

Full Length Research Paper

Protective effects of *Launaea procumbens* against oxidative adrenal molecular, hormonal and pathological changes in rats

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The aim of the study was to investigate the protective effects of *Launaea procumbens* methanolic extract (LM) against CCl₄-induced molecular, hormonal and pathological abnormalities in rats. Male Sprague Dawley rats were provided by National Institute of Health (NIH) Islamabad and orally fed with 100, 200 mg/kg body weight of LM after 48 h of CCl₄ treatment (3 ml/kg body weight, 30% in olive oil) biweekly for 4 weeks. The results showed that the administration of LM significantly improved the CCl₄-induced serum level of hormones, argyrophilic nucleolar organizer regions (AgNORs) and DNA damages. Histopathology showed that LM reduced the incidence of adrenal lesions induced by CCl₄ in rats. These results suggest that LM could protect adrenal against the CCl₄-induced oxidative damage in rats.

Key words: Carbon tetrachloride, *Launaea procumbens*, adrenal histopathology.

INTRODUCTION

The imbalance between the reactive oxygen species and the ability of biological system to detoxify these reactive intermediate or easily repair the resulting damage causes by them is called oxidative stress. All living organisms maintain a reducing environment within their cells by a system of antioxidant enzymes. This imbalance causes toxic effects through the rapid production of peroxides and free radicals that damage cell and macromolecules including proteins, lipids and nucleic acids. Carbon tetrachloride has molecular formula CCl₄, and its molecular weight is 153.8 g/mol, has been used as solvent in varnishes, resins and as starting material of many industrial organic compounds, and it is estimated that the average daily intake of CCl₄ for a general population is 0.1 µg (Abraham et al., 1999; ATSDR, 2003). Exposure to such toxic chemical through inhalation, ingestion or skin absorption is distributed throughout the body with

high concentration in liver, muscles, fat tissue brain, kidney and blood (Ogeturk et al., 2004), and damages various tissues especially liver (Khan and Ahmed, 2009).

Carbon tetrachloride induces reactive oxygen species (ROS) and oxidative DNA damages, with the formation of DNA adducts, genetic mutation, strand breakage and chromosomal alterations. DNA strand breaks are especially important in inducing mutations, such as deletions and translocations in affected cells undergoing replication with error-prone repair or without proper repair. Moreover, extensive DNA strand breaks without prompt repair may cause cell death and compensatory cell regeneration (Khan et al., 2010a, b).

Nuclear morphology can be evaluated histologically using a newly developed silver (argyrophilic) staining method for nucleolar organizer regions (NORs), the so-called AgNOR technique. NORs are composed of

chromosomal sites endowed with ribosomal DNA (rDNA) and complexes with a set of non-histone proteins characterized by high affinity for silver (Trere et al., 1996; Khan et al., 2010c) used for identification of normal cells from neoplastic cells (Cheah et al., 1996). Medicinal plant play crucial role in improving various pathogenesis (Sahreen et al., 2010; Khan et al., 2009; Khan et al., 2010c). *Launaea procumbens* is locally used in Pakistan in adrenal dysfunction. Therefore the present study was arranged to evaluate the protective function of *L. procumbens* versus carbon tetra chloride induced oxidative damages in rats.

MATERIALS AND METHODS

Plant collection and extraction

L. procumbens at maturity was collected from Wah Cantt District Rawalpindi (Pakistan), identified and its ariel parts (leaves, stem, flowers and seeds) were shade dried at room temperature, grinded mechanically and extracted with methanol to get crude methanolic extract. Methanolic extracts were stored at 4°C for *in vivo* screening.

Animals

Six week old, 30 Sprague Dawley male rats (190 to 200 g) were provided by National Institute of Health Islamabad and were kept in ordinary cages at room temperature of $25 \pm 3^\circ\text{C}$ with a 12 h dark/light cycle. They were allowed to standard laboratory feed and water. The study protocol was approved by Ethical Committee of Quaid-i-Azam University Islamabad for laboratory animal feed and care.

Experimental design

To study the protective effects of LM, rats were equally divided into 5 groups (6 rats). Group 1 received only raw water and free access to food materials. Group 2 received olive oil intraperitoneally (Monday and Thursday) and dimethyl sulphoxide (DMSO) intragastric (Wednesday and Saturday) at a dose of 3 ml/kg body weight. Group 3 received CCl_4 3 ml/kg (30% in olive oil) intraperitoneally (Monday and Thursday). Group 4 and 5 received 100, 200 mg/kg body weight of LM after 48 h of CCl_4 (Wednesday and Saturday), respectively. Experimental period was of four weeks. After 24 h of the last treatment, all the animals were weighted, sacrificed; with their blood collected, weighted and perfuse adrenal in ice-cold saline solution. Half of adrenal tissues were treated with liquid nitrogen for further enzymatic and DNA damage analysis while the other portion was processed for histology.

Assessment of serum markers

Serum hormonal analysis of adrenal gland was carried through kits.

Histopathological studies

For microscopic evaluation adrenal glands were fixed in a fixative (absolute ethanol 60%, formaldehyde 30%, glacial acetic acid 10%) and embedded in paraffin, sectioned at 4 μm and subsequently

stained with hematoxylin/eosin. Sections were studied under light microscope (DIALUX 20 EB) at 40 and 100 magnifications. Slides of all the treated groups were studied and photographed.

DNA fragmentations

DNA fragmentation (%) assay was conducted using the procedure of Wu et al. (2005) with some modifications. The adrenal tissue was homogenized in TE solution pH 8.0, centrifuged and separates the intact chromatin (pellet, B) from the fragmented DNA (supernatant, T). The pellet and supernatant fractions were assayed for DNA content using a freshly prepared DPA (Diphenylamine) solution for reaction. Optical density was read at 620 nm.

AgNORS analysis

Silver staining technique was used according to Trere et al. (1996) with some modifications. The cells were examined under light microscope at 100 \times magnification and number of NORs was counted per cell.

DNA ladder assay

DNA was isolated by using the methods of Wu et al. (2005) to estimate DNA damages. 5 μg of rats DNA was loaded in 1.5% agarose gel containing 1.0 $\mu\text{g}/\text{ml}$ ethidium bromide including DNA standards (0.5 μg per well).

Statistical analysis

Data were expressed as mean and standard error (SE) and analysis of variance (ANOVA) test was used to analyze the difference among various treatments, with least significance difference (LSD) at 0.05 and 0.01 as a level of significance. SPSS ver. 14.0 (Chicago, IL, USA) and Microsoft Excel 2007 (Roselle, IL, USA) were used for the statistical and graphical evaluations.

RESULTS

Effect of *L. procumbens* on serum level of adrenalin, nor adrenalin and cortisol in rat

The protective effects of *L. procumbens* against CCl_4 intoxication on serum level of adrenalin, nor adrenalin and cortisol in rat are shown in Table 1. Administration of CCl_4 significantly ($P < 0.01$) elevated the serum level of adrenalin, nor adrenalin and cortisol as compared to the control group. Serum level of adrenalin, nor adrenalin and cortisol was reversed towards the control group in a dose dependent manner by the treatment of methanolic fraction of *L. procumbens*.

Effect of AgNORs count and DNA fragmentation

Changes in the effect of *L. procumbens* against the CCl_4 on AgNORs count and DNA fragmentation in adrenal gland of rat are shown in Table 2. CCl_4 treatment significantly ($P < 0.01$) AgNORs count and DNA damages

Table 1. Effect *L. procumbens* on serum level of adrenalin, nor adrenalin and cortisol in rat.

Treatment	Adrenalin (mg/dl)	Nor adrenalin (mg/dl)	Cortisol (mg/dl)
Control	35±3.8 ⁺⁺	17.8±0.45 ⁺⁺	63.±3.65 ⁺⁺
Olive oil+DMSO	36±2.93 ⁺⁺	18.86±0.50 ⁺⁺	64±3.23 ⁺⁺
3 ml/kg CCl ₄	61±3.26 ^{**}	27.3±0.58 ^{**}	103±2.12 ^{**}
100 mg/kg LM+CCl ₄	48±3.1 ^{***}	21±0.41 ^{***}	81±3.12 ^{***}
200 mg/kg LM+CCl ₄	36±2.81 ⁺⁺	18±0.18 ⁺⁺	66±5.25 ⁺⁺

Mean ±SE (n=6 number). *, ** indicate significance from the control group at P<0.05 and P<0.01 probability level, respectively. +, ++ indicate significance from the CCl₄ group at P<0.05 and P<0.01 probability level, respectively.

Table 2. Effect of various fractions of *L. procumbens* on adrenal AgNORs count and DNA damages.

Treatment	AgNORs (NORs/cell)	% DNA fragmentation
Control	1.4950±0.0448 ⁺⁺	46.67±1.28 ⁺⁺
Olive oil+DMSO	1.6017±0.0744 ⁺⁺	47.67±1.09 ⁺⁺
3 ml/kg CCl ₄	6.595±0.367 ^{**}	67.333±0.803 ^{**}
100 mg/kg LM+CCl ₄	3.835±0.159 ^{***}	57.500±0.885 ^{***}
200 mg/kg LM+CCl ₄	2.102±0.215 ^{***}	48.17±1.30 ⁺⁺

Mean ±SE (n=6 number). *, ** indicate significance from the control group at P<0.05 and P<0.01 probability level, respectively. +, ++ indicate significance from the CCl₄ group at P<0.05 and P<0.01 probability level, respectively.

as compared to the control group. Treatment of *L. procumbens* significantly ($P < 0.01$) ameliorated the CCl₄ intoxication and reduced the number of NORs per cell and DNA fragmentation in a dose dependent manner

Effect of *L. procumbens* on DNA damages

Protective effects of different fractions of *L. procumbens* versus CCl₄ induced DNA damages in the adrenal tissues of rats are shown by DNA ladder assay in Figure 1. Extensive DNA breakages in adrenal gland were depicted by the treatment of CCl₄ to rats. Post-administration of *L. procumbens* reduced the DNA damages dose dependently as shown by DNA bands of different groups as compared to CCl₄ group.

Effect of different fractions of *L. procumbens* on histopathology of adrenal glands in rat

The microscopic evaluation of adrenal gland sections in control rats showed normal architecture having uniform basophilic nuclei and lack pleiomorphism. CCl₄ treatment caused necrosis of adrenal cortex, degradation of modularly cells, accumulation of fatty droplets, damage of structural proteins and the breakage of nuclear membrane. Adrenal medulla showed hypertrophy, hyperplasia

and dilation of blood vessels with CCl₄ treatment. Post-administration of *L. procumbens* in the CCl₄ intoxication and reversed necrosis cortex started to attain the normal shape and size and the amount of lipid droplets were also decreased. The nuclear membrane started to repair, medulla size was normal and blood vessels were less dilated than CCl₄ group as shown in Table 3 and Figure 2.

DISCUSSION

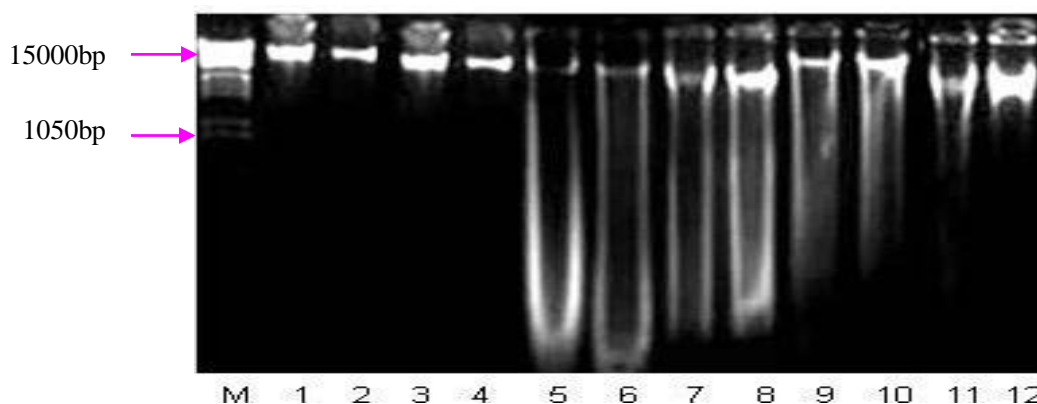
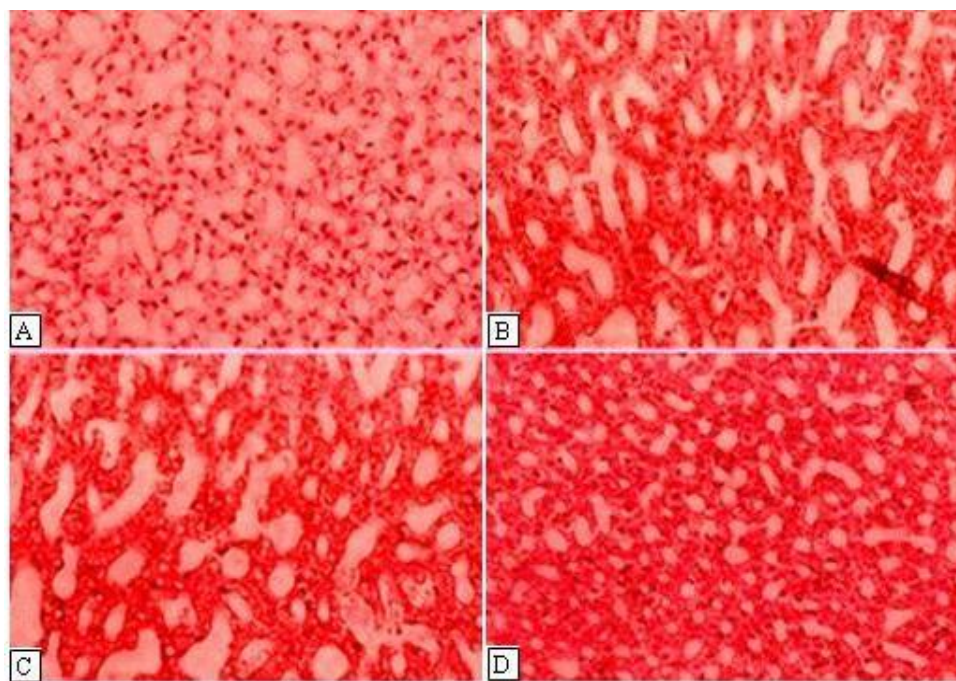
Our results show that CCl₄ treatment causes significant increase in the secretion of epinephrine, nor epinephrine and cortisol. These changes are markedly restored by treatment of plant extracts. Stern and Brody (1963) reported that the oral administration of 2.5 ml/kg CCl₄ in peanut oil to rats elevated free epinephrine and nor epinephrine levels in plasma and urine, which supports our results. Similarly, results of Rubinstein (1962) are in accordance with our investigations, and reported that intraduodenal administration of carbon tetrachloride to rats for 2 h caused increase in serum epinephrine level.

According to the Marnett (2000), the product of lipid peroxidation, MDA react with DNA to form the adduct M1G, the mutagenic pirimidinepurine adduct of deoxyguanosine. According to Shimoda et al. (1994), it is very important to identify risk factors for genomic instability

Table 3. Effect of *L. procumbens* on histopathology of adrenal glands in rat.

Treatment	Adrenal cortex necrosis	Fatty changes	Accumulation of cells	Blood vessels dilation
Control	-	-	-	-
Olive oil+DMSO	-	-	-	-
3 ml/kg CCl ₄	+++	+++	++	++
100 mg/kg LM+CCl ₄	-/+	-/+	-/+	-/+
200 mg/kg LM+CCl ₄	-	-	-/+	-

-, normal; -/+, mild; ++, medium; +++, severely damaged.

**Figure 1.** Agarose gel showing DNA damage by CCl₄ and preventive effect of *Launaea procumbens* extracts in different groups. Lanes (from left) DNA marker (M), Control (1- 4), CCl₄ (5, 8), 100 mg/kg LM (9, 10) 200 mg/kg LM (11, 12).**Figure 2.** Histopathological changes caused by CCl₄ and preventive effect of *Launaea procumbens* extracts in different groups. Slides (from left) Control (A), CCl₄ (B, C), 200 mg/kg LM (D).

which is responsible for the occurrence of genetic alterations for carcinogenesis. The data of the present study revealed that the treatment of CCl₄ causes significant oxidative DNA damage in adrenal gland which are visualized on agarose gel by staining with ethidium bromide. Treatment with *L. procumbens* plant extracts significantly reduces these damages. Similar investigation was reported by Khan et al. (2009) during study of protective effects against carbon tetrachloride induced toxicity in rats.

The microscopic evaluation of adrenal glands showed that CCl₄ treatment caused necrosis of adrenal cortex, degradation of cells, accumulation of fatty droplets, modularly hypertrophy, hyperplasia and blood vessels dilation. Similar histopathological changes were reported that carbon tetrachloride causes necrosis to the adrenal cortex after initiation of lipid peroxidation, which requires a CYP-catalysed bioactivation (Rosol et al., 2001).

Conclusion

From data it was inferred that protective effects are due to the presence of bioactive compounds in the extract, which might be responsible in modulating the effects of CCl₄-induced toxicity and concomitantly near to normal rats as *L. procumbens* treated groups.

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