5.1. Materials

HPLC standardized TSE, nitric oxide assay kit (Biovision, USA, catalog # 262-200) Streptozotocin (Sigma Chemicals U.S.A), Metformin (Ranbaxy Ltd, Gudgaon, India) superoxide dismutase (SOD), glutathione, and catalase (Biovision, USA) HDL and LDL/VLDL Cholesterol Quantification Kit (Catalog #K613-100; Biovision, USA)

5.2. Animals

Male Wistar rats weighing between 150-200g used for study were obtained from Animal House of Delhi Institute of Pharmaceutical Sciences and Research (IAEC protocol No-2/2010). Rats were housed in colony cages (4 rats per cage), at an ambient temperature of 25°C with 12 h light: 12 h dark cycle. Rats had free access to standard food and water *ad libitum*. The Principles of Laboratory Animal Care (NIH, 1985) were followed throughout the duration of experiment and instruction given by our institutional ethical committee was followed during experimentation.

5.3. Induction of hypertension superimposed on NIDDM

STZ was injected at dose level of 90 mg/kg I.P. in 0.1 M freshly prepared citrate buffer pH 4.5 to 2 day old neonatal rat. Control was injected with equivalent volume of citrate buffer. After 6 week of injection, animals were evaluated for fasting blood glucose level. The fasting glucose level of 140 mg/dl was the criteria for selection of diabetic rats.

Furthermore, rats were anesthetized with ketamine hydrochloride at 50 mg/kg, intraperitoneally. The left kidney was removed by taking incision below the left flank region, with care being taken to avoid adrenalectomy. An incised area was then sutured with cat gut for muscles (absorbable suture) followed by nylon for skin tissue. All operated rats received an injection of ampicillin (10 mg/kg, i.m.) daily for 5 days and local application of neosporin-H to prevent infection. Animals were given 1% w/v NaCl solution as drinking water. DOCA was injected subcutaneously twice a week at a dose of 40 mg/kg (s/c) in cotton seed oil for period of 4 weeks to all animals except sham control group (normotensive) (Selye and Bois 1957).

All animals in treatment groups received *T. indica* seed extract daily for period of 4 weeks after surgery at the dose level of 120 and 240 mg/kg body weight orally.
5.4. Experimental Design

The animal groups in the present study were composed of six animals per group and were designated as follows

**Group I:** Normotensive Control (sham control), unilateral nephrectomised animals received daily injection of 0.1ml of sterilized cotton seed oil subcutaneously (s.c) for 4 weeks.

**Group II:** Unilateral nephrectomised animals received single dose DOCA injection (40 mg/kg/week) for 4 weeks, dissolved in cotton seed oil (s.c).

**Group III:** Unilateral nephrectomised animals received DOCA injection (40 mg/kg/week, s.c.) with TSE at 120 mg/kg body weight for 4 weeks orally.

**Group IV:** Unilateral nephrectomised animals received DOCA injection (40 mg/kg/week, s.c.) with TSE at 240 mg/kg body weight for 4 weeks orally.

**Group V:** STZ induced diabetes + unilateral nephrectomised animals received DOCA injection (40 mg/kg/week, s.c.)

**Group VI:** STZ induced diabetes + unilateral nephrectomised animals received DOCA injection (40 mg/kg/week, s.c.) + TSE at 120 mg/kg body weight for 4 weeks orally

**Group VII:** STZ induced diabetes + unilateral nephrectomised animals received DOCA injection (40 mg/kg/week, s.c.) + *T. Indica* seed extract at 240 mg/kg body weight for 4 weeks orally.

5.5. Blood Pressure Measurement

At the end of experiments, all rats were fasted for 12 hours and the systolic, diastolic and mean blood pressure was measured at day 0, 5th and 8th week of the study by Non Invasive Blood Pressure (NIBP) tail cuff method (Kent Scientific Ltd, USA).

The animals were sacrificed after blood pressure measurement under light ether anesthesia. The guideline of our institutional ethical committee for this purpose was followed strictly. The rats were sacrificed by overdose of ether anaesthesia and blood was collected from dorsal aorta and serum was separated by centrifugation at 3000 g for 5 min. The serum samples were stored at -20 °C for the biochemical assay of LDL, HDL, NO and antioxidant enzymes viz, SOD, catalase, and glutathione concentration.
5.6. Glutathione

Glutathione (GSH) is the major intracellular low-molecular-weight thiol that plays a critical role in the cellular defense against oxidative stress in mammalian cells. BioVision’s ApoGSHTM Glutathione colorimetric assay kit provided a convenient, colorimetric method for analyzing either total glutathione or the reduced form glutathione alone using a microtiter plate reader. The assay was based on the glutathione recycling system by DTNB and glutathione reductase. DTNB and glutathione (GSH) react to generate 2-nitro-5-thiobenzoic acid which has yellow colour. Therefore, GSH concentration determined by measuring absorbance at 412 nm. The kit included the 5-Sulfosalicylic acid (SSA) for the removal of proteins from samples and for the protection of GSH oxidation and γ-glutamyl transpeptidase reaction.

5.7. Superoxide dismutase (SOD)

Superoxide dismutase (SOD) is one of the most important antioxidative enzymes. It catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen. The sensitive SOD assay kit employed in present study (Biovision) utilized WST-1 that produces a water-soluble formazan dye upon reduction with superoxide anion. The rate of the reduction with a superoxide anion was linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD.

5.8. Catalase

Catalase is a ubiquitous antioxidant enzyme that is present in nearly all living organisms. It functions to catalyze the decomposition of hydrogen peroxide (H$_2$O$_2$) to water and oxygen. BioVision’s Catalase Assay Kit provided a highly sensitive, direct, and HTS-ready assay for measuring Catalase activity in biological samples (plasma, serum). In the assay, catalase first reacts with H$_2$O$_2$ to produce water and oxygen, the unconverted H$_2$O$_2$ reacts with OxiRed™ probe to produce a product, which can be measured at 570 nm (Colorimetric method).

5.9. Serum HDL/LDL and nitric oxide

The determination of serum HDL/LDL and NO was according to the standard operating procedure of ELISA kit (Bio Vision, USA) and the principle employed in the method is described above.
5.10. Statistical analysis

All values are means ± SEM. Data analysis was done with one-way analysis of variance (ANOVA) followed by Dunnet’s multiple test where a diabetic group was considered a positive control (Sigma Plot, USA 11). Group means were considered to significantly differ at $P < 0.05$, as determined by Dunnet’s multiple range analysis.