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Enhanced antioxidant enzyme activity in tissues and reduced total oxidative stress in plasma by the effect of *Swietenia macrophylla* king seeds in type II diabetes rats

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ABSTRACT
The complications of diabetes are mainly as a result imbalance between oxidative stress and antioxidant defence. Several plant products are being reported to be effective in this condition including *Swietenia macrophylla* king. The aim of this study was to find out whether the aqueous extract of these seeds have any effect on the antioxidant enzyme activity in liver and kidney tissues and on the total oxidative stress in plasma in the experimental type II diabetic rats. Streptozotocin was used to induce diabetes except in the control group. The activities of the antioxidant enzymes in the tissues extracts. The activities of the antioxidant enzymes in the tissues extracts and total oxidative stress in plasma were estimated using standardized methods.The extract fed rats show significantly increased activity of superoxide dismutase (U/mg), Catalase (U/mg) and glutathione peroxidise (U/mg) in the liver (5.12±0.21, 45.03±1.19, 2.69±0.02 respectively) and kidney tissues (5.66±0.06, 89.61±0.27, 2.93±0.01, respectively) than the diabetic control animals (3.08±0.21, 33.01± 1.36, 1.94±0.08 in liver and 3.60±0.07, 40.42±1.73, 2.21±0.05 in kidney respectively). Total oxidative stress was found significantly decreased (82.5±0.05 FORT U) in the animals treated with extract compared to the diabetic controls (100±0.63 FORT U). We conclude that the extract of *Swietenia macrophylla* king seeds effectively reduce the oxidative stress in plasma as well as increase the antioxidant enzyme defence in the antioxidant enzyme defence in the tissues of experimental diabetic rats.

Keywords: Diabetic, *Swietenia macrophylla* king seeds, Modified FORT, Antioxidant enzymes, SOD, Catalase and Gpx.

1. Introduction
Diabetes Mellitus is a major problem not only in India but all over the world. Our country is undergoing an epidemic stage of this non-communicable disorder. Though modern treatment is available with insulin and several hypoglycemic drugs along with dietary and lifestyle management, the disease seems to be spreading heavily in the community. The complications of diabetes are mainly as a result imbalance in between oxidative stress and antioxidant defense [1-3]. This is now becoming a great social burden especially for the developing countries including India. Cost of the treatment and medications are increasing day by day. Therefore, the searches for natural remedies in the use of herbs are gaining attention by researchers all over the world. Several plants and plant products are being reported to be effective in this condition [4]. *Swietenia macrophylla* is one such plant which was reported earlier to be effective in several other diseases including Diabetes Mellitus [5]. Earlier we have reported that the extracts of the seeds of *Swietenia macrophylla* is a nontoxic one [6]. Recently we have reported the extracts of the seeds have hypoglycemic and antioxidant effect in the blood of experimental diabetic rats [7]. In the current study we wanted to find out whether the seeds have any effect on the antioxidant enzymes at the tissue levels in the diabetic rats as well as on total oxidative stress in their plasma. Assessment of antioxidant enzymes in the kidney and liver tissues along with estimation of total oxidative stress in plasma was done and compared with control groups.

2. Materials and Methods
2.1 Plant material
Swietenia macrophylla king seeds were collected from west Midnapore dated 1st December 2009 and authenticated by Botanical Survey of India, Government of India, Howrah dated 4th February A voucher specimen (Ref. no. CNH/I-I/54/2009/Tech.II /154) has been kept in our laboratory for further reference.

2.2 Preparation of extract

The seeds of *Swietenia macrophylla* king seeds were separated, washed, shed-dried at room temperature, powdered and sieved through 40 meshes. One ml of distilled water was added to 200 mg powder to make the solution. After that the solution was centrifuged at 3000 r.p.m for 15 minutes. The supernatant was filtrated and collected. The pure extract of *Swietenia macrophylla* seeds was stored in glass vial sealed by air tight lid [8].

2.3 Animal models

Thirty wister rats (180-240 gms) were selected for the experiment. The animals were kept under standard condition of 12:12 hr light & dark cycle in a polythene cages and they were fed with standard laboratory diet and water ad libitum. The principle of laboratory Animals care [9] and the instructions given by our institutional ethics committee were followed throughout the experiment. Streptozotocin was used through intraperitoneal route for the induction of diabetes to all the rats except the control group. Diabetes was induced in overnight fasted adult Wistar strain albino rats weighing 180-240 g by a single intraperitoneal injection of 65 mg/ kg streptozotocin in a volume of 1 ml/ kg body weight [10]. Due to the instability of streptozotocin in aqueous media, the solution was made in citrate buffer (pH4.5) just before injection [11]. Hyperglycemia was confirmed by the elevated glucose level in plasma, determined at 48 hours after injection. The rats which were found hyperglycemic were screened for the study along with the normal control group except in Group I rats the normal control.

2.4 Experimental Design

Animal were divided into five groups of six rats each and further experiments carried out using six rats in each group.

- Group I (Normal Control, NC): Normal rats administered double distilled water for 30 days.
- Group II (Diabetic Control, DC): Diabetic control rats administered distilled water.
- Group III (Diabetic rats with extract, D with E): Diabetic rats administered aqueous extract of *Swietenia macrophylla* king seeds (2 gm/kg body weight) daily for 30 days.
- Group IV (Diabetic rats with Metformin, D with M): Diabetic rats administered aqueous extract of Metformin (10 mg/ kg body weight) daily for 30 days.
- Group V (Diabetic rats with extract, soya peptide, D with E+P): Diabetic rats administered aqueous extract of *Swietenia macrophylla* seeds (1gm / kg) + peptide (1gm / kg) daily for 30 days. Peptide was used as an adjuvant.

2.5 Collection of Blood

After completion of treatment period, blood samples from rats were collected under anesthesia by cardiac puncture in heparinized tubes and centrifuged at 3000 g for 15 min at 4 ^{0}C. Plasma samples were stored at -20 ^{0}C in aliquots until analysis.

2.6 Assay of antioxidant enzymes in tissue extract

The animals were sacrificed. Livers and kidneys were excised immediately, washed with ice-cold physiologic formalin 10% solution and weighed. The livers and kidneys were cut into small pieces and homogenized with an Ultra Turrax homogenizer in cold appropriate buffers (TBS, pH7.4). Tissue were centrifuged at 9000 g for 15 min at 4 ºC. Supernatants (S1) were collected in aliquots and stored at -20 ºC until use for enzyme assays**.**

2.7 Assessment of Antioxidant Enzymes in Tissues

The antioxidant enzyme catalase, SOD and GPX enzyme activity were measured by the following methods.

2.8 Catalase activity:

Catalase (CAT) activity was determined spectrophotometrically as per the method of Patterson et al., 1984. The rate of disappearance of H_2O_2 was measured at 250 nm considering $\Delta\epsilon$ (molar extension coefficient) at 240 nm is 43.6 mol/lit^{-1}/cm^{-1}.The reaction mixture content 50 mmol/L potassium phosphate (pH 7.0), 0.1 mmol/L H_2O_2. The reaction was run at 25 ^{0}C for 2 min, after adding the enzyme extract containing 20 µg of protein, and the initial linear rate of decrease in absorbance at 240 nm was used to calculate the activity.

2.9 Superoxide dismutase activity

Superoxide dismutase (SOD), activity in the supernatant was measured by the method of Beyer and Fridovich (1987). The reaction mixture was prepared by mixing 25 ml of 50 mmol/L potassium phosphate buffer, pH 7.8, 35 mg of L-methionine (9.9 mM/L), 7.5 µl of Triton X- 100 (0.025%) and 4 mg NBT (57 µmol/L). One ml of this mixture was delivered into small glass tube, followed by 20 µL of enzyme extract and 10 µL of Riboflavin (4.4 mg/100 ml). The cocktail was mixed and then illuminated for 7 minutes in an aluminium foil-lined box, containing two 20 w florescent tubes. A control tube in which the sample was replaced by 20 µl of buffer was run in parallel. A560 was measured in both the tubes. The test tubes containing the reaction mixture were exposed to light immersing the glass tubes in a cylindrical glass container three fourth filled with clean water and maintained at 25 ^{0}C and placed in between two 20w florescent tubes. The increase in absorbance due to formazan formation was read at 560 nm. Under the described conditions, the increase in absorbance without the enzyme extract was taken as 100% and the enzyme activity was calculated by determining the percentage inhibition per min. Fifty percent of inhibition was taken as equivalent to 1unit of SOD activity.

2.10 Glutathione peroxidase activity (GPX)

(GPX; EC 1.11.1.9) activity was measured following the method of Flohe´ and Gunzler (1984) [12]. The GSSG generated by GPX was reduced by GR, and NADPH oxidation was monitored at 340 nm. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.1), 3.6 mM GSH, 3.6 mM sodium azide, 1 IU/mL glutathione reductase, 0.2 mM NADPH, and 1.2 mM H_2O_2.

2.11 Assay of Total Oxidative Stress by Modified FORT method.

Free Oxygen Radical Test) is a colorimetric test based on the ability of transition metals, such as iron, to catalyse the breakdown of hydroperoxides into derivative radicals, according to Fenton's reaction. Once they are formed in cells, ROOH maintain their chemical reactivity and oxidative capacity to generate proportional amounts of alkoxyl (RO·) and peroxyl (ROO·) radicals. These derivative radicals are then preferentially trapped by a suitably buffered chromogen (an amine derivative, ($CrNH_2$) and develop, in a linear kinetic based reaction at 37 ^{0}C, a colored fairly long-lived radical cation photometrically detectable. The intensity of the color correlates directly with the quantity of radical compounds, according to the Lambert-Beer's law, and it can be related to the oxidative status of the sample.

$$R\text{-}OOH+Fe^{2+}\rightarrow R\text{-}O^{\cdot}+OH^{-}+Fe^{3+}$$
$$R\text{-}OOH+Fe^{3+}\rightarrow R\text{-}OO^{\cdot}+H^{+}+Fe^{2+}$$
$$RO^{\cdot} + ROO^{\cdot} + 2CrNH_2 \rightarrow RO^{-} + ROO^{-} + [Cr\text{-}NH_2^{+\cdot}]$$

The visible spectrum of the $ChNH_2$ radical cation, shows two peaks of absorbance at 505 and 550 nm. The overall spectral intensity increases with time.

Preparation of Buffers and Reagents

1. The Chromogen Reagent solution: 10 mMolar working reagent solution (4-AMINO-N-ETHYL-N-ISOPROPYLANILINE HYDROCHLORIDE , Fluca, Sigma), prepared by dissolving 21.5 mg in 10 ml of 20 m Molar PBS buffer (pH 7.4) and kept in 4-8° C for use during assay.
2. Acetate Buffer (pH 5.2): Prepared by mixing 0.1N acetic acid (570 µl to 500 ml deionized water) and 0.1N sodium acetate (2.72gm in 200ml) in 42: 158 proportions (Pearse, 1980).
3. 20 m Molar PBS (pH 7.4)
4. 7.6 Molar (0.26 mg/l) H_2O_2 prepared by adding 88 µl of 30% H_2O_2 to 100 ml of deionised water

Assay System: When 100 µL of plasma/ serum was dissolved in one ml of acetate buffer in a test tube, the hydroperoxides reacted with the transition metal ions liberated from the proteins in the acidic medium and were converted to alkoxy ($RO^{\cdot}$) and peroxy ($ROO^{\cdot}$) radicals. 10 µL of working Chromogen solution added to the mixture. The radical species produced by the reaction interact with the chromogen that forms a colored, fairly long-lived radical cation evaluable by spectrophotometer at 505 nm (linear kinetic-based reaction, 37°C). The intensity of the color correlates directly to the quantity of radical compounds and to the hydroperoxides concentration and, consequently, to the oxidative status of the sample according to the Lambert-Beer law. The results are expressed as FORT units, whereby 1 FORT U corresponds to 1mmol/l of H_2O_2. A calibration curve was developed concentrations of H_2O_2. The intraassay and interassay coefficients of variation were 3.7% and 6.2%, respectively, for FORT, whereas the linearity ranged from 1.22 to 4.56 mmol/L H_2O_2. The assay is completed in 6 minutes [13].

3. Results

Table 1: Results of Modified FORT Assay

Groups	Modified FORT Unit (One FORT Unit Eqv to 1 mmol/L H_2O_2)
Control (I)	90 ± 0.63
Diabetes mellitus (II)	100 ± 0.63
Metformin (III)	93.11 ± 0.01
Extract (IV)	82.5 ± 0.054[b]
Extract + peptide (V)	88.01 ± 0.70 [a,b]

a indicate P <0.05 compare to healthy control.
b indicate P <0.05compare to diabetic control.

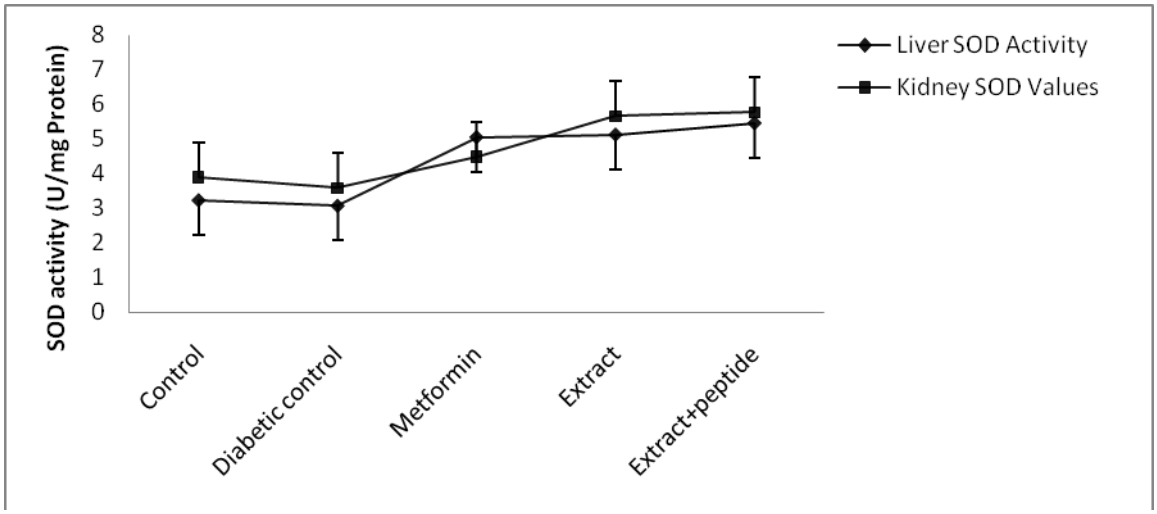

Fig 1: Changes in the activity of SOD in liver and kidney of experimental diabetic rats

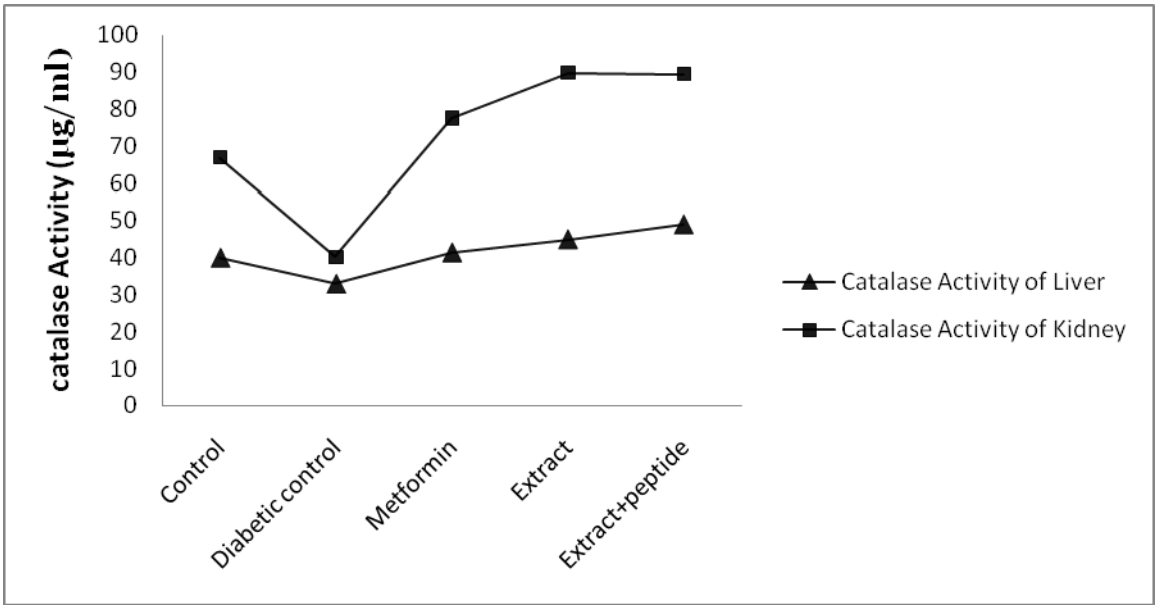

Fig 2: Changes in activity of Catalase in liver and kidney of experimental diabetic rats

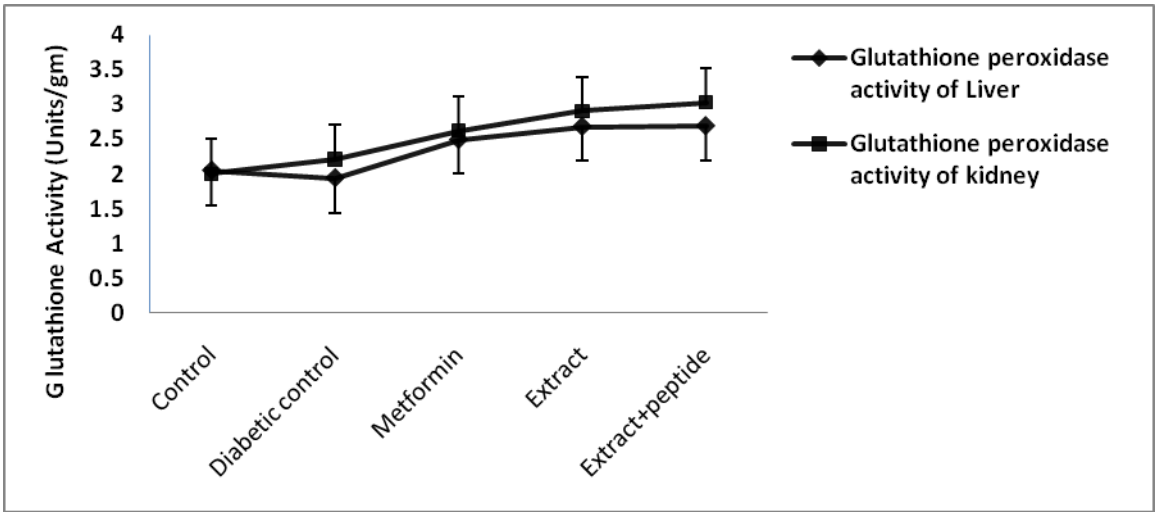

Fig 3: Changes in activity of Glutathione peroxidase in liver and kidney of experimental

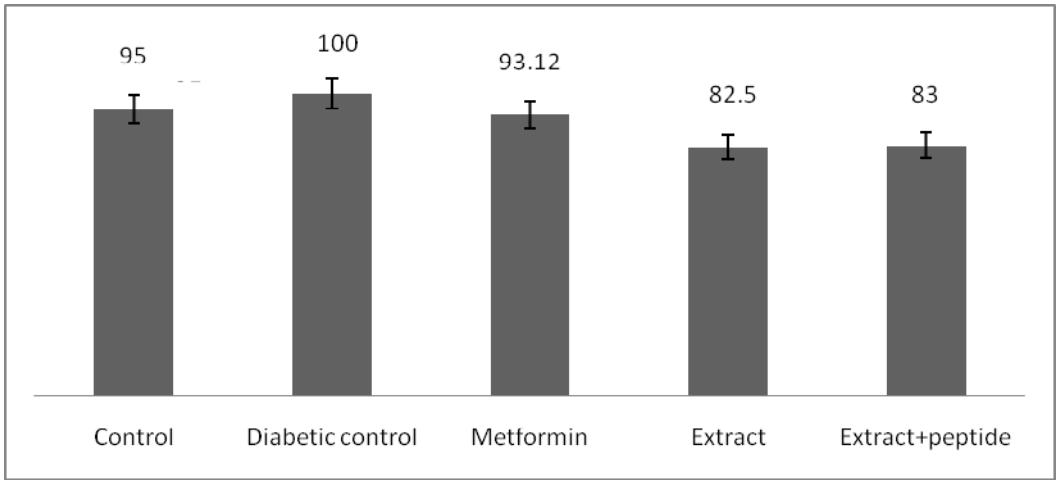

Fig 4: Total oxidative stress in plasma of the experimental rats by Modified FORT

In this study we wanted to find out the effect of *Swietenia macrophylla* king seeds on the antioxidant defense at the tissue levels by assessment of antioxidant enzymes in the kidney and liver tissues of the diabetic rats as well as on total oxidative stress in their plasma. The antioxidant enzyme activities in liver and kidney tissues of different groups of experimental rats have been depicted in Table 1. The diabetic rats show deficient antioxidant activity in comparison to healthy controls. This observation is similar with earlier reports [14]. The time course changes in the SOD activities in liver and kidney tissues are shown in Fig. 1. Our results exhibit significant increase in SOD activity in the extract and extract plus peptide treated rats compared to the diabetic control group. The catalase and GPx activities also show the similar trends (Table 1, Fig. 2 and 3). The extract and extract treated rats (Gr. IV & V) both have increased activity of these enzymes in their tissue. Although metformin treated rats (Gr.III) show increased activity of these enzymes than the diabetic control group (Gr.II), but the activity is significantly lower than the groups (Gr. IV and Gr. V) treated with extracts and extract plus peptide. The results of the total oxidative stress assessment by modified FORT method in plasma of the different groups have been depicted in Table 2 and Fig. 4. The diabetic control groups (Gr. II) are under maximum stress. This observation is in agreement with the previous studies [15]. The oxidative stress is always associated with diabetes mellitus and the complication of diabetes mellitus is mainly as a result of long continued oxidative stress [16, 17, 18]. In the current study both extract (Gr.IV) and extract plus peptide (Gr.V) treated animals exhibit significantly reduced total oxidative stress in plasma compared to the diabetic control group (Gr II). This implies that *Swietenia macrophylla* king seeds are beneficial in combating the stress associated with diabetes.

4. Discussion

We have earlier reported that the extract of *Swietenia macrophylla* king seeds not only have significant hypoglycemic property but it also improves the total antioxidant defense in plasma measured by modified FORD (Free Oxygen Radical Defense) test in the experimental diabetic rats [7]. Our experiment also reveals that there is no significant difference in total oxidative stress between the control group (I) and the rats treated with metformin (Gr.III). This signifies that though metformin effectively lower the blood glucose levels but it does not help to reduce the existing oxidative stress. Many of the present oral hypoglycemic drugs like metformin effectively reduce the blood glucose levels. But very few of them are effective in reducing the oxidative stress related damage to the tissues and organs in this condition [19] Therefore, very few of the modern anti-diabetic medicine like metformin can effectively prevent long term complications.

Our results indicate that the extract of *Swietenia macrophylla* king seeds have significant antioxidant property. It not only helps to reduce the existing oxidative stress in plasma associated with diabetes but also helps to increase the antioxidant enzyme activities at the tissue levels. Therefore, along with its hypoglycemic property reported earlier [7], *Swietenia macrophylla* king seeds may effectively restore the balance between oxidative stress and antioxidant defense and thereby may be used to prevent the complications of diabetes mellitus.

5. Conclusion

Although our study consist of less number of samples but it shows that the extract of *Swietenia macrophylla* king seeds, which is nontoxic, effectively reduce the oxidative stress as well as increase the antioxidant defence in experimental diabetic rats. However further study with large number of samples with the different components of the extract are needed in this direction to establish the effectiveness of the extract in diabetes mellitus.

6. Acknowledgment

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7. Conflicts of interest

The authors have declared that there is no conflict of interest.

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