RESEARCH ARTICLE

Editorial Process: Submission:10/10/2021 Acceptance:02/19/2022

Effect of an Extract from the Egyptian Sea Cucumber, *Bohadschia marmorata*, on Methotrexate-Induced Hepatorenal Toxicity in Male Mice

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Abstract

Background: The sea cucumber, *Bohadschia marmorata*, is a marine echinoderm consumed and used as a medication. Extract of this species displays a broad spectrum of bioactivity, such as antifungal, antibacterial, immunomodulatory, and cytotoxic properties. This investigation explored sea cucumber extract for hepatorenal protection against the toxicity of methotrexate (MTX). **Methods:** Four groups of mice were divided into G1: control, G2: MTX treated, G3: B. marmorata extract-treated daily for 14 days, and G4: B. marmorata extract and MTX treated. **Results** and **Conclusions:** Biochemical analysis and histopathological examination of liver tissue showed that administration of MTX increased serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), lowered levels of serum albumin, total protein, Superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH). Administration of B. marmorata extract to MTX- injected mice significantly reversed the increase in serum levels of liver enzymes and induced a significant elevation in serum albumin and total protein levels. SOD, CAT, and GSH levels returned to nearly normal levels. Histopathological examination indicated fewer signs of toxicity in liver and kidney tissues of mice treated with both extract and MTX compared to MTX treatment alone. An extract of B. marmorata will protect mice from hepatorenal toxicity induced by MTX.

Keywords: *Bohadschia marmorata*- histopathological examination- antioxidant enzymes- methotrexate

Asian Pac J Cancer Prev, **23 (2)**, 703-713

Introduction

MTX is an antimetabolite antineoplastic medication. At high doses, it is used to treat a variety of cancers; at low doses; it is used to treat several autoimmune diseases, including rheumatoid arthritis and psoriasis (Widemann et al., 2004). MTX acts as an anti-inflammatory, antiproliferative, and immunomodulatory folic acid antagonist (Chan and Cronstein, 2013; Daggulli et al., 2014).

Unfortunately, MTX therapy is sometimes restricted due to side effects, such as nausea, fever, increased risk of infection, leucopenia, lymphocytosis, skin rash, infertility, and hepatic and pulmonary toxicity (Kivity et al., 2014; Shea et al., 2013; Widemann et al., 2004). MTX injection raises intracellular polyglutamate levels, and folic acid deficiency causes hepatocyte necrosis (Kamen et al., 1981). Patients receiving long-term therapy need frequent monitoring for adverse effects (Chan and Cronstein, 2013). Mechanisms for MTX-induced organ dysfunction and toxicity in rats and humans are mostly attributable to oxidative stresses (Armagan et al., 2008; Daggulli et al., 2014).

MTX causes hepatic and renal toxicity (Abdel-Daim et al., 2017). The drug is processed in the liver, causing inflammation, injury, and release of liver enzymes into the circulation (Çakır et al., 2015). More than 90% of MTX is eliminated in the urine (Izzedine et al., 2005). MTX-mediated renal toxicity is due to the generation of reactive oxygen species (ROS). The most common form of toxicity at high doses is acute renal failure and MTX precipitation. Patients receiving high doses require adequate urine production and urinary alkalinisation to avoid acute response (Asvadi et al., 2011) .

Treatment of liver syndrome remains a challenge because no effective means exist to counter reduced liver function, regenerate hepatic cells, or otherwise protect the liver from injury. The use of corticosteroids and immunosuppressive medicines is limited since both exhibit several negative side effects. Thus, alternative pharmaceuticals are needed to protect against liver dysfunction to replace drugs of questionable efficacy and safety (Adewusi and Afolayan, 2010). Natural products or derivatives of natural products are candidates for such pharmaceuticals because of their varied biological activities. Significant interest has developed for exploring natural compounds from marine animals, plants, and microorganisms as possible therapeutic agents.

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Herbal and marine sources are important sources; 65 % of patients in the United States and Europe rely on herbal remedies for the management of liver syndrome (Zhang et al., 2013). Recently, marine species have been used as nutritional supplements and as a source of substances to treat some disorders (Tuwo, 2004). The sea cucumber, *B. marmorata*, is one such species in the Holothuriidae family. Triterpene glycosides (saponins) are the most abundant secondary metabolites in sea cucumber extracts and exhibit a wide range of bioactivity, including antibacterial, antifungal, immunomodulatory and cytotoxic effects (Aminin et al., 2001; Tian et al., 2005; Zou et al., 2005; Eissa et al., 2020). The antioxidant characteristics of coelomic fluids from three species – *B. marmorata* vitiensis, Stichopus variegatus, and Stichopus badionotus – have been described (Hawa et al., 1999).

Little information on the protective and curative effects of ethanol extracts of B marmorata on liver and kidney dysfunction induced by immune suppressant drugs is available, despite extensive study of the species. Limited research on Holothuria atra protein extract does suggest notable protective, curative, and antioxidant activity against hepatorenal dysfunction in rats (Dakrory et al., 2015). Further, *H. atra* extract relieved MTX-induced oxidative stress and subsequent dysfunction (Saad et al., 2018). An extract from, *H. scabra*, reduced the severity of liver damage in mice (Abdulkadir and Tungadi, 2017). Holothuria scabra, at doses of 500 mg/kg bw, reduced hepatic damage caused by paracetamol in mice (Abdulkadir and Tungadi, 2018).

The goal of the current investigation was to determine the biochemical and histological impact of an ethanol extract from *B. marmorata* on MTX-induced liver and kidney dysfunction, antioxidant status, and apoptosis.

Materials and Methods

Sample collection and preparation of B. marmorata extract

B. marmorata (Order: *Aspidochirotida*, Family: *Holothuriidae*) was collected from deep water (35 m maximum depth) off the Red Sea coast of Hurghada via SCUBA diving and from shallow coral flat areas via snorkeling and hand collection. Specimens were transported to the laboratory in an icebox filled with seawater and frozen at −20°C for later examination. Taxonomic identification was validated using published information (Eissa et al., 2017). Internal organs and body fluids were extracted from specimens via an abdominal incision after thorough washing.

Body walls were blended in 96 % ethanol in a volume equivalent to twice the tissue weight. Homogenized tissues were extracted twice for one day at room temperature then filtered. A rotary evaporator was used to remove the ethanol and the residue was dried in a vacuum oven at 40°C for 48 hours. The resulting powdered extract was stored at −20°C until further use. The purity of the extract was verified with thin-layer chromatography (TLC). Spots were visualized under UV light and by spraying with 10% sulfuric acid until maroon-dark purple spots appeared, indicating the presence of saponins (Bahrami et al., 2014). Saponins from *B. marmorata* were identified previously using Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC–MS) (Omran et al., 2020).

Ethical considerations

Tanta University's Faculty of Science and institutional Animal Care authorized the experimental protocols and procedures. Experimental procedures followed the International Laboratory Animal Care and Use guidelines. The ethical approval number is IACUC-SCI-TU-0255

Experimental animals

Male albino mice (CD-1) weighing 30–35 ± 5 g were procured from the National Research Center (Dokki, Giza). Animals were housed six per cage at the well-ventilated animal house of Tanta University's Department of Zoology, Faculty of Science. Mice were kept in a comfortable setting at room temperature for 12 hours (22°C–25°C) with free access to water and food and acclimated for seven days before the start of the experiment. Mice were divided into four groups based on a previous study (Armagan et al., 2008): G1 mice served as a negative control, G2 mice were injected intraperitoneally with MTX, 20 mg/kg bw, as a single dose on day seven, G3 mice received a sublethal dose of *B. marmorata* extract, 30 mg/kg bw, by gavage daily for 14 days based on a previous study (Dakrory et al., 2015), and G4 mice received the G3 dose of *B. marmorata* extract orally for one week before receiving the G2 dose of MTX on day seven, followed by administration of *B. marmorata* extract for an additional seven days. Animals were sacrificed under light diethyl anesthesia at the end of the 14-day trial and bled from the orbital plexus to collect blood for biochemical assessment. Liver and kidneys were immediately collected.

Biochemical assessment

Biomarkers for liver function

Levels of serum ALT, AST (Reitman and Frankel, 1957), serum albumin, and total protein (Tietz et al., 1994) were determined using Biodiagnostic kits (Giza, Egypt) following the manufacturer's instructions.

Assessment of oxidative stress markers and tissue preparation

Liver samples were homogenized in ice-cold phosphate buffer (50 mM phosphate pH 7.4) at 10% w/v using an Omni international homogenizer (USA) at 22000 rpm for 20 seconds at 10 second intervals. The homogenate was centrifuged at 2000g for 15 minutes at 4°C (Hettich, Germany). The supernatant was freeze-thawed twice to completely destroy mitochondria (Salach, 1978), then centrifuged at 6,000g at 4°C for 15 minutes, and the supernatant, which including cytosolic and mitochondrial enzymes was used immediately for enzyme assays. Enzyme concentrations and GSH levels were measured using the Automated Elisa System Chemwell 2099 from the Gama Trade Company. SOD, (CAT) and, (GSH) were assessed with research kits KT-50849 from Kamiya Biomedical Company, `038818 96th from MyBioSource, and E0943Hu from MyBioSource, respectively.

Histopathological preparation

Small portions of liver and kidney were fixed in 10% neutral buffered formalin for 24 hrs. Tissue samples were dehydrated in ascending concentrations of ethyl alcohol, cleared with xylene, washed to remove excess fixative, and embedded in paraffin wax. Five µm sections were mounted and stained with hematoxylin and eosin (H&E) (Bancroft and Layton, 2012).

Statistical analysis

All data are presented as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to evaluate differences between study groups. If a significant difference between means was observed, Tukey post hoc comparisons among different groups were performed. p-values ≤0.05 were considered statistically significant. Data and statistical analysis were performed using Excel 365 (Microsoft Corporation, USA) and Minitab version 19.

Results

Determination of B. marmorata triterpene glycosides using UPLCMS

Sea cucumbers metabolic compounds were identified using UPLC–MS (Omran et al., 2020). The identified compounds are holothurin A, 24-dehydroechinoside A, holothurin B, bivittoside C, and bivittoside D. The ions at a mass to charge ratio (m/z + Na)+ of 1,243.47, 1,227.48, 905.36, 1,433.24, and 1449.68 were identified from the investigated extracts of sea cucumber body wall (Figures 1-5). The (m/z) was detected using the positive and negative ionization mode. UPLC-MS base peak chromatograms of crude extracts from *B. marmorata* showed peaks against the retention time 0-19 min and 10-14 min, respectively.

As shown in Table 1, *B. marmorata* body wall extract contains three sulfated triterpene glycosides: Holothurin A, 24-dehydroechinoside A, and Holothurin B. Also, it contains two non-sulfated triterpene glycosides, namely bivittoside C, and bivittoside D.

Liver biomarkers

MTX-injected mice had significantly higher serum levels of AST and ALT ($p < 0.05$) compared to control mice, Table 2. Treatment of MTX-injected animals with *B. marmorata* extract resulted in a substantial reversal of this increase in serum enzyme levels ($p < 0.05$). In contrast, total protein level in plasma of MTX-treated mice was considerably less than in comparable control animals (p

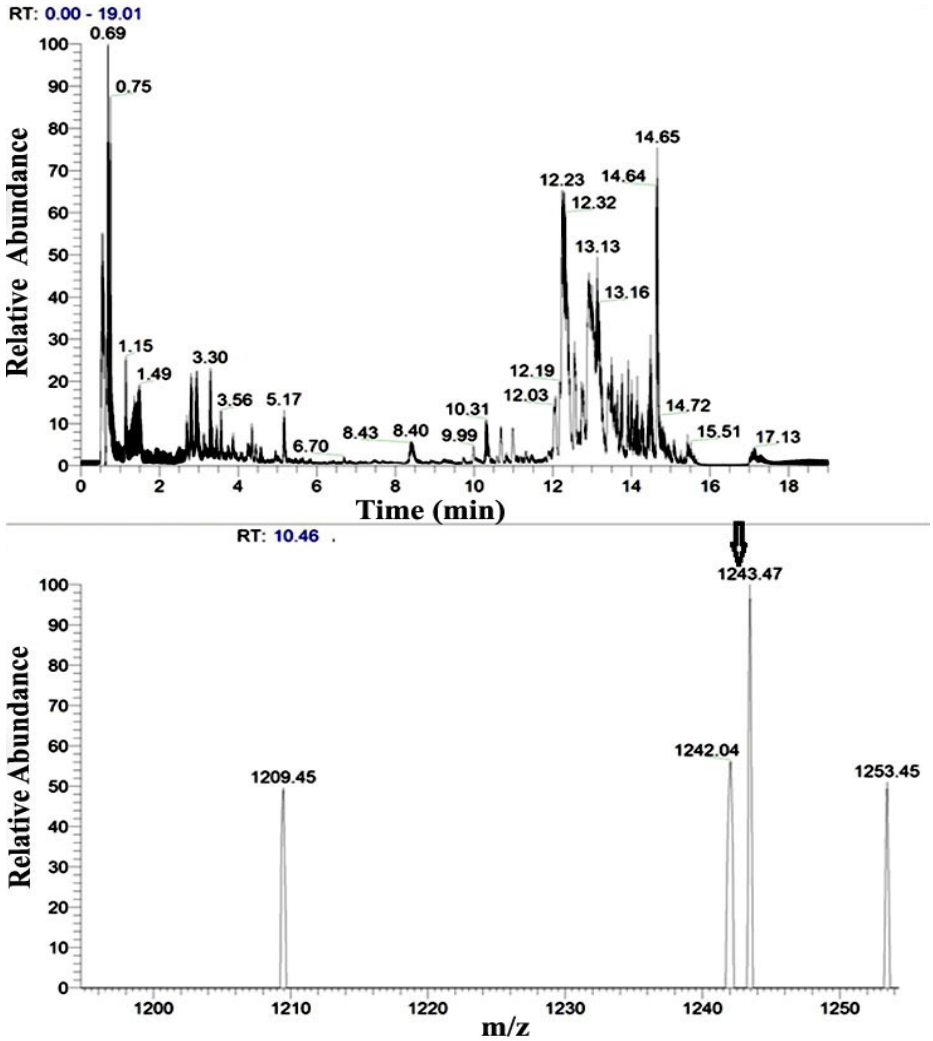

Figure 1. UPLC-MS Chromatograms of Ion Beaks (m/z + Na)+ 1243.47 were Acquired from *B. Marmorata* Body Wall Extract

Table 1. Triterpene Glycosides Identified from *B. marmorata*

Triterpene glycosides name	Retention time (min)	(M+Na) +	formula
Holothurin A	10.46	1243.47	C54H85NaO27S
24-dehydroechinoside A	10.88	1227.48	C54H85NaO26S
Holothurin B	11.09	905.36	C41H63NaO17S
Bivittoside C	8.26	1433.24	C67H110O31
Bivittoside D	12.72	1449.68	C67H110O32

< 0.05). Prior administration of *B. marmorata* extract to MTX-injected mice normalized serum albumin and raised total protein levels.

Protective effect of B. Marmorata extract on antioxidant status in liver tissue

Treatment with *B. marmorata* extract (G3) did not show induce significant changes in SOD, CAT, and GSH levels when compared to controls (G1). MTX-injected mice (G2) showed a substantial decrease in SOD, CAT, and GSH levels. Treatment of MTX-injected animals with *B. marmorata* extract ameliorated the impact of MTX on these biomarkers, as indicated by substantial rescue of SOD, CAT, and GSH levels (G4). *B. marmorata* extract proved to be efficacious when administered both pre- and post- insult (Figures 6–8).

Liver and kidney histopathology

Examination of H&E-stained sections of livers from control mice (G1) showed normal hepatocyte architecture with hepatic lobulation. Hepatic strands alternated with narrow blood sinusoids lined by an endothelial cell layer containing Kupffer cells, typical of lobules (Figure 9a). Livers from MTX-injected mice (G2) displayed severely disrupted hepatic architecture, with vacuolated and degraded cytoplasm, substantial numbers of pyknotic nuclei, and obliteration of blood sinusoids with activated Kupffer cells (Figure 9b). Liver sections from mice administered *B. marmorata* extract (G3) exhibited typical hepatic architecture, normal radiating hepatocytes with

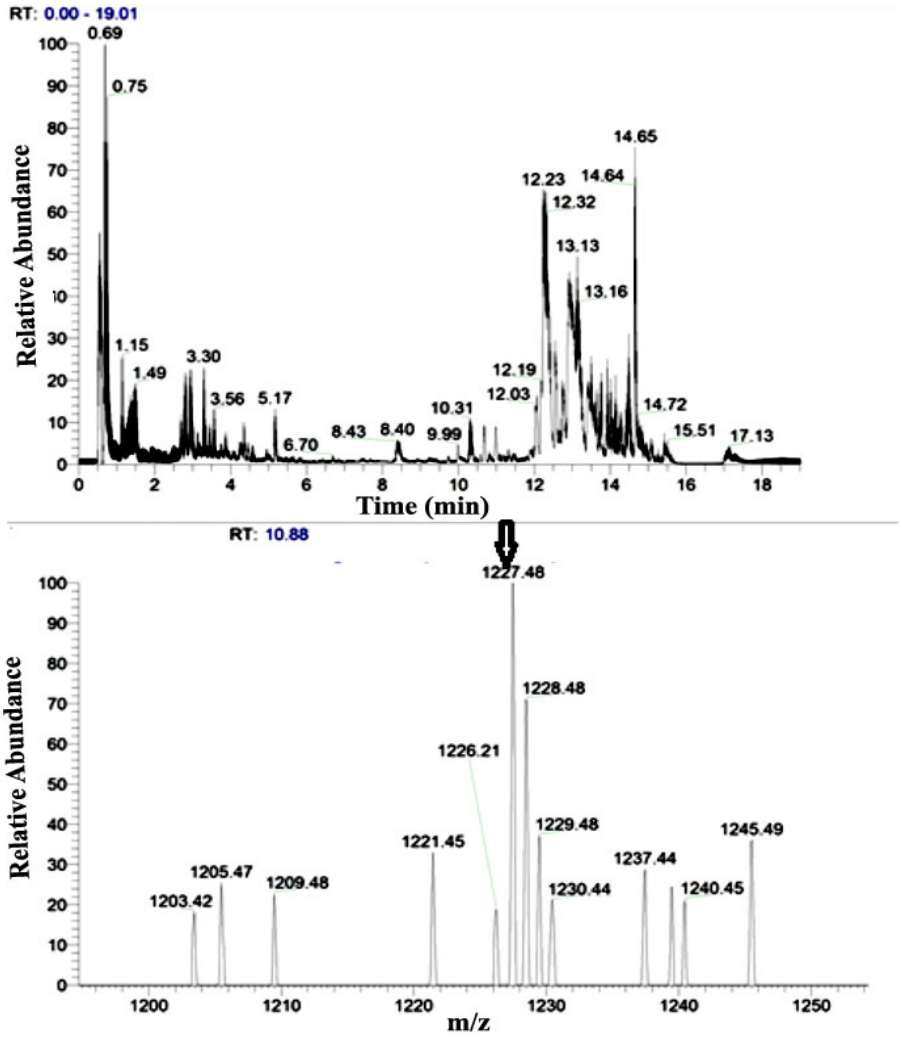

Figure 2. UPLC-MS Chromatograms of Ion Beaks (m/z + Na)+ 1227.48 were Acquired from *B. marmorata* Body Wall Extract

Table 2. Protective Effects of *B. marmorata* Extract on Methotrexate (MTX) Induced Changes in Liver Biomarkers, Serum Albumin, and Total Protein

Groups	ALT (IU/L)	AST (IU/L)	Albumin Serum (g/dl)	Total Protein Serum (g/dl)
Control	69 ± 3.98 [c]	65 ± 9.99 [c]	2.8 ± 0.10 [b]	5.83 ± 0.15 [b]
B. marmorata	89 ± 5.65 [c]	90 ± 23.16 [c]	3.43 ± 0.30 [a]	7.4 ± 0.40 [a]
MTX	266 ± 5.99 [a]	356.3 ± 34.64 [a]	1.6 ± 0.20 [c]	3.77 ± 0.25 [c]
B. marmorata + MTX	130 ± 9.89 [b]	160 ± 16.61 [b]	2.7 ± 0.15 [b]	4.77 ± 0.10 b [c]

Data are means ± standard deviation (SD). Means that do not share a letter are significantly different at $p < 0.05$

normal cytoplasm; some hepatocytes did show pyknotic (arrows), and others karyolytic nuclei (thick arrows), slight widening of central veins, and obliteration and narrowing of blood sinusoids (Figure 9c). Liver sections from animals administered both *B. marmorata* extract and MTX showed noticeable improvement in hepatic architecture, typical central veins, some hepatocytes with eosinophilia, others with pyknotic nuclei, and increased activity of Kupffer cells (Figure 4d).

Examination of H&E-stained sections of kidneys (G1) of mice showed typical renal cortex architecture with normal renal glomeruli and renal tubules (Figure 10a). Kidneys from MTX-injected mice (G2) showing marked disorganisation of kidney structures; irregular, collapsed, and disorganized glomeruli with irregular mesangial areas. Most renal tubules were damaged and had lost their characteristic appearance. Lining epithelial cells were poorly distinguished and their contents were intermixed. These cells exhibited cloudy swelling with separation from the underlying basement membrane and intertubular hemorrhage (Figure 10b). Kidney sections from animals treated with *B. marmorata* extract (G3) showed regular structure for most glomeruli as well as characteristic appearance of mesangial areas. Sections from mice treated with *B. marmorata* extract and MTX (G4) exhibited some improvement in kidney structures. Some glomeruli still showed irregular mesangial areas. Most renal tubules were normal with typical lining epithelium. Others showed poorly distinguished cells with intermixed contents; some intertubular hemorrhages were

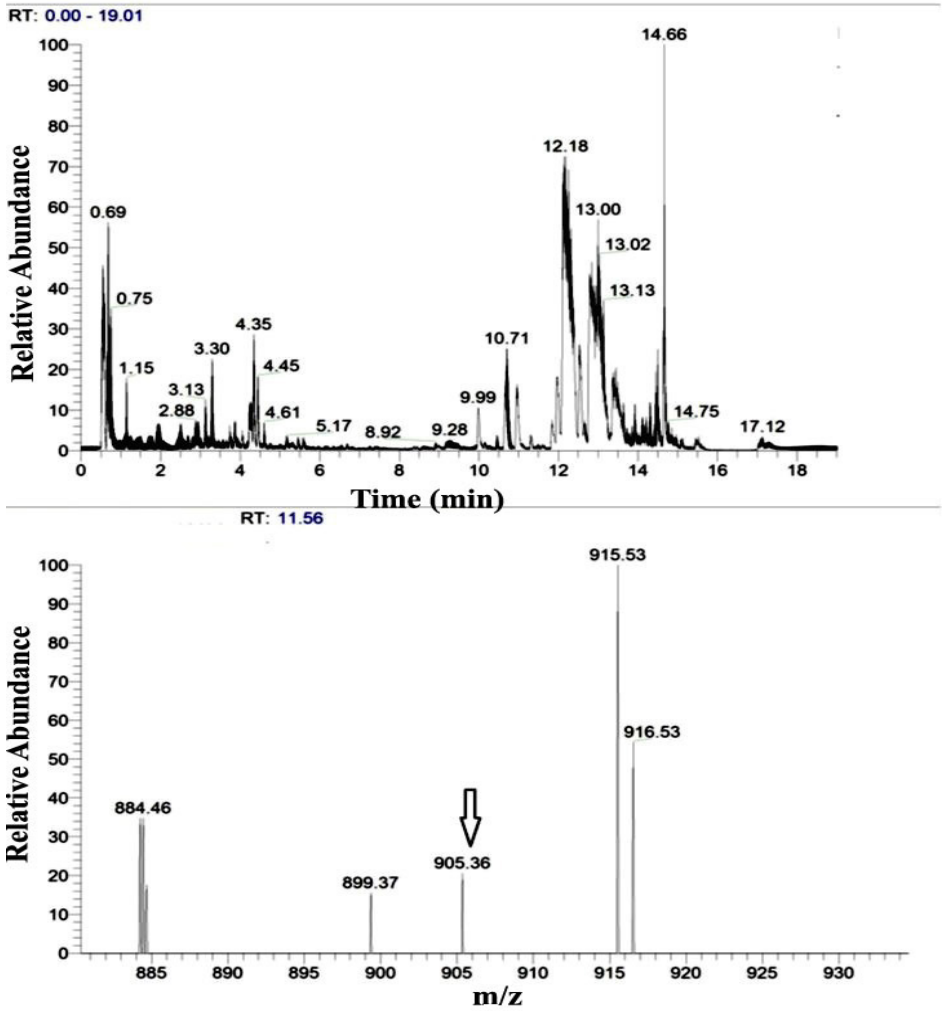

Figure 3. UPLC-MS Chromatograms of Ion Beaks (m/z + Na)+ 905.36 were Acquired from *B. marmorata* Body Wall Extract.

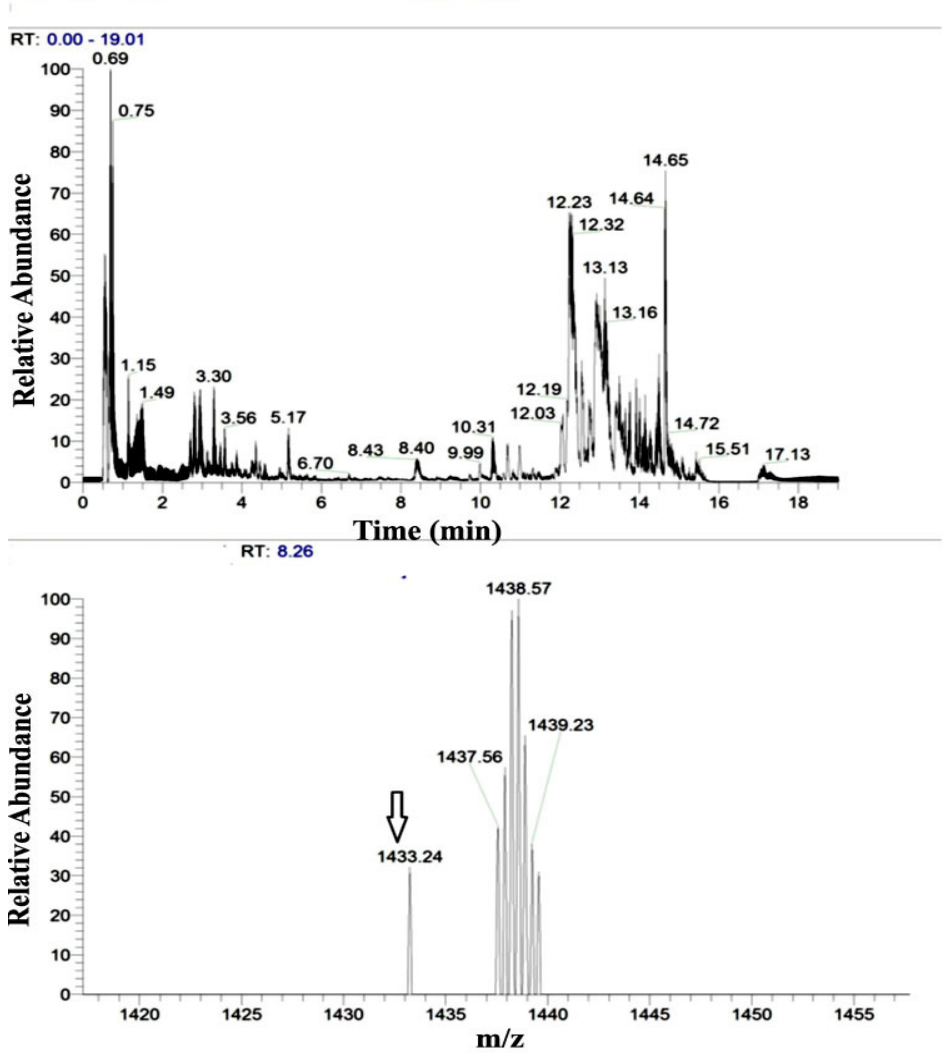

Figure 4. UPLC-MS Chromatograms of Ion Beaks (m/z + Na)+1433.24 were Acquired from *B. marmorata* Body Wall Extract

noticed (Figure 10d).

Discussion

Novel chemotherapeutic drugs could be critical for treatment of resistant diseases. Chemicals produced by marine species have sparked interest in elucidating structures, synthesis, and cytotoxicity as initial steps toward understanding their pharmaceutical potential (Schwartsmann, 2000; Schwartsmann et al., 2001). Marine creatures and their metabolites are thought to be rich sources of bioactive molecules. This study examined an extract of *B. marmorata* for protective and curative properties against MTX-induced liver and kidney damage, as measured using biomarkers at biochemical and histological levels.

The greater susceptibility of the liver to chemical cytotoxicity is likely due to its critical role in xenobiotic metabolism (Al-Attar, 2004). MTX causes hepatic (Abdel-Daim et al., 2017) and renal (Yuksel et al., 2016) damage. MTX metabolites in the liver induce inflammation, injury, and release of liver enzymes into the circulation (Çakır et al., 2015). MTX administration in this study produced considerable increases in serum AST and ALT activity, and previous administration of *B. marmorata* extract to MTX-treated animals significantly ameliorated this impact. Similar findings were also reported for an extract from Holothuria atra (Dakrory et al., 2015; Saad et al., 2018).

The notable decrease in total protein levels in the blood of MTX-injected mice is a helpful indicator of the severity of cellular dysfunction in chronic liver disorders. The most common cause of metabolic failure during pathogenesis is oxidative damage to sensitive amino acids (Bandyopadhyay et al., 1999). The current study found that giving *B. marmorata* to MTX-injected mice balanced serum albumin and improved total protein levels.

Links between in vivo MTX exposure and oxidative stress are likely due to metabolic activation and detoxification in the liver (Çakır et al., 2015). Mice injected with MTX exhibited significantly lower levels of SOD, CAT, and GSH than control mice. In contrast, administration of *B. marmorata* extract did not produce significant changes in these parameters. Treatment with extract before and after MTX injection ameliorated the effects on antioxidant biomarkers, significantly reversing the drop in SOD, CAT, and GSH levels. Thus, *B. marmorata* extract when administered before and after MTX challenge is effective in maintaining antioxidant defenses. Saad et

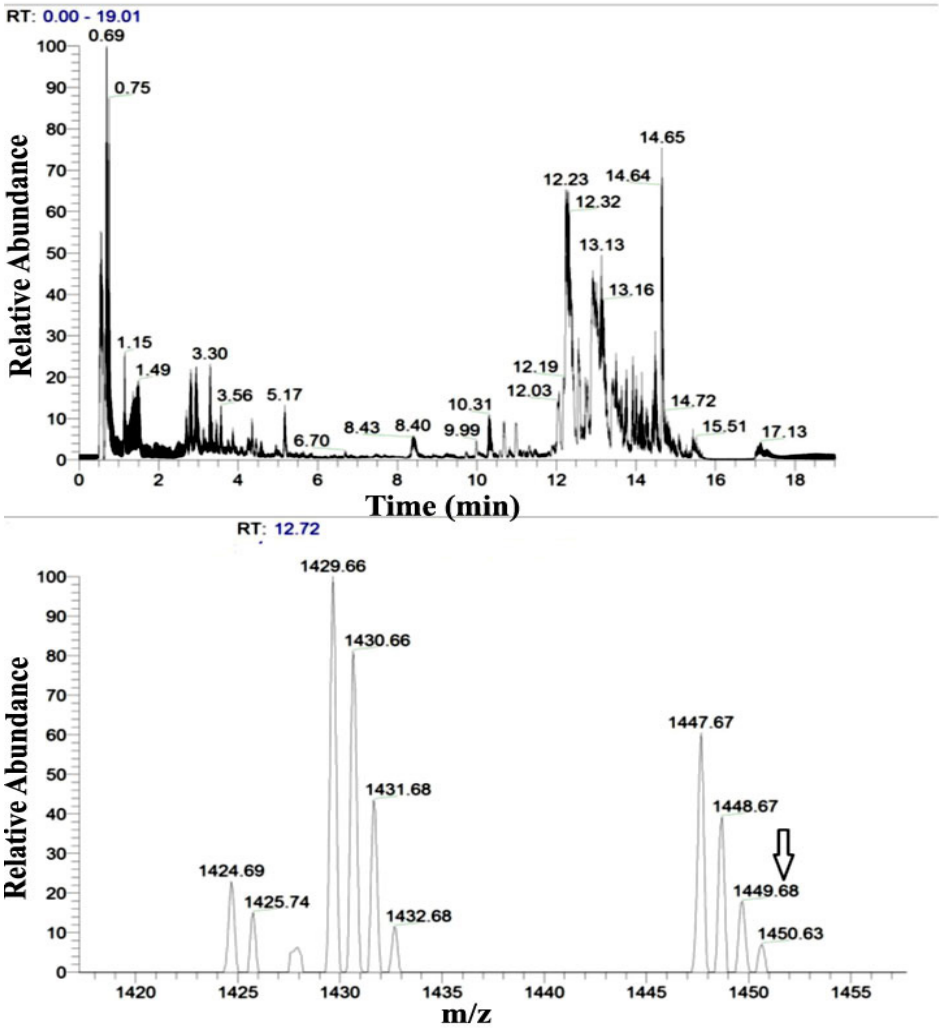

Figure 5. UPLC-MS Chromatograms of Ion Beaks (m/z + Na)+1449.68 were Acquired from *B. marmorata* Body Wall Extract

al. (2018) reported that MTX administration caused a decrease in SOD and CAT levels and an increase in serum levels of malondialdehyde (MDA) and total antioxidant capacity (TAC). Administration of H. atra extract to MTX-injected rats countered these adverse effects.

SOD and CAT are antioxidative enzymes that work sequentially for protection against ROS (Cerutti et al., 1994). Suppression of SOD and CAT activity is likely correlated with increased levels of the peroxidation end product, malondialdehyde (MDA), that inhibits protein synthesis and certain enzymes (El Kholy et al., 2013). *B. marmorata* extract may increase SOD and CAT

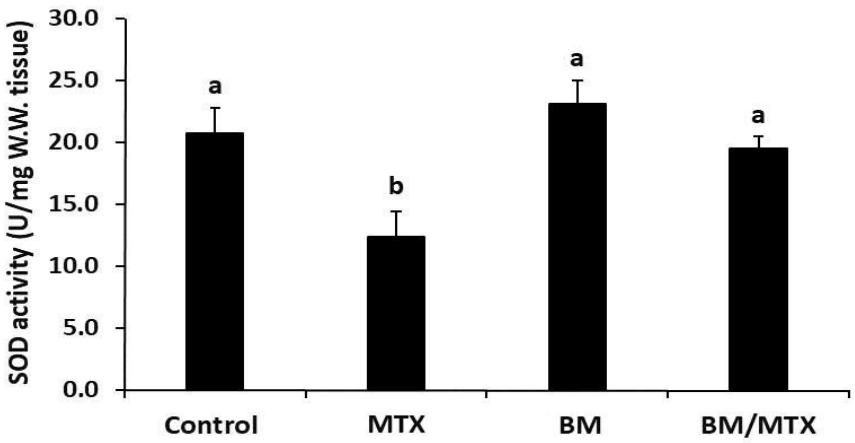

Figure 6. SOD Activity in Liver Tissue of Mice; Control, Methotrexate (MTX) Treated, *B. marmorata* (BM), Extract treated, BM + MTX treated. Means that do not share a letter are significantly different (Turkey´s test, $p < 0.05$)

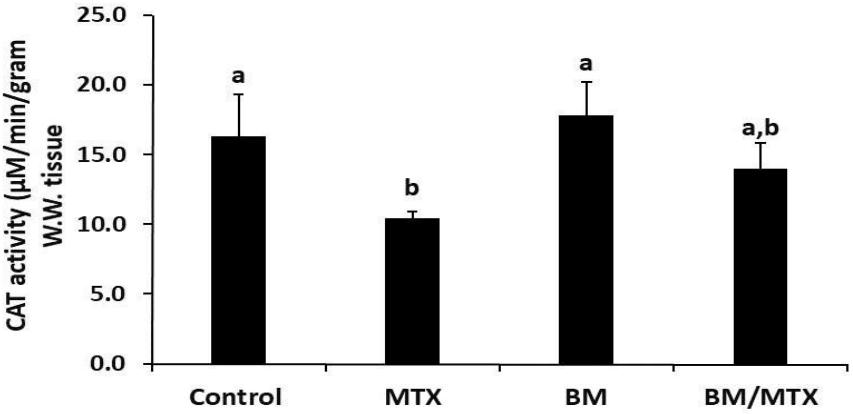

Figure 7. Catalase (CAT) Activity in Liver Tissue of All Groups of Mice; Control, Methotrexate (MTX) Treated, *B. marmorata* (BM) Extract treated, and BM + MTX treated. Means that do not share a letter are significantly different (Turkey's test, $p < 0.05$)

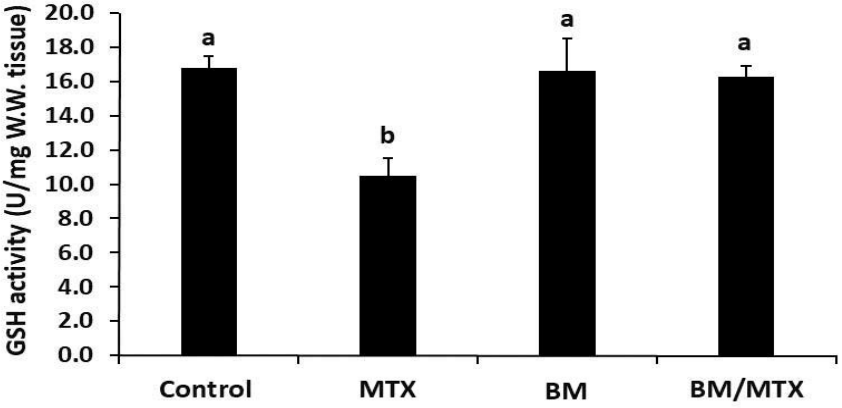

Figure 8. Reduced Glutathione (GSH) Concentration in Liver Tissue of All Groups of Mice; Control, Methotrexate (MTX) treated, *B. marmorata* (BM) extract treated, and BM + MTX treated. Means that do not share a letter are significantly different (Turkey´s test, $p < 0.05$)

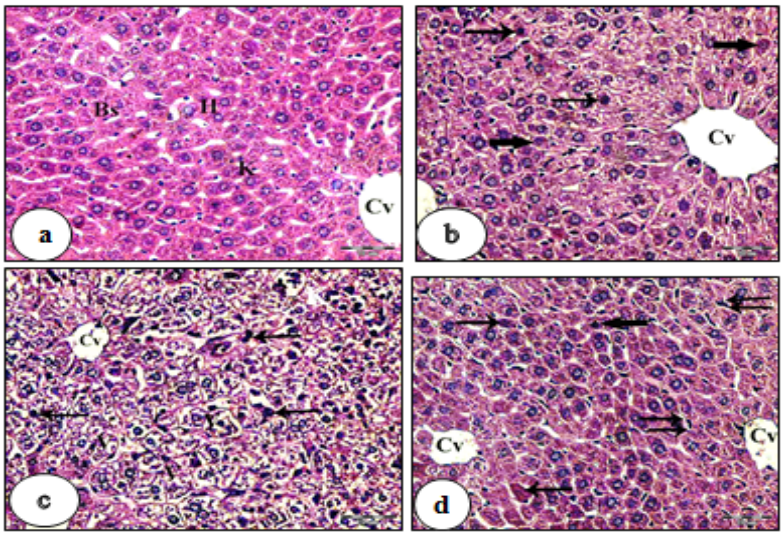

Figure 9. Photomicrographs of H&E-stained Liver Sections from Mice from Different Experimental Groups. a: Highly magnified sections of control liver showing standard hepatic architecture: central vein (Cv), radiating polygonal hepatocytes (H), and normal narrow blood sinusoids (Bs) lined by endothelial and Kupffer cells (K). b: Liver sections from MTX-injected mice showing disorganized hepatic structure: most hepatocytes vacuolated with degenerated cytoplasm (V), a large number of pyknotic nuclei (arrows), and obliterated blood sinusoids with activated Kupffer cells (K). c: Liver sections of B. marmorata extract-treated animals showing typical hepatic architecture, hepatocytes with normal cytoplasm. Some hepatocytes with pyknotic (arrows), others with karyolitic nuclei (thick arrows), a slight widening of central veins (Cv), and obliteration of blood sinusoids. d: Liver sections of mice treated with B. marmorata extract + MTX) exhibiting a noticeable improvement of hepatic architecture with typical central veins (Cv). Some hepatocytes show eosinophilia (arrows), others pyknotic nuclei (thick arrows), and activated Kupffer cells (K). (400×).

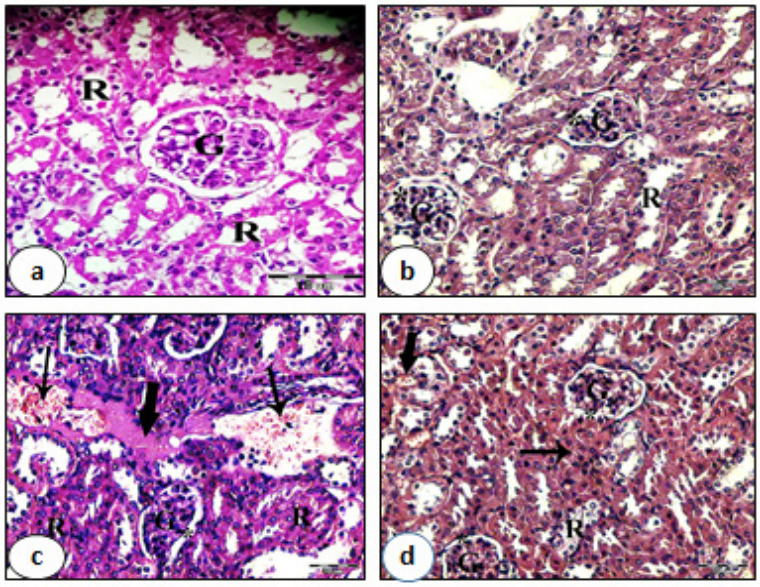

Figure 10. Photomicrographs of H&E-Sstained Kidney Sections from Mice from Different Experimental Groups. a: Highly magnified section of control kidney showing typical renal cortex structure with normal glomerular architecture (G) and renal tubules (R). b: Highly magnified section of the kidney after MTX treatment showing disorganized kidney structures: disorganized and atrophied glomeruli (G) with irregular mesangial areas (star), congested renal blood vessels (bv), most renal tubules with damage (thick arrows), intertubular hemorrhage (arrows), and accumulation of hyaline casts in intertubular spaces (double arrows). c: Highly magnified section of the kidney from animals after treatment with B. marmorata extract (BM) exhibiting disorganized glomeruli (G) with irregular bowman's space (star), and elongated and distended renal tubules (R). d: Highly magnified section of kidney after combined treatment with BM + MTX showing mild improvement of the kidney structures: glomeruli (G) with irregular mesangial areas (star), most of renal tubules are normal, others are poorly distinguished with intermixed contents. A few intertubular hemorrhages are seen (thick arrow) (400×).

activity and limit the concentration of free radicals. GSH deficiency promotes oxidative stress and ROS, resulting in a cascade of effects that compromise the functional and structural integrity of cell and organelle membranes (Singh et al., 2011). Restoration of GSH levels by *B. marmorata* extract from upregulation of glutathione de novo synthesis or regeneration (Park et al., 2008). Further, the extract may act directly to relieve reactive oxidative stress or may work with existing antioxidant chemicals to prevent loss of antioxidant capacity.

MTX-induced histological alterations in the liver tissue not seen in control mice and animals treated with *B. marmorata* extract, including disorganization of hepatic architecture, hepatocellular degeneration, and pleomorphic hepatic nuclei with pyknosis and karyolysis. Similarly, a single dose of MTX (20 mg/kg) in rats induced an increase in microscopic damage score in liver tissue (Kose et al., 2012). Mice treated with both MTX and extract significantly restored damaged hepatic tissue and promoted typical central veins. Some hepatocytes still showed eosinophilia; others exhibited pyknotic nuclei and increased Kupffer cell activity.

Also, mice injected with MTX displayed histological alterations in kidney tissues with disorganized glomeruli and irregular mesangial areas, congestion of renal blood vessels, damage and destruction of renal tubules, and intertubular hemorrhage. Similar results were reported by Asvadi et al. (2011). Conversely, kidney sections from animals treated with both MTX and *B. marmorata* extract exhibited mild improvement in kidney structures. Glomeruli with irregular mesengial areas were still observed, but most renal tubules were normal with typical lining epithelium. Still, other epithelial cells were poorly distinguished with intermixed content. A few intertubular hemorrhages were also seen. Dakrory et al., (2015) found that H. atra extract is a helpful natural product that can reduce hepatorenal toxicity caused by DMBA hydrocarbon exposure, consistent with current findings.

Chemotherapy is widely employed for the treatment of cancer and chronic inflammatory illnesses, but often caused significant undesired organ damage and immune suppression (Arnon et al., 2001) due to oxidative stress (Armagan et al., 2008; Padma et al., 2012). The current study proposes *B. marmorata* extract as a nutritional supplement capable of alleviating MTX cytotoxicity toward liver and kidney, modulating immunity, and decreasing oxidative stress. Further, *B. marmorata* extract is beneficial for patients receiving immunosuppressive medications, especially MTX.

Author Contribution Statement

Manar kandeil: conceptualization, data curation, methodology, software, validation, formal analysis, investigation, resources, writing-original draft preparation, writing-review, and editing, visualization, supervision. Eman El-Nahass: data curation, methodology, software, validation, formal analysis, investigation, resources, writing-original draft preparation, writing-review, and editing, visualization, supervision. Mona Elwan: data curation, methodology, software, validation, formal analysis, investigation, resources, writing-original draft

preparation, writing-review, and editing, visualization, supervision. All authors have read and agreed to the published version of the manuscript.

Acknowledgments

We express our sincere thanks to members of Physiology Laboratory, Faculty of Science, Tanta University.

Conflicts of interest

There are no conflicts of interest to declare.

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