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Research Article
Effectiveness of *Moringa oleifera* L. Leaves Extract Against Methotrexate-induced Acute Hepatotoxicity in Male Rats

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Abstract
Background and Objective: Methotrexate (Meth), is one of the most commonly utilized anticancer agents. Besides, Meth has been widely used to treat rheumatoid arthritis as well as a vast variety of inflammatory disorders. The clinical use is obstacles by its hepatotoxicity. This work aimed to investigate the hepatoprotective action of *Moringa oleifera* Lam (*M. oleifera*) leaves extract against Meth-induced hepatotoxicity in rats. **Materials and Methods:** Thirty-two male albino Wistar rats were used in this study. Rats were randomly divided into four groups, control, hepatotoxic (Meth) and *M. oleifera* (500 and 750 mg kg^{-1}) pre-treatment Meth groups. Hepatotoxicity was induced by a single intraperitoneal (i.p.) injection of Meth (20 mg kg^{-1}) while the pre-treatment groups received either *M. oleifera* (500 or 750 mg kg^{-1}) 21 days before Meth and 5 days thereafter. Serum concentration of liver enzymes and inflammatory cytokines were measured. Liver contents of oxidative stress markers and antioxidant enzymes were determined. Hematoxylin and eosin (H and E) stained liver sections from all groups were also examined. **Results:** Both *M. oleifera* doses significantly reduced serum liver enzymes, serum inflammatory cytokines and liver oxidative stress markers compared to Meth group. Besides, the two doses of *M. oleifera* significantly increased liver antioxidant enzymes activity compared to Meth group. In comparison, there were no differences between the two *M. oleifera* doses in concern to the biochemical measurements. On the other hand, *M. oleifera* (500 mg kg^{-1}) decreased Meth-induced pathological changes in liver while *M. oleifera* (750 mg kg^{-1}) perfectly protected the liver against Meth-induced pathological changes. **Conclusion:** This study provided an evidence for the protective action of *M. oleifera* extract against acute liver damage induced by Meth through an antioxidant and anti-inflammatory mechanism.

Key words: *Moringa oleifera*, methotrexate, hepatotoxicity, oxidative stress, cytokines, histopathology

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Methotrexate (Meth), a folic acid antagonist is one of the most commonly utilized anticancer agents. Meth is used extensively to treat many tumors type including leukemia and lymphoma. Nowadays, Meth as an immunosuppressant agent has been widely used to treat rheumatoid arthritis as well as a vast variety of inflammatory disorders[1-4]. As antiproliferative agent, Meth causes much toxicity to the body systems including, lung, blood and liver[1,2,5].

Although folic acid is considered by many researchers to prevent Meth-induced various toxicities, opposite opinion about its real benefits has been documented. In addition, there is no guidelines that regulate folic acid dose and frequency of administration with Meth[6-8].

As the hepatotoxicity of Meth is the most common one, researchers do their best to explore the mechanism(s) of Meth-induced hepatotoxicity. To date, the mechanisms of Meth-induced hepatotoxicity have not been reached but free radicals and oxidative stress are likely to play the greatest role[3,9,10].

Moringa oleifera Lam (*M. oleifera* L.) is a species of Moringaceae family that ordinarily developed in tropical and subtropical countries[11]. There are many researches that documented the anti-oxidant role played by *M. oleifera* L. leaves extract through a variety of bioactive compounds, the principal of them are the polyphenols, generally, the flavonoids myrecytin, quercetin and kaempferol[12-17]. Numerous studies reported a hepatoprotective effect of *M. oleifera* L. leaves extract against several hepatotoxic drugs and chemicals including acetaminophen[18], alloxan[19], nickel[20], diclofenac sodium[21], carbon tetrachloride[22] and alcohol[23].

The current research theory relies on the *M. oleifera* L. extract to contain many antioxidants, which are believed to cause a protective effect against hepatotoxicity induced by Meth.

The present study aims to evaluate the possible hepatoprotective effect of *M. oleifera* L. leaves extract against Meth-induced hepatotoxicity. In addition, the mechanism of the suggested effect will be studied regarding the potential antioxidant properties of the *M. oleifera* L. leaves extract.

MATERIALS AND METHODS

Chemicals: Meth (50 mg^{-2} mL), MYLAN S.A.S., France. Leaves of *M. oleifera* L. were obtained from Prof El-Ghadban, EA, Professor of Medicinal and Aromatic Plants Research Department, Horticultural Research Institute, Agricultural Research Centre. The Plant was identified by Dr El-Gibali, MA, Senior Botanist, Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Giza, Egypt. All other chemicals were of analytical grade. This study was conducted on May, 2017 and the experimental protocol took about 8 months.

Extraction procedure: About 500 g air dried powdered *M. oleifera* L. leaves was macerated at room temperature with 1000 mL 80% ethanol. They were mixed using magnetic stirrer at 100 rpm speed for 2 days. The ethanolic extract was concentrated to nearly dryness under vacuum using rotary evaporator at 40°C, then the condensed residue was further freeze-dried. The extract was stored in non-permeable glass containers at 4°C[24].

Phytochemical screening of *M. oleifera* L. leaves extract: The phytochemical analysis of *M. oleifera* L. leaves extract has been performed to find out that the plant contains some important chemical components including alkaloids, flavonoids, glycosides, saponins, tannins, resins and triterpenoids using standard procedures of analysis[25].

Ethical approval: This study was approved by Deanship of Scientific Research Committee, King Abdulaziz University, reference no (J-422-253-38). The experiment was conducted at King Fahd Medical Research Center (KFMRC), KAU.

Animals: Thirty-two male albino Wistar rats weighing 170-200 g purchased from animal house of KFMRC, KAU, Saudi Arabia. Animals were caged at the standard laboratory conditions and we left them free to eat and drink. Animals were handled under the rules of Canadian ethical approval from the Local Biomedical ethical committee of KAU.

Experimental protocol: Rats were randomly divided into four groups. Group I (Contr), rats received normal saline orally. Group II (Meth), rats in this group received a single intraperitoneal (i.p.) dose of Meth (20 mg kg^{-1})[26]. Group III (*M. Oleifera* L. 500 mg+Meth), rats in this group received *M. Oleifera* L. (500 mg kg^{-1}) orally for 21 days[27] followed by a single i.p., dose of Meth (20 mg kg^{-1}). Group IV (*M. Oleifera* L. 750 mg+Meth), rats in this group received *M. Oleifera* L. (750 mg kg^{-1}) orally for 21 days followed by a single i.p., dose of Meth (20 mg kg^{-1}).

Samples collection: After 5 days of Meth injection, the rats were anesthetized using diethyl ether then blood samples were collected directly from the heart. Serum was then

separated and stored at -80°C. The liver samples were then collected and each sample was divided into two parts, one of them was stored in a freezer at -80°C and the other part was preserved in neutral buffer formaldehyde solution.

Determination of serum liver functions: This determination use double-sandwich ELISA technique to determine the concentrations of liver enzymes, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) using the rat ELISA kits obtained MyBioSource USA following the instructions and steps contained in the internal kits bulletin. A rat monoclonal antibody specific for ALT, AST, ALP and GGT were utilized and the detecting antibody was polyclonal antibody with biotin labeled.

Determination of liver oxidative stress markers and antioxidant enzymes: Liver concentrations of malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were measured in the liver homogenates using the rat ELISA kits obtained MyBioSource USA following the instructions and steps contained in the internal kits bulletin. All the kits apply double-sandwich ELISA technique. The pre-coated antibody was rat specific monoclonal antibody and the detecting antibody was polyclonal antibody with biotin labeled.

Determination of serum proinflammatory cytokines: The concentrations of serum tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) were measured using the rat ELISA kits obtained MyBioSource USA following the instructions and steps contained in the internal kits bulletin. This experiment used double-sandwich ELISA technique. The pre-coated antibody was either rat TNF-alpha monoclonal antibody or rat IL-1b monoclonal antibody whereas the detecting antibody was polyclonal antibody with biotin labeled.

Histopathological study: The formaldehyde preserved liver was paraffin-embedded, cut into sections, fixed then stained with hematoxylin-eosin (H and E). The slides examined under the light microscope.

Statistical analysis: Data were presented as Mean$\pm$SE. The values were compared by one-way analysis of variance (ANOVA), L.S.D. comparison using SPSS software version 22. The $p\leq 0.05$ indicate significance difference.

RESULTS

Phytochemical screening of *M. oleifera* L. leaves extract: Phytochemical analysis of *M. oleifera* L. leaves extract revealed that it contained small amount of anthraquinone, glycosides, resins, triterpenoids and saponins. In addition, it contains moderate amounts of alkaloids, tannins and terpenes. It also contained large amount of flavonoids and phenolic. The results also showed that the extract contains no steroids (Table 1).

Effect of *M. oleifera* L. on liver function: Meth treatment increased the concentrations of serum liver enzymes ALT, AST, ALP and GGT nearly to double the control values ($p\leq 0.001$). These concentrations were found to be decreased significantly in both Meth groups pretreated with *M. oleifera* L. extract compared to the Meth only group ($p\leq 0.001$). There was no significant difference observed between *M. oleifera* L. extract 500 mg kg^{-1} group and *M. oleifera* L. extract 750 mg kg^{-1} (Table 2).

Effect of *M. oleifera* L. on liver histopathology: Liver of rats from Control group showing the centrally located vein and the radially arranged hepatocytes with its eosinophilic cytoplasm, appearance of basophilic granules and the intervening blood sinusoids (Fig. 1a). Meth treatment caused loss of the radial organization of hepatocytes around the central vein with marked congestive areas alternating with focal areas of degenerated hepatocytes, that were replaced by inflammatory cells. There were also different nuclear changes ranging from nuclear fading to pyknotic and karyolytic nuclei (Fig. 1b). Meth treatment also caused congestion of hepatoportal blood vessels and hepatic blood sinusoids (Fig. 1c). These pathological changes were found to be decreased in *M. oleifera* L. extract 500 mg kg^{-1}

Table 1: Phytochemical screening of *Moringa oleifera* L. (*M. oleifera* L.) leaves extract

Phytoconstituents	Inference
Phenolics	+++
Flavonoids	+++
Steroids	-
Saponins	+
Terpenoids	+
Alkaloids	++
Tannins	++
Terpenes	++
Resins	+
Glycosides	+
Anthraquinone	+

The intensity of compounds in *M. oleifera* L. leaves extract: (+) slightly, (++) moderate and (+++) largely amount. (-) absence of the constituents

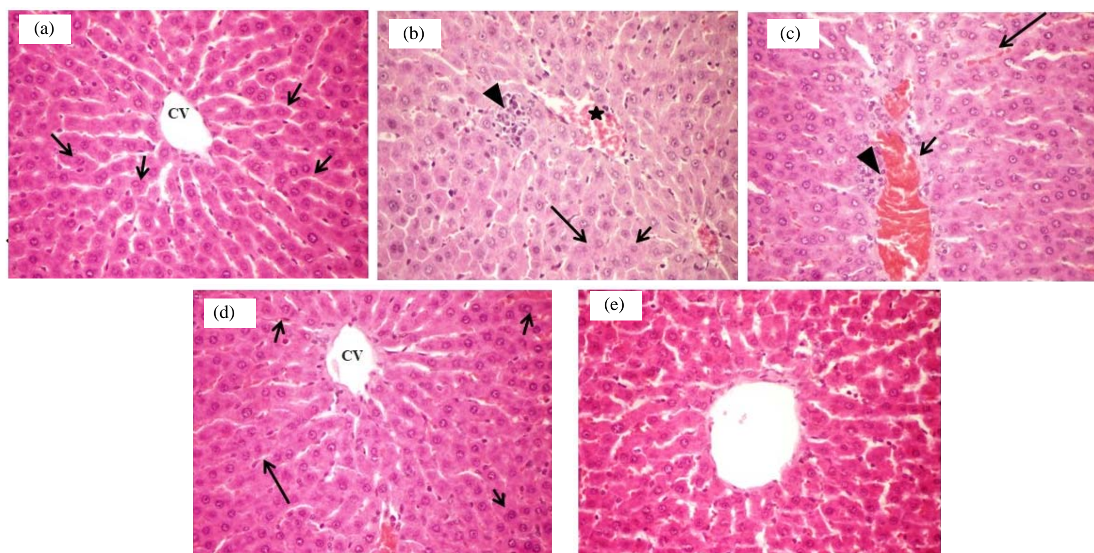

Fig. 1(a-e): (a) Liver of rats from Contr group showing the centrally located vein (CV) and the radially arranges hepatocytes with its eosinophilic cytoplasm with appearance of basophilic granules and the intervening blood sinusoids. Notice the normal vesicular nuclei with prominent nucleolus (arrow), (b) Liver of rats from Meth group showing loss of the radial organization around central vein with marked congestive areas (star) alternating with focal areas of degenerated hepatocytes, that is replaced by inflammatory cells (arrow head). Notice there are different nuclear changes from nuclear fading (small arrow) to pyknotic and karyolytic (large arrow), (c) Liver of rats from Meth group showing congestion of hepatoportal blood vessels (small arrow) and hepatic blood sinusoids (large arrow). Notice the periportal mononuclear cells (arrow head), (d) Liver of rats from M. oleifera 500 mg kg^{-1}+Meth group showing the evident decrease in the sinusoidal blood congestion, binucleated hepatocytes (small arrow) and prominent sinusoidal kupffer cells (large arrow) are still remarkable and (e) Liver of rats from M. oleifera 750 mg kg^{-1}+Meth group showing radial organization around central vein with no congestive areas. Notice the similar appearance to control sections. (H and E x 400)

Table 2: Effect of *Moringa oleifera* L. (*M. oleifera* L.) 500 and 750 mg kg^{-1} on serum liver enzymes measured in methotrexate (Meth)-induced hepatotoxicity in rats

Experimental groups	ALT (U L^{-1})	AST (U L^{-1})	ALP (U L^{-1})	GGT (U mL^{-1})
Contr	42.19±3.18	139.09±7.91	110.97±6.95	4.24±0.30
Meth	91.03±5.53^{a}	211.95±8.93^{a}	190.82±8.43^{a}	6.46±0.21^{a}
M. oleifera 500 mg+Meth	60.07±4.48^{b}	171.81±6.04^{b}	142.31±9.87^{b}	5.12±0.22^{b}
M. oleifera 750 mg+Meth	51.20±3.59^{b}	156.06±8.19^{b}	131.06±6.86^{b}	4.75±0.31^{b}

Data are represented as Mean±SE (n = 8). ^{a}Significant versus control (Contr) group, ^{b}Significant versus Meth group ($p \le 0.05$)

group (Fig. 1d). Liver of rats from *M. oleifera* L. 750 mg kg^{-1} group showed apparently normal appearance (Fig. 1e).

Effect of *M. oleifera* L. on oxidative stress measures: Meth treatment increased the concentrations of liver oxidative stress measures MDA and NO nearly to double the control values ($p \le 0.001$). These concentrations were found to be decreased significantly in both Meth groups pretreated with *M. oleifera* L. extract compared to the Meth only group ($p \le 0.001$). There was no significant difference observed between *M. oleifera* L. extract 500 mg kg^{-1} group and *M. oleifera* L. extract 750 mg kg^{-1} (Fig. 2 and 3).

Effect of *M. oleifera* L. on antioxidant enzymes: Meth treatment significantly decreased the concentrations of liver antioxidant enzymes SOD and GSH-Px nearly to half the control values ($p \le 0.001$). These concentrations were found to be increased significantly in both Meth groups pretreated with *M. oleifera* L. extract compared with the Meth only group ($p \le 0.001$). There was no significant difference observed between *M. oleifera* L. extract 500 mg kg^{-1} group and *M. oleifera* L. extract 750 mg kg^{-1} (Fig. 4 and 5).

Effect of *M. oleifera* L. on inflammatory cytokines: Meth treatment increased the concentrations of serum TNF-α and

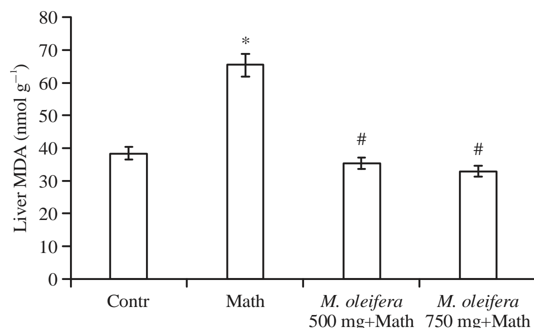

Fig. 2: Effect of *Moringa oleifera* (*M. oleifera*) 500 and 750 mg on liver malondialdehyde (MDA) concentration measured in methotrexate (Meth)-induced hepatotoxicity in rats
Data are represented as Mean±SE (n = 8), *Significant versus control (Contr) group, #Significant versus Meth group (p = 0.05)

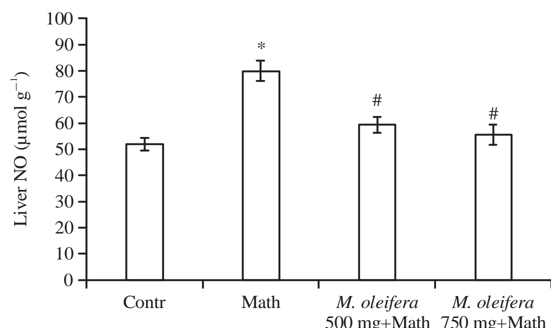

Fig. 3: Effect of *Moringa oleifera* (*M. oleifera*) 500 and 750 mg on liver nitric oxide (NO) concentration measured in methotrexate (Meth)-induced hepatotoxicity in rats
Data are represented as Mean±SE (n = 8), *Significant versus control (Contr) group, #Significant versus Meth group (p = 0.05)

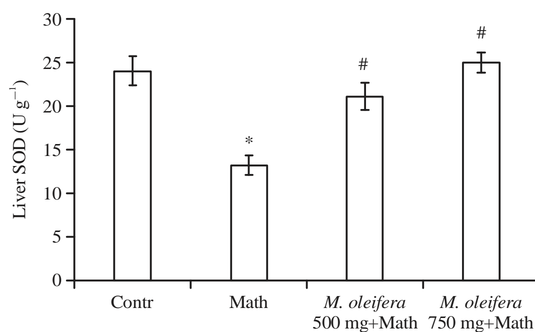

Fig. 4: Effect of *Moringa oleifera* (M. oleifera) 500 and 750 mg on liver superoxide dismutase (SOD) activity measured in methotrexate (Meth)-induced hepatotoxicity in rats
Data are represented as Mean±SE (n = 8), *Significant versus control (Contr) group, #Significant versus Meth group (p = 0.05)

IL-1β nearly to double the control values (p<0.001). These concentrations were found to be decreased significantly in

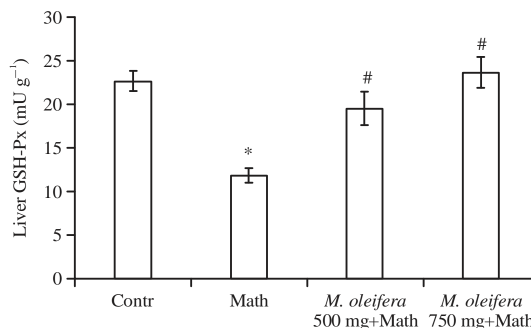

Fig. 5: Effect of *Moringa oleifera* (*M. oleifera*) 500 and 750 mg on liver glutathione peroxidase (GSH-Px) activity measured in methotrexate (Meth)-induced hepatotoxicity in rats
Data are represented as Mean±SE (n = 8) *Significant versus control (Contr) group, #Significant versus Meth group (p = 0.05)

Table 3: Effect of *Moringa oleifera* (*M. oleifera*) 500 and 750 mg kg^{-1} on serum tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1b) measured in methotrexate (Meth)-induced hepatotoxicity in rats

Experimental groups	TNF-α (Pg mL^{-1})	IL-1b (Pg mL^{-1})
Contr	9.25±0.68	37.86±2.44
Meth	18.80±1.27[a]	82.59±3.37[a]
M. oleifera 500 mg+Meth	13.33±1.22[b]	47.41±2.98[b]
M. oleifera 750 mg+Meth	11.15±1.03[b]	41.34±2.26[b]

Data are represented as Mean±SE (n = 8). [a]Significant versus control (Contr) group, [b]Significant versus Meth group (p<0.05)

both Meth groups pretreated with *M. oleifera* L. extract compared to the Meth only group (p<0.001). There was no significant difference observed between *M. oleifera* L. extract 500 mg kg^{-1} group and *M. oleifera* L. extract 750 mg kg^{-1} (Table 3).

DISCUSSION

The results of this study showed the protective effect of *M. oleifera* L. extract against Meth-induced liver damage in male Wister rats, which was confirmed by reducing the level of liver enzymes, ALT, AST, ALP and GGT as well as preventing Meth-induced hepatocellular necrosis. In addition, this study showed that pre-treatment of Meth treated rats with the *M. oleifera* L. extract decreased levels of oxidative stress markers, MDA and NO as well as increased levels of antioxidant enzymes, SOD and GSH-Px. As far as we know there are no studies on the effect of *M. oleifera* L. extract on Meth-induced liver damage.

Meth is used in the treatment of many diseases such as cancer, immunological diseases and inflammatory diseases[28]. It is documented that Meth has many side effects, the most common of which is its hepatotoxicity and its bone marrow toxicity[29]. On the contrary, the *M. oleifera* L. extract has been

known to have an anti-hepatotoxic effect against many chemicals (carbon tetrachloride), heavy metals (cadmium and nickel) and drugs (paracetamol, isoniazid, rifampicin and pyrazinamide)-induced liver damage[20,30-34]. There are many previous studies that documented the protective effect of the *M. oleifera* L. extract against the hepatic toxicity caused by streptozotocin and alloxan-induced diabetes[19,35].

To this point, the potential prevention of *M. oleifera* L. extract against the toxic effect of Meth on the liver has not been investigated. Therefore, this study was designed to investigate the potential protective effect of the *M. oleifera* L. extract against the Meth-induced hepatotoxicity, highlighting the effect of *M. oleifera* L. as antioxidant and anti-inflammatory remedy.

In this study model, the results revealed that, compared to control group Meth treatment induced significant increased levels of serum liver enzymes ALT, AST, ALP and GGT. This confirmed the destruction caused by Meth on the liver cells. Liver enzymes are the main determinant of the death of liver cells as the destruction of cell membranes, leading to the leakage of enzymes from hepatocytes to the serum[36-38]. The Meth-induced destruction of liver cells was confirmed by examining the pathological changes caused by Meth. In histopathological results, several changes were observed in Meth treatment group confirming the necrotic cell death in different parts of the liver. A large number of mononuclear cells were infiltrated which together with blood vessel congestion indicated presence of inflammation. Meth in the liver is converted into its active metabolite Meth polyglutamates (MethMPG). MethMPG accumulates in the liver cells causing its necrosis[39,40]. In the same direction as our results, previous studies have obtained similar results that confirm hepatic damage when injecting Meth into experimental animals[41,42]. The results of this study showed the protective effect of *M. oleifera* L. extract against Meth-induced liver damage, which was confirmed by reducing the level of liver enzymes as well as weakening the pathological damage.

Oxidative stress is one of the most important mechanisms that cause hepatotoxicity of Meth. The metabolism of Meth is accompanied by the release of many free radicals that affect the balance between oxidative stressors and antioxidants[43]. Under the current research conditions, Meth-induced severe liver damage was found to be associated with increased hepatic content of the lipid peroxidation product, MDA and the oxidative factor, NO and the reduction of hepatic tissues content of the antioxidant enzymes SOD and GSH-Px. These results are similar to the data of Khafaga and El-Sayed[38]. In this study, improved oxidative stress measurements and increased levels of antioxidant enzymes were observed in the case of pre-treatment with the *M. oleifera* L. extract. The results of this study support previous reports that showed the ability of *M. oleifera* L. extract to reduce and protect against oxidative stress caused by many drugs. Furthermore, previous researches suggest that the significant protective effect of *M. oleifera* L. extract against cadmium-induced hepatic toxicity and oxidative stress is due to the presence of total phenolics and flavonoids such as quercetin and kaempferol and ascorbic acid[12,31,44]. Like many previous findings[45,46], the results of this study confirmed the presence of large quantities of phenolics and flavonoids in *M. oleifera* L. extract. Phenolics and flavonoids are the common antioxidants known in plants[24,47]. The results of this study showed that the hepatic toxicity of Meth was accompanied by a marked increase in serum levels of proinflammatory cytokines TNF-α and IL-1β. Our results have coincided with recent research that documented the proinflammatory effect of Meth[48,49]. In this study, pre-treatment with the *M. oleifera* L. extract significantly decreased serum levels of TNF-α and IL-1β. Earlier studies have attributed the anti-inflammatory effect of *M. oleifera* L. extract to its high contents of flavonoids, polyphenols and antioxidants[50,51].

The results of this study showing that pre-treatment with the *M. oleifera* L. extract reduces the level of liver enzymes, weakens the pathological damage, improves oxidative stress measurements and decreases proinflammatory cytokines induced by Meth treatment. Therefore, it can be used to safeguard against hepatotoxicity in patients receiving Meth-therapy.

CONCLUSION

In conclusion, this study provided an evidence for the protective action of *M. oleifera* L. extract against Meth-induced acute liver damage through an antioxidant and anti-inflammatory mechanism.

SIGNIFICANCE STATEMENT

The results of this study showed for the first time the protective effect of *M. oleifera* L. extract against the hepatic damage caused by Meth, the drug used clinically in the treatment of many cancers and immune diseases. This finding will be beneficial to many patients. Also, it opens the search field in front of researchers to search the protective effect of the *M. oleifera* L. extract in human beings.

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