A 43 kD Protein Isolated from the Herb *Cajanus indicus* L Attenuates Sodium Fluoride-induced Hepatic and Renal Disorders *in Vivo*

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The herb, *Cajanus indicus* L, is well known for its hepatoprotective action. A 43 kD protein has been isolated, purified and partially sequenced from the leaves of this herb. A number of *in vivo* and *in vitro* studies carried out in our laboratory suggest that this protein might be a major component responsible for the hepatoprotective action of the herb. Our successive studies have been designed to evaluate the potential efficacy of this protein in protecting the hepatic as well as renal tissues from the sodium fluoride (NaF) induced oxidative stress. The experimental groups of mice were exposed to NaF at a dose of 600 ppm through drinking water for one week. This exposure significantly altered the activities of the antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione reductase (GR) and the cellular metabolites such as reduced glutathione (GSH), oxidized glutathione (GSSG), total thiols, lipid peroxidation end products in liver and kidney compared to the normal mice. Intraperitoneal administration of the protein at a dose of 2 mg/kg body weight for seven days followed by NaF treatment (600 ppm for next seven days) normalized the activities of the hepato-renal antioxidant enzymes, the level of cellular metabolites and lipid peroxidation end products. Post treatment with the protein for four days showed that it could help recovering the damages after NaF administration. Time-course study suggests that the protein could stimulate the recovery of both the organs faster than natural process. Effects of a known antioxidant, vitamin E, and a non-relevant protein, bovine serum albumin (BSA) have been included in the study to validate the experimental data. Combining all, result suggests that NaF could induce severe oxidative stress both in the liver and kidney tissues in mice and the protein possessed the ability to attenuate that hepato-renal toxic effect of NaF probably via its antioxidant activity.

**Keywords:** Antioxidant, *Cajanus indicus* L, Hepatic and renal oxidative stress, Sodium fluoride, 43kD protein

**Introduction**

Fluorine, a naturally occurring element never exists in its elemental state in nature because it is the most reactive non-metal and most electronegative element. So it occurs in environment in combination with other elements, except oxygen and noble gases, as a fluoride compounds. Human beings are exposed to fluoride through food (Latifah et al., 1989; Stannard et al., 1991; Dabek et al., 1995), drinking water (Zhao et al., 1996) and inhalation (ASTDR, 1993; Gritsan et al., 1995). The United States National Academy of Sciences recognized fluoride as an essential nutrient (National Research Council, 1980). Daily intake of fluoride at a dose of 1-3 mg prevents dental caries whereas its long-term exposure at higher doses (5-10 mg) cause deleterious effects on tooth enamel and bone (WHO, 1984; CCIS, 1994). National Cancer Institute Toxicological Program classified fluoride to be an equivocal carcinogen (Mauter et al., 1990). Various mainstream organizations documented the devastating toxic effects of fluorides (U.S. Department of Health and Human Services, 1993; Yiamouyiannis, 1993). Frequent absorption of the fluoride causes tooth decay (Neurath et al., 2005), damage of kidneys (Lantz et al., 1987), bones (Bezerra de Menezes et al., 1994), nerves (Shivajishankara et al., 2002) and muscles (Cieck et al., 2005). The adverse toxic effects of fluoride arise due to a) enzyme inhibition b) collagen break down c) gastric damage and d) disruption of the immune system (Ahmad et al., 2000). Along with the other toxic effects, fluoride treatment also induces oxidative stress causing significant depletion of the activities of the antioxidant enzymes and enhancement of lipid peroxidation (Shanthakumari et al.,...
Oxidative stress due to fluoride exposure is reduced by the simultaneous treatment of various antioxidants like vitamin E and vitamin C (Bendich et al., 1986; Susheela et al., 2002). In alternative medicine various plant extracts are used for the treatment of different toxin induced disorders in many organs like liver (Guo et al., 2003), kidney (Sharma et al., 1998) and heart (Shin et al., 2001) as these extracts possess various therapeutically important compounds. Medicinal uses of many plants like Silybum marianum (Campos et al., 1989; Muriel et al., 1992); Curcuma longa (Donatus et al., 1999); Phyllanthus niruri (Unander et al., 1995); Terminalia arjuna (Karthikeyan et al., 2003) Andrographis paniculata (Choudhuri et al., 1984); Cajanus indicus (Ghosh et al., 1973) and many others have been reported in the literature. Our recent studies suggest that the active constituents of the herbs, Phyllanthus niruri and Terminalia arjuna possessed antioxidant properties and protect liver and kidneys against toxin-induced oxidative stress (Sarkar et al., 2005; Chatterjee and Sil, 2006a; Chatterjee and Sil, 2006b; Bhattacharjee and Sil, 2006a; Bhattacharjee and Sil, 2006b; Bhattacharjee and Sil, 2006c; Manna et al., 2006; Bhattacharjee and Sil, 2007). The herb Cajanus indicus has been extremely used in folk and medicine in India and many other countries for the treatment of various hepatic disorders.

In our laboratory a protein has been isolated and purified to homogeneity from the leaves of this herb (Sarkar et al., 2006). The molecular mass of the protein has been determined as 43 kD by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration techniques. Following MALDI-TOF as well as LC-MS analyses, peptide fragments of the protein generated by trypsin cleavage were subjected to the determination of the amino acid sequence. Partial amino acid sequence of the hepatoprotective protein has been carried out from three peptide fragments. Among these sequences, one of the peptide fragment showed strong sequence homology with a tomato protein present in the NCBI non-redundant database. The third one, on the other hand, is a unique peptide as it did not show any sequence homology with any known protein in the database. Various biochemical as well as the histological experiments established that the protein possessed protective roles against various drug and toxin - (such as carbon tetrachloride, chloroform, thioacetamide and acetaminophen) induced hepatic disorders in vivo and cytoprotective effects in hepatocytes (Sarkar et al., 2005; Sarkar and Sil 2006a; Ghosh et al., 2006; Ghosh and Sil 2006; Sarkar and Sil 2006b). The free radical scavenging activity of this protein was determined from its 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical quenching ability and data were compared to those obtained from a known free radical scavenger, vitamin C (Ghosh et al., 2006). Results of the experiment showed that the protein possess potent free radical scavenging activity in cell free system comparable to that of vitamin C. In the present study we aimed to find out the potential efficacy of that protein against fluoride induced toxicity in liver and kidney. For this purpose we take sodium fluoride (NaF) as the source of the fluoride ion donor. The protective role of this protein was evaluated by measuring a) the activity of the intracellular antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione-S-transferase (GST) b) the levels of the reactive oxygen species scavengers like reduced glutathione (GSH) and total thiol as well as c) the levels of the oxidized glutathione (GSSG) and lipid peroxidation end products. Hepato-renal protective effects of the protein were also compared with a known antioxidant vitamin E (α tocopherol) and a non-relevant protein, bovine serum albumin (BSA).

Materials and Methods

Plant. Cajanus indicus is a shrub belonging to the family leguminose and subfamily papilionacea. Fresh young leaves were collected from Bose Institute experimental farm.

Animals. Swiss albino mice (male, body weight 20 ± 2 g) were used for the experiments. The animals were acclimatized under standard laboratory conditions for a fortnight prior to the commencement of the treatment. They were provided with standard diet and water ad libitum. The animals were divided into several groups, each group having six mice.

Chemicals. Bovine serum albumin (BSA) and Bradford reagent were purchased from Sigma-Aldrich Chemical Company, (St. Louis) USA. Ammonium sulphate [(NH₄)₂SO₄], 1-chloro-2,4-dinitrobenzene (CDNB), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB, (Ellman's reagent)), disodium hydrogen phosphate (Na₂HPO₄), ethylene diamine tetraacetic acid (EDTA), glacial acetic acid, hydrogen peroxide (H₂O₂), N-ethylmaleimide (NEM), nicotinamide adenine dinucleotide reduced (NADH), nitro blue tetrazolium (NBT), oxidized glutathione (GSSG), phenazine methosulphate (PMT), potassium dihydrogen phosphate (KH₂PO₄), reduced glutathione (GSH), sodium dihydrogen phosphate (NaH₂PO₄), sodium fluoride (NaF), sodium pyrophosphate, trichloroacetic acid (TCA), thiobarbituric acid (TBA), tris buffer, vitamin E were bought from Sisco research laboratory, India.

Preparation of the homoheous protein for the experiments. Preparation of the homogeneous biologically active protein has been described in detail by Sarkar et al (Sarkar et al., 2006). Briefly, the leaves of Cajanus indicus were homogenized in 20 mM tris-HCl buffer, pH 7.4 and the supernatant was saturated with 60% (NH₄)₂SO₄. The pellet was reconstituted and dialysed in tris-HCl buffer, passed through DEAE Sephadex column and eluted in linear gradient of 0-1 M NaCl in tris buffer. The active fraction eluted at 0.2 M NaCl was concentrated and applied on a Sephadex G-50 column. The bioactive fraction obtained was subjected to a C₈ hydrophobic column for reverse phase column chromatography. The homogeneity of preparation was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

The protein concentration was measured according to the method of Bradford (Bradford, 1976) using crystalline BSA as standard.
Pre treatment with the protein. The pre-treatment group was divided into five sub-groups each consisting of six mice. The first group served as normal control. The second group served as toxin control, received drinking water containing 600 ppm NaF for 7 days. The third group of mice was pre-treated with the protein at an intraperitoneal dose of 2 mg/kg body weight for 7 days followed by drinking water containing 600 ppm sodium fluoride for another 7 days. The animals of all the study groups were anesthetized in ether, sacrificed and livers and kidneys were collected. The organs were extensively perfused in situ in isotonic salt solution (0.9% NaCl) to get rid of blood and used for the study. A well-known antioxidant agent vitamin E, (α tocopherol, administered orally at a dose of 200 mg/kg body weight) and a non-relevant protein, BSA (administered intraperitoneally, at a dose of 2 mg/kg body weight) were used as the positive and negative controls respectively for the study.

Determination of time-dependent curative effect of the protein. To determine the time needed for the protein to exhibit maximum curative activity against NaF induced hepatic and renal disorders, a time course study was conducted where six mice in each group were injected with the protein for 1, 2, 3, 4, 5 and 6 days after the administration of the toxin for 7 days. Mice were anesthetized in ether and sacrificed on 3rd, 4th, 5th, 6th and 7th day respectively. In another set, six mice in each group were treated with the toxin only for 7 days and sacrificed on 2nd, 3rd, 4th, 5th, 6th and 7th day in addition, six mice were kept as normal control. The organs were collected as described earlier. The study was carried out by measuring the activity of antioxidant enzyme, SOD in liver and kidney tissue homogenates of all the experimental mice.

Post-treatment with the protein. The post-treatment group was divided into four sub-groups each consisted of six mice. The first group served as normal control. The second group received drinking water containing 600 ppm NaF for 7 days and served as the toxin control. The third group received drinking water containing same dose of fluoride for 7 days followed by intraperitoneal injection with the protein at a dose of 2 mg/kg body weight for 4 days. The fourth group received fluoride containing drinking water for 7 days and then they are kept for another 4 days without any treatment to see whether there is any natural recovery or not. Mice were anesthetized in ether, sacrificed and livers and kidneys were collected. The organs were perfused in situ in isotonic salt solution (0.9% NaCl) as described earlier and used for the study.

Preparation of liver and kidney homogenates. About 200 mg of liver and kidney (cortex) tissues were homogenized separately in 10 volume of 100 mM KH2PO4 buffer containing 1 mM EDTA, pH 7.4 and centrifuged at 12,000 g for 30 minutes at 4°C. The supernatant was collected and used for following experiments. The protein concentration of the supernatant was measured according to the method of Bradford (Bradford, 1976) using crystalline BSA as standard.

Assay of antioxidant indices in the liver and kidney homogenates. Estimation of lipid peroxidation end products. The extent of lipid peroxidation in terms of thiobarbituric acid reactive substances (TBARS) formation was measured according to the method of Esterbauer and Cheeseman (Esterbauer and Cheeseman, 1990). As 99% of the TBARS is malondialdehyde (MDA), TBARS concentrations of the samples were calculated using the extinction co-efficient of MDA, which is 1.56 × 10⁻⁶ M⁻¹ cm⁻¹.

SOD assay. The activity of SOD was measured following the method originally developed by Nishikimi (Nishikimi et al., 1972) and then modified by Kakkar (Kakkar et al., 1984). One unit of SOD activity is defined as the enzyme concentration required for the inhibition of chromogen production by 50% in one minute under the assay conditions.

CAT assay. The CAT activity was measured by the method of Bonaventura (Bonaventura et al., 1972). One unit of CAT activity is defined as the amount of enzyme, which reduces 1 µmol of H2O2 per minute.

GST assay. GST catalyzes the conjugation reaction with glutathione in the first step of mercapturic acid synthesis. The activity of GST was measured by the method of Habig and Jakoby (Habig and Jakoby, 1974). One unit of GST activity is 1 µmol product formation per minute.

Assay of GR. GR activity was measured according to the method of Smith (Smith et al., 1988). The enzyme activity was calculated using molar extinction coefficient of 13,600 M⁻¹ cm⁻². One unit of enzyme activity is defined as the amount of enzyme, which catalyzes the oxidation of 1 µmol NADPH per minute.

GSH assay. GSH level was measured according to the method of Ellman (Ellman, 1959). A standard curve was drawn using different known concentrations of GSH solution. With the help of this standard curve, GSH contents of the homogenates were calculated.

GSSG assay. GSSG level was measured according to the method of Hissin and Hilf (Hisin and Hilf, 1976). The absorbance of the sample was measured at 412 nm.

Assay of total thiols. The total thiols (total sulfhydryl groups) content was measured according to the method of Sedlak and Lindsay (Sedlak and Lindsay, 1968) with some modifications. Absorbance was measured at 412 nm. The content of total thiols was calculated using molar extinction coefficient of 13,600 M⁻¹ cm⁻².

Statistical analysis. All the values are represented as mean ± S.D. (n = 6). Data on biochemical investigation were analyzed using analysis of variance (ANOVA) and the group means were compared by Duncan’s Multiple Range Test (DMRT). P values of 0.05 or less were considered significant.

Results

Preventive role of the protein against sodium fluoride induced toxicity

Effect on the products of lipid peroxidation. Figure 1 shows the levels of MDA in liver and kidney tissue homogenates
obtained from the normal control mice, NaF treated mice, and separately protein, vitamin E and BSA treated mice followed by NaF administration. MDA level in liver tissue homogenates of NaF treated mice (50.26 ± 1.08 nmol/g tissue) was found to be higher than that level compared to normal control mice (30.12 ± 0.81 nmol/g tissue). Pre treatment with the protein followed by toxin treatment decreased that level (31.66 ± 0.73 nmol/g tissue). In the kidney tissue homogenates the MDA level in NaF treated group (36.63 ± 0.97 nmol/g tissue) was higher than the normal control group (19.51 ± 0.42 nmol/g tissue). Treatment of the protein prior to NaF treatment decreased the MDA level (16.53 ± 0.49 nmol/g tissue).

**Effect on SOD activity.** The activities of SOD in the tissue homogenates of all experimental mice are shown in Fig. 2. In the liver homogenates, reduction of SOD activity was observed in NaF treated mice (72.81 ± 2.33 unit/mg protein) compared to the normal control (132.16 ± 3.37 unit/mg protein). SOD activity has been found to increase in the protein treated mice (181.57 ± 3.81 unit/mg protein) prior to NaF administration. In the kidney tissue homogenate the SOD activity in toxin treated mice (41.62 ± 1.37 unit/mg protein) was reduced compared to the normal control (82.21 ± 2.79 unit/mg protein). Mice receiving the protein prior to NaF showed an increase in SOD activity (77.22 ± 2.16 unit/mg protein) compared to NaF treated mice.

**Effect on CAT activity.** CAT activities in the liver and kidney homogenates of all experimental mice are shown in the Fig. 3. The CAT activity in the liver tissue homogenates of NaF treated mice (197.51 ± 4.34 µmol/min/mg protein) was much lower than that of normal control mice (343.14 ± 7.89 µmol/min/mg protein). In the protein-treated mice, CAT activity was significantly higher (440.76 ± 15.43 µmol/min/mg protein) compared to NaF treated mice. The CAT activity in the kidney homogenates of NaF treated mice (67.69 ± 1.95 µmol/min/mg protein) was lower than the normal control mice (211.54 ± 7.19 µmol/min/mg protein). The mice treated with the protein prior to NaF intoxication showed increased CAT activity (189.75 ± 5.88 µmol/min/mg protein), compared to NaF treated mice.

**Effect on GST activity.** GST activities as measured from the liver and kidney tissue homogenates of all the experimental mice have been shown in Fig. 4. In the liver homogenates the values show decreased activity of GST in NaF treated mice (5.77 ± 0.18 µmol/min/mg protein) compared to the normal control group (8.66 ± 0.21 µmol/min/mg protein). Protein treatment prior to NaF intoxication increased GST activity (8.89 ± 0.25 µmol/min/mg protein). In the kidney homogenates the normal mice showed a GST activity of 5.07 ± 0.14 µmol/min/mg protein. This was reduced to 1.25 ± 0.05 µmol/min/mg protein in NaF treated mice, while the activity increased to
3.84 ± 0.12 µmol/min/mg protein in mice treated with the protein prior to NaF treatment.

**Effect on GR activities.** GR activities in the liver and kidney homogenates of all experimental mice are shown in the Fig. 5. GR activity in the liver tissue homogenates of NaF treated mice (84.76 ± 2.15 nmol/min/mg protein) was lower than that of normal control mice (197.62 ± 5.14 nmol/min/mg protein). In the pretreated mice, GR activity was significantly higher (236.94 ± 7.46 nmol/min/mg protein) compared to NaF treated mice. GR activity in the kidney homogenates of NaF treated mice (156.03 ± 3.51 nmol/min/mg protein) was lower than the normal control group (409.21 ± 12.14 nmol/min/mg protein). Mice treated with the protein prior to NaF intoxication showed increased GR activity (439.34 ± 11.57 nmol/min/mg protein), compared to NaF treated mice.

**Effect on GSH levels.** Figure 6 shows the levels of GSH in liver and kidney homogenates of all experimental mice groups. In liver homogenate NaF treatment caused significant decrease in GSH level (6.49 ± 0.15 µg/mg protein) compared to the normal control group (8.71 ± 0.21 µg/mg protein). Pretreatment of the protein followed by NaF intoxication increased the level of GSH (8.76 ± 0.17 µg/mg protein) compared to toxin control group. In the kidney tissue homogenate the GSH level in NaF treated group (6.54 ± 0.17 µg/mg protein) was lower than the normal control group (8.48 ± 0.25 µg/mg protein). Treatment of the protein prior to the NaF treatment increased the GSH level (8.65 ± 0.22 µg/mg protein).

**Effect on GSSG levels.** Figure 7 shows the levels of GSSG in liver and kidney homogenates of all experimental mice groups. In liver homogenate NaF treatment caused significant enhancement of GSSG level (~163%) compared to the normal control group. Pretreatment of the protein followed by NaF intoxication decreased the level to normal control group. In the kidney tissue homogenate the GSSG level in NaF treated group was significantly higher (~94%) than the normal control group. Treatment of the protein prior to the NaF treatment decreased the GSSG level to normal level.

**Effect on the levels of total thiols.** Figure 8 shows the levels of total thiols in liver and kidney homogenates of all experimental mice groups. In liver homogenate NaF treatment decreased the level of total thiols (305.42 ± 7.22 nmol/mg protein) compared to the normal control group (623.45 ± 13.49 nmol/mg protein). Pretreatment of the protein followed by NaF intoxication increased the level of total thiols (362.45 ± 13.49 nmol/mg protein). Pretreatment of the protein followed by NaF intoxication increased the level of total thiols (560.93 ± 17.45 nmol/mg protein) compared to toxin control group.

3.84 ± 0.12 µmol/min/mg protein in mice treated with the protein prior to NaF treatment.
level of total thiols in kidney homogenates of NaF treated mice (177.94 ± 4.12 nmol/mg protein) was lower than that of normal control mice (274.31 ± 6.43 nmol/mg protein). For the pretreated group, level of total thiols was significantly higher (272.25 ± 5.48 nmol/mg protein).

In all the experiments Vitamin E treatment prior to NaF intoxication prevented the change in levels of the antioxidant indices studied in both the liver and the kidney homogenates, although BSA did not show any preventive activity.

Curative role of the protein against sodium fluoride induced toxicity

Time-dependent curative activity of the protein. Figure 9 (A and B) shows the time-dependent curative effects of the protein against NaF induced toxicity. Administration of the protein after NaF intoxication significantly attenuated the disorders caused by the toxin as evident from the increased activity of the antioxidant enzyme, SOD in both the liver and kidney tissue homogenates. The maximum curative effect on both the organs was observed when the protein was administered for 4 days after toxin treatment. Although liver recovered to some extent by its own with the help of self-recovery mechanisms after the oxidative stress, the recovery process was rapid when the mice were post-treated with the protein. For kidney, on the other hand, practically no natural recovery was observed. From these experiments we found that the protein exhibits optimum curative effect against NaF-induced hepatic and renal oxidative stress at the dose of 2 mg/kg body weight for 4 days post to this particular toxin administration. This dose and time were, therefore, used for all the subsequent post treatment experiments described in this paper.

Effect on the products of lipid peroxidation. The contents of MDA in the liver and kidney homogenates of normal control mice, NaF treated mice, mice treated with protein after NaF administration and mice kept for normal recovery after toxin treatment are shown in Fig. 10. MDA level in liver tissue homogenates of NaF treated mice (50.26 ± 1.08 nmol/g tissue) was higher than that compared to normal control mice (30.12 ± 0.81 nmol/g tissue). Post treatment with the protein decreased that level (29.49 ± 0.72 nmol/g tissue). In the kidney tissue homogenates the MDA level in NaF treated group (36.63 ± 0.97 nmol/g tissue) was higher than the normal control group (19.51 ± 0.42 nmol/g tissue). Treatment of the protein after NaF intoxication decreased the MDA level (15.01 ± 0.39 nmol/g tissue).
Effect on CAT activity. CAT activities in the liver and kidney homogenates of all experimental mice are shown in the Fig. 11. The CAT activity in the liver homogenates of NaF treated mice (197.51 ± 4.34 µmol/min/mg protein) was lower than that of normal control mice (343.14 ± 7.89 µmol/min/mg protein). In the post-treated mice CAT activity was significantly higher (338.02 ± 7.12 µmol/min/mg protein) compared to NaF treated mice. In the kidney homogenates of NaF treated mice (67.69 ± 1.95 µmol/min/mg protein) was lower than the normal control mice (211.54 ± 7.19 µmol/min/mg protein). The mice treated with the protein after NaF intoxication showed increased CAT activity (240.25 ± 5.17 µmol/min/mg protein), compared to NaF treated mice.

Effect on GST activities. GST activities as measured from the liver and kidney tissue homogenates of all the experimental mice have been shown in Fig. 12. In the liver homogenates the values show decreased activity of GST in NaF treated mice (5.77 ± 0.18 µmol/min/mg protein) compared to the normal control group (8.66 ± 0.21 µmol/min/mg protein). Protein treatment after NaF intoxication increased GST activity (9.09 ± 0.25 µmol/min/mg protein). In the kidney homogenates the normal control mice showed a GST activity of 5.07 ± 0.14 µmol/min/mg protein. This was reduced to 1.25 ± 0.05 µmol/min/mg protein in NaF treated mice, while the activity increased to 5.21 ± 0.11 µmol/min/mg protein in mice treated with the protein after NaF treatment.

Effect on GR activities. GR activities in the liver and kidney homogenates of all experimental mice are shown in the Fig. 13. GR activity in the liver tissue homogenates of NaF treated mice (84.76 ± 2.15 nmol/min/mg protein) was much lower than that of normal control mice (197.62 ± 5.14 nmol/min/mg protein). In the post-treated mice GR activity was significantly higher (160.81 ± 3.46 nmol/min/mg protein). GR activity in the kidney homogenates of NaF treated mice (156.03 ± 3.51 nmol/min/mg protein) was lower than the normal control mice (409.21 ± 12.14 nmol/min/mg protein). The mice treated with the protein after NaF intoxication showed increased GR activity (412.49 ± 12.46 nmol/min/mg protein), compared to NaF treated mice.

Effect on GSH levels. Figure 14 shows the levels of GSH in liver and kidney homogenates of all experimental mice groups. In liver homogenate NaF treatment caused significant depletion of GSH level (6.49 ± 0.15 µg/mg protein) compared to the normal control group (8.71 ± 0.21 µg/mg protein). Post treatment of the protein after NaF intoxication enhanced the level of GSH (8.81 ± 0.18 µg/mg protein) compared to toxin control group. In the kidney tissue homogenate the GSH level
A Protein from *C. indicus* Attenuates NaF-induced Hepato-renal Disorders

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in NaF treated group (6.54 ± 0.17 µg/mg protein) was lower than the normal control group (9.48 ± 0.25 µg/mg protein). Treatment of the protein after NaF intoxication increased the GSH level (10.02 ± 0.31 µg/mg protein).

**Effect on GSSG levels.** Figure 15 shows the levels of GSSG in liver and kidney homogenates of all experimental mice groups. Like pretreatment, post treatment of the protein after NaF intoxication decreased the NaF-induced GSSG level to that of the control group. Similarly, in the kidney tissue

Fig. 9. Time-dependent curative effect of the protein against NaF induced toxicity in the liver (A) and kidney (B). The study has been carried out to determine the effect only on SOD activity. Cont: normal mice, Recovery: NaF administered (600 ppm through drinking water) and sacrificed on 2nd, 3rd, 4th, 5th, 6th and 7th day, respectively. NaF+Protein: protein administered once daily i.p. at a dose of 2 mg/kg body weight after NaF intoxication for 1, 2, 3, 4, 5 and 6 days and sacrificed on 2nd, 3rd, 4th, 5th, 6th and 7th day, respectively. "a" indicates the significant difference between the normal control and normal recovery of toxin treated groups, "b" indicates the significant difference between the normal recovery of toxin treated groups and protein induced recovery of the toxin treated groups, (P<0.01, P<0.001).

Fig. 10. Curative effect of the protein on the TBARS formation against NaF induced oxidative damage in the livers and kidneys of the experimental mice. Left panel shows effects of the protein on liver and right panel shows that on the kidney against NaF induced MDA contents. For experimental detail, see the materials and methods. Cont: MDA content in normal mice, NaF: MDA content in only NaF treated mice, NaF + Protein: MDA level in protein post-treated mice, Recovery: MDA level in mice kept for normal recovery. Each column represents mean ± SD, n = 6; "a" indicates the significant difference between the normal control and toxin treated groups and "b" indicates the significant difference between the toxin treated and protein treated groups, (P<0.01, P<0.001).

Fig. 11. Curative effect of the protein on the CAT activity against NaF induced oxidative damage in the livers and kidneys of the experimental mice. Left panel shows effects of the protein on liver and right panel shows that on the kidney against NaF induced CAT activities. Cont: CAT activity in normal mice, NaF: CAT activity in only NaF treated mice, NaF + Protein: CAT activity in protein post-treated mice, Recovery: CAT activity in mice kept for normal recovery. Each column represents mean ± SD, n = 6; "a" indicates the significant difference between the normal control and toxin treated groups and "b" indicates the significant difference between the toxin treated and protein treated groups, (P<0.01, P<0.001).
**Fig. 12.** Curative effect of the protein on the GST activity against NaF induced oxidative damage in the livers and kidneys of the experimental mice. Left panel shows effects of the protein on liver and right panel shows that on the kidney against NaF induced GST activities. Cont: GST activity in normal mice; NaF: GST activity in only NaF treated mice; NaF + Protein: GST activity in protein post-treated mice; Recovery: GST activity in mice kept for normal recovery. Each column represents mean ± SD, n = 6; “a” indicates the significant difference between the normal control and toxin treated groups and “b” indicates the significant difference between the toxin treated and protein treated groups, (P<0.01, P<0.01).

**Fig. 13.** Curative effect of the protein on the GR activity against NaF induced oxidative damage in the livers and kidneys of the experimental mice. Left panel shows effects of the protein on liver and right panel shows that on the kidney against NaF induced GR activities. Cont: GR activity in normal mice; NaF: GR activity in only NaF treated mice; NaF + Protein: GR activity in protein post-treated mice; Recovery: GR activity in mice kept for normal recovery. Each column represents mean ± SD, n = 6; “a” indicates the significant difference between the normal control and toxin treated groups and “b” indicates the significant difference between the toxin treated and protein treated groups, (P<0.01, P<0.01).

**Fig. 14.** Curative effect of the protein on the GSH level against NaF induced oxidative damage in the livers and kidneys of the experimental mice. Left panel shows effects of the protein on liver and right panel shows that on the kidney against NaF induced GSH levels. Cont: GSH level in normal mice; NaF: GSH level in only NaF treated mice; NaF + Protein: GSH level in protein post-treated mice; Recovery: GSH level in mice kept for normal recovery. Each column represents mean ± SD, n = 6; “a” indicates the significant difference between the normal control and toxin treated groups and “b” indicates the significant difference between the toxin treated and protein treated groups. (P<0.01, P<0.01).

**Fig. 15.** Curative effect of the protein on the GSSG level against NaF induced oxidative damage in the livers and kidneys of the experimental mice. Left panel shows effects of the protein on liver and right panel shows that on the kidney against NaF induced GSSG levels. Cont: GSSG level in normal mice; NaF: GSSG level in only NaF treated mice; NaF + Protein: GSSG level in protein post-treated mice; Recovery: GSSG level in mice kept for normal recovery. Each column represents mean ± SD, n = 6; “a” indicates the significant difference between the normal control and toxin treated groups and “b” indicates the significant difference between the toxin treated and protein treated groups. (P<0.01, P<0.01).
Administration of the protein either prior or post to NaF treatment attenuated the hepatic and renal disorders as indicated by the levels of various parameters determined in this particular study.

The term reactive oxygen species (ROS) include superoxide anion radical (O$_2^-$), H$_2$O$_2$, and the highly reactive hydroxyl radical (OH) (Buechter, 1988). During cellular respiration ROS are collectively produced via the sequential biological reduction of the molecular oxygen (Scandalios, 2002). Thus, this endogenous production of ROS is an unavoidable consequence of aerobic respiration (Touyz, 2000; Wilcox, 2002). The exogenous sources by which ROS are generated include exposure to cigarette smoke, environmental pollutants, consumption of alcohol in excess, exposure to ionizing radiation etc (Gustafsson et al., 2000; Van Vleet et al., 2003; Novitskiy, et al., 2006; Guelman, et al., 2005). Oxidative stress has been defined as a disturbance in the pro-oxidant-antioxidant balance in favour of the former, leading to potential damage (Sies, 1991). Thus, oxidative stress is imposed on cells as a result of the two factors i) a reduction in antioxidant enzyme activity ii) an increase in the reactive species. Oxidative stress plays a crucial role to induce net disorders on the normal body functions and may result in different clinical conditions such as diabetes, cancer, nephritic syndromes, rheumatoid arthritis, malignant diseases, atherosclerosis, Parkinson's disease and ischemia-reperfusion injury (Tsutsui, 2006; Cotone et al., 2006; Gulsussen et al., 2006; Singh et al., 2006; Firuzi et al., 2006). ROS can also damage cell membrane (by reacting with lipids in cellular membranes), DNA, protein, tumor suppressor genes and enhanced expression of proto-oncogenes (Suji et al., 2006; Sicinska et al., 2006). A recent review by Chlubek (Chlubek, 2003) summarized the works of various groups on NaF induced oxidative stress. Although there are some discrepancies among the results of different groups, one point is clear, NaF can induce oxidative stress through the generation of ROS.

Antioxidants are the molecules that can bind and or inactivate ROS, provide protection against oxidative stress and prevent cellular damage. Human body contains various types of antioxidants, namely, the intracellular enzymes like SOD, CAT, GR and the endogenous molecules such as GSH, total sulphydryl groups etc. In addition to these molecules, vitamins (vitamin C, vitamin E), dietary compounds (bioflavonoids, pronthiocyanidins) and many herbal extract possess antioxidant capacity (Koyu et al., 2005; Shi et al., 2006; Hsu, 2006). SOD reduces the radical superoxide (O$_2^-$) to oxygen (O$_2$) and H$_2$O$_2$ (Fridovich, 1972), which is further reduced to water by the antioxidant enzymes CAT and glutathione peroxidase (GPx) along with the nonprotein thiol, GSH (Jones et al., 1968; De levé et al., 1991). The GSSG produced in the second step of the process is then reduced by another antioxidant enzyme, namely, GR (Ketterer, 1986). GR is essential for glutathione redox cycle that maintains adequate levels of cellular GSH. GSSG is reduced by a multi-step reaction in which GR (Carlberg and Mannervik, 1985) is initially reduced by NADPH.
forming a semiquinone of flavin adenin dinucleotide (FAD) a sulphur radical and a thiol. The reduced GR reacts with a molecule of GSSG resulting in a disulphide interchange which produces a molecule of GSH and GRred-SG complex. An electron rearrangement in GRred-SG complex results in a second disulphide interchange, splitting of second molecule of GSH and restoring GR to the oxidized form. In addition, GSH directly involves in the detoxification by the liver. It binds to toxins, such as heavy metals, solvents, and pesticides, and transforms them into a form that can be excreted in urine or bile. GSH is also an important antioxidant. The GSH/GST system is also a critical factor in protecting cells and organs against toxicity and disease. The thiol containing metabolites react with GSH and the enzyme GST catalyzes the binding of a large variety of electrophiles to the sulphydryl group of GSH, generally resulting in less harmful and more water soluble molecules (Hayes and Pullford, 1995).

In the present study, we observed that NaF treatment at a dose of 600 ppm for 7 days through drinking water caused a significant reduction in the activities of antioxidant enzymes like SOD, CAT, GST, and GR along with the depletion in the levels of GSH and total thiols in the liver and kidney tissue homogenates. NaF treatment at the same dose also caused the enhancement of the levels of lipid peroxidation and GSSG in these tissue homogenates. Treatment of the protein at a dose of 2 mg/kg body weight for 7 days prior to the NaF treatment normalized the activities of the antioxidant enzymes and the levels of the antioxidant molecules as well as prevent the increase in the levels of GSSG and lipid peroxidation in the hepatic and renal tissue homogenates. The increased activities of the antioxidant enzymes, SOD, CAT, GST, and GR as well as the increased levels of the non-protein thiol, GSH and total thiols in association with the decreased levels of lipid peroxidation and GSSG due to protein treatment suggests that the protein itself being a strong radical scavenger has an efficient protective mechanism in response to ROS. Post treatment with the protein for 4 days after NaF intoxication recovered the damaged liver tissues faster than the natural recovery as evidenced from the levels of various parameters determined in the study. For the kidney, similar results have been obtained after protein post treatment although no significant natural recovery has been detected for this particular organ. The exact mechanism by which the protein protects toxin-induced hepatic and renal damages is not clear yet, although it can be said that intraperitoneal administration of the protein may allow it to go through the blood stream and helps it to get direct contact with the cells of these organs. Determination of the exact pathways utilized by the protein in the molecular and cellular level for its action needs further studies.

A number of very recent reports (Tsoi et al., 2005; Oh et al., 2006; Lee et al., 2006) revealed that protein molecules from various plant sources possess antioxidant and hepatoprotective activities like the protein of our interest although these reports lack the knowledge of the mechanism of such actions. Based on the reports of Bruck et al., 2002; Lu et al., 1999; Muller et al., 1991; Schreck et al., 1992, it is known that antioxidative agents like N-acetylcyesteine, vitamin E, pyridoxine dithiocarbamates and heavy metal chelators can suppress the activation of NF-κB, a ubiquitous transcription factor and is activated in pathophysiological states like ROS generation. Since the protein of our interest reduces ROS generation, it is likely that it would also reduce the NF-κB activation during its hepatorenal protective action. Future studies on the effect of the protein on NF-κB activation will address this issue and the result would help to elucidate its mechanism of action related to hepato-renal protection.

Combining all, we would like to say that the protein obtained from the leaves of the herb Cajanus indicus L possesses preventive as well as curative role against NaF induced oxidative stress in liver and kidney. This protective and curative role of the protein seems to be due to its antioxidative property. Further works are needed to define the exact mechanisms by which the protein exhibits its biological activity.

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