Original Article

Microbial Biosystems 10(3) (2025) 2025.387368
10.21608/mb.2025.387368.1325

Contents lists available at Egyptian Knowledge Bank
Microbial Biosystems
Journal homepage: http://mb.journals.ekb.eg/

In vivo study of the expression of some immune markers in BALB/c mice exposed to *Aspergillus fumigatus* mycotoxin

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ARTICLE INFO

Article history
Received 20 May 2025
Received revised 17 June 2025
Accepted 14 August 2025
Available online 1 September 2025

Corresponding Editors
Omran, B.
Mohammed, S. H.

Keywords
Amyloid aggregation,
gliotoxin,
histopathology,
immunohistochemistry,
matrix metalloproteinases.

ABSTRACT

Gliotoxin (GT) is sulfur-containing mycotoxin within the 2,5-diketopiperazines class. First discovery from Gliocladium. Later discovered from different strains belonging to *Aspergillus fumigatus* mainly those have glicluster. This study outlines a study on the histological effects of gliotoxin (GT) on mouse brain and spleen tissues using light and electron microscopy, with a focus on its interaction with matrix metalloproteinases (MMPs). Histopathological changes through MMPs expressing variability estimated by using Immunohistochemistry (IHC). Mature mice were injected intraperitoneally with acute doses determined by data response analysis (EC50/IC50) as (125, 250, and 500 µg/ml) of GT and compared with a control group that received (methanol 10%). GT highlights specific histopathological changes, such as amyloid aggregation, apoptosis, and MMP expression. Evidence of infected spleen appears as Amyloid (insoluble protein) aggregates on red pulp, accumulation of phagocytic cells, and apoptosis of lymphocytes in white pulp. On the other hand, tissue vacuolation and atrophy of glial cells, necrosis of neuronal cells, and damage to Purkinje fibers on infected brains. IHC analysis showed MMP1 and MMP7 expression induction on mice spleen treated with different concentrations of GT. MMP-1 expression was induced 1.3 times when treated with 500 µg/ml compared with control, while the induction on MMP7 expression reached up to 3 times when treated with 500 µg/ml compared with control. Based on the information provided, it can be inferred that any harmful effects caused by toxins will impact and change the normal physiological expression of matrix metalloproteinases 1 & 7

Published by Arab Society for Fungal Conservation

Introduction

Mycotoxins refers to dangerous secondary metabolic products resulting through fungal cellular metabolism, with the effectivity to disrupt human and animal validity (Bennett & Klich 2003, Darwish 2019, Abo Nouh et al. 2020, Abed et al. 2020, Abdelmotilib et al. 2021).

The introduction of a research document focused on gliotoxin (GT), a mycotoxin produced by fungi like *Aspergillus fumigatus* and *Candida* species. Gliotoxin (GT), considered as major virulence factors for *A. fumigatus*, different species belong to *Candida* and other genera of fungi (Al-Dahlaki et al., 2023). Gliotoxin is appropriately identified as a significant virulence factor

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and is a potent mycotoxin. Its effects on the immune system, oxidative stress, and cellular function. GT's disulfide bridge and quinoid moiety contributing to its hydrophobicity and bioactivity is scientifically valid. The diversity about GT harmful impact related to hydrophobic ring which exhibits mainly on immune system. Previously, Montes de Oca *et al.* (2022) demonstrate the ability of GT to inhibit macrophage and PMNs cell on murine model, also its effect on phagocytosis process (Montes de Oca *et al.*, 2022, Latge JP 1999, Waring1969).

Many additional investigations have been conducted to detect key enzymes involved through apoptosis. The inhibition of macrophages and PMNs, apoptosis induction, alterations in RBC and WBC counts, and hepatic damage are well-grounded effects supported by referenced studies.

Analysis clarifies ability of purified GT to induced reduction in monocytes, lymphocyte ratio, also confirmed that gliotoxin induced apoptosis (Stanzani *et al.* 2005). Several investigations that have experienced the effects of GT on mice's liver indicate reducing total red blood cell (RBC) count and many histological variations in hepatic tissue including necrosis, and blood vessel congestion also showed an increase in white blood cell (WBC) count (Talib, 2024).

Recently studies focused on the neurotoxic implications of gliotoxin in human neuronal functions, neuroblastoma cell line cells are used as an *invitro* model to observe the mechanisms of GT combination to exert toxicity at the neuronal level by monitorization of intracellular ROS and studying the expression of different pro-apoptotic gene expression (Olcina *et al.*, 2025).

Previous studies have demonstrated that oxidative stress increases MMP production (Griselda et al., 2020). The present study utilizes MMP markers (matrix metalloproteinases) to assess the effect of GT on oxidative stress induction and the subsequent modulation of MMP expression.

Materials and Methods

Gliotoxin preparation and determination models of animals

Acute cytotoxic gliotoxin concentrations were prepared from previously extracted and quantified stock gliotoxin solution by culturing *Aspergillus fumigatus* on different culture media like yeast extract sucrose (YES). In determining the optimum cytotoxic concentrations of GT for being applied in this study, many recent and previous studies focused on determining LD50 of GT on mature mice and have established the following graduated concentrations as 125, 250, and 500 µg/ml were verified using high performance liquid chromatography (HPLC) for detection expression diversity (Anaam, 2020).

In this experiment, twenty mature mice male were employed. Animals were raised on house (animal house) of Al-Nahrain University were stable at roughly [25 C^0]. The animals were kept and fed food that was appropriate for their growth and upkeep (Makun, 2010).

Experimental design

Twenty mature mice weighted (21-23 grams) were divided into four groups G1, G2, G3, and control, each consisting of five mice, to investigate the histopathological and MMP1& MMP7 expression changes of GT. Groups A, B, and C were given a single intraperitoneal dose of prepared GT at different concentrations of 500, 250, and 125 µg/ml consequently, and the last group received a 10% intraperitoneal dosage of methanol it diluted in saline as a solvent (control). On the seventh day, all of the mice were sacrificed (Kwon-Chung & Sugui 2009).

Depending on the protocol proved by Jun *et al* (2006), the effect of Gliotoxin on Body weight and survival rate of mice with intraperitoneally administered dosage determined. The three groups of mice were administered I.P. injection with 682 µg/ kg refer as 500 µg/ml , 341 µg/ kg refer as 250 µg/ml and 170682 µg/ kg refer as 125 µg/ml of gliotoxin respectively on day 1, then monitor the body weight after injection. Control group was weighed as well.

Tissues (spleen & brain) preparation for histopathological study

Every mouse underwent dissection (Kwon-Chung and Sugui, 2009). To eliminate blood clots and specimen shrinkage, the spleen and brain were removed and placed in a petri dish with a physiological solution (8.5 g NaCl/ 1000ml D.W.) that had been adjusted to a pH of 7. According to Bancroft and Stevens (1982), detailed step-by-step fixation, dehydration, embedding, sectioning, and staining procedures was followed. Inclusion of Mayer's Albumin for slide adhesion and use of H&E is standard and reliable. After fixation with 10% formalin for 24hr, the samples were rinsed under running water, left in 70% ethanol for the night, and then dehydrated for two hours at each concentration using a graded sequence of alcohol (70%, 90%, 100%, and 100% ethanol).

The organ samples were then immersed in a block of paraffin (which has a melting point of 58°C), cleaned in xylene for 30 minutes, and cut into ribbon by a microtome 5– 7.5 µm in thickness. To mount slices of tissue on the slides Mayer's Albumin was applied, and they were then dried for one to two hours at 24 C in the oven. After using

Xylene to dewax the tissue slice on the slides, it was quickly cleaned in three changes of 100% alcohol, followed by 95%, 70%, and 30% alcohol. Staining with hematoxylin and eosin H&E for seconds then rinsed for 2–3 minutes with D.W and allowed to be dry. Slides were then covered with Distyrene-Plasticizer-Xylene DPX after being in xylene for 15 to 30 minutes. Power microscope magnification of 40 X used for histopathological evaluation (Abdulateef, 2024).

Tissues preparation for the immunohistochemistry study

Following the processing mentioned above tissues were fixed using the paraffin wax. Samples of Slices spleen were deparaffinized in a hot incubator at 80°C using positively charged adhesion microscope slides for 70 min for before being rehydrated in graded alcohol. Slides submerged step by step on the following solutions as [60 minutes of Xylene, 5 minutes of graduated ethanol from absolute then 90%,70% , 50% finally to 5 minutes of distilled water and then allowed for cooling at room temperature. Leakage prevention during the IHC staining, liquid blocker (pap ped) was used for sections borders defined (Al-Mudallal 2023).

Estimation and evaluation of matrix metalloproteinase (MMP) 1 and 7 using immunohistochemistry

Specific MMP1 and MMP7 antibodies were added to tissue sections at 40µl (catalog number ab5263; ab5706; Abcam- Cambridge-UK) followed the instructions mentioned by manufacture, spleen slicestreated with 40µL of primary anti-MMP1, anti-MMP7 separately and incubated at 37°C for 30 min. The following day, slides were rinsed with buffer bath for five minutes, drained, and gently blotted. Then, 20µL of the secondary antibody (the complement) was applied to the sections, incubated for ten minutes at 37°C, rinsed and put in the same washing buffer bath. Any extra buffer was then drained and gently blotted. Finally, 20µL of HRP (horseradish peroxidase) conjugate was added to each tissue section, and the sections were incubated for fifteen minutes at 37°C in a humidified room with 3,3'-diaminobenzidine tetrachloride (DAB) (Nouri, 2015). The expression of MMP1 and MMP7 was examined by histological scoring that became ideal for capturing variations in expression.

Assign intensity values to stained cells as (0 represent no color, 1= little to weak, 2= medium, 3= strong effective staining) that determine the percentage of cells at each intensity level, the following formula allows detection of MMP expression changes by quantifying both intensity and extent.

$$H-SCORE = \Sigma[INTENSITY * PERCENTAGE\ OF\ CELLS]$$

Statistical analysis

The data was analyzed using the statistical software SPSS-27 (Statistical Packages for Social Sciences, version 27). The data was presented using basic measures of mean and standard deviation. The significance of the difference between different means (quantitative data) was assessed using the students' test for the difference between two independent means. When the P-value was equal to or less than 0.05, statistical significance was assessed.

Results

Estimation histopathological study in infected Mice spleen

Histopathological section of mice spleen treated with125 µg/ml of toxin appeared to contain amyloid-like depose in the red pulp that already become congested (Fig:1-A), while the results appeared many inflammatory cells especially mononuclear cells infiltrated on red pulp that also being congested (Fig:1-B).

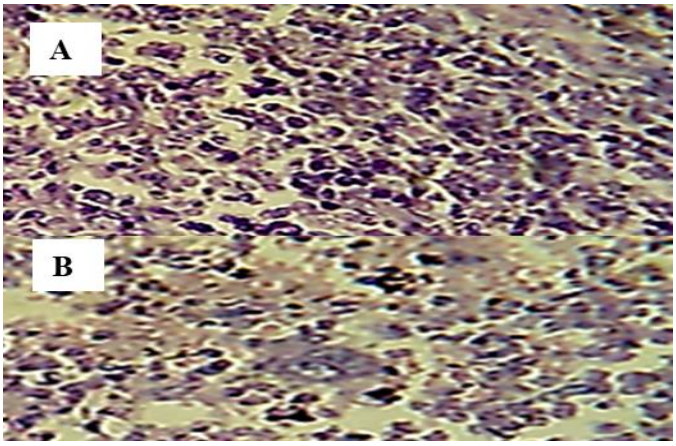

Fig 1. Histopathological section of mice spleen treated with (125 µg/ml). **(A):** Amyloid-like depose, **(B):** Infiltration of inflammatory cells, particularly mononuclear cells, (H&E stain, 40X).

The Extensive lymphocyte apoptosis in white pulp was observed clearly in histopathological sections of the Mice spleen injected with **250 µg/ml** that were characterized by cellular debris in multiple irregular spaces(Fig 2-A). Also, fatty changes were shown (Fig: 2-B).

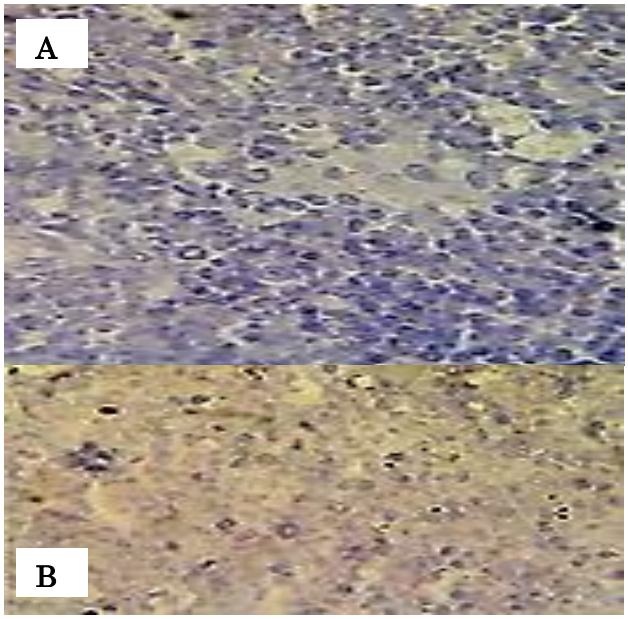

Fig 2. Histopathological section of the spleen from mice treated with 250 µg/ml of toxin. (A) Presence of cellular debris within numerous irregular cavities. (B) Evidence of fatty changes. Hematoxylin and eosin stain, 40×.

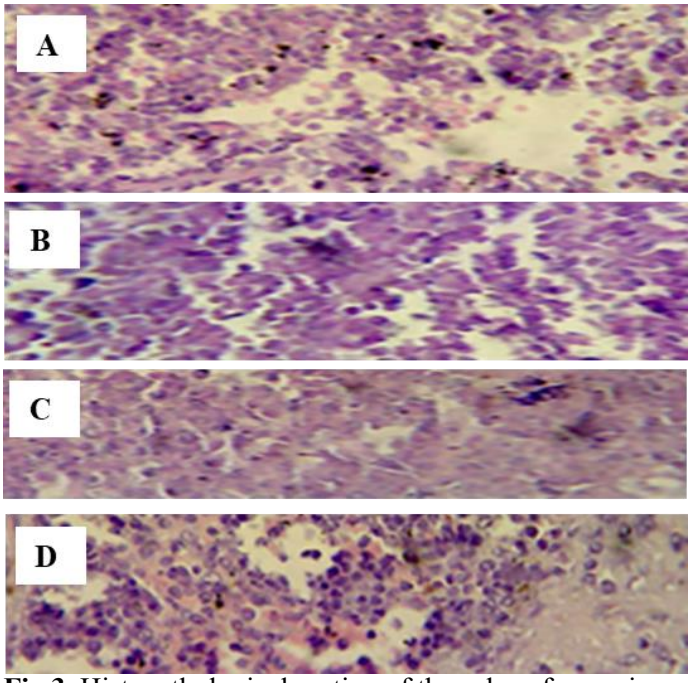

Fig 3. Histopathological section of the spleen from mice treated with 500 µg/ml of toxin. (A) Mononuclear cells in crowded vessels with white pulp atrophy. (B) Mononuclear cells within engorged blood vessels. (C) Apoptotic lymphocytes with accumulated cellular debris. (D) Amyloid-like aggregates in the white pulp. Hematoxylin and eosin stain, 40×.

The results were shown the histological examination of the spleen from injected animals with 500 µg/ml (higher GT dose) reveals mononuclear cells appearance in congested vessels alongside atrophy in the white pulp (Fig 3-A). It also shows mononuclear cells in congested blood vessels accompanied by depletion of the white pulp (Fig 3-B). Additionally, lymphocyte apoptosis within the white pulp is noted, characterized by the presence of cellular debris in various irregular spaces (Fig 3-C). Finally, there is an accumulation of amyloid-like substances surrounding the atrophied areas of the white pulp (Fig 3-D).

Estimation histopathological study in infected mice brain

The results of light microscopy elucidates the histopathological effect of GT in the mice cerebella subjected to 125 µg/ml of toxin. The figure below shows perivascular edema with the proliferation of astrocytes (Fig. 4).

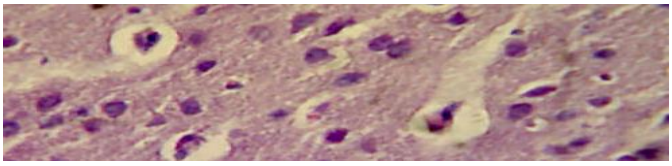

Fig 4. Histopathological section of the cerebellum from mice treated with 125 µg/ml of toxin, showing perivascular edema with astrocyte proliferation. Hematoxylin and eosin stain, 40×.

The central chromatolysis of Purkinje cells in the gray matter, along with perivascular edema in the white matter, was observed in cerebellar sections of animals treated with 250 µg/ml of toxin (Fig 5 - A). Additionally, edema accompanied by the proliferation of microglial cells was noted (Fig 5-B).

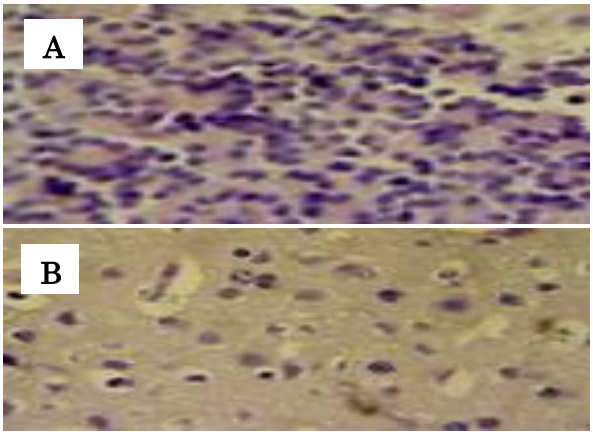

Fig 5. Histopathological section of the cerebellum from mice treated with 250 µg/ml of toxin. (A) Purkinje cells in the gray matter. (B) Proliferation of microglial cells. Hematoxylin and eosin stain, 40×.

The histopathological examination of cerebellar sections from animals treated with 500 µg/ml of toxin reveals the presence of perivascular edema, liquefactive degeneration surrounding neuronal cells, significant proliferation of astrocytes and oligodendroglia cells, and numerous vacuoles within the neutrophil (Fig 6).

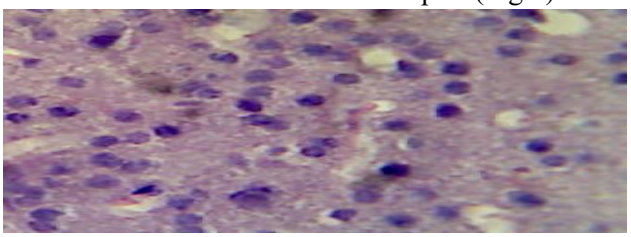

Fig 6. Histopathological section of the cerebellum from mice treated with 500 µg/ml of toxin. Hematoxylin and eosin stain, 40×.

Estimation of MMP1 and MMP7 using immunohistochemistry technique

Immunohistochemistry revealed an increase in MMP1productivity and also MMP7 in mice spleen inoculation with different concentrations of gliotoxin compared with the control group. MMP1 in spleen sections was expressed as 93.00 ± 52.07 when treated with125µg/ ml. However, the following table explain expression was induced to 124.20 ± 10.44 and 179.21 ± 3.10, after adding 250µg/ ml and 500µg /ml in succession, comparing with control (Table 1). The results in table (2) show that the differences between groups were statistically significant ($F(3, 16) = 14.182$, $p < 0.001$), indicating that GT significantly modulates MMP1 expression in spleen tissue based on dosage.

Table 1 MMP1 expression in mice spleen after treatment with different concentrations of gliotoxin

Stained cells (%) (Mean±S.E.)			
Control	125µg/ml	250µg /ml	500µg/ ml
72± 3.23*	93.00 ± 52.07*	124.20 ± 10.44*	179.21 ± 3.10*

Table 2 ANOVA results for MMP-1 expression in the spleen of mice treated with different concentrations of gliotoxin.

Source of Variation	Sum of Squares (SS)	df	Mean Square (MS)	F-value	P-value
Between Groups	35,414.45	3	11,804.82	14.182	0.000 **
Within Groups	13,317.94	16	832.371		
Total	48,732.39	19			

The ANOVA test at the 0.05 level indicates a significant difference between MMP1 expressions among the treated groups at ($p < 0.05$).

Immunostaining assay for MMP1 in different mice tissues was performed depend on cytoplasmic absorbance capability of these tissues to DAB. Figure 7-(B), (C), and (D) shows the differences between spleen section tissues subjected to graduated concentrations of gliotoxin compared with control, Fig 7(A).

A remarkable induction in MMP1 expression was observed in mice spleen tissues after treatment with GT in comparison with the control group. MMP7 in spleen sections was expressed as 79.00 ± 9.01 when treated with 125µg/ ml. However, the expression was induced to 88.25 ± 3.10, after adding 500µg /ml, compared with the control group expressed as 32.00 ± 3.34. The expression of MMP7 was reduced in very low percent as 73.30 ± 4.28 when compared with the lowest conc. of GT, (Table 3). But In Table (4), the results show that the differences between groups were statistically significant ($F(3,16) = 80.373$, $p < 0.001$), indicating that GT significantly modulates MMP7 expression in spleen tissue based on dosage. The ANOVA test at the 0.05 level indicates a significant difference independent means as in Table (4). A remarkable induction in MMP7 expression was observed in mice spleen tissues after treatment with 125, 250, and 500µg/ ml of GT in comparison with the control group (fig 8). In general, Gliotoxin stimulates the expression and activation of both studied MMPs. Matrix metalloproteinases (MMP1) are highly expressed in mice spleen when treated with different concentrations of GT in comparison with MMP7 and control.

Table 3 Expression of MMP-7 in the spleen of mice treated with different concentrations of gliotoxin.

Stained cells (%)(Mean±S.E.)			
Control	125µg/ml	250µg /ml	500µg/ ml
32.00 ± 3.34	79.00 ± 9.01*	73.30 ± 4.28*	88.25 ± 3.10*

Discussion

Components and their corresponding intrinsic metabolic pathways, which are critical for elucidating the potential pathological consequences that may arise. GT is a better - known naturally created mycotoxin, considered as secondary metabolites of most medical fungi. Gliotoxin cyto-genotoxicity is generated through the metabolic steps that involving catalysis, hydroxylation then sulfurization. (Scharf et al., 2011, Dolan et al., 2015). The sulfur bond is crucial for the toxic effects of gliotoxins. Formation of a disulfide bond directly affect both cell viability and proliferation across different cell lines (DeWitte-Orr 2005). On the other hand, excessive production of reactive oxygen species those arise though above-mentioned metabolic pathways acts as major reasonable for many neuro- genic conditions and cellular apoptosis especially when ROS eliminator production reduced and the cellular

Table 4 ANOVA results for MMP-7 expression in the spleen of mice treated with different concentrations of gliotoxin.

Source of Variation	Sum of Squares (SS)	Df	Mean Square (MS)	F-value	Sig. (p-value)
Between Groups	9775.185	3	3258.395	80.373	0.000 **
Within Groups	648.651	16	40.541		
Total	10423.835	19			

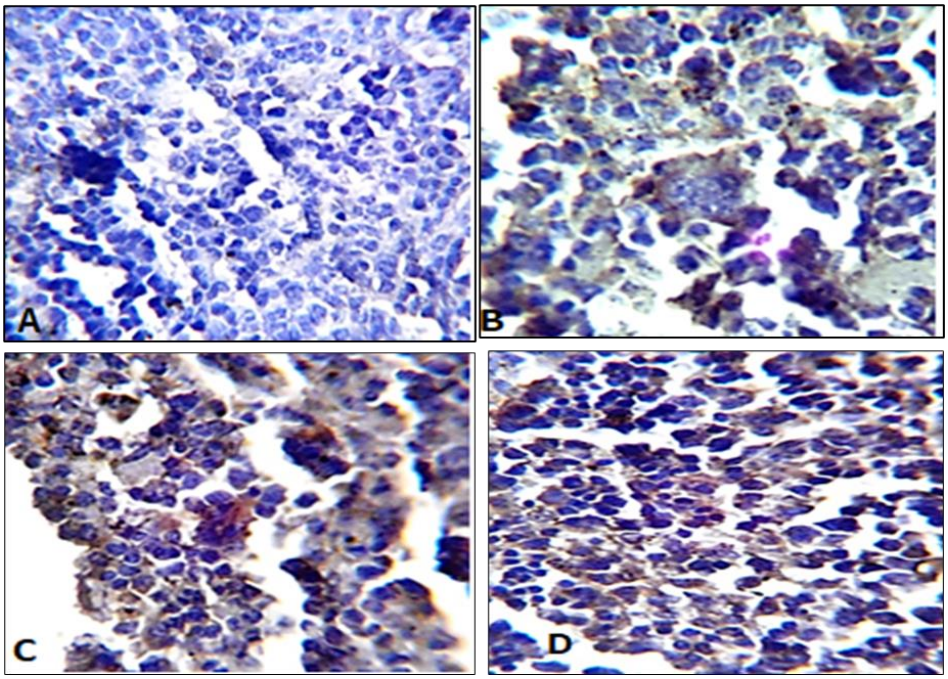

Fig 7. MMP-1 expression in spleen sections of mice. (A) Control. (B) Treatment with GT 125 µg/ml. (C) Treatment with GT 250 µg/ml. (D) Treatment with GT 500 µg/ml. Hematoxylin stain (40×), counterstained with DAB (brown).

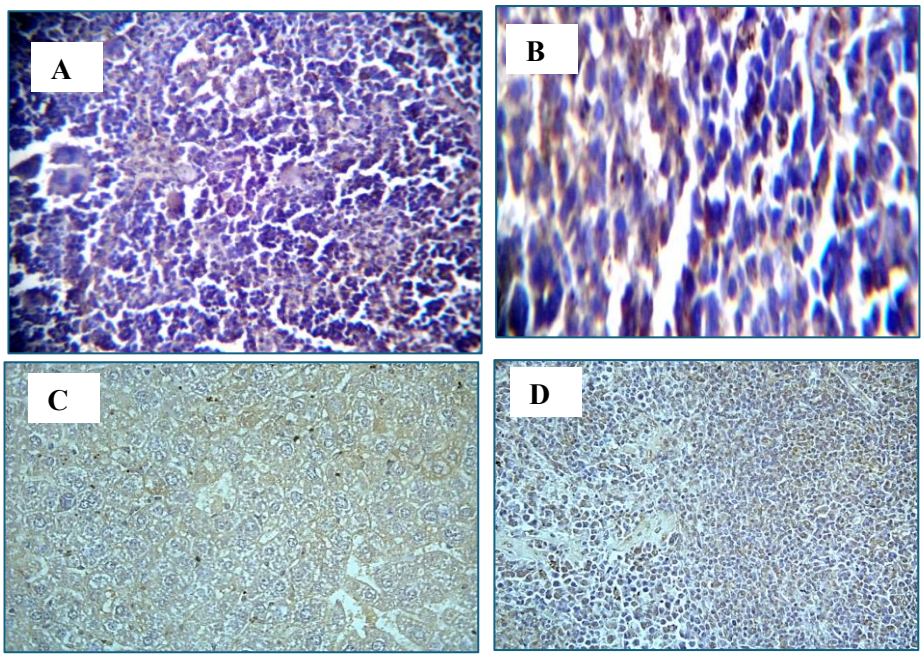

Fig 8. MMP-7 expression in spleen sections of mice. (A) Control. (B) Treatment with GT 125 µg/ml. (C) Treatment with GT 250 µg/ml. (D) Treatment with GT 500 µg/ml. DAB stain, 40×.

imbalance become dominant resulting oxidative stress, (Choi et al., 2005, Halliwell, 2011). Several studies proved GT cytotoxicity on human neutrophile through accumulation of superoxide and diminishing of NADPH oxidase action, (Yoshida et al., 2000, W Ye *et al.,* 2021). Also, cellular oxidative stress triggers DNA, protein and cell membrane integrity abnormality leading apoptosis disturbance followed by necrosis (Zhou et al., 2022; Shalini et al. 2015. Elmore 2007). In the Current study histopathological effect of GT on spleen and brain directly correlated to the chemical effectivity of toxin and the imbalance that generated specifically on tissues that subjected to different concentration of toxin. Also, immune-histochemistry assay results clarify the relation between expression variation of the two main markers belong to matrix – metallo- proteinases= (MMPs) with gliotoxin cellular exposing at different doses. All Cytotoxic, pathogenic effects influenced by toxin will be reflected and alter natural physiological express of both MMP1, MMP7. Specialized research on the linkage between the effects of gliotoxin and MMPs are limited, otherwise the MMPS play important role in cancer progression through facilitate extracellular matrix degradation when produced irregularly, (Claudia et al., 2009).

Conclusion

Variable histopathological effects were indicated in both studied mice organs (spleen and brain) when treated with different concentrations of GT by the effect of GT intracellular metabolism. Expression induction for both studied markers (MMP1 and MMP7) was confirmed in treated mice spleens. Expression diversity is directly proportional with toxin concentration.

Conflict of interest: The authors declare that they have no conflict of interest

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