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Research article

Moringa extract reduces DNA fragmentation and ameliorates Tamoxifen®-induced hepatotoxicity in rats

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Key words: Hepatotoxicity, Tamoxifen®, *Moringa oleifera*, DNA, Rat.

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Abstract

Breast cancer is the most common cancer among women, and it causes the most deaths after lung cancer. Tamoxifen® (TMX) is a known chemopreventive agent that widely used for treatment of breast cancer; however it presents several side effects. The objective of this study was to elucidate the efficacy of Moringa aqueous extract (MAE) in reduction of DNA-fragmentation and amelioration of the hepato-pathophysiological complications sided by TMX. Adult male Wistar rats (150-170g) were randomly divided into four groups (10 animals each group) as follows: group (1): Rats administrated with saline and served as control, group (2): Rats orally administrated with MAE (300 mg/Kg b.wt./day), group (3) Rats intoxicated with TMX (3mg/Kg/3days), and group (4); Rats daily treated with MAE in combination with TMX. After consecutive six weeks, the results revealed that the administration of MAE markedly restored the pathophysiological deteriorations resulted from TMX-intoxication; This was evidenced by the marked reduction in serum ALAT, ASAT, GGT, ALP and bilirubin as well as the improvement in the serum level of total protein, albumin, creatinine, urea, Na and K. Moreover, the microscopic examinations showed marked regeneration of the hepatocytes. These findings were in line with the reduction of DNA-fragmentation and could be mechanized through the antioxidant and anti-inflammatory battery of moringa constituents. In conclusion, MAE could play a beneficial role for prevention of TMX-induced pathophysiological distortions. So, it may be worthy to consider the beneficial use of moringa extract as a supplement with the TMX therapy.

Introduction

Cancer breast is one of the most common invasive cancers in females; it affects nearly 12% of women around the world [1]. Tamoxifen® is an anti-estrogenic compound, which has been confirmed to significantly reduce the rate and time to reoccurrence of the breast cancer [2]. Recently, the TMX and its derivatives can also be used in prevention of the breast cancer [3]. However, with these beneficial effects, it can increase the risk for endometrial cancer in these patients [4]. The risks for endometrial cancer associated with TMX therapy are very similar to the risks for breast cancer associated with hormone replacement therapy [5]. In both cases, the long term administration of an estrogen or estrogen like compound results in a small but significant increase risk for cancer [6].

The *Moringa oleifera* is belonging to the family of Moringaceae, it is an effective remedy for malnutrition. Moringa has a variety of essential phytochemicals present in its leaves, pods and seeds. In fact, moringa provide 7 times more vitamin C than oranges, 10 times more vitamin A than carrots, 17 times more calcium than milk, 9 times more protein than yoghurt, 15 times more potassium than bananas and 25 times more iron than spinach [7].

The presence of phytochemicals makes it a good medicinal agent. Several studies have shown that, moringa can act as an anti-diabetic agent. It has been shown that the aqueous extracts of MO can cure streptozotocin-induced Type 1 diabetes and also insulin resistant Type 2 diabetes in the rats [8]. Cancer treatments like surgery, chemotherapy and radiation are expensive and have serious side effects. The MAE can also be used as an anticancer agent, as it is natural, reliable and safe.

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The moringa can be used as an anti-neoproliferative agent, thereby inhibiting the growth of cancer cells. Soluble and solvent extracts of Moringa leaves have proven its effective role as anticancer agents. Furthermore, the research papers have suggested that the anti-proliferative effect of cancer might due to its ability to induce reactive oxygen species in the cancer cells. The reactive oxygen species (ROS) induced in the cells leads to apoptosis, proved by the up regulation of caspase 3 and caspase 9, which are part of the apoptotic pathway [9]. Moreover, the ROS production by moringa is specific, and targets only the cancer cells, making it an ideal anticancer agent.

Moringa oleifera leaves were utilized in treatment of different diseases. Also, Moringa leaves can be employed for humans and animals treatment; can be taken as food supplementary. Moringa leaves include about 27% protein, vitamins and beneficial phytoactives [10].

The main goal of this study was to investigate the efficiency of Moringa aqueous extract (MAE) in reduction of DNA-fragmentation and amelioration of the pathophysiological complications sided by TMX in a strategy to minimize drug side effects and enhance patients' body tolerance.

Materials and methods

Chemicals

Tamoxifen citrate (TAM) was a kind gift obtained from medical union pharmaceutical drug company (MUP), Egypt. All the other chemicals were of analytical grade and purchased from Sigma (St. Louis, USA) and Fluka (Buchs, Switzerlands).

Herb extraction

This study dealt with the aqueous extract of the herb rather than that of organic solvents; this due to the possible effects of the organic solvents on the conformation and configuration structure of the extract components. *Moringa oleifera* was purchased from the stores of the local supplier, Abd El-RahmanHarraz (Bab El-Khalk zone, Cairo, Egypt).The herb was scientifically clearly identified by a special botanist, faculty of pharmacy, Cairo University and was found carry taxonomic serial number (TSN 503874). The aqueous extract was carried out according to the method of Berkovich *et al.* [11]. Fifty g of powdered dry herb leaves were soaked in 500 ml boiling distilled water for 3 hours; then filtered through sterile Whatman filter paper number 42 (Whatman International Ltd, Maidstone, England) and lyophilized using freeze drier (Snijders- Scientific-tilburg, Holland) under pressure, 0.1- 0.5 mBar and temperature -35- 41°C conditions; the dry extract was stored in a dark bottle at -80°C until usage.

Determination of total extract yield

The filtrate was transferred to a quick fit round bottom flask with a known weight (W_1), then freeze-dried and weighed again (W_2). Finally, the yield was calculated from the following formula:

$$\text{Yield (g/ g crude herb)} = \frac{W2 - W1}{W3}$$

Where, W_1 is the weight of clear and dry quick fit flask in grams, W_2 is the weight of the flask with the extract after lypholization in grams, and W_3 is the weight of the crude powdered herb in grams.

Determination of total phenolics content

The content of total phenolic compounds in the aqueous extract was analyzed spectrophotometrically using modified Folin–Ciocalteu colorimetric method of Jayaprakasha *et al.* [12]. In brief, 5 mg of the extract was dissolved in a 10 ml mixture of acetone and water (6:4 v/v). - Samples (0.2 ml) mixed with 1.0 ml of 10-folds diluted Folin-Ciocalteu reagent and 0.8 ml of sodium carbonate solution (7.5%). After 30 min at room temperature, the absorbance was measured at 765 nm using UV – 160 1PC UV-visible spectrophotometer (Shimadzu, Japan). Total phenolic content as catechin equivalents (CE) was monitored from standard curve.

Determination of radical scavenging activity (RSA)

The capacity of antioxidants in the extracts to quench DPPH radical was determined using the method of Nogala-Kalucka *et al.* [13]. In this method, dissolve a certain weight of the extract in methanol (MeOH) to obtain a concentration of 200 ppm. A volume of 200µl from this solution was made up to 4 ml by MeOH. Add 1 ml of DPPH (6.09 x 10-5 mol/l) solution (in MeOH), and after 10 minutes the absorbances of both tested and control sample [1 ml of DPPH solution (6.09 x 10-5 mol/l) and 4 ml MeOH] were measured at 516 nm using spectrophotometer (UV-Visible, Shimadzu, Japan) using. The radical scavenging activity of the extract was calculated according to the following equation:

RSA% = [absorbance of control sample - absorbance of tested sample]/absorbance of sample) x 100.

Animals and experimental design

Adult male albino rats (150-170 g) were obtained from the Animal Colony, National Research Centre, Egypt. Animals were maintained on free access under standard laboratory condition (25±2 °C; 12h/12h light/ dark cycles) to food and water for a week before starting the experiment for acclimatization. Along the experimental duration, all animals received human care in compliance with the standard institutions' criteria for the care and use of experimental animals according to ethical committee of National Research Centre (FWA 00014747); however, this study was approved by this ethical committee. After being acclimatized, the animals were divided randomly

into four groups (10 animals each): group (1) included healthy rats administrated orally with saline for six weeks and served as control, group (2) included healthy rats orally administrated with MAE (300 mg/kg/day) for six weeks, group (3) included rats intoxicated with Tamoxifen (TMX) alone (3 mg/kg/3days) for six weeks, and group (4) included rats daily administrated with MAE in combination with TMX at the same routes, doses and duration.

Blood and tissue sampling

At the end of treatment period (six weeks), rats were weighed then fasted overnight. Following anesthesia (inhalation with diethyl ether), blood specimens were withdrawn from the retro-orbital plexus using heparinized and sterile glass capillaries, left 20 minutes till clot, and cool-centrifuged at 3000 rpm for 10 minutes; the sera were separated, divided into aliquots and stored at -80°C till biochemical measurements were carried out as soon as possible. Immediately after blood collection, the animals were sacrificed by decapitation; then the liver of each animal was dissected out; one part of the liver was washed in saline, dried, rolled in a piece of aluminum foil and stored at -80°C for determination of both DNA-fragmentation and biochemical measurements; second part was soaked in formaldehyde-saline (10%) mixture buffer for histopathological processing and microscopic examination.

Tissue homogenization

A specimen from the liver organ was homogenized in ice-cold phosphate buffer (50 mM, pH 7.4) to give 10% homogenate (w/v). The homogenate was centrifuged at 10,000 rpm for 20 minutes to remove the cellular ghosts and nuclear and mitochondrial fractions; the supernatant was divided into aliquots and stored at -80°C till the biochemical measurements could be carried out.

Biochemical determinations

Biochemical measurements were carried out using spectrophotometry and ELISA techniques. Serum ASAT, ALAT, ALP and GGT activities were determined using kits purchased from Human Gesell Schaft fur Biochemical und Diagnostic mbH, Germany; total proteins, albumin, total cholesterol, triglycerides, LDL and HDL concentrations were evaluated using reagent kits purchased from Dia Sys Diagnostic systems GmbH, Germany; bilirubin level was measured with the reagent kits purchased from Diamond Diagnostics MDSS GmbH Schiffgraben 4130175 Hannover, Germany; urea and creatinine levels were assessed using reagent kits purchased from Diamond Diagnostic, MDCS GmbH Schiffgraben, Hannover, Germany; Sodium and Potassium level was estimated using MEDICA Easylyte Na+/K+ANALYZER, USA and reagent kits purchased from Easylyte, USA. Hepatic GSH and NO levels as well as SOD activities were estimated using regent kits obtained from Biodiagnostic, Dokki, Giza, Egypt. Using ELISA system (Dynatech Microplate Reader Model MR 5000, 478 Bay Street, Suite A213 Midland, ON, Canada), serum TNF-α, IL1β & IL10 levels were determined using ELISA rat reagent kits (SG-10057, SG-10179 and SG-10127, respectively) purchased from SinoGeneClon Biotech Co., Ltd, No.9 BoYuan Road, YuHang District 311112, Hang Zhou, China.

Hepatic MDA

Malondialdehyde (MDA), the most abundant individual aldehyde resulting from lipid peroxidation breakdown in biological systems and used as an indirect index for lipid peroxidation, level was determined chemically as described by Ruiz-Larnea *et al.* [14] through MDA reaction with thiobarbituric acid (TBA) forming a pink complex that can be measured photometrically. In this method, 0.5 ml liver homogenates' supernatant was added to 4.5 ml working reagent [0.8 g TBA was dissolved in 100 ml perchloric acid 10%, and mixed with TCA 20% in a volume ratio 1:3, respectively). In a boiling and shaking water bath, the sample-reagent mixture was placed for 20 minutes; then carried to cool at room temperature and centrifuged for 5 minutes at 3000 rpm. Finally, the absorbance of the clear pink supernatant was measured at 535 nm against reagent blank (0.5 ml distilled water + 4.5 ml working reagent); MDA level (nmol/g tissue) was calculated according to the following formula [MDA (nmol/g tissue) = [{$A_{535}\times 10^9$ / (1.56×10^5) × 10^3} x AD] × 10], where, 1.56x105 $M^{-1}L^{-1}cm^{-1}$ is MDA extinction coefficient and AD is assay dilution (10).

DNA fragmentation percentage

The degree of DNA fragmentation was determined by separating the cleaved DNA from the intact chromatin by centrifugation and measuring the amount of DNA present in the supernatant and pellet using the diphenylamine assay according to the quantitative method used for grading the DNA damage [15]. The degree of DNA fragmentation refers to the ratio of DNA in the supernatant to the total DNA in the supernatant and pellet. The liver tissues were lysed in 0.5 ml of hypotonic lysis buffer containing 10 mMTris–HCl (pH 8), 1 mM EDTA and 0.2% Triton X-100, and centrifuged at 14,000 ×g for 20min at 4°C. The pellets were resuspended in hypotonic lysis buffer. To the resuspended pellets and the supernatants, a 0.5 ml of 10% trichloroacetic acid was added. The samples were centrifuged for 20 min at10, 000 ×g at 4°C, and the pellets were suspended in 500 µl of 5% TCA. Subsequently, each sample was treated with a double volume of diphenylamine (DPA) solution [200mg DPA in 10 ml glacial acetic acid, 150µl of sulfuric acid and 60µl acetaldehyde] and incubated at 4°C for 48h. The proportion of fragmented DNA was

calculated from the absorbance reading at 578 nm using the following equation:

$$\text{DNA fragmentation \%} = \frac{\text{A supernatant}}{\text{A supernatant} + \text{A pellet}}$$

Histopathology

Paraffin sections of 5µm thick were stained with hematoxylin and eosin [16] and investigated by light microscope.

Statistical analysis

All data were statistically analyzed by analysis of Variance (ANOVA) using the general linear model procedure of the statistical analysis system (SAS). The significance of the differences among means was determined by Waller-Duncan k-ratio [17]. All statements of significance were based on probability of $p \leq 0.05$. The test was carried out using statistical analysis system (SAS) program software; copyright (c) 1998 by SAS Institute Inc., Cary, NC, USA.

Results

The yield, total phenolic content (TPC) and radical scavenging activity (RSA) of the aqueous extract of moringa are illustrated in figure 1. The obtained data revealed that aqueous *Moringa oleifera* extract possesses high content of phenolic compounds as well as great RSA, which is one of the antioxidant mechanisms.

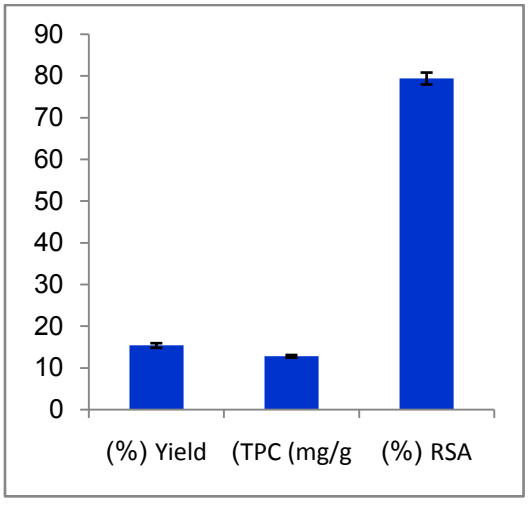


Figure 1. Yield, total phenolic content (TPC), and radical scavenging activity (RSA) of Moringa aqueous extract (MAE).

Body weight gain of MAE-treated group didn't change from that of control group, confirming that MAE has not any unfavorable effects on the animals' metabolic rates, while that of TMX group recorded a significant reduction from the control indicating sever disturbances in the body physiology, assimilation and metabolism. Favorably, administration of MAE together with TMX significantly inhibited the deterioration in the body weight progression; proving that MAE is very effective and succeeded in restoring the body weight slightly close to control (Figure 2).

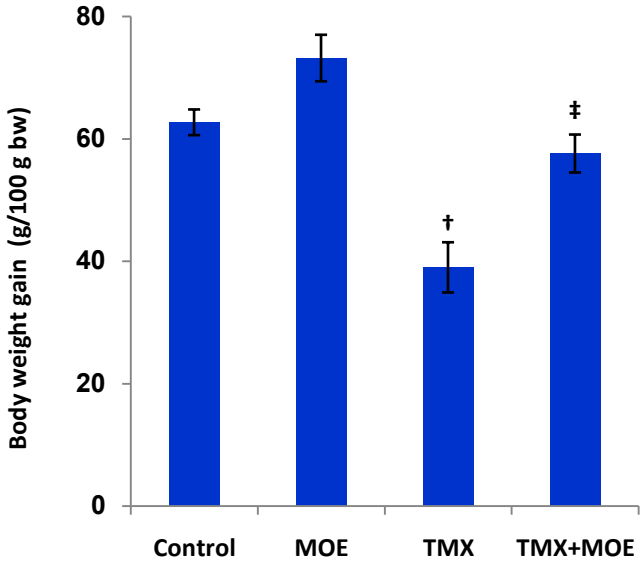


Figure 2. shows the body weight gain (%) of treated and control rats; (†) significant from control group, while (‡) is significant from TMX group.

Table 1 indicated that TMX administration induced significant elevations in ASAT, ALAT, ALP and GGT activity, while MAE alone hasn't damage these parameters as compared with those of normal rats. Administration of TMX in line with MAE ameliorated significantly the TMX-induced changes in the mentioned parameters.

Comparing with control group, the current study showed that the daily oral administration of rats with MAE resulted in non-significant changes in serum total proteins, albumin, and globulin levels, and A/G ratio, while intoxication with TMX led to a significant decrease in this proteins profile. In contrast, animals supplemented orally with MAE besides to Tamoxifen® for six weeks revealed a significant improvement in that protein profile (Table 2).

Also, oral administration of rats with MAE did not adverse level of serum bilirubin types, while intoxication with TMX increased bilirubin significantly, when both groups were compared with the control group. In comparison with Tamoxifen®-treated group, the treated orally rats with MAE extract in combination with TMX for six weeks (prophylactic animal group), showed a significant decrease in the concentration of serum total, direct and indirect bilirubin, respectively table 3.

The results of lipid profile in Table 4 illustrated that administration of rats with MAE similarly didn't unfavorably affect serum total cholesterol, triglycerides, LDL or HDL, but Tamoxifen®-intoxication significantly increased total cholesterol, triglycerides and LDL, and

markedly reduced HDL level when both groups were compared with corresponding values of control group. Fortunately, animals ingested with MAE combined with Tamoxifen® showed a significant reduction in cholesterol, triglycerides and LDL coupled with obvious elevation in HDL.

Serum creatinine, urea, Na^+ and K^+ levels after daily oral administration of rats with MAE and those of control group were similar, while administration of TMX revealed a significant increase in serum levels of creatinine, urea and Na^+ coupled with marked drop in K^+ level. In addition, the rats treated orally with MAE in combination with TMX for six weeks recorded a significant reduction in serum of creatinine, urea, and Na^+ levels coupled with increased in K^+ compared to the untreated TMX-intoxicated animals group (Table 5).

With respect to control group, the obtained results in Table 6 showed no unfavorable changes in the hepatic oxidative markers (MDA, NO, GSH and SOD); however the intoxication with Tamoxifen® drug resulted in marked elevations in both MDA and NO associated with a significant depletion of GSH and inhibition of SOD. On the other side, supplementation of the animals with MAE besides Tamoxifen® induced a marked decrement in the oxidative stress voltage (MDA and NO) concomitant with significant improvement in the anti-oxidative battery (GSH & SOD) in compare to Tamoxifen® group.

The results of serum cytokines and hepatic DNA fragmentation came parallel to the other measurements; as MAE-administration nether deteriorate serum TNF α, IL1β and IL10 nor hepatic DNA-fragmentation values; however, treatment of rats with MAE in line with TMX succeeded significantly to restore serum cytokines levels and hepatic DNA-fragmentation percentage, those were deteriorated as a consequence to TMX administration (Figure 3-4).

Table 1. Serum ALAT, ASAT, ALP and GGT activity of control, Tamoxifen®-intoxicated and MAE-treated male albino rats.

	Control	MAE	TMX	TMX + MAE
ALAT (U/L)	61.4 ± 1.87^{B}	60.7 ± 2.87^{B}	102.2 ± 9.01^{A}	78.2 ± 10.3^{B}
ASAT (U/L)	105 ± 3.94^{C}	101 ± 5.01^{C}	201 ± 19.65^{A}	126 ± 9.12^{BC}
GGT (U/L)	10.5 ± 0.5^{C}	11.0 ± 0.4^{C}	24.8 ± 0.8^{A}	12.5 ± 0.63^{C}
ALP (U/L)	124 ± 20^{D}	127 ± 11.8^{D}	391 ± 38^{A}	138.7 ± 19^{D}

Data are presented as mean ±standard error; within each column, means with superscript different letters are significantly different at $p\leq0.05$ using one way ANOVA followed by Duncan post hoc test; MAE (Moringa aqueous extract) and TMX (Tamoxifen®) drug.

Table 2. Serum total proteins, albumin, globulin, and A/G ratio of control, Tamoxifen®-intoxicated and MAE-treated male albino rats.

	Control	MAE	TMX	TMX + MAE
T. Protein(g/dl)	6.6 ± 0.11^{B}	6.8 ± 0.12^{AB}	5.9 ± 0.18^{C}	7.2 ± 0.14^{A}
Albumin (g/dl)	3.4 ± 0.04^{BC}	3.5 ± 0.04^{AB}	2.9 ± 0.09^{E}	3.6 ± 0.05^{A}
globulin (g/dl)	3.2 ± 0.11^{BC}	3.3 ± 0.11^{ABC}	3.0 ± 0.12^{C}	3.5 ± 0.12^{A}
A/G Ratio	1.1 ± 0.05^{A}	1.1 ± 0.04^{AB}	0.9 ± 0.03^{B}	1.0 ± 0.03^{AB}

Data are presented as mean ±standard error; within each column, means with superscript different letters are significantly different at $p\leq0.05$ using one way ANOVA followed by Duncan post hoc test; MAE (Moringa aqueous extract) and TMX (Tamoxifen®) drug.

Table 3. Illustrates serum total, direct and indirect bilirubin of control, Tamoxifen®-intoxicated and MAE-treated male albino rats.

	Control	MAE	TMX	TMX + MAE
T. bilirubin (mg/dl)	0.163 ± 0.02^{B}	0.157 ± 0.01^{B}	0.313 ± 0.01^{A}	0.176 ± 0.02^{B}
D. bilirubin (mg/dl)	0.043 ± 0.002^{C}	0.042 ± 0.002^{C}	0.056 ± 0.002^{A}	0.048 ± 0.004^{B}
Ind. bilirubin (mg/dl)	0.120 ± 0.005^{B}	0.115 ± 0.005^{B}	0.157 ± 0.006^{A}	0.118 ± 0.02^{B}

Data are presented as mean ±standard error; within each column, means with superscript different letters are significantly different at $p\leq0.05$ using one way ANOVA followed by Duncan post hoc test; MAE (Moringa aqueous extract) and TMX (Tamoxifen®) drug.

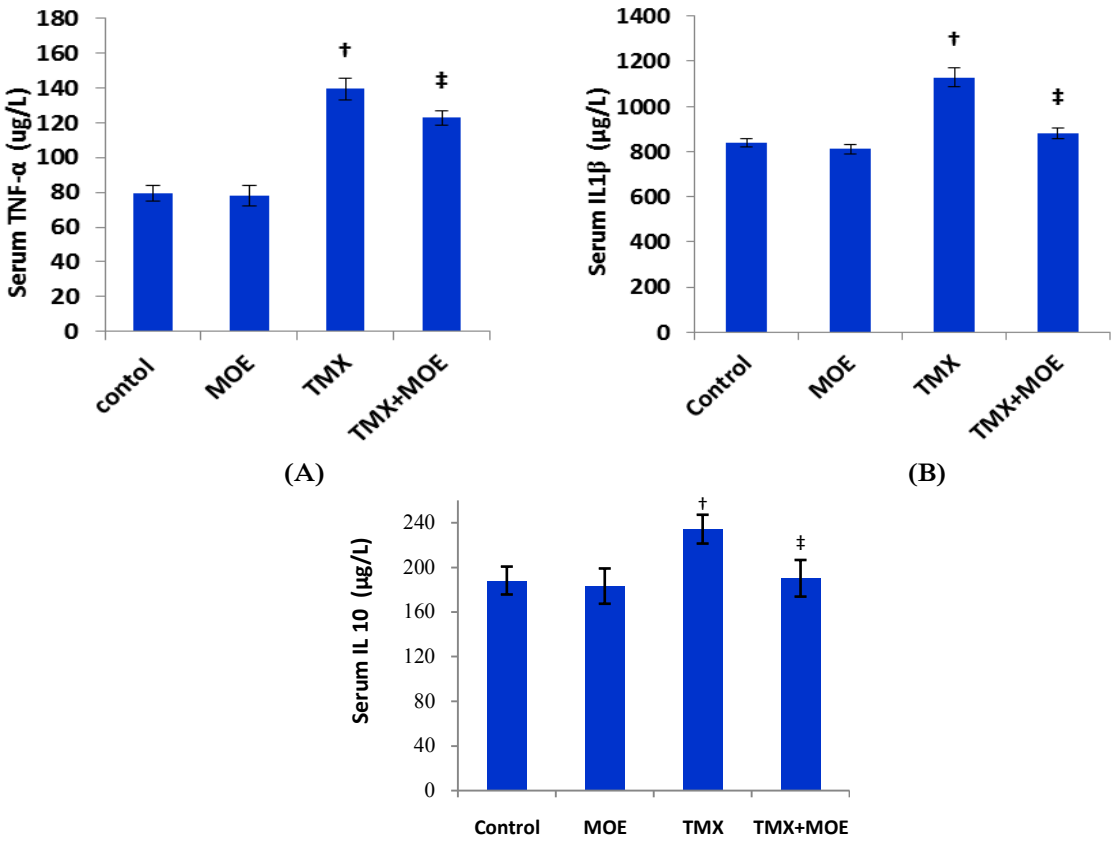


Figure 3. Shows serum levels of (A) TNFα, (B) IL1β and (C) IL10 of control, TMX-intoxicated and MAE-treated rats' groups.(†) significant from control group, while (‡) is significant from TMX group.

Table 4. Shows serum cholesterol, triglyceride, HDL and LDL levels of control, Tamoxifen®-intoxicated and MAE-treated male albino rats.

	Control	MAE	TMX	TMX + MAE
Cholesterol (mg/dl)	110 ± 4.4^{C}	111 ± 4.1^{C}	162 ± 6.1^{A}	140 ± 3.7^{B}
Triglyceride (mg/dl)	101 ± 3.8^{C}	98.9 ± 3.2^{C}	158 ± 4.9^{A}	137 ± 3.2^{B}
LDL-c(mg/dl)	50.2 ± 3.6^{C}	49.7 ± 3.6^{C}	87.1 ± 4.4^{A}	59 ± 3.3^{B}
HDL-c(mg/dl)	39 ± 4.1^{A}	41 ± 3.6^{A}	26 ± 2.1^{C}	33.9 ± 2.3^{B}

Data are presented as mean ±standard error; within each column, means with superscript different letters are significantly different at $p\leq0.05$ using one way ANOVA followed by Duncan post hoc test; MAE (Moringa aqueous extract) and TMX (Tamoxifen®) drug.

Table 5. Shows serum creatinine, urea, Na^+ and K^+ of control, Tamoxifen®-intoxicated and MAE-treated male albino rats.

	Control	MAE	TMX	TMX + MAE
Creatinine (mg/dl)	0.71 ± 0.06^{B}	0.7 ± 0.06^{B}	1.0 ± 0.08^{A}	0.8 ± 0.02^{B}
Urea (mg/dl)	57 ± 2.8^{B}	53 ± 2.8^{B}	121 ± 14.0^{A}	92 ± 11.0^{A}
K^+(mmol/l)	5.9 ± 0.4^{AB}	6.0 ± 0.3^{A}	5.1 ± 0.3^{B}	$6.0\pm0.3A^{B}$
Na^+ (mmol/l)	46 ± 3.5^{B}	48 ± 3.7^{B}	63 ± 3.7^{A}	49 ± 2.9^{B}

Data are presented as mean ±standard error; within each column, means with superscript different letters are significantly different at $p\leq0.05$ using one way ANOVA followed by Duncan post hoc test; MAE (Moringa aqueous extract) and TMX (Tamoxifen®) drug.

Table 6. Shows hepatic redox markers (MDA, NO, SOD and GSH) of control, Tamoxifen®-intoxicated and MAE - treated male albino rats.

	Control	MAE	TMX	TMX + MAE
MDA(mmol/g)	128.4±5.1^{C}	126.5±5.2^{C}	283.6±6.6^{A}	189.5±6.2^{B}
NO(mmol/g)	70.2±4.8^{C}	68.5±4.4^{C}	164.9±6.2^{A}	113.2±5.1^{B}
SOD(U/g tissue)	2345±67^{A}	2398±55^{A}	1032±57^{C}	1816±61^{B}
GSH(mg/g)	88.2±3.6^{A}	91±3.3^{A}	45.4±2.9^{C}	77.8±3.1^{B}

Data are presented as mean ±standard error; within each column, means with superscript different letters are significantly different at $p \leq 0.05$ using one way ANOVA followed by Duncan post hoc test; MAE (Moringa aqueous extract) and TMX (Tamoxifen®) drug.

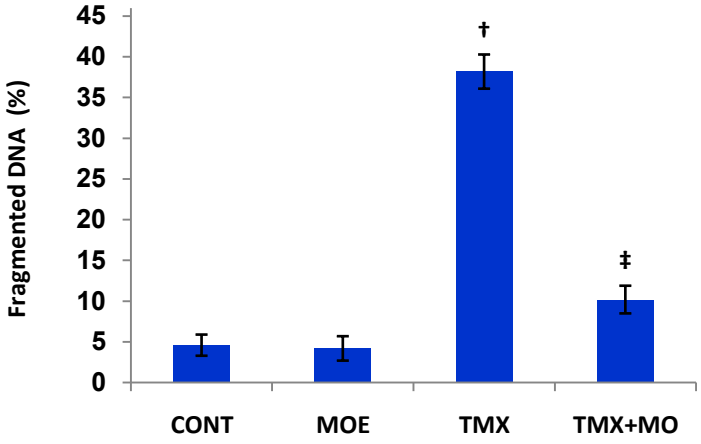


Figure 4. shows percentage of DNA fragmentation of control, TMX-intoxicated and MAE-treated rats' groups.(†) significant from control group, while (‡) is significant from TMX group.

Histological results of liver

Results of histological examination of liver sections of the study groups are described and illustrated by figures (5-8).

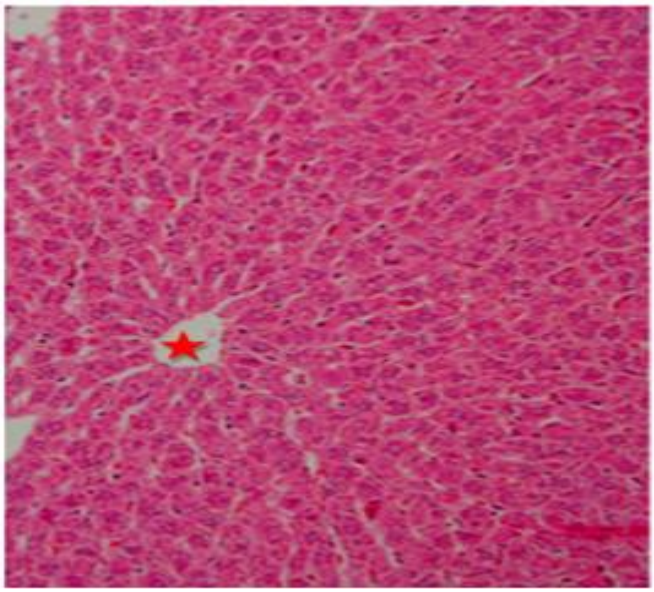


Figure 5A. Section in the liver of control rat showing normal histological structure of hepatic lobules and central vein (Star) (Hx&Ex100).

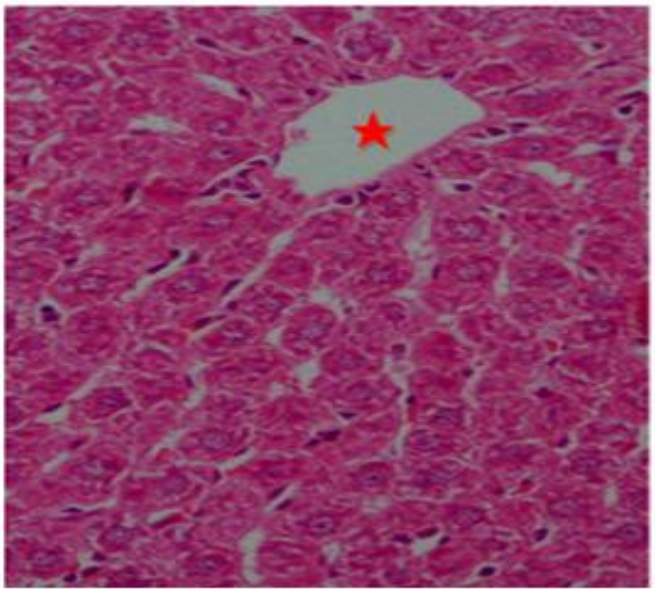


Figure 5B. Section in the liver of control rat showing normal histological structure of hepatic lobules and central vein (Star) (Hx&Ex400)

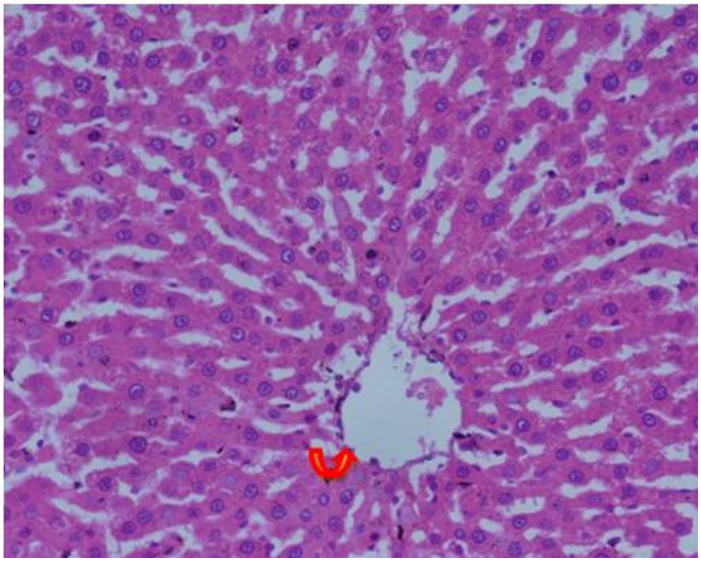


Figure 6. Section of the liver of a rat treated with Moringa aqueous extract (MAE) only showing normal histological structure, (Hx&Ex200).

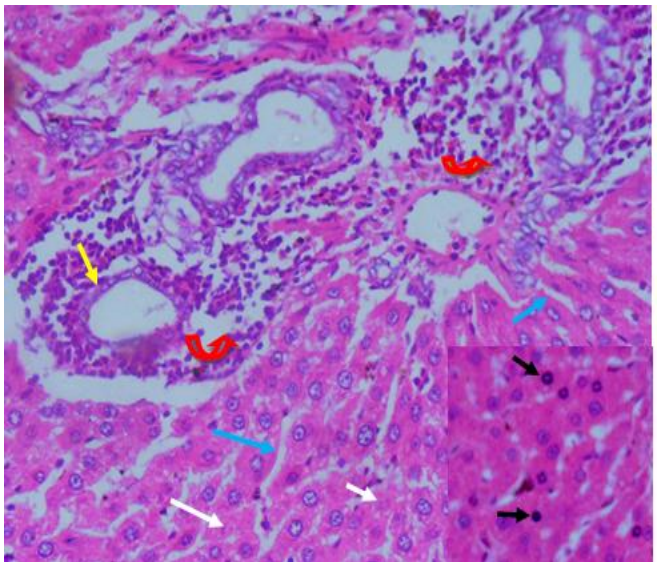


Figure 7A. Section of the liver of a rat treated with Tamoxifen® only showing signs of degeneration in the form of pyknosis (black arrow), karyolysis (white arrow) and cellular infiltration around bile duct (red curved arrow). Dilated blood sinusoid (blue arrow) was observed (Hx&Ex400).

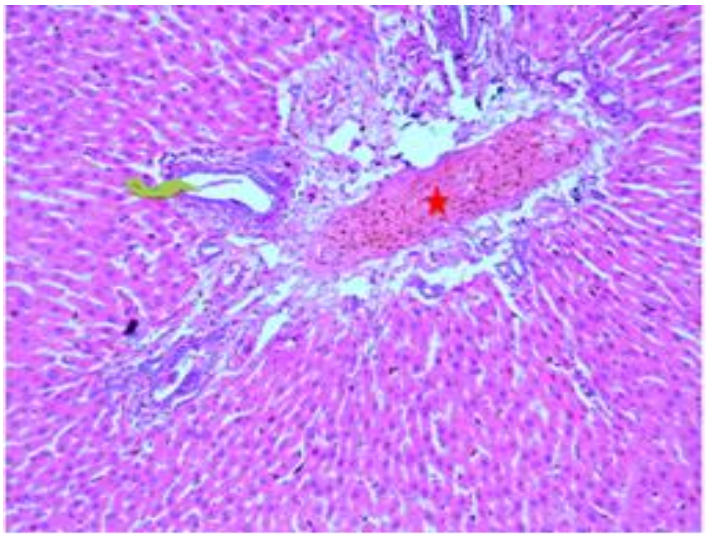


Figure 7B. Another section of the liver of a rat treated with Tamoxifen® only showing dilated congested portal vessel (star), dilated bile duct (green curved arrow), (Hx&Ex100).

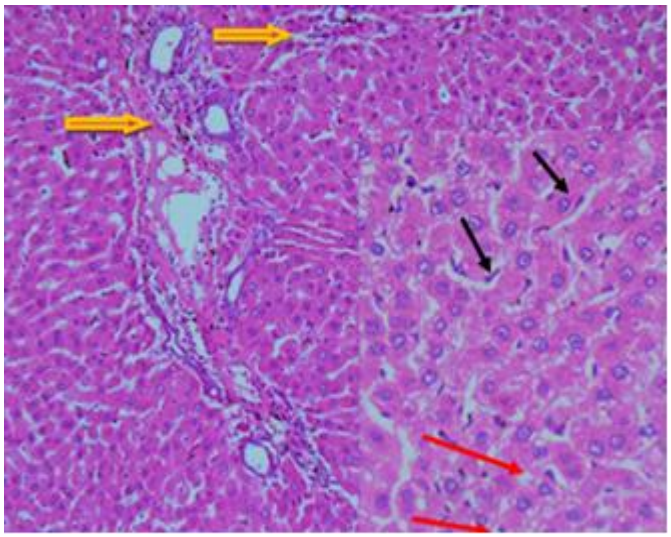


Figure 7C. Another section of the liver of a rat treated with Tamoxifen® only showing fibrosis (orange arrow) (Hx&Ex100), hypertrophy of kupher cells and vacuolar degeneration (red arrow) at right of figure, (HX&Ex200).

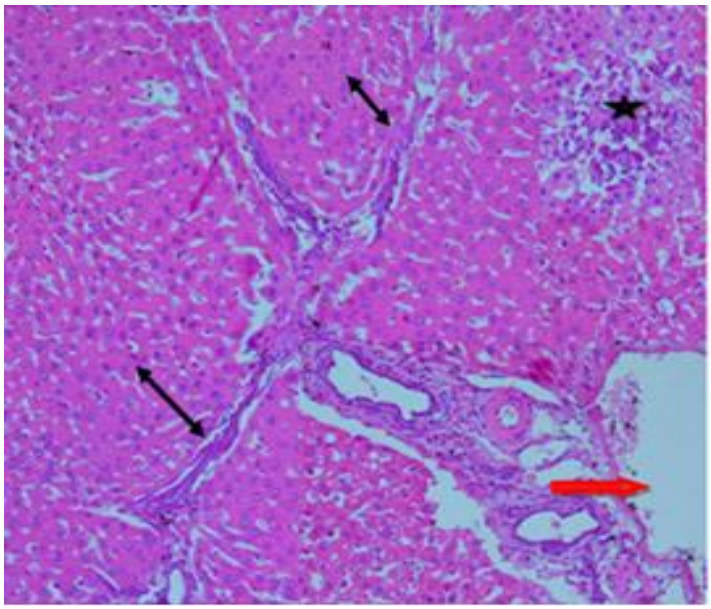


Figure 8A. Section of the liver of a rat treated with Tamoxifen® along with Moringa aqueous extract (MAE) showing fibrosis (black arrow) dilated bile vessel (red arrow), aggregation of cellular infiltration (star), (Hx&Ex100).

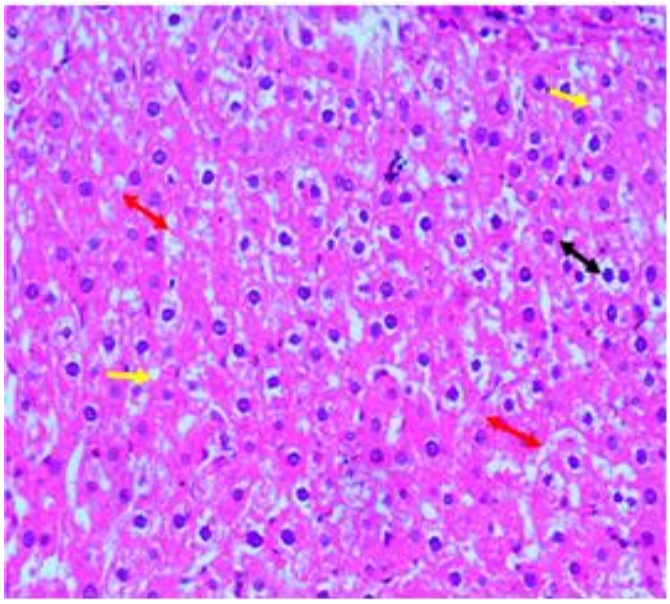


Figure 8B. Another field of the liver of rat treated with Tamoxifen® along with Moringa aqueous extract (MAE) showing dilated blood sinusoid (red arrow), vacuolar degeneration (yellow arrow) and pyknosis (black arrow), (Hx&Ex200).

Discussion

Tamoxifen® (TMX) is a non-steroidal anti-estrogen that is commonly used in the prevention and treatment of cancer breast. Many studies provided significant evidence that 5-year TMX therapy could improve the 5-year survival rate, particularly in postmenopausal women. Also, the initial results from the first International Cancer Breast Intervention Study-I revealed that prophylactic use of TMX reduced the risk of invasive ER-positive tumors by 31% in females who were at an increased risk for developing cancer breast [2]. However, the adverse effects have restricted the long term use of TMX to a large extent. Studies reported that long-term use of TMX can cause many side effects such as hot flashes, night sweats, gynecological symptoms (vaginal dryness and vaginal discharge), sleep alterations, memory loss, depression, weight gain and diminished sexual function [18]. Among all these, hepatic injury or even hepatocellular carcinoma can be one of the most serious side effects, which restricted its long-term use in many cases [19]. This study aimed to illustrate the protective role of MAE on the hepatotoxicity resulted as a consequence to TMX.

This study showed that TMX treatment caused a significant increase in serum level of ALAT, ASAT, GGT and ALP activity; these findings are in agree with previous reports of Hassanein *et al.* [20] and El-Kashef and El-Sheakh [21]. This elevation could be due to hepatocyte structural-damage as these enzymes are normally present in the cytoplasm of the hepatocyte and are released into circulation after disruption of cell-membrane permeability as result of the oxidative stress and lipid peroxidation due to the cytotoxic effects of TMX. Alkaline phosphatase is a specific indicator of biliary epithelium affection and may reflect compression of intrahepatic biliary canaliculi by inflammatory cells in portal tracts [22].

TMX doses increased the oxidative stress markers in the liver as measured by the high level of MDA, NO and significant depletion in GSH and SOD values compared to normal control group; this explains the observed leakage of cellular ALAT, ASAT, GGT and ALP into the circulation that suggest the hepatocellular damage [23].

A remarkable elevation in serum TG, cholesterol, urea, bilirubinand creatinine levels was observed in TMX-intoxicated rats, whereas the level of total proteins was reduced due to the TMX-induced hepatotoxicity. The reduction is attributed to the initial damage produced and localized in the endoplasmic reticulum, which results in the loss of CYC-P450 activity leading to its functional failure with a decrease in protein synthesis and accumulation of TG leading to fatty liver; inhibition of bile acids synthesis from cholesterol leading to increase in cholesterol level. Suppression of cholesterol levels by MAE suggests that bile acids synthesis was reversed. The decrease in antioxidant defense systems of TMX-intoxicated rats renders them more susceptible to hepatotoxicity [24].

Tamoxifen® (TMX) increases the intra-mitochondrial ionized Ca^{2+} which stimulates mitochondrial nitric oxide synthase activity, increases NO production and hampers mitochondrial respiration [25]. The produced NO reacts with superoxide anion (O_2^-)to produce peroxynitrite

which increases lipid peroxidation leading to oxidative stress and cytochrome-C release into cytoplasm and activates caspase-9, which in turn, leading to DNA fragmentation [26]. Furthermore, TMX induces endoplasmic reticulum stress intensifies various types of damage, leading to oxidative stress, inflammation and apoptosis [27, 28]. In the current study, TMX administration induced increase in hepatic MDA and NO level coupled with decreases of SOD and GSH activities. These results provide a further evidence and support previous studies that TMX hepatotoxicity seem to favor the mechanism of toxic injury being related to mitochondrial injury and/or endoplasmic reticulum stress, resulting in oxidative stress, inflammatory response and activation of apoptotic cascade leading to hepatocyte injury and necrosis.

Interestingly, Co-administration of MAE with TMX resulted in a significant reduction in serum ALAT, ASAT, ALP and GGT activity as well as hepatic MDA and NO level and increase in the antioxidant battery (GSH and SOD) providing the antioxidant property of MAE. This exhibition is in agreement with the study done by Essawy *et al.* [29] and Qasim & Baraj [30]. The reversal of elevated activity of serum hepatic enzymes herein as a consequence to MAE may be attributed to the re-stabilization of the hepatocyte membrane that preventing enzyme leakages as earlier postulated [31]. Previous study reported that hepatoprotective exhibition of Moringa was due to presence of quercetin and kaempferol [32]. However, MAE prevents permeation of the toxins by contending for the same peroxidation suscepable-sites on cell membranes; this is possibly due to a blend of two focal mechanisms; 1) an modification of cell membranes, such that only slight quantities of toxins may infiltrate into the cell; 2) speeding up of protein synthesis, thus exciting cellular regeneration [33]. This finding goes in line with Islam and Alam [34] who attributed thateffect to the presence of flavonoids (quercetin and kaempferol), vitamin A, and ascorbic acid.

Treatment with MAE significantly elevated serum total protein concentrations, which is possibly associated with a decreased affinity of albumin. The restoration of proteins levelcould be due to regeneration of protein synthetic capacity as a consequence to activate ingredients (moringinine, quercetin and chlorogenic acid) that inhibited liver cell injury. These results are in agreement with recent study which revealed that MAE reduced the toxicity due to elimination of the toxic products of TMX in rat [35].

It was stated that *M. oleifera* containing a variety of chemical constituents (phenols, coumarins, lignans, essential oil, monoterpenes, carotinoids, glycosides, flavonoids, organic acids, lipids, alkaloids and xanthene) that possess antioxidant properties, so responsible for this effect [33].

In the present study, TMX administration induced significant increase in serum TNF-α, IL-1β, IL-10 levels and DNA fragmentation amount. These findings are similar to those observed in recent studies [20, 21]; where they reported marked increase in circulatory levels of pro-inflammatory cytokines in TMX treated rats. In terms of inflammation; infiltration of various inflammatory cells and up-regulation of inflammatory mediators (such as TNF-*α*, IL-1β and IL-10) play a key role in promoting the progression of steatosis to fibrosis, cirrhosis, and cancer [36]. Tamoxifen® induces liver injury including inflammation resembling that of alcoholic hepatitis, stimulation of IL-1β production [37] and increase of TNF-α level [23]. ROS, via NF-κB nuclear translocation, cause hepatocyte expression of Fas ligand leading to Fas/Fas ligand mediated apoptosis. The elevated serum TNF-α, IL-1β and IL-10 levels of TMX-intoxicated group could be pertinent to TMX-induces mitochondrial injury, ROS and lipid peroxidation [38] leading to increase generation of several cytokines TNF-α, IL-1β and IL-10 that play sundry roles in the pathogenesis of liver injury [39].

These interesting findings provide strong evidence on the anti-inflammatory efficacy of MAE, which is one of the significant mechanisms for prevention of drug induced hepatotoxicity. The anti-inflammatory and hepatoprotective effects of MAE may explain MAE as rich source of antioxidants due to the presence of various phytochemicals such as polyphenolics, carotenoids, α-tocopherol, ascorbic acid, and several amino acids [40]. The bioavailability of these metabolites in MAE has been directly linked to various biological profiles, especially viaits scavenging activity which helps to ameliorate the damaging effects caused by oxidative stress in TMX drugs.

TMX-induced histopathological degenerations of hepatocytes may be due to the fact that TMX drug strongly induce hepatotoxicity through the increase in generating lipid peroxidation in hepatic tissue [41] which destroys cell nuclei and cellular membranes; also, generation of ROS by TMX drug interferes with the anti-toxin defense system that produces oxidative damage in different tissues, causing dysfunction in liver cells. Favorably, treatment with MAE significantly decreased TMX-induced hepatotoxicity, indicating that MAE possess hepatoprotective effect which confirmed herein by the improved liver and kidney functions, decreased inflammatory cytokines, improved antioxidant battery, reduced DNA fragmentation (which is an indicator of decreased apoptosis and tissue damage), and regeneration of the hepatic image compared to TMX -intoxicated group. Interestingly, many phytoconstituents (flavonoids, phenolics and ascorbic acid) were identified in the aqueous extract of moringa leaf [35]. These secondary metabolites have been identified as natural antioxidants [42]. Thus, the observed free radical scavenging and

hepatoprotective activities of the aqueous extract of moringa leaf may be attributable to the antioxidant phytoconstituents.

Conclusion

TMX induces high percent of DNA-fragmentation and many pathophysiological complications. Its co-administration with TMX, MAE succeeded in minimizing the drug side effects; this could be mechanized through the presence of large amount antioxidant constituents in MAE. So, it may be worthy to consider the beneficial use of MAE as a supplement with Tamoxifen® therapy reduction of DNA-fragmentation and amelioration of the pathophysiological complications sided by TMX in a strategy to minimize drug side effects and enhance patients' body tolerance.

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