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Protective and Therapeutic Effect of *Moringa oleifera* Leaf Extract on DNA Damage, Cytogenetic Changes, Sperm Abnormalities and High Level of MDA Induced by CCL4 in Rats.

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ABSTRACT

The present study was investigated to evaluate the protective and therapeutic role of Moringa oleifera leaf extract (MOLE) against CCL4-induced toxicity in rats . Male Albino rats of eleven groups (eight animals each) were used in this study : negative control ; control of olive oil ; positive control received CCL4 in olive oil for 12 weeks ; groups 4-7 received CCl4 in olive oil + MOLE at doses 1.3, 2.0, 2.6 and 4.0 gm /kg (used as a protective agent) for 12weeks . Groups 8-11 received MOLE alone (used as a therapeutic agent) for 3 weeks after cessation of CCL4 (12 weeks) treatment . DNA damage and level of Melanodialdehyde (MDA) in liver tissue , as well as cytogenetic changes (frequency of micronuclei and chromosome aberrations) in bone marrow cells and sperm abnormalities were analyzed . The calculation of DNA fragmentation using spectrophotometer and analysis of genomic DNA by fractionation on agarose gel were used to assay the DNA damage . The results showed that the CCL4 treatment caused significant induction of genetic aberrations (DNA damage , micronuclei in polychromatic erythrocytes and chromosome aberrations) and sperm abnormalities as well as significant elevation of MDA level was observed as compared to control . MOLE treatment (as a protective or therapeutic agent) resulted in significant reduction of genetic aberrations, sperm abnormalities and the level of MDA , in contrast to alterations in all these parameters in rats received CCL4 alone .These results were dose dependent . But better results were obtained by using MOLE as therapeutic agent especially the treatment with the highest dose 4.0 gm / kg., where the percentages of DNA fragmentation , micronuclei , chromosome aberrations and sperm abnormalities and MDA level reverted to near normal . In conclusion , the present investigation is important in presenting data suggesting considerable promise for Moringa oleifera leaf ethanolic extract as a therapeutic agent in CCL4 induced oxidative stress in the rats .

Keywords: Moringa oleifera, CCL4, Genetic alterations, Sperm abnormalities, MDA, Rats.

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INTRODUCTION

The liver is a highly sensitive organ which plays a major role in maintenance and performance of homeostasis in the body. It is the chief organ where important processes life metabolism and detoxification take place. Thus the liver is proned to injury due to the chronic exposure to drugs, environmental toxicants and other xenobiotics (Amacher, 2002; Ezejindu et al., 2013).

Excessive exposure to chemical or pharmacological substances might cause acute liver injury characterized by abnormality of hepatic function, degeneration, necrosis or apoptosis of hepatocytes (Pang et al., 1992; Abdou et al., 2012,b) . CCL4-induced liver injury is one of the well established systems for xenobiotic – induced hepatotoxicity and is a commonly used model for screening of the anti-hepatotoxic and / or hepatoprotective activities of the drugs (Pang et al., 1992; Abdou et al., 2012,b ; Ezejindu et al., 2013).

Because hepatic cells are involved in a variety of metabolic events and all the biochemical pathways related to growth, fight against disease, nutrient supply, energy provision and reproduction (Ward and Daly, 1999; Abdou et al., 2012,b), the establishment of liver protective / therapeutic agents is of paramount importance in the protection from liver damage.

Natural remedies from traditional plants are seen as effective and safe alternative treatments for hepatotoxicity. Several studies have shown that hepatoprotective effects are associated with phytoextracts / phytocompounds rich in natural antioxidants (Fakurazi et al., 2008; Sabir and Rocha, 2008; Bhaskar and Balakrishnan, 2010; Huang et al., 2010 ; Nayak et al.,2011). Many bioactive compounds and extracts from plants have thus been investigated for hepatoprotective and antioxidant effects against hepatotoxin – induced liver damage (Choi et al., 2009; Yousef et al., 2010). Phenolic compounds are commonly found in both edible and other traditional medicinal plants and they have been reported to have multiple biological activities, including free radical scavenging activity (Kahkonen et al., 1999). Antioxidants in food appear to play an essential role in the prevention of oxidative stress – related diseases / disorders and in the reduction of total mortality (Perez – Jimenez and Saura – Calixto, 2008). Typical phenolies that possess antioxidant activity are mainly phenolic acids and flavonoids. Flavonoids commonly accumulate in the epidermal cells of plant organs such as flowers, leaves, stems, roots seeds and fruits, being found in glycosidic form (glycosides) and non – glucosidic form (Pietta, 2000).

Moringa oleifera is considered to be a main medicinal plant. It is commonly known as drumstick tree that has been used as antiulcer, diuretic, anti-inflammatory and for wound healing (Caceres et al., 1991; Udupa et al., 1994; Pal et al., 1995; Bassey et al., 2013).

Many phytochemicals have been isolated from various parts of the plant, viz, phenolic compounds such as quercetin and kaempferol, flavonoids, anthocyanins, carotenoids, vitamins, minerals, amino acids, sterols, glycosides and alkaloids. It contains unique group of compounds called glucosinolates and isothiocyanates (Fahey et al., 2001; Bennett et al., 2003). Recently isothiocyanates and niacimicin from this plant were shown to be potent inhibitors of cancer (Murakami et al., 1998; Guevara et al., 1999; Fahey et al., 2004). Niazimicin also inhibited tumor promotion in a mouse two-stage DMBA – TPA tumor model (Bharali et al., 2003). Seed pod extracts of Moringa oleifera have been demonstrated to prevent skin tumors in mice (Bharali et al., 2003).

On the other hand, M. oleifera leaves were found to be highly nutritious and have potent antioxidant as compared to other part of plant. This is because the leaves are being a significant source of B-carotene ,Vitamin C, protein, iron, potassium , ascorbic acid and flavonoid pigments such as kaempferol, isoquercitrin and kaempferitrin as well as they have diverse curative properties (Pal et al., 1995; 1996; Tahiliani and Kar, 2000; Siddhuraju and Becker, 2003) . Also, leaves are used as nutritional supplement and growth promoters (Makkar and Becker, 1996; Freiberger et al., 1998; Nambiar and Seshadri, 2001; Lakshminarayana et al., 2005; Sanchez et al., 2006).

Ethanolic extract of leaves has shown antimicrobial activity (Chuang et al., 2007; Nepolean et al., 2009). The protective effect of leaves against radio hazards has been established wherein radiation – induced chromosomal aberrations and micronuclei were suppressed by pre–treatment with methanolic extract (Rao et al., 2001) .

Ethanolic extracts of M. oleifera leaves possessed antigenotoxic phytoconstituents in mice, the high percentages of micronuclei and DNA damage induced by cyclophosphamide were decreased in animals pre – dosed with the extract (Sathya et al.,2010) . Moreover, M. oleifera leaves possess tremendous antioxidant properties that ameliorate the deleterious effect of alcohol on pre- pubertal tests of rats (Bassey et al., 2013) .

Also, M. oleifera leave aqueous extract was observed to have a therapeutic action against radiation hazards through enhancing of liver enzyme activities (AST, ALT and ALK), decreasing the malondialdehyde (MDA) and reduction of genetic alterations (micronuclei and DNA damage) in irradiated rats by gamma irradiation (Eshak and Osman, 2013).

So, the present study was designed to evaluate the protection role and therapeutic effect of the putative antioxidant action of ethanol extract of the Moringa oleifera leaves in an experimental model of CCL4 – induced hepatotoxicity in rats. The study was investigated through determination the percentages of DNA fragmentation and levels of malondialdehyde (MDA) in liver tissue. The assaying of frequencies of micronuclei and chromosome aberrations were also evaluated in bone marrow cells. Moreover, the sperm abnormalities were analyzed .

MATERIALS AND METHODS

Chemicals

Carbon tetrachloride (CCL4)

CCL4 is a colorless non –flammable liquid , of molecular weight 153 : 84 was obtained from El- Nasr Pharmaceutical Chemical Co., A.R.E. laboratory chemical division .

CCL4 is one of the most commonly used hepatotoxins in the experimental studies of liver diseases . This compound induced hepatotoxicity as judged from serum marker enzymes and antioxidant levels in liver tissues (Palanivel et al., 2008) Preparation of Moringa oleifera leaves extraction (MOLE):Ethanolic extract of Moringa oleifera leaves was prepared according to Ugwu et al . (2013) as follows :

The fresh leaves of Moringa oleifera plant were picked from trees grown on sand soil in El- Sharkia governorate , Egypt . The leaves were washed thoroughly with distilled water and dried under room temperature at (29°C – 35°C) for three weeks , after which the leaves were pulverized into coarse form with acrestor high speed milling machine . The coarse form (1000g) was then macerated in absolute ethanol . This was left to stand for 48 h .After that the extract was filtered through muslin cloth on a plug of glass wool in a glass column . The resulting ethanol extract was concentrated and evaporated to dryness using rotary evaporator at optimum temperature was between 40 and 45 °C to avoid denaturation of the active ingredients. The concentrated extract was diluted to 1000 ml using a polysaccharide as a carrier and stored in the refrigerator.

Experimental animals

Male albino rats of Sprague –Dawley strain weighing 120-150 g were obtained from the animal house , National Research Centre , Egypt . Animals were housed in an ambient temperature of 25 ± 3.2 °C on light / dark cycle of 12/12 hours . All rats were kept in clean polypropylene cages and administered food and water *ad libitum*.

Experimental design

The rats were divided into 11 equal groups, 8 rats each

Group 1, (n= 8) received saline .
Group 2, received the vehicle (olive oil) at 2.8 ml / kg.
Group 3, received CCl4 in olive oil (1:1, vol/vol) at a dose of 2.8 ml / kg through
orogastric tube and then the rats were administered half the initial dose of CCL4 , twice weekly after the first administration of CCL4 for 12 weeks so as to maintain hepatic damage .

Groups 4-7 , received CCl4 in olive oil in the same dose and way previously mentioned and for the same period . Starting on the first day of CCL4 administration, rats were treated (orally) with Moringa oleifera leaves extract (1.3 gm/kg, 2.0 gm/kg, 2.6 gm/kg, 4.0 gm/kg) of the crude material that are equivalent to (221, 340, 442 and 680 mg) of the extract respectively as each gram of the crude material yields 170 mg of the extract. These groups (4-7) used to evaluate the protective role of MOLE against CCL4.

Groups 8-11, received CCl4 in olive oil in the same dose and way previously mentioned and for the same period , then the rats were treated with Moringa oleifera leaves extract (1.3 gm/kg, 2.0 gm/kg, 2.6 gm/kg, 4.0 gm/kg) for three weeks. These groups (8-11) were used to evaluate the therapeutic effect of MOLE against CCL4.

Rats had free access to food and drinking water during the study . At the end of the experiment, rats were sacrificed by cervical dislocation for studying of DNA damage, micronuclei, chromosome aberrations , sperm abnormalities and also for assaying MDA level .

The used doses of CCL4 for rats were equivalent to the human dose according to Paget and Barnes (1964) and Abdel Salam et al. (2010 ; 2012).

Evaluation of DNA damage

Assaying of DNA fragmentation using spectrophotometer

Liver samples were collected immediately after sacrificing the animals. The tissues were lysed in 0.5ml lysis buffer containing 10mM tris-HCL (pH, 8) 1mM EDTA, 0.2% triton X-100 centrifuged at 10000 r.p.m. (Eppendorf) for 20 minutes at 4°C. The pellets were resuspended in 0.5 ml of lysis buffer. To the pellets (P) and supernatant (S), 1.5 ml of 10% trichloroacetic acid(TCA) was added and incubated at 4°C for 10 minutes. The samples were centrifuged for 20 minutes at 10000 r.p.m. (Eppendorf) at 4°C and the pellets were suspended in 750 ul of 5% TCA, followed by incubation at 100°C for 20 minutes. Subsequently to each sample 2 ml of DPA solution [200 mg DPA in 10 ml glacial acetic acid, 150ul of sulfuric acid and 60 ul acetaldehyde] was added and incubated at room temperature for 24 hour (Gibb et al., 1997). The proportion of fragmented DNA was calculated from absorbance reading at 600 nm using the formula:

$$\text{DNA fragmentation} = \frac{\text{OD of fragmented DNA(S)}}{\text{OD of fragmented DNA (S) + OD of intact DNA (P)}} \times 100$$

Analysis of genomic DNA on agarose gel

Genomic DNA was isolated from the rat liver tissue . This genomic DNA was fractioned on agarose gel (1.5%) and stained with ethidium bromide according to Surzycki, (2000).

Cytogenetic Analysis

Micronucleus Test

Bone marrow slides were prepared according to the method described by Krishna and Hayashi (2000). The bone marrow was washed with 1 ml of fetal calf serum and then smeared on clean slides. The slides were left to air dry and then fixed in methanol for 5 minutes, followed by staining in May-Grunwald-Giemsa for 5 minutes then washed in distilled water and mounted. For each animal, 2000 polychromatic erythrocytes (PCEs) were examined for the presence of micronuclei.

Chromosome Preparations

For chromosome analysis both treated and control animals were sacrificed by cervical dislocation at the end of experiment. One hour and half or two hours before sacrifice, rats were injected i. p with 0.5 gm colchicine / kg. b.w. Femurs were removed and the bone marrow cells were aspirated using saline solution. Metaphase spreads were prepared using the method of Preston et al. (1987). Fifty metaphase spreads per animal were analyzed, for scoring the different types of chromosome aberrations.

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Sperm Analysis

For sperm-shape analysis, the epididymis excised and minced in about 8 ml of physiological saline, dispersed and filtered to exclude large tissue fragments. Smears were prepared after staining the sperms with Eosin Y (aqueous), according to the methods of Wyrobek and Bruce (1978) and Farag et al. (2002). At least 3000 sperms per group were assessed for morphological abnormalities. The sperm abnormalities were evaluated according to standard method of Narayana (2008).

Measurement of Lipid Peroxidation

Liver tissues were homogenized in 20mm Tris-HCl (pH 7.4). Homogenates were centrifuged at 6000g for 30 min. Malondialdehyde (MDA) levels in the supernatants were determined using a spectrophotometric assay kit according to the manufacturer's instructions.The absorbance of the resultant pink product was measured at 534 nm (Ohkawa et al.,1979). The lipid peroxidation values were expressed as nm MDA/mg tissue.

Statistical Analysis

Statistical analysis was performed with SPSS software. Data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan ´s post hoc test for comparison between different treatments. Results were reported as mean ± S.E. and differences were considered as significant when $P < 0.05$.

RESULTS

Results of DNA damage

The present results showed (table 1) that the treatment with CCL4 caused significant massive fragmentation the DNA as compared to control groups. The DNA damage was decreasing by using Moringa oleifera leaves extract as a protective agent, as the proportion of DNA fragmentation significantly reduced with all doses used (1.3, 2.0, 2.6 and 4.0 g/Kg). From the results, it was found the reduction of DNA fragmentation damage was increased by increasing the dose level, where, the dose 4.0 gm/Kg gave the lowest proportion of DNA damage. Using Moringa oleifera leaves extract as a therapeutic agent (table 2) gave most better results. It significantly decreased the proportion of DNA fragmentation in a dose dependent manner. The percentages of DNA damage (given with CCL4) were decreased in all animals groups treated with all doses of MOLE . By using the highest dose. (4.0 gm/Kg) the proportion of DNA fragmentation markedly reduced as compared to other lower doses.

Figure (1) illustrated the different lanes profiling the genomic DNA on agarose gel (1.5%).As revealed from figure, the genomic DNA of control groups showed definite bands, while damaged bands in genomic DNA of CCL4 group were observed. On the other hand, the DNA bands in rat groups supplemented with Moringa extraction as protective or therapeutic agent were relatively similar with control groups and they were also relatively similar with each other.

Table 1 : Effect of MOLE as a protective agent on hepatic DNA damage in CCL4 intoxicated rats

Treatment	% of DNA Fragmentation	
	Range	M±S.E.
NC	8.65 – 10.6	9.64±0.45 [a]
CO	10.56– 12.45	11.56 ± 0.44 [a]
CCL4	22. 5 –26.14	24.31±0.74 [c]
CCL4+1.3 gm/kg MOLE	19.98 – 23.54	21.56±0.79 [b]
CCL4+2.0 gm/kg MOLE	19.4 – 23.06	21.51±0.78 [b]
CCL4+2.6 gm/kg MOLE	18.83 – 21.21	20.0±0.5 [b]
CCL4+4.0 gm/kg MOLE	17.99 – 21.59	19.5±0.79 [b]

Data were expressed as mean ± S.E.
Means with different superscript letters (a, b, c) are significantly different (P<0.05).
NC : Negative control , rats received saline. CO : Control of rats received only the vehicle (olive oil). MOLE: Moringa oleifera leaves extract .

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Table 2 :Effect of MOLE as a therapeutic agent on hepatic DNA damage in CCL4 intoxicated rats

Treatment	% of DNA Fragmentation	
	Range	M±S.E.
NC	8.65 – 10.6	9.64±0.45 [a]
CO	10.56– 12.45	11.56 ± 0.44 [b]
CCL4	22. 5 – 26.14	24.31±0.74 [d]
CCL4 then1.3 gm/kg MOLE	13. 8 – 16.26	14.63 ±0.44 [c]
CCL4 then2.0 gm/kg MOLE	13.2 – 16.15	14.58 ±0.56 [c]
CCL4 then 2.6 gm/kg MOLE	13.35 – 15.35	14.38 ±0.62 [c]
CCL4 then 4.0 gm/kg MOLE	10.69 – 13.0	11.69±0.48 [b]

Data were expressed as mean ± S.E.
Means with different superscript letters (a, b, c,d) are significantly different (P<0.05).
NC : Negative control , rats received saline. CO : Control of rats received only the vehicle (olive oil). MOLE: Moringa oleifera leaves extract.

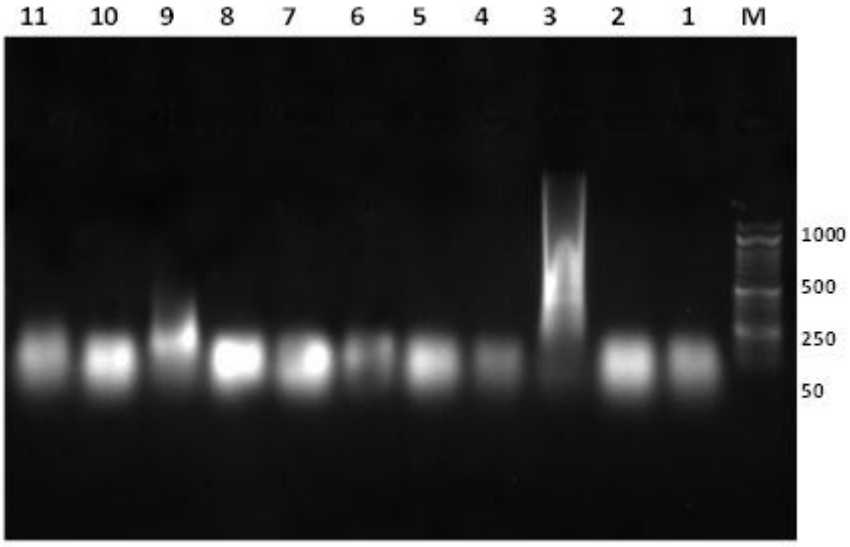

Figure 1: Genomic DNA of liver tissue fractioned on agarose gel electrophoresis

(1.5 %). (M) Marker (50 bp): (lane 1) Negative control ; (lane 2) control of olive oil ; (lane 3) CCL4; (lanes of 4-7) CCL4 + 1.3, 2.0, 2.6, and 4.0 gm / kg of MOLE (as a protective agent) for 12 weeks; (lanes of 8-11) CCL4 for 12 weeks then 1.3, 2.0, 2.6, and 4.0 gm / kg of MOLE (as a therapeutic agent) for three weeks.

Micronuclus results

The present results (table 3) indicated that the treatment with CCL4 significantly induced MNPCE in respect to control groups. Using Moringa oleifera leaves extract as a protective agent reduced the mutagenic effect in a dose dependent manner. Dose 1.3 gm/kg insignificantly reduced the genetic damage (given with CCL4). Whereas, using the other doses (2.0, 2.6and 4.0 gm/Kg) significantly decreased the inducing of MNPCE, this reduction of mutagenic effect increased by increasing the dose level where the highest dose (4.0 gm/Kg) markedly reduced the inducing of MNPCE as compared to other lower doses.

Better results were given by using Moringa oleifera leaves extract (table 4) as a therapeutic agent . It is significantly reduced the proportions of MNPC5 in a dose dependent manner. The dose 4.0 gm/ Kg greatly improved the genetic damage and gave the lowest proportion of MMPCE in respect to other lower doses.

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Table 3: Effect of MOLE as protective agent on the frequency of MNPCE in CCL4 intoxicated rats

Treatment	Number of examined cells	Mean values of MNPCE
NC	2000	2.40 ±0.24 a
CO	2000	3.00 ± 0.31 a
CCL4	2000	19.6 ± 0.81 e
CCL4+1.3 gm/kg MOLE	2000	18.8 ± 0.37 e
CCL4+2.0 gm/kg MOLE	2000	12.40 ± 0.40 d
CCL4+2.6 gm/kg MOLE	2000	8.80± 0.48 c
CCL4+4.0 gm/kg MOLE	2000	5.40 ± 0.60 b

Data were expressed as mean ± S.E.
Means with different superscript letters (a, b, c, d, e) are significantly different (P<0.05)
NC : Negative control , rats received saline.
CO: Control of rats received only the vehicle (olive oil).
MOLE: Moringa oleifera leaves extract .
MNPCE : Micronucleated polychromatic erythrocyte

Table 4: Effect of MOLE as a therapeutic agent on the frequency of MNPCE in CCL4 intoxicated rats

Treatment	Number of examined cells	Mean values of MNPCE
NC	2000	2.40 ±0.24 a
CO	2000	3.00 ± 0.31 ab
CCL4	2000	19.6 ± 0.81 e
CCL4 then1.3 gm/kg MOLE	2000	9.20 ± 0.66 d
CCL4 then2.0 gm/kg MOLE	2000	6.20 ± 0.58 c
CCL4 then 2.6 gm/kg MOLE	2000	5.60 ± 0.60 c
CCL4 then 4.0 gm/kg MOLE	2000	4.60 ± 0.60 bc

Data were expressed as mean ± S.E.
Means with different superscript letters (a, b, c, d, e) are significantly different (P<0.05). NC : Negative control , rats received saline.
CO: Control of rats received only
the vehicle (olive oil). MOLE: Moringa oleifera leaves extract .
MNPCE : Micronucleated polychromatic erythrocytes .

Chromosome results

The results of this study (table, 5) revealed that the treatment with CCL4 significantly induced structural and numerical chromosome aberrations as compared to control groups. This mutagenic effect was reduced by using Moringa oleifera extract as a protective agent in a dose dependent manner. The using of dose 1.3 gm/Kg slightly decreased such mutagenic effect, while dose 2.0 mg/Kg or dose 2.6 gm/Kg markedly decreased the inducing of chromosome aberrations (given with CCL4). Moreover, dose 4.0 gm/Kg greatly ameliorated the genetic damage and gave the lowest frequencies of structural and numerical chromosome aberrations as compared to other lower doses.

Better results were obtained (table ,6) by using Moringa oleifera extract as a therapeutic agent. These results were dose dependent as the lowest dose 1.3gm/Kg significantly reduced the structural chromosome aberrations and did not reduce the numerical chromosome aberrations. Also, dose 2.0 gm/Kg significantly decreased the structural chromosome aberrations and slightly reduced the numerical chromosome aberrations, while dose 2.6 gm/Kg markedly reduced both structural and numerical chromosome aberrations. Whereas, the using dose 4.0 gm/Kg greatly ameliorated the genetic damage and led to the presence of the lowest proportions of such chromosome of aberrations in respect to other lower doses

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Table 5 :Effect of MOLE as a protective agent on the frequency of chromosome aberrations in CCL4 intoxicated rats

treatment	Structural chromosomal aberrations					Total structural aberrations	Numerical chromosomal aberrations			Total numerical aberrations	Total Chromosomal aberrations
	Gaps	Breaks	Fragments	Deletions	Centromeric attenuations		Hypoploid	Hyperploid	Polyploid		
NC	0.60 ± 0.24 a	0.20 ± 0.20 a	0.40 ± 0.24a	00 a	1.20 ± 0.37 a	2-4±.50 0a	0.40 ± 0.24a	0.20 ± 0.20a	00 a	0.60±0.24 a	3.00 ± 0.31 a
CO	1.20 ± 0.37ab	0.40 ± 0.24 a	00 a	00 ab	1.40 ± 0.24ab	3.00±0.31a	1.20 ± 0.37abc	00 a	00 a	1.20±0.37 a	4.20 ± 0.37 a
CCL4	5.60 ± 0.60 e	4.20 ± 0.66 b	4.20 ± 0.48 d	6.40 ± 0.24 c	5.80 ± 0.37e	26.20±1.15e	2.20 ± 0.37 cd	1.20 ± 0.37b	1.00 ± 0.44 b	4.40±0.74 b	30.60 ± 0.92 e
CCL4+ 1.3gm/kg MOLE	4.60 ± 0.40 de	4.00 ± 0.31 b	4.40 ± 0.24 d	6.00 ± 0.44 c	5.80 ± 0.37e	24.80±0.37e	2.40 ± 0.24d	1.40 ± 0.24bc	0.60 ± 0.24ab	4.40±0.50 b	29.20 ± 0.37 e
CCL4+ 2.0gm/kg MOLE	3.60 ± 0.67 cd	3.20 ± 0.37 b	2.40 ± 0.24 c	5.40 ± 0.40 c	3.40 ± 0.50cd	18.00±0.70d	1.80 ± 0.37bcd	1.20 ± 0.37bc	0.40 ± 024ab	3.40±0.40 b	21.40 ± 0.74 d
CCL4+ 2.6gm/kg MOLE	2.40 ± 0.24 bc	1.40 ± 0.40 a	1.40 ± 0.24 b	3.80 ± 0.37 b	3.60 ± 0.40d	12.60±0.81c	1.80 ± 0.37bcd	1.60 ± 0.24c	0.60 ± 0.24ab	4.00±0.70 b	16.60 ± 0.74 c
CCL4+ 4.0gm/kg MOLE	1.60±0.24 ab	1.00 ± 0.31 a	1.40 ± 0.50 b	2.80 ± 0.37 b	2.40 ± 0.24bc	9.20±0.37b	1.00 ± 0.44ab	0.60 ± 0.40ab	0.20 ± 0.20a	1.80±0.66 a	11.00 ± 0.89 b

Data were expressed as mean ± S.E.
Means with different superscript letters (a, b, c, d, e) are significantly different (P<0.05).
NC : Negative control , rats received saline. CO : Control of rats received only the vehicle (olive oil). MOLE: Moringa oleifera leaves extract

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Table 6 :Effect of MOLE as a therapeutic agent on the frequency of chromosome aberrations in CCL4 intoxicated rats

treatment	Structural chromosomal aberrations					Total structural aberrations	Numerical chromosomal aberrations			Total numerical aberrations	Total Chromosomal aberrations
	Gaps	Breaks	Fragments	Deletions	Centromeric attenuations		Hypoploid	Hyperploid	Polyploid		
NC	0.60 ± 0.24 a	0.20 ± 0.20a	0.40 ± 0.24 a	00 a	1.20 ± 0.37 a	2-4±0.50 a	0.40 ± 0.24 a	0.20 ± 0.20a	00 a	0.60±0.24 a	3.00 ± 0.31 a
CO	1.20 ± 0.37ab	0.40 ± 0.24 a	00 a	00 ab	1.40 ± 0.24 ab	3.00±0.31 a	1.20 ± 0.37 abc	00 a	00 a	1.20±0.37 a	4.20 ± 0.37 a
CCL4	5.60 ± 0.60 e	4.20 ± 0.66 b	4.20 ± 0.48 d	6.40 ± 0.24 c	5.80 ± 0.37e	26.2±1-15 e	2.20 ± 0.37 cd	1.20 ± 0.37b	1.00 ± 0.44 b	4.40±0.74 d	30.60 ± 0.92 e
CCL4 then 1.3 gm/kg MOLE	1.60 ± 0.60 a	2.00 ± 0.54 b	1.60 ± 0.24 b	3.60 ± 0.67 b	4.60 ± 0.40 de	13.40±0.67 c	2.60 ± 0.37 e	0.80 ± 0.37 ab	1.00 ± 0.31 b	4.40±0.50 d	17.80 ± 0.58d
CCL4 then 2.0 gm/kg MOLE	1.40 ± 0.24 a	1.00 ± 0.44 ab	1.60 ± 0.40 b	4.60 ± 0.24 b	3.80 ± 0.48 cd	12.40±0.50 c	2.00 ± 0.31 cde	1.20 ± 0.20 b	0.60 ± 0.24ab	3.80±0.37 cd	16.20 ± 0.80 cd
CCL4 then 2.6 gm/kg MOLE	1.00 ± 0.31 a	0.60 ± 0.24 a	1.60 ± 0.24 b	4.00 ± 0.44 b	4.80 ± 0.58 de	12.00±0.70 c	1.60 ± 0.24 bcd	0 .80 ± 0.31ab	0.40 ± 0.24ab	2.80±0.37bc	14.80 ± 0.58 c
CCL4 then 4.0 gm/kg MOLE	0.60 ± 0.24 a	0.40 ± 0.24 a	0.60 ± 0.24 a	3.40 ± 0.24 b	2.60 ± 0.40 bc	7.60±0.60 b	1.00 ± 0.31 ab	0.60 ± 0.24 ab	0.20 ± 0.20ab	1.80±0.58 ab	9.40 ± 0.81 b

Data were expressed as mean ± S.E .
Means with different superscript letters (a, b, c, d, e) are significantly different (P<0.05) . NC : Negative control , rats received saline. CO : Control of rats received only the vehicle (olive oil). MOLE: Moringa oleifera leaves extract .

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Table 7 :Effect of MOLE as a protective agent on the frequency of sperm shape abnormalities in CCL4 intoxicated rats

Treatment	Head abnormalities						Tail abnormalities				Total sperm abnormalities
	Amorphous	Without hock	Banana	Double head	Small head	Total head abnormalities	Middle piece defect	Coiled	Double Tail	Total tail abnormalities	
NC	1.00±0.40 a	1.75±0.47 a	00 a	00 a	00 a	2.75±0.62a	1.75 ±0.47 a	5.00± 0.70 a	00 a	6.75±0.62 a	9.50 ± 0 .28 a
CO	2.25± 0.47 ab	1.75±0.47 a	00 a	00 a	00 a	4.00±0.40 ab	2.25±0.25 ab	5.25±0.25 a	00 a	7.50±0.28 a	11.50 ± 0.50 a
CCL4	4.50±0.68 c	3.50±0.28 b	0.50±0.28 a	0.25±0.25 a	0.25 ±.25 a	9.00±0.40 c	3.75 ±0.47 b	9.75±0 .85 d	0.50±0.50 b	14.00±1.08 d	23.00 ± 1.41 d
CCL4 +1.3 gm/kg MOLE	4.25±0.75 c	3.50±0.50 a	0.50±0.28 a	00 a	00 a	8.25±1.03 c	3.50±0.64 b	9.25±0.47 cd	00 a	12.75±0.47 d	21.00 ± 1.2 cd
CCL4 +2.0 gm/kg MOLE	3.75±0.62 bc	3.25±0.62 a	0.25±0.25 a	00 a	00 a	7.25±0.85 c	3.25±0.47 b	7.75± 0.47 bc	00 a	10.75±0.25 c	18.00 ± 0.81 bc
CCL4 +2.6 gm/kg MOLE	3.25±0.47 bc	3.25± 0.85 a	0.25± 0.25 a	00 a	00 a	6.75±1.43 bc	2.75±0.47 ab	7.25±0 .47 b	00 a	10.00± 0.40 bc	16.75 ± 1.22 b
CCL4 +4.0 gm/kg MOLE	2.25±0.75 ab	1.75±0.85 a	00 a	00 a	00 a	4.00±1.08 ab	2.50± 0.28 ab	6.00±0.47 ab	00 a	8.50±0.95 ab	12.50 ± 1.3 a

Data were expressed as mean ± S.E.

Means with different superscript letters (a, b, c, d) are significantly different (P<0.05). NC : Negative control , rats received saline. CO : : Control of rats received only the vehicle (olive oil) . MOLE: Moringa oleifera leaves extract .

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Table 8 :Effect of MOLE as a therapeutic agent on the frequency of sperm shape abnormalities in CCL4 intoxicated rats

Treatment	Head abnormalities						Tail abnormalities				Total sperm abnormalities
	Amorphous	Without hock	Banana	Double head	Small head	Total head abnormalities	Middle piece defect	Coiled	Double Tail	Total tail abnormalities	
NC	1.00±0.40 a	1.75±0.47 a	00 a	00 a	00 a	2.75±0.62 a	1.75 ±0.47a	5.00± 0.70 a	00 a	6.75±0.62 a	9.50±0 .28 a
CO	2.25± 0.47 ab	1.75±0.47 a	00 a	00 a	00 a	4.00±0.40 ab	2.25±0.25 a	5.25±0.25 a	00 a	7.50±0.28 a	11.50 ± 0.50 a
CCL4	4.50±0.68 d	3.50±0.28 b	0.50±0.28 a	0.25±0.25 a	0.25±0.25 a	9.00±0.40 e	3.75±0.47 b	9.75±0 .85 d	0.50±.50 b	14.00±1.08 b	23.00 ± 1.41 d
CCL4 then1.3gm/kg MOLE	4.00±0.40 cd	3.50±0.65 b	0.25±0.25 a	0.25 ±0.25 a	0.25±0.25 a	8.25±0.47e	2.50±0.28a	9.50±0.86 bc	00 a	12.00±0.70 b	20.25 ± 0 .25 c
CCL4 then2.0 gm/kg MOLE	3.50±0.64bcd	3.25± 0.62 ab	0.25±0.25 a	0.25 ±0.25a	0.25±0.25 a	7.50±0.64 de	2.25±0.47 a	9.25±0.85 c	0.00 a	11.50±1.32 b	19.00 ± 1.08 c
CCL4 then 2.6gm/kg MOLE	2.75±0.25 bc	3. 25±0.47 ab	0.25± 0.25 a	00 a	00 a	6.25±0.47cd	1.75±0.25 a	7.25± 0.47 b	00 a	9.00± 0.57 a	15.25 ± 0.62 b
CCL4 then4. 0gm/kg MOLE	2.50±0.28 b	2.25± 0.25 ab	00 a	00 a	00 a	4.75±1.60 bc	1.50± 0.50 a	5.25±0.47 ab	00 a	6.75±0.85 a	11.50 ± 0.50 a

Data were expressed as mean ± S.E.
Means with different superscript letters (a, b, c,.d) are significantly different (P<0.05)
NC : Negative control , rats received saline. CO : : Control of rats received only the vehicle (olive oil) .MOLE: Moringa oleifera leaves extract.
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Results of sperm abnormalities

The present results (table, 7) showed that treatment with CCL4 significantly induced sperm abnormalities (head + tail) (23.00 ± 1.41) in respect to normal control (9.50 ± 0.28). These total sperm abnormalities were reduced by using Moringa oleifera leaves extract as a protective agent in a dose dependent manner. The using of doses 1.3 , gm/Kg slightly decreased the sperm abnormalities. Whereas, the doses of 2.0 and 2.6 gm/Kg lowered the total sperm abnormalities, this reduction was significant , the highest dose 4.0 gm/Kg resulted in the lowest proportion of sperm abnormalities, and the statistical analysis showed significant differences ($P < 0.001$) between this group of rats that supplemented with CCL4 + 4.0 gm/Kg of Moringa oleifera leaves extraction and the group of rats that supplemented with CCL4 alone.

Using Moringa oleifera leaves extract as a therapeutic agent (table, 8) resulted in better findings. Doses 1.3 or 2.0 gm/Kg significantly reduced ($p< 0.05$) the total proportions of sperm abnormalities (head + tail) that given with CCL4. Whereas, the dose 2.6 gm /Kg caused high significant decrease ($P < 0.01$) of sperm abnormalities (head + tail) given with CCL4. Moreover, dose 4.0 gm/Kg markedly ameliorated the such reproductive parameter and gave the lowest frequencies of sperm abnormalities (head + tail) as compared to other lower doses.

Effect of M. oleifera leaves extract on hepatic MDA level (LPO content):

The present results (table 9) indicated that the CCL4 treatment elevated the level of Malondialdehyde (MDA) in liver tissue, this elevation was very high significant as compared to negative controls.

On the other hand, the effect of test extract of M. oleifera leaves on CCL4 mediated lipid peroxidation (LPO) was examined through monitoring the level of MDA. The used doses of Moringa extract (1.3, 2.0, 2.6 and 4.0 gm/Kg) as a protective agent significantly reversed the CCL4 intoxicated elevation of hepatic MDA level. The reduction of MDA level was increased by increasing the dose level. The highest dose 4.0 gm/Kg caused the lowest level of MDA

Best results of MDA analysis were obtained using Moringa oleifera leaves extraction as a therapeutic agent (table 10). There were considerable decreases of hepatic MDA level by using the all test doses (1.3, 2.0, 2.6 and 4.0 gm/Kg). These decreases were found to be significant statistically. The decreasing of MDA level was increased by increasing the dose level. The dose 4.0 gm/Kg greatly reduced the MDA level and gave findings relatively near with those found in normal controls.

Table 9 : Effect of MOLE as a protective agent on MDA level in CCL4 intoxicated rats

Treatment	Malondialdehyde(MDA)nM/mg protein
NC	2.30 ±0.4 a
CO	2.90 ± 0.54 a
CCL4	59.10 ± 0.89 e
CCL4+1.3 gm/kg MOLE	44.50 ± 1.14 d
CCL4+2.0 gm/kg MOLE	35.90 ± 0.84 c
CCL4+2.6 gm/kg MOLE	15.60± 1.06 b
CCL4+4.0 gm/kg MOLE	14.34 ± 0.71 b

Data were expressed as mean ± S.E. .
Means with different superscript letters (a, b, c, d, e) are significantly different ($P<0.05$).
NC : Negative control , rats received saline. CO : : Control of rats received only the vehicle (olive oil) . MOLE: Moringa oleifera leaves extract .

Table 10: Effect of MOLE as a therapeutic agent on MDA level in CCL4 intoxicated rats.

Treatment	Malondialdehyde(MDA)nM/mg protein
NC	2.30 ±0.4 a
CO	2.90 ± 0.54 a
CCL4	59.10 ± 0.89 e
CCL4 then 1.3 gm/kg MOLE	13.60 ± 0.31 e
CCL4 then 2.0 gm/kg MOLE	7.30 ± 0.3 d
CCL4 then 2.6 gm/kg MOLE	4.60 ± 0.63 c
CCL4 then 4.0 gm/kg MOLE	3.90 ± 0.16 bc

Data were expressed as mean ± S.E. .
Means with different superscript letters (a, b, c ,d, e) are significantly different (P<0.05).
NC : Negative control , rats received saline. CO : Control of rats received only the vehicle (olive oil) . MOLE: Moringa oleifera leaves extract .

DISCUSSION

The present study showed that the administration of CCL4 to rats significantly elevated the each of the percentages of DNA damage, the frequencies of micronucleated erythrocytes, the frequencies of chromosome aberrations and sperm abnormalities as well as the level of MDA, which are evidence of its toxicity when compared to normal control. The inducing of genetic alterations and sperm abnormalities were similar with the reported in other studies, where CCL4 treatment in rats induced significantly higher frequencies of chromosome aberrations, DNA damage and sperm abnormalities (Abdou et al., 2012 a , b). Concerning the MDA, the present findings were in agreement with that reported by Jayakumar et al. (2006) ; Nanjappaiah and Hugar (2012) who found significant elevation of MDA in rats treated with CCL4 as compared to negative controls. Also Sreelatha and Padma (2010) observed significant increase in the level of lipid peroxides of liver slices treated with CCL4 in vitro in respect to normal control.

Carbon tetrachloride (CCL4) is a well –known hepatotoxic agent. The basis of its hepatotoxicity lies in its biotransformation by the cytochrome P450 system to two free radicals. The first metabolie, a trichlorometyhyl free radical, forms covalent adducts with lipids and proteins ; it can interact with O_2 to form a second metabolite, a trichloromethlperoxy free radical, or can remove hydrogen atoms to form chloroform. This sequence of events leads to lipid peroxidation of membranes and consequent liver injury. In response to this hepatocellular injury, "activated" hepatic Kupfer cells release increased quantities of active oxygen species (ROS) and other bioactive agent (EISisi et al., 1993; Jayakumar et al ., 2006). These ROS may attack soluble cell compounds as well as membranes, eventually leading to the impairment of cell functionality, cytotoxicity and genotoxicity (Towner et al., 2003; Berg et al., 2004), that have been confirmed in our findings by the presence of more frequencies of DNA fragmentation, micronuclei and chromosome aberrations in CCL4 group than those of negative control.

On the other hand, micronucleus assaying as of chromosome aberration is a cytogenetic form that measures chromosomal damage, thus it is only effective when both DNA strands are broken (Slamenova et al., 1997; Zeni and Searfi, 2010). Therefore, the significant increases of micronuclei which observed in the present study proved also the mutagenic effect of CCL4 compound that consequently led to inducing of high frequencies of chromosome aberrations in CCL4 group.

Evaluating the genotoxic effects of any agent in an organism, it is much relevant to study the genotoxic effect on chromosomal aberrations and the germinal cells because this will give information on transmissible genetic damage from one generation to another (Au and Hsu, 1980). Chemicals that showed positive response in the sperm abnormality tests are also proved to be carcinogenic (Wyrobek et al ., 1983).In the present study a significant increase in the number of morphologically abnormal sperms occurred in animals treated with CCL4. The consequently high incidence of DNA fragmentation and chromosome

aberrations as a result of potential generation of ROS during metabolic processing of CCL4 in liver may be indicative of a general susceptibility of animals in the present study for inducing DNA fragmentation and consequently chromosome aberrations in gonadal cells causing sperm shape abnormalities (Sakkas et al., 1999; Aitken and Krausz, 2000) . Furthermore, in other studies, DNA fragmentation of human genomic of gonadal cells was due to an excessive production of ROS by oxidative stress and led to damage in sperm morphology (Twigg et al., 1998; De Lamirande and Gagnon, 1999; Aitken and Krausz, 2000, Muratori et al., 2003). Sperm abnormalities in CCL4 group might be also due to attack of generated ROS to polyunsaturated fatty acid residues of phospholipids of cell membrane occurring lipid peroxidation (LPO). Since the sperms have a high content of polyunsaturated fatty acids in the plasma membrane, So they are highly sensitive to oxidative stress. Increased LPO and altered membrane can affect the sperm DNA leading sperm abnormalities (Tramer et al., 1998; Sanchez et al., 2006).

The CCL4 damaged liver toxicity was associated with marked increase in MDA level. The MDA elevation has been accepted reliable marker of lipid peroxidation. Malondialdehyde (MDA) is a major reactive aldehyde resulting from the peroxidation of biological membrane polyunsaturated fatty acid (PUFA) (Vaca et al.,1988). This component is a secondary product of lipid peroxidation and it is used as an indicator of tissue damage involving a series of chain reactions (Ohkawa et al., 1979). It reacts with thiobarbituric acid, producing red - colored products. lipid peroxidation has been implicated in the pathogenesis of increased membrane rigidity osmotic fragility, reduced erythrocyte survival and perturbations in lipid fluidity. It has been hypothesized that one of the principal causes of CCL4- induced hepatotoxicity is lipid peroxidation of hepatocyte membranes by free radical derivatives of CCL4 (Recknagel et al., 1989; 1991; Jayakumar et al., 2006). The observation of elevated levels of hepatic MDA in rats administered CCL4 alone in the present study is consistent with this hypothesis.

MOLE Treatment

The current results showed that the animals treated with MOLE had significant lower of DNA fragmentation, micronuclei, chromosome aberrations and morphological sperm abnormalities as well as significant reduction of MDA level. These findings indicated that MOLE have a protective and therapeutic role in body cells against the observed mutagenic effect of CCL4.

The protective or therapeutic effect may be attributed to the potential involvement of the phytomolecules (natural antioxidants) of the extract to interfere with enzymes participating in the biotransformation of CCL4 to cytotoxic metabolites (Sathya et al., 2010).Generally the mode of action of many phytochemicals might be due to binding with mutagens or inhibition of activated of cytochrome system – mediated N- hydroxylation enzymes with a consequent reduction of genetic material (DNA or chromosomes) and sperm abnormalities (Aitken and Krausz, 2000; Farag et al., 2002; Wang et al., 2004, Devaraj et al., 2008).

Free radical scavenging represents one of the important strategies in antimutagenesis and anticarcinogensis (Steinmetz and Potter, 1991a and b) . MOLE rich amount of antioxidants (Iqbal and Bhanger, 2006; Chumark et al., 2008). A possible explanation for the protective or therapeutic effect recorded in the present investigation could be the involvement of its antioxidant and scavenging properties. Antioxidants provide protection or remediation by scavenging reactive oxidative species (ROS) that damage DNA and initiate diseases such as cancer (Sathya et al., 2010).

There is an evidence indicated that some components of MOLE such as Vitamin C and B- carotene have antimutagenic effect against toxicants. These constituents were found in other studies to have antimutagenic effects against smoke induced – lymphocyte DNA damage of smokers (Duthie et al., 1996). Also vitamin C was known to be immune system booster and reduces the free radicals in the body (Abdou et al.,2012 b).

Also, many components of MOLE such polyphenols and various carotenoids were observed to improve the immune system, scavenge of free radical and reduce the production of DNA mutations in different mammalian cells that were previously exposed to variety of oxidative conditions (Nicolle et al., 2003; Van Breda et al., 2005; Srinivasan et al., 2007, Devaraj et al., 2008). Furthermore, polyphenols which are present in MOLE were shown in other studies to inhibit a specific protein found in bone marrow and which is responsible for cancer in bone and increased the production of antioxidants in the sperms (Abdou et al., 2012,a).

Moreover, many of micro- constituents of MOLE were considered to be anti-carcinogenesis , they were showed in other studies to reduce the risk of ovarian cancer, lung cancer and prostate cancer in human and mice (Cramer et al., 2001; Van Breda et al., 2005; Gitenay et al. 2007). The high percentages of radiation-induced genetic alterations (chromosome aberrations and micronuclei) were suppressed by pre- treatment with ethanolic extract of MOLE (Rao et al., 2001) . The antigenotoxic phytoconstituents of MOLE were found to minimize the high percentages of micronuclei and DNA damage induced by cyclophosphamide in mice pre-dosed with the extract (Sathya et al., 2010). Also, MOLE decreased the MDA level and reduced the genetic alterations (micronuclei and DNA damage) in irradiated rats by gamma irradiation (Eshak and Osman 2013).

So, the reduction of DNA mutation (DNA fragmentation and micronuclei) induced in the present study by MOLE led to a reduction in chromosome abnormalities as a result of a decrease in disturbance of DNA replication (Sugimura and Wakabayashi, 1990; Sinha and Prasad 1990; Breneman et al., 1996; Starvic et al., 1997). Consequently this led to a reduction in sperm abnormalities (Sailer et al ., 1995; Sakkas et al .,1999; Aitken and Krausz, 2000; Farag et al., 2002) .

In the present study, CCL4 significantly increased MDA level as compared to normal control. Treatment with MOLE (as a protective or therapeutic agent) significantly decreased MDA level. This indicates the anti- lipid peroxidation of MOLE which acted against the damaging effects of free radicals produced by CCL4. High content of polyphenols and alkaloids in MOLE (Fahey et al., 2001; Bennett et al.,2003) contributes free radicals scavenging and antioxidant (Sathya et al., 2010). Sreelatha and Padma (2010) evaluated the efficacy of MOLE against CCL4 – treated liver slices in vitro. Their results showed that the treatment with MOLE effectively suppress CCL4-induced oxidative stress by significant reduction of lipid peroxide levels and thiobarbituric acid-reacting substances (TBARS) as well as significant increase of the activities of antioxidant enzymes and glutathione. Prasanna and Sreelatha (2014) reported that MOLE treatment was capable for reducing ROS (H202)-induced cytotoxicity and lipid peroxidation in Saccharomyces cerevisiae cells, this treatment attenuated the oxidative stress induced by ROS (H2O2) and significantly enhanced the levels of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) that are capable of removing oxygen radicals and their products and/or repairing the damage caused by oxidative stress.

Ethanolic extract of Moringa oleifera leaves was used in this study . Aqueous and methanolic extract of M. oleifera leaves have been reported only to limited extent for their antioxidant properties (Siddhuraju and Becker, 2003) . In that regard , the anti-genotoxic effect delivered by the ethanolic extract of Moringa oleifera leaves in the present study could probably be attributed to the appreciable amount of antioxidant constituents (Sathya et al., 2010).

In conclusion , the present investigation is important in presenting data suggesting considerable promise for Moringa oleifera leaf ethanolic extract as a therapeutic agent in CCL4 induced oxidative stress in the rats

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