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Original Research Article

In vivo anti-tumor effect of Egyptian scorpion *Leiurus quinquestriatus* venom in Ehrlich ascites carcinoma-bearing mice

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Abstract

Purpose: *To investigate the anti-tumor efficacy of scorpion Leiurus quinquestriatus venom (LQV) in Ehrlich ascites carcinoma (EAC) mouse model.*
Methods: *Mice were divided into 4 groups (n = 8), with Group 1 as negative control. Groups 2 to 4 mice were inoculated with EAC cells (1 x 10^6/mouse) intraperitoneally (ip). Group 2 mice were untreated while groups 3 and 4 mice were injected with 2 mg/kg of cisplatin (Cis) and 0.025 mg/kg of LQV ip, respectively, for 7 days. Tumor profile, as well as hematological and biochemical analyses were carried out.*
Results: *Tumor volume and tumor cell counts decreased significantly ($p < 0.05$) following LQV treatment. Also, LQV potentiated necrosis, apoptosis and arrested tumor cells in the G0 and S-phases. Furthermore, it upregulated apoptotic-related gene (Bax and caspase-3) expressions and down-regulated anti-apoptotic gene (bcl-2) expression in EAC cells ($p < 0.05$).*
Conclusion: *LQV has potential anti-tumor activity against EAC cells in mouse models. Therefore, it should be further investigated in vivo as an anti-tumor agent.*

Keywords: *Anti-tumor, Leiurus quinquestriatus venom, Ehrlich ascites tumor, Apoptosis, Cell cycle, Gene expression*

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INTRODUCTION

Cancer is a public health problem worldwide. Classical chemotherapeutic agents are cytotoxic on normal cells and tissues [1]. Thus, new strategies for cancer treatment are needed. Several compounds derived from natural products have been considered candidates for developing new anti-cancer treatments [2]. Numerous pharmacologically active molecules isolated from animal venoms have been tested as anti-cancer agents. For instance, invertebrate animal venoms have many bioactive compounds such as chlorotoxin, charybdotoxin-2 and α-toxin that exert their anti-cancer effects [3].

Scorpion venom (SV) is a mixture of non-protein and protein compounds in nature, produced for defense and capturing prey [2]. SV has been screened on different human carcinoma cell lines

[4]. In cancer cells, SV toxicity may be due to its efficacy to induce apoptosis or necrosis [5]. For example, *Odontobuthus doriae* venoms decreased viability, induced necrosis, apoptosis and DNA fragmentation in human neuroblastoma [4]. Furthermore, a previous study reported that *Androctonus amoreuxi* venom downregulated VEGF expression in Ehrlich solid tumors and decreased tumor volume and size [6]. Till now, there is no known study on *Leiurus quinquestratus* venom (LQV) efficacy in EAC-bearing mice. Therefore, this work was conducted to estimate the anti-cancer effect of LQV on EAC-bearing mice and to investigate its protective effect against hepato-renal toxicities induced by EAC-cells in mice.

EXPERIMENTAL

Mice

Female CD1 mice (22 ± 2 g) were obtained from Helwan University and maintained under optimal laboratory conditions at 22 ± 1 °C and 55 ± 5 % relative humidity. The National Institutes of Health's guidelines for the care and use of laboratory animals and the National Research Center Ethics Committee's guidelines were followed in handling the laboratory animals. The institutional Animal Care Committee approved the study (approval no. IACUC-Sci- TU 0130).

Animal groups and design

Four groups of mice (n = 8) were used. Group 1 was negative control. Groups 2, 3 and 4 were injected with EAC-cells (1 x 10^6/mouse) intraperitoneally (ip) with Group 2 left untreated. Post 24 h of EAC inoculation, Group 3 was injected with 2 mg/kg of Cis, and Group 4 was injected with 0.025 mg/Kg of LQV ip for 7 consecutive days. Via orbital plexus, blood samples were collected for determination of biochemical and hematological parameters.

Chemicals

Cisplatin (Cis) was obtained from Sigma-Aldrich, France and re-constituted as 2 mg/kg in phosphate-buffered saline (PBS). Different biochemical kits including liver transaminases (alanine transferees, ALT and aspartate transferees. AST), creatinine and urea as well as antioxidant/oxidant biomarkers namely catalase (CAT), superoxide dismutase (SOD) and malondialdehyde (MDA) were obtained from Bio-diagnostic Company, Egypt. Annexin-V and Propidium iodide (PI) were obtained from e-bioscience, USA.

Collection of scorpions and venom preparation

Scorpions were obtained from Aswan City (Egypt) in July 2021. Specimens were placed in plastic cages, then identified by an Animal Taxonomist. SV was collected via electrical stimulation (12 – 17 V) of their stinging process and then lyophilized as described by Salama *et al* [7].

EAC cells expansion and inoculation

Tumor cells were obtained from Cairo University, Egypt (National Cancer Institute). Each mouse was injected with 1 × 10^6 EAC-cells ip. For tumor progression, EAC-injected mice were monitored daily for abdominal distention, distress and illness.

Determination of percentage body weight changes (B. wt)

At the beginning (Ib) and end of experiment (Fb), animals were weighed and percentage body weight changes (% B.wt) were calculated using Eq 1.

B. wt (%) = {(Fb-Ib)/Ib}100 …… (1)

Determination of hematological and biochemical parameters

Complete blood parameters were evaluated using blood counter (Mendary, China). Serum ALT, AST, SOD, and CAT were determined using Biodiagnostic kits (Cairo, Egypt). Serum urea, creatinine and MDA levels were colorimetrically determined using the Bio-diagnostic kits.

Determination of apoptosis and cell cycle analysis

Tumor cells were harvested from untreated and LQV-treated EAC-bearing mice and then re-suspended as 1 × 10^6 cells/mL in PBS. A hundred microliters (100 µL) of tumor cell suspension were mixed with Annexin-V and PI (5 µL/each) and then incubated for 15 min in the dark at 25 ˚C. Thereafter, PBS (400 µL) was added. Using a BD FACS Canto™ II flow cytometer, stained cells were aspirated and analyzed.

Cell cycle analysis was performed according to Weir *et al* [8]. Tumor cells from untreated and treated groups were fixed in 70 % cold ethanol at 4 ˚C overnight. Thereafter, cell pellets were washed and centrifuged, then resuspended in

PI/RNase staining buffer. Stained cells were analyzed and the cell-cycle stage was determined using CELLQUEST software (Becton Dickinson, San Jose, CA).

Gene expression analysis for Bax, Caspase-3 and Bcl-2 in EAC-cells

Tumor cells were collected from different groups of EAC-bearing mice. Total RNA was extracted and complementary DNA (cDNA) was synthesized using RNeasy Mini kit (Qiagen, Germany) and Quant script reverse transcriptase, respectively. As shown in Table 1, gene-specific primers (Bax, caspase-3, and Bcl-2) were used. Amplification of cDNA was done and relative change in gene expression was calculated according to the method of Diaz-Garcia *et al* [9].

Table 1: Forward and reverse primers of bax, caspase-3 and bcl-2 genes used in qPCR amplification

Gene	Primer sequence (5'->3')
Bax	(F) GGACGAACTGGACAGTAACATGG
	(R) GCAAAGTAGAAAAGGGCGACAAC
Caspase-3	(F) AGAACTTAGG CATCTGTGGGC
	(R) ATCCAGGGGCATTGTAGCAC
Bcl-2	(F) CAGGTCCTCTCAGAGGCAGATAC
	(R) CCTCT CCAGGGACCTTAACG
β-actin	(F) CCTTCCTGGGCATGGAGTCCTG
	(R) GGAGCAATGATCTTGATCTTC

Histological examination

The liver and kidneys were collected and fixed in formalin (10 %) for 24 h, then dehydrated in different concentrations of ethanol. Thereafter, tissues were cleared and embedded in wax. Tissue sections (5 µm) were prepared for mounting and staining with Hematoxylin and Eosin (H&E) [10].

Data analysis

For data and statistical analysis, Excel 2013 and Minitab version 18 were used. Data are presented as mean ± standard deviation (SD). Comparison between groups was performed by one-way ANOVA. Statical significance was set at $p < 0.05$.

RESULTS

LQV treatment decreased changes in body weight of tumor-bearing mice

After two weeks of EAC-cells inoculation, the % B. wt change in tumor-bearing mice was 39.7 %. Cis or LQV treatments decreased the % B. wt to 20.1 and 17.6 %, respectively (Figure 1 A).

LQV inhibited EAC progression

Cisplatin treatment significantly decreased the volume of tumor (0.55 ± 0.19 ml/mouse). Also, LQV treatment exhibited an anti-tumor activity evidenced by a decrease in tumor volume to 3.35 ± 0.16 mL/mouse (Figure 1 B). Treatment with Cis showed a significant reduction (95.3 %) in the tumor cell count (TCC). In addition, treatment with LQV decreased the TCC by 57.7 % (Table 2). Furthermore, Cis treatment reduced the viable tumor cells (increased dead tumor cells). In LQV-treated mice, the % of reduction in EAC-live and dead cells were 68.8 and 29.4 %, respectively (Table 2).

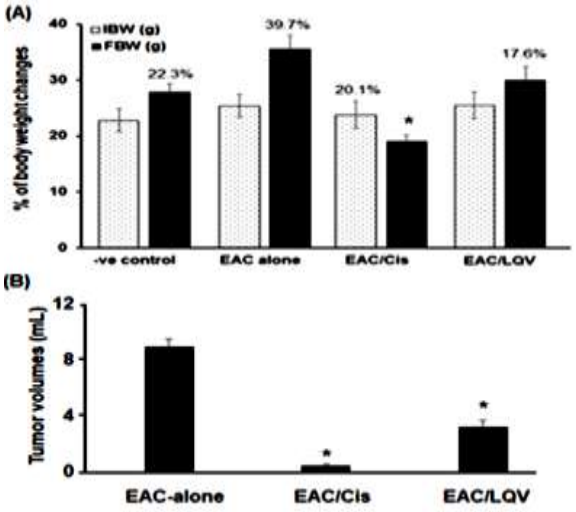

Figure 1: (A) Treatment of EAC-bearing mice with LQV increased percentage of body weight changes. (B) Treatment of EAC-bearing mice with LQV decreased total volume of ascitic fluid. *$P < 0.05$ versus control

Table 2: Tumor cells count (TCC), live (TLC) and dead (TDC) EAC-cells in different groups of mice under study

Group	TCC (×10^6)/Mouse		TLC (×10^6)/Mouse		TDC (×10^6)/Mouse	
	M±SD	r (%)	M±SD	r (%)	M±SD	r (%)
EAC-alone	603±16^{a}	-	552±36^{a}	91.5	48±8.1^{a,b}	7.96
EAC/Cis.	28±3.2^{c}	95.3	9.0±2.0^{c}	32.1	18±4.2^{b}	64.3
EAC/LQV	255±10.5^{b}	57.7	175±24^{b}	68.8	75±7.5^{a}	29.4

EAC: Ehrlich ascites carcinoma, Cis: Cisplatin (2 mg/kg/6 days), LQV: Leiurus quinquestratus venom (0.025 mg/Kg/6 days), r (%); percentage of reduction. Groups that don't share the same letter are significantly different ($p < 0.05$)

Table 3: Hematological profile n different groups of mice

Group	RBCs (×10^6/µL)	Hb (g/dL)	Hct (%)	Platelets (×10^3/µL)
Negative control	7.9±0.95^a	13.8±1.1^a	34.4±1.7^b	935±52.5^b
EAC-alone	4.5±1.5^b	8.6±2.5^b	28.2±3.5^a	840±60^b
EAC/Cis	5.2±1.2^{a,b}	9.5±2.1^{a,b}	29.6±2.5^b	1454±71^a
EAC/LQV	7.3±1.4^{a,b}	12.6±1.5^{a,b}	31.1±3.6^b	630±44^c

EAC: Ehrlich ascites carcinoma, Cis: Cisplatin (2 mg/kg/6 days), LQV: Leiurus quinquestratus venom (0.025 mg/Kg/6 days). Groups that don't share the same letter are significantly different ($p < 0.05$)

LQV treatment ameliorated hematological alterations in tumor-bearing mice

The results of hematological parameters are presented in Table 3. Cis treatment had no significant alteration ($p > 0.05$) on red blood cell (RBCs) count, hemoglobin (Hb) level and percentage hematocrit (Hct), but increased total platelets count significantly in tumor-bearing group ($p < 0.05$) when compared to untreated group. However, in the untreated tumor-bearing group, only RBCs count, Hb level and Hct, but not total platelet count, were significantly reduced ($p < 0.05$) compared to negative control. LQV treated group showed a significant increase ($p < 0.05$) in RBC count, Hb and Hct, but platelets count was significantly decreased compared to untreated group. WBCs count was reduced post-Cis treatment, however, LQV restored the count of WBCs close to normal (Figure 2).

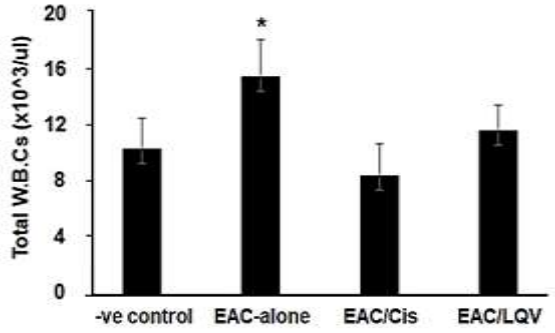

Figure 2: Total white blood cells count in groups of EAC-bearing mice. *$P < 0.05$ vs. control

Treatment with LQV ameliorated liver and kidney dysfunctions in tumor-bearing mice

There was a significant ($p < 0.05$) increase in liver transaminases (ALT and AST) in tumor-bearing mice compared to the control (Figure 3 A and B). Treatment with Cis or LQV decreased the tumor-induced increase in liver enzymes albeit, non-significantly ($p > 0.05$). A similar trend was observed in the kidney function parameters (urea, creatinine and blood urea nitrogen (BUN)) assayed (Figure 4).

LQV enhanced total protein and bilirubin levels in tumor-bearing mice

Total protein and bilirubin were decreased in EAC-bearing mice groups (Table 4). However, there was a significant increase ($p < 0.05$) of total protein and bilirubin in LQV treated group.

Treatment with LQV enhanced antioxidant/ oxidant homeostasis

The activities of SOD and CAT enzymes were significantly reduced in EAC-treated mice. Conversely, MDA level was significantly increased in tumor-bearing mice compared to the negative control. Cis treatment, however, increased activities of SOD, CAT and decreased levels of MDA. Furthermore, LQV treatment led to a substantial increase in SOD and CAT activities with a substantial decrease in MDA levels (Figure 5).

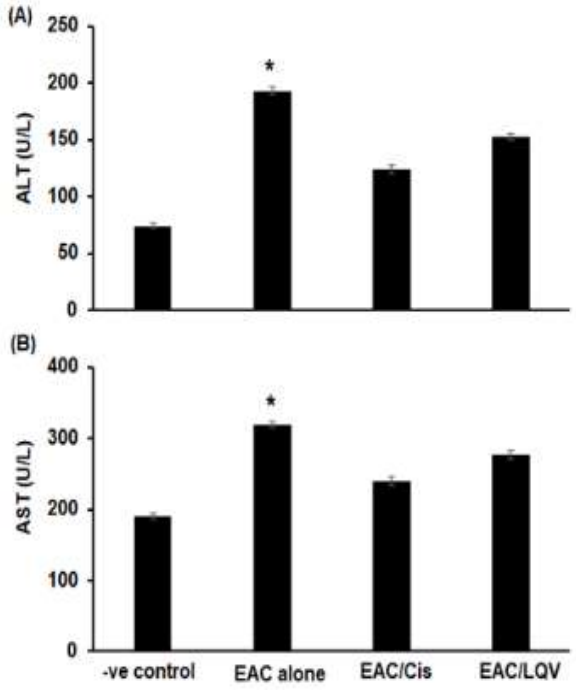

Figure 3: Treatment of EAC-bearing mice with LQV improves liver function enzymes. (A) Alanine transaminase (ALT) (B) Aspartate transaminase (AST) in different groups under study. *$P < 0.05$ vs. control.

Table 4: Total protein and bilirubin levels in different groups of EAC-bearing mice

Group	TP (g/dL)	Alb (mg/dL)	Glb (mg/dL)	TB (mg/dL)	DB (mg/dL)	IB (mg/dL)
Negative control	8.4±0.56[a]	3.9±0.15[a]	3.36±0.14[a]	0.9±0.13[c]	0.15±0.04[c]	0.3±0.06[c]
EAC-alone	4.5±0.35[c]	1.5±0.13[d]	3.06±0.19[a]	1.4±0.15[a,b]	0.38±0.03[a,b]	0.9±0.05[a]
EAC/Cis	5.4±0.45[b,c]	2.1±0.15[c]	3.11±0.4[a]	1.7±0.14[a]	0.42±0.05[a]	0.8±0.09[a,b]
EAC/LQV	6.2±0.69[b]	2.5±0.16[b]	2.9±0.17[a]	1.1±0.11[b,c]	0.29±0.06[b]	0.7±0.08[b]

EAC: Ehrlich ascites carcinoma, Cis: Cisplatin (2 mg/kg/6 days), LQV: Leiurus quinquestratus venom (0.025 mg/Kg/6 days), TP: Total protein, Alb: Albumin, Glb: Globulin, TB: Total bilirubin, DB: Direct bilirubin, IB: Indirect bilirubin. Groups that don't share the same letter are significantly different (p < 0.05)

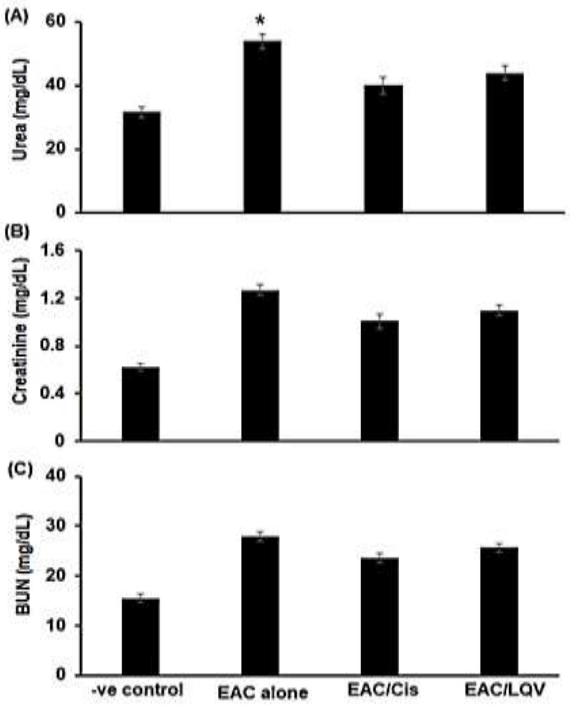

Figure 4: Treatment of EAC-bearing mice with LQV improves kidney function. (A) Urea, (B) Creatinine, and (C) Blood urea nitrogen (BUN) in different groups (*P < 0.05 vs. control)

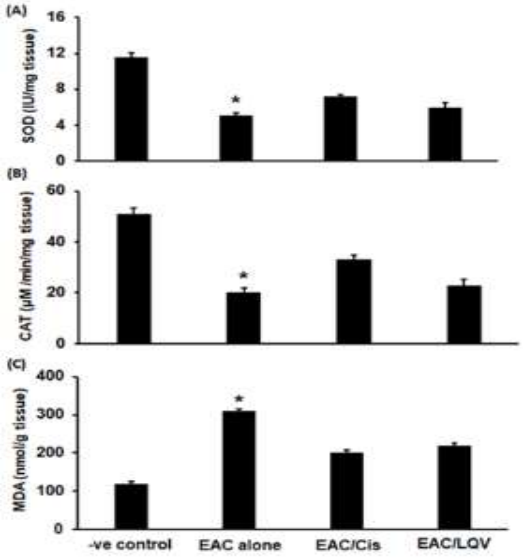

Figure 5: Treatment of EAC-bearing mice with LQV enhanced antioxidant/oxidant status. (A) Superoxide dismutase (SOD) (B) Catalase (CAT) (C) Malondialdehyde (MDA) in different experimental groups (*P < 0.05 vs. control)

LQV increased the percentage of necrotic, apoptotic, and arresting EAC-cells in G_0 and S phases

The percentage of necrosis was 0.42 and 2.07 % in tumor cells collected from untreated and LQV/EAC-bearing mice, respectively. The percentage of early and late apoptosis in tumor cells harvested in tumor-bearing mice was 3.4 and 2.7 %. However, the percentage of tumor cells harvested in LQV/EAC-bearing mice were 24.08 and 3.75 %, respectively (Figure 6 A). The % of cells in G_0, G_1, S, and G_2/M phases in tumor cells harvested from untreated tumor-bearing mice were 3.41, 61.29, 19.44, and 19.27 %, respectively. However, treatment with LQV led to an increase in the percentage of cells in G_0 phase from 3.41 to 29.7 % with a significant increase in S phase (Figure 6 B).

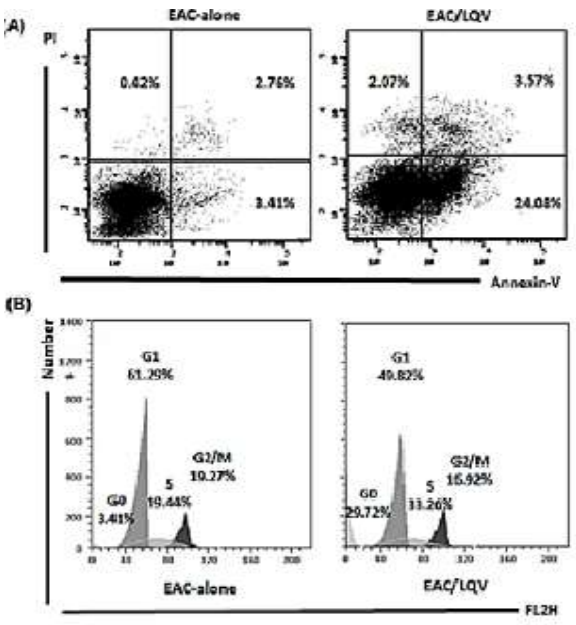

Figure 6: (A) The percentages of apoptotic and necrotic cells, (B) Cell cycle analysis in different experimental groups

LQV upregulated expressions of Bax, caspase-3, and down-regulated Bcl-2 gene

Expressions of Bax and caspase-3 were down-regulated, while, the expression of Bcl-2 was upregulated in EAC-cells harvested from untreated tumor-bearing mice (Figure 7). Conversely, treatment with LQV led to up-regulation in the expression of Bax and caspase-3 genes and a downregulation in Bcl-2 gene expression (Figure 7).

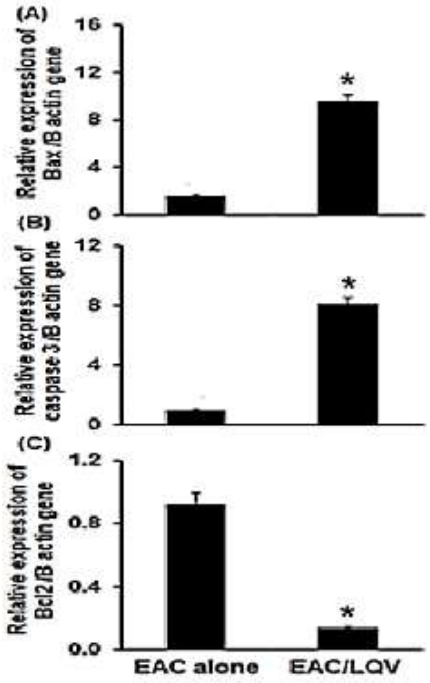

Figure 7: Real-time quantitative PCR analysis of the expression of Bax (A), caspase-3 (B), and Bcl-2 (C) in the untreated and treated EAC-bearing mice

LQV ameliorated liver and kidney histopathological changes

In the liver section of control mice groups, hepatic lobules comprise a central vein surrounded with hepatocytes cords and separated by narrow blood sinusoids (Figure 8 A). Tumor-bearing mice liver sections revealed hepatocytes degeneration with loss of boundaries and blood sinusoids were extended (Figure 8 B). Cis treatments increased hepatocytes degeneration by losing cell boundaries and inflammatory cells. On the other hand, LQV treatment ameliorated liver tissue changes in EAC-bearing mice (Figures 8 C and D). In control kidneys' sections, normal architecture of cortical tissue was observed (Figure 9 A). Tumor-bearing mice kidney sections showed inflammatory cells in interstitial spaces (Figure 9 B). Cis-treated EAC-bearing mice exhibited toxicity evidenced by tube degeneration and congestion of corpuscles. The kidney structure of LQV in tumor-bearing mice appears like normal structure (Figures 9 C and D).

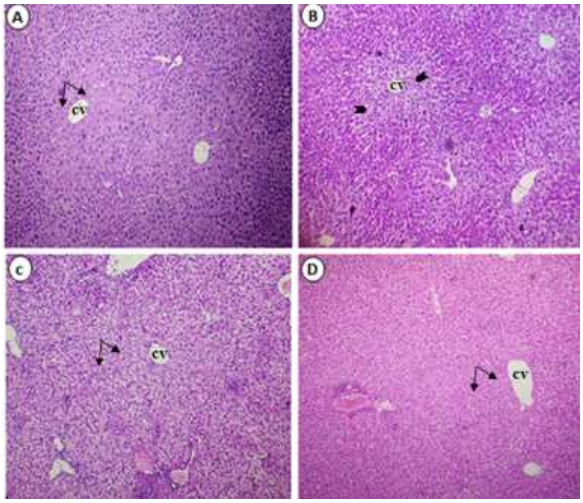

Figure 8: Light micrograph of liver sections in (A) control untreated mice show normal architecture of hepatic lobules (black arrows) surrounding central vein (CV) (B) EAC-bearing mice show cytoplasmic degeneration of hepatocytes (arrows head). (C) EAC-bearing mice treated with Cis shows degeneration of hepatic lobules (black arrows) (D) Liver section of EAC-bearing mice treated with LQV shows improvement in the hepatic lobules (black arrows) (H&E, scale bar 100 µm, magnification ×200)

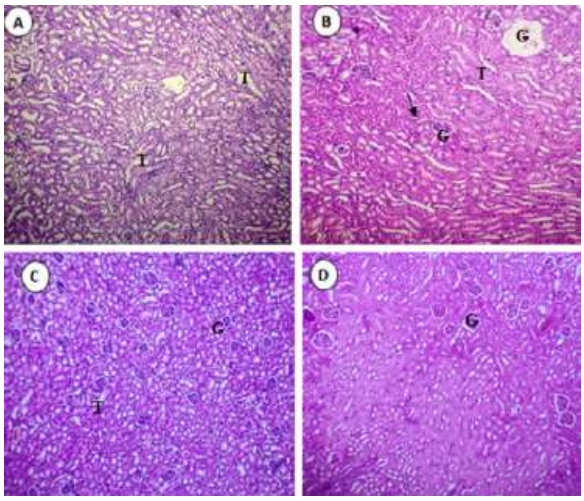

Figure 9: Light micrograph of kidney sections in (A) normal control mice shows renal corpuscle, renal tubules (T), and glomerulus (G). (B) EAC-bearing mice show degeneration, shrinkage in the glomerulus (G), and inflammatory infiltration (arrow) in interstitial spaces. (C) EAC-bearing mice treated with Cis shows shrinkage of the glomerulus (G) size and increase in its number as well as degeneration of renal tubules (T) (D) EAC-bearing mice treated with LQV showing near normal structure. (H&E, scale bar 100 µm, magnification ×200).

DISCUSSION

Scorpion venoms (SV) consist of proteins, neurotoxins and lipids, with pharmaceutical and biomedical applications [3]. SV have potential cytotoxic effect against various cancer cell lines [2]. *In vivo,* the anti-cancer effect of LQV against tumor-bearing mice was evaluated in this study. Cis treatment resulted in a significant decrease in percentage body weight changes. This finding is consistent with the report of El-Naggar *et al* [11]. A significant decrease in the percentage of body weight changes was also noticed post-LQV treatment. This finding suggests that LQV could possess an anti-tumor effect. In addition, treatment with Cis decreased tumor volume and increased death of tumor cells. This finding is consistent with a study that reported that Cis treatment directly affected tumor cells [11]. Also, LQV treatment decreased total tumor volume and viable cells. These findings are consistent with the work of Nafie *et al* [12] who reported that *A. australis* venom had a potent cytotoxic effect against tumor cells. In this study, the elevated total WBCs count was observed in EAC-bearing mice but Cis treatment decreased WBCs count. However, the WBCs count in LQV-treated mice was comparable to normal control. Amelioration of hematological alterations by LQV treatment could be due to protecting the hematopoietic system similar to the effect of *A. australis* venom [12]. Treatment with LQV reduced the liver and kidney histological alterations and restored their architecture close to normal. These findings are consistent with those of Nafie *et al* [12], who found that SV ameliorated the toxic effect of tumor on hepatic tissue. According to Bekheet *et al* [18], bradykinin potentiating factor (BPF) isolated from *Buthus occiantus* venom was able to protect liver tissues from gentamicin toxicity. Liver transaminase enzymes (ALT and AST) are commonly used as markers of hepatocellular damage. The proliferation of EAC-cells in experimental mice significantly increased liver dysfunctions represented by an increase in these enzymes [11]. There was no liver toxicity reported upon LQV treatment. This could be due to the fact that this venom has a protective property against liver injury (supported by liver histopathology). Interestingly, Nadjia and Fatima [13] previously reported that *A. australis* venom treatment has a beneficial effect on hepatic toxicity induced by fumonisin. Furthermore, SOD and CAT activities were decreased in EAC-bearing mice as a result of tumor propagation. These results agree with the work of Marklund and Marklund [14]. Treatment with LQV, however, led to the elevation of CAT and SOD activities in tumor-bearing mice. This increase may be due to the antioxidant properties of LQV. Nafie *et al* [12] also reported an increase in SOD and CAT activities in liver homogenate of tumor-bearing mice treated with *A. australis* venom. Furthermore, urea and creatinine levels in tumor-bearing mice were found to increase due to the effect of tumor progression on the kidney. However, treatment of tumor-bearing mice with LQV gradually returned these kidney biomarkers levels close to normal levels.

LQV induced cell apoptosis and this finding is in agreement with the report of Salama and El-Naggar [2]. Furthermore, it has been shown that *A. crassicauda* venom induced apoptosis in human neuroblastoma cell lines and arrested cell cycle in S-phase [4,15]. Treatment of EAC-bearing mice with LQV arrested the EAC-cells in G_0 and S-phases. In the G_2/M transition, cyclin-dependent kinase 1 (CDK1)/ Cyclin B complex is the main regulator responsible for progression from G_2 to the M phase. In addition, p21, is a key regulator that modulates CDK1 activities and arrests tumor cells at G_2/M phase [16].

Rhopalurus junceus venom has been shown to regulate Bcl-2, induce caspase 3, 9 and up-regulate BAX expression [17]. Therefore, this study postulated that treatment with LQV could protect both liver and kidney tissues against EAC-induced toxicity. Histopathological changes in liver and kidney tissues of tumor-bearing mice could be due to elevated levels of free radicals induced by EAC cells or from chemotherapy. The ameliorative effect of LQV treatment could be due to its potent antioxidant activity which led to eradication or decreasing levels of free radicals, which in turn resulted in a decrease in oxidative stress on both liver and kidney tissues.

CONCLUSION

Leiurus quinquestriatus venom is a potential anti-tumor agent that has been shown in this study to decrease tumor volume. Its anti-tumor effect is achieved by inducing apoptosis, blocking tumor cell cycle progression, upregulating apoptotic genes and downregulating anti-apoptotic genes in EAC-cells. Further studies are required to address the functionality and characterization of the active molecules of LQV responsible for its anti-cancer properties.

DECLARATIONS

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None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims related to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Wesam M. Salama and Sabry A. El-Naggar conceived, designed the study, and wrote the manuscript, whereas Barakat El Rashdi collected and analyzed the data.

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