PREVENTION OF CCL_4-INDUCED OXIDATIVE DAMAGE IN ADRENAL GLAND BY *DIGERA MURICATA* EXTRACT IN RAT

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ABSTRACT

Digera muricata (L.) Mart. is a weed and commonly found in waste places, road sides and in maize fields during the summer season. It possesses antioxidant capacity and is locally used for various disorders such as inflammation, urination, as refrigerant, aperient and in sexual anomalies.

In this study antioxidant potential of *Digera muricata* methanol extract (DMME) and n-hexane extract (DMHE) was evaluated against CCl_4-induced oxidative stress in adrenal gland of Sprague-Dawley male rats. 42 rats were equally divided into 7 groups of 6 rats in each. Group I remained untreated, while Group II treated with vehicles. Group III received only CCl_4 (1 ml/kg b.w., 10% in olive oil) once a week for 16 weeks. Group IV and VI received DMME and DMHE at a dose of 200 mg/kg b.w. along with CCl_4. Animals of Group V and VII administered with DMME and DMHE alone at a dose of 200 mg/kg b.w. once a week for 16 weeks.

Lipid peroxidation significantly increased while activities of antioxidant enzymes (CAT, SOD, GST, GSR and GSH-Px) were reduced in adrenal gland samples by the administration of CCl_4. Glutathione (GSH) concentration was significantly decreased whereas DNA fragmentation% and AgNORs count was increased in adrenal gland by CCl_4 administration. Treatment of rat by both the extracts (DMME, DMHE) and CCl_4 increased the glutathione level and activities of antioxidant enzymes while reduced the lipid peroxidation, DNA fragmentation percent and AgNORs count in adrenal gland.

These results indicate that *Digera muricata* extract is able to ameliorate oxidative stress in adrenal gland induced by CCl_4 in rat.

Keywords: *Digera muricata*, carbon tetrachloride, adrenal gland, antioxidant enzymes.

INTRODUCTION

Carbon tetrachloride (CCl_4) is known to induce damage in liver, lungs, kidneys, adrenals and central nervous system in humans and experimental animals (Rechnagel *et al*., 1989). The initial step in the tissue injury induced by carbon tetrachloride is its cytochrome P450-mediated formation of trichloromethyl radical ($\bullet CCl_3$) and trichloromethyl peroxyl ($\bullet CCl_3OO.$) free radical (Slater, 1984; Halliwell and Gutteridge, 2007). With respect to its molecular characteristics CCl_4 does induce oxidative stress with the production of free peroxy radicals and lipoperoxides thereby damaging proteins, DNA and lipids.

The adrenal gland is exquisitely sensitive to toxic assault. It has been reported that the most frequently observed site of endocrine lesion is the adrenal gland (Ribelin, 1984; Harvey, 1999). There are two features of the adrenal gland which make it vulnerable to toxic assault (Hinson and Raven, 1999). It is a discrete gland and its high vascularity, facilitates the delivery of toxins and metabolic substrates as well as the efficient removal of steroid products (Vinson and Hinson, 1992). The adrenal has a high capacity for lipid peroxidation, which is implicated in the toxic effects of carbon tetrachloride on this tissue (Brogan *et al*., 1984).

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Endogenous antioxidants such as polyphenolic compounds, ascorbic acid and monosaccharides in medicinal plants may constitute antioxidative defense by scavenging free radicals possibly increase the longevity of biological systems (Khan and Ahmed, 2009). Chemical characterization of *Digera muricata* indicated the presence of flavonoids, tannins, alkaloids, saponins, phenols, and terpenes (Mathad and Meti, 2010). Rutin and hyperoside flavonoids have been identified in this plant. Antioxidant properties of *Digera muricata* methanol extract against the CCl_4-induced toxicity in kidneys and testis had been well documented (Khan *et al*., 2009; Khan and Ahmed, 2009). Alterations induced with CCl_4 in kidneys were suppressed with *Digera muricata*, as were evident by the higher activities of antioxidant enzymes while lower concentration of lipid peroxides. It also inhibited the genotoxicity and suppressed the activity of telomerase enzyme induced with CCl_4 in kidneys of rat (Khan *et al*., 2009). Similarly, its protective effects against the CCl_4 induced liver and testiculat toxicity have been characterized (Khan and Ahmed, 2009). *Digera muricata* restored the disruptions induced with CCl_4 for various male hormones in rat (Khan and Ahmed, 2009). This plant is used as an alternative in secondary infertility (Chettleborough *et al*., 2000). In Pakistan, *Digera muricata* is used as an alternative in the treatment of renal disorders (Anjaria *et al*., 2002; Khan *et al*., 2009), aperient, and refrigerant (Hocking, 1962) and in sexual

disorders (Khan and Ahmed, 2009). Antimicrobial activity of *Digera muricata* had been well documented (Mathad and Meti, 2010). This study was carried out to evaluate the protective effects of *Digera muricata* (L.) Mart. methanol and n-hexane extracts against CCl_4 induced adrenal toxicity. The main objectives were to determine the changes in antioxidant enzymes, lipid peroxidation, antioxidant enzymes and glutathione, argyrophilic nucleolar organizer regions (AgNORs) changes, and to determine the effects on genomic DNA of adrenal gland of rat.

MATERIALS AND METHODS

Preparation of plant extract

Digera muricata (L.) Mart. locally named as "Tandla" at maturity were collected from the campus of Quaid-i-Azam University Islamabad during July 2006. After identification a voucher specimen (125127) was deposited in the Herbarium of Pakistan at Quaid-i-Azam University Islamabad, Pakistan.

Aerial parts were shade dried for two weeks and powdered in a Willy Mill to 60-mesh size. Briefly, 750 g powder was extracted separately with 5 litres of methanol and n-hexane at 25 °C for a week. After extraction the mixture was filtered, methanol and n-hexane solution was evaporated in a rotary evaporator (Panchun Scientific Co., Kaohsiung, Taiwan) at 40 °C and stored at 4 ºC. Methanol extraction yielded 19 g while n-hexane extraction gave 7 g of extract.

Animals and treatment

Forty two healthy Sprague-Dawley male rats, weighing 250±10g, were provided by the Animal House of National Institute of Health (NIH) Islamabad and were maintained at the Primate Facility at Quaid-i-Azam University, Islamabad. Food and fresh water was available to the rats. Animals were equally divided into seven groups with 6 animals in each. Group I was control, group II was treated with olive oil (1 ml/kg b.w., i.p.) followed by DMSO (1 ml/kg b.w., intragastric) once a week for 16 weeks. Group III was injected intraperitoneally once a week for 16 weeks with CCl_4 (1 ml/kg b.w., 10% in olve oil). Group IV and V were treated both with CCl_4 and the extracts (DMME; DMHE; 200 mg/kg b.w., intragastric). Group VI and VII were treated with the extracts (DMME; DMHE 200 mg/kg b.w., intragastric) only. Both the extracts were administered once a week for 16 weeks.

At the end of 16 weeks, 24 h of the last treatment, all the animals were anesthesized in an ether chamber. Blood was collected by cardiac puncture and serum obtained by blood centrifugation at 1500 × g for 10 min, at 4 °C. The adrenal gland was removed after perfusion with ice cold saline at 4°C. Half of the adrenal gland was stored at -70°C to study the DNA damages, and biochemical parameters while the other portion was processed for the AgNORs count.

Assessment of antioxidant enzymes

Adrenal gland samples were centrifuged at 12,000 × g for 30 min at 4°C after homogenizing in 10 volume of phosphate buffer; 100 mM KH_2PO_4, 1 mM EDTA (pH 7.4). The supernatant obtained was used for the estimation of activity level of different enzymes and the endogenous glutathione and lipid peroxidation concentration.

To estimate the catalase (CAT) activity in adrenal gland hydrogen peroxide as substrate was used according to the method of Chance and Maehly (1955). Activity level of superoxide dismutase (SOD) was determined according to Kakkar *et al*. (1984) method. Glutathione-S-transferase (GST) activity was estimated according to Habig *et al*. (1974) procedure. Assay of glutathione reductase (GSR) activity was carried out according to Carlberg and Mannervik (1975). However, glutathione peroxidase (GSH-Px) activity level was estimated by using the method of Mohandas *et al*. (1984).

Reduced glutathione (GSH) and lipid peroxidation assay

Endogenous level of reduced glutathione (GSH) was find out by following Jollow *et al*. (1974) method whereas method of Iqbal *et al*. (1996) was used to determine the lipid peroxidation by measuring malondialdehyde (MDA) contents in adrenal gland.

DNA fragmentation percent assay

In adrenal gland samples DNA fragmentation was determined according to Wu *et al*. (2005) method. Briefly, adrenal gland (50 mg) was homogenized in TE buffer (pH 8; 5 mM Tris HCl and 8 mM EDTA) and 0.2% triton X-100 and centrifuged at 27,000 × *g* for 20 min. Intact chromatin was obtained as pellet B while fragmented DNA was obtained as supernatant T. Both B and T were used to estimate the DNA by using diphenylamine solution and optical density was measured at 620 nm. The results were expressed as amount of % fragmented DNA by the following formula:

$$\% \text{ fragmented DNA} = T \times 100/(T+B)$$

Argyrophilic Nucleolar Organizer's Region (AgNORs) count

Nucleoli were silver stained according to the technique of Trere *et al*. (1996). Fixed slides were dewaxed in xylene and after hydration in descending order of ethanol concentration (90, 70 and 50%) were washed for 10 min in distilled water and dried. Slides were incubated at 35ºC for about 8-12 min after addition of one drop of colloidal solution (2% gelatin; 1% formic acid) and two drops of 50% $AgNO_3$ solution. Reaction was stopped by washing in distilled water and 1% sodium thiosulphate (1 min) to get golden colored nuclei and brown/black NORs. Slides were examined under microscope to count the number of AgNORs per cell.

STATISTICAL ANALYSIS

Mean and standard deviation (SD) of the data was determined. One way analysis of variance was applied and *post hoc* comparison in terms of least significance difference (LSD) at 0.05, 0.01 and 0.001 was used to analyze the difference among various treatments by using the Microsoft SPSS Ver. 14.0.

RESULTS

Effect of DMME and DMHE on antioxidant enzymes

Activity levels of CAT, SOD, GSH-Px, GST and GSR were significantly ($p < 0.001$) lowered in adrenal gland samples of CCl_4 treated group compared with control group (table 1). Administration of DMME and DMHE to rats along with CCl_4 ameliorated the oxidative stress induced with CCl_4 and increased the activities of antioxidant enzymes; CAT, SOD, GSH-Px, GST and GSR in adrenal gland samples compared with the group given CCl_4 only. DMME and DMHE administration alone did not significantly change the activity levels of CAT, SOD, GSH-Px, GST and GSR in adrenal gland samples when compared with controls.

Effect of DMME and DMHE on glutathione, lipid peroxidation, AgNORs count and DNA fragmentation

Carbon tetrachloride treatment to rats significantly ($p<0.001$) increased the MDA level an indicator of lipid peroxidation whereas the contents of GSH, an endogenous antioxidant were significantly ($p<0.001$) decreased as compared to the control group (table 2). Equally, DNA fragmentation% and AgNORs count were enhanced with CCl_4 treatment against the controls. The level of MDA, AgNORs count and DNA fragmentation% was significantly lowered in the groups administered both with CCl_4 and DMME, DMHE (200 mg/kg b.w.) whereas significantly increased the GSH levels ($p<0.001$) in adrenal gland samples compared to the group given CCl_4 only. DMME and DMHE administration alone did not statistically change the MDA, GSH, DNA fragmentation % and AgNORs levels in the adrenal gland samples when compared to the controls.

DISCUSSION

Carbon tetrachloride induced alterations in antioxidant enzymes and lipid peroxidation in Sprague-Dawley rats of this study appears to suggest that CCl_4 is able to cause oxidative stress in adrenal gland of these animals. Antioxidant enzymes such as CAT, SOD, GSH-Px, GST and GSR are the endogenous defenses; actively involved in scavenging of free radicals to maintain the steady state level and consequently integrity and functionality of cells. Superoxide dismutases are specific for catalytic removal of superoxides by converting them into H_2O_2 (Halliwell and Gutteridge, 2007). Hydrogen peroxide is catalytically dismutated by catalase into ground-state oxygen and water (Reiter *et al*., 2000). GSH is a predominant endogenous antioxidant and used as a cofactor to remove

Table 1: Effect of DMME and DMHE on antioxidant enzymes in adrenal gland of rat

Treatment	CAT U/min	SOD U/mg protein	GSH-Px (nM/mg protein)	GST(nM/min /mg protein)	GSR(nM/min /mg protein)
Control	6.80±0.3 [a]	1.63±0.18 [a]	15.5±0.91 [a]	1.35±0.10 [a]	43.8±1.7 [a]
Olive oil+DMSO	6.85±0.4 [a]	1.60±0.16 [a]	16.2±0.72 [a]	1.45±0.16 [a]	44.2±1.4 [a]
CCl_4 (1 ml/kg b.w.)	4.11±0.4 [B]	0.55±0.08 [B]	10.6±0.99 [B]	0.77±0.08 [B]	26.6±2.0 [B]
CCl_4+DMME (200 mg/kg b.w.)	4.55±0.4 [B a]	1.17±0.13 [B a]	14.6±0.60 [a]	0.90±0.05 [B]	36.7±1.0 [B a]
DMME (200 mg/kg b.w.)	6.85±0.4 [a]	1.70±0.08 [a]	15.4±1.01 [a]	1.41±0.14 [a]	44.0±1.9 [a]
CCl_4 +DMHE (200 mg/kg b.w.)	5.50±0.5 [B a]	0.90±0.12 [B a]	14.2±0.82 [A a]	0.88±0.05 [B]	33.2±2.1 [B a]
DMHE (200 mg/kg b.w.)	6.93±0.4 [a]	1.61±0.16 [a]	16.2±0.86 [a]	1.41±0.14 [a]	43.9±1.3 [a]

Table 2: Effect of DMME and DMHE on glutathione, lipid peroxidation, DNA fragmentation% and AgNORs count in adrenal gland of rat

Treatment	GSH (µM/mg tissue)	MDA (nM/min /mg protein)	DNA fragmentation %	AgNORs (number/cell)
Control	18.0±0.8 [a]	22.8±1.0 [a]	0.26±0.02 [a]	1.40±0.14 [a]
Olive oil+DMSO	18.2±0.7 [a]	22.4±1.1 [a]	0.25±0.01 [a]	1.41±0.11 [a]
CCl_4 (1 ml/kg b.w.)	09.9±0.7 [B]	33.8±1.4 [B]	0.38±0.04 [B]	8.45±0.72 [B]
CCl_4+DMME (200 mg/kg b.w.)	15.9±0.9 [B a]	27.5±0.8 [B a]	0.28±0.02 [a]	2.00±0.20 [A a]
DMME (200 mg/kg b.w.)	17.7±1.0 [a]	22.3±1.3 [a]	0.24±0.01 [a]	1.45±0.10 [a]
CCl_4 +DMHE (200 mg/kg b.w.)	15.8±1.1 [B a]	29.2±1.0 [B a]	0.28±0.01 [a]	1.96±0.19 [A a]
DMHE (200 mg/kg b.w.)	17.5±1.1 [a]	22.2±1.1 [a]	0.26±0.01 [a]	1.46±0.10 [a]

[A, B] indicate significance from control group at $p<0.01$ and $p<0.001$. [a]indicate significance from CCl_4 group at $p<0.001$

hydrogen peroxide and lipoperoxides by the GSH-Px family during which GSH is converted into oxidized form of glutathione (GSSG). Oxidized glutathione is converted back into GSH by another rate controlling enzyme the glutathione reductase (GSR) thereby maintain the intracellular GSH levels. This optimum level of GSH is an utmost criterion in maintaining the structural integrity and physiology of cell membranes. Another multifunctional antioxidant enzyme of the redox cycle; glutathione-S-transferase plays a key role as a thioltransferase-like redox regulator of hydrophobic compounds (Sheweita *et al*., 2001).

CCl_4-intoxication to rats in this experiment evidently induces decline in the activity of antioxidant enzymes (CAT, SOD, GSH-Px, GSR, GST) suggesting severe oxidative injuries to adrenal gland. Determination of activity level of these antioxidant enzymes is an appropriate indirect way to assess the pro-oxidant antioxidant status in tissues (Halliwell and Gutteridge, 2007; Priscilla and Prince, 2009).

GSH along with other endogenous antioxidants such as ascorbic acid and α-tocopherol plays a central role in eliminating free radicals and other reactive species. Antioxidant activities of GSH are mainly attributed to its side chain sulfhydryl (-SH) residue. The treatment of DMME and DMHE are able to restore the level of antioxidant enzymes such as CAT, SOD, GST, GSR and GSH-Px which are decreased in CCl_4 treated group. The protective effects of DMME and DMHE in maintaining the GSH level towards control have increased the capacity of endogenous antioxidant defense and increased the steady state of GSH and/ or its rate of synthesis that confers enhanced protection against oxidative stress (Khan and Ahmed, 2009; Khan *et al*., 2009).

Metabolites of CCl_4 such as trichloromethyl radical ($\bullet CCl_3$) and trichloromethylperoxyl ($\bullet CCl_3OO.$) are the major free radicals (Slater, 1984; Halliwell and Gutteridge, 2007) which cause oxidative stress and lipid peroxidation of polyunsaturated fatty acids. It has been hypothesized that our plant extracts afford protection by impairing CCl_4 mediated lipid peroxidation, through amelioration of oxidative stress, as evident from the alleviated malondialdehyde (MDA) level. The group treated with CCl_4 only is more vulnerable to oxidative injuries and thereby high lipid peroxidation, whereas the group received the co-administration of DMME and DMHE exhibited significant protection. These results indicated the antioxidant potential of DMME and DMHE against the reactive oxygen species generated by the metabolic conversion of CCl_4. Similar testicular and nephroprotective effects of *Digera muricata* are also reported (Khan and Ahmed, 2009; Khan *et al*., 2009). Administration of CCl_4 causes DNA damages as evident by the higher level of DNA fragmentation percentage (Weber *et al*., 2003; Khan *et al*., 2009; Khan *et al*., 2010). However, DNA fragmentation induced by CCl_4 was restored by the DMME and DMHE treatment.

Nucleolar organizer regions (NORs) are the areas of secondary constriction and are primarily involved in the synthesis of ribosomal RNA (rRNA). Chemically these sites are characterized of ribosomal DNA (rDNA) and non-histone proteins having high affinity for silver (Trere *et al.,* 1996). Treatment of CCl_4 to rats induces genotoxicity by increasing the number of nucleoli (NORs) (Bocking *et al*., 2001; Khanna *et al.,* 2001; Khan *et al*., 2009) and structural deformities in adrenal gland samples to complement other oxidative injuries. Supplement of DMME and DMHE to rats ameliorated the genotoxicity on AgNORs and revert the AgNORs count towards the control group in conformity to other studies (Khan *et al*., 2009).

CONCLUSION

It is envisaged from the present investigation that the altered antioxidant profile, glutathione, lipid peroxidation, DNA fragmentation percent and AgNORs count due to CCl_4 exposure is reversed towards normalization by DMME and DMHE. These results suggest that there are some active compounds present in the plant extracts which are responsible for the observed antioxidant activity. Further studies are required to isolate and characterize the active compounds responsible for antioxidant effects.

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