxidant Enzyme Assays in Organ Homogenates

SOD activity was assayed using the nitro blue tetrazolium (NBT) reduction method,[13] based on the ability of the enzyme to inhibit the reduction of NBT salt by superoxide (O_2^-), which is generated from the reduction of photo reduced riboflavin with O_2^+. Total protein content was determined in the homogenates by Lowry[14] method using bovine serum albumin as standard. The level of reduced GSH content in tissue homogenates was measured on the reaction with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB),[15] and the GPx activity assay was done based on the oxidation of GSH in the presence of H_2O_2.[16]

Myocardial Marker Enzyme Assays

Assays for cardiac marker enzymes in serum and the heart tissue supernatant were done using standard commercial diagnostic kits supplied by Euro Diagnostic Systems Pvt. Ltd., Chennai, and Linear Chemicals SL, Spain. Cardiac marker enzymes CKMB and LDH were assayed as per kit protocols, and the optical density was measured at 340 and 412 nm, respectively.

Analysis of Data

The results are expressed as mean values ± SD. One way ANOVA with posthoc Tukey's honest significant difference (HSD) test was used to compare significant differences between group means using SPSS software version 20 (2012). A level of $p < 0.05$ was regarded as statistically significant.

Results

In acute toxicity studies, no mortality was observed in all groups except group IV (500 mg/kg), where the mortality rate was 100% in this category. However, exposure to subacute doses did not make any significant changes in physical parameters, feeding habits, and mortality rates ($p < 0.05$) of all treated groups (25, 50, and 100 mg/kg).

Relative Organ Weight

The normal morphological nature of vital organs indicated that the extract did not affect their metabolism adversely. Statistically analyzed data between control versus all three treated groups showed no significant variation in liver, kidney, and heart weight ($p < 0.05$), but, there was a significant decrease in brain weight ($p < 0.01$)

of group IV animals (0.68 ± 0.06) compared to control group (0.8 ± 0.02).

The effect of seed extract on enzymatic and non-enzymatic antioxidants was analyzed in kidney, liver, heart, and brain tissue homogenates (Table 1). The activity of SOD in liver was found to be significantly increased to 3.15 ± 0.58 ($p < 0.05$) in 100 mg/kg treated group when compared to control animals (2.23 ± 0.46). Similarly, the activities of GPx enzyme also showed a significant increase from 30.14 ± 5.12 (control) to 42.32 ± 5.02 ($p < 0.05$) and 46.55 ± 4.79 ($p < 0.01$) in group III and IV animals. A significant dose-dependent increase was observed in GSH activity among all studied groups, especially in the higher dosage group ($p < 0.01$).

In kidney homogenates, the activities of antioxidant enzyme group, SOD showed a slight elevation from 2.94 ± 1.05 (control group) to 3.95 ± 1.36 (100 mg/kg group). But, the increase in GPx level shown by group IV animals was significant at $p < 0.05$ level. Similar elevation noticed in GSH level of control and all treated groups increased in a dose-dependent manner, but the deviation was statistically insignificant ($p < 0.05$).

The activities of SOD in brain homogenates were increased substantially on extract administration as compared to control animals (1.39 ± 0.38). The SOD activity was found as 1.97 ± 0.32 in group II ($p < 0.05$), but its level elevated significantly in group III (2.17 ± 0.24) and group IV (2.51 ± 0.27) animals ($p < 0.05$ and $p < 0.01$). The GPx enzyme level also increased dosedependently from 20.71 ± 0.93 (control) to 29.74 ± 4.27 (group II, $p < 0.05$), 36.79 ± 5.83 (group III, $p < 0.05$), and 41.71 ± 7.29 (group IV, $p < 0.01$), but the level of GSH was found an insignificant hike compared to control group.

The heart, supposed to be the most adversely affected organ on cardiac glycoside administration, showed undisturbed values of enzyme levels with the control group. In group IV animals, a slight decline in SOD ($p < 0.05$) was recorded, but the reduction in GPx and GSH enzyme activity from 22.88 ± 2.22 to 16.84 ± 4.72, and 35.19 ± 4.82 to 29.20 ± 3.35 in the tissue homogenates of 100 mg/kg group animals, were found as an insignificant variation ($p < 0.05$).

A noticeable elevation was observed in the serum CKMB and LDH enzyme levels in a dose-dependent manner, but the extract did affect only the higher dosed animals (100 mg/kg) significantly ($p < 0.05$), and the enzymes present in the lower and medium dosage groups were within the reference range ($p < 0.05$). In contrary to that, tissue homogenates of subacute animals under study showed a markedly significant hike in both CKMB and LDH levels, as shown in Table 2. As the mortality rates of acute

Table 1: Effect of seed extract on antioxidant enzyme levels of tissue homogenates during subacute studies

Antioxidant assays	*Group I (Control)*	*Group II (25 mg/kg)*	*Group III (50 mg/kg)*	*Group IV (100 mg/kg)*
Liver-SOD	2.23 ± 0.46	2.73 ± 0.16	3.01 ± 0.45*	3.15 ± 0.58*
GPx	30.14 ± 5.12	39.29 ± 5.93	42.32 ± 5.02*	46.55 ± 4.79**
GSH	15.87 ± 0.27	16.39 ± 0.94	16.67 ± 1.07	18.20 ± 0.56**
Kidney-SOD	2.94 ± 1.05	3.04 ± 0.94	3.29 ± 1.08	3.95 ± 1.36
GPx	28.90 ± 5.38	36.51 ± 5.32	39.02 ± 6.25	41.50 ± 7.04*
GSH	7.24 ± 2.09	8.64 ± 2.44	8.99 ± 2.98	9.02 ± 0.71
Brain-SOD	1.39 ± 0.38	1.97 ± 0.32	2.17 ± 0.24*	2.51 ± 0.27**
GPx	20.71 ± 0.93	29.74 ± 4.27	36.79 ± 5.83**	41.71 ± 7.29**
GSH	31.2 ± 0.83	35.58 ± 2.21	37.75 ± 4.52	37.29 ± 5.28
Heart-SOD	1.65 ± 0.23	1.52 ± 0.13	1.44 ± 0.05	1.33 ± 0.05*
GPx	22.88 ± 2.22	20.09 ± 3.11	18.79 ± 2.51	16.84 ± 4.72
GSH	35.19 ± 4.82	32.45 ± 1.68	29.60 ± 2.23	29.20 ± 3.35

SOD-superoxide dismutase; GSH-glutathione; GPx-glutathione peroxidase; values are mean ± SD; all values are $p < 0.05$; *$p < 0.05$; **$p < 0.01$

Table 2: Effect of seed extract on myocardial enzyme levels (CKMB, LDH) in serum and heart tissue homogenates

	Serum (U/L)		*Tissue homogenate*	
Category/ treatments	*CKMB*	*LDH*	*CKMB*	*LDH*
Acute (500 mg/kg)	1,239	2,083	-	-
Subacute (control)	171.67 ± 5.44	236.10 ± 9.54	688.16 ± 74.09	882.91 ± 81.91
25 mg/kg	195.16 ± 9.56	294.12 ± 3.82	1,395.01 ± 29.51**	1,512.83 ± 53.59
50 mg/kg	233.39 ± 4.58	361.58 ± 15.26	2,501.08 ± 45.49**	2,288.28 ± 160.54*
100 mg/kg	276.34 ± 11.15*	485.70 ± 38.16*	3,302.16 ± 61.87**	3,963.35 ± 253.52**

CKMB-creatinine kinase; LDH-lactate dehydrogenase; values are mean ± SD; all values are $p < 0.05$; *$p < 0.05$; **$p < 0.01$

animals in group IV is cent percent, heart tissues were not used for homogenization.

Discussion

Toxic plants with medicinal properties can be used as herbal drugs in small amounts and correct proportions.[8] When ingested in sufficient quantity, it can be harmful or fatal to man or other animals, which can be assayed using serum biochemical tests. These tests are frequently used in diagnosis diseases of hearts, liver, kidney, and cardiovascular system, etc. They are also widely used in monitoring the response to exogenous toxic exposure.

After determining the lethal effect of the seeds via acute studies, subacute experiments were carried out. The studied doses (25, 50, and 100 mg/kg) showed normal morphological behavior of all vital organs. Organ damages were assessed by the determination of antioxidant enzyme levels in the serum since necrosis or membrane damage releases enzymes into circulation. Mammalian cells are endowed with extensive antioxidant defense mechanisms that counteract the damaging effects of toxic, reactive oxygen species (ROS) produced during the oxidative stress.[17] ROS are kept at physiologically optimal levels with antioxidant defense systems, which include an array of cellular and mitochondrial superoxide dismutases (SOD), catalase (CAT), and glutathione peroxidase (GPx) enzymes, and non-enzymatic antioxidants such as reduced glutathione (GSH), ascorbic acid, and vitamin E.[18] The equilibrium between these enzymes is an important process for the effective removal of oxygen stress in intracellular organelles.[19]

In the present study, the activity of SOD in liver cells was found increased significantly ($p < 0.05$) in 100 mg/kg animals, whereas GPx enzyme levels were found high in both 50 and 100 mg/kg treated animals ($p < 0.01$), which appears to be an adaptive mode in the form of increased antioxidant defense. Glutathione peroxidase scavenges the superoxide anion to form hydrogen peroxide and thus diminishing the toxic effect caused by this radical.[20] SOD is an important enzyme in living cells for maintaining normal physiological conditions and for coping with stress conditions.

Glutathione plays an important role in the regulation of a variety of cell function and in cell protection from oxidative injury. Acute oxidative stress has been reported to enhance glutathione levels and GPx activities, which also can be attributed to the adaptive response of the tissues to the oxidant challenge resulting from exposure of extracts in rats.[21,22] GSH is considered to be one of the most important components of the antioxidant defense of living cells,[23] which directly scavenges free radicals and protects the bio-molecules from their attack.[24]

Rat brain had significantly higher levels of SOD and GPx activities in 50 and 100 mg/kg animals than those recorded in their control animals. Brain is more vulnerable to oxidative damage due to its high oxygen consumption and due to the presence of high levels of polyunsaturated fatty acids.[25] The significant increase in SOD and GPx enzyme activity noticed, maybe to cope up with excess amount of free radicals generated to give a protective effect to brain tissue. As evident from the present results, cardiac glycosides have a marked potency to induce a large amount of SOD production in vital organs, to protect the body against the damaging effects of free radicals.

According to Yu,[26] free radicals may also induce the expression of antioxidant enzymes, thereby enhancing the neuronal resistance to subsequent oxidative challenges. The action of SOD, therefore, is to protect the biological integrity of the cells and tissues against harmful effects of superoxide free radical.[27] Similarly, an increase in activities of GSH peroxidase system observed in this study could be a part of the biochemical mechanism of tolerance development.[28] Wang *et al*.[29] has previously shown that neriifolin, which is structurally related to oleandrin, provide robust neuroprotection to brain tissues damaged by oxygen, and whole animal models with ischemic stroke.[30] Usually, lipid-soluble glycosides showed a higher accumulation and even distribution throughout all brain areas.[31]

Decrease in SOD activity in heart tissues, one of the most important enzymes in the enzymatic antioxidant defense system, is a sensitive index in tissue damage. Digoxin, the prominent cardiac glycoside used in therapy, also acts indirectly on the failing heart by depressing the activated neuroendocrine system.[32] On the other hand, an insignificant reduction in the activities of GPx with a concomitant decline in the level of reduced GSH observed in the heart tissues of treated groups indicated the inhibition of GSH synthesis and/or increased utilization of GSH for detoxification of the toxicant-induced free radicals.[24] The use of toxins seems to have produced many physiological, biochemical, and behavioral changes in man and other non-target organism by influencing both the activities of many enzymes and other cellular processes.[33]

As the name suggests, cardiac glycosides affect the heart muscles, either most effectively or adversely. Level of cardiac marker enzymes CKMB and LDH in the serum, and the antioxidant status in heart tissues were analyzed to evaluate the effect of the seed kernel extract on the heart muscles. Higher level of CKMB (1,239 U/L) and LDH (2,083) enzymes present in the serum of the collapsing heart of an LD_{100} animal (500 mg/kg) revealed its brutality.

A significant climb noticed in CKMB and LDH levels in serum and heart tissue homogenates of 50 and 100 mg/kg animals, maybe temporary, caused due to the daily dosage and may back to normal when the dosage is terminated. Ashley *et al.*[34] found digitoxin and similar cardiac glycosides were excreted or eliminated through the kidneys of human beings and rats. In urine, it was found either as a metabolic product in greater amount or

unchanged form in lesser amount. An analysis of rabbit urine after digitoxin injection revealed a delayed urination and subsequent metabolism of cardiac glycosides, i.e., conversion of digitoxin into more polar metabolites was found subsequently hindered.[35]

Creatinine kinase and lactate dehydrogenase, the two valuable indicators found in the heart muscles, usually began to leach out into the bloodstream following myocardial infarction. The determinations of CKMB and LDH isoenzymes in the serum have been quite useful in the diagnosis of specific tissue damage in the heart, skeletal muscle, and liver disorders in humans.[36] These enzymes were recorded in a range of 80 to 260 and 128 to 467 U/L for CKMB and LDH in the serum of normal untreated Wistar rats. Regular intake of the extract did not affect the muscle fibers of myocardial tissues. Proper functioning of internal organs may possibly be resulted in the metabolism and subsequent degradation of cardiac glycosides, thereby preventing the accumulation and damage induced to heart muscles. Kuhlmann *et al*.[31] pointed out that, the glycoside concentration in the atria is lower than in the ventricles, and the left heart areas showed higher concentrations than the right areas, so a higher level of enzyme accumulation in heart tissue homogenates depends on the segment used for homogenization.

Cardiac glycosides are beneficial in treating cardiac conditions, depending on time and dosage, they can also be toxic as they regularly cross the blood-brain barrier.[37] Endogenous cardiotonic steroids may control not only cardiac and kidney function, salt metabolism, and hypertension but also, cell proliferation, cell half-life, and, more generally, cell function.[38]

Intoxication of toxic plants are a significant cause of morbidity and mortality. Significant variations observed in the antioxidant marker enzyme levels were considered as a detoxifying effect for bio-protection. Results revealed that single-dose would damage heart tissues at higher concentrations, which is considered as an "overdose," but for cumulative doses, cardiac glycosides were detoxified by vital organs of the body. Since all the plant parts are a treasure of cardiac glycosides, use of effective methods to purify this compound is significant to the pharmaceutical point of view. In order to minimize accidental intoxication, creation of awareness among general population is needed.

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