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Original Article

The Hepatoprotective Role of *Balanites Aegyptiaca* Extract and its Nano-Formulation against Methomyl-Induced Toxicity and Oxidative Stress in Mice via Overexpression of *Nrf2*

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Abstract

Introduction: Methomyl (MET) is a monomethyl carbamate insecticide that is widely used around the world. MET is highly toxic to humans via the oral exposure and mainly affects the liver tissue. It actually induces toxicity through overproduction of Reactive Oxygen Species (ROS) leading to oxidative stress with subsequent destruction of lipids, proteins and nucleic acids.

Materials and Methods: Various plant extracts have been primarily screened for their antioxidant activities by measuring the free radical scavenging ability. Later, the plant extract with the highest antioxidant efficacy has been further formulated into a nanosuspension and the antioxidative effect has also been investigated against MET. Additionally, liver, kidney and heart function biomarkers, liver tissue oxidative stress parameters and total antioxidant capacity were assessed. Moreover, RT-PCR was applied to measure the *Nrf2* expression.

Results: The antioxidant screening data showed that *balanites* extract (BLT, *Balanites aegyptiaca*) had the most potent antioxidant activity. Besides, BLT showed dose-dependent improvement in liver, heart and kidney functions in experimental mice treated with MET. The antioxidant biomarkers in liver tissue and total antioxidant capacity were elevated as compared to the MET-treated group. Furthermore, BLT significantly ameliorated MET-induced toxicity *via* the induction of *Nrf2* and MET hepatic clearance. This study suggests the potential use of BLT extract as a natural antioxidant for the safe management of MET-induced hepatotoxicity and oxidative stress.

Conclusions: Based on the presented data in this study, it can be concluded that BLT or BLT-NS can be used as a safe drug for methomyl toxicity.

Keywords: Methomyl, Methomyl-Induced Toxicity, Oxidative Stress, *Balanites, Balanites* Nanosuspension

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Introduction

Carbamate insecticides are generally used in various agricultural sectors, particularly crop protection. The higher global order for pesticides has created a market that is worth billions of dollars.[1,2] Methomyl (S-methyl-N-(methylcarbamoyloxy)-thioacetimidate) or MET, an oxime insecticide in the carbamate class, is widely used to control adults of different pests.[3] However, MET contact with humans may cause serious toxic effects. According to the United States Environmental Protection Agency (US EPA), human exposures to MET fall into three different types of toxicity depending on the route of exposure: the first type, oral exposure which is extremely toxic; second type, inhalation is moderately toxic; and the third type, dermal exposure is slightly toxic.[4]

The metabolism of MET in mammals produces a Mercapturic Acid Derivative (MAD) via conjugation with glutathione and replacement of the S-methyl groups, which can be removed by liver and kidney. Failure to eliminate MAD from blood cells generate nephrotoxic diseases. MAD is quickly broken down in the blood to carbon dioxide causing hypoxia and respiratory malfunction. Moreover, accumulation of MET leads to its hydrolysis to produce S-methyl- N-hydroxy thioacetimidate which is also rapidly broken down to carbon dioxide.[5]

Moreover, MET causes ROS overproduction leading to oxidative stress with a subsequent destruction of lipids, proteins and nucleic acids.[6] Thus, lipid peroxidation has been incriminated as a main mechanism involved in toxicity of carbamate.[7] Under normal conditions, cells are provided

with enzymatic and non-enzymatic pathways maintaining cellular redox homeostasis.[8] This includes glutathione (GSH) and the antioxidant enzymes: catalase (CAT), glutathione peroxidases (GPx) and superoxide dismutase (SOD). Disruption of this homeostasis by either the inhibition of the antioxidant enzymes or the consumption of antioxidant molecules such as GSH, catalyzes ROS formation.[9] This leads to cellular malfunction and associated apoptosis.[10]

Nuclear erythroid 2-related factor 2 (Nrf2) is a key regulator of oxidative stress in many cell types including hepatic cells.[11-16] In the absence of oxidative stress, Nrf2 is located in the cytoplasm where it interacts with Keap1 (Kelch-like ECH-associated protein 1) and is quickly degraded by the ubiquitin-proteasome pathway.[17,18] However, under oxidative stress, phosphorylation of Nrf2 leads to its separation from Keap1 and following translocation to the nucleus.[14,15] Herein, it binds to Antioxidant Response Element (ARE) sequence and, in association with other nuclear proteins, catalyzes the transcription of ARE-responsive genes to mount strong antioxidant and cytoprotective responses.[19,20]

Herbal medicines have increasingly gained attention due to rising costs of treatments with artificial western medicine, various side effects of allopathic drugs, drug resistance and availability of these herbal medicines.[21] In addition, plant extracts have been widely used due to their potent antioxidant properties and their marked effects in the prevention of oxidative stress due to their high contents of polyphenolic compounds.[22]

Conventional drug delivery systems have several challenges such as low bioavailability, *in vivo* instability, poor absorption, lack of specificity or poor targeting capacities and increased adverse effects of drugs. Thus, the need for new drug delivery systems that can restraint these challenges and hence improve the efficacy and safety of drugs is required. One of these new drug delivery systems that specially ameliorate targeted delivery is nanotechnology. Nanotechnology is a technique that helps to elevate the therapeutic value by decreasing toxicity and increasing the bioavailability in addition to reducing repeated doses. Nanoparticles are also able to protect the drug from breaking down in the gastrointestinal tract, release the incorporated drug in a controlled method so reducing side effects.[23]

Various plants with well-known antioxidant activities have screened in this study including *Balanites aegyptiaca* Del. (Zygophyllaceae) (whole date), *Amrtemisia absinthium* L. (Asteraceae) (seeds), *Zingiber officinale* Rosc. (Zingiberaceae) (roots), *Curuma Longa* L. (Zingiberaceae) (roots), *Citrus paradise* Macfad. (Rutaceae) (fruits), *Moringa Oliefera* Lam. (Moringaceae) (leaves), *Ammi visnaga* (L.) Lam. (Apiaceae) (whole plant), *Cymbopogon proximus* STAPF. (Gramineae) (roots), *Olea europaea* L. (Oleaceae) (leaves) and *Glycyrrhiza glabra* Linn.

As will be discussed later, this study has mainly focused on Balanites (BLT), *Balanites aegyptiaca* (L.) Del., also known as desert dates, a popular plant from Sudan and is widely used in Sudanese folk medicine. BLT has been reported by many workers to possess antioxidant and antimicrobial activities.[24] Due to its antioxidant effect, BLT extract has been proposed in the current study as one of the plants antioxidant agents to mitigate the toxicity of MET caused by free radicals.

Therefore, the objective of the present study was to investigate the antioxidant efficacy of various plant extracts on MET-induced oxidative stress in Swiss albino mice. To this end, various plant extracts have been screened for their free radical scavenging ability. Later, the plant extract that exhibited the highest antioxidant efficacy has been further formulated into a nanosuspension and assessed for antioxidant efficacy as well. Moreover, hepatotoxicity, nephrotoxicity and cardiotoxicity of MET and the protective effects of the most powerful antioxidant plant extract were assessed.

Materials and Methods

Chemicals and Plant Materials

Methomyl, Tween 20 (Polyethylene glycol sorbitan monolaurate) and acetonitrile were purchased from Sigma-Aldrich, Saint Louis, MO, USA. Methanol was obtained from EL-Gomhouria Chemicals Company, Cairo, Egypt. Superoxide dismutase kit was purchased from Bio-diagnostic Company, Dokki, Giza, Egypt. Various plant extracts have been used in the study including: *Balanites aegyptiaca* Del. (Zygophyllaceae) (whole date), *Amrtemisia absinthium* L. (Asteraceae) (seeds), *Zingiber officinale* Rosc. (Zingiberaceae) (roots), *Curuma Longa* L. (Zingiberaceae) (roots), *Citrus paradise* Macfad. (Rutaceae) (fruits), *Moringa Oliefera* Lam. (Moringaceae) (leaves), *Ammi visnaga* (L.) Lam. (Apiaceae) (whole plant), *Cymbopogon proximus* STAPF. (Gramineae) (roots), *Olea europaea* L. (Oleaceae) (leaves) and *Glycyrrhiza glabra* Linn. (Fabaceae) (roots). All plants were obtained from a local herbal market in Mansoura City, Egypt.

Preparation of Plant Extracts

Each plant was collected, washed with water and grounded into powder. Methanolic extract was prepared by macerating 250 g of the grounded plant in pure methanol (3×1 L). Later, the extract was filtered, and marc was discarded. Finally, organic solvent was allowed to evaporate to dryness, and the extract was collected and kept in a refrigerator till further use.[25]

Preliminary Phytochemical Investigation of the Plants

By utilizing standard techniques, the plants extracts were tested for the presence of bioactive compounds as follow: carbohydrates content was determined according to Molish's test.[26] The level of flavonoids was estimated with few drops of sodium hydroxide according to the Alkaline reagent test.[27] The level of saponins were determined

according to foam formation, and the level of tannins were examined by using 2% solution of $FeCl_3$.[28] Glycosides content was determined according to legal's test and the level of alkaloids was determined according to Wagner's test and Hager's test.[29] Steroids content was detected by the reaction of Liebermann.[30] The level of terpenoids were tested according to Salkowski's test where the formation of reddish brown color at the interface is indicative for the presence of terpenoids.[31] The total flavonoids and total phenolic content of each plant extract were determined spectrophotometrically by using quercetin and gallic acid as a reference compound, respectively.[32,33]

Estimation of Antioxidant Activity of the Plant Extracts Using the DPPH and SOD Activity- Like Assay

The antioxidant activity of the plants extracts was determined based on the radical scavenging ability in reacting with stable DPPH (2,2-diphenyl-1-1picrylhydrazyl) free radical spectrophotometrically at λ_{517} nm.[34,35] The SOD-like activity was measured according to the manual of the kit of superoxide dismutase.

Preparation of Balanites Nanosuspension

Balanites nanosuspension (BLT-NS) was prepared using high-pressure homogenization in the presence of a non-ionic surfactant as a stabilizer as follows.[36] In brief, BLT was ground using glass mortar and pestle. Then, accurately weighed amount of BLT (1.5 mg/mL) was dispersed in 50 mL deionized water and the mixture was stirred using high pressure homogenizer (FA25, Shanghai Fluko Co., Ltd., China) at 20000 rpm for 5 min. Later, the stabilizer tween 20 was rapidly added to the BLT dispersion at a concentration level of 5% (v/v). Afterwards, the mixture was homogenized using high pressure for 15 min.

Characterization of BLT-NS

Particle Size, Size Distribution and Zeta Potential Measurements

The developed BLT-NS was characterized for average particle size, size distribution (polydispersity index, PDI) and zeta potential by Dynamic Light Scattering (DLS) using Zetasizer (Nano-ZS90, Malvern Instruments Ltd., UK). Measurements were carried out at room temperature. BLT-NS was properly diluted with deionized water before measurement to maintain a count rate in the range of 180-250 KCP.

Transmission Electron Microscopy (TEM)

The size and morphology of the prepared BLT-NS were observed using TEM. The TEM samples were prepared by dropping gold colloids on carbon-coated copper grids and dried at room temperature. Then, the samples were observed using a JEM-100CXII transmission electron microscope (JEOL Ltd, Tokyo, Japan).

Study of LD50 of Balanites

The animals were allocated into 11 groups of six animals. They were treated for one month. Group 1: Healthy mice, group 2: plain group, group 3: BLT (5 mg/kg) once/week /oral, group 4: BLT (10 mg/kg) once/week/oral, group 5: BLT-NS (5 mg/kg)/week/oral, group 6: BLT-NS (10 mg/kg)once/week/oral, group 7: MET (5 mg/kg)/single dose/i.p [39], group 8: MET (5 mg/kg)/single dose/i.p. + BLT (5 mg/kg)/2nd day of MET injection once/week/oral, group 9: MET (5 mg/kg)/single dose/i.p. + BLT (10 mg/kg)/2nd day of MET injection once/week/oral, group 10: MET (5 mg/kg)/single dose/i.p. + BLT-NS (5 mg/kg)/2nd day of MET injection once/week/oral. Group 11: MET (5 mg/kg)/single dose/i.p. + BLT-NS (10 mg/kg)/2nd day of MET injection once/week/oralin nine groups each containing four adult female Swiss albino mice weighting 25-30 gm. Mice were obtained from Helwan breeding farm (Egypt). The animals were kept under good ventilation and received a balanced diet and supplied with water *ad libitum.* Throughout the experimental period, they were caged in an environment of 12-hour light/dark cycle and 25 ± 2 °C air temperature. Mice were acclimatized for one week before the start of the experiment. All mice were handled according to the guidelines of the Animal Ethics Committee at Zoology Department in the Faculty of Science, Mansoura University, Egypt. All animals were treated with a single dose of 100, 200, 400, 500, 1000, 2000, 3000, 4000, 5000 mg/kg. All the animals were kept under continuous observation for 24 h after the administration of the dose, for any change in behavior or physical activities and rate of mortality in each. At the end of the experiment, animals were counted. The arithmetic method of Karber was used for the determination of LD50.[37,38]

Study of The Hepato-Protective Activity of BLT-NS

The animals were allocated into 11 groups of six animals. They were treated for one month. Group 1: Healthy mice, group 2: plain group, group 3: BLT (5 mg/kg) once/week/oral, group 4: BLT (10 mg/kg) once/week/oral, group 5: BLT-NS (5 mg/kg)/week/oral, group 6: BLT-NS (10 mg/kg) once/week/oral, group 7: MET (5 mg/kg)/single dose/i.p,[39] group 8: MET (5 mg/kg)/single dose/i.p. + BLT (5 mg/kg) /2nd day of MET injection once/week/oral, group 9: MET (5 mg/kg)/single dose/i.p. + BLT (10 mg/kg)/2nd day of MET injection once/week/oral, group 10: MET (5 mg/kg)/single dose/i.p. + BLT-NS (5 mg/kg)/2nd day of MET injection once/week/oral. Group 11: MET (5 mg/kg)/single dose/i.p. + BLT-NS (10 mg/kg)/2nd day of MET injection once/week/oral.

Blood Collection and Tissue Samples

At the end of treatment period, the animals were left one day

before decapitation. Blood samples were collected in Serum Separator Tubes and were allowed to clot for 10 to 15 minutes, centrifuged at 500 g. The serum was transferred to a clean Eppendorf tube and stored at -20 °C. The liver was immediately dissected out and washed in sterile ice-cold saline. A small section was stored immediately in the liquid nitrogen for gene expression and biochemical analysis. Another part of the liver, kidney, lung and spleen was fixed in buffered formalin for pathological examination. The fixed tissues were processed and embedded in paraffin. Fifty µm section was deparaffinized and rehydrated then stained with Hematoxylin and Eosin (H&E). The stained sections were examined for histopathological changes under light binocular (OPTIKA B-150, Italy).

Biochemical Estimation

The serum was used to measure the level of liver enzymes (aspartate aminotransferase (AST/GOT), alanine aminotransferase (ALT/GPT) and γ- glutamyl transferase (GGT)), the level of albumin, kidney function tests (urea and creatinine), and heart function tests (lactate dehydrogenase (LDH)) and alkaline phosphatase (ALP).

Determination of MET Residual in Liver Tissue Using High Performance Liquid Chromatography (HPLC)

For being the main target of MET toxicity, it is extremely crucial to measure the residual amount of MET in liver using a suitable analytical method as previously reported.[40,41] In brief, 50 mg of liver tissue from groups 7, 8, 9 10 and 11 was homogenized in 1 ml of ice-cold 1x Phosphate Buffer Saline (PBS) by sonication (Sonics & Materials Inc. Danbury, Connecticut, USA) for 1 min. The homogenate was centrifuged at 10000 g for 30 min at 4 °C to remove cell debris and nuclei. The MET content in each sample was measured using a validated HPLC method. The supernatant was filtered using syringe filter (450 nm, Sartorius Minisart®) before injection into an HPLC system (Shimadzu, model UFLC-LC 20A). The injection volume was 10 µL and elution was carried out on Promosil C18 reverse-phase (RP) column at flow rate of 1 mL/min. The mobile phase was a mixture of acetonitrile and water (25:75 v/v). The peak was detected at a wavelength of 210 nm using UV-visible detector (Model SPD-20A).[40,41]

Evaluation of NRF-2 Gene Expression by RT-PCR

The total RNA was extracted from liver tissue (20 mg) with RNA Spin™ total RNA Extraction Kit (iNTRON Biotechnology, Cat no. 17211). The concentration of the RNA was measured spectrophotometrically at ʎ260 nm and ʎ280 nm. The RNA integrity was evaluated by using bleach gel.[42] The cDNA was synthesized by using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific™, Cat. No. K1622). Specific primers for *Nrf-2* and the housekeeping gene (*Rps3*) were designed by using Primer3 (Table). The PCR mixture (20 µl) consisted of 10 µl MyTaq™ Red Mix (Bioline, BIO-25043), 0.1 nmol reverse primer, 0.1 nmol forward primer, 500 ng cDNA and nuclease-free water. The gene was amplified by using SensoQuest Labcycler at the following conditions: initial denaturation at 95 °C for 3 mins, denaturation at 95 °C for 30 s, annealing at 60.5 °C for 30 s, extension at 72 °C for 30 s (30 cycles) and final extension at 72 °C for 10 mins. The PCR product was visualized by UVP PhotoDoc-it™ Imaging System (Analytik Jena AG, Germany). Target gene expression was normalized to the housekeeping gene *RpS3*.

Statistical Analysis

Statistical analysis was performed by One-way ANOVA by using IBM SPSS statistics 17 (SPSS Inc. Released 2008. SPSS Statistics for Windows, Version 17.0. Chicago: SPSS Inc.) All data were expressed as mean ± (standard error) SE. The $P < 0.05$ was considered statistically significant.

Table 1. Primers Used in this Study

Gene Name	Sequence (5`-3`)	Accession Number	Product Size (bp)
Nrf2	Fr: TCTCCTAGTTCTCCGCTGCT	NM_010902.4	240
	Re: GTTTGGGAATGTGGGCAACC		
RpS3	Fr: CTGCTATGGTGTGCTTCGGT	NM_012052.2	271
	Re: CACATGATCAGGCAGAGGCT		

Results

Balanites aegyptiaca Possesses the Highest Phytochemical Content and Antioxidant Activity

Ten plant species belonging to nine different families were phytochemically screened to detect eight naturally occurring classes (carbohydrates, saponins, flavonoids, glycosides, steroids, alkaloids, tannins and finally terpenes). Table 2 clearly revealed that phenolics (flavonoids and tannins) were widely distributed in *Amm.visnaga* L, *Balanites aegyptiaca* (L.) Del, *Glycyrrhiza glabra* Linn, *Moringa Oliefera* Lam and *Zingiber officinale* Roscoe methanolic extracts. The highest glycosides content was recorded in *Balanites aegyptiaca* (L.) Del, *Curcuma Longa* L and *Glycyrrhiza glabra* Linn while, the saponins content was rich in *Artemisia.absinthium* L., *Glycyrrhiza glabra* Linn, *Zingiber officinale* Roscoe and *Balanites aegyptiaca* (L.) Del. All methanolic plant extracts were rich in carbohydrates except *Glycyrrhiza glabra* Linn and the highest content of terpenes was found in *Curcuma Longa* L.

Quantitative analysis was performed to estimate the Total

Phenolic Content (TPC) and flavonoids content (TFC) in each methanolic extract. The comparative data for both (TPC and TFC) is presented in Table 3. The total flavonoids content of the methanolic extracts significantly varied (P < 0.05) from 255.6 ± 1.23 to 14.9 ± 0.24 µg/ml. *Curcuma Longa* L, *Balanites aegyptiaca* (L.) Del and *Olea europare* L. had the highest flavonoid content (255.6 ± 1.23, 56.6 ± 0.24 and 48.6 ± 0.40 µg/ml, respectively). Regarding the total phenolic content, the data significantly ranged from 299.09 ± 5.84 to 46.69 ± 0.62 µg/ml. The plant extracts with the highest phenolic contents were *Glycyrrhiza glabra* Linn, *Curcuma Longa* L and *Balanites aegyptiaca* (L.) Del (299.09 ± 5.84, 255.43 ± 4.27 and 227.90 ± 7.05 µg/ml, respectively).

The DPPH radical scavenging activity and SOD-like activity were evaluated for all methanolic plant extracts, and vitamin C was used as a standard antioxidant. The results showed that *Balanites aegyptiaca* (L.) Del extract possess the highest radical scavenging activity among the tested plant extracts (Table 3) followed by *Zingiber officinale* Roscoe, *Curcuma Longa* L, *Olea europare* L, *Glycyrrhiza glabra* Linn, *Cymbopogon proximus*, *Citrus paradise*, *Artemisia.absinthium* L, *Amm.visnaga* L. *and Moringa Oliefera* Lam. Moreover, SOD-like assay revealed that *Balanites aegyptiaca* (L.) Del extract has also the highest antioxidant activity followed by *Amm.visnaga* L., *Olea europare* L. *Glycyrrhiza glabra*, *Zingiber officinale* Roscoe, *Curcuma Longa*, *Cymbopogon proximus*, *Artemisia.absinthium* L and *Moringa Oliefera* Lam.

Table 2. Phytochemical Investigation of Plant Extracts

Plant/Test	Carbohydrates	Saponins	Flavonoids	Glycosides	steroids	Alkaloids	Tannins	Terpenes
Amm.visnaga L.	++	+	+	-	-	+	+++	-
Artemisia.absinthium L.	+++	++	+	-	+	+	+	-
Balanites aegyptiaca (L.) Del	++	+	+++	+++	+	-	+++	-
Citrus paradise	+++	-	+	-	-	-	-	-
Curcuma Longa L.	+++	-	+	++	-	++	++	+++
Cymbopogon Proximus	++	+	+	-	-	-	+	+
Glycyrrhiza glabra Linn.	-	+++	+++	+++	-	++	+++	++
Moringa Oliefera Lam.	+++	+	++	-	+	+	+++	-
Olea europare L.	+++	+	+	+	+	+	++	-
Zingiber officinale Roscoe	++	+++	+++	-	-	+	+	+

The sign (-) indicates a negative test, (+) indicates a weak positive test, (++) indicates a moderate positive test and (+++) indicates heavy positive test.

Table 3. Total Flavonoids, Phenolic Contents and Antioxidant Activity of the Selected Plant Extracts

Plant Name	Part	Yield (wt/wt)	Flavonoids Content (µg/ml) mean± SE	Phenolic Content (µg/ml) mean± SE	Flavonoids: Phenolic Ratio	DPPH (I%)	SOD (I%)
Amm.visnaga L.	Whole plant	0.04	37.1 ± 2.85^{ef}	69.34 ± 7.41^{fg}	0.54	14.05%	70.43%
Artemisia.absinthium L.	Seeds	0.21	21.3 ± 0.15^{g}	111.41 ± 4.28^{e}	0.19	21.13%	39.13%
Balanites aegyptiaca (L.) Del	Whole date	0.09	56.6 ± 0.24^{b}	227.90 ± 7.05^{c}	0.25	89.78%	94.78%
Citrus paradise	Fruits	0.04	40.9 ± 0.84^{de}	46.69 ± 0.62^{h}	0.88	23.73%	56.52%
Curcuma Longa L.	Roots	0.26	255.6 ± 1.23^{a}	255.43 ± 4.27^{b}	1.00	41.34%	47.82%
Cymbopogon Proximus	Roots	0.06	14.9 ± 0.24^{h}	72.57 ± 4.28^{f}	0.21	31.41%	46.08%
Glycyrrhiza glabra Linn.	Roots	0.19	39.9 ± 1.32^{e}	299.09 ± 5.84^{a}	0.13	34.20%	65.21%
Moringa Oliefera Lam.	Leaves	0.22	44.9 ± 1.99^{cd}	54.78 ± 5.60^{gh}	0.82	0.71%	23.48%
Olea europare L.	Leaves	0.14	48.6 ± 0.40^{c}	70.96 ± 8.56^{fg}	0.68	40.01%	68.26%
Zingiber officinale Roscoe	Roots	0.17	35.3 ± 1.21^{f}	203.63 ± 7.05^{d}	0.17	53.92%	60%
Vitamin C						91.94%	40.86%

Data are expressed as mean ± standard error, values in the same column with different letters are significantly different at (P<0.05).

Particle Size, Size Distribution and Zeta Potential Measurements of BLT-NS

Table 4 shows the average particle size, PDI and zeta potential of the prepared BLT-NS. As it can be seen, the average particle size of the prepared BLT-NS formulation was found to be 654.2 ± 75.84 nm with narrow size distribution as indicated by the low PDI value (0.469). In addition, the zeta potential was almost neutral (-1.73 mV).

Transmission Electron Microscopy (TEM) of BLT-NS

The morphology of the prepared BLT-NS was observed using TEM. Figure 1 shows that BLT-NS exhibited a semi-spherical to polygonal particles. Moreover, particles aggregation was noticed during the microscopic observation. This finding was evidenced by the low zeta potential value as previously demonstrated. This lower stability and particles aggregation of the formulated BLT-NS may suggest the use of another stabilizer as co-surfactants (eg. lecithin).

Interestingly, the average particle size obtained by TEM was approximately 20 nm, which was significantly smaller than that obtained by DLS measurements. This finding may be contributed to the presence of aggregated particles and, due to the existence of hydrodynamic layers surrounding the particles in the DLS analysis. In contrast, TEM detects only the electron dense mass of the particles without the surrounding layer of water and electrolytes.

Table 4. The Average Particle Size, PDI and Zeta Potential of the Prepared BLT-NS

Average Particle Size (nm)	Polydispersity Index (PDI)	Zeta Potential (mV)
654. 23 ± 75.84	0.469 ± 0.064	-1.73 ± 0.64

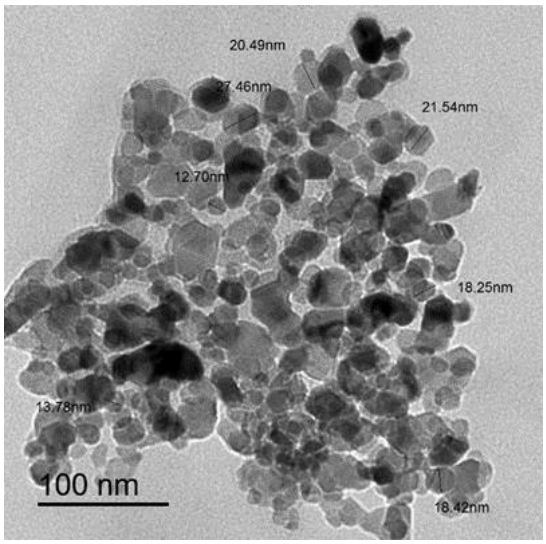

Figure 1. The TEM Image of the Prepared BLT-NS.

Balanites aegyptiaca was Safe and Ameliorated the Toxicity of MET

In order to evaluate the effectiveness of *Balanites* treatment in the hepatoprotection from MET, the Lethal Dose that kills 50% of mice (LD50) was calculated according to Karber et al. The analysis showed that the LD50 of oral administration of *Balanites aegyptiaca* was 100 mg/kg body weight. The toxicity of BLT to mice was evaluated by measuring biochemical markers for liver (ALT, AST albumin and GGT), kidney (creatinine and urea) and heart (ALP and LDH). Firstly, the safety of BLT alone was evaluated by oral administration of BLT or BLT-NS, the analysis revealed insignificant changes in serum levels of ALT, AST, ALP, LDH, urea and creatinine but GGT level showed a significant ($P < 0.05$) decrease after administration of BLT-NS at high dose (10 mg/kg) as compared to the control group (Table 5). Administration of low dose of BLT (5 mg/kg) caused insignificant changes in serum level of albumin as compared to the control group, while administration of BLT-NS at low dose (5 gm/kg) significantly ($P < 0.05$) elevated the level of serum albumin. Administration of high dose of either BLT or BLT-NS (10 mg/kg) resulted in a significant ($P < 0.05$) improvement in liver function, which was reflected by increasing in serum albumin.

The hepatotoxicity of MET was evaluated and the biochemical markers for liver, heart and kidney were exhibited at a significant ($P < 0.05$) elevation in serum levels of ALT, AST, GGT, ALP, LDH, urea and creatinine but caused a significant ($P < 0.05$) reduction in the serum level of albumin as measured with respect to the control group (Table 5).

Treatment with low dose of either BLT or BLT-NS (5 mg/kg) showed a slight improvement in liver function, which was reflected by an insignificant reduction in serum levels of ALT, AST and GGT and a significant ($P < 0.05$) increasing serum albumin as measured with respect to the methomyl group (positive group). This is while treatment with a high dose of either BLT or BLT-NS (10 mg/kg) showed moderate improvement in liver function, which was reflected by a significant ($P < 0.05$) reduction in serum levels of ALT, AST and GGT and significant ($P < 0.05$) increasing in serum albumin as measured with respect to MET group (positive group). Treatment with BLT-NS at high dose significantly ($P < 0.05$) decreased the levels of AST and ALT and also revealed a significantly ($P < 0.05$) increasing level of albumin as compared to the group treated with BLT at a low dose. Treatment with BLT or BLT-NS showed improvement in heart and kidney function, which was reflected by a significant ($P < 0.05$) reduction in serum levels of ALP, LDH, urea and creatinine as measured with respect to the MET group (positive group) (Table 5). Treatment with BLT-NS at a high dose significantly ($P < 0.05$) decreased the level of LDH and creatinine as compared to the group treated with BLT at either low dose or high dose (Table 5**Error! Reference source not found.**).

BLT Treatment Increases the Antioxidant Parameters

The effect of BLT treatment on the antioxidant parameters (SOD, GPx, GST, total antioxidant capacity and catalase) in the liver was evaluated. Oral administration of BLT (5 mg/kg) caused insignificant changes in liver oxidative stress biomarker activities such as SOD, GPx, GST, total antioxidant and catalase as compared to the control group. On the other hand, the administration of a high dose of BLT (10 mg/kg) significantly ($P > 0.05$) elevated the level of GST activity and the total antioxidant capacity and caused insignificant changes in the activity of SOD, GPx and catalase with respect to the normal group.

The administration of a low dose of BLT-NS (5 mg/kg) showed insignificant changes in liver antioxidant biomarker activities SOD, GPx and catalase but the level of GST activity and total antioxidant capacity significantly ($P > 0.05$) increased compared to the control group. This is while the administration of a high dose of BLT-NS (10 mg/kg) caused a significant ($P > 0.05$) increase in SOD, GST, catalase activities and total antioxidant capacity ($P > 0.05$), but GPx showed insignificant change in its activity as compared to the healthy group. Administration of BLT or BLT-NS in at either low dose (5 mg/kg) or at high dose (10 mg/kg) significantly ($P > 0.05$) reduced in liver MDA content as compared to the control group (Table 6).

Table 5. The Effect of *Balanites* on Liver, Heart and Kidney Function

Group/Parameters	ALT(U/L) mean± SE	AST(U/L) mean± SE	Albumin(g/dt) mean± SE	GGT(U/L) mean± SE	ALP(U/L) mean± SE	LDH(U/L) mean± SE	Urea (mg/dl) mean± SE	Creatinine (mg/dl) mean± SE
Normal (n=6)	13.8±1.2^{a}	25.3±0.9^{ac}	5.8±0.1^{a}	2.±0.9^{a}	183.7±4.1^{a}	27.7±3^{ad}	22.2±1.2^{a}	0.6±0.01^{a}
Plain (n=6)	14±1.1^{a}	23±1.3^{ac}	5.9±0.1^{a}	1.8±0.3^{ae}	183.1±2.7^{a}	27±3.3^{ad}	22.2±1.2^{a}	0.6±0.02^{a}
BLT (5 mg/kg) (n=6)	13.8±1.37^{a}	24.8±1.1^{ac}	6±0.02^{ad}	1.5±0.1^{ad}	181.1±1.9^{a}	25.6±4.2^{a}	22±0.8^{a}	0.6±0.04^{a}
BLT (10 mg/kg) (n=6)	11.5±0.7^{a}	22.3±1.1^{ac}	6.1±0.02^{d}	1.4±0.1^{ad}	182.9±1.4^{a}	22.3±2.3^{a}	21.3±0.9^{a}	0.8±0.03^{a}
BLT-NS (5 mg/kg) (n=6)	13.3±0.9^{a}	22±1^{ac}	6.7±0.1^{g}	1.5±0.2^{ad}	182.7±2.1^{a}	23.4±1.3^{a}	21.6±0.6^{a}	0.5±0.03^{a}
BLT-NS (10 mg/kg) (n=6)	10.3±0.9^{a}	18.8±1.1^{a}	7.1±0.02^{f}	1.3±0.1d	182.6±4.1^{a}	21.8±0.8^{a}	20.7±0.3^{a}	0.7±0.01^{a}
Methomyl (5 mg/kg) (n=6)	45.3±2.7^{b}	83.5±3.3^{b}	1.4±0.1^{b}	5.2±0.4^{b}	256.8±1.5^{b}	73.5±4^{b}	36.7±0.6^{b}	1.9±0.1^{b}
Methomyl (5 mg/kg) + BLT (5 mg/kg) (n=6)	34.8±0.9^{d}	60.5±1.3^{d}	3.2±0.1^{e}	4.4±0.3^{cb}	189.1±2.5^{a}	46.5±3^{c}	24.6±1.4^{a}	0.9±0.03^{c}
Methomyl (5 mg/kg) + BLT (10 mg/kg) (n=6)	28.3±1.4^{ce}	54.8±0.9^{de}	3.8±0.1^{c}	3.9±0.4^{c}	186.2±2.9^{a}	41.2±4.^{dc}	22.8±1.1^{a}	0.7±0.1^{ac}
Methomyl (5 mg/kg) + BLT-NS (5 mg/kg) (n=6)	29.3±1.1^{cd}	50.3±1.4^{e}	3.9±0.2^{c}	4.2±0.3^{cb}	187.1±4.1^{a}	35.8±2.3^{ac}	23.7±0.6^{a}	0.7±0.01^{c}
Methomyl (5 mg/kg) + BLT-NS (10 mg/kg) (n=6)	22.3±1^{e}	29.3±1.1^{c}	4.2±0.1^{c}	3.4±0.3^{c}	182.4±2^{a}	31.7±2.3^{a}	22.1±0.7^{a}	0.6±0.02^{a}

Data are expressed as mean± standard error, values with the same column with different letters are significantly different at P<0.05.

Table 6. The Effect of BLT on Oxidative Stress Including SOD, GPX, GST, Total Antioxidant and Catalase

Group/Parameters	SOD (I%) mean± SE	GPx (U/g tissue) mean± SE	GST (U/g) mean± SE	Total Antioxidant Capacity mean± SE	Catalase(U/g) mean± SE	MDA (n.mol/g.tissue) mean± SE
Normal (n=6)	93±1.9^{ad}	24.3±3.1^{a}	31±1^{a}	3.4±0.02^{a}	12.9±0.2^{af}	115±3.6^{a}
Plain(n=6)	92.5±1.7^{ad}	23.6±1^{a}	28.7±1.7^{ca}	3.4±0.03^{a}	13±0.1^{f}	113.7±2.8^{a}
BLT (5 mg/kg) (n=6)	94±2.1^{ad}	24.1±0.7^{a}	32.7±2.7^{acd}	3.4±0.03^{a}	12.9±0.1^{f}	104.7±0.7^{f}
BLT (10 mg/kg) (n=6)	95.5±1^{d}	24.5±0.8^{a}	35.9±0.4^{d}	4.5±0.01^{d}	13.3±0.2^{af}	98.3±2.6^{d}
BLT-NS (5 mg/kg) (n=6)	95±1.1^{ad}	25.5±1.4^{a}	35.8±1.5^{df}	4.5±0.04^{d}	14.1±0.1^{a}	84.1±1.3^{j}
BLT-NS (10 mg/kg) (n=6)	96.8±0.5^{d}	26.8±1.5^{a}	40.3±1.8^{d}	6.4±0.03^{f}	15.1±0.1^{d}	68.6±4.3^{g}
Methomyl (5 mg/kg) (n=6)	28.8±3.6^{b}	4.5±1.5^{b}	18.3±0.5^{b}	0.3±0.01^{b}	5.1±0.7^{b}	237.1± 6.9^{b}
Methomyl (5 mg/kg) + BLT (5 mg/kg) (n=6)	78±1.9^{c}	14.6±1.6^{c}	24.1±0.9^{e}	1.09±0.04^{e}	8.1±0.2^{b}	184.3±3.7^{e}
Methomyl (5 mg/kg) + BLT (10 mg/kg) (n=6)	82.5±2.5^{acd}	19.4±2.6^{ac}	26.3±1.5^{c}	1.5±0.01^{c}	10.5±0.2^{c}	148.4±2^{c}
Methomyl (5 mg/kg) + BLT-NS (5 mg/kg) (n=6)	87±1.29^{ac}	22.3±1^{a}	29±1.6^{a}	2.4±0.03^{g}	11±0.2^{ec}	138.8±0.9^{h}
Methomyl (5 mg/kg) + BLT-NS (10 mg/kg) (n=6)	90.5±1.5^{ad}	24.4±0.8^{a}	31.2±2.4^{aef}	3.3±0.04^{a}	12.1±0.1^{ac}	119.3±2.7^{a}

Data are expressed as mean ± standard error, values in the same column with different letters are significantly different at P<0.05.

Balanites Ameliorated the MET Toxicity in Treated Mice

It has been reported that MET treatment leads to generation of free radicals and lipid peroxidation. In this study, the content of MDA was significantly (P > 0.05) elevated in the MET-treated group with a significant (P > 0.05) reduction in the level of SOD, GPx, GST and catalase as well as total antioxidant capacity with respect to the control group (Table 6).

Treatment of mice with high a dose of BLT or BLT-NS after MET injection significantly (P < 0.05) elevated the antioxidant biomarker in liver tissue (SOD, GPX, GST and catalase) as well as total antioxidant capacity as compared to the MET group. At the same time, the treatment with a high dose of BLT or BLT-NS significantly (P < 0.05) reduced liver tissue contents of MDA. Treatment with a low dose (5 mg/kg) of either BLT or BLT-NS showed moderate improvement in liver, which was reflected by a significant (P < 0.05) increase in the level of SOD, GST and GPx activities in addition to total antioxidant capacity and significantly (P < 0.05) reduced liver tissue contents of MDA. However, the level of catalase activity caused insignificant change as compared to the MET group. Treatment with a high dose (10 mg/kg) of BLT-NS caused significant (P < 0.05) improvement in antioxidant biomarkers SOD, GPx, GST and catalase levels in addition to the total antioxidant capacity and significantly (P > 0.05) reduced liver tissue contents of MDA as compared to the group treated with BLT at a low dose (Table 6). Moreover, the treatment with BLT-NS showed significant (P <0.05) reduction in MDA content and significantly (P < 0.05) increased the total antioxidant capacity as compared to group treated with BLT (Table 6).

The attenuation of oxidative stress induced by MET treatment was found to be via the induction of *Nrf2* expression. The relative expression of *Nrf2* gene was slightly increased in the MET group and significantly increased in groups received MET and treated with low dose of either BLT or BLT-NS. Additionally, *Nrf2* expression was increased in group received MET and treated with high dose of either BLT or BLT-NS as compared to control group (Figure 2).

Histopathological Results

Microscopic examination of H&E-stained liver sections showed normal hepatocytes having centrally located nuclei with normal sinusoids, portal areas and hepatic cords radially arranged around the central vein in the control group

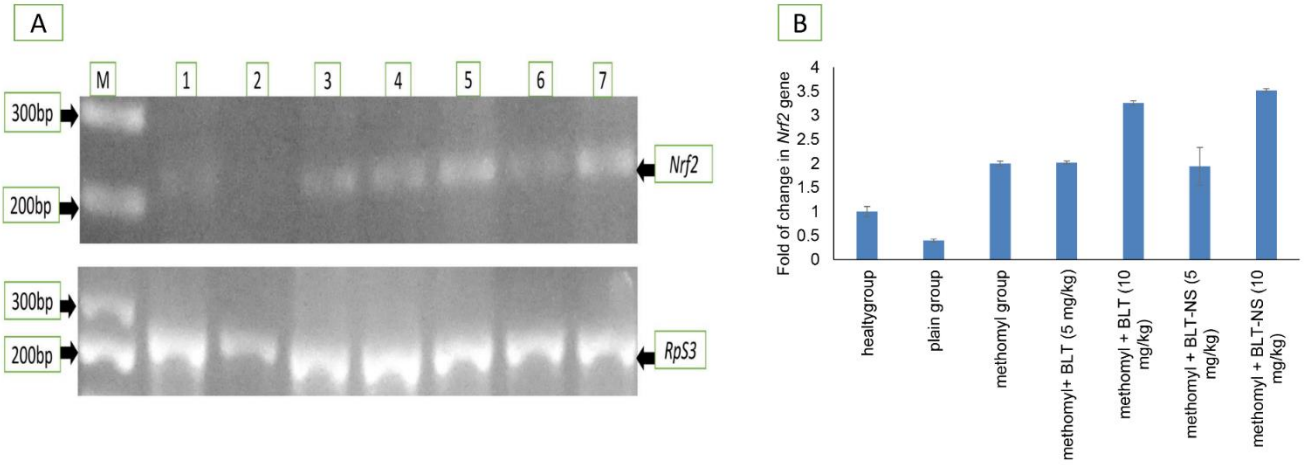

Figure 2. The effect of Balanites aegyptiaca on the expression of Nrf2 gene. (A) the relative expression of Nrf2 gene and housekeeping gene (RpS3) (M) DNA marker, (1) healthy group, (2) plain group, (3) methomyl group, (4) methomyl + BLT (5 mg/kg), (5) methomyl + BLT (10 mg/kg), (6) methomyl + BLT-NS (5 mg/kg) and (7) methomyl + BLT-NS (10 mg/kg) &. (B) fold of change in Nrf2 gene in different treated groups.

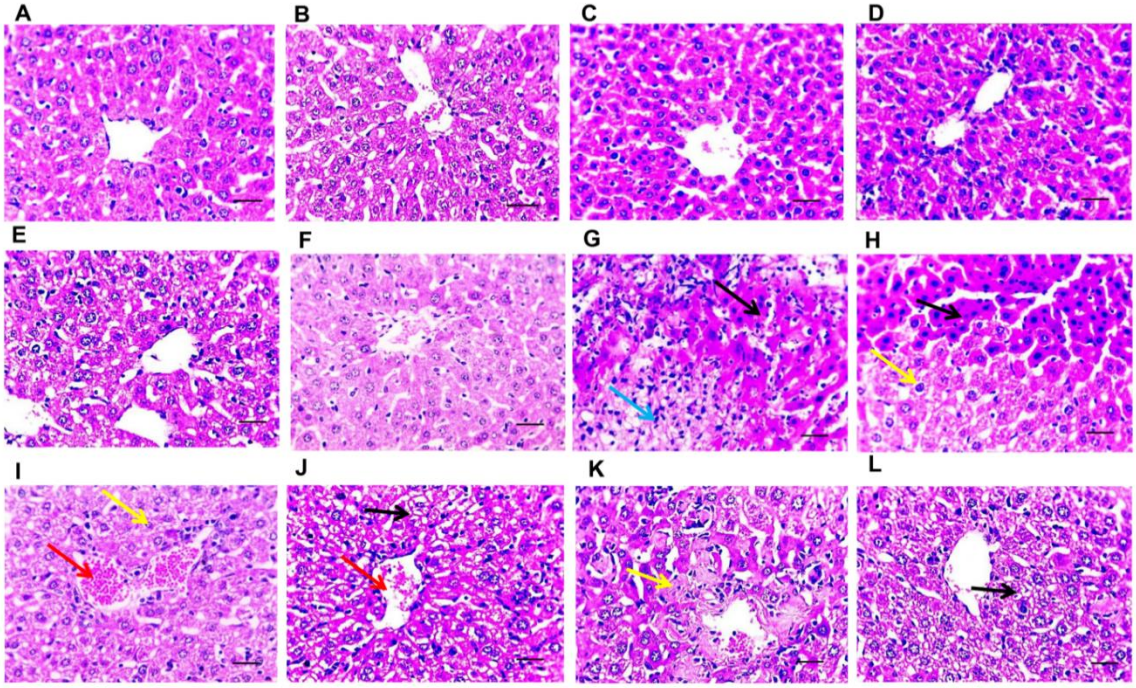

Figure 3. Microscopic Pictures of H&E Stained Liver Sections Showing Normal Hepatocytes Having Centrally Located Nuclei with Normal Sinusoids, portal areas and hepatic cords radially arranged around the central vein in control group (A), plain group (B), groups received only low (5 mg/kg) (C) or high (10 mg/kg) (D) dose of BLT and groups received only low (E) or high (F) dose of BLT-NS. Meanwhile, liver sections from untreated MET group showed focal areas of necrosis (black arrow), fibrosis (blue arrow) (G). Liver sections from group received MET and treated with low dose of BLT (5 mg/kg) showed slight improvement in histopathological picture where hydropic degeneration in hepatocytes (yellow arrow), focal areas of necrosis (black arrow) were seen (H). However, liver sections from group received MET and treated with high dose of BLT (10 mg/kg) show moderate improvement in histopathological picture where hydropic degeneration in hepatocytes (yellow arrow) and congested blood vessels (red arrow) were seen (I). liver sections from group received MET and treated with low dose of BLT-NS (5 mg/kg) showed hydropic degeneration in hepatocytes (black arrow), congested blood vessels (red arrow) (J) and amyloid deposits (yellow arrow) (K). However, liver sections from group received MET and treated with high dose of BLT-NS (10 mg/kg) showed marked improvement in histopathological picture where very mild hydropic degeneration in hepatocytes (black arrow) (L). Magnification was 400X and Scale bar was 50 µm.

(Figure 3 A), plain group (Figure 3 B), groups receiving low (5 mg/kg) or high doses of BLT (Figure 3 C and D) and groups receiving low or high (5 mg/kg or 10 mg/kg) doses of BLT-NS (Figure 3 E and F).

Meanwhile, liver sections from the MET group showed degeneration in the hepatocytes, multifocal areas of necrosis, fibrosis and congested blood vessels (Figure 3 G). Liver sections from the group which had received MET treated

with a low dose of BLT (5 mg/ml) showed slight improvement in histopathological picture where hydropic degeneration in hepatocytes, focal areas of necrosis and congested blood vessels were seen (Figure 3 H). However, liver sections from the group which had received MET treated with a high dose of BLT (10 mg/ml) showed moderate improvement in histopathological picture where hydropic degeneration in hepatocytes and congested blood vessels were seen (Figure 3 I). liver sections from the group which had received MET treated with a low dose of BLT-NS (5 mg/kg) showed hydropic degeneration in hepatocytes, congested blood vessels and amyloid deposits (Figure 3 J and K). However, liver sections from the group which had received MET treated with high dose of BLT-NS (10 mg/kg) showed marked improvement in histopathological picture where very mild hydropic degeneration in hepatocytes were seen (Figure 3 L).

BLT Treatment Decreased the Accumulation of Methomyl in the Liver

The histopathological alteration in liver was correlated to the accumulation of MET in the liver. HPLC was used to detect the level of MET toxicant in liver. The analysis showed that MET was completely undetectable in liver homogenate in the groups treated either wit BLT or BLT-NS (at both doses 5 and 10 mg/kg) (Figure 4). The decrease in the level of MET may be contributed to the improvement in liver antioxidant capacity and the pathological alterations.

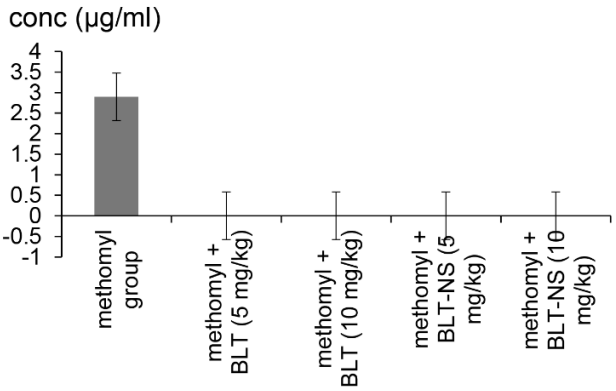

Figure 4. Liver tissue concentrations of methomyl in various groups.

Discussion

The present study focused on the treatment of the MET toxicity in mice due to the generation of free radicals. Currently, plant phenolic compounds (flavonoids, tannins, phenolic acids, etc.) have gained attention due to their natural antioxidant potential.[43] It has been reported that the antioxidant activity of plant phenolic compounds is due to their ability to donate hydrogen atoms or electrons and by scavenging free radicals.[44] In our study, the tested plants revealed antioxidant activity that is consistent with various reports.[45-54] The results showed that *Curcuma Longa L.* has the highest flavonoids content and *Glycyrrhiza glabra Linn*. has the highest phenolic content. However, BLT showed the highest antioxidant activity as evidenced by the DPPH radical scavenging and SOD-like assays. This may be attributed to the synergistic effect of saponins with the estimated phenolics. Diosgenin, a steroidal saponin, is the main constituent identified in *B. aegyptiaca* and has antioxidant activity.[55,56]

As one of the objectives of the current study was to investigate the efficacy of BLT extract and its nano-formulation, a nanosuspension of BLT extract was prepared and characterized for size and morphology as previously demonstrated. Reports have shown that the higher the zeta potential value, the greater the probability that the studied nanosuspension will be stable. In contrast, a small value of the zeta potential (from +5 to −5 mV) indicates a less stable system.[57] In the current study, the zeta potential value of the prepared BLT-NS was low which may suggest the probability of low stability and tendency of particles for aggregation.

The induction of liver toxicity by MET was indicated by the increase in ALT, AST and GGT into the blood stream. The AST and ALT levels are normally located in the cytosol of hepatocytes and are involved in the breakdown of the amino acids into α-keto acids. The GGT normally catalyzes the transfer of γ-glutamyl groups to amino acids and short peptides. Higher level of AST, ALT and GGT in the serum indicates the cellular leakage and loss of integrity of permeability.[58] The administration of the *balanites* alone or after MET treatment protected the liver from damage and decreased the level of enzymes.

In reference to the biochemical markers of heart function, the present study showed that serum ALP and LDH level significantly increased in MET-administrated mice and the pathological alterations were reverted in *balanites*-treated mice. This finding is in concurrence with previous research which have reported that the exposure to insecticides led to cardio-toxicity in experimental animals.[59,60]

In regards to kidney toxicity, a variety of specific biochemical parameters were measured such as urea and creatinine as blood wastes that showed an increase in their levels. However, after treatment with *balanites* a decrease was observed in their levels. Creatinine is an end product of creatinine metabolism, whose measurement provides a useful marker of the kidney function.[61] According to Gilman et al.,[62] the increase in urea could be due to an increase in nitrogen retention and/or owing to renal dysfunction. A marked elevation was observed in the serum urea level in severe defect of glomerular filtration.[63]

The oxidative damage of hepatic cells caused by MET exposure has been documented by a number of researchers.[64] It has been indicated that lipid peroxidation was significantly elevated in the liver of mice treated with MET associated with an increase in MDA levels.[65] Several mechanisms can

be used to defend oxidative damage which include antioxidant machinery such as SOD, GPx, GST and catalase.[66] MET induces oxidative stress and also suppresses the levels of antioxidants (enzymatic and non-enzymatic) in the liver. This was confirmed in the present study where a significant elevation of MDA tissue as well as reduction of tissue SOD, GPX, GST, catalase and total antioxidant was detected in mice which had only received MET as compared to healthy mice (control group). Meanwhile, the group treated with BLT or BLT-NS showed an enhancement in the level of the parameters of antioxidant which was reflected by a decrease in the level of MDA and an increase in enzymatic and non-enzymatic antioxidant in liver tissue as compared to the MET group.

On the molecular mechanism of MET toxicity, the level of expression of *Nrf2* gene was measured. In hepatocytes, oxidative stress frequently triggers antioxidant response by activating nuclear erythroid 2-related factor 2 *(Nrf2*), a transcription factor,[67,68] which up regulates various cytoprotective genes such as superoxide dismutase and hemooxygenase-1. The BLT-treatment attenuated the oxidative damage-induced by MET via overexpression of *Nrf2* and its downstream SOD.

The current biochemical results were supported by histopathological changes including degeneration in hepatocytes, multifocal areas of necrosis, fibrosis and congested blood vessels as well as accumulation of MET residue in mice injected with MET alone. These histopathological changes in our study were improved in the group of mice administrated MET then treated with BLT at high dose. However, this improvement was slight in group treated with low dose of BLT. In addition to these improvements, the residue of MET was not detected in groups treated with BLT or BLT-NS either at high dose or low dose. This needs further studies to evaluate the mechanism of MET metabolism during BLT treatment.

Conclusion

The current study showed that *Balanites Aegyptiaca* extract possess marked antioxidant activity without notable toxicity in experimental mice. In addition, BLT has a dose-dependent improvement in liver, heart and kidney functions in animals treated with MET. Furthermore, BLT ameliorated the antioxidants biomarkers levels in liver tissue and total antioxidant capacity. Data have also shown that BLT managed MET-induced toxicity and oxidative stress via significant induction of *Nrf2* gene expression and hepatic clearance. In conclusion, the findings of this study suggest the potential use of BLT extract as a natural antioxidant for the management of MET-induced toxicity and oxidative stress. Future studies should be conducted to explore and understand the mechanistic approaches of BLT against other types of environmental pollutants and toxins.

Authors' Contributions

OE described the work plan to carry out this research. YE performed the methods, collected the data and performed the analysis. MEM helped in phytochemical identification. FE helped in gene expression and HPLC analysis. FMA helped in the preparation of nanoformulation and HPLC analysis. MR helped in biochemical estimation. All authors read and approved the final manuscript.

Conflict of Interest Disclosures

All authors declare that they do not have any conflict of interest in this study or personal relationships that could have appeared to influence the work reported in this paper.

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