Tribulus terrestris ameliorates aluminium chloride–induced alterations in oxidative status and functional markers in the liver, kidney, brain, and testis of the laboratory mouse

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The ameliorating potential of fruit extract of Tribulus terrestris L. (Tt) against aluminium chloride (AlCl3)-induced alterations in oxidative status and functional markers were studied in the liver, kidney, brain, and testis of the laboratory mouse. Adult male mice were divided into six groups of six each. Group I served as control while that of II - IV received various doses of AlCl3 (25, 50, 100 mg/kg body wt./day, respectively) orally, for 30 days. The mice of groups V and VI were administered with Tt (100 mg/kg body wt./day) only and AlCl3 (100 mg/kg body wt./day) along with Tt (100 mg/kg body wt./day) respectively, for the same duration. Metal estimation, MDA level, the activities of antioxidant enzymes and functional markers were studied in the liver, kidney, brain, and testis. Among three doses of AlCl3, only the high dose (100mg/kg body wt. for 30 days) caused significant increase in the accumulation of aluminium and MDA level, accompanied by significant decrease in the activities of antioxidant enzymes (SOD, CAT, GPx) in all these organs. AlCl3 at the same dose also caused a significant alteration in the functional markers of liver, kidney, brain and testis. These parameters were reinstated near to the control values following administration of AlCl3 (100mg/kg body wt./day) along with fruit extract of Tt (100mg/kg body wt./day) for the same duration. The results suggested that chelating property of Tt against aluminium accumulation might have resulted in the reinstatement of the lipid peroxidation, activities of antioxidant enzymes and the levels of the functional markers in the liver, kidney, brain, and testis.

Keywords: Aluminium Chloride, Antioxidant enzymes, Bindii, Functional markers, Lipid peroxidation, Metal accumulation, Puncture vine, Tackweed

In recent years impact of aluminium intoxication on human health has been increasingly alarming. People are frequently exposed to aluminium from various food additives, therapeutic treatments, and environment1. Continuous exposure of aluminium through different sources may cause its accumulation in several tissues such as kidney, liver, heart, blood, bones, brain and testis2-5 resulting in neurotoxicity6, hematotoxicity7,8, hepatotoxicity9, nephrotoxicity10, cardiotoxicity11 and reproductive toxicity12,13. Accumulation of aluminium in tissues has a significant toxic potential in human and animals which is attributed to its deleterious effects on lipid peroxidation and induction of the oxidative stress14 leading to various biochemical and physiological dysfunctions15.

Oxidative stress by free radicals generation can be prevented by the use of several antioxidants such as ascorbic acid, tocopherols, carotenoids and certain plants16. Several medicinal plants are considered as potential sources of antioxidants17 which can encounter the oxidative stress in the biological system. The medicinal plants have proved the existence of antioxidant properties against various experimental conditions. For example, hepatotoxicity caused by heavy metals (Hg, Pb, Cd), DMBA and iron is protected by Zingiber officinale18, Azadirachta indica19, and Clerodendrum colebrookianum20, respectively. Likewise, Withania somnifera21 protects the gamma radiation- induced nephrotoxicity while Bacopa monnieri22 and Argemone mexicana23 prevent the PBDE-209 and lithium-pilocarpine-induced neurotoxicity, respectively. Also, the antioxidant property of Allium cepa24 also protects the aluminium-induced testicular toxicity.

Tribulus terrestris, a flowering plant of family Zygophyllaceae, is native to warm temperate and tropical regions. It has long been used in the traditional systems of Chinese and Indian medicine for the treatment of various ailments. Tt is used as a tonic, aphrodisiac, astringent, analgesic, stomachic, antihypertensive, diuretic and urinary anti-septic25. The fruit extract of Tt contains various active constituents
such as saponin, glycoside, flavonoid, alkaloid, resin, tannin, sugar, steroid and essential oil\textsuperscript{26}. Among them, the flavonoid is one of the active constituents, bearing antioxidant properties\textsuperscript{27}. \textit{T. tilmannii} and its related species have been reported to exhibit antioxidative effects in various forms of experimentally-induced oxidative stress. The fruit extract of this plant prevents the cadmium-induced hepatic and renal toxicity\textsuperscript{28}, acetaminophen–induced hepatic toxicity\textsuperscript{29} and oxalate–induced renal toxicity\textsuperscript{30} in the rat and mercuric chloride induced nephrotoxicity\textsuperscript{31} in the mouse. It also protects the testicular injuries caused by cadmium\textsuperscript{32} and cypermethrin\textsuperscript{33} in rat and metronidazole\textsuperscript{34} in the mouse by providing its antioxidative effects.

Plants with metal chelating activity are also most useful for reduction in lipid peroxidation reaction and therefore play a vital role in the medicinal practice\textsuperscript{35}. Chelation of the metals is an effective method of treatment of the metal-induced toxicity as it enhances the mobilization and excretion of metallic cations\textsuperscript{36,37}. There are lots of natural chelators, essential for metal discharge with moderate side effects hence are the important tool for prevention of metal-storage diseases\textsuperscript{38, 39}. Extract of \textit{T. tilmannii} also possesses a chelation property which is helpful in reducing the metal load from the tissue\textsuperscript{12} because as like synthetic chelators, there is a possibility of \textit{T. tilmannii} also to bind with a metal ion and produce metal–chelator complex to remove the metals from the body\textsuperscript{38,39}. Thus in light of such properties of \textit{T. tilmannii}, the present study has been designed to investigate the efficacy of \textit{T. tilmannii} on AlCl\textsubscript{3}–induced alterations in the oxidative stress and functional markers in the liver, kidney, brain and testis of the laboratory mouse.

**Materials and Methods**

**Plant and Extract Preparation**

The fruits of \textit{T. tilmannii} were collected from local market of Varanasi and morphologically identified by Prof. NK Dubey, Department of Botany, BHU, Varanasi. Coarse powder of shade dried fruits of \textit{T. tilmannii} was extracted with 70\% ethanol (1:10) in a Soxhlet apparatus, and extract was evaporated in the water bath at 60°C until a consistent solid material was formed. This extract was stored at 4°C in air tight bag and dissolved in distilled water for animal treatment.

**Animals and treatments:**

Thirty-six adult male mice (25-35g) belonging to Swiss strain were obtained from Institute of Medical Sciences, BHU, Varanasi and maintained in the animal house under a controlled environment at 22°C. A 12 h light and 12 h dark cycle was ensured during which they were allowed to acclimatize under optimum feed and water access for 2 weeks before the commencement of the experiment. Approval from Institutional Ethical Committee was obtained vide F.Sc./IAEC/2014-15/0333. The animals were randomly divided into six groups of six each (n=6). Mice of group I were administered with distilled water that served as vehicle-treated control. Different doses of AlCl\textsubscript{3} (25, 50, 100 mg/kg body wt./day) were administered orally in the mice of groups II to IV for thirty consecutive days, respectively. Mice of groups V and VI were administered with \textit{T. tilmannii} (100 mg/kg body wt./day) alone and AlCl\textsubscript{3} (100 mg/kg body wt./day) along with \textit{T. tilmannii} (100 mg/kg body wt./day) for the same duration, respectively. Twenty-four hours after the last treatment, mice were humanely sacrificed. Liver, kidney, brain and testis were quickly dissected, rinsed in ice-cold saline to remove them from blood and stored in −80°C for conducting various studies.

**Blood serum collection:**

Blood samples were collected by cardiac puncture at the beginning of the experiment. Blood serum was obtained by centrifugation at 3500 rpm for 20 minutes and stored at −20°C until utilized for the study.

**Metal Estimation**

For metal estimation, the liver, kidney, brain and testis were weighed and then digested in an aqua regia, a mixture of HNO\textsubscript{3}: HCl (1:3), diluted with distilled water and read by inductively coupled plasma mass spectrometry (ICP-MS).

**Preparation of Tissue Homogenate**

10\% (w/v) homogenate of the various organs (liver, kidney, brain and testis) were prepared with the aid of 50mM phosphate buffer (pH- 7.0), as per requirement. The whole homogenate was first centrifuged at 10000 X g for 20 min at 4°C\textsuperscript{40}. The supernatant was collected and stored at −20°C for further investigation. The supernatant was used for the enzymes assay after estimating the protein content by the method of Lowry\textsuperscript{41} using bovine serum albumin as a standard.

**Estimation of oxidative stress markers:**

**Lipid peroxidation**

The concentration of malonaldehyde (MDA) was measured in the supernatant by the method of Okhawa\textsuperscript{42}.The reaction mixture was prepared by adding 200µL supernatant, 100 µL of 0.8% BHT
(Butylated Hydroxy Toluene), 200 µL SDS (Sodium dodecyl sulfate), 1.5 mL of 20% acetic acid, and 0.8% aqueous solution of TBA (Thiobarbituric acid). The mixture was then heated at 95°C in a water bath for 60 minutes, and absorbance was measured in a spectrophotometer at 532 nm against a blank containing all the reagents except the test sample. The values were expressed as nanomole MDA produced per mg protein.

**Superoxide dismutase**

The method of Marklund assayed superoxide dismutase (SOD)\(^4\). The assay mixture contained 2.4 mL of Tris–HCl (50mM), 1.0 mM EDTA (pH 7.6), 300 µL pyrogallol (0.2mM) and 100 µL enzyme source. The increase in absorbance was measured immediately in a spectrophotometer at 420 nm, against a blank containing all the components except the enzyme source and pyrogallol, at 10 sec interval for 3 min. The enzyme activity was expressed as unit per milligram protein.

**Catalase**

The method of Claiborne assayed catalase (CAT)\(^4\). The assay mixture contained 2.4 mL of phosphate buffer (50mM, pH 7.0), 10 µL of H\(_2\)O\(_2\) (19mM) and 50 µL enzymes source. The decrease in absorbance was measured immediately in a spectrophotometer at 240 nm, against a blank containing all the components except the enzyme source, at 10 sec interval for 3 minutes. The enzyme activity was expressed as micromoles H\(_2\)O\(_2\) consumed per minute per milligram protein.

**Glutathione peroxidase**

The method of Flohe assayed glutathione peroxidase (GPx)\(^4\). Enzyme assay was carried out by pipetting 750 µL of potassium phosphate buffer (0.1M, pH 7.0), 60 µL of NADPH (2.25 mM in 0.1% NaHCO\(_3\)), 15 µL of Glutathione reductase (7.1 µL/mL) and 25 µL of reduced glutathione (11.52mg/mL) in 1.0 mL cuvette. The enzymatic reaction was started by adding 50 µL of supernatant, and 100 µL of H\(_2\)O\(_2\) and the extinction of the sample was recorded at 340 nm every minute for a period of 120 sec. The glutathione peroxidase activity was expressed in units per mg of protein.

**Liver functional markers**

Liver function was assessed by determining the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) by IFCC method (ENZOPAK kit) and the level of albumin by BCG method (BEACON kit) in the blood serum.

**Kidney functional markers**

Kidney function was assessed by determining the level of creatinine using alkaline picrate method and of urea by modified Berthelot method in the blood serum, using reagent kit of ENZOPAK (Reckon Diagnostic Pvt. Ltd.).

**Brain functional markers**

The activities of lactate dehydrogenase (LDH) and creatine kinase (CK) were measured by IFCC method in the supernatant of brain tissue using infinite LDH kit (Accurex Biomedical Pvt. Ltd.) and ENZOPAK diagnostic kit, respectively.

**Testicular functional markers**

The activity of lactate dehydrogenase (LDH) was determined using infinite LDH kit (Accurex Biomedical Pvt. Ltd.) while p-NPP method was adopted to determine the activity of alkaline phosphatase (ALP) in the tissue supernatant by using ENZOPAK diagnostic kit.

**Statistical analysis:**

Data were presented as mean ± standard error of mean (SEM) of the six animals. They were analyzed statistically by one-way ANOVA followed by Newman-Keul’s test. Values were considered significant at \(P<0.05\).

**Results**

**Metal estimation**

Oral administration of AlCl\(_3\) (50 and 100 mg/kg body wt./day) led to a significant increase in the accumulation of aluminium in the liver and kidney as compared with the control, whereas in the brain and testis, significant increase of this metal was found only in high dose of AlCl\(_3\)(100 mg/kg body wt./day) treated mice. However, a significant decrease was noticed in the accumulation of aluminium in these vital organs and the testis of mice treated with AlCl\(_3\) (100 mg/kg body wt./day) along with \(Tt\) (100 mg/kg body wt./day) and hence attained the control values (Fig. 1).

**Antioxidant enzymes**

Administration of AlCl\(_3\) (100 mg/kg body wt./day) caused a significant increase in the level of MDA along with significant decrease in the activities of antioxidant enzymes such as SOD, CAT and GPx in the liver, kidney, brain and testis. The significant increase in the level of MDA along with significant decrease in the
activities of CAT were also found in the liver of 50 mg/kg body wt./day of AlCl₃- treated mice whereas the level of CAT and the activity of GPx was reduced significantly in the brain at 50 mg/kg body wt./day of AlCl₃-treated mice. No significant alterations were found in the activity of SOD in the liver, kidney, brain, and testis of low doses (25mg and 50 mg/kg body wt./day) of AlCl₃-treated mice. However, administration of AlCl₃ (100 mg/kg body wt./day) along with Tt (100 mg/kg body wt./day) resulted in significant restorations in the level of MDA and the activities of SOD, CAT and GPX in all the organs studied (Fig. 2-5).

Liver and Kidney function tests
Administration of AlCl₃ (100 mg/kg body wt./day) caused the significant increase in the levels of serum AST, ALT, creatinine and urea along with significant decrease in serum albumin as compared with the control. Significant increase in the levels of serum AST and creatinine were also found in 50 mg/kg body wt. AlCl₃-treated mice. However, significant restorations
were observed in the levels of these functional markers following administration of AlCl3 (100 mg/kg body wt./day) along with Tt (100 mg/kg body wt./day), thus attained the control values (Fig. 6 and 7).

**Brain function tests**

The significant increase in the activity of LDH was found in the brain of all the AlCl3-treated mice as compared with the control while the activity of CK showed significant increase only in 50 mg and 100 mg/kg body wt./day of AlCl3-treated mice as compared with the control. However, major restorations were found in the activities of LDH and CK in the mice administered with AlCl3 (100 mg/kg body wt./day) along with Tt (100 mg/kg body wt./day), as compared with AlCl3 (100 mg/kg body wt./day)-treated group alone, hence attained the values similar to that of the control (Fig. 8).

**Testicular function tests**

The activity of LDH in the testis exhibited a significant increase in the mice administered with AlCl3 at the doses of 50 and 100 mg/kg body wt./day as compared with the control whereas, a significant increase in the activity of ALP was found only in AlCl3-treated (100 mg) mice. However, in AlCl3 + Tt–treated (100 mg) mice, significant reductions in the activities of LDH and ALP were found as compared with AlCl3-treated (100 mg) mice, hence attained the values similar to that of the controls (Fig. 9).
Discussion

The present study was carried out to investigate the ameliorating potential of *Tt* on AlCl₃ – induced alterations in oxidative status and the functional markers in the liver, kidney, brain, and testis of the mice. Oral administration of AlCl₃ at the doses of 50 and 100 mg/kg body wt./day, for 30 days resulted in significant increase in aluminium concentration in the liver and kidney while only 100mg/kg body wt./day of AlCl₃ caused substantial increase in the same in the brain and testis. This supports the observation of Shrivastava et al. who reported the accumulation of aluminium in the liver, kidney, and brain of the rat and Guo et al. in the testis of the mouse. Reduction in aluminium accumulation in these organs following administration of AlCl₃ (100 mg/kg body wt./day) along with *Tt* (100 mg/kg body wt./day) suggests the metal chelating property of the extract of this plant. *Tt* binds with a metal ion and produces metal–chelator complex to remove the metals from the different organs. Metal chelating properties of this plant have also been earlier by DhanaLakshmi in the liver and kidney and Rajendran in the testis against Cd-induced toxicity in the rat.

In high dose (100 mg/kg body wt./day) of AlCl₃-treated mice, the level of MDA was significantly elevated whereas the activities of SOD, CAT, and GPx were significantly declined in the liver, kidney, brain and testis. Earlier authors have reported consistent findings, suggesting the condition of oxidative stress in the various organs following aluminium exposure. The decrease in enzyme activities could be the result of a reduced synthesis of the enzyme’s protein attributed to the higher intracellular concentration of aluminium, however, increased lipid peroxidation in the tissue is due to inhibition of SOD and CAT activities.

Restorations in the level of MDA and the activities of antioxidative enzymes following administration of AlCl₃ (100 mg/kg body wt./day) along with *Tt* (100 mg/kg body wt./day) suggest the antioxidant properties of flavonoids present in its fruit extract, thus reducing the oxidative stress in the various organs. Antioxidant properties of *Tt*, as noticed in the current study are also reported against oxidative stress, induced by mercury in the kidney, cadmium in the liver and kidney of rat and metronidazole in the testis of the mouse.

Significant alterations as noticed in the functional markers of the liver (ALT, AST, and albumin) and kidney (creatinine, urea) following administration of AlCl₃ is in agreement with the findings of previous authors. Tissue peroxidation induced by aluminium accumulation leads to leakage of these markers from the tissues into the blood stream, reflected by their significant alterations in the serum. Alterations in the functional markers in the blood serum in AlCl₃-treated mice are considered as a significant marker of liver and kidney dysfunctions.

Significantly increased functional markers of the brain, such as LDH and CK as noticed in the AlCl₃-treated mice, is consistent with the findings reported earlier in the rat that might be attributed to neuronal lipoperoxidative damage.

Likewise, the significant increase in the activities of testicular LDH and ALP as noticed following AlCl₃ administration is by the findings of earlier authors in rat and rabbit. The elevated LDH activity might be due to its leakage in the seminiferous tubular fluid from the germ cells which are damaged by the increased oxidative stress. The increase in the ALP activity may also be attributed to the cellular leakage caused by chemical-induced injury of the tissue.

In the present study, administration of *Tt* in AlCl₃ treated mice restored the functional markers significantly in the liver, kidney, brain and testis. *Tt*-induced amelioration in altered activities/levels of functional markers has also been observed in the liver and kidney of rat treated with cadmium and acetaminophen. Similar observations have also been reported in cadmium-treated rat and metronidazole-treated mouse. These results possibly arise due to the existence of flavonoids possessing antioxidant properties present in *Tt*. Restored functional markers indicate that *Tt* probably tends to stabilize the cell membrane, leading to prevent the damage and thus suppresses the leakage of enzymes through the cellular membrane.

The findings of the present study, therefore, indicate that aluminium accumulation-induced free radical generation results in significant alterations in oxidative status and the functional markers in the liver, kidney, brain, and testis that can be reinstated by administration of the fruit extract of *Tt* through scavenging the free radicals.

Conclusion

The findings suggest that fruit extract of *Tt* may emerge as an effective natural, an herbal source in correcting the hazardous health effects of aluminium.

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