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EFFECT OF GLIOTOXIN ON MICE LIVER AND DETOXIFICATION BY CLOVE (SYZYGIUM AROMATICUM)

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ABSTRACT

Gliotoxin (GT) is a major and highly potent mycotoxin produced by Aspergillus fumigatus. This study aimed to investigate the effects of GT on mice and its detoxification by clove (Syzygium aromaticum). Aspergillus fumigatus exhibited the highest production of GT on Yeast Extract Sucrose medium, with a concentration of 23.823 µg/kg using HPLC. Mice were divided into four groups: the control group, the GT-treated group (16.75 mg/kg body weight, orally), the clove-treated group (200 mg/kg body weight, orally), and the combined GT and clove-treated group (16.75 mg/kg GT and 200 mg/kg clove, orally). The treatments were administered for three months. This study evaluated the impact of GT on liver function by analyzing blood parameters, liver enzyme activities (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]), and histopathological changes in the liver. The results indicated a significant decrease in hemoglobin concentration, packed cell volume (PCV%), and total red blood cell (RBC) count, leading to normocytic normochromic anemia in the GT-treated group. Additionally, changes in hepatic tissue included severe lymphocytic cuffing around blood vessels, dilated and congested blood vessels, and degeneration and necrosis of hepatocytes. This group also showed a significant increase in white blood cell (WBC) count and elevated ALT and AST activities. Conversely, no significant differences were observed in the groups treated with either the combined GT and clove extract or clove extract alone. Histopathological examination of liver sections supported the serum analysis findings. In conclusion, gliotoxin adversely affected the liver of mice, whereas clove extract effectively mitigated these effects.

Keywords: Gliotoxin, *Syzygium aromaticum*, AST, ALT, *Aspergillus Fumigatus*

INTRODUCTION

Aspergillus fumigatus is a ubiquitous fungus that produces airborne spores (conidia) constantly inhaled from both indoor and outdoor environments. It is the primary causative agent of aspergillosis, a group of diseases affecting animals and humans. Aspergillosis, candidiasis, and cryptococcosis are opportunistic fungal infections that predominantly affect immunocompromised hosts (Ali and Ibrahim, 2023). Some fungi are toxigenic, synthesizing one or more mycotoxins (Abdulbaqi *et al.*, 2018), which can elicit harmful effects on other organisms (Abad and Al-Haddad, 2017). These molds can

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produce mycotoxins in several ways, causing a range of harmful effects such as sudden poisoning, suppression of the immune system, or cancer (Alnaemi *et al.,* 2023).

Gliotoxin is a secondary metabolite of A. fumigatus (Nouri, 2015; Abdulhadi and Abdulbaqi, 2018). One of the most abundant mycotoxins, gliotoxin belongs to the epipolythiodioxopiperazine family, characterized by a disulfide bridge across a piperazine ring, which is essential for its toxicity. Interestingly, the majority of mutants generated through the deletion of genes encoding enzymes necessary for the production of certain toxins have not exhibited a decrease in virulence (Ojaimi *et al.,* 2023). Gliotoxin triggers apoptosis in cultured hematopoietic stem cells (HSCs) and simultaneously suppresses the activity of NF-κB (Abu-Seidah, 2003). The compound has several histopathological effects on both human and animal cells and tissues, including the brain (Bertossi *et al.,* 2003), colon (Pan and Harday, 2007), and apoptotic hepatic non-parenchymal cells (NPCs) (Nejak-Bowen *et al.,* 2013). Diets contaminated with multiple mycotoxins can cause physiological abnormalities in the liver, blood, and biochemistry, as well as growth depression in animals (Gowda *et al.,* 2008). Consequently, the presence of mycotoxins in poultry feeds results in significant financial losses for the animal industries (Awad *et al.,* 2006).

Syzygium is the largest genus within the Myrtaceae family, comprising approximately 1,200 to 1,800 species of flowering plants widely distributed across tropical and subtropical regions of Asia, Africa, Madagascar, and throughout the Pacific and Oceanic islands (Cock and Cheesman, 2018). Syzygium aromaticum, commonly known as clove, is an aromatic medicinal plant belonging to the Myrtaceae family (Chaieb *et al.,* 2007). It contains numerous biologically active compounds, including eugenol, eugenol acetate, and β-caryophyllene (Sebaaly *et al.,* 2015), which render it antifungal, antibacterial, antiviral, and insecticidal. Eugenol, the most biologically active component, accounts for 70-80% of clove oil. Clove extract has demonstrated hepatoprotective activity by restoring normal concentrations of cytoplasmic enzymes (aspartate aminotransferase and alanine aminotransferase) in the serum (Thuwaini et al., 2016), indicating its potential in treating liver diseases.

This research aimed to study the effect of gliotoxin on the blood and liver of mice and to explore the medicinal use of clove extract to reduce and mitigate the toxic effects of gliotoxin.

MATERIALS AND METHODS

Fungal isolation

A fungal specimen was collected from various veterinary clinics and stray bovines in the Baghdad province. The specimen was cultured on Czapek Dox Agar and identified using conventional morphological evaluation techniques in the laboratory (Al-Saffy and Abdulshaheed, 2021).

Production of gliotoxin

The 30 Aspergillus fumigatus isolates were cultured on Czapek Dox Agar plates for 7 days at 37°C. To produce gliotoxin on Yeast Extract Sucrose (YES) medium, certain modifications were made to the process (Hussain *et al.,* 2020).

Extraction of Gliotoxin

According to Kosalec *et al.* (2005), 50 mL of chloroform was used to extract the biomass. The biomass was then pulverized into smaller fragments using an electric homogenizer operating at 3,500 revolutions per minute for 10 minutes. The resulting mixture was filtered using Whatman No.1

filter paper, followed by extraction with 2 × 25 mL of chloroform. The chloroform fractions were combined and treated with anhydrous Na_2SO_4 to remove any residual water. The chloroform extract was then evaporated using a rotary evaporator under reduced pressure at 60°C until dry. The dried extracts were diluted in 500 µL of chloroform and stored at 4°C until high-performance liquid chromatography (HPLC) analysis for gliotoxin detection. The organic phase was evaporated to dryness using a nitrogen stream and reconstituted in 1000 µL of the mobile phase. Finally, a 100 µL sample was introduced into the HPLC system for analysis.

Extraction of Clove

According to Tajuddin *et al.* (2004), dried clove flower buds were procured from the Baghdad market, crushed into powder, and extracted using a Soxhlet apparatus with 50% ethanol at a 1:3 w/v ratio for six hours. The extract was then filtered, and the solvent was removed by rotary evaporation under reduced pressure and low temperature, yielding a yellowish extract with a pleasant aroma.

Animals and Design of Experiments

Ethics Approval

The current study was approved by the ethics committee at the College of Veterinary Medicine, University of Baghdad, Iraq (P.G 1909).

Animals and Design of Experiments

Forty albino mice, approximately 3 months old and weighing 25.3 ± 0.9 g, were randomly assigned to different groups. The mice were maintained on a standard laboratory diet with access to water and were kept in temperature-controlled conditions at the animal house laboratory of the College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq. The mice were divided into four groups, with 10 mice per group, as follows:

- **G1**: Negative control mice (untreated).
- **G2**: Mice treated orally with gliotoxin alone at a dose of 16.75 mg/kg body weight every 48 hours for 3 months.
- **G3**: Mice treated orally with clove extract alone at a dose of 200 mg/kg body weight every 48 hours for 3 months.
- **G4**: Mice treated orally with a combination of gliotoxin (16.75 mg/kg body weight) and clove extract (200 mg/kg body weight) every 48 hours for 3 months.

Blood Collection

After anesthetizing the mice with chloroform, blood samples were collected from each group. Blood was drawn into sterile plastic test tubes containing an anticoagulant for hematological analysis. Additional blood samples were collected in test tubes without anticoagulant, allowed to stand upright at room temperature, and then used to obtain serum. The serum was separated by centrifugation at 3000 rpm for 15 minutes to maximize the serum yield. The collected serum was stored in a deep freezer at -18°C for biochemical analysis, including measurements of aspartate aminotransferase (AST) and alanine aminotransferase (Ageel and Abdul-Shaheed, 2022).

Liver Function Tests

The evaluation of liver function involved measuring blood parameters, specifically the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase. These enzymes are key indicators of liver function in mice.

Histopathological Examination

Liver tissue samples were collected from mice anesthetized with chloroform and subsequently sacrificed. The liver specimens were fixed in a 10% formalin solution and processed using a tissue-processing apparatus (Histokinette). The processed tissues were embedded in paraffin blocks, sectioned with a microtome, and stained with hematoxylin and eosin. The stained

sections were examined under a light microscope at the highest achievable magnification (Bancroft and Stevens, 1996).

Statistical Analysis

The data were analyzed statistically using the SPSS program. Both Analysis of Variance (ANOVA) and Least Significant Difference (LSD) tests were employed to evaluate the results.

RESULTS

The results demonstrated that Yeast Extract Sucrose (YES) medium was highly effective in promoting gliotoxin production. The ability of A. fumigatus isolates to produce gliotoxin was evaluated using a Czapek-Dox broth medium. The presence of gliotoxin was confirmed through High-Performance Liquid Chromatography (HPLC), with a concentration measured at 23.823 μg/kg.

Effects on Blood Picture

The study revealed that gliotoxin alone caused a significant decrease ($p \leq 0.05$) in hemoglobin (Hb), packed cell volume (PCV%), and red blood cell (RBC) counts (Table 1). In contrast, no significant differences ($p \leq 0.05$) were observed in mice treated with clove extract alone or those treated with a combination of clove extract and gliotoxin, compared to the control group.

Table 1: Effects of Gliotoxin Alone or in Combination with Clove Extracts on Mice RBCs, Hb, and PCV (mean ± SE)

Parameters	G1 (mean ± SE)	G2 (mean ± SE)	G3 (mean ± SE)	G4 (mean ± SE)	ANOVA	LSD 0.05 (P value)
RBC (10^6/mm³)	8.77 ± 0.21 **a**	6.36 ± 0.20 **c**	8.16 ± 0.03 **b**	8.32 ± 0.09 **b**	45.86	<0.001 **HS**
Hb (gm/dl)	12.61 ± 0.36 **a**	8.08 ± 0.08 **c**	12 ± 0.11 **b**	11.62 ± 0.13 **b**	98.33	<0.001 **HS**
PCV (%)	35.55 ± 0.59 **b**	24.80 ± 1.04 **c**	42.0 ± 0.47 **a**	34.60 ± 0.33 **b**	113.71	<0.001 **HS**

S: Significant difference between groups (p-value < 0.05)
HS: High significant difference between groups (p-value < 0.01)
NS: Non Significant difference between groups (p-value < 0.05)
Groups with different letters within the same raw are significantly different

Effect of Gliotoxin on Serum Levels of ALT and AST

The serum biochemical analysis (Table 2) revealed that gliotoxin (GT) treatment led to a significant increase in the activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) ($P \leq 0.05$). In contrast, there were no significant differences in ALT and AST levels in mice treated with clove extract alone or in combination with gliotoxin when compared to the control group ($P > 0.05$).

Table 2: Effects of GT alone or in combination with clove extracts WBCs and differential count of leucocytes on mice (mean ± SE)

Parameters	Groups (mean ± SE)				ANOVA $LSD_{0.05}$ (P value)
	G1	G2	G3	G4	
WBCs (10^9/L)	6.16 ±0.25 b	9.09 ±0.45 a	6.32 ±0.17 b	6.62 ±0.22 b	21.64<0.001 **HS**
Neutrophil %	31.32 ±0.36a	42.44 ± 0.69 c	32.16 ± 0.31 b	31.88 ± 0.43 b	127.33 <0.001 **HS**
Monocyte %	8.54 ±0.59	8.70 ±0.21	8.42 ±0.08	8.40 ±0.12	2.19 0.105 **HS**

S: Significant difference between groups (p-value < 0.05)
HS: High significant difference between groups (p-value < 0.01)
NS: Non Significant difference between groups (p-value < 0.05)
Groups with different letters within the same raw are significantly different

Effect of Gliotoxin on Serum Levels of ALT and AST

The serum biochemical analysis presented in Table 3 indicated that GT treatment resulted in a significant increase ($P \leq 0.05$) in ALT and AST activity. In contrast, no significant differences ($P > 0.05$) were observed in mice treated with clove extract alone or in combination with gliotoxin compared to the controls regarding the ALT and AST parameters tested.

Table 3: Effects of gliotoxin alone or in combination with clove extracts on mice liver enzymes (means± SE)

Parameters	Groups (mean ± SE)				ANOVA
	G1	G2	G3	G4	$LSD_{0.05}$ (P value)
ALT (IU/L)	31.60 ± 0.34 b	81.60 ± 0.68 a	31.40 ± 0.34 b	42 ± 0.47 b	92.72 <0.001HS
AST (IU/L)	76.7 ± 1.15 b	203.8 ± 0.82 a	75.8 ±1.01 b	88 ± 0.47 b	78.29 <0.001HS

S: Significant difference between groups (p-value < 0.05)
HS: High significant difference between groups (p-value < 0.01)
NS: Non Significant difference between groups (p-value < 0.05)
Groups with different letters within the same raw are significantly different

Histopathological Examination

Histopathological examination of the liver in control (untreated) mice demonstrated no significant pathological changes, as shown in Fig. 1A, compared to the other groups. In G2, severe lymphocytic cuffing around blood vessels, dilated and congested blood vessels, and degeneration and necrosis of hepatocytes were observed (Fig. 1B). In G3, a dilated central vein, an increase in Kupffer cells, and degeneration of some hepatocytes were noted (Fig. 1C). In G4, there was an increase in Kupffer cells, degeneration of some hepatocytes, and focal aggregation of lymphocytes (Fig. 1D).

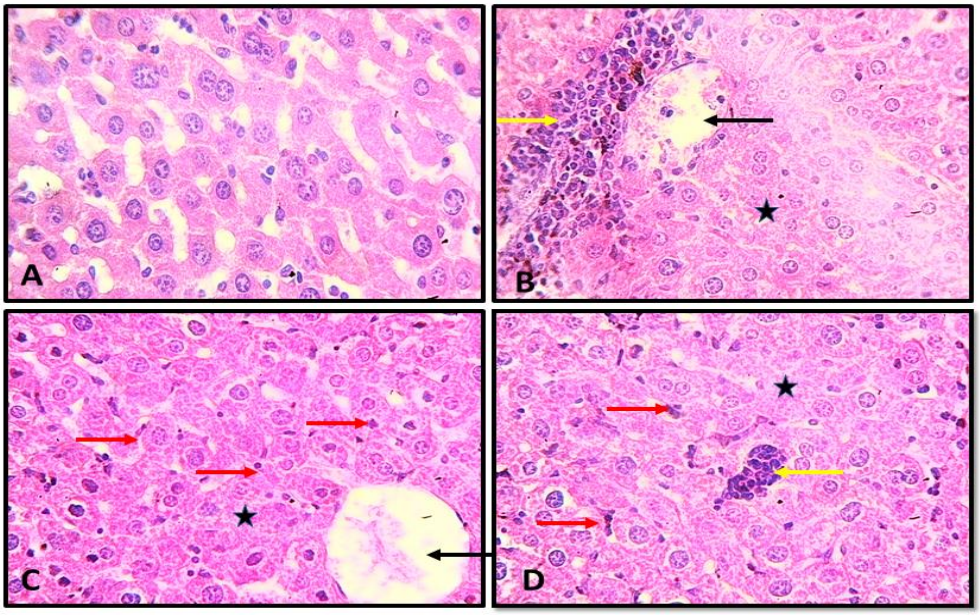

Figure 1: Photomicrograph of the mice liver of the studied group showing A) Section in mouse liver (G1) showing normal liver tissue. B) Section in mouse liver (G2) showing severe lymphocytic cuffing around blood vessels (yellow arrow), dilated and congested blood vessels (black arrow), and degeneration of hepatocytes (star). C) Section in mouse liver (G3) showing a dilated central vein (black arrow), proliferation of Kupffer cells (red arrows), and mild degenerated hepatocytes (star). D) Section in mouse liver (G4) showing Kupffer cells proliferation (red arrows), degenerated hepatocytes (star), and focal aggregation of lymphocytes (yellow arrows). (H&E stain, x400).

DISCUSSION

The results showed that the YES (Yeast Extract Sucrose) medium was highly effective in promoting gliotoxin production, consistent with Kosalec's earlier findings.

In our study, gliotoxin altered biochemical parameters, including decreased concentrations of red blood cells (RBC), hemoglobin (Hb), and packed cell volume (PCV%), while increasing white blood cell (WBC) and neutrophil levels, as well as serum enzyme levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). These changes suggest that gliotoxin has a direct toxic effect on the hemostatic blood system and hepatocytes.

Mycotoxins have a broad spectrum of harmful effects on animal health, manifesting as immunosuppression, hepato-toxicity, nephrotoxicity, neurotoxicity, genotoxicity, and damage to the hemostatic blood system (Abbès *et al.*, 2006). Blood can serve as a valuable biomarker for various pathological and physiological alterations in animal health (Jorum *et al.*, 2016). Hematological indices such as hemoglobin, hematocrit, and erythrocyte and white blood cell counts can be utilized as biomarkers of toxicity. Changes in these parameters are considered significant indicators of physiological stress. Hematological and biochemical variables are widely used to assess nutritional and health status (Sehonova *et al.*, 2018). Alterations in hematopoietic parameters might result from various factors, including suppression of protein synthesis, as evidenced by reduced blood albumin and serum total proteins (Abdel-Wahhab and Aly, 2005). Mycotoxin may lead to hypochromic macrocytic anemia, which inhibits red blood cell maturation (Abdel-Tawwab *et al.*, 2020).

There was a significant increase in white blood cell (WBC) count, predominantly consisting of neutrophils. The increased WBC count and neutrophil percentage suggest an inflammatory response induced by the toxin, affecting bone marrow and immune system function (Rompelberg, 2011).

Similarly, Abbès et al. (2006) reported that mice treated with 500 mg/kg of ZEN showed significant increases in WBC, hematocrit (Hct), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and mean platelet volume (MPV), alongside decreased RBC levels. Other studies also indicate that mycotoxins cause damage to the hemostatic blood system, with Ageel and Abdul-Shaheed (2022) showing significant decreases in hemoglobin concentration, packed cell volume (PCV%), and total RBCs, as well as significant increases in WBC count and activities of ALT and AST in mice treated with OTA.

The liver is the primary target organ in cases of mycotoxin poisoning, though other organs may also be affected (Gaddawi *et al.*, 2022). Several studies have documented histopathological changes in hosts with aspergillosis (Shafiq and Al-Joofy, 2010), and enzymes such as glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), alkaline phosphatase (ALP), and creatine kinase play established roles in various diseases affecting the liver, kidneys, and bones (Lieberman *et al.*, 2008).

To assess liver damage, enzyme levels such as serum AST and ALT are widely used. The ALT levels were significantly higher ($P \leq 0.05$) in the group without clove treatment (G2: 81.60 ± 0.68) compared to the groups treated with clove (G1: 31.60 ± 0.34, G3: 31.40 ± 0.34, and G4: 42 ± 0.47). These results align with those of Fahmy *et al.* (2014), who reported the highest concentration of gliotoxin in mice infected with A. fumigatus conidia. Elevated ALT levels may indicate liver inflammation and necrosis (Jasim and Abdul-Shaheed, 2023).

The AST levels also showed significant differences ($P \leq 0.05$) in G2 without clove treatment (203.8 ± 0.82) compared to the groups treated with clove (G1: 76.7 ± 1.15, G3: 75.8 ± 1.01, and G4: 88 ± 0.47). High serum AST levels suggest liver damage. These results are consistent with those of Shafiq and Al-Joofy (2010), who observed significant increases in serum levels of AST and ALT, while Shen *et al.* (2011) indicated that gliotoxin-induced damage was associated with more severe liver damage. Herbal dietary supplements, such as clove extract, have potential benefits in mitigating hepatotoxicity. Clove extract, known for its analgesic, anti-nociceptive, antibacterial, and anticancer properties (Venugopal *et al.,* 2017), has been shown to have anti-inflammatory effects (Sharma *et al.,* 2017) and antifungal properties against Fusarium oxysporum and F. lycopersici. It also offers protection against aflatoxicosis in rats exposed to aflatoxin (Abdel-Wahhab and Aly, 2005).

Significant histopathological changes were observed in the liver tissue of mice treated with gliotoxin, as shown in Figure 1B. Gliotoxin, a fungal metabolite classified under the epipolythiodioxopiperazine class, has been documented to induce apoptosis in hepatic stellate cells (HSCs) through mitochondrial permeability, the release of cytochrome c, and the activation of caspase-3 (Kweon *et al.,* 2003). Gliotoxin has been observed to cause apoptosis in various cell types, including thymocytes, peripheral lymphocytes, macrophages, P815 mastocytoma cells, L929 fibroblasts, and HSCs, in both laboratory settings and living organisms, including the thymus, spleen, lymphatic nodules of the mesentery, and liver (Hagens *et al.,* 2008). Gliotoxin also demonstrates immunosuppressive properties in hepatocytes (Stanzani *et al.,* 2005) and affects the survival of Kupffer cells and endothelial cells in the liver, impacting the non-parenchymal cell (NPC) population (Nejak-Bowen *et al.,* 2013).

The group treated with clove extract significantly reduced the elevation of serum ALT and AST activity, suggesting that clove extract may restore liver function and histopathology. Clove extracts, particularly eugenol and caryophyllene, possess antioxidant and anti-inflammatory properties (Zhang *et al.,* 2017). Eugenol and ethanolic clove extracts have been shown to have anti-inflammatory effects (Sharma *et al.,* 2017). Additionally, clove extract has antifungal properties against Fusarium oxysporum and F. lycopersici and provides protection against aflatoxicosis in rats exposed to aflatoxin (Abdel-Wahhab and Aly, 2005).

CONCLUSION

The active clove extract demonstrated significant activity against gliotoxin produced by Aspergillus fumigatus in vivo. This was evidenced by improvements in blood parameters, liver function markers such as alanine aminotransferase (ALT) and aspartate transaminase (AST), and histopathological alterations in the liver of the mice.

RECOMMENDATIONS

To minimize mycotoxin production, it is crucial to reduce conditions that favor fungal growth, particularly in animal feed and agricultural settings. Additionally, using clove extract as a treatment is recommended, as it is a natural plant with demonstrated therapeutic effectiveness.

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تاثير سم الكلايوتوكسين على الكبد وانزيماته وازالة السمية بواسطة القرنفل

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الكلايوتوكسين هوالسم الرئيسي والأقوى الذي يفرزه فطر الاسبرجلس . يهدف هذا البحث إلى دراسة تأثير سم الكلايوتوكسين على الفئران وازالة سميته بواسطة القرنفل. أظهر فطر الاسبرجلس أعلى إنتاج لـسم الكلايوتوكسين باستخدام وسط مستخلص الخميرة والسكروز،وتم قياس تركيز سم الكلايوتوكسين هو ٢٣,٨٢٣ mg\kg بواسطة جهاز HPLC. تم عمل التجربة بتقسيم الحيوانات الفئران الى اربع مجاميع: المجوعة الاولى الضابطة غير المعالجة بأي شي، المجموعة الثانية تم اعطائها الكلايوتوكسين، المجموعةالثالثة تم معالجتها بواسطة القرنفل، المجموعة الرابعة تم اعطائها سم الكلايوتوكسين والقرنفل وتم الاعطاء بجميع المجاميع عن طريق الفم . تم تجريع الحيوانات لمدة ثلاث اشهر لدراسة تاثير السم واستخدام القرنفل كعلاج لتقليل سمية الكلايوتوكسين على كبد الفئران من خلال ملاحظة التغيرات في الدم وكذلك التغيرات بانزيمات الكبد ،اضافة الى التغيرات النسيجية .اظهرت النتائج فقر الدم نتيجة قلة كريات الدم الحمراء والهيموجلوبين بلاضافة الى زيادة كريات الدم البيضاء و زيادة انزيمات الكبد في مجموعة الفئران التي تم اعطائها السم فقط بينما لم تلاحظ فروق معنوية في المجموعة المعالجة بالكلايوتوكسين مع مستخلص القرنفل أو بمستخلص القرنفل وحده. وخلصت النتائج الى حدوث تاثيرات السم على كبد الفئران وفعالية القرنفل العلاجية في ازالة تاثير السم على الكبد .