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THE ROLE OF *MORINGA OLEIFERA* EXTRACT IN MAINTENANCE OF BRAIN AND TESTIS TISSUES AGAINST AFLATOXIN B_1 (AFB_1) TOXICITY ON LIGHT OF MOLECULAR GENETIC AND HISTOLOGICAL STUDIES IN RATS

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ABSTRACT

Moringa oleifera is a promising plant that accounts for the medicinal uses such as a vital antioxidants, antibiotics and nutrients including vitamins and minerals. The present study was conducted to quantify the genotoxic and histopathological effects of AFB_1 treatment on brain and testis tissues of rats and investigate the protective and curative role of *M. oleifera* leaf extract (MOLE) against AFB_1 toxicity in rats. 72 rats were involved in this study and divided into 9 groups (8 each), normal control (G1), control of DMSO (G2), positive control that injected with AFB1 in DMSO (G3), G 4 to G6 were injected with AFB1 in DMSO plus MOLE as a protective agent at doses 3.3, 4.0 and 4.7 mg/kg respectively, while G7 to G9 of rats received MOLE with the same three doses, post treatment with AFB1, as a therapeutic groups. Molecular genetic and histopathological assessment of brain and testis tissues and immunohistochemical studies were involved. Exposure to AFB1 caused significant elevation of DNA damage, upper-regulation of studied genes in brain and testis tissues, respectively and induced massive damage in cerebral cortex and cerebellum in brain tissues as well as testicular degeneration of seminefrous tubules in testis tissues that confirmed by immunohistochemical results. Protective or therapeutic treatment with MOLE significantly

decreased the DNA damage, suppress the over expression studied genes and histopathological and immunohistochemical damage. Best findings were given with the highest dose of MOLE as curative agent in rats. **Conclusion:** MOLE effectively alleviates AFB1 induced over expression of 5a-R3 and LHR genes and improved the histopathological damages in brain and testis tissues in rats. The consumption of MOLE-basal diet could maintain the integrity of genetic materials and histological architectures against AFB1 toxicity.

KEYWORDS: Aflatoxin B1, MOLE, genotoxicity, histological changes.

INTRODUCTION

Aflatoxins (AFs) were found to be produced as secondary metabolites of fungal strains, *Aspergillus flavus* and *Aspergillus parasiticus*. These fungi can grow on a variety of food and feed commodities.[1] The mentioned fungi can produce AFs in developing crops of rice, wheat, and others.[2] AFB_1 was found to have a low molecular weight, so it passive diffusion into enterocyte and therefore this is suggested as a mechanism of absorption, thus it was found to be efficiently absorbed in the intestinal tract, after ingestion and the duodenum was observed to be the main region of absorption.[3, 4] **Verma and Chaudhari**[5] revealed that the main method for distribution of absorbed AF has been found to be via the blood circulation and it binds with albumin proteins forming AF-albumin adduct.

Also, **Parhizkar *et al.***[6] and **Bonsi *et al.***[7] noted that the binding of AFB_1 to functional proteins cause suppression of protein activity especially in the case of enzymes. Their results also showed that AFB_1 could suppress protein kinase C and Ca^{2+}-ATPase as well as inhibite the cAMP and cGMP hydrolytic activity that was revealed in brain, lung, kidney, liver, heart and spleen tissue extracts. On the other hand, several studies revealed that nitrogen and oxygen in the organic bases of nucleic acid were shown to be susceptible to attack by metabolites of mycotoxins, inducing covalent adducts which cause impairment of DNA and RNA template activity leading to suppression of the DNA, RNA and protein synthesis.[8, 9, 10] Furthermore, **Smela *et al.***[11] noted that these adducts could induce error-prone DNA repair causing base pair substitutions, single-strand breaks leading to frame shift mutations and consequently cause heritable genetic changes. Also these authors reported that mutational profile of AFB_1 was shown to be dominated by one genetic alteration: GC to TA transversion and considered the P53 gene codon 249 at the third position is hotspot for AFB_1-induced mutation, specifically AGGC to AGTC. **Hussain *et al.***[12] revealed in human HCC in the

regions with high exposure of AFs, up to 50% of tumours were observed to be a specific AGG to AGT point mutation in P53 tumor suppressor gene at codon 249. In vitro study, **Abbas *et al.*[13]** noted that the addition of AFB_1 to the cellular of Caco-2 cell had induced DNA fragmentation. In previous study, **Sengstag[14]** found in *S. cerevisiae* yeast strains that exposure to AFB_1 caused mitotic recombination leading to chromosome translocation and events of gene conversion. The exposure to AFs not only induces damage in the liver but also cause injury in other body organs including brain and testes. **Oyelami *et al.*[15]** and **Bonsi *et al.*[7]** reported in brain autopsies of children that those live in regions with high exposure of Aflatoxin the presence of such toxin was 81% of the cases studied and deregulation of signal transduction activity of neuroblastoma cells. Also, in vitro study, **Qureshi *et al.*[16]** observed cytotoxic effect of AFB_1 that represented in the formation of DNA adducts in primary human brain microvascular endothelial cells (HBMEC), which constitute the blood barriers of brain and human umbilical vein endothelial cells (HUVEC). These authors also showed 85% and 22% cell death of HBMEC and HUVEC, respectively. Moreover, **Behrens *et al.*[17]** investigated the effect of fusarium mycotoxins on the blood barrier of porcine brain by cultivating the primary brain capillary endothelial cells (PBCEC) in vitro. Their results showed cytotoxic effects in such cultivated cells and reduction of barrier integrity. **Waggas and Rawi[18]** showed that the ingestion of AFB_1 of Sparague-Dawley rats led to production of brain injury in vivo. Concerning the effect of AFs on reproductive system has been investigated: **Ibeh and Saxena[19] and Agnes and Akbarsha[20]** revealed that the exposure to AFs could induce deleterious effects on spermatogenesis, seminal enzymes, sperms and fertility, as well as reduction of litter size in mothers that mated with males exposed to AFB_1. In adult roosters, **Ortatatli *et al.*[21]** and **Salem *et al.*[22]** observed that the treatment with Aflatoxins (B1, B2, G1 and G2) might inhibit spermatogenesis inducing aberrations in spermatozoa, reduction of serum testosterone, sperm motility. The pathogenesis of brain and testicular damage after or following the treatment with AFB_1 has been generally ascribed to oxidative damage. The exposure to AFB_1 might cause lipid peroxidation and reduction the enzyme activity that protect against oxidative damage in these tissues.[16, 23]

The antioxidants administration such vitamins, flavonoids, carotenoids, niazimicin, amino acids, isothiocyanates, minerals and others might protect against xenobiotic-induced damage.[24, 25] *Moringa oleifera* extracts are known as antioxidants that have been shown to suppress various types of diseases.[26] *Moringa oleifera* leaf extract (MOLE) has attracted considerable attention because it has a strong antioxidant properties and free radical

scavenging capacity.[27, 28] This extraction (MOLE) has been observed to decrease the amount of oxidative DNA damage and also decrease the lipid peroxidation in different mammalian cells in vivo.[28, 29]

The 5 alpha-reductase (5α-reductase) are a family of enzymes in the central nervous system that consisted of three subfamilies and five isozymes which are 5α-R1, 5α-R2, 5α-R3, GPSN2 and GPSN2L proteins that carry out a crucial role in the biosynthesis of androgens by catalyzing the irreversible conversion of testosterone to dihydrotestosterone that determines the male characteristics[30] but 5α-R3 gene which found in the prefrontal cortex of the brain still needs more studies. Additionally, the luteinizing hormone receptor (LHR) is belonging to the three glycoprotein hormone receptors and play very important rule in male sex differentiation, gonadal steroidogenesis, oocyte maturation and ovulation[31] and regulated by 5 alpha-reductase type 3. So, study of gene expression for 5α-R3 and LHR genes are very important.

Cysteinyl aspartate-specific proteases (Caspase) are signaling enzymes, when they activated it can be used as a marker for cell death depending on the subtype and organ involved. The subtype caspase-3 stain pathway is used for detection of apoptosis and cellular assays to quantify inhibitors and activators of the death cascade.[32]

Resultant to these antioxidant properties, MOLE was selected for utilization in this work. So, the objective of this study was to evaluate the role of MOLE in maintenance of brain and testis tissues against AFB_1 toxicity on the light of measurements of DNA damage, gene expression alterations and histological changes in brain and testis tissues of rats. The measurements of DNA damage included the analyses of genomic DNA on agarose gel and by using spectrophotometer. Semi quantitative-PCR method was used to measurement the gene expression of 5a-R3 and LHR genes in brain and testis tissue, respectively. Whereas, the histological analyses had been performed by using H and E stain and immunohistochemical marker through caspase-3 stain.

MATERIALS AND METHODS

Chemicals

Aflatoxin B1 (AFB_1)

Aflatoxin B_1 powder was purchased from Sigma-Aldrich. 5mg of AFB_1 were dissolved in 250 ml of DMSO solvent (Art Nr. 7029.1; 98% by GLC).[33]

Preparation of Moringa oleifera leaves extraction

Ethanolic extract of MOLE was prepared by picking fresh leaves of *Moringa oleifera* plant from trees grown on sand in El-Sharkia governorate, Egypt. The leaves were washed with distilled water and dried under room temperature at (29^{o}C-35^{o}C) for three weeks, after which the leaves were pulverized into coarse form with acrestor high speed milling machine. The coarse form (1000 g) was then macerated in absolute ethanol. This was left to stand for 48h. MOLE was filtered through muslin cloth on a plug of glass wool in a glass column, concentrated and evaporated to dryness using rotary evaporator at optimum temperature between 40^{o} and 45^{o}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.[34]

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. The experimental rats were kept in clean polyprophylene cages and administered food and water ad libitum. All experimental procedures involving animals were conducted in accordance to the ethical guidelines of the Medical Ethical Committee of the National Research Centre in Egypt.[35]

Experimental design

A total of 72 rats were used and divided into 9 equal groups.

G1 (control group): received (i.p.) saline at dose of 0.7 gm/kg. (about 4 times each week for one month).

G2 (DMSO group): received (i.p.) DMSO at dose of 0.7 gm/kg. (about 4 times each week for one month).

G3 (AFB_1 group): received (i.p.) AFB_1 in DMSO (5 mg of AFB_1 were in 250 ml of DMSO) at dose of 0.7 gm/kg.b.wt. (about 4 times each week for one month).

G4 (AFB_1 + M1), G5 (AFB_1 + M2) and G6 (AFB_1 + M3): received (i.p.) AFB_1 in DMSO at the same dose and way previously mentioned and for the same period. Starting on the first day of AFB_1 administration, rats in 4-6 groups were treated (orally) daily for one month with MOLE (3.3 gm/kg M1, 4.0 gm/kg M2 and 4.7 gm/kg M3, respectively) of the crude material that are equivalent to (561. 680 and 799 mg), respectively of the extract, as each gram of the

crude material yields contains 170 mg of the extract. These groups (4-6) used to evaluate the protective role of MOLE against AFB_1.

G7 (AFB_1 then M1), G8 (AFB_1 then M2) and G9 (AFB_1 then M3): received (i.p.) AFB_1 in DMSO in the same dose and way previously mentioned and for the same period, then the groups were treated with MOLE (3.3 gm/kg M1, 4.0 gm/kg M2 and 4.7 gm/kg M3, respectively) for 15 days. These groups (7-9) were used to evaluate the therapeutic effect of MOLE against AFB_1.

Genomic DNA analysis on agarose gel

According to method of **Surzycki**[36], genomic DNA has been isolated from brain and testis tissues of rats, fractioned on agarose gel (1.5%), stained with ethidium bromide and analyzed using gel documentation system.

DNA fragmentation

Liver samples were collected immediately after sacrificing the animals. The tissues were lysed in 0.5 ml of lysis buffer containing, 10 mM tris-HCl (pH 8), 1 mM EDTA, 0.2% triton X-100, centrifuged at 10 000 r.p.m. (Eppendorf) for 20 min at 4°C. The pellets were resuspended in 0.5 ml of lysis buffer. To the pellets (P) and the supernatants (S), 1.5 ml of 10% trichloroacetic acid (TCA) was added and incubated at 4°C for 10 min. The samples were centrifuged for 20 min at 10 000 r.p.m. (Eppendorf) at 4°C and thepellets were suspended in 750 µl of 5% TCA, followed by incubation at 100°C for 20 min. Subsequently, to each sample 2 ml of DPA solution [200 mg DPA in 10 ml glacial acetic acid, 150 µl of sulfuric acid and 60 µl acetaldehyde was added and incubated at room temperature for 24 h.[37] The proportion of fragmented DNA was calculated from absorbance reading at 600 nm using the formula.

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

Gene expression analysis

Extraction of total RNA and cDNA syntheses

Total RNA was extracted from brain and testis tissues of male rats using TRIzol® Reagent Kit (Invitrogen, Germany) according to the manufacturer's protocol of the Kit. Approximately 50 mg of the brain and testes tissues were homogenized in 750 µl of TRIzol® Reagent in autoclaved mortar. Afterwards, total RNA was dissolved and preserved in diethylpyrocarbonate (DEPC)-treated water and preserved at -80°C up to use. To assess the

RNA yield and purity of the total RNA, A small drop of isolated RNA was examined spectrophotometrically at 260 nm. The purity of total RNA was determined between 1.8 and 2.1 to be good purified when it examined by spectrophotometer at the 260/280 nm ratio. RNA was stored at -80°C up to use. To synthesize the complementary DNA (cDNA), isolated RNA from brain and testes tissues was reverse transcribed into cDNA using Oligo $(dT)_{15}$ Primer Maxime RT PreMix Kit (iNtRON Biotechnology). The reaction volume was carried out in 20 µl. The reaction volume was prepared according to the Kit instructions. The reverse transcription (RT) reaction was performed for 60 min at 45^{o}C then the RTase inactivation step was terminated for 5 min at 95°C. The PCR products containing the cDNA were kept at -20°C up to use for DNA amplification.[38, 39] Primer sequence of the brain 5α-R3 gene and testes LHR gene are listed in table 1.[40, 41]

PCR amplification

Polymerase chain reaction was used to amplify the cDNA of male rats to detect the expression values of the tested genes 5α-R3 and LHR. To perform the PCR reaction, a volume of 25 µL of reaction mixtures was prepared containing 12.5 µl of PCR master mix containing (1 µl of 10 pmol of each forward and reverse primers, and 2 µl of the synthesized cDNA. The cDNA was propagated using reaction program consisted of 3 steps. In the first step the PCR tubes was incubated at 95°C for 3 min. In the second step the reaction program consisted of 40 cycles. Each cycle of them consisted of 3 steps: (a) 15 sec at 95°C; (b) 30 sec at 52°C for 5α-R3 gene and 57°C LHR gene; and (c) 30 sec at 72°C. PCR termination was performed at 72°C for 5 min. The relative quantification of the target genes was determined by using the reference (β-actin) gene.

Histological studies

The brain and testis tissues of different groups were removed and fixed in 10% formalin, embedded in paraffin blocks then two sections 4um thick were cut from each block, one section was stained by hematoxylin and eosin (H and E) for histopathological diagnosis, the other section were mounted on positively charged glass slide for immunohistochemical staining (stained with caspase-3) and investigated by light microscope, images were captured and processed using Adobe Photoshop version 8.0.[42]

Immunohistochemical stain for Caspase-3

Paraffin-embedded brain and testis sections were deparaffinized and hydrated. Immunohistochemistry was performed with mouse monoclonal antibodies against caspase-3 for detection of the caspase cleavage. The paraffin sections were heated in a microwave oven (25 min at 720 W) for antigen re-trieval and incubated with either anti-caspase antibodies (1:50 dilution) overnight at 4°C. After washing with PBS, followed by incubation with biotinylated goat-anti-rabbit immunoglobulin G secondary antibodies (1:200 dilution; Dako Corp, Carpinteria, CA, USA) and streptavidin/alkaline phosphatase complex (1:200 dilution; Dako) for 30 min at room temperature, the binding sites of anti-body were visualized with 3,3′-diaminobenzidine DAB (Sigma–Aldrich). After washing with PBS, the samples were counterstained with H&E for 2–3 min, and dehydrated by transferring them through increasing ethanol solutions (30%, 50%, 70%, 80%, 95%, and 100% ethanol). Following dehydration, the slices were soaked twice in xylen at room temperature for 5 min, mounted, examined, and evaluated by a high-power light microscope.

Statistical analysis

Data of gene expression and DNA fragmentation were analyzed using Gel Qutant Net program[43] followed by one way ANOVA and two-way ANOVA to assess significant differences between groups. The values are expressed as mean±SEM. All statements of significance were based on probability of ($P \leq 0.05$). The significance of the differences among treatment groups was determined.[44]

RESULTS

Results of genomic DNA analysis on agarose gel of brain and testis tissues

The analysis of the profile of genomic DNA on agarose gel (Fig. 1, 2) in brain and testis tissues showed different band lanes. In the control groups, the genomic DNA was definite band. Whereas, in AFB_1 group the genomic DNA was observed as damaged bands. In rat groups that received MOLE as protective or therapeutic agent, the bands of genomic DNA were approximately similar with those in control groups and also they were approximately similar with each other.

Results of DNA fragmentation in brain tissues

The present findings (Table 2, Diagram 1) observed high value of DNA fragmentation (24.09 ± 0.69) in rats received AFB_1 as compared to normal control (7.25 ± 0.37). Statistical analysis showed that this value of DNA fragmentation in AFB_1 group was highly significant.

Whereas, in cases of rat groups (G4, G5, G6) that treated with MOLE as protective agent, the harmful of AFB_1 was significantly reduced in respect to rat group that received AFB_1 alone, however the values of DNA damage in protective groups were still more than normal or healthy control. On the other hand, in therapeutic groups (G7, G8, G9), the percentages of DNA fragmentation were more significantly decreased compared with the MOLE ingestion, these values of DNA fragmentation were found to be around those of normal or healthy control.

Results of DNA fragmentation in testis tissues

Concerning the effects of AFB_1 on genomic DNA in rat testis tissues, the results of table 2 and diagram 2 observed that the level of DNA fragmentation (20.61 ± 0.23) was significantly elevated than those of normal control (7.90 ± 0.34). The treatment with MOLE as a protective agent in AFB_1-intoxicated rats of groups G4, G5 and G6, led to significant reduction of DNA fragmentation, and this reduction was observed to be increased by increasing the ingested level of MOLE. The same trend, the rats of DNA fragmentation in therapeutic groups G7, G8 and G9, were more significantly alleviated than of AFB_1 group or as compared to those in protective groups. The best amelioration of genetic material (by reducing the level of DNA fragmentation) was revealed for the last group (The ninth group).

Gene expression of 5α-Reductase Type 3 (5α-R3) gene in brain tissues

The effects of AFB_1 and MOLE treatments on levels of mRNA expression of gene 5α-R3 in male rats were determined in figure 3, table 3 and diagram 3. Compared to normal or healthy control, overexpression levels of 5α-R3 gene for AFB_1 treatment were observed (1.5 VS.5.4), and statistical analysis showed these over-expressions were highly significant ($p<0.001$). In contrast, the levels of mRNA expression were significantly down-regulated for AFB_1-intoxicated rats that received MOLE as protective agents as compared to those found in AFB_1 treatment alone. This reduction of gene expression was noted to be increased by increasing of the ingested dose of MOLE, however, the levels of m RNA expression were still more than those observed in healthy control. On the other hand, the results showed no statistical differences between AFB_1-intoxicated rats that ingested the highest dose of MOLE (as a protective agent) and normal control for mRNA expression. In AFB_1-intoxicated rats that received MOLE as a therapeutic agent, the levels of mRNA expression were markedly reduced as compared to AFB_1 group alone ($p<0.01$ or $p<0.001$). Interestingly, the present findings showed no significant differences for mRNA expression between healthy control

and AFB_1-intoxicated rats that ingested MOLE at three doses. The level of mRNA expression in the last group that received MOLE at the highest dose was found to be around to those of normal control.

Gene expression of Luteinizing Hormone Receptor (LHR) gene in testis tissues

The effects of AFB_1 and MOLE of gene LHR in male rats were determined in Figure 4, table 3 and diagram 4. The present findings revealed upper-regulation of gene expression of LHR gene in rats that exposed to AFB_1 as compared to those found in the normal control (8.4 VS.0.76), and statistical analysis showed that the levels of mRNA expression in AFB_1 group was highly significant ($p<0.001$). Whereas, the levels of mRNA expression in AFB_1-intoxicated rats that received MOLE as a protective agent were notably decreased and statistical analysis showed that these decreases were significant as compared to those observed in the AFB_1 treatment alone ($p<0.05$ or $p<0.01$). However, the results observed that the rates of mRNA expression in the three protective groups were high as compared to those in the normal control. The levels of mRNA expression in AFB_1-intoxicated rats that ingested the MOLE as a therapeutic agent had been obviously reduced ($p<0.001$) than those noted in AFB_1 treatment alone. No significant differences were observed between AFB_1-intoxicated rats that received MOLE at low, medium or the highest doses and normal control for the levels of mRNA expression of LHR gene. The best results for the amelioration of gene expression of LHR gene was found in last group of rats that ingested the highest dose of MOLE.

Histopathological assessment of brain tissue

Cerebral cortex

The findings of this work observed that AFB_1 treatment had induced in cerebral cortex necrotic area, vacuolization, scattered apoptotic cells and neuronal degeneration (Fig. 5C and D). However, in the intoxicated rats with AFB_1 that treated with MOLE as a protective agent, the damaging effect was reduced and this reduction was noted to be increased by increasing the dose of MOLE, where the using of high dose preserved the normal structure of brain tissues in spite of presence of some apoptosis and vacuolated cells (Fig. 5E). Best findings were revealed by utilizing MOLE as a curative agent. The medium and high dose improved the damaging effect more than the low dose. The using of medium dose (Fig. 5F) showed normal cerebral structure with few vacuolated cells. Moreover, intoxicated rats with AFB_1

that received a high dose of MOLE (Fig. 5G), the cerebral cortex structure retained its normal structure.

Cerebellum

The results of this research noted that the AFB_1 treatment had caused distortion of structure and degeneration of purkinje cells (Fig. 6B). In contrast, in the intoxicated rats with AFB_1 that treated with MOLE as a protective or therapeutic agent, the damaging effect was decreased by increasing the dose of MOLE. The using of high dose gave the best results.

Normal cellular arrangement were shown but with purkinje cells degeneration (Fig. 6C) in rats received high dose of MOLE as a protective agent. On the other hand, better results were obtained by using the high dose of MOLE as a curative agent, where the cerebellum structure had been retained to its normal structure (Fig. 6D) and it became close to normal.

Immunohistochemical results

Caspase-3 immunoreactivity

From the present results, it was observed that the using of caspase-3 stain had confirmed the histopathological results. AFB_1 treatment showed positive caspase (Fig. 7B). However, the intoxicated rats with AFB_1 that treated with MOLE as a protective agent, the results showed mild positive caspase (Fig. 7C). Better results were noted in intoxicated rats with AFB_1 and treated with MOLE as atherapeutic agent, where the results showed negative reaction for caspase-3 (Fig. 7D).

Histopathological assessment of testicular tissue

The present examination revealed that AFB_1 treatment induced testicular degeneration of some seminefrous tubules that diagnosed by reducing number of spermatogenic cells and buckling of seminefrous tubule basement membrane (Fig. 9) in comparison to the control group (Fig. 8).

In contrast, the intoxicated rats with AFB_1 that treated with MOLE as a protective agent, the damaging effect was decreased and this decrease was increased by increasing the dose of MOLE, where the using of high dose led to improvement of testicular tissue with moderate changes as buckling of basement membrane and vacuolation in testicular tissue (Fig. 10A and B). On the other hand, better results were obtained by using MOLE as curative agent, where the testicular tissue became normal especially with high dose (Fig. 11A and B).

Immunohistochemical caspase-3

The present findings noted that the using of caspase-3 stain had confirmed the histopathological results. AFB_1 treatment showed positive caspase (Fig. 12B). However, the intoxicated rats with AFB_1 that treated with MOLE especially with high dose as a protective or therapeutic agent (Fig. 12D), the examination showed normal testicular tissues.

Table 1: Primer used for Semi quantitative-PCR amplification.

Gene	Primer sequences (5'-----'3)	
5α-R3	F- TGCCCATCAGTATAAGTGCC	R- TCACCATAAAGCTCGAACCAG
LHR	F- CATTCAATGGGACGACTCTA	R- GCCTGCAATTTGGTGGA
β-actin	F- TCGTGCGTGACATTAAAGAG	R- ATTGCCGATAGTGATGACCT

Table 2: The values of DNA fragmentation in brain and testis tissues of rat groups received AFB1 and treated with *Moringa oleifera* leave extract (MOLE).

Groups	DNA fragmentation (M±S.E.)	
	Brain	Testis
G1	7.25 ± 0.37^{a}	7.90 ± 0.34^{a}
G 2	8.06 ± 0.25^{a}	8.40 ± 0.41^{a}
G 3	24.09 ± 0.69^{e}	20.61 ± 0.23^{d}
G4	14.86 ± 0.34^{d}	13.42 ± 0.41^{c}
G5	13.79 ± 0.63^{cd}	12.33 ± 0.42^{c}
G6	13.11 ± 0.66^{c}	12.14 ± 0.51^{c}
G7	10.90 ± 0.29^{b}	10.27 ± 0.49^{b}
G8	8.47 ± 0.24^{a}	10.24 ± 0.54^{b}
G9	8.28 ± 0.18^{a}	9.86 ± 0.56^{b}

Data expressed as mean±SE. Values followed by different superscript letters are significantly different from one another within the same columns.

Table 3: Gene expression of 5α-R3 and LHR genes levels in brain and testis tissues of male rats administer AFB_1 and treated with MOLE.

Tissue	G1	G2	G3	G4	G5	G6	G7	G8	G9
Brain	1.5^{a}	1.9^{a}	5.4^{d}	2.92^{c}	2.64^{bc}	2.2^{ab}	1.98^{ab}	1.86^{a}	1.56^{a}
Testis	0.76^{a}	0.9^{a}	8.4^{e}	6.1^{d}	6.0^{d}	2.26^{c}	1.48^{b}	1.3^{ab}	1.08^{ab}

***Data expressed as mean±SE. Values followed by different superscript letters are significantly different from one another within the same columns.**

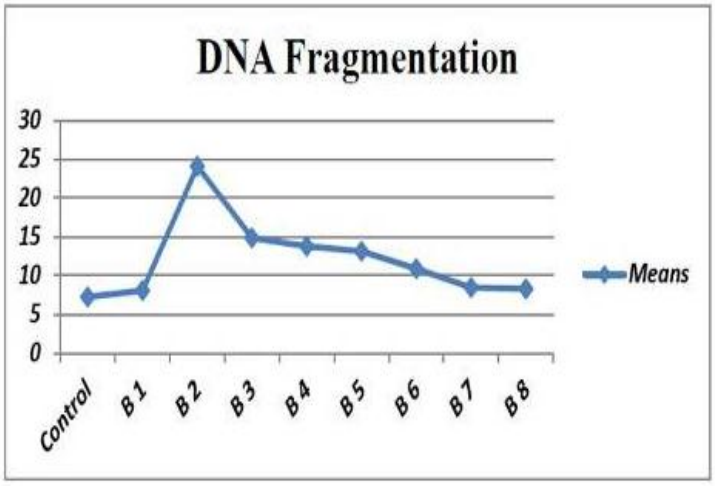

Diagram 1: DNA fragmentation analysis of brain tissues of male rats treated with *Moringa oleifera* leave extract (MOLE) against AFB_1. B1= Solvent (DMSO). B2= AFB1. B3= AFB_1+ low dose of MOLE. B4= AFB_1+ Medium dose of MOLE. B5= AFB_1+ High dose of MOLE. B6= AFB_1 then low dose of MOLE. B7= AFB_1 then medium dose of MOLE. B8= AFB_1 then high dose of MOLE.

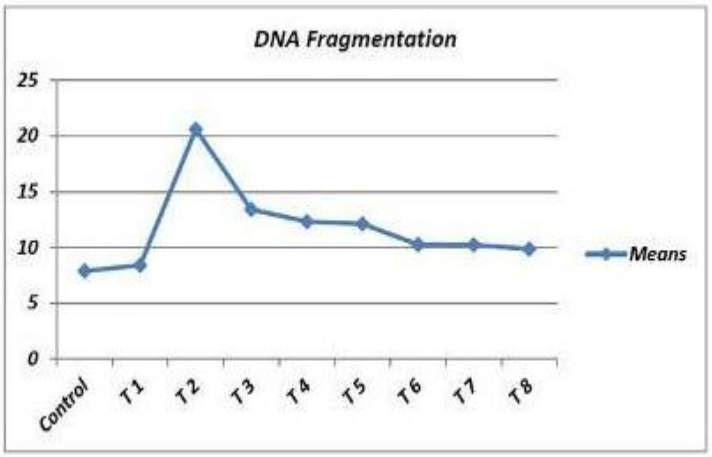

Diagram 2: DNA fragmentation analysis of testis tissues of male rats treated with *Moringa oleifera* leave extract (MOLE) against AFB_1. T1= Solvent (DMSO). T2= AFB_1. T3= AFB_1+ low dose of MOLE. T4= AFB_1+ Medium dose of MOLE. T5= AFB_1+ High dose of MOLE. T6= AFB_1 then low dose of MOLE. T7= AFB_1 then medium dose of MOLE. T8= AFB_1 then high dose of MOLE.

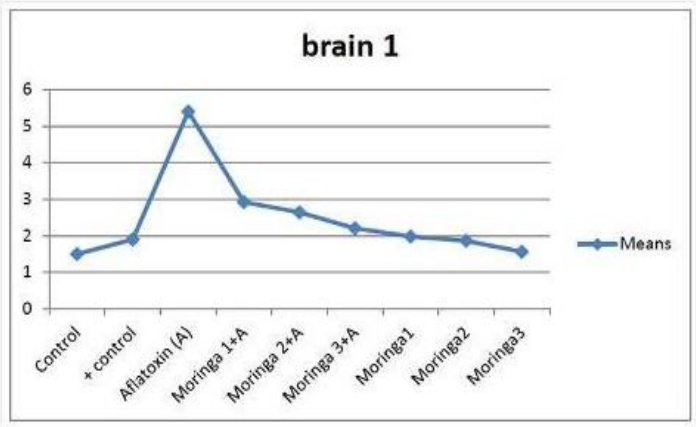

Diagram 3: Shows the expression levels of 5α-R3 gene in the brain tissues of male rats treated with *Moringa oleifera* leave extract (MOLE) against AFB_1 determined by Semi quantitative PCR. M= DNA Marker. Lane 1= Control. Lane 2= Solvent (DMSO). Lane 3= AFB_1. Lane 4= AFB_1+ low dose of MOLE. Lane 5= AFB_1+ Medium dose of MOLE. Lane 6= AFB_1+ High dose of MOLE. Lane 7= AFB_1 then low dose of MOLE. Lane 8= AFB_1 then medium dose of MOLE. Lane 9= AFB_1 then high dose of MOLE.

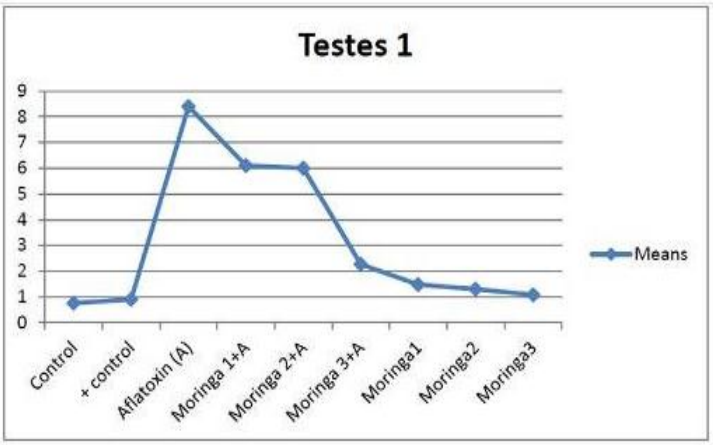

Diagram 4: Shows the expression levels of LHR gene in the testis tissues of male rats treated with *Moringa oleifera* leave extract (MOLE) against AFB_1 determined by Semi quantitative PCR. M= DNA Marker. Lane 1= Control. Lane 2= Solvent (DMSO). Lane 3= AFB_1. Lane 4= AFB_1+ low dose of MOLE. Lane 5= AFB_1+ Medium dose of MOLE. Lane 6= AFB_1+ High dose of MOLE. Lane 7= AFB_1 then low dose of MOLE. Lane 8= AFB_1 then medium dose of MOLE. Lane 9= AFB_1 then high dose of MOLE.

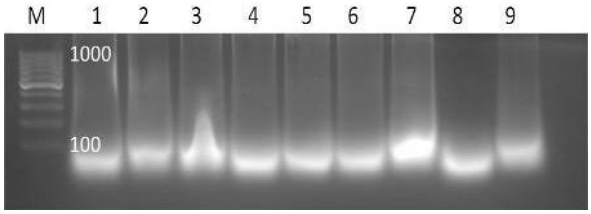

Fig. 1: Genomic DNA analysis of brain tissues on agarose gel of male rats treated with *Moringa oleifera* leave extract (MOLE) against AFB_1. M= DNA Marker. Lane 1= Control. Lane 2= Solvent (DMSO). Lane 3= AFB_1. Lane 4= AFB_1+ low dose of MOLE. Lane 5= AFB_1+ Medium dose of MOLE. Lane 6= AFB_1+ High dose of MOLE. Lane 7= AFB_1 then low dose of MOLE. Lane 8= AFB_1 then medium dose of MOLE. Lane 9= AFB_1 then high dose of MOLE.

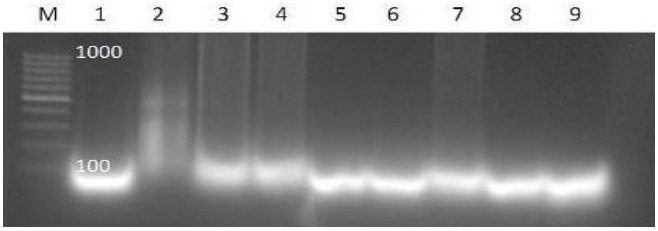

Fig. 2: Genomic DNA analysis of testis tissues on agarose gel of male rats treated with Moringa oleifera leave extract (MOLE) against AFB1. M= DNA Marker. Lane 1= Control. Lane 2= AFB1. Lane 3= Solvent (DMSO). Lane 4= AFB1+ low dose of MOLE. Lane 5= AFB1+ Medium dose of MOLE. Lane 6= AFB1+ High dose of MOLE. Lane 7= AFB1 then low dose of MOLE. Lane 8= AFB1 then medium dose of MOLE. Lane 9= AFB1 then high dose of MOLE.

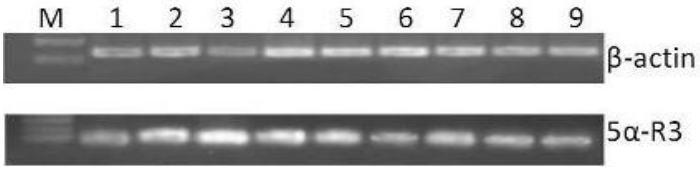

Fig. 3: Shows the expression levels of 5α-R3 gene in the brain tissues of male rats treated with *Moringa oleifera* leave extract (MOLE) against AFB_1 determined by Semi quantitative PCR. M= DNA Marker. Lane 1= Control. Lane 2= Solvent (DMSO). Lane 3= AFB_1. Lane 4= AFB_1+ low dose of MOLE. Lane 5= AFB_1+ Medium dose of MOLE. Lane 6= AFB_1+ High dose of MOLE. Lane 7= AFB_1 then low dose of MOLE. Lane 8= AFB_1 then medium dose of MOLE. Lane 9= AFB_1 then high dose of MOLE.

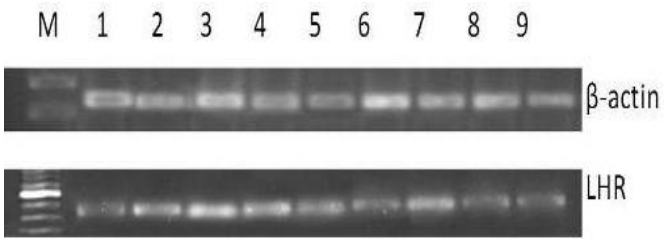

Fig. 4: Shows the expression levels of LHR gene in the testis tissues of male rats treated with *Moringa oleifera* leave extract (MOLE) against AFB_1 determined by Semi quantitative PCR. M= DNA Marker. Lane 1= Control. Lane 2= Solvent (DMSO). Lane 3= AFB_1. Lane 4= AFB_1+ low dose of MOLE. Lane 5= AFB_1+ Medium dose of MOLE. Lane 6= AFB_1+ High dose of MOLE. Lane 7= AFB_1 then low dose of MOLE. Lane 8= AFB_1 then medium dose of MOLE. Lane 9= AFB_1 then high dose of MOLE.

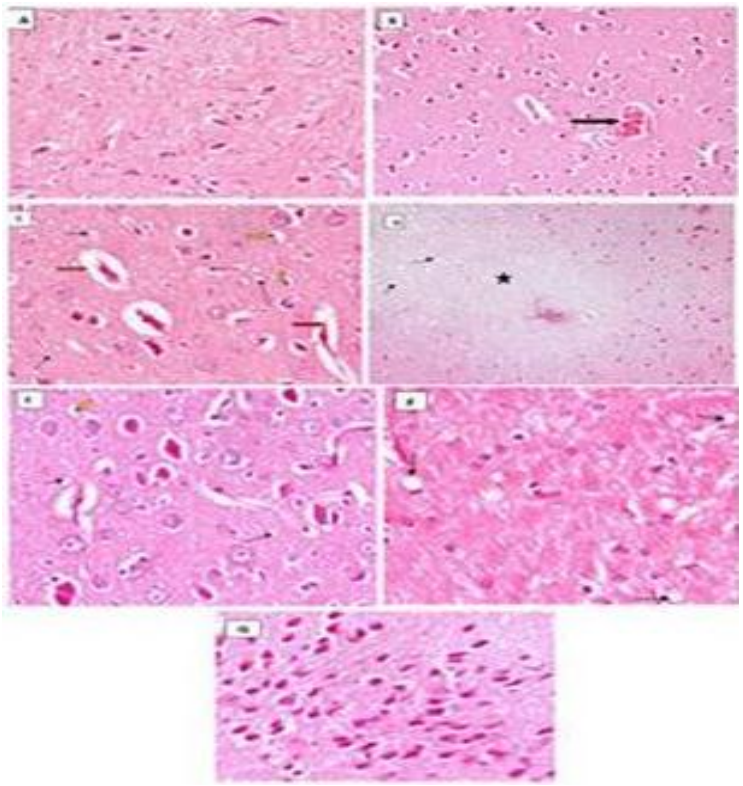

Fig 5: Photomicrographs of representative brain sections from the cerebral cortex of rats. In (A): Control group showed normal structure. In (B): DMSO treated group showed vascular congestion (black arrow). In (C& D): AFB_1 treated group, the examination showed necrotic area (star), vacuolization (thin arrow), scattered apoptotic cells (yellow arrow) and marked neuronal degeneration (red arrow). In (E): The intoxicated rat with AFB_1 and treated with MOLE (4.7 mg/kg) as a protective agent, the examination showed less vacuolated cells (thin arrow) and apoptotic cells (yellow arrow). In (F): Brain tissues of rats treated with MOLE (4.0 mg/kg) as a curative group

after cessation of AFB_1 treatment showed normal cerebral structure with few vacuolated cells (thin arrow). In (G): Brain tissues of rats treated with MOLE (4.7 mg/kg) as a curative group after cessation of AFB_1 treatment showed normally appearing neuron (H and E 100,200).

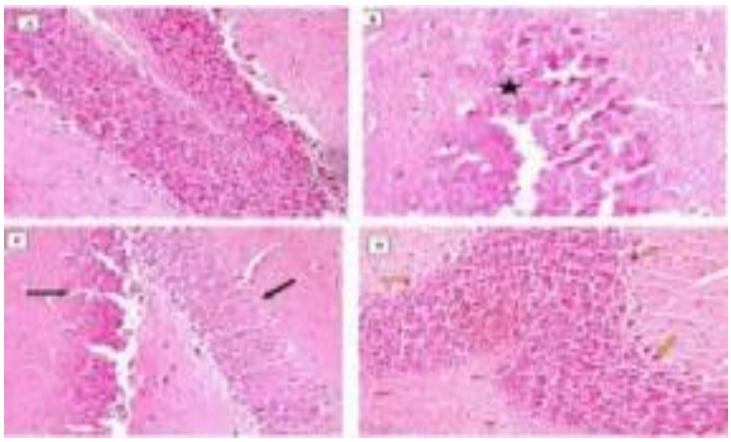

Fig 6: Photomicrographs of representative brain sections from the cerebellum of rats. In (A): Control group showed normal Purkinje cells. In (B): AFB_1 treated group showed distortion in cell arrangement with loss of purkinje cells (star). In (C): AFB_1 and MOLE (4.7 mg/kg) treated protective group showed normal cellular arrangement with marked degeneration of purkinje cells (balck arrow). In (D): AFB_1 then MOLE (4.7 mg/kg) as curative treated group showed presence of purikinje cells (yellow arrow). (H and E 100,200).

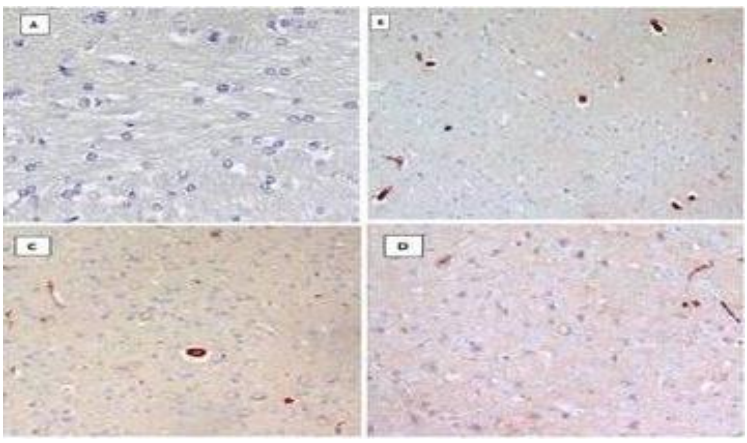

Fig 7: Caspase-3 expression in the cytoplasm of the brain cells in the cerebral cortex. In (A): Normal negative control. In (B): AFB_1 group showed positive stain. In (C): In groups of intoxicated rats with AFB_1 and received MOLE as a protective agent showed mild positive stain. In (D): Brain tissues of rats treated with MOLE after cessation of AFB_1 treatment showed negative stain. (caspase-3 sain 100,200).

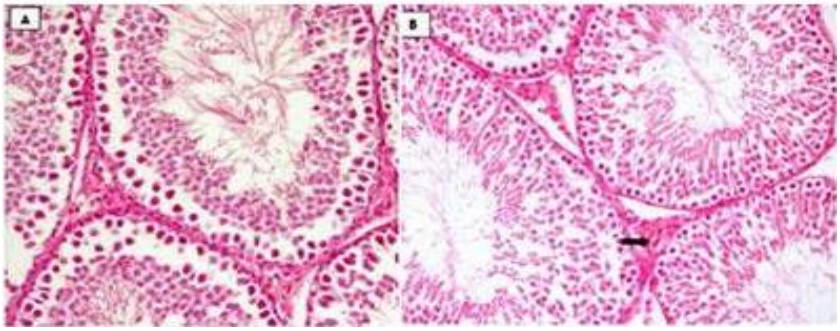

Fig 8: Photomicrograph of control group of testis tissues showed normal testicular structure in (A): DEMSO group in (B): Testis tissues showed normal testicular structure with vascular congestion in interstitial tissue (H and E 200,100).

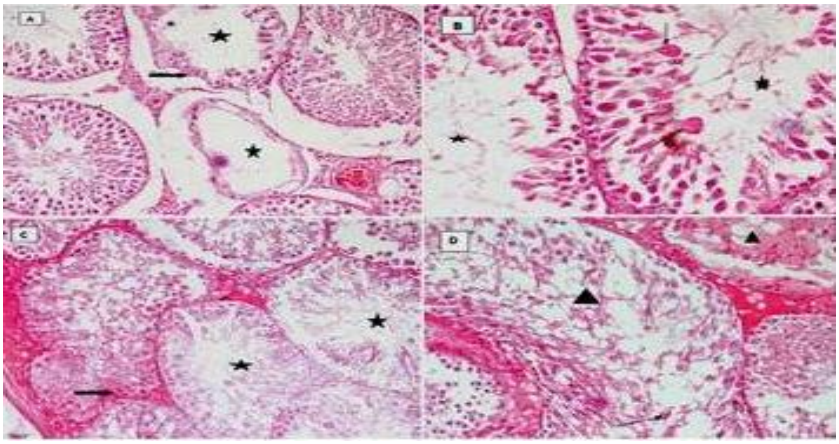

Fig 9: Photomicrograph of testicular tissue of rats treated with AFB_1 in (A to D) showing Peritubular oedema and atrophy of some tubules with decrease number of spermatogenic cells (star), buckling of basement membrane (thick arrow), appear of giant multinucleated giant cells (thin arrow) and vacuolation of spermatogenial cells (H and E 100.200).

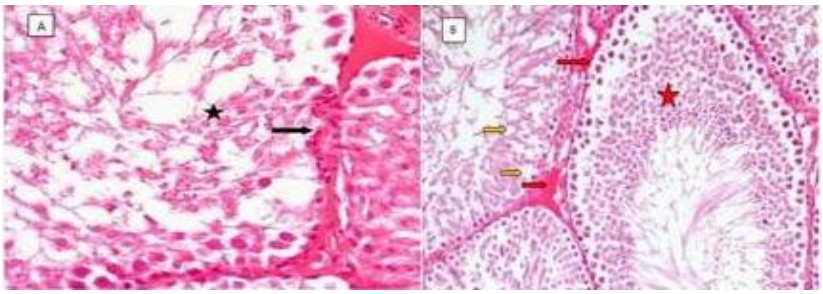

Fig 10: Photomicrograph of testicular tissue of rats treated with AFB_1 and MOLE. In (A): The intoxicated rat with AFB_1 and treated with MOLE (4.0 mg/kg) as a protective agent, the examination showed mild regression of normal architecture of testicular tube (black star) with mild buckling of basement membrane (thick arrow). In (B): The

intoxicated rat with AFB_1 and treated with MOLE (4.7 mg/kg) as a protective agent, the examination showed few tubules with mild vacuolation (yellow arrow), deposition of hyaline material between tubules (red arrow) and some of tubules return normal (star) (H and E 200.100).

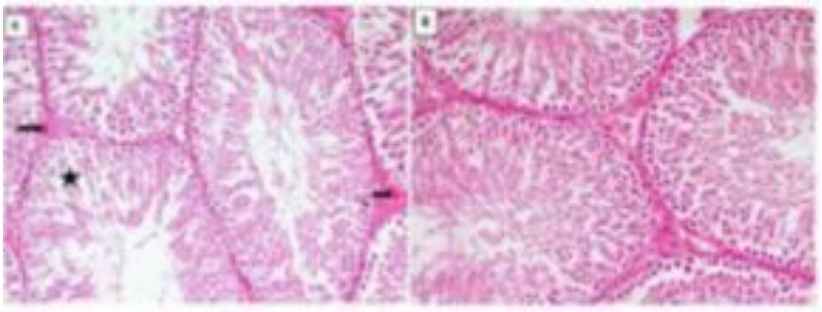

Fig 11: Photomicrograph of testicular tissue treated with AFB1 then MOLE. In (A): Testis tissues of rats treated with MOLE (4.0 mg/kg) as a curative group after cessation of AFB_1 treatment showed normal testicular tube in most of section except few tubules with distorted cellular pattern (star) and minimal hyaline deposition (arrow). In (B): Testis tissues of rats treated with MOLE (4.7 mg/kg) as a curative group after cessation of AFB_1 treatment showed normal testicular tissue (H and E 100).

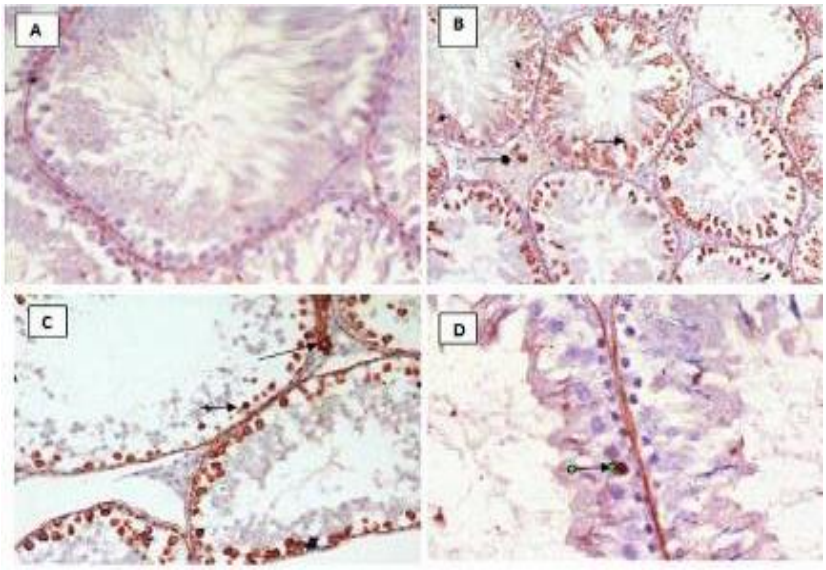

Fig 12: Photomicrograph of testicular tissue stained with caspase-3. In (A): Normal tissue with negative stain. In (B and C): Testis tissues in AFB1 treatment showed positive caspase stain in the tube and interstial tissues (arrows). In (D): Testis tissues in rat group that received MOLE showed normal testicular tissues.

DISCUSSION

The present investigation used 5α- R3 and LHR genes to test the toxic effect of AFB1 on their gene expressions and to evaluate the protective and therapeutic role of MOLE agianst AFB toxicity. 5α-reduced neurosteroid of progestrone is considered to be among the strongest known ligands of the ɣ-aminobutyric acid type A receptor complex in the central nervous system. This factor was found to have important role for protecting neurons against apoptosis.

Also, the 5α- R3 gene was found to have a masculinizing role in some regions of brain converting testosterone into the more potent androgen dihydrotestosterone.[40, 45]

The luteinzing hormone receptor (LHR) is considered to be a glycoprotein that found in the cell membrane of testis and ovary cells. The LHR has a main role in reproductive physiology. Also, it was revealed that there are two interaction activities of LHR with pathway of intracellular signals that include the production of adenylyl cyclase-stimulate cAMP inositol phosphates and increase of intracellular free Ca^{2+}.[46, 47]

The present work showed that the exposure to AFB_1 cauced significant elevation of DNA damage in brain and testis tissues. Also, the treatment with AFB_1 significantly uper-regulated of the gene expression of each 5α-R3 gene in brain tissue and LHR gene in testis tissues.

In brain tissue, the present results were supported by the report of **Qureshi *et al.*[16]**, who revealed that AFB_1 could pass the blood-brain barriers by affecting its integrity. These authors treated in vitro the primary human brain microvascular endothelial cells (HBMEC) (that constitute the blood-brain barrier) and human umbilical vein endothelial cells (HUVEC) with AFB_1. Their results found that HBMEC were more susceptible to cytotoxic effect of AFB_1 followed by HUVEC by inducing AFB_1-N7-guanine adducts (AFB1 DNA adducts) formation. This toxic effect was markedly increased by increasing the concentration of the toxin. In vivo studies, **Oyelami *et al.*[15]** reported that the lipophilic nature of AFB_1 allow its storage in children brain tissues causing cell damage. Also, **Waggas and Rawi[18]** reported that the ingestion of AFB_1 for Sprague-dawley rats induced brain injury in vivo. In previous study, **Bonsi *et al.*[7]** reported that this toxin (AFB_1) had been known to deregulate the activity of signal transduction of neuroblastoma cells. In fibroblast cells of chicken, **Iwaki *et al.*[48]** observed that most incorporated AFB_1 had been isolated from lipid fractions as opposed to DNA, RNA and protein incorporation in chicken primary hepatocytes.

Also, in testis cells, the present results were similar with that noted by **Abd El-Rahim *et al.*[49]**, who reported that the rate of DNA fragmentation was found to be significantly increased in testis cells of mice that exposed to AFB1 treatment.[49, 50] Also, the inducing of DNA fragmentation in testis and ovarian cells of mice that exposed to AFB1 was observed to be high frequencies as compared to those noted in the control.[50] The exposure to AFB_1 significantly induced DNA fragmentation in Coco-2cells in vitro.[51] Also high rates of DNA fragmentation were also observed in quail fed basal diet polluted with AFB1.[52]

Concerning the alternations in gene expression of the studied genes in the present work, the our findings were similar with that revealed by **Bbosa *et al.*[53]** who observed that the oxidative stress due to the AFB treatment had induced overexpression of some nuclear receptors including arylhydrocarbon receptor (AHR), pregnant x receptor (PXR) and constitutive androstane receptor (CAR). In previous study, **Wang *et al.*[54]** reported that AFB_1 treatment could induce DNA adduct causing gene mutation and altering gene expression in the testis. **Yang *et al.*[55]** observed abnormal gene expression of cancer-related miRNA in rat liver tissues that exposed to AFB_1.

Also in another studies, the inducing of oxidative stress due to different toxicant treatments such cytokines, propiconazole, phenols and CCL_4 had been found to cause overexpression of different types of cytochrome P450 genes in livers of different animal species.[50, 56, 57, 68]

AFs had been found to be highly liposoluble, the lipophilic property make these toxins readily absorbed through gastrointestinal tract once there are injected or ingested, then enter the blood-stream and reach the liver.[16] In liver, the biotransformation enzymes (cytochrome P450 group) converte the AFB1 into its epoxide form. This epoxide form can reeach blood circulation and affect other body organs and cells including brain and testis.[15, 16, 23, 59, 60]

The reactive epoxide was found to have ability to bind with serum albumin resulting in albumin adduct[61] and it can also bind with guanine residues of DNA structure forming AFB1-N7-guanine adduct and alterating guanine to thiamine mutation.[62] These adducts could induce DNA damage and consequently alter the gene expression.[59] So, AFB_1 treatment has the ability to modulate the activity of genomic DNA by forming DNA lesions leading to abnormal genetic events and genomic instability including gene expression alterations.[57]

The present data noted that the treatment with MOLE to intoxicated rats significantly decreased each of DNA damage and overexpression of LHR and 5α-R3 genes as compared to AFB_1 treatment alone. Similarly, **Eshak *et al.*[50]** noted that the treatment with MOLE to intoxicated rats with CCL4 significantly decreased each of DNA damage and overexpression of CYP1A2 and CYP2B1 with respect to treatment with CCL_4 alone.

The present results indicated that MOLE has capable to make the protective and therapeutic role against AFB_1 mutagenicity in brain and testis tissues. This genetic amelioration of MOLE might be related to its containing on rich natural antioxidants[63, 64, 65] that can interfere with cytochrome enzymes (P450) participating in biotransformation of AFB_1 to genotoxic metabolities[63, 66] leading to prevention or decrease the oxidative stress and consequently reduce the inducing of DNA damage and upper-regulation of gene expression of LHR and 5α-R3 genes.

The MOLE was observed to enhancement the stimulation of antioxidant enzymes including Catalase (CAT) and superoxide dismutase (SOD) that can remove or scavenge reactive oxygen species (ROS) or oxygen radicals and repair genetic damage induced by oxidative stress.[67] Also, MOLE was found to have polyphenols, these components are considered to be strong antioxidants that have significant capability for regulation of gene expression of different genes.[67, 68]

Abdou *et al.*[69] reported that polyphenols can suppress gene expression in androgen-independent protest cancer cells and inhibit the growth of cancer cells through interfering with its genetic factors. Moreover, MOLE has a high content of alkaloids that were observed to act as scavenging the ROS or oxygen radicals avoiding and repairing the genetic damage induced by oxidative stress through ROS formation and consequently could improve the immune defense and reduce the risk of different diseases.[50, 63]

The present histopathological examination clarified that AFB_1 treatment could induce sever damage effects on brain and testis tissues. In the rat brain that treated with AFB_1, the histopathological results showed degeneration of most neuronal cells, scattered apoptosis and necrotic area. Also, in testis tissues, AFB_1 treatment caused degeneration of most seminefrous tubules, absence in tubular lumen and congested blood vessels. Immunohistochemical results by using caspase-3 stain confirmed the histopathological examination. Similar histopathological changes in rat brains that exposed to AFB_1 had been

previously reported by **Soliman *et al.*[70]** and **Bahy *et al.*[71]**, who proved that AFB_1 had induced many histopathological changes in rat brain tissues such cellular degeneration, blood vessels dilatation and decrease in the neuronal cell number.

Moreover, the changes of testicular tissues that were observed in this study are in agreement with previous studies on rabbits[72, 73, 74], rats[23, 75, 76], where AFB_1 had induced alot of regression alterations of different intensity in the germinal epithelium of seminefrous tubules resulting in a many dystrophic changes of the spermatogenic epithelium along with edematous alterations in the interstitial tissue of adult male animals.

Oxidative stress generally induced following AFB_1 exposure by generating of oxygen-derived species and formation of different organs of the body involving brain and testis tissues.[16, 26, 77]

In brain tissues, **Qureshi *et al.*[16]** revealed that the AFB_1 treatment could pass the blood-brain barriers causing brain tissue injury. Previously, **Oyelami *et al.*[15]** noted that lipophilic nature of AFs allow their storage in brain tissues and consequently inducing histopathological conditions.

In testis tissues, **Atessahin *et al.*[78]** and **Tas *et al.*[23]** reported that the formation of reactive oxygen species (ROS) and lipid peroxidation due to the exposure to AFB_1 could induce reduction of the antioxidant enzyme activities and consequently cause pathological cases of testis organ. Moreover, **Tas *et al.*[23]** found significant increases of MDA concentration in rat testis tissues that exposed to AFB_1. This increase of rate of MDA confirmed the impaired immunonodulation that lead to induce DNA damage and cell injury.

In the present histological examination sever damage affects that observed with AFB_1 treatment on brain and testis tissues of rats had been partially prevented and markedly reduced using MOLE as a protective or therapeutic agent, especially with high dose. Also, immunohistochemical results using caspase-3 stain confirmed the amelioration of MOLE on brain and testis tissues of intoxicated rats with AFB_1. Similarly, **Omotoso *et al.*[79]** found that *Moringa oleifera* extracts had protective effect on brain cells against nicotine toxicity. Also, **Zade *et al.*[80]** observed improvements of sexual activity of male Albino rats that administrated *Moringa oleifera* seeds as compared to control. This amelioration of brain and testis tissues that noted in the present study might be due to antioxidant components in

MOLE as reported by **Bertling *et al.***[81], who recorded 1.8 mg/sup as a total antioxidants, 2.0 mg/sup as leaf-ascorbic acid and 64.1 mg/sup as a total phenol. Also, **Hamza**[82] stated that *Moringa oleifera* extracts had significant suppression or scavenging effect on inducing 1.1-diphenyl-2-picryl-hydrazol free radical, this finding indicates that such medicinal plant could act against AFB_1-induced brain and testis injury in rats through mechanism that related to its antioxidant properties and anti-inflammatory effect.

Moreover, **Chadamas *et al.***[83] reported that *Moringa oleifera* extracts could ameliorate liver enzymes including in the reaction of enzymes of phase 1 (cytochrome bs and cytochrome P450) and phase 11 (glutathione S-transferase), where these enzymes had been observed to be responsible for the detoxification of xenobiotic substances, thus these findings suggested that MOLE can reduce the brain and testis injury induced by AFB_1. **Sreelatha and Padma**[84] found that the administration of MOLE to intoxicated rats with CCL_4 could reduce the high levels of phospholipids, cholesterol, triglycerides and free fatty acids and restore to their normal levels. These results indicated that MOLE could reduce the massive damage induced by AFB_1 on brain and testis tissues. In another study, **Khalafalla *et al.***[32] revealed that MOLE had capability for killing 70-86% of leukemia cells that were obtained of some patients and cultured in vitro.

Conclusion: The present work could supply with a scientific rational to utilization of MOLE in the management of brain and testis diseases especially induced by Aflatoxins contamination. MOLE effectively alleviates AFB_1 induced over expression of 5a-R3 and LHR genes and improved the histopathological damages in brain and testis tissues in rats. The consumption of MOLE-basal diet could maintain the integrity of genetic materials and histological architectures against AFB_1 toxicity.

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