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Protective effects of *Balanites aegyptiaca* extract, Melatonin and Ursodeoxycholic acid against hepatotoxicity induced by Methotrexate in male rats

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ABSTRACT

Objective: To compare the degree of ameliorative effects of Melatonin (MEL), Ursodeoxycholic acid (UDCA) and *Balanites aegyptiaca* (BA) against hepatotoxicity induced by MTX for one month.

Methods: Eighty adult male rats (Sprague Dawely) weighing (190 ± 10 g), were randomly divided into eight equal groups: Control, MTX, MEL, BA, UDCA, MTX + MEL, MTX + BA, MTX + UDCA. Liver function biomarker enzymes, liver tissue oxidative stress parameters, together with total antioxidant capacity and tumor necrosis factor (TNF-α) were determined. Histopathological and immunohistochemistry examinations for TNF-α were also done.

Results: MTX showed significant increase in alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), total and direct bilirubin, as well as TNF-α levels, oxidized glutathione (GSSG), malodialdehyde (MDA) and nitric oxide (NO). Whereas total protein, albumin, total antioxidant capacity, reduced glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT) levels were significantly decreased in MTX treated group. These alterations were improved by MEL and BA treatment, whereas no improvement was noticed in UDCA treatment.

Conclusions: BA may be as promising as MEL in the hepatoprotection against MTX toxicity through their antioxidant and radical scavenging activities. In addition, it is not recommended to co-administer UDCA with MTX as it enhanced inflammation and damage to the liver.

1. Introduction

Methotrexate (MTX), a folic acid competitor, used for chemotherapy in the treatment of diverse malignancies (acute lymphoblastic leukemia) as well as diverse inflammatory diseases [1]. MTX has also been used successfully in psoriasis since 1951, and over the past ten years in psoriatic and rheumatoid arthritis [2]. It is on the World Health Organization's List of Essential Medicines [3].

The efficacy of MTX is restricted by its toxicity and severe side-effects including hepatitis, liver cirrhosis and fibrosis, hepatocytes hypertrophy, hepato-cellular necrosis and death [1]. MTX toxicity has serious drawbacks on the hematopoietic system and liver enzymes [1,4]. It is toxic to the liver, kidneys, respiratory and reproductive systems for long term therapy even at very low doses [5].

MTX causes lipid peroxidation indicated by high levels of thiobarbituric acid reactive substances. It also lowered the levels of antioxidant enzymes like superoxide dismutase, catalase, and glutathione reductase, indicating oxidative stress and distressing the antioxidant enzyme defense system [1].

Melatonin (N-acetyl-5-methoxytryptamine) is synthesized principally by the pineal gland and is proposed to have antioxidant and protective effects in opposition to oxidative stress [6]. It has powerful antioxidant activity thus protecting cells, tissues, and organs against oxidative damage of DNA, proteins, and lipids that causes pathogenesis [7,8]. It also protects the liver

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from oxidative damage as a result of ischemia reperfusion [9]. Because melatonin has excellent antioxidative properties, there is a likely possibility that it would protect against MTX toxicity.

Balanites aegyptiaca (BA) is a common wild plant. It is known as Desert date. Mesocarp of fruit contains 1.5% protein and 37% sugars, 15% organic acids. The phytochemical composition of different parts of the plant showed that it contains high amounts of saponins, moderate amounts of tannins, flavonoids, cardiac glycosides, besides fatty acids and sterols [10–12]. BA stem bark aqueous extract protected the hepatocytes in biliary duct ligated rats demonstrated by dose-reliant reduction in serum bilirubin levels [13–15]. The fruit mesocarp of desert dates ameliorated hepatotoxicity driven by CCl_4 in rats [16]. The effect of ethanolic extracts of bark dates against paracetamol [17] and CCl_4 [14] driven hepatotoxicity in rats was analogous to silymarin. The purified fractions of BA own considerable antioxidant [18] and anti-inflammatory [19] activities.

Ursodeoxycholic acid (UDCA) is a secondary bile acid (metabolic byproduct of intestinal bacteria) and it has anti-oxidative powers [20]. UDCA enhanced liver function in various biliary disorders [21]. UDCA protected the hepatocytes from amoxicillin-clavulanic acid toxicity in rats, as it possesses anti-oxidant properties [22]. This study aims to compare the degree of ameliorative effects of Melatonin (MEL), Ursodeoxycholic acid (UDCA) and *B. aegyptiaca* (BA) against hepatotoxicity induced by MTX for one month.

2. Materials and methods

2.1. Animals

Adult male rats (*Sprague Dawely*), weighing (190 ± 10) g, were purchased from the National organization of drug control and research, Egypt. The experimental protocol was approved by Institutional Animal Care and Use Committee (IACUC), Faculty of Science, Cairo University (Egypt) (CUFS/F/PHY/14/15). All the experimental procedures were carried out in agreement with international guiding principles for care and use of laboratory animals. Animals were maintained at a controlled temperature (23–25) °C and on a 12 h: 12 h light dark cycle. They had open access to water and standard diet ad libitum and were acclimatized to laboratory conditions for 7 d before the commencement of the experiment.

2.2. Experimental design

Rats were arbitrarily divided into eight groups, each comprises 10 rats. They were treated for one month as follows: Control (treated with daily oral dose of 0.5 mL carboxy methyl cellulose (CMC) and 1 mL saline intraperitoneal weekly); MTX (treated with weekly intraperitoneal dose of 13.4 mg/kg b.w); MEL (treated with daily oral dose of 10 mg/kg b.w. MEL); BA (treated with daily oral dose of 100 mg/kg b.w. BA); UDCA (treated with daily oral dose of 20 mg/kg b.w. UDCA); MTX + MEL (treated with weekly intraperitoneal dose of 13.4 mg/kg b.w. MTX and daily oral dose of 10 mg/kg b.w. MEL); MTX + BA (treated with weekly intraperitoneal dose of 13.4 mg/kg b.wt. MTX and daily oral dose of 100 mg/kg b.w. BA); MTX + UDCA (treated with weekly intraperitoneal dose of 13.4 mg/kg b.w). MTX and daily oral dose of 20 mg/kg b.w. UDCA.

2.3. Dosages

MTX was purchased as 50 mg injection vials, from Shanxi PUDE pharmaceutical Company (Shanxi, China) given as intraperitoneal injection of 13.4 mg/kg body weight [1]; MEL was purchased as 3 mg tablets, from Puritan's Pride (USA) given orally as 10 mg/kg body weight [23]; BA was purchased from National Research Center (NRC, Giza, Egypt), department of phytochemistry and plant systematic, its voucher specimen is maintained by the Herbarium of NRC with registration number (CAIRC 3665) given orally as 100 mg/kg body weight [10]; UDCA was purchased from SEDICO company (Cairo, Egypt) given orally as 20 mg/kg body weight [24].

At the end of the experiment animals in all groups were anaesthetized and sacrificed by cervical decapitation. Blood samples were collected and centrifuged at 3 000 rpm for 20 min for serum separation. The livers of rats were instantly dissected out.

2.4. Preparation of tissue homogenates

A part of the liver tissues was homogenized in phosphate buffer saline (PBS), pH 7.4, using a homogenizer to obtain 20% homogenate. The homogenate was centrifuged at 3 000 rpm for 15 min at 4 °C and the resultant supernatant was used for biochemical analysis. A second part was homogenized in 75% methanol for the estimation of oxidative stress parameters.

2.5. Biochemical determination

Serum separated was used for the determination of biochemical parameters. Aspartate transaminase (AST), alanine transaminase (ALT) [25], serum total protein [26] and total antioxidant capacity [27] were measured (colorimetrically) using reagent kits of Biodiagnostic Company (Dokki, Giza, Egypt). Alkaline phosphatase, serum albumin, total and direct bilirubin levels [28–30] were assayed by a quantitative method, using commercial kits (Salucea, Haansberg, Netherlands). γ-glutamyl transferase (GGT) activity was determined using a commercial kit (Centronic GmbH, Munich, Germany) [31]. TNF-α was assayed using Rat TNF-α ELISA Kit purchased from Koma Biotech (Korea) [32].

2.6. Measurement of oxidative stress parameters in tissue

Oxidized and reduced glutathione (GSSG and GSH) contents were estimated by HPLC method developed by Jayatilleke and Shaw [33] for the measurement of oxidized and reduced glutathione in biological samples. MDA was estimated according to the method of Karatepe [34] using HPLC with a UV detector. NO was assayed by anion exchange HPLC, with UV detector according to Papadoyannis *et al* [35]. The activities of Glutathione peroxidase (GPx), Glutathione reductase (GR), Glutathione S-transferase (GST), Superoxide dismutase (SOD) and Catalase (CAT) [36–40] were estimated according to the manufacturer's instructions using kits from Biodiagnostic company (Dokki, Giza, Egypt).

2.7. TNF-α immunohistochemistry

The detection of TNF-α in liver tissue was done by Immunohistochemistry (IHC) using a streptavidin-biotin method by

Santa Cruz Biotechnology kit, USA [41]. Tissue sections were deparaffinized in xylene, rehydrated in diminishing concentrations of alcohol then washed with water. Endogenous peroxidase was blocked using hydrogen peroxide 3% afterwards they were blocked with normal goat serum (37 °C, 15 min). Then they were incubated with TNF-α polyclonal antibody (37 °C, 60 min), washed with PBS, and then incubated with a biotinylated horse peroxidase-conjugated secondary antibody and 0.1% 3, 3-diaminobenzidine (DAB) substrate, using the standard streptavidin-biotin-based method. Positive expression of TNF-α was indicated by brown granule. Sections were examined with an optical image analyzer and photographed with a digital Olympus camera (SC100, Japan) connected to the computer with high power (100-fold magnification).

2.8. Tissue sampling and histopathological examination

Part of liver tissue separated from either normal or treated groups of rats was fixed in 10% formal saline for twenty-four hours. The fixed tissues were processed habitually, embedded in paraffin, sectioned, deparaffinized and rehydrated then stained with hematoxylin and eosin (H & E) using typical techniques [42] for histopathological examinations.

2.9. Statistical analysis

Statistical analysis was performed by one way ANOVA by means of the IBM SPSS statistics 20 program (SPSS Inc., Chicago, IL, USA). All data were expressed as mean ± SE. $P < 0.05$ was considered statistically significant. Letters not sharing a common letter superscript are significantly different ($P < 0.05$).

3. Results

3.1. Biochemical parameters

3.1.1. Serum levels of liver function biomarker enzymes

MTX and UDCA administration to group of male rats resulted in a significant ($P < 0.05$) elevation in serum levels of liver function biomarker enzymes ALT, AST, ALP and GGT as measured with respect to control group. While, administration of MEL and BA caused insignificant changes in serum levels of ALT, AST, ALP and GGT as compared to the control group. Co-administration of MEL or BA extract with MTX significantly ($P < 0.05$) alleviated the liver biomarker enzymes activities, which was reflected by a significant ($P < 0.05$) reduction in serum levels of ALT, AST, ALP and GGT as measured with respect to MTX. However, co-administration of UDCA with MTX showed insignificant changes in the activities of serum ALT, AST, ALP and GGT as measured with respect to MTX (Table 1).

3.1.2. Serum levels of total protein, albumin, total and direct bilirubin

Data in Table 1 showed that MTX administration, as well as UDCA administration, to groups of male rats resulted in a significant ($P < 0.05$) reduction in serum levels of total protein and albumin as measured with respect to control group. However, total and direct bilirubin levels in serum showed a significant ($P < 0.05$) elevation as measured with respect to control. Meanwhile, administration of MEL and BA caused insignificant changes in serum levels of total protein, albumin, total and direct bilirubin as compared to the control group.

Combined administration of MEL or BA extract with MTX significantly ($P < 0.05$) elevated the serum levels of total protein and albumin and significantly ($P < 0.05$) reduced the serum levels of total and direct bilirubin as measured with respect to MTX. However, co-administration of UDCA with MTX showed insignificant changes in serum levels of total protein, total and direct bilirubin as measured with respect to MTX.

3.2. Oxidative stress parameters in liver tissue

3.2.1. Non-enzymatic parameters

Liver tissue contents of MDA, NO and GSSG were significantly ($P < 0.05$) elevated in MTX and UDCA administrated groups of male rats, whereas liver tissue content of GSH was significantly ($P < 0.05$) reduced as measured with respect to

Table 1
Protective effect of daily administration of MEL, BA and UDCA against MTX hepatotoxicity for one month on liver biomarker enzymes in serum.

Group	ALT (U/mL)	AST (U/mL)	ALP (U/100 mL)	GGT (U/L)	Total protein (g/dL)	Albumin (g/dL)	Total Bilirubin (mg/dL)	Direct Bilirubin (mg/dL)
Control	45.19 ± 0.79	61.18 ± 1.47	24.74 ± 0.81	3.46 ± 0.10	7.49 ± 0.02	4.21 ± 0.10	0.028 ± 0.001	0.013 ± 0.0002
MTX	90.64 ± 1.72[a]	139.21 ± 1.38[a]	55.54 ± 1.07[a]	6.07 ± 0.19[a]	5.11 ± 0.04[a]	2.62 ± 0.04[a]	0.043 ± 0.003[a]	0.031 ± 0.0010[a]
MEL	44.00 ± 0.41	58.66 ± 0.84	22.43 ± 0.89	3.57 ± 0.12	7.63 ± 0.11	4.09 ± 0.10	0.028 ± 0.001	0.012 ± 0.0006
BA	47.02 ± 1.26	61.28 ± 1.63	23.13 ± 0.68	3.44 ± 0.10	7.33 ± 0.14	4.36 ± 0.10	0.030 ± 0.003	0.012 ± 0.0002
UDCA	59.23 ± 1.22[a]	70.56 ± 1.63[a]	34.62 ± 0.47[a]	5.74 ± 0.24[a]	5.36 ± 0.10[a]	3.09 ± 0.10[a]	0.040 ± 0.001[a]	0.022 ± 0.0016[a]
MTX + MEL	47.11 ± 1.04[b]	115.24 ± 2.10[ab]	26.81 ± 0.53[b]	3.86 ± 0.19[ab]	7.79 ± 0.17[b]	3.87 ± 0.06[ab]	0.033 ± 0.001[b]	0.012 ± 0.0004[b]
MTX + BA	47.63 ± 0.65[b]	119.15 ± 0.86[ab]	28.06 ± 0.64[ab]	4.17 ± 0.14[ab]	7.21 ± 0.13[b]	3.44 ± 0.04[ab]	0.031 ± 0.002[b]	0.015 ± 0.0007[b]
MTX + UDCA	89.05 ± 2.56[a]	129.47 ± 2.76[ab]	60.89 ± 1.77[ab]	7.66 ± 0.43[ab]	5.07 ± 0.21[a]	2.89 ± 0.14[ab]	0.042 ± 0.001[a]	0.030 ± 0.0009[a]

n = 6 in each group. Data are represented as mean ± SE. [a]$P < 0.05$ compared with control. [b]$P < 0.05$ compared with MTX. Letters not sharing a common letter superscript are significantly different ($P < 0.05$).

control group. On the other hand, administration of MEL and BA caused insignificant changes in liver MDA, NO, GSSG and GSH contents as compared to the control group.

MEL or BA co-administration with MTX significantly ($P < 0.05$) reduced liver tissue contents of MDA, NO and GSSG and significantly ($P < 0.05$) increased liver tissue content of GSH as measured with respect to MTX. However, co-administration of UDCA with MTX revealed insignificant ($P > 0.05$) changes in liver tissue contents of MDA, NO, GSSG and GSH as measured with respect to MTX (Table 2).

3.2.2. Enzymatic parameters

Results in Table 2 showed that MTX, as well as UDCA, administration to group of male rats resulted in a significant ($P < 0.05$) reduction in liver tissue levels of oxidative stress biomarker enzymes activities GPx, GR, GST, SOD and CAT as measured with respect to control group. While administration of MEL and BA caused insignificant changes in liver oxidative stress biomarker activities GPx, GR, GST, SOD and CAT as compared to the control group.

MEL or BA extract co-administrated with MTX significantly ($P < 0.05$) elevated the oxidative stress biomarker enzymes activities in liver tissue GPx, GR, GST, SOD and CAT as measured with respect to MTX. However, co-administration of UDCA with MTX revealed insignificant changes in liver tissue levels of GPx, GR, GST, SOD and CAT as measured with respect to MTX (Table 2).

3.3. Total antioxidant capacity in serum

Moreover, MTX administration to group of male rats resulted in a significant ($P < 0.05$) reduced in serum TAC as measured with respect to control group, which was also observed in UDCA group. Administration of MEL and BA caused insignificant changes in serum TAC as compared to control group. While, MEL or BA extract co-administrated with MTX significantly ($P < 0.05$) elevated serum TAC as measured with respect to MTX, whereas co-administration of UDCA with MTX showed insignificant changes in serum TAC as measured with respect to MTX (Table 2).

3.4. Tumor necrosis factor (TNF-α) in serum

Our results also showed that MTX and UDCA administration to groups of male rats resulted in a significant ($P < 0.05$) elevation in serum levels TNF-α as measured with respect to control group which was supported by strong positive expression of Tumor necrosis factor alpha in immunohistochemical staining of liver tissue sections of MTX treated group of rats (Table 2).

3.5. Immunohistochemical staining of TNF-α in liver tissue

Immunohistochemical staining of liver tissue sections of groups of rats co-administered MTX and MEL (MTX + MEL) showing mild to no positive expression of TNF-α, which was in accordance with the significant ($P < 0.05$) reduction in serum levels of TNF-α as measured with respect to MTX. However, immunohistochemical staining of liver tissue sections of group

Table 2
Protective effect of daily administration of MEL, BA and UDCA against MTX hepatotoxicity for one month on liver oxidative stress parameters in tissue, serum total antioxidant capacity and TNF-α.

Group	MDA (nmol/g)	NO (nmol/0.1 g)	GSSG (mg/g)	GSH (mg/g)	GSH/GSSG ratio	GPx (U/g)	GR (U/L)	GST (U/g)	SOD (U/0.1 g)	Catalase (mmol/min/g)	TAC (mM/L)	TNF-α (pg/mL)
Control	31.01 ± 1.60	76.40 ± 2.17	190.00 ± 18.17	314.03 ± 14.04	1.70 ± 0.12	37.39 ± 0.36	52.25 ± 1.29	0.15 ± 0.006	490.98 ± 5.15	645.60 ± 12.38	1.68 ± 0.12	10.66 ± 0.05
MTX	46.27 ± 3.67[a]	106.76 ± 1.66[a]	239.41 ± 4.65[a]	189.06 ± 6.92[a]	0.79 ± 0.03[a]	19.56 ± 0.42[a]	36.37 ± 1.47[a]	0.07 ± 0.001[a]	389.63 ± 8.66[a]	395.80 ± 24.21[a]	0.83 ± 0.08a	18.98 ± 0.15[a]
MEL	31.29 ± 0.74	75.90 ± 0.88	191.14 ± 14.78	317.42 ± 6.50	1.73 ± 0.19	36.36 ± 0.88	53.50 ± 0.44	0.16 ± 0.001	484.23 ± 2.86	631.53 ± 15.78	1.50 ± 0.10	10.55 ± 0.10
BA	29.57 ± 0.77	78.07 ± 1.24	194.51 ± 3.65	305.20 ± 9.91	1.57 ± 0.07	36.11 ± 0.40	51.35 ± 1.02	0.16 ± 0.002	496.63 ± 3.71	635.55 ± 13.01	1.51 ± 0.06	10.89 ± 0.07
UDCA	40.37 ± 1.71[a]	83.37 ± 2.57[a]	258.31 ± 12.53[a]	185.44 ± 4.64[a]	0.73 ± 0.05[a]	20.86 ± 0.61[a]	42.02 ± 0.82[a]	0.08 ± 0.004[a]	395.25 ± 6.16[a]	570.66 ± 14.61[a]	0.73 ± 0.06[a]	17.10 ± 0.16[a]
MTX + MEL	31.31 ± 1.86[b]	72.12 ± 1.54[b]	186.21 ± 3.88[b]	305.68 ± 15.07[b]	1.65 ± 0.11[b]	32.27 ± 0.79[ab]	54.93 ± 1.47[b]	0.15 ± 0.002[b]	479.73 ± 15.41[b]	652.07 ± 19.89[b]	1.53 ± 0.05[b]	11.08 ± 0.21[b]
MTX + BA	33.72 ± 1.82[b]	71.50 ± 1.70[b]	203.59 ± 9.71[b]	320.15 ± 3.73[b]	1.59 ± 0.08[b]	31.56 ± 0.55[ab]	48.27 ± 0.51[ab]	0.12 ± 0.002[ab]	504.50 ± 7.54[b]	564.18 ± 21.00[ab]	1.57 ± 0.09[b]	11.10 ± 0.05[b]
MTX + UDCA	48.98 ± 1.29[a]	103.30 ± 2.70[a]	227.08 ± 11.05[a]	196.64 ± 5.27[a]	0.88 ± 0.06[a]	17.80 ± 0.36[ab]	35.92 ± 0.84[a]	0.06 ± 0.001[a]	369.38 ± 11.65[a]	315.22 ± 21.88[ab]	0.9 ± 0.03[a]	19.53 ± 0.32[ab]

$n = 6$ in each group. Data are represented as mean ± SE. [a]$P<0.05$ compared with control. [b]$P < 0.05$ compared with MTX. Letters not sharing a common letter superscript are significantly different ($P < 0.05$).

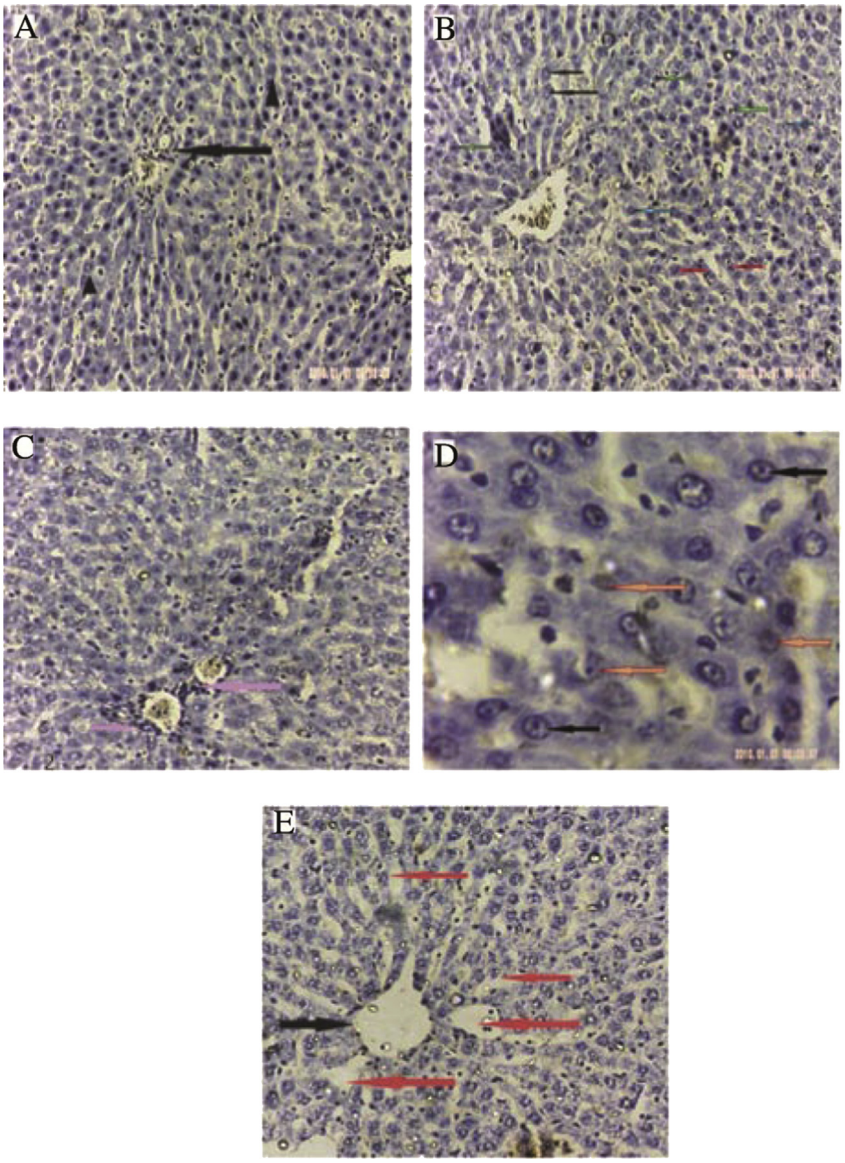

Figure 1. Photomicrographs of liver sections showing expression of TNF-α immunopositivity indicated by brown color.
(A): Control group, Arrow pointed to portal tract, Arrow heads pointed to hepatocytes. Negatively stained sections with preserved hepatic architecture (×100); (B): MTX group, Black filled arrows pointed to vesicular nuclei in active viable cell. Red filled arrows pointed to karyorrhexitic nuclei. Blue filled arrows pointed to karyolitic nucleus. Green filled arrows pointed to pyknotic nuclei. Hepatocytes' nuclei revealed pyknosis, karyorrhexis and karyolysis. Scattered positively stained cells at variable studied fields (×100); (C): MTX + MEL group, Purple arrows pointed to inflammatory cells along portal tracts. Preserved hepatic architecture, most nuclei are viable; active and vesicular (×100); (D): MTX + BA group, Black arrows normal active vesicular nuclei. Pink arrows positively stained cells (×400); (E): MTX + UDCA group, Black arrow central vein. Red arrows widened sinusoidal spaces. Most nuclei are small dark or lysed (×100).

of rats co-administered MTX and BA (MTX + BA) showed moderate positive expression of TNF-α as was reflected by a significant ($P < 0.05$) reduction in TNF-α levels in serum of this group. However, co-administration of UDCA with MTX (MTX + UDCA) showed insignificant ($P > 0.05$) changes in serum levels of TNF-α as measured with respect to MTX, which was also confirmed with strong positive expression in immunohistochemical staining (Figure 1).

3.6. Histopathological results

The biochemical results were supported by the histopathological results (Figure 2) showing severe histopathological changes in liver sections of MTX group. These include cytoplasmic vacuolization of hepatocytes. Fibrous tissue in the portal triad around the bile duct, steatosis and necrosis of sporadic hepatocytes were also noticed.

As a result of combined administration of MEL and MTX histological examination of liver sections revealed improvement in the histopathological picture which was denoted by slight granularity of the cytoplasm and binucleation of hepatocytes.

Liver sections of the group co-administered BA and MTX revealed congestion of the central vein and hepatic sinusoids, as well as slight focal hepatic necrosis and slight vacuolization of hepatocytes indicating reduction in hepatic damage. However, liver sections of the group co-administered UDCA and MTX revealed steatosis of hepatocytes, necrosis of sporadic hepatocytes of some hepatocytic nuclei.

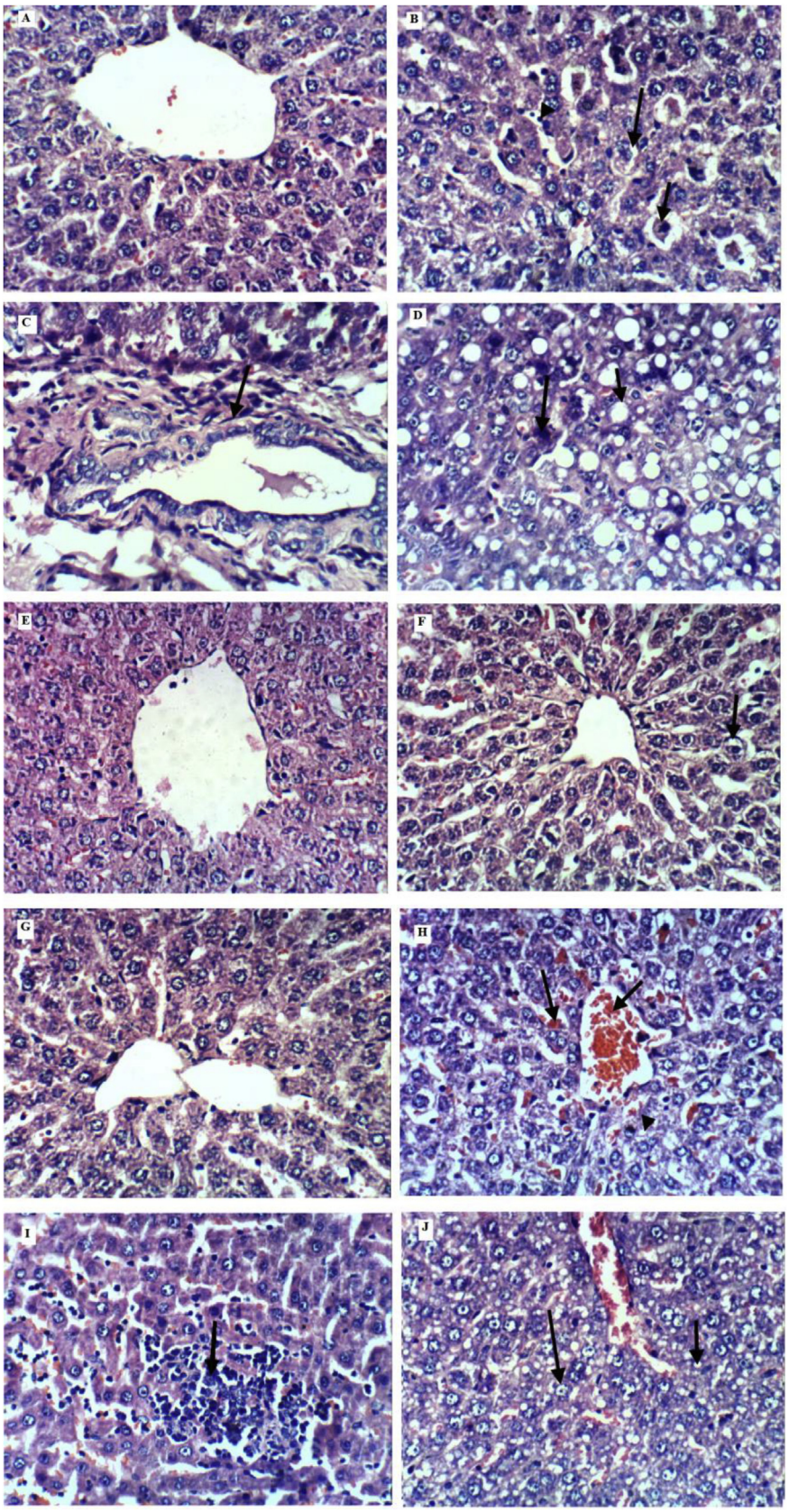

Figure 2. Liver sections of male rats showing histopathological changes.
(A): control group showing normal central vein and normally arranged hepatocytes of hepatic lobule (H&E ×400); (B): MTX treated group showing cytoplasmic vacuolization of hepatocytes (H&E ×400); (C): MTX treated group showing fibrous tissue in the portal triad around the bile duct (H&E ×400); (D): MTX treated group showing steatosis of hepatocytes and necrosis of sporadic hepatocytes (H&E ×400); (E): MEL treated group showing apparently normal hepatocytes. (H&E ×400); (F): MEL and MTX treated group showing slight granularity of the cytoplasm of hepatocytes (H&E ×400); (G): BA treated group showing normal hepatocytes of hepatic lobule (H&E ×400); (H): BA and MTX treated group showing congestion of central vein and hepatic sinusoids as well as slight vacuolization hepatocytes (H&E ×400); (I): UDCA treated group showing focal hepatic necrosis associated with inflammatory cells inflammation as well as sinusoidal leukocytosis (H&E ×400); (J): UDCA and MTX treated group showing congested non dialated central vein and steatosis of hepatocytes (H&E ×400).

4. Discussion

Being a folic acid antagonist and a cytotoxic agent, Methotrexate (MTX) was originally used in the remedy of malignancies [43]. Presently it is known as an anti-inflammatory and immunosuppressant [44], although its use is impeded by its toxicity at different sites including the liver [45].

MTX acts through the inhibition of dihydrofolate reductase, involved in DNA synthesis and repair, causing depletion of reduced folates [44]. Low-moderate to high doses of MTX induce various drawbacks including liver cirrhosis or fibrosis. [1] MTX generates oxidative stress by increasing ROS production causing tissue injury, which may be the main cause of its drawbacks [44]. The harmful effect of MTX treatment was reflected by the increase in TNF-α level, which is a pro-inflammatory cytokine. The increase in TNF-α secretion is due excessive ROS formation, which leads to neutrophil infiltration and the release of pro-inflammatory cytokine triggering apoptosis, cell damage and death of liver cells [46].

In the evaluation of liver toxicity by Methotrexate (MTX), the release of ALT and AST into the blood flow indicates liver damage, as they are normally located in the cytosol of hepatocytes and are involved in the breakdown of amino acids into α-keto acids, their release is due to cellular leakage and loss of functional integrity of cell membranes; therefore, they can be detected in serum [45].

The current biochemical results were supported by severe histopathological changes including cytoplasmic vacuolization of hepatocytes. Fibrous tissue in the portal triad around the bile duct, steatosis and necrosis of sporadic hepatocytes were also noticed, as was observed by Patel *et al* [5]. It was reported that increased necrosis in the liver is marked by increase in leakage of enzymes into the blood flow [47].

These histopathological changes observed in our study were significantly improved in groups of rats co-administered MTX with either MEL or BA with more improvement in the MEL group showing slight granularity of the hepatocytes cytoplasm. In addition, Co-administration of MEL or BA extract with MTX significantly alleviated the liver biomarker enzymes activities, which was reflected by a significant reduction in serum levels of AST, ALT, ALP, GGT, total and direct bilirubin, as well as a significant elevation of serum levels of total protein and albumin as measured with regard to MTX.

These results were in agreement with Suky *et al* [18] and Salihu *et al* [16], they showed that the fruit mesocarp of BA has hepatoprotective properties against CCl_4 in rats. This result may be owed to the sterols stabilization of cell membranes, preventing leakage of the intracellular enzymes [48], Normalization of serum levels of transaminases occurs as the hepatic parenchyma heals and the hepatocytes regenerate. BA conditions the hepatocytes and protects the membrane integrity against CCl_4 induced leakage of marker enzymes into the blood flow [18]. Moreover, the reduction in total protein level in MTX treated group indicates pathological damage of the liver [49]. Furthermore, MEL enhances hepatic differentiation [50] and stops the formation of pro-inflammatory cytokines such as TNF-α [51,52].

MTX induces a dose dependent increase in peroxide levels as it alters redox-properties of the cell leading to consumption of the cellular and mitochondrial glutathione [53], which gives MTX its immunosuppressive characteristics. Oxidative stress damage results from the imbalance between oxidants and antioxidants leading to many pathological changes including cellular damage. MTX toxicity results from free radical creation and lipid peroxidation [45], deriving oxidative stress or oxidative cellular injury. It also suppresses the levels of antioxidants (enzymatic and non-enzymatic) and increases the levels of oxidants in the liver, kidney and gut tissues [54].

This was confirmed in our results showing a significant elevation of tissue MDA, NO and GSSG, as well as a significant reduction of tissue GSH [45,55]. Moreover, our results showed significant reduction of tissue enzymatic oxidative stress parameters including GPx, GR, GST, SOD and CAT.

Reduction of tissue oxidative stress parameters was supported by a significant drop in serum total antioxidant capacity (TAC). Oxygen radicals and hydrogen peroxides are the main cause of many side effects of MTX [45]. They cause cell damage through binding to cellular macromolecules, especially membrane lipids resulting in the release of ALT and AST from cells to serum. They also cause shape and structural changes of the nucleus due to DNA fragmentation and denaturation, thus initiating apoptosis [55].

Inhibition of RNA, DNA and protein synthesis, as well as the release of adenosine account for the toxic actions of MTX. It also competitively inhibits dihydrofolate reductase (DHFR), inhibit regeneration of tetrahydrafolate (FH_4) from dihydrofolate (FH_2) affecting purine and pyrimidine synthesis [53]. Results in our study showed that co-administration of MEL with MTX alleviated its toxic effects on the liver. MEL stabilizes the lipid bilayers, as well as the mitochondrial inner membranes thus, improving the electron transport chain activity through increasing the electron flow, and ATP production [56]. Also MEL acts as an anti-apoptotic agent in mitochondrial ROS/RNS- mediated cell death [57].

Melatonin is capable of limiting hepatic damage as it restores tissue antioxidant enzyme activities through directly neutralizing as well as up-regulating anti-oxidative enzymes including SOD, GPx and glutathione (GSH). Thus, protects the cells before the free radicals, reactive oxygen as well as nitrogen species carry out their destructive activities. This way MEL protects the lipid bilayer of cell membranes, proteins in the cytoplasm and DNA inside the nucleus, which is facilitated by its ability to crossover all morphophysiological barriers [45,57,58].

Also, the co-administration of BA with MTX showed improvement against MTX toxicity in liver tissue, suggesting the functional improvement of hepatocytes as a result of the antioxidant effect BA extract. This antioxidant activity is due to the presence of saponins and flavonoids which are known to exhibit anti-inflammatory and antioxidant activities. Administration of BA was able to restore the decrease in tissue antioxidant enzymes activities and the scavenging activity of radicals, thus protecting the liver cells [18].

Although UDCA is known to be a hepatoprotective agent, it enhanced inflammation and damage of the liver in our model of liver toxicity. UDCA acid worsened small intestinal inflammation in the model of indomethacin induced intestinal inflammation [59]. Results of our study may be due to retention of bile constituents like lithocholic (LCA) within the hepatocytes triggering the release of growth factors, cytokines and lipid peroxidation products that promote the inflammatory reaction, injury of neighboring cells [60] and apoptosis [61]. Moreover, bile acids act like detergents on lipid bilayer leading to the disruption of cell membranes [62], generating ROS and leading to hepatocyte apoptosis [63]. In addition, it has been reported

that hepatocyte necrosis is the predominant form of cell death in certain models of bile acids toxicity [64]. UDCA toxicity is associated with its obstruction to drug detoxification which inhibits cellular functions. Moreover, there is a fine range between the approved and toxic dose of UDCA and it transforms into LCA bringing about DNA strand breakage and promoting cell transformation [65].

BA may be as promising as MEL in the hepatoprotection against MTX toxicity through their antioxidant and radical scavenging activities. In addition, it is not recommended to co-administer UDCA with MTX as it enhanced inflammation and damage to the liver.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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