

The Protective Effect of *Moringa Oleifera* Leaves Extract on Intestinal Injury Caused by Amethopterin Administration in Mice

Adel Q Alkhedaide

Laboratory Technology Department, Turabah University College, Taif University, Saudi Arabia.

*Corresponding Author E-mail: adelglhk@hotmail.com

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Amethopterin (methotrexate) is a well-known chemotherapy agent that has been used for the treatment of different types of cancers, such as leukemia and breast cancer. It is also used for the treatment of rheumatoid arthritis as an immunosuppressor; however, its numerous adverse effects limit its use in clinical settings. The aim of this study was to determine the protective effect of *Moringa oleifera* leaves extract on the intestinal toxicity caused by amethopterin exposure. Forty adult male mice were divided into four groups (10 mice each): the control group, a group treated orally by moringa extract (300 mg/kg) daily, a group treated by a single intraperitoneal injection dose of amethopterin at (20mg/kg) on the seventh experimental day, and a group given orally moringa extract (300 mg/kg) orally daily and a single intraperitoneal injection dose of amethopterin (20mg/kg) on the seventh experimental day. A serum analysis of TNF- α and CRP showed a significant elevation of both parameters in amethopterin-treated mice, while the co-treatment of moringa extract protected the intestines and restored both to normal control values. The immunohistochemistry and histopathology images indicated inflammation in the intestinal lamina as a response to the amethopterin toxicity supported by the downregulation of GLP2 and TGF β 2 genes in the laminal tissues. These structural alterations were prevented by moringa extract co-treatment in the fourth group. In conclusion, *Moringa oleifera* leaves extract exerts an anti-inflammatory and anti-stress effect on intestinal tissues caused by amethopterin toxicity exposure in experimental mice.

Keywords: Methotrexate, *Moringa*, Intestinal injury, Amethopterin, GLP2, TGF β 2.

Amethopterin, or methotrexate as it is commercially known, is a synthetic organic compound, and its chemical name is 2,4-diamino-N10-methyl propyl glutamic acid¹. It was introduced in 1950 as a treatment for acute lymphocytic leukemia¹. Amethopterin has been widely used as a chemotherapy agent and for rheumatoid arthritis treatment as an immunosuppressor²⁻⁴; however, it has some side effects that vary in severity, such as nausea, fatigue, recurrent infections, liver injury,

and renal failure⁵⁻⁸. In addition, J A Taminiou et al. have reported that amethopterin caused an intestinal injury in rats that were injected with just a single dose, which in turn caused malnutrition in experimented animals⁹. Another study revealed that amethopterin caused significant damage in the small intestinal epithelium in experimental mice¹⁰. A study has also shown that amethopterin causes intestinal mucositis that delays gastric emptying in treated rats¹¹. Therefore, doctors are highly cautious

when prescribing this medication, and they always prescribe vitamins as well, such as folic acid, vitamin A, or vitamin D analogues to avoid these side effects¹²⁻¹⁴.

In another context, some traditional medicinal plants can be used in place of these supplements to provide more protection with fewer side effects. *Moringa oleifera* Lam is a fast-growing tree belonging to the family Moringaceae¹⁵. The leaves of this plant possess several beneficial organic compounds. They are rich in vitamins, minerals, flavonoids, and polyphenols that consumers benefit from as nutrients and antioxidants^{16, 17}. This plant has long been used for medical purposes in some ancient nations¹⁵. It has been reported that *Moringa oleifera* Lam has some medicinal attributes, such as antimicrobial activity and an antiulcer effect as well as antispasmodic, anti-inflammatory, and anti-tumor growth effects¹⁸⁻²¹. Therefore, the objective of this study was to investigate the efficiency of *Moringa oleifera* Lam leaves extract as an anti-inflammatory and an antioxidant caused by amethopterin administration to mice and to what extent it can protect the intestinal lining of these animals after two weeks of co-treatment.

MATERIALS AND METHODS

Materials

Ethanol 99.8% hplc grade, dNTPs, Oligo (dT), Reverse transcriptase, and Dimethyl ether were purchased from Sigma Aldrich St. Louis, Missouri, United States. The hematoxylin, eosin, anti-Bcl2 antibody, Rabbit Anti-Mouse IgG H&L (HRP), C-reactive protein Elisa kit, and TNF-alpha Elisa kit were purchased from Abcam, Discovery Drive, Cambridge, UK. Amethopterin (Methotrexate) was purchased from Biovectra, Charlottetown, Canada. Primers were purchased from Macrogen, Beotkkot-ro, Geumcheon-gu, Seoul, South Korea. The real-time PCR kit and biozol reagent were purchased from Qiagen, Skelton house, Manchester, UK.

Moringa ethanolic extraction

Fresh *Moringa Oleifera* leaves were collected from the local markets in Taif, Saudi Arabia. The plant was identified by a botanist colleague from the College of Science, Taif University, Saudi Arabia. The leaves were washed with distilled water and dried in the dark and then

powdered using a high-speed electrical grinder. 25 grams of the powder were mixed with 70% of ethanol and stored in the dark for 48 hours at room temperature. The mixture was then filtered using Whatman No1 filter paper, and the filtrate was dried and concentrated at a reduced pressure at a controlled temperature.

Experimental design

All animal model experimental protocols were approved by the Scientific Research and Ethical Committee (SREC) of the University Collage of Tarabah, Taif University. Forty adult male mice were purchased from the animal house of the King Fahad Research Center, Jeddah, Saudi Arabia. The mice were accommodated for two weeks prior to beginning the experiment and were divided into four groups. Group 1 was the negative control (10 mice had free access to food and water). Group 2 had 10 mice exposed to a single intraperitoneal injection dose of Amethopterin (Methotrexate) of 20 mg/kg on the 7th day. Group 3 had 10 mice that were orally administered *Moringa Oleifera* leaves extract (MOLE) at 300mg/kg daily for the 12 experimental days. Group 4 had 10 mice that were orally administered *Moringa Oleifera* leaves extract (MOLE) at 300mg/kg daily for the 12 experimental days with exposure to a single intraperitoneal injection dose of Amethopterin (Methotrexate) at 20 mg/kg on the 7th day. On the 13th day, the mice inhaled dimethyl ether and were slaughtered for serum and intestinal tissue collection^{22, 23}.

Serum analysis

The serums were collected via centrifugation of the whole blood samples at 4000rpm. The samples then were treated using sandwich ELISA technology commercial kits. At the end of the procedure according to the manufacturer instructions, the reactions were read using a Bio-Rad xMark™ Microplate Absorbance Spectrophotometer at 450nm.

Gene expression analysis

The intestinal tissues were collected and immersed in 1ml of biozol reagent and frozen immediately at -20°C. On the day of total RNA extraction, the samples were homogenized and centrifuged at 4°C in a chloroform solution. In equal volumes, Isopropanol was added to each supernatant. The samples then were centrifuged at a high speed, and the supernatants were removed.

The precipitates (total RNAs) were rinsed with 70% ethanol and dissolved in DEPC water. For cDNA forming, the extracted total RNAs were mixed with oligo dT and incubated for 5 min at 70°C in a thermal cycler. The denatured RNAs were then mixed with 10mM of dNTPs, 100U of M-MuLV, the reverse transcriptase, and 2uL of 10X RT-buffer for each sample. The mixtures were incubated at 37°C for one hour in the thermal cycler followed by 10 min at 90°C to stop enzyme activity. The primers of the genes investigated listed in table 1 were used for the qRT-PCR analysis. The PCR reactions were conducted by following the optimized protocol provided with the kit. The reactions were analyzed using a Bio-Rad CFX Real-Time PCR Detection System.

Histopathological investigation

Intestinal tissues were collected from the mice of different groups and immediately fixed in 10% of formalin. The specimens were then washed under running tap water, dehydrated by alcohol, and then cleared in xylene. In paraffin, the

samples were embedded, casted, and sectioned into five micron sections. Finally, routine staining was performed using the H&E stain for the microscopic investigation.

Immunohistochemical examination of Bcl2

The intestinal tissue slides were washed twice with xylene for three min each followed by serial washes in gradient ethanol concentrations for deparaffinization. The slides were then washed under cold tap water to remove the remaining ethanol. The slides were treated with 3% H₂O₂ for 10 min for peroxidases suppression and then heated in a 10-mM citrate buffer at 121 °C for 30 min for antigen retrieval. The next step was blocking in a 5% normal serum for 15 min. The slides were incubated with a rabbit polyclonal anti-Bcl2 antibody (1:100; sc-EPR17509; Abcam, Discovery Drive, Cambridge, CB2 0AX, UK) in phosphate-buffered saline (PBS) overnight at 4 °C. The slides were then washed three times with PBS and incubated with a Rabbit polyclonal Secondary Antibody to Mouse IgG - H&L (HRP) for 15 min

Table 1. Real time PCR primers used in this study

Gene	Primer sequence	Product size	Annealing temp.(°C)	
GLP-2R	GLP-2-F	GCGGCCTGAAGAGGACTTG	128	60
	GLP-2-R	CCTCTGGCGCCCTTCACA		
TGF-b2	TGFb-2-F	GCGGAGGGTGAATGGCTCTC	122	60
	TGFb-2-R	GACGGCACGAAGGTACAGCA		
b-actin	b-actin-F	CCAGCCTTCCTTCTTGGGTA	143	60
	b-actin-R	CAATGCCTGGGTACATGGTG		

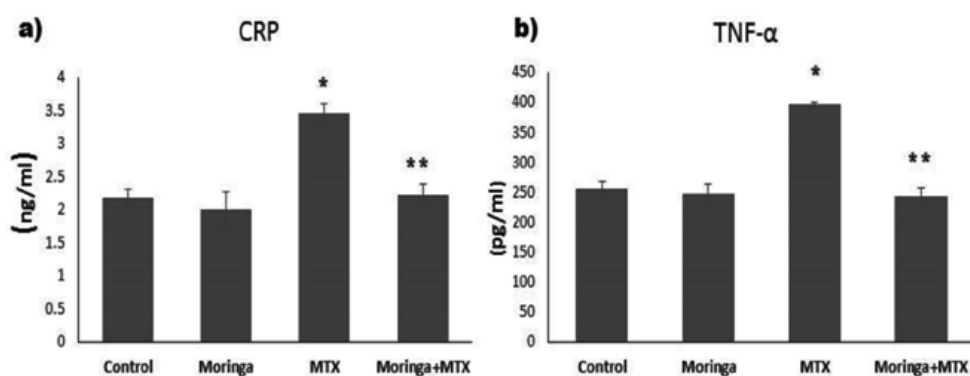


Fig. 1. Serum analysis of CRP and TNF- α in experimental mice. a) Serum C-reactive protein (CRP) levels in ng/ml. b) Serum tumor necrosis factor alpha (TNF- α) levels in pg/ml. Data presented in mean \pm SEM. * indicated P values < 0.05 corresponding to control, ** indicated P value < 0.05 corresponding to methotrexate treated group

at room temperature. Samples then underwent further incubation with diamino-benzidine (DAB) and were counterstained with hematoxylin for 10 s at room temperature.

Statistical analysis

Data are presented as means \pm standard error of means for 10 animals of each group. A two-way ANOVA was performed using SPSS data analysis software. P values < 0.05 were considered significant.

RESULTS AND DISCUSSION

Methotrexate (amethopterin) is an anti-cellular growth agent used for treating different types of cancers, such as breast cancer, leukemia, and melanoma²⁴. It is also used for treating

rheumatoid arthritis and psoriasis^{25, 26}. To some extent, exposure to methotrexate can cause severe toxicity that injures the liver, kidneys, and nervous system (22). In addition, it harms the intestinal lamina and the intestinal mucosa absorptive area^{27, 28}. In a study published in 2013, scientists reported that exposure to methotrexate for just four days can lead to intestinal mucositis, which is indicated by TNF- α and other cytokines²⁹. In fact, the challenge is determining how to manage the beneficial effects of methotrexate while avoiding its serious side effects, such as intestinal injury. Interestingly, reviewing several reports on the antioxidant and health benefits of *Moringa oleifera* Lam Leaves extract (MOLE) encouraged our lab to examine the positive effects of this extract on methotrexate toxicity. The health benefits of MOLE could be due

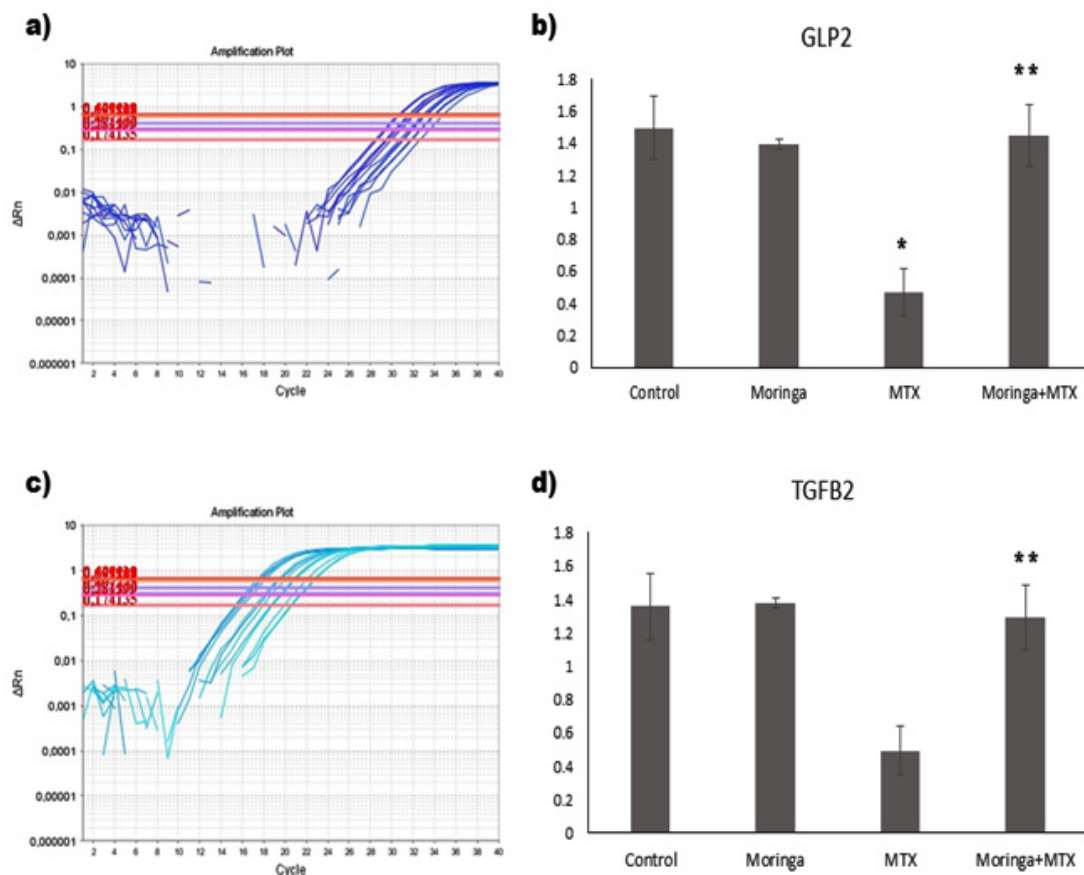


Fig. 2. Real Time PCR analysis of GLP2 and TGF β 2 genes. a) Graphical reading of GLP2 gene expression. b) Numerical analysis of the real time reaction of GLP2 gene. c) Graphical reading of TGF β 2 gene expression. d) Numerical analysis of the real time reaction of TGF β 2. * indicated P values < 0.05 corresponding to control, ** indicated P value < 0.05 corresponding to methotrexate treated group

to some of its valuable organic components, such as vitamins, short chain fatty acids, flavonoids, and polyphenols^{30, 31}.

Moringa oleifera leaves extract (MOLE) reduces serum levels of both CRP and TNF- α that are elevated by methotrexate (MTX) exposure

In the current study, the serum levels of both CRP and TNF- α were estimated for all experimental mice as inflammatory condition markers. CRP is a protein produced by hepatocytes, clinically known as an acute-phase inflammatory marker when its concentration is increased in the plasma. In addition, TNF- α is a small protein released mainly by macrophages, natural killer cells, and T-lymphocytes and is known as a proinflammatory cytokine^{32, 33}. Figure 1 clearly shows a significant increase in both markers. CRP and TNF- α in mice exposed to a single dose of MTX at 20 mg/kg on the seventh experimental day both had P values of $d > 0.05$ corresponding to normal control mice; however, figure 1 shows there was almost no difference between the control

group and mice administered Moringa Oleifera leaves extract (MOLE) at 300mg/kg daily over the 12 experimental days, which might mean there is no possible toxicity at that dose for that period. Interestingly, the serum levels of both the CRP and the TNF- α of mice treated with Moringa Oleifera leave extract (MOLE) at 300mg/kg daily over the 12 experimental days with exposure to a single dose of amethopterin (Methotrexate) at 20 mg/kg on the 7th day were significantly reduced to the normal levels, and the P values were $d > 0.05$ corresponding to mice treated with MTX alone. These findings may indicate that the inflammatory response caused by MTX toxicity can be prevented by either pre- or co-treatment of Moringa Oleifera leaves extract (MOLE).

Co-treatment of moringa oleifera leaves extract (MOLE) prevents the downregulation of both GLP2 and TGB2 genes caused by methotrexate (MTX) treatment

Two important genes have been investigated: the Glucagon-like peptide-2 (GLP2)

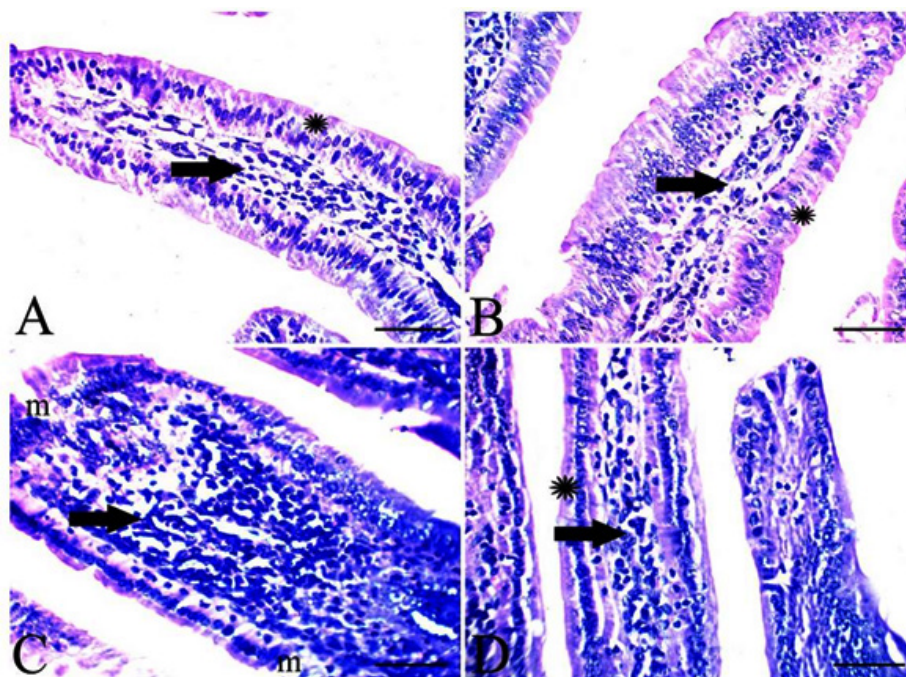


Fig. 3. Microscopic pictures of histopathological examination A. & B Intestinal villi of control and moringa-administered groups respectively showing normal columnar lining epithelium (*) with normal lamina propria (arrow). C. The intestine of the methotrexate-administered group showing metaplasia of lining epithelium into goblet cells (m) with massive infiltration of round cells into lamina propria (arrow). D. The intestine of the methotrexate-administered group treated with moringa showing regeneration of lamina epithelialis (*) and lamina propria (arrow). Scale bar= 50 μ m

gene and the transforming growth factor beta 2 (TGF β 2) gene. Glucagon-like peptide-2 (GLP2) is a polypeptide produced by intestinal cells to enhance the absorption process and to protect the gut epithelium^{34, 35}. Transforming growth factor beta 2 (TGF β 2) is an anti-inflammatory cytokine released by different cell types to exert a sort of control on the immune response and the bowel anti-inflammatory cytokine³⁶. Several studies have reported that both proteins are endogenous intestinal lamina protectors against any oxidative stress caused by different types of medications³⁷⁻⁴⁰. B van't Land and colleagues found that TGF β 2 exerts some protection in the small intestine of rats when co-treated with methotrexate⁴¹. In another study, GLP2 was reported to be a useful treatment for enteritis caused by chemotherapy⁴².

Herein, a single dose of methotrexate significantly downregulated both GLP2 and TGF β 2 gene expression in the intestinal tissues. P values of $p < 0.05$ corresponding to normal control mice, while MOLE significantly kept the expression levels at almost normal whereas the P values were $p > 0.05$ corresponding to mice treated with MTX alone, as clearly shown in figure 2.

Histopathological investigation

Figures 3A and 3B show a normal histological depiction of the intestinal samples of the control group and the moringa-administered group, which strikingly show normal submucosa, normal lamina epithelials, and normal lamina propria. In the 3C picture of figure 3, methotrexate is shown to have caused a massive infiltration of round cells in the lamina propria accompanied

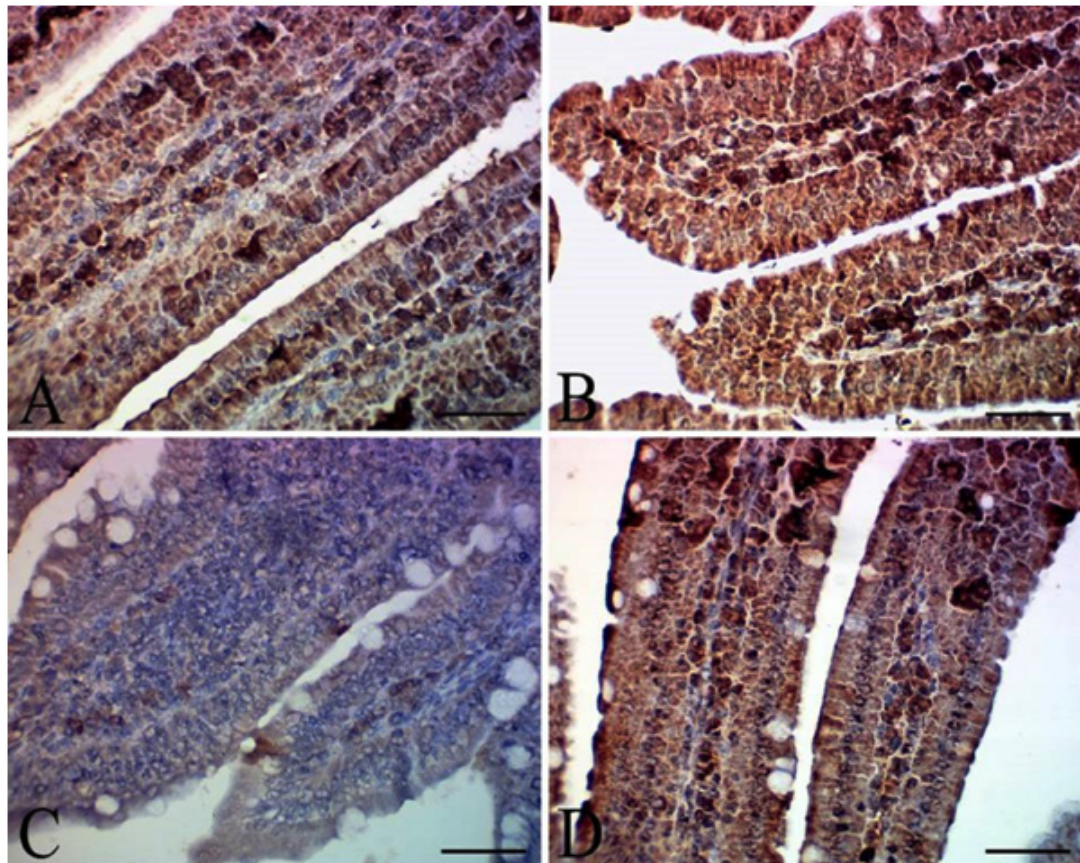


Fig. 4. Microscopic pictures of immunohistochemical examination A. Intestine of control group showed strong expression of Bcl2. B. The intestine of the moringa-administered group showed overexpression of Bcl2 in the villus mucosa. C. The intestine of the methotrexate administered group showed weak expression of Bcl2 with goblet cells metaplasia. D. Intestine of methotrexate-administered group treated with moringa showed restoration of normal elevated Bcl2 expression. Scale bar= 50 μ m

with goblet cell metaplasia and the desquamation of lamina epithelials. The abnormal depiction caused by methotrexate was entirely prevented in the intestinal tissues of mice that were co-treated with moringa leaves extract, as can be observed in figure 3D.

Immunohistochemical investigation of Bcl2

In the current study, the intracellular Bcl2 levels were examined to investigate the extent of MTX toxicity for cellular apoptosis. Bcl2, or B-cell lymphoma 2, is a family member of proteins that regulate the apoptosis process. Some previous studies have shown that MTX treatment downregulates the expression of the Bcl2 gene⁴³.⁴⁴ Figure 4C demonstrates a significant decrease in cellular Bcl2 in the intestinal tissues of mice as a response to MTX toxicity, while MOLE prevented the reaction, as shown in figure 4D. These findings indicate a fairly apoptotic process in these tissues, which is consistent with previous reports as well as with both serum CRP and TNF- α data and the histological depictions presented in figures 1 and 2. These results add further technical validation to the data that demonstrated both the toxicity effect of methotrexate on the intestinal lamina and the strong prevention of moringa leaves extract against the toxicity.

CONCLUSION

In conclusion, this study clarified that a single dose of amethopterin (Methotrexate) at 20 mg/kg can cause moderate to severe intestinal injury and Moringa leaves Extract has powerful content that can protect the gut from such toxicity.

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