

## Pharmacological Profile of *Diospyros melanoxylon* Methanolic Extract

Sarvani Palaparthy\*

Acharya Nagarjuna University, Guntur, Andhra Pradesh, India

\*Corresponding author: Sarvani Palaparthy, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India, Tel: +91 9885610011; E-mail: palaparthy.sarvani@gmail.com

Received date: October 30, 2018; Accepted date: January 28, 2019; Published date: February 1, 2019

Copyright: © 2018 Palaparthy S. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

### Abstract

Present study was aimed to investigate the *in-vitro* antioxidant, anti-inflammatory and *in-vivo* nephroprotective activity novel herbal extracts. Initially the test extracts were evaluated for antioxidant potential by performing the DPPH assay. Few extracts displayed potent antioxidant activity by DPPH free radical scavenging activity. Among all the extracts Methanolic extracts of *Diospyros melanoxylon* was found to be more potent for antioxidant potential. Presence of phenols and flavonoid in the test extracts might be contributed to their potent antioxidant activity. The test extracts were also evaluated for anti-inflammatory activity by carrageenan induced paw edema model. RX- has shown moderate anti-inflammatory activity. Based on preliminary *in-vitro* antioxidant activity results *Diospyros melanoxylon* was selected for further to evaluate Nephro-protective activity against Potassium dichromate induced model. Acute oral toxicity test was performed to find out the safe dose of test extract before going to *in vivo* evaluation (Potassium Dichromate induced nephrotoxicity). Acute toxicity study of test extract was conducted in wistar rats to find the Maximum tolerated dose. The test extract did not show any toxicity and mortality symptoms during the study at the different doses studied. The Maximum Tolerated Dose (MTD) of the test extract was found to be >2000 mg/kg in rats.

*In-vivo* nephroprotective activity was conducted in wistar rats by Potassium dichromate-induced nephrotoxicity model. During the study period test extract (250 mg/kg, 500 mg/kg) were administered by oral route for the period of 7 days followed by potassium dichromate administration (15 mg/kg). At the end of the study blood samples were collected and used for estimation of kidney biochemical parameters. Results showed that significant increase was observed in biochemical parameters (BUN, CR) in PDC group compared to vehicle control. The test extract displayed significant reduction in blood urea nitrogen and serum creatinine at the dose of 500 mg/kg. Kidney tissue samples were collected on termination day of all rats and subjected for measurement of antioxidant enzymes and lipid Peroxidation to check the organ toxicity. Significant increase in lipid Peroxidation and decrease in antioxidant enzyme levels were observed in PDC control whereas test extract prevented the kidney toxicity by decreasing TBARS production and normalization of antioxidant defense enzymes at the doses studied. Gain in body weight and organ weight compared to PD control also revealed the Nephroprotective effect of *D. melanoxylon* extract at both the doses. All the data showed that both biochemical antioxidant parameters correlated together and supported the protective effect of the Herbal extract (*D. melanoxylon*) against potassium dichromate induced nephrotoxicity.

**Keywords:** *Diospyros melanoxylon*; *Xestospongia muta*; Alkaloids; Anti-dormant

### Introduction

In The recent years, many researchers have examined the effects of plants used traditionally by indigenous healers and herbalists to support kidney function and treat diseases of the kidneys. In most cases, research has confirmed traditional experience and wisdom by discovering the mechanism and mode of action of these plants as well as reaffirming the therapeutic effectiveness of certain plants or plant extracts in clinical studies. Several hundred plants have been examined for use in a wide variety of disorders. Just a handful has been fairly well researched.

### Aim

The aim of the present study is to evaluate *in-vitro* antioxidant, anti-inflammatory and nephroprotective activities of novel herbal extracts.

### Objectives

To study the *in-vitro* antioxidant activity of alcoholic extracts of *Diospyros melanoxylon* Leaves.

To evaluate the anti-inflammatory activity by carrageenan induced model in wistar rats.

Acute oral toxicity studies will be conducted for active compounds as per OECD Guidelines 420.

To study the effect of methanolic extracts of *D. melanoxylon* against potassium –dichromate induced nephrotoxicity in rats. In the present study Nephrotoxicity will be assessed by measuring biochemical parameters like Blood Urea Nitrogen (BUN) Creatinine. Antioxidant parameters will also be evaluated in tissues at the end of the study (CAT, MDA, GSH, GST, NO) to determine the protective effect of the herbal extract. The plant is shown in Figure 1.

### Review of plant

Botanical name: *Diospyros melanoxylon*

Family: Ebenaceae



**Figure 1:** Picture of plant.

## Description

Coromandel Ebony or East Indian Ebony (*D. melanoxylon*) is a species of flowering tree in the family Ebenaceae that is native to India and Sri Lanka and that has a hard, dry bark. Its common name derives from Coromandel, the coast of southeastern India. Locally it is known as temburini or by its Hindi name tendu. In Odisha and Jharkhand it is known as kendu. The leaves can be wrapped around tobacco to create the Indian beedi, which has outsold conventional cigarettes in India [1-4].

### Taxonomy:

Name of the: *Diospyros melanoxylon*

Synonym: Coromandel Ebony

Family: Ebenaceae

Genus: *Diospyros*

Species: *melanoxylon*

Vernacular names:

(Bengali): Kend, Kendu

(Hindi): Abnus, Kendu, Tendu, Timburni

(Nepali): Abnush, Tendu

(Sanskrit): Dirghapatraka

(Tamil): Karai, Karundumbi, Tumb

(Trade name): Ebony

## *Diospyros melanoxylon* plant and other parts

Botanic description: *D. melanoxylon* is a medium-sized tree or shrub up to 25 m and 1.9 m girth. The bark is pelican in color, exfoliating in rectangular scales. The primary root is long, thick and fleshy at first, afterwards woody, greyish, often swollen in upper part near ground level. The roots form vertical loops in sucker-generated plants. Leaves opposite or alternate and coriaceous, up to 35 cm long, to mentose on both sides when young, becoming glabrous above when fully grown. Male flowers are mauve in color, tetramerous to sextamerous, 1-1.5 cm [5].

Long, sessile or nearly sessile in short peduncles, mostly 3-flowered. Female flowers mauve, mostly extra-axillary or sometimes solitary, axillary generally 2, opposite each other, larger than the male flowers.

Fruits olive green, ovoid or globose 3-4 cm across; 1-, 2-, 3-, 4-, 5-, 6-, or 8-seeded berries. Pulp yellow, soft and sweet. Seeds compressed, oblong, shiny, often banded. The generic name is derived from the Greek 'dios' (divine), and 'pyros' (fruit), referring to the excellent fruit of the genus. The specific name is Greek and means 'dark wood' [6].

Biology: The tree is deciduous or evergreen depending on its habitat. In a dry locality, it is leafless for a short time in the hot weather, regaining its leaves in May-June. In a moist locality, it evergreen. The flowers appear from April to June on new shoots and the fruit ripens after 1 year. The edible fruits are largely eaten and disseminated by fruit bats and birds, notably hornbills. The tree produces good seed in alternate years.

## Reported pharmacological activities of *Diospyros melanoxylon*

Anti-hyperglycemic effect of *D. melanoxylon* (Roxb.) bark against alloxan-induced diabetic rats: The anti-hyperglycemic activity of *D. melanoxylon* (Roxb.) bark was evaluated with scientific approach including biochemical parameters and histopathological studies of pancreas. The ethanolic extracts of the powdered bark was tested for its efficacy in alloxan-induced diabetic rats. The extracts were also evaluated for acute oral toxicity studies and its effect on different biochemical parameters. An effect of extracts was compared to that of standard glibenclamide [7]. It was revealed that ethanolic extracts has significantly ( $p < 0.01$ ) reversed the diabetes-induced hyperlipidemia compared to standard drug. Histopathological studies of pancreas revealed its significant effects on  $\beta$ -cells count. The extracts showed significant anti-hyperglycemic activity as compared to standard drug. Ethanolic extract (200 mg/kg) showed beneficial effects on blood glucose and hyperlipidemia associated with diabetes, which might be due to presence of steroids, tannins, alkaloids and triterpenoids present in that extract. Thus ethanolic extract could serve as good adjuvant to other oral hypoglycemic agents and seems to be promising for the development of phytomedicines for diabetes mellitus.

This endemic plant of India and Ceylon is used in various ways. Besides being the source of Indian ebony, its wood is also utilized for making boxes, combs, ploughs and beams. The fruits are eaten and sold commercially. The bark is burnt by tribals to "cure" small-pox. The seeds are prescribed as cure for mental disorders, palpitation of heart and nervous breakdown. Above all, the leaves of this plant constitute one of the most important raw materials of the "Bidi" (Indian cheap smoke) industry.

Antimicrobial activity of *D. melanoxylon* bark from Similipal Biosphere Reserve, Orissa, India: The antimicrobial activity of five extracts of *D. melanoxylon* Roxb. bark collected from Similipal Biosphere Reserve, Orissa was evaluated against human pathogenic bacteria and fungi. The extracts including both polar and non-polar solvents; petroleum ether, chloroform, ethanol, methanol and aqueous were evaluated for their antimicrobial activity against three gram positive and five gram negative bacteria as well as three fungal strains. Although, all the five extracts exhibited promising antibacterial activities, yet maximum activity was observed in ethanol extract. In case of antifungal activity, except petroleum ether extract none of the extracts were found to be active against the fungal strains. MIC values for most of the extracts ranged from 1.5 to 6 mg/ml, while MBC values varied from 3 mg/ml to values greater than 12 mg/ml. Phytochemical analysis exhibited the presence of steroids, alkaloids, glycoside, proteins, tannins, phenolic compounds, carbohydrates, gums and

mucilage in acetone, methanol and ethanol extracts with maximum phytochemicals in ethanol extract. Least phytochemicals was observed in case of petroleum ether. These results, so obtained, demonstrate the broad spectrum activity of *D. melanoxylon* bark extracts which may be useful in treatment of various microbial infections. However, the active components responsible for antimicrobial activity need to be evaluated [8,9].

## Material and Methods

### Chemicals

Chemicals used in the experiment are given in Table 1.

| S.NO | Chemicals                                | Source                   |
|------|--|--------------------------|
| 1    | 1,1-Diphenyl-2-picryl-hydrazyl (DPPH)    | Sigma                    |
| 2    | Alcohol                                  | SD fine chem. Ltd        |
| 3    | Ascorbic acid                            | Sigma                    |
| 4    | Acetic acid                              | SD fine chem. Ltd        |
| 5    | Aluminium Hexahydrate                    | SD fine chem. Ltd        |
| 6    | Dichlorofluoresceindiacetate (DCF-DA)    | Sigma                    |
| 7    | DTNB/Elman's Reagent                     | Biochemika Fluka         |
| 8    | Dimethyl Sulphoxide (DMSO)               | Merck                    |
| 9    | Folin-Ciocalteu Reagent                  | Sigma                    |
| 10   | Formaldehyde                             | Loba Chemie              |
| 11   | Gallic acid                              | Sigma                    |
| 12   | Gum Acacia                               | Loba Chemie              |
| 13   | Hydrochloric acid                        | Rankem                   |
| 14   | Hydrogen Peroxide                        | Fischer Scientific       |
| 15   | Naphthyl ethylene diaminedihydrochloride | SD fine chem. Ltd        |
| 16   | Potassium monohydrogen phosphate         | Qualigens fine chemicals |
| 17   | Potassium Dihydrogen Phosphate           | SD fine chem. Ltd        |
| 18   | Phosphoric acid                          | SD fine chem. Ltd        |
| 19   | Rutin hydrate                            | Sigma                    |
| 20   | Sodium Carbonate                         | SD fine chem. Ltd        |
| 21   | Sodium Hydroxide (NaOH)                  | SD fine chem. Ltd        |
| 22   | Sodium Chloride (NaCl)                   | SD fine chem. Ltd        |
| 23   | Sodium Lauryl Sulphate (SLS)             | Loba Chemie              |
| 24   | Sodium nitrite (NaNO <sub>2</sub> )      | Sigma                    |
| 25   | Sulfanilamide                            | Loba Chemie              |
| 26   | Superoxide Dismutase (SOD) Assay kit     | Sigma                    |
| 27   | Thio-Barbituric Acid (TBA)               | Sigma                    |
| 28   | Tris Buffer                              | Sigma                    |

|    |                      |       |
|----|----------------------|-------|
| 29 | Carrageenan          | Sigma |
| 30 | Potassium Dichromate | -     |

**Table 1:** Chemicals used for experiment.

Apparatus used are given in Table 2.

| S.NO | Instruments                  | Source                              |
|------|------------------------------|-------------------------------------|
| 1    | Micro plate reader           | BioTek instruments, Synergy 4, USA  |
| 2    | UV/Visible Spectrophotometer | Perkin Elmer, Lambda 25, USA        |
| 3    | Laboratory Micro Centrifuge  | Biofuge stratus, Germany            |
| 4    | Auto Blood Analyzer          | Dimension X pand plus, USA          |
| 5    | Tissue Homogenizer           | Heidolph, Silent crusher S, Germany |
| 6    | PH meter                     | pH 540 GLP, WTW, Germany            |
| 7    | Digital Balance              | Precisa                             |

**Table 2:** Apparatus used for experiment.

### Experimental animals

Wistar Albino Rats weighing between 160-180 g were procured from NIN, Hyderabad, India. The animals were housed in a bio-safe temperature-controlled environment with a 12:12 light/dark cycle with standard conditions of temperature ( $25 \pm 20^\circ\text{C}$ ) and relative humidity (30-70%) during the experimental period. The animals were fed with standard pellet diet and water ad libitum. All the animals were acclimatized under laboratory conditions for a week before the commencement of experiments. The Institutional Animal Ethics Committee (IAEC), IICT, and Hyderabad, India approved the study (Protocol No: IICT/PHARM/SRK/Feb/2013/11). The norms for Good Laboratory Practice (GCP) were followed for care of laboratory animals. The animals were maintained in accordance with the CPCSEA guidelines.

### Test compound preparation

Required quantity of alcoholic extracts of *D. melanoxylon* Leaf were weighed and made suspension with gum acacia (2%) in de-ionized water.

*Potassium dichromate:* Required quantity of potassium dichromate powder (250 mg/kg of rat body wt 500 mg/kg) weighed and made suspension with gum acacia (2%) in de-ionized water.

### DPPH (1, 1-Diphenyl-2-picryl-hydrazil) free radical scavenging activity

*In-vitro* studies were performed to evaluate antioxidant activity of novel herbal extracts

Preparation of sample: Stock solution of methanolic extract (for antioxidant activity) of *D. melanoxylon* leaf was prepared by dissolving in Dimethyl Sulphoxide (DMSO) at a concentration of 5 mg/ml.

Preparation of DPPH solution: 2.8 mg of DPPH was dissolved in 13 ml of methanol.

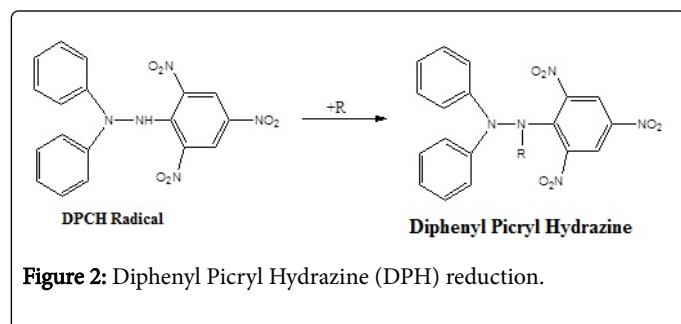
Preparation of Tris buffer (pH 7.2): 0.605 gm of Tris powder dissolved in 50 ml of distilled water.

Preparation of samples: 5 mg of sample (alcoholic extracts of bark and leaf) dissolved in 1 ml of dimethyl sulphoxide (5 mg/ml). From this stock solution, further dilutions of each sample were made.

Preparation of standard: 5 mg of ascorbic acid dissolved in 1 ml dimethyl sulphoxide (5 mg/ml). From this stock solution, further dilutions were made.

### Principle of the assay

DPPH reduction: DPPH is one of a few stable and commercially available organic nitrogen radicals with a UV-Vis absorption maximum at 517 nm. When DPPH reacts with an antioxidant compound, which can donate hydrogen, DPPH gets reduced as shown in Figure 2 changes the color from deep violet to light yellow. The change in color (from deep violet to light yellow) is directly proportional to antioxidant potential of the compound, which was measured at 517 nm on a UV/Visible light spectrophotometer. DPPH is nitrogen centered free radical that react similarly peroxy radicals and represents a model free radical originating in lipophilic medium. DPPH is the parameter of antioxidant activity [10].



Assay procedure: DPPH free radical scavenging activity tested with 25 µl of test sample (5 mg/ml DMSO), 100 µl of 0.1M Tris HCL buffer (pH 7.2) and 125 µl of 0.5 mM DPPH (1,1- diphenyl-2-picrylhydrazyl; sigma chemicals, USA, dissolved in methanol) were mixed and shaken well. After 30 min, absorbance was recorded Spectrophotometrically (Biotek synergy, USA) at 517 nm. The free radical scavenging potential was determined as the percent discoloration of DPPH due to test samples and calculated as  $(1-B/A) \times 100$ , where A is absorbance of DPPH control with solvent and B is absorbance of decolorized DPPH in the presence of test compound. All the analysis was done in triplicates; ascorbic acid was taken as reference compound. Several dilutions of primary solutions (5 mg/ml in DMSO) were made and assayed accordingly to obtain concentration of sample required for scavenging of DPPH free radical (Table 3) [11].

| Assay Performed | Concentration of solvent used |               |                 |
|-----------------|-------------------------------|---------------|-----------------|
| Blank           | 25 µl DMSO                    | 100 µl Buffer | 125 µl Methanol |
| Control         | 25 µl DMSO                    | 100 µl Buffer | 125 µl DPPH     |
| Standard        | 25 µl ASA                     | 100 µl Buffer | 125 µl DPPH     |
| Sample          | 25 µl sample                  | 100 µl Buffer | 125 µl DPPH     |
| PSB             | 25 µl sample                  | 100 µl Buffer | 125 µl Methanol |

**Table 3:** Concentrations used for assay.

### Determination of Total Phenolic Content

Preparation of samples: 5 mg of sample (alcoholic extracts of bark and leaf) dissolved in 1 ml of dimethyl sulphoxide (5 mg/ml).

Preparation of standard: 5 mg of gallic acid dissolved in 1 ml dimethyl sulphoxide (5 mg/ml). From this stock solution, further dilutions were made.

Preparation of reagents: Folin-Ciocalteu Reagent (FCR): 2.5 ml of FCR dissolved in 2.5 ml distilled water.

20% Na<sub>2</sub>CO<sub>3</sub>: 2 g Sodium Carbonate was dissolved in 10 ml distilled water.

### Assay procedure

Total phenolic content in the extracts (bark and leaf of *D. melanoxylon*) was measured using Folin-Ciocalteu reagent. Briefly, 25 µL (5 mg/ml DMSO solution) of the extract was diluted with 2.5 mL of distilled deionized water followed by addition of 250 µL of Folin-Ciocalteu reagent (1M) and 250 µL of Na<sub>2</sub>CO<sub>3</sub> (20% w/v). After incubation for 60 min at room temperature absorbance was measured spectrophotometrically at 765 nm (BioTeksynergy4 multi-mode microplate reader, BioTek Instruments, Inc.). Quantification was performed with respect to the standard curve of Gallic acid. Results were expressed as milligrams of Gallic Acid Equivalent (GAE) per 100 g of the extract as shown in Table 4.

| Reagents                                 | Blank | Control | Pseudo blank | Test |
|--|-------|---------|--------------|------|
| Sample (µL)                              | -     | -       | 25           | 25   |
| Distilled Water (µL)                     | 2775  | 2525    | 2750         | 2500 |
| FCR (µL)                                 | -     | 250     | -            | 250  |
| 20% Na <sub>2</sub> CO <sub>3</sub> (µL) | 250   | 250     | 250          | 250  |

**Table 4:** Assay procedure for experimentation.

### Determination of Total Flavonoids Content

Preparation of samples: 5 mg of sample (alcoholic extracts of bark and leaf) dissolved in 1 ml of dimethyl sulphoxide (5 mg/ml).

Preparation of standard: 5 mg of rutin dissolved in 1 ml of dimethyl sulphoxide (5 mg/ml). From this stock solution, further dilutions were made.

Preparation of reagent: 2% AlCl<sub>3</sub> (Aluminum chloride hexahydrate): 0.1 g AlCl<sub>3</sub> dissolved in 5 ml methanol.

### Assay procedure

Total flavonoids content in the extracts (leaf of *D. melanoxylon*) was measured using Aluminum chloride hexahydrate reagent. Briefly, 125 µL (5 mg/ml DMSO solution) of the extract followed by the addition of 125 µL of Aluminum chloride hexahydrate reagent. After incubation for 10 minutes at room temperature, Absorbance was measured at 450



nm. Quantification was performed with respect to the standard curve of Rutin. Results were expressed as milligrams of Rutin per 100 g of the extract. All determinations were performed in duplicates.

## Acute Oral Toxicity

The acute oral toxicity study conducted as per OECD guidelines by fixed dose method adopted by OECD (420).

## Principle of the study

The acute oral toxicity study was conducted as per OECD guidelines by fixed dose method adopted by OECD (420). The study involved a preliminary sighting study using small number of animals in order to derive the dose effect relation for toxicity and mortality and to provide information on dose selection for the main study. In the preliminary sighting study, the effect of various dosed administered to single animals of each sex was investigated in a sequential manner. The sighting study generally yields information on the dose– toxicity relationship including an estimate of the minimum lethal dose. In the main study, the test article is administered to groups of 5 males and 5 female animals at one of the fixed doses (5, 50, 300 and 2000 mg/Kg) [12-14].

## Sighting study

In the sighting study, the effect of various doses was investigated in single animal of each sex. Dosing was sequential allowing at least 24 h. before dosing the next animal. All animals were carefully observed for signs and symptoms of toxicity continuously up to 24 h. Later up to 7 days. The sighting study was conducted with sequential doses of 5, 50, 300 and 2000 mg/kg of the test article. If the initial dose chosen did not produce severe toxicity, the next higher dose was selected. In this sighting study, the dose that produced evident toxicity but not death was identified. Dose escalation was continued up to 2000 mg/kg.

## Main study

At least 10 animals (5 males and 5 females) for each species were used for the dose level in this study. Animals were fasted overnight and the test articles were administered as 1% gum acacia suspension by oral route. The dose used in this study is selected from one of the four levels 5 mg/kg, 50 mg/kg, 300 mg/kg and 2000 mg/kg i.e. the dose that produced evident toxicity but not mortality from sighting study [15]. The animals were observed for following signs and symptoms of toxicity apart from the cage side observations which include changes in skin and fur, eyes, mucous membrane, respiratory, circulatory, and autonomic and central nervous systems. The different biochemical and hematology parameters observed on “0” day and on the termination day during the main study physical parameters like feed consumption and body weight increase were also recorded as given in Table 5.

| Observations                         | Physical examination             |
|--------------------------------------|----------------------------------|
| Bizarre physical position            | Altered muscle tone              |
| Bizarre tail position                | Catatonnia                       |
| Muscle tremors                       | Aggressive ness towards observer |
| Aggressiveness towards other animals | Coma                             |
| Inactivity                           | Convulsions to touch             |

|                         |   |
|-------------------------|---|
| Spontaneous Convulsions | Alterations in cardiac rhythms and rate |
| Dyspnoea                | Paralysis                               |
| Sedation                | Change in pupil size                    |
| Nystagmus               | Sensitivity to pain                     |
| Cyanosis                | Skin lesions                            |
| Abnormal Excreta        | Corneal opacity                         |
| Salivation              | Loss of righting reflex                 |
| Nasal Discharge         | Grasping reflexes                       |
| Piloerection            | Pinna reflexes                          |
| Phonation               | Death                                   |

**Table 5:** Toxicity symptoms in acute oral toxicity.

## Potassium-dichromate Induced Nephrotoxicity Experimental Procedure

Wistar rats weighing 160-180 g randomly selected for the present study and they were divided into four groups consisting 6 rats in each. They are as follows:

Group I: Normal control rats, received distilled water for 7 days.

Group II: potassium dichromate Control: Rats received distilled water and served as a negative control and on the 7<sup>th</sup> day received potassium dichromate (15 mg/kg).

Group III: Treatment group: Rats received low dose (250 mg/kg) of test extract Herbal Extract (HE) p.o. from first day to seventh day followed by potassium dichromate (15 mg/kg) on day 7.

Group IV- Treatment Group: Rats received high dose (500 mg/kg) of herbal extract Herbal Extract (HE) p.o. from first day to seventh day followed by potassium dichromate (15 mg/kg) on day 7.

At the end of the study blood samples were collected from all the groups of rats and used for estimation of biochemical parameters. Later animals were sacrificed by cervical dislocation and kidney tissue samples were retained for further antioxidant enzyme evaluation. During the study, body weights were recorded on day 1 and on termination day. Percentage organ weights were calculated by taking terminal body weights of all animals. All the animals were observed for toxicity signs during the study.

## Serum sample preparation

At the end of the experimental period (9 days) blood samples were collected using heparinized capillary tubes from retro orbital plexus of the animals (rats and mice) of all groups. Blood samples were left to clot for 1 h at room temperature, and then centrifuged by using centrifuge (Biofuge stratos, Germany) at 4000 rpm for 15 min at 25°C to separate the sera, which were stored at -80°C until analysis could be completed. Serum was used for estimation of BUN and Creatinine using Auto Blood analyzer (Dimension x pand plus; USA).

## Tissue preparation and homogenization

Animals were sacrificed by cervical dislocation with light ether anesthesia and kidney tissues were removed from rats. Tissues washed

thoroughly with ice-cold normal saline and weighed. Then immediately part of the tissues were stored in formaline and used for Histopathological study. Remaining tissues were stored at -80°C and used for further estimations. Tissues were cut in to small pieces and homogenized with a Homogenizer (Heidoph, Silent crusher S, Germany) in ice-cold phosphate buffer saline (PBS) (0.05M, pH 7) to obtain 1:9 (w/v) (10%) whole homogenate. Homogenate was mixed with equal volume of 10% Trichloroacetic acid (TCA) and centrifuged at 5000 rpm for 10 min and supernatant was used for the determination of MDA, Vitc. Then remaining homogenate was centrifuged at 17,000 g for 60 min at 4°C, and supernatants were used for the measurement of antioxidant parameters, NO, CAT, GSH, GST.

## Total Lipid Peroxidation

The concentration of MDA in homogenate as an index of lipid peroxidation and it was determined.

### Principle of the assay

Melonaldehyde (MDA) is one of many low molecular weight end products of lipid hydro peroxides decomposition and is the most often measured as an index of lipid peroxidation. In this assay, one molecule of Melonaldehyde (MDA) reacts with two molecules of 2-Thiobarbituric Acids (TBA) at pH 3.5 to form pink chromogen, which is measured spectrophotometrically at 532 nm with extinction coefficient of  $156 \text{ M}^{-1}\text{cm}^{-1}$ .

### Reagents

- A: 8.1% SLS (Sodium Lauryl Sulphate)
- B: 20% Acetic acid (9.5 ml of Glacial acetic acid dilute up to 50 ml with DDW (pH 3.5 with 4N NaOH)
- C: 0.8% aqueous solution of TBA (2-Thiobarbituric Acid)

### Method

All the reagents were freshly prepared and to the 0.2 ml of experimental sample, 0.2 ml of 8.1% SLS, 1.5 ml of 20% Acetic acid (pH 3.5) and 1.5 ml of 0.8% aqueous solution of TBA was added and made the volume up to 4 ml with double distilled water [16]. Then heat the mixture at 95 °C for 60 min in water bath on hot plate to develop light pink Color. The mixture was allowed to cool and measure the absorbance spectrophotometrically at 532 nm using micro plate reader (BioTek instruments, synergy 4, USA) and Calculate the MDA content with extinction coefficient of  $156 \text{ nM}^{-1}\text{cm}^{-1}$  and was expressed as nmol/g wet tissue. Finally MDA content was calculated by following formula:

$$\text{Lipid proxides (Nmol MDA/g Tissue (Liver/Heart))} = (\text{Abs}/156) \times [\text{Total Volume (4 ml)}/\text{Sample Volume (0.2 ml)}] \times \text{Dilution Factor (10)} \times 1000$$

## Nitric Oxide Assay (NO)

The nitric oxide (NO) production was measured by estimating the accumulation of nitrites ( $\text{NO}_2^-$ ) in the supernatant by the method with slight modification of Griess Reagent System (Promega technical bulletin, USA).

### Principle

Nitrite ( $\text{NO}_2^-$ ) is one of two primary, stable and nonvolatile breakdown product of Nitric Oxide. The Griess Reagent System is based on the diazotization chemical reaction which uses sulfanilamide and N-1-Naphthylethylenediaminedihydrochloride (NED) under acidic (phosphoric acid) conditions according to the following reactions [17,18].

### Reagents

- A: Sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid)
- B: NED Solution (0.1% N-1-Naphthylethylenediaminedihydrochloride in water)
- C: Nitrite Standard (0.1 M Sodium Nitrite)

### Method

Sulfanilamide Solution, NED Solution and Nitrite Standard (0.1M Sodium Nitrite) were prepared and allowed to equilibrate to room temperature (15-30 min). A standard curve was prepared with nitrite concentration range from 0-100  $\mu\text{M}$  (100, 50, 25, 12.5, 3.13 and 1.56, 0  $\mu\text{M}$ ), using sodium nitrite ( $\text{NaNO}_2$ ) as standard. To the wells containing (duplicate) 50  $\mu\text{l}$  of standard nitrite sample and experimental sample, 50  $\mu\text{l}$  of the Sulfanilamide Solution was added and incubated 5-10 min at room temperature, protected from light. Then 50  $\mu\text{l}$  of the NED Solution was added to all wells and incubated at room temperature for 5-10 min, protected from light. A purple/magenta color thus formed was measured by taking absorbance at 540 nm using micro plate reader (BioTek instruments, synergy 4, USA) and interpolated to a standard curve of  $\text{NaNO}_2$  (0-100  $\mu\text{M}$ ) to calculate the nitrite content. The nitrite level expressed as  $\mu\text{mol/g}$  wet tissue.

## Glutathione Assay (GSH)

Tissue GSH concentration was measured by method described. Although this method will measure all acid soluble thiols, glutathione represents more than 90% of the reactive thiol groups.

### Principle

The assay is based on the reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) by SH groups of glutathione to form 2-nitro-5-thiobenzoate (NTB.) per mole of glutathione, which ionizes to  $\text{NTB}_2^-$  dianion (yellow color) in water at neutral and alkaline pH. The  $\text{NTB}_2^-$  is quantified in a spectrophotometer by measuring the absorbance at 412 nm and was expressed as  $\mu\text{mol/g}$  wet tissue [19].

### Reagents

- A: 0.1 M potassium phosphate pH 8.4 (5.225 g  $\text{K}_2\text{HPO}_4$  in 100 ml DDW, pH 8.4, adjust with 1N HCl)
- B: DTNB (0.002%) (2 mg/100 ml of 1% sodium citrate solution)
- C: Reduced Glutathione.

### Method

Reduced Glutathione was taken as reference standard for preparation of Standard graph. To 2 ml of 0.1 M potassium phosphate pH 8.4, 0.1 ml of standard or experimental sample (deproteinized with 10% TCA), 0.5 ml of DTNB were added and made the volume up to 3

ml with double distilled water. Then the mixture was incubated for 10 min at room temperature and measured the absorbance at 412 nm and calculated the GSH content from standard graph.

## Catalase Assay (CAT)

Catalase activity tissues was determined by measuring the rate of decomposition of hydrogen peroxide at 240 nm, according to the method.

## Principle

Catalase is a common enzyme in all-living organisms. Which catalyze the decomposition of  $H_2O_2$  to water and oxygen and activity was measured by measuring the rate of decomposition of hydrogen peroxide at 240 nm. The difference in absorbance ( $\Delta E$  240) per unit time is a measure of the catalase activity.

## Reagents

Phosphate Buffer (50 mM, pH 7)

Dissolved 6.81 g  $KH_2PO_4$  in double distilled water to make 1000 ml.

Dissolved 7.091 g  $Na_2HPO_4$  in double distilled water to make 1000 ml.

Mix both solutions in 1:1.55.

Hydrogen Peroxide (30 mM) (Dilute 0.34 ml of 30%  $H_2O_2$  with phosphate buffer up to 100 ml).

## Method

To The 1.95 ml phosphate buffer (50 mM, pH 7), 50  $\mu$ L of experimental sample were added. Then changes in absorbance were recorded at 240 nm by addition of 1 ml hydrogen peroxide (30 mM) for 1 min at 15 sec interval and then the activity was calculated by following formula.

Catalase activity (K/min) =  $(1/\Delta t) \times \ln (s_1/s_2) = (2.3/\Delta t) \times \log (s_1/s_2)$

Where,  $\Delta t$  =  $t_2 - t_1$  (time interval)

$S_1$  and  $S_2$  =  $H_2O_2$  concentrations at times  $t_1$  and  $t_2$ .

## Ascorbic Acid Assay

## Principle

Ascorbic acid was assayed by the method in which Ascorbic acid is oxidized to dehydro ascorbic acid and 2,3-dioxogluconic acid with copper sulphate [20]. Further after reaction with 2,4-dinitrophenyl hydrazine it forms tris 2,4-dinitrophenyl hydrazone which on treatment with ice cold sulfuric acid forms a complex which is quantified colorimetrically at 520 nm.

## Reagents

5% Trichloroacetic acid.

DTC Reagent (3 g Dinitrophenyl hydrazine, 0.4 g thiourea, 0.05 g Urea and 0.05 g copper sulphate made upto 100 ml with 9 N sulfuric acid).

65% Sulfuric acid.

## Procedure

To 0.1 ml of tissue homogenate, 1 ml of Trichloroacetic acid was added and centrifuged at 3000 rpm for 10 min to 0.5 ml of the supernatant, 0.2 ml of DTC reagent was added and incubated at  $37^\circ C$  for 3 h. To the resultant, 1.5 ml of ice cold Sulfuric acid (65%) was added and again incubated at  $37^\circ C$  for 3 h. Absorbance of resultant solution was observed at 520 nm.

## Statistical Analysis

The intergroup variation between various groups were analyzed statistically using one-way analysis of variance (ANOVA) using the Graph Pad Prism version 5.0, followed by Turkey's Multiple Comparison Test (TMCT). Statistical significance was evaluated at  $P < 0.05$ . The experimental results expressed as the Mean  $\pm$  S.E.M or as percent activity compared to control animals.

## Results and Discussion

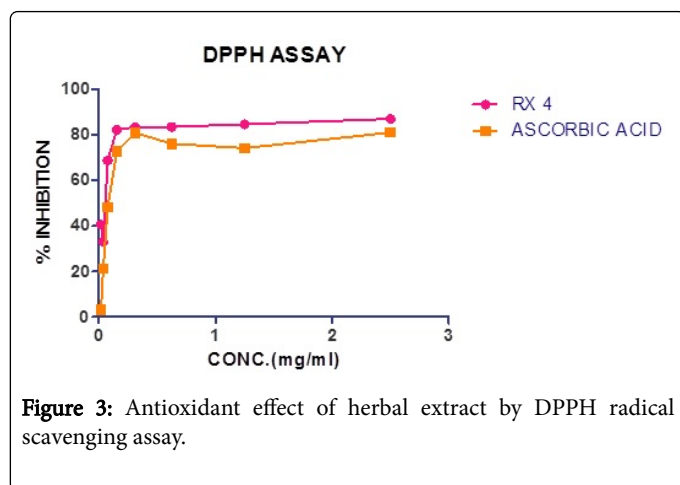
### DPPH free radical scavenging activity

The *in-vitro* antioxidant activity can be evaluated on the basis of their capability to scavenge free radical like DPPH and by estimating total phenolic content and total flavonoid content. Increased consumption of fruits, vegetables and herbal products significantly reduce the incidence of chronic diseases, such as cancer, cardiovascular diseases and other aging-related pathologies. Photochemical, especially antioxidants of these natural products are suggested to be the major bioactive compounds for these health benefits. The details are given in Table 6.

| Test extract        | IC-50 (mg/ml) |
|---------------------|---------------|
| Ascorbic acid       | 0.6673        |
| Herbal Extract (HE) | 0.564         |

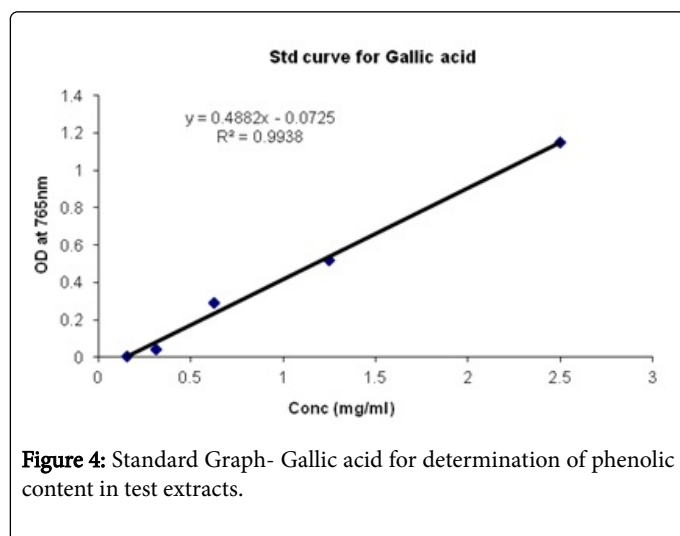
**Table 6:** IC-50 values of standard and test extracts for DPPH radical assay.

DPPH assay evaluates the ability of antioxidants to scavenge free radicals. Hydrogen-donating ability is an index of the primary antioxidants. These antioxidants donate hydrogen to free radicals, resulting in non-toxic species and therefore inhibiting the propagation phase of lipid oxidation. Antiradical antioxidants act by donating hydrogen atoms to lipid radicals. Radicals obtained from antioxidants with molecular structures such as phenols are stable species and will then stop the oxidation chain reaction. The assay has been performed with eight dilutions made serially of both the standard and the sample (Methanolic extracts of *D. melanoxylon* leaf). The values represent the mean and standard deviation of the triplicates done at each point. The percentage scavenging activity was calculated by comparing the absorbance of control and test. Below graph was obtained by plotting the concentration on the X-axis versus the scavenging activity on the Y-axis. The test extracts has shown significant and dose dependent antioxidant activity at all the concentrations used in the assay. Antioxidant potential of the test extracts was comparable with the standard ascorbic acid at all the concentrations used as shown in Figure 3.



### Total phenolic content

Several studies report the linear correlation between the phenolic content present in the sample and their antioxidant capacity. In our study, the total polyphenolic content of the samples was estimated using Gallic acid as the reference standard and was expressed as gallic acid equivalents. The average absorbance and their respective concentrations of gallic acid were plotted to obtain the standard curve as shown in Figure 4 and Table 7.



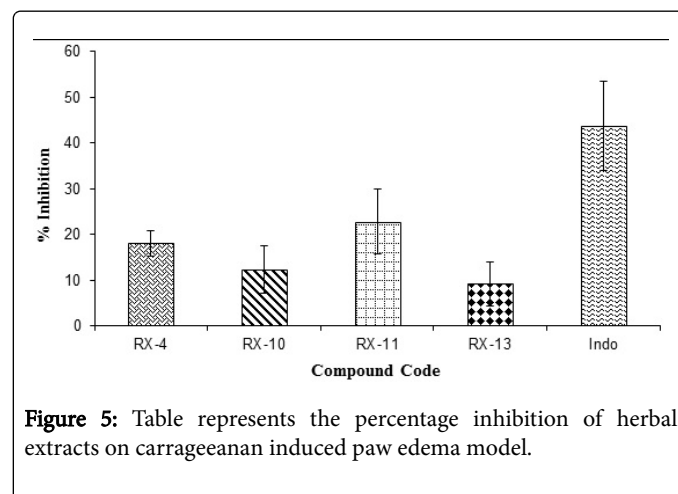
| Test Extract        | Total phenolic content (mg gallic acid equi/g of extract) |
|---------------------|---|
| Herbal Extract (HE) | 3.75  |

**Table 7:** Herbal extract used for experimentation.

Regression equation was obtained as  $y = 0.4882x - 0.0725$ ;  $R^2 = 0.9938$ , the absorbance values average of samples were substituted as y and their respective Gallic Acid equivalent was calculated as  $GAE = (y + 0.0725) / 0.4882$ .

### Anti-inflammatory effect of herbal extracts by carrageenan induced model

Carrageenan-induced paw edema is a suitable experimental animal model for evaluating the anti-inflammatory effect of new agents. Edema developed following injection of carrageenan is an index of acute inflammatory changes, and it can be determined from differences in the paw volume measured immediately after carrageenan injection. Edema induced by carrageenan is believed to be biphasic: the first phase involves the release of serotonin and histamine and the second phase is mediated by prostaglandins, cyclooxygenase products [15]. This model is the standard experimental model of acute inflammation, and it is the phlogestic agent of choice for testing anti-inflammatory drugs. Moreover the experimental model exhibits high degree of reproducibility. In the present study the herbal extracts were evaluated for anti-inflammatory activity by carrageenan induced model in wistar rats. The test compounds were administered by oral route prior to inflammatory agent. Percentage inhibition of the test compounds was calculated using control. Among all the extracts RX-11 has shown significant ( $p < 0.05$ ) inhibition compared to control. Rest all extracts showed mild inhibition in paw volume which is shown in Figure 5.



### Acute toxicity study acute oral toxicity

**Sighting study:** In the present study test compound was evaluated for different *in-vitro* activities and it was found to be potent. Based on these results the compounds were evaluated for maximum tolerated dose in the animals by conducting acute oral toxicity study before proceeding to *in-vivo* Nephroprotective activity by Potassium-dichromate-induced nephrotoxicity model.

The test extract did not show any toxic symptoms and mortality at different doses 5, 50, 300, and 2000 mg/kg throughout the study. Based on sighting study, a dose 2000 mg/kg was selected for the main study in rats for the herbal extract.

**Main study:** The extract was found to be safe at the dose of 2000 mg/kg and did not show any toxic symptoms. No abnormal behavior was observed in both rats and mice during the study. During the study physical parameters, body weight was measured and uniform increase in body weight was observed in all the compound groups of animals.



Based on all above observations the test extract compounds was safe up to 2000 mg/kg. Therefore, the maximum tolerated dose was found to be >2000 mg/kg in both rats shown in Table 8.

| S. No | Parameters                    | Result             |
|-------|-------------------------------|--------------------|
| 1     | Toxic signs                   | Absent             |
| 2     | Pre-terminal deaths           | Nil                |
| 3     | Body weight                   | No specific change |
| 4     | Cage side observation         | Normal             |
| 5     | Motor activity                | Normal             |
| 6     | Tremors                       | Absent             |
| 7     | Convulsions                   | Absent             |
| 8     | Straub reaction               | Absent             |
| 9     | Righting reflex               | Present            |
| 10    | Lacrimation and Salivation    | Normal             |
| 11    | Unusual vocalization          | Absent             |
| 12    | Sedation                      | Absent             |
| 13    | Body temperature              | Normal             |
| 14    | Analgesia                     | Absent             |
| 15    | Ptois                         | Absent             |
| 16    | Diarrhoea                     | Absent             |
| 17    | Skin color                    | Normal             |
| 18    | Respiration                   | Normal             |
| 19    | Scratching                    | Absent             |
| 20    | Grooming                      | Normal             |
| 21    | Aggressiveness & Restlessness | Absent             |

**Table 8:** Acute Toxicity Study Observations.

### Effect of herbal extract on potassium dichromate induced nephrotoxicity model

Potassium dichromate ( $K_2Cr_2O_7$ ) is a chemical compound widely used in metallurgy, chrome plating, chemical industry, textile manufacture, wood preservation, photography and photoengraving, refractory and stainless steel industries and cooling systems. The oxidation state and solubility of chromium (Cr) compounds determine their toxicity. In contrast to Cr (III), which is a naturally occurring form and an essential trace element for humans and others mammals, Cr(VI) compounds are highly toxic  $K_2Cr_2O_7$  is a hexavalent form of Cr and has been demonstrated to induce oxidative stress and carcinogenic in nature. In the present study the herbal extract was evaluated for nephroprotective activity by Potassium Dichromate induced model in Wister rats. The test compounds were administered prophylactically to all the test group of rats for the period of 7 days followed by Potassium dichromate administration.

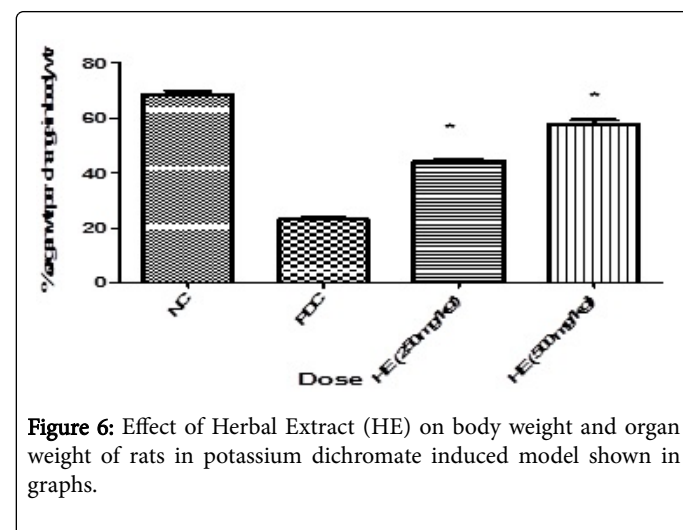
### Effect of herbal extract on potassium dichromate induced nephrotoxicity model

Body weight and organ weights were used to assess the toxicity effect of test compounds. During the study period, body weights were taken on every alternative day. At the end of the study, all the animals were sacrificed and liver tissues were collected. Percentage change in body weights was calculated by taking the termination day and zero day body weights. Percentage organ (kidney) weights were calculated by taking terminal body weights of rats. In the present study rats were treated with test extracts for the period of 7 days prophylactically by per oral route at the dose of 500 mg/kg. Significant ( $P < 0.01$ ) decrease in body weight and organ weight was observed in potassium dichromate group compared to vehicle control. Significant ( $P < 0.05$ ) gain was observed in test extract compared to potassium dichromate group. Significant restoration in body weights indicated the nephroprotective effect of test extracts. No evidence of toxicity symptoms were observed throughout the study. The details are shown in Figure 6 and effect of herbal extract are given in Table 9 respectively.

| Groups         | Change in body weight (g) | Organ weight (Kidney) (g) |
|----------------|---------------------------|---------------------------|
| Normal Control | $3.4 \pm 0.32$            | $1.925 \pm 0.05$          |
| PD Control     | $7.8 \pm 0.215$           | $1.856 \pm 0.08$          |
| Group 3        | $4.4 \pm 0.245$           | $1.93 \pm 0.05$           |
| Group 4        | $2.8 \pm 0.125$           | $1.892 \pm 0.07$          |

Values are expressed as Mean  $\pm$  S.E.M; n=6

**Table 9:** Effect of Herbal Extract (HE) on body weight and organ weight of rats in potassium dichromate induced model.



**Figure 6:** Effect of Herbal Extract (HE) on body weight and organ weight of rats in potassium dichromate induced model shown in graphs.

### Effect of herbal extract on biochemical parameters in potassium dichromate induced model

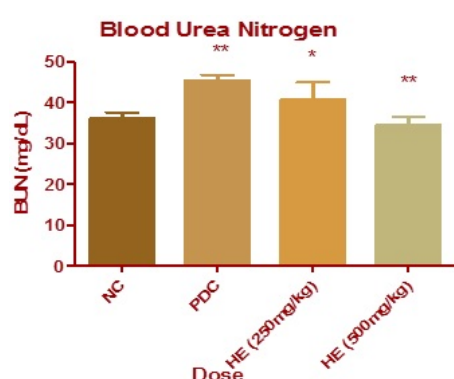
It is reported that the kidney is the principal route of Cr excretion and acute exposure induces an increase in Cr kidney content on  $K_2Cr_2O_7$  treated rats. Exposition to Cr (VI) produced anatomical lesions at the level of the proximal tubular cells and lipid peroxidation in human kidney. Creatinine and blood urea nitrogen levels reported to be elevated in kidney dysfunction [17]. On termination animals

were sacrificed and kidney tissue samples were collected and estimated for antioxidant enzymes. Blood samples were collected and analyzed for kidney biochemical parameters. Significant increase in creatinine and blood urea nitrogen levels was observed in PD control compared

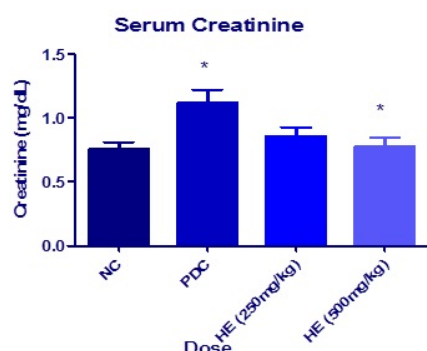
to normal control which indicates nephrotoxicity. The herbal extract at both the doses (250, 500 mg/kg) possessed significant ( $p < 0.05$ ) decrease in BUN levels when compared to PD. The details are shown in Figure 7a, 7b and Table 10.

| S. No | Parameter  | Normal Control | Potassium control | Dichromate | Herbal Extract (250 mg/kg) | Herbal Extract (500 mg/kg) |
|-------|------------|----------------|-------------------|------------|----------------------------|----------------------------|
| 1     | Bun        | 36.2 ± 1.356   | 45.5 ± 1.26       |            | 40.75 ± 4.19               | 34.5 ± 2.062               |
| 2     | Creatinine | 0.76 ± 0.114   | 1.12 ± 0.228      |            | 0.86 ± 0.152               | 0.748 ± 0.148              |

**Table 10:** Table represents biochemical parameters in potassium dichromate induced nephrotoxicity model.



**Figure 7a:** Effect of herbal extract on blood urea nitrogen levels in potassium dichromate induced model. Blood Urea Nitrogen values of test extract Herbal Extract (HE). Values are expressed as Mean ± S.E.M; n=6. \*\*  $P < 0.01$ , \*  $P < 0.05$ .



**Figure 7b:** Effect of herbal extract on serum creatinine levels in potassium dichromate induced model. Blood Urea Nitrogen values of test extract Herbal Extract (HE). Values are expressed as Mean ± S.E.M; n=6. \*\*  $P < 0.01$ , \*  $P < 0.05$ .

### Effect of herbal extract on kidney antioxidant parameters in potassium dichromate induced model

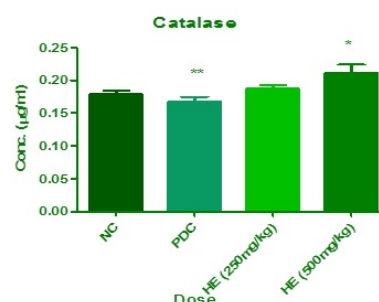
The body has effective mechanism to prevent and neutralize the free radical induced damage. This is accomplished by a set of endogenous antioxidant enzymes, such as SOD, CAT, GSH, etc. When the balance

between ROS (Reactive Oxygen Species) production and antioxidant defense is lost, oxidative stress results leads to deregulation of the cellular function leading to various pathological conditions. A major systemic event that occurs in the rat following the induction of inflammation is marked alteration in the cellular defense mechanism. It is also reported that Oxygen free radicals have been implicated as mediators of tissue damage. In the present study kidney samples were collected on termination day and estimated the antioxidant enzyme levels and lipid peroxidation respectively to assess the oxidative stress.

### Catalase

Catalase (CAT) is an enzymatic anti-oxidant widely distributed in all animal tissue and the highest activity is found in the red cells and in liver. It decomposes hydrogen peroxide and protects the tissue from highly reactive hydroxyl radicals [12]. Therefore, reduction in the activity of these enzymes may result in a number of deleterious effects due to the assimilation of super-oxide radicals and hydrogen peroxide. Significant decrease (Catalase) was observed in PD control compared to normal control whereas Herbal extract at the dose of 500 mg/kg showed significant increase compared to PD control as shown in Figure 8.

### Catalase assay

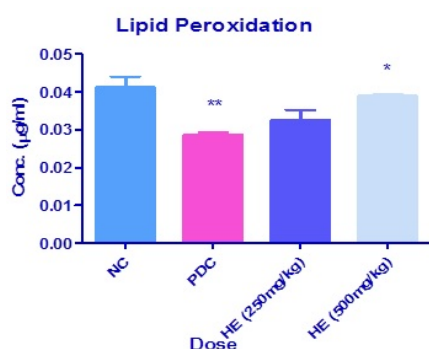


**Figure 8:** Effect of herbal extract on antioxidant enzymes CAT level in potassium dichromate -induced nephrotoxicity model. Where Values are expressed as Mean ± S.E.M; n=6. \*\*  $P < 0.01$ , \*  $P < 0.05$  as in Figure 8.

### MDA (TBARS)

Lipid peroxidation has been postulated as being the destructive process in kidney injury due to potassium dichromate administration

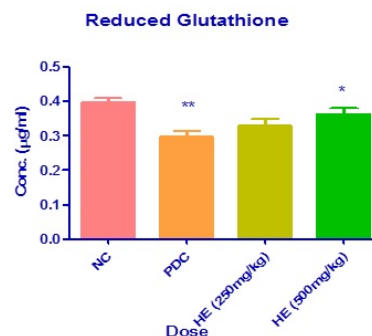
[20]. In our study, elevations in the levels of TBARS in kidneys of rats treated with potassium dichromate were observed. The increase in TBARS levels in kidney suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. In biological systems, MDA, a degradation product of lipid hydro peroxides, is considered as an index of lipid peroxidation. Accumulating evidence suggests that ROS initiates lipid peroxidation, which not only deteriorates cellular structure and function but also produces Melondialdehyde (MDA) a potent carcinogen. In the present study, kidney tissues were collected at the end of the treatment and used for estimation of lipid Peroxidation and different antioxidant enzymes. Significant decrease (GSH) was observed in PD control compared to normal control whereas Herbal extract at the dose of 500 mg/kg showed significant increase compared to PD control as shown in Figure 9.



**Figure 9:** Effect of herbal extract on antioxidant enzymes MDA level in potassium dichromate -induced nephro toxicity model. Where Values are expressed as Mean  $\pm$  S.E.M; n=6. \*\* P<0.01, \*P<0.05 as in Figure 9.

#### Effect of herbal extract on antioxidant enzymes on reduced glutathione level in potassium dichromate- induced nephrotoxicity model

Glutathione (GSH) is one of the most abundant tri-peptide, non-enzymatic biological antioxidants. Its functions are concerned with the removal of free radical species such as hydrogen peroxide, superoxide radicals, alkoxy radicals, and maintenance of membrane protein thiols and as a substrate for Glutathione Peroxidase (GPx) and GST [10]. Glutathione redox cycle is the most important intracellular antioxidant system which maintains cell integrity and participation in the cell metabolism. In our present study, the decreased level of GSH has been associated with an enhanced lipid peroxidation in Potassium dichromate treated rats compared to vehicle control group. The decrease may be because of potassium dichromate induced oxidative stress, which inactivates the enzyme Gaama-Glutamyl Cysteine Synthetase ( $\gamma$ -GCS) a rate limiting enzyme in *de novo* GSH synthesis. Significant decrease (GSH) was observed in PD control compared to normal control whereas Herbal extract at the dose of 500 mg/kg showed significant increase compared to PD control as shown in Figure 10.



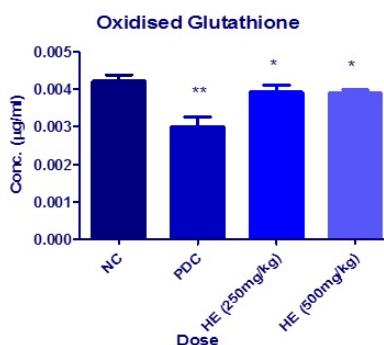
**Figure 10:** Effect of herbal extract on antioxidant enzymes Reduced Glutathione level in potassium dichromate- induced nephro-toxicity model.

Where Values are expressed as Mean  $\pm$  S.E.M; n=6. \*\* P<0.01, \*P<0.05 as in Figure 10.

#### Effect of herbal extract on antioxidant enzymes on oxidized glutathione level in potassium dichromate- induced nephrotoxicity model

The principle involves oxidation of GSH by the sulfhydryl reagent 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) to form the yellow derivative 5'-Thio-2-Nitrobenzoic Acid (TNB), measurable at 412 nm. The Glutathione Disulfide (GSSG) formed can be recycled to GSH by glutathione reductase in the presence of NADPH. GST assay can be extended for drug discovery/pharmacology and toxicology protocols to study the effects of drugs and toxic compounds on glutathione metabolism. Significant decrease (Oxidized Glutathione) was observed in PD control compared to normal control whereas Herbal extract at both doses (250, 500 mg/kg) showed significant increase compared to PD control as shown in Figure 11.

#### Oxidized glutathione assay



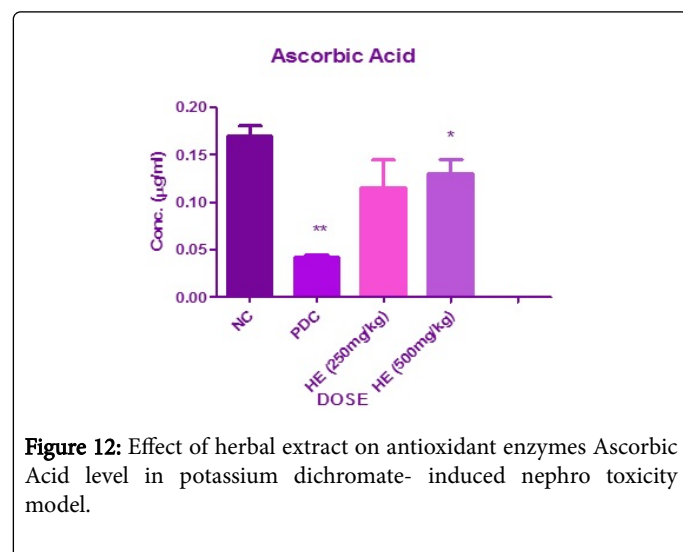
**Figure 11:** Effect of herbal extract on antioxidant enzymes Oxidized Glutathione level in potassium dichromate- induced nephro-toxicity model.

Where Values are expressed as Mean  $\pm$  S.E.M; n=6. \*\* P<0.01, \*P<0.05 as in Figure 11.

### Effect of herbal extract on antioxidant enzymes on ascorbic acid level in potassium dichromate- induced nephrotoxicity model

Ascorbic acid was assayed by the method in which Ascorbic acid is oxidized to dehydro ascorbic acid and 2,3-dioxogluconic acid with copper sulphate. Further after reaction with 2,4-dinitrophenyl hydrazine it forms tris 2,4-dinitrophenyl hydrazone which on treatment with ice cold sulfuric acid forms a complex which is quantified colorimetrically at 520 nm. Significant decrease (Ascorbic Acid) was observed in PD control compared to normal control whereas Herbal extract at the dose of 500 mg/kg showed significant increase compared to PD control as shown in Figure 12.

Ascorbic acid assay



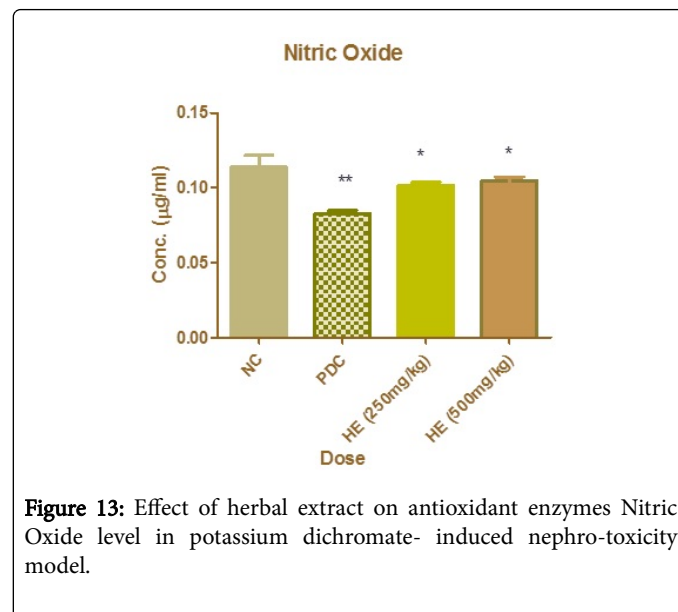
**Figure 12:** Effect of herbal extract on antioxidant enzymes Ascorbic Acid level in potassium dichromate- induced nephro toxicity model.

Values are expressed as Mean  $\pm$  S.E.M; n=6. \*\* P<0.01, \*P<0.05 as in Figure 12.

### Effect of herbal extract on antioxidant enzymes on nitric oxide levels in potassium dichromate- induced nephrotoxicity model

Nitric Oxide (NO), a short-lived free radical generated endogenously, exerts influence on a number of functions including vasodilation, neurotransmission, synaptic plasticity and memory in the central nervous system. Besides mediating normal function, NO has been implicated in pathophysiological states. Overproduction of NO can mediate toxic effects, like DNA fragmentation, cell damage and neuronal cell death. Significant decrease (Nitric Oxide) was observed in PD control compared to normal control whereas Herbal extract at both doses (250, 500 mg/kg) showed significant increase compared to PD control as shown in Figure 13.

### Nitric oxide assay



**Figure 13:** Effect of herbal extract on antioxidant enzymes Nitric Oxide level in potassium dichromate- induced nephro-toxicity model.

Values are expressed as Mean  $\pm$  S.E.M; n=6. \*\* P<0.01, \*P<0.05 as in Figure 13.

### Conclusion

This study was aimed to investigate the *in-vitro* antioxidant, anti-inflammatory and *in-vivo* nephroprotective activity novel herbal extracts. Initially the test extracts were evaluated for antioxidant potential by performing the DPPH assay. Few extracts displayed potent antioxidant activity by DPPH free radical scavenging activity. Among all the extracts Methanolic extracts of *D. melanoxylo*n was found to be more potent for antioxidant potential. Presence of phenols and flavonoid in the test extracts might be contributed to their potent antioxidant activity. The test extracts were also evaluated for anti-inflammatory activity by carrageenan induced paw edema model. RX- has shown moderate anti-inflammatory activity. Based on preliminary *in-vitro* antioxidant activity results *D. melanoxylo*n was selected for further to evaluate Nephro-protective activity against Potassium dichromate induced model. Acute oral toxicity test was performed to find out the safe dose of test extract before going to *in-vivo* evaluation (Potassium Dichromate induced nephrotoxicity). Acute toxicity study of test extract was conducted in Wistar rats to find the Maximum tolerated dose. The test extract did not show any toxicity and mortality symptoms during the study at the different doses studied. The MTD of the test extract was found to be >2000 mg/kg in rats.

*In-vivo* nephroprotective activity was conducted in wistar rats by Potassium dichromate-induced nephrotoxicity model. During the study period test extract (250 mg/kg, 500 mg/kg) were administered by oral route for the period of 7 days followed by potassium dichromate administration (15 mg/kg). At the end of the study blood samples were collected and used for estimation of kidney biochemical parameters. Results showed that significant increase was observed in biochemical parameters (BUN, CR) in PDC group compared to vehicle control. The test extract displayed significant reduction in blood urea nitrogen and serum creatinine at the dose of 500 mg/kg. Kidney tissue samples were collected on termination day of all rats and subjected for measurement of antioxidant enzymes and lipid Peroxidation to check the organ



toxicity. Significant increase in lipid Peroxidation and decrease in antioxidant enzyme levels were observed in PDC control whereas test extract prevented the kidney toxicity by decreasing TBARS production and normalization of antioxidant defense enzymes at the doses studied. Gain in body weight and organ weight compared to PD control also revealed the Nephroprotective effect of *D. melanoxylon* extract at both the doses. All the data showed that both biochemical antioxidant parameters correlated together and supported the protective effect of the Herbal extract (*D. melanoxylon*) against potassium dichromate induced nephrotoxicity.

The results of this study demonstrated that *D. melanoxylon* has shown strong antioxidant potential. Significant protective effect on potassium dichromate induced nephrotoxicity in rats revealed that the herbal extract *D. melanoxylon* may be find good therapeutic use in nephrotoxicity. Further studies are needed to find the exact possible mechanism of the protective effect of the plant extract.

## References

1. Purohit S, Vyas P (2004) Medicinal Plant Cultivation: A Scientific Approach. Agro bios India p: 1.
2. Mukherjee P (2002) Quality control of herbal drugs: An approach to evaluation of botanicals. New Delhi: Business horizons pharmaceuticals publishers p: 13.
3. Thannickal V, Fanburg B (2000) Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol 279: 1005-28.
4. Castro L, Freeman B (2001) Reactive oxygen species in human health and disease. Nutrition 17: 161-65.
5. Bhende Y (2002) General pathology. 6th edn Mumbai: Popular Prakashan Private Limited Pp: 198-202.
6. Mohan H (2000) Textbook of pathology. (4th edn), medical publishers, Jaypee brothers, New Delhi.
7. Rubin E, Gorstein F, Rubin R, Schwarting R, Stayer D, et al. (2004) Rubin's pathology: Clinicopathological Foundation of Medicine. 4th ed. Philadelphia: Lippincott Williams and Wilkins 18: 747-62.
8. Drew B, Leeuwenburgh C (2002) Aging and the Role of Reactive Nitrogen Species. Ann NY Acad Sci 959: 66-81.
9. Valko M, Leibfritz D, Moncol J, Cronin M, Marzur M, et al. (2006) Free radical and antioxidants in normal physiological functions and human disease. J Biocel 7: 45-78.
10. Prakash J, Gupta S, Kochupillai V, Singh N, Gupta Y, et al. (2001) Chemo preventive activity of Withania somnifera in experimentally induced fibro sarcoma tumors in Swiss albino mice. Phytother Res 15: 240-4.
11. Curtis S, Moritz M, Snodgrass P (1972) Serum enzymes derived from liver cell fraction and the response to carbon tetrachloride intoxication in rats. Gastroenterology 62: 84-92.
12. Chance B, Green Stein D, Roughton R (1952) The mechanism of catalase action- steady state analysis. Arch Biochem Biophys 37: 301-39.
13. Vilasrao J, Joshi Y, Sawant H, Jadhav T (2010) Free radical scavenging activity of aqueous solution of black salt. J Pharm Pharm Sci 2: 94-95.
14. Siddique N, Mujeeb M, Abdul K, Najmi, Khan H, et al. (2010) Evaluation of antioxidant activity, quantitative estimation of phenols and flavanoids in different parts of Aegle marmelos. J Saudi Chem Soc 14:1-8.
15. Vinegar R, Schreiber W, Hugo R (1969) Biphasic development of carrageenin oedema in rats. J Pharmacol Exp Ther 166: 96-103.
16. Aslam M, Dayal R, Javed K, Parray S, Jetley S, et al. (2013) Nephroprotective Effects of Methanolic Extract of Peucedanum grandeagainst Acute Renal Failure Induced by Potassium dichromate in Rats. Inter J Pharmaceutical Sciences and Drug Research 5: 45-49.
17. Bandyopadhyay U, Das D, Banarjee R (1999) Oxidative damage and pathogenesis. Current Sci 77: 515-6.
18. Joshi SG (2006) Medicinal plants. Saujanya Books Ltd, New Delhi p: 145.
19. Warriar P, Nambier V, Ramankutty C. Indian Medicinal Plants: a compendium of 500 species. Orient Blackswan publications 5: 277.
20. Sashidharan N (2004) Biodiversity documentation for Kerala-Flowering Plants. C Khare Indian Medicinal Plants: an illustrated dictionary. Springer publications. New Delhi. p: 655.