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The Effects of *Citrobacter freundii* Isolated from Cattle on the Immunological and Histopathological Changes in Mice

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Abstract:

Citrobacter species are opportunistic invasive bacterium and possible zoonotic pathogens that can cause infections in both humans and animals. A study was conducted in Baghdad City to isolate and identify *Citrobacter freundii* bacteria from cattle feces and to identify them by a different method, including biochemical testing, PCR analysis, VITEK 2, and sequencing of 16S rRNA, as well as assessing the immune response to the injection of the mice with *C. freundii* antigens. Thirty Swiss mice were divided into three general groups, injected with KWCA and SWCA immunized killed and sonicated with whole cell antigen. Immune response evaluation was done through the DTH-skin test and TAT. The results showed that the biochemical test revealed 20 isolates, comprising 20% of *C.freundii*. Vitek 2 results confirmed the positive selection of *C.freundii* isolates, equivalent to 97%, while 16S rRNA sequencing subjected the isolated bacteria to 99.74% similarity with GenBank references. This result registered one isolate of *C. freundii* in GenBank by accession number (OR766039). TAT results: The positive control and the second group (SWCA) have indicated an antibody titer of 1280. The first group KWCA has indicated 320. DTH results: The diameter of induration increases from 24 h to 48 h followed by a decrease at 72 h. KWCA results for 24 h, 48 h, and 72 h are 2.46 ± 0.001mm, 3.78± 0.008 mm, and 3.1± 0.0008 mm, respectively. The SWCA has an induration of 3.2± 0.576 mm, 4.08± 0.07 mm, and 3.35± 0.06 mm for 24 h, 48 h, and 72 h, respectively. The statistical difference is $P<0.05$. The Histopathological alterations indicated changes in both groups. The impact level is similar in all groups, from mild to moderate. In conclusion, the study observed that SWCA and KWCA promoted humoral and cellular immune responses.

Keyword: ***Citrobacter freundii*,PCR,DTH,TAT**

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Introduction:

Citrobacter is a genus of facultative anaerobic, Gram-negative bacteria belonging to the *Enterobacteriaceae* family (1). *Citrobacter* species are potential zoonotic pathogens and opportunistic nosocomial bacteria, in humans are associated with urinary tract infections, wound infections, pneumonia abscesses, septicemia, meningitis, and endocarditis in adults, as well as septicemia, meningitis, and brain abscesses in neonates (2,3). In animals, can cause respiratory tract infections, septicemia, and encephalitis in sheep (4,5). The correct identification of bacteria plays an important role in the diagnosis of infectious diseases, conventional identification is not always thought to be a clear-cut, accurate marker for Citrobacter identification (6). Microbiological diagnostic labs have demonstrated the remarkable variety of *C. freundii* in terms of colony form, biochemistry, antigenicity, and pathogenicity. Due to its phenotypic flexibility, *C. freundii* becomes difficult to distinguish using traditional techniques and is frequently confused with both *S. enterica* and *E. coli* (7). The molecular tools are more reliable for the identification *C. freundii* than cultural and biochemical tests (7), 16S rRNA is a powerful tool for identifying and conducting phylogenetic analyses of many bacteria, including *C. freundii* (8,9).

Three antigenic structures are present in *Citrobacter* species: the capsular antigen K, the somatic O, and the flagellum H (10). They also produce lipopolysaccharide (LPS), the outer membrane, and strong toxins (Shiga-like toxins). When this bacterium infects a host, it stimulates the production of CD4+ T cells, which in turn stimulates the humoral immune response and the production of both IgM and IgG from B cells, thereby inducing both a systemic and local immune response. Through the process of opsonization, these immunoglobulins help the bacterium become more susceptible to being eliminated by phagocytosis. In addition, T cells help produce cytokines such as IL-12, IL-17, IL-6, IFNγ, and άTNF-. The importance of T cells in the response of innate and systemic immunity to eliminate infection with this bacterium has been the subject of numerous investigations (11).

This study aims to identify *Citrobacter freundii* by bacteriological and molecular characterization, prepare and evaluate the immune response of mice immunized by whole cell sonicated antigen and killed whole cell antigen by hemagglutination tube and skin test and study the histopathological changes in the vital organs (liver, kidney, spleen, and intestine) in mice.

Material and method:

Ethical approval:

Ethical approval was granted through the local committee of animal care and use at the College of Veterinary Medicine within the University of Baghdad (Number P-G\652,24\3\2024) during this study.

Isolation and identification:

Collect from different regions in Baghdad city 100 samples from sheep (feces). Details such as gender, and age, and then transport

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in a cold container and brought to a laboratory in under two hours (12). A sterile test tube was filled with one gram of each fecal sample, ten milliliters of normal saline, and 0.1 milliliters of each sample suspension inoculated on either MacConkey agar (HiMedia\India) , Salmonella shigella agar (HiMedia\India) and Xylose Lysine Deoxycholate (XLD) (HiMedia\India), at 37°C in incubator for 24 - 48 hours (12). Using conventional morphological, biochemical (Oxidase and Catalase), vitek2 compact system, antibiotic susceptibility, and pathogenicity tests, such as PCR assay, the Citrobacter isolates were identified at the species level. Following the manufacturer's instructions, the Vitek 2 system (bioMérieux, Lyon, France) was used to identify the bacterial isolates and perform an antimicrobial susceptibility test. DNA was extracted using a bacterial DNA Kit by the manufacturer's instructions (Geneaid, KOBA) to identify *Citrobacter freundii* by PCR. Thermo Scientific Inc., USA's NANODROP-2000 spectrophotometer was used to determine the concentration of DNA. The amplification size of the 16S rRNA primer is 1500 bp. F5'-AGAGTTTGATCCTGGCTCAG-3'R5'-TACGGTTACCTT GTTACGACTT-3' (13)

A total volume of 25µl was used for the PCR amplification, which included 12.5µl of the Promega Master Mix, 1µl each of the Forward and Reverse Primers, 8.5µl of nuclease-free water, and 2µl of DNA template. The PCR condition protocol involved five minutes of initial denaturation at 95°C, thirty seconds of denaturation at 95°C, thirty seconds of annealing at 60°C, one minute of 72°C extension, and seven minutes of final extension at 72°C. DNA sequencing was done on the PCR product tubes containing the sample and the forward and reverse primers of 16S rRNA.

Antigens preparations:

Both the Sonicated Whole Cell Antigen (SWCA) and the *C. freundii* Killed Whole Cell Antigen (KWCA) were produced (14). Following a 48h incubation at 37°C on nutrient agar to harvest bacteria, the bacteria were stained with Gram's stain and scrutinized under a microscope to verify complete morphology. After centrifuging for 20 minutes at 3000 rpm, they were rinsed three times with PBS (pH 7.2), then added formalin (0.3%) with PBS (pH 7.2), incubated for one to two hours at 37 °C, and then spent the night at 4 °C. After three PBS (pH 7.2) cleanings, the bacterial suspension was centrifuged for 20 minutes at 3000 rpm for KWCA. The suspension was then maintained in its frozen state until further notice. Using an ultrasonicator set to operate at a rate of 15 KHZ/sec on ice, the suspension was treated to intermittent sonication intervals during the SWCA, consisting of one minute of sonication followed by one minute of rest. After centrifuging the sonicated bacterial solution for 20 minutes at 3000 rpm, the product was filtered through a (Millipore filter 0.45 µ). After that, the suspension was placed in a refrigerator at -20 °C until the protein content was determined.

While the McFarland tube method was used to assess the colony-forming unite for the KWCA, (15) was utilized to measure the protein concentration for the SWCA

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Experimental design:

Thirty Swiss mice, weighing between 17 and 18 grams, were composed of three groups at random (10 mice each) as follows: The first group received 1.5 $\times 10^9$CFU\ml, S/C, of the KWCA vaccine. The second group received a 1000 µg/mL S/C immunization with SWCA. One milliliter of PBS (pH 7.2, S/C) was put into the third group (negative control). On day 14 after immunization, the first and second doses of the antigen booster were given. Day 21 after immunization saw the collection of blood samples, and on Day 18 after immunization, all inoculated mice underwent a delayed-type hypersensitivity test (TAT) to estimate their antibody titer. The Tube Agglutination Test was made according to (16). The stock tube was made by tenfold serial dilution adding 0.9 ml of PBS and 0.1 ml of serum then by twofold serial dilution, ten sterilized test tubes were used each containing 0.5 ml of PBS. and 0.5 ml of diluted serum and 0.5 ml of particulate antigen of killed whole cell *C.freundii*. The tubes were then incubated for two hours at 37°C, and then overnight at 4°C. The negative control was made by adding 0.5 ml of antigen suspension to 0.5 ml of PBS (pH of 7.2), and the positive control was made by adding 0.5 ml of serum dilution to 0.5 ml of PBS and 0.5 ml of antigen suspension in a tube. The clump formation in the tube bottom was observed as a network to assess the positive results.

Following (17), the DTH test involved taking approximately 0.1 ml of the (SWCA) that had been previously prepared and injecting it intradermally (I/d) into the right hind footpad of mice, while the left hind footpad of all immunized groups received 0.1 ml of sterilized PBS (pH 7.2) by injection. measured the induration of skin during (24, 48, and 72) hours post-injection by using a caliper.

After 21 days of post-immunization of mice. All of the animals were infected with 1×10^6 CFU⁄ml of *C. freundii* (18). They were monitored every 6 to 8 hrs. for 7 days for signs of clinical illness. The gross pathological changes for the infected and control mice were recorded for 7 days, these included location, color, size, shape, consistency, and appearance of the cut surface, Specimens were taken from the liver, kidney, spleen, and intestine. The tissues were fixed in a 10% formalin solution immediately after removal (19).

Statistical analysis:

The SAS (20) application, which stands for Statistical Analysis System, was utilized to determine how various factors affected the study parameters. To compare between means, the analysis of variance-ANOVA test with the least significant difference (LSD) was employed (20).

Results:

The results of one hundred fecal samples 20(20%) as shown in table (1).

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Table (1): Source and isolate rates of *C.freundii* isolates

Studies \months	No. of samples	No. of isolates	Positive percentage %
October-December 2023	100	20	20%

All isolates appeared similarly on SS agar as black colonies because they could produce H2S on S.S agar after 24 hours. Because of the lactose fermenter, Citrobacter isolates displayed as pink colonies on MacConkey agar; however, on XLD Citrobacter changed the media's color to yellow fig1. All isolates are oxidase-negative and catalase-positive.

Vitek 2 compact system and antimicrobial susceptibility were used to confirm the identification of *Citrobacter* spp. The results indicated that the bacterium isolated in this investigation was *Citrobacter*, specifically the species *freundii*, as indicated in fig1.

Identification Information																
Analysis Time: 3.85 hours													**Status:** Final			
Selected Organism							97% Probability **Bionumber:**						**Citrobacter freundii**			
ID Analysis Messages																
Biochemical Details																
2	APPA	-	3	ADO	-	4	PyrA	+	5	lARL	-	7	dCEL	-	9	BGAL	+
10	H2S	+	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	+	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	+
40	ILATk	-	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	-
46	GlyA	+	47	ODC	-	48	LDC	-	53	lHISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Ticarcillin	<= 8	S	Amikacin	<= 4	S
Ticarcillin/Clavulanic Acid	<= 8	S	Gentamicin	<= 3	S
Piperacillin	<= 4	S	Tobramycin	<= 4	S
Piperacillin/Tazobactam	<= 4	S	Ciprofloxacin	<= 0.25	S
Ceftazidime	<= 2	S	Pefloxacin		
Cefepime	<= 3	S	Minocycline	4	S
Aztreonam	<= 1	S	Colistin		
Imipenem	<= 1	S	Rifampicin		
Meropenem	<= 0.25	S	Trimethoprim/Sulfamethoxazole	<= 20	S

+= Deduced drug *= AES modified **= User modified

Fig (1): Vitek 2 compact system and antimicrobial susceptibility for *Citrobacter* spp.

16S rRNA was found to be present in the isolate. Therefore, the isolate tested positive for the 16S rRNA gene by 1.5% agarose gel electrophoresis and monoplex PCR amplification. Figure (2)

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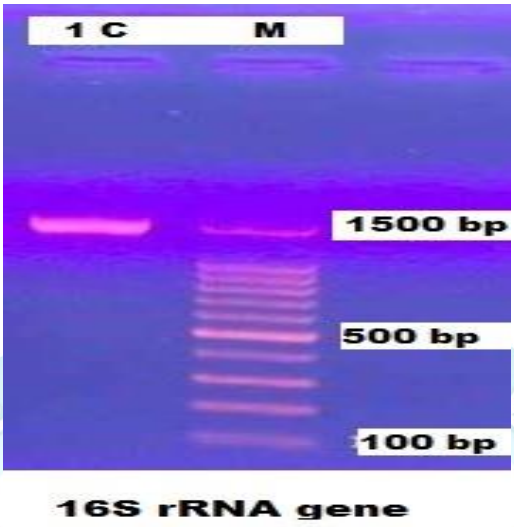
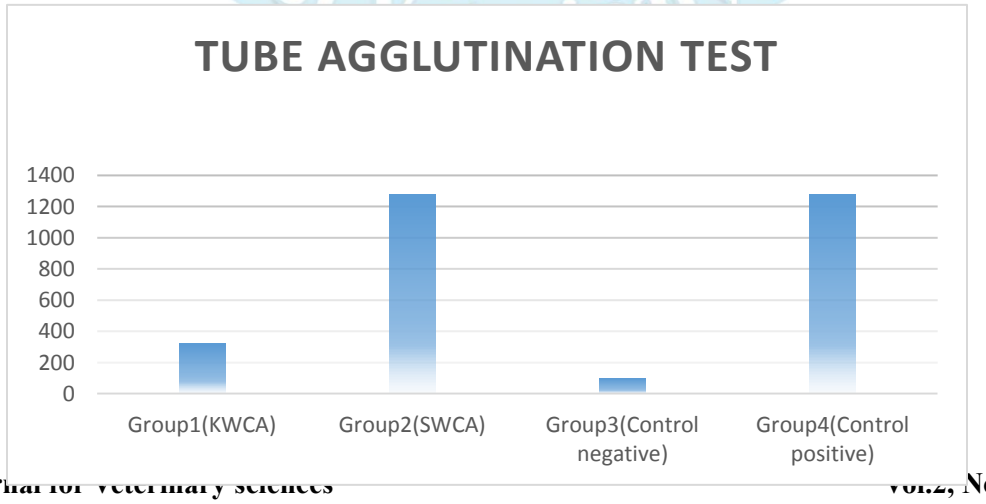


Fig (2): The 16S rRNA gene's amplified PCR products (1500 bp): M: The DNA molecular weight marker (100 bp ladder), and 1C: The *Citrobacter freundii* 16SrRNA amplified PCR product

NCBI website (http://www.ncbi.nlm.nih.gov) was used to analyze the sequences. There was 99.74% homology between the *Citrobacter freundii* reference strains and the isolated strains in GenBank (Accession No. MT421943.1). The isolate of *Citrobacter freundii* was registered by GenBank under Accession No. OR766039.

After 21 days following immunization, the Tube Agglutination Test findings revealed higher antibody titers in the immunized groups when compared to the negative-positive control groups. The first group (KWCA) showed a titer of 320 followed by the second group (SWCA) exhibited a higher titer (1280). In contrast, a control positive showed the same titer as the second group, and the negative control showed a titer (100) as shown in Fig (3).

Fig (3): Tube agglutination test for Citrobacter antigens



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demonstrated that all immunized groups' right hind footpads had more induration than the mice's control left hind footpads. The KWCA group showed an increase of induration in the diameter at (24 and 48) h, followed by a decrease at 72 h (2.46 ± 0.001mm, 3.78± 0.008mm, and 3.1± 0.0008mm), respectively. In contrast, the results of SWCA at 24 h were (3.2± 0.576 mm, 48 h were 4.08± 0.07 mm), and at 72 h were 3.35± 0.06 mm with a significant difference (P<0.05). and Table (2)

Table (2): induration diameter (mm) of mice immunized b *Citrobacter freundii* antigens in DTH-Skin test

Time Hours / Groups	Diameter skin test (mm)		
	24	48	72
G1(KWCA)	2.46 ± 0.001 **a **A	3.78± 0.008 **a **B	3.1± 0.0008 **a **C
G2(SWCA)	3.2± 0.576 *a A	4.08± 0.07 **b **AC	3.35± 0.06 **b **AB
G3 (Control negative PBS)	1.33± 0.150 b A	1.35± 0.081 c A	1.21± 0.069 c A
** P<0.01 Capital letters horizontal comparison between groups * P<0.05 Lowercase letters vertical comparison between groups			

Following a 7-day challenge, a histological examination revealed that all groups under investigation had varying histopathological alterations. Group1: The liver showed a normal central vein, mild intravascular hemolysis, normally distributed hepatic cords and sinusoids, and minimal localized necrosis with inflammatory aggregation, all seen in the liver's histological (fig. 4). The thoracic portal sinusoid showed the infiltration of polymorphic nuclear leukocytes, whereas the hepatic portal triad displayed the proliferation of bile ductulus together with portal aggregation of mononuclear leukocytes (fig. 5). Kidney showed a renal cortex's histological (figs. 6 and 7) revealed focal tubular necrosis with MNC aggregation, vacuolar degeneration of the renal tubules' lining cells, and a normal glomerulus. The spleen showed severe congestion with distension of the red pulp's sinusoid and reticular tissue, a substantial

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increase in hemosiderin-laden macrophages, and marked hypoplasia with atrophy of lymphoid follicles (figs. 8 and 9).

Group2: the liver revealed normal hepatic cords and significant perivascular cuffing together with significant congestion and dilatation of the portal vein (fig.10). The hepatic portal triad revealed modest portal vein dilatation along with normal bile ductulus (fig.11). Figures 12 when magnified revealed modest hypercellularity in Kupffer cells and normal hepatocytes. The kidney showed focal tubular necrosis with MNC aggregation, vacuolar degeneration with renal tubule lining cell necrosis, and glomerular congestion (figs. 13& 14). the spleen histological findings (figs. 15&16) revealed normal reticuloendothelial tissue and red and white pulp appearance. Group 3: The liver revealed severe congestion, portal vein dilating, widespread hepatocyte production, and necrosis (fig. 17 & 18). Kidney, the renal cortex's histological findings (figs. 19 and 20) revealed focal tubular necrosis with MNC aggregation and tubular cast formation, severe tubular dilatation with flattening of the renal tubule lining cells, and congestion of the glomerulus. while the spleen within the splenic portion, normal white pulp lymphoid follicles were observed, along with severe sinusoidal congestion with distension and a notable increase in megakaryocytes and hemosiderin-laden macrophages (fig. 21 and 22).

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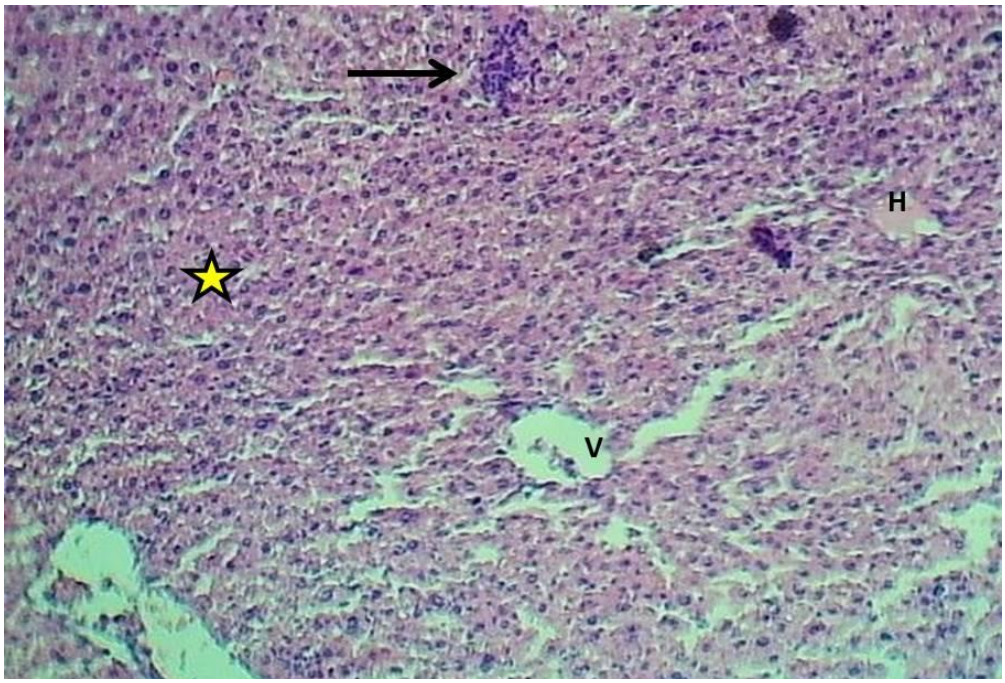

Figure4: section of liver (G1) shows: normal central vein (V) & mild intravascular hemolysis (H) normal arranged hepatic cords and sinusoid (Asterisk) & little focal necrosis with inflammatory aggregates (Arrow). H&E stain.100x.

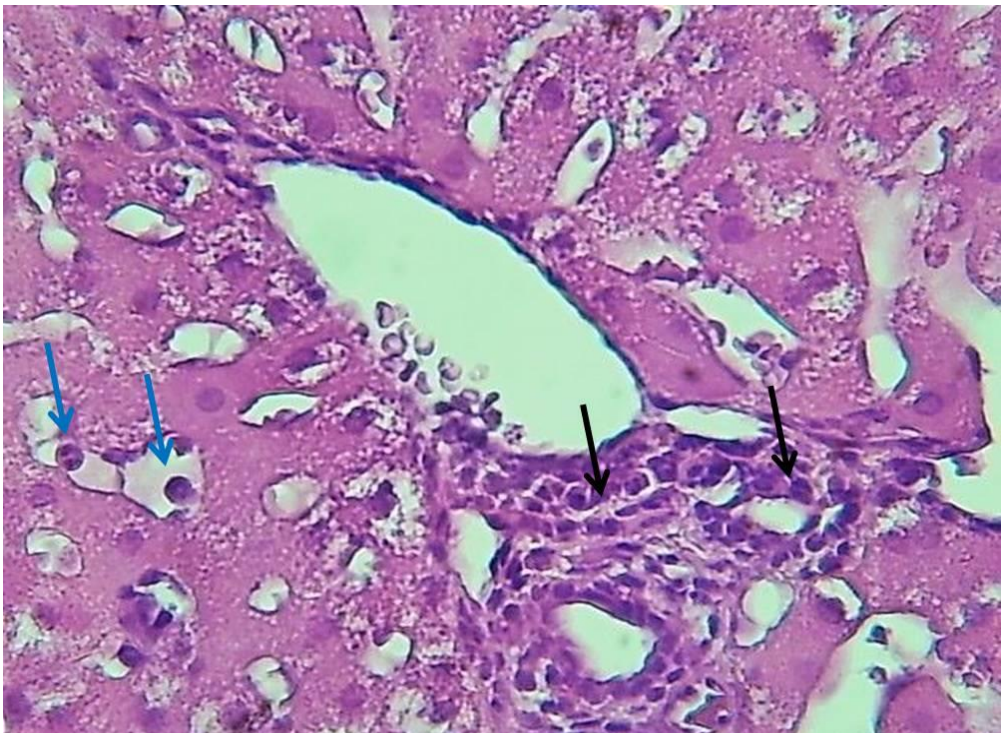

Figure 5: Section of hepatic portal trid (G1) shows: a proliferation of bile ductulus with portal aggregation of mononuclear leukocytes (Black arrows) & sinusoid with infiltration of polymorphic nuclear leukocytes (Blue arrows). H&E stain.400x.

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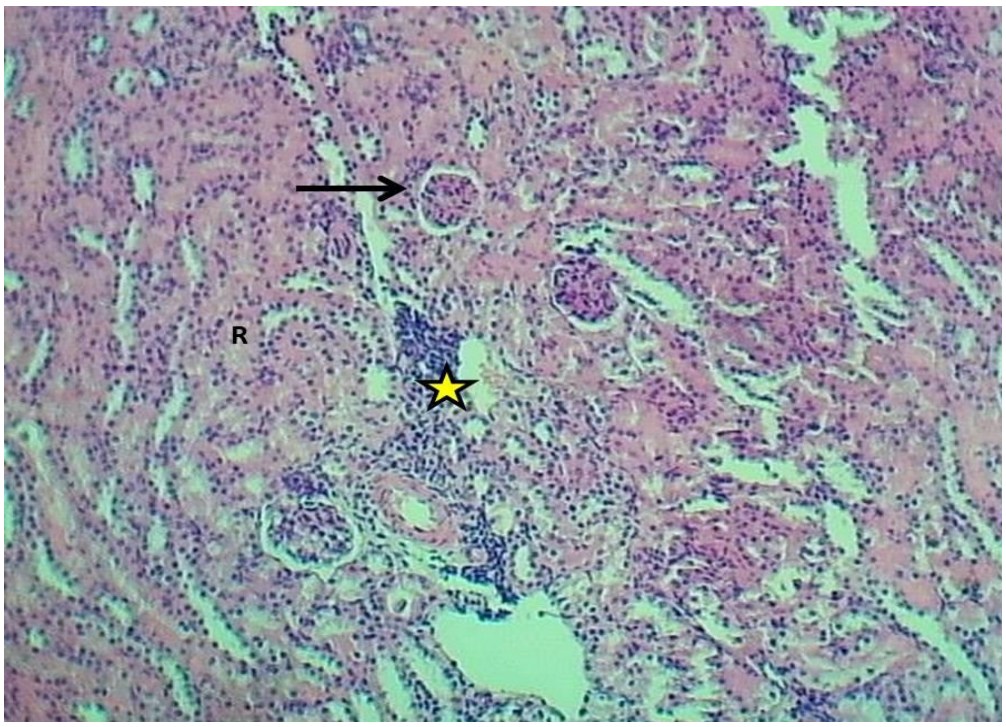

Figure 6: Section of renal cortex (G1) shows: normal glomerulus (Arrow), vacuolar degeneration of the lining cells of the renal tubules (R) & focal tubular necrosis with aggregation of MNCs (Asterisk). H&E stain.40x

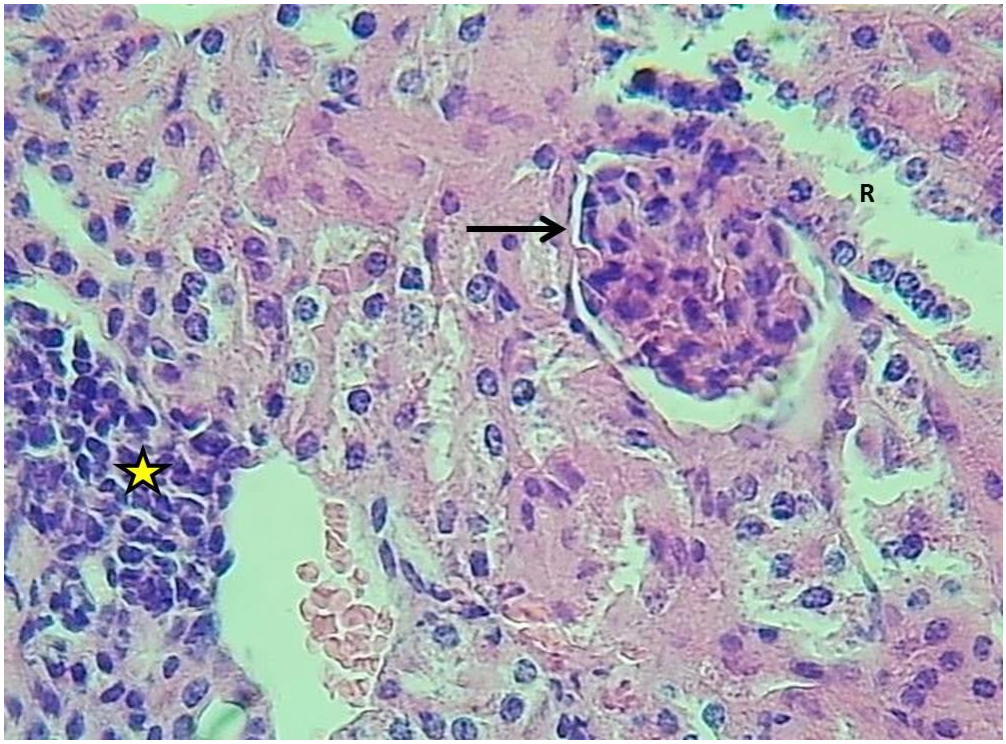

Figure 7: Section of renal cortex (G1) shows: normal glomerulus (Arrow), vacuolar degeneration of the lining cells of the renal tubules& focal tubular necrosis with aggregation of MNCs (Asterisk). H&E stain.400x

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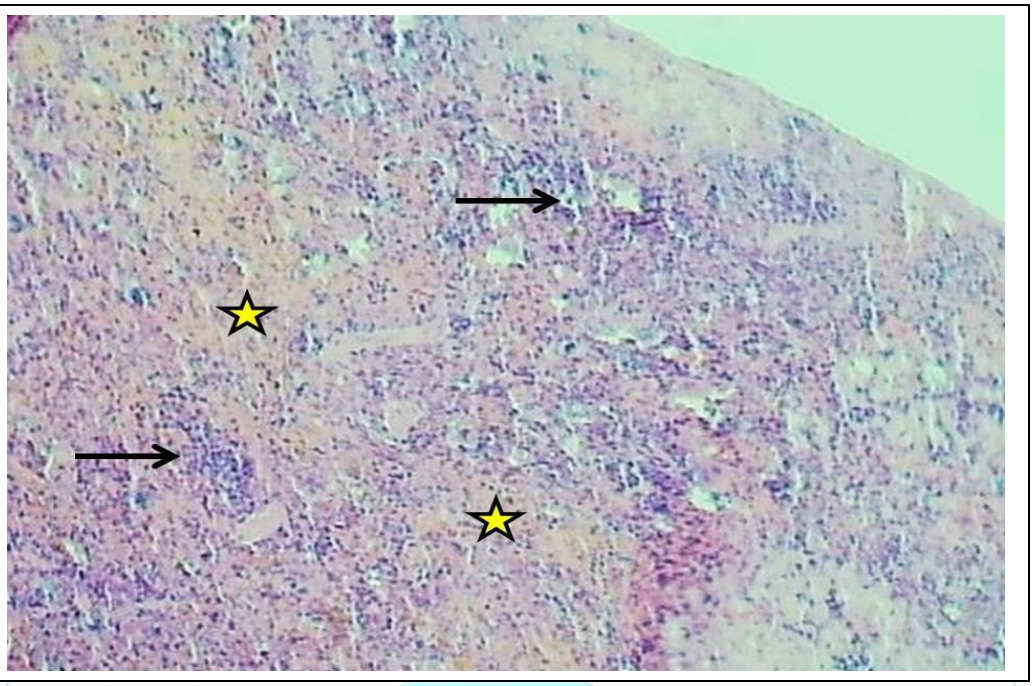

Figure 8: section of spleen (G1) shows: marked hypoplasia with atrophy of lymphoid follicles (Arrows) and sever congestion with distension of sinusoid and reticular tissue of red pulp (asterisks). H&E stain.100x.

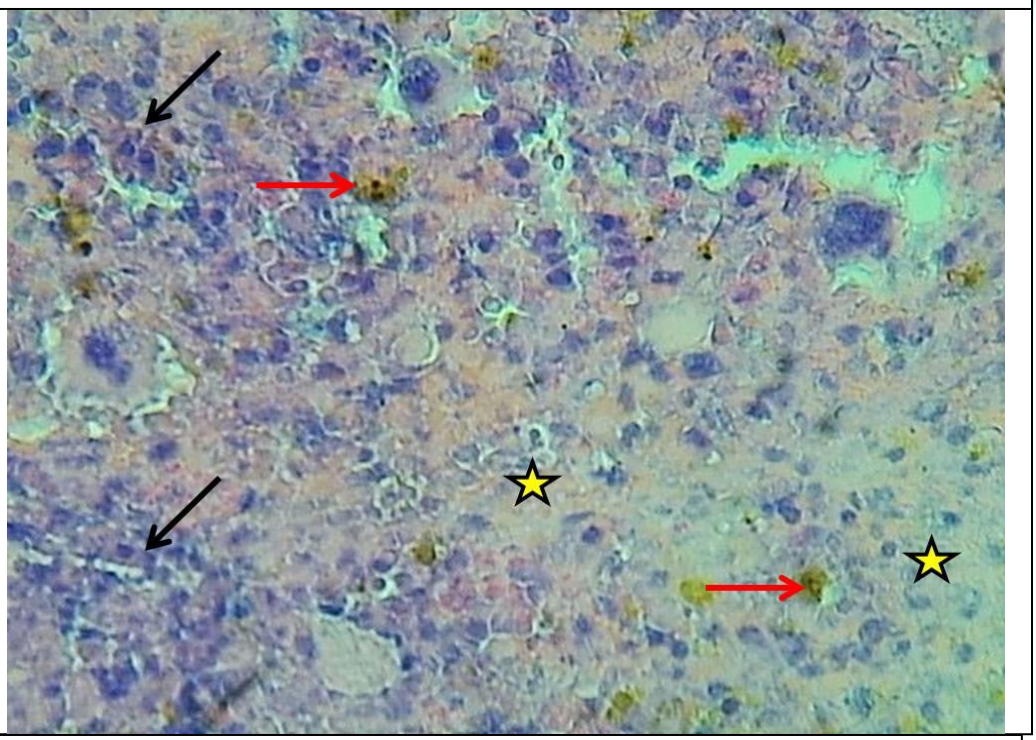

Figure 9: section of spleen (G1) shows: marked hypoplasia with atrophy of lymphoid follicles (Black arrows) and sever congestion with distension of sinusoid and reticular tissue of red pulp (asterisks) & marked increase of hemosiderin laden macrophages (red arrows). H&E stain.400x.

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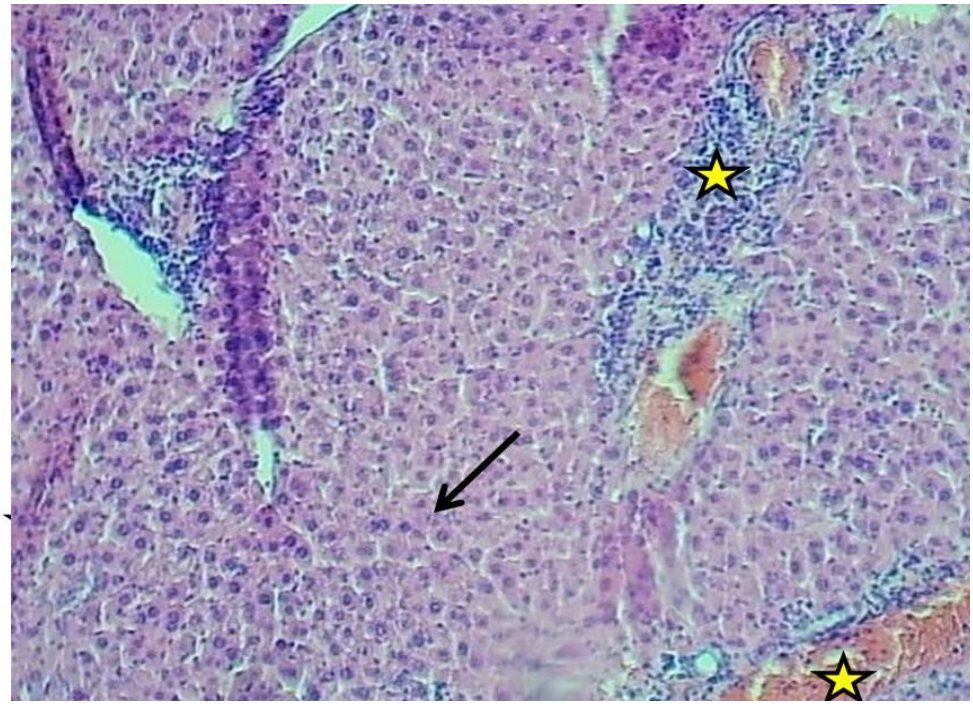

Figure 10: section of liver (G2) shows: marked congestion with dilation of portal vein with marked peri vascular cuffing (Asterisks) & normal hepatic cords (Arrow). H&E stain.100x.

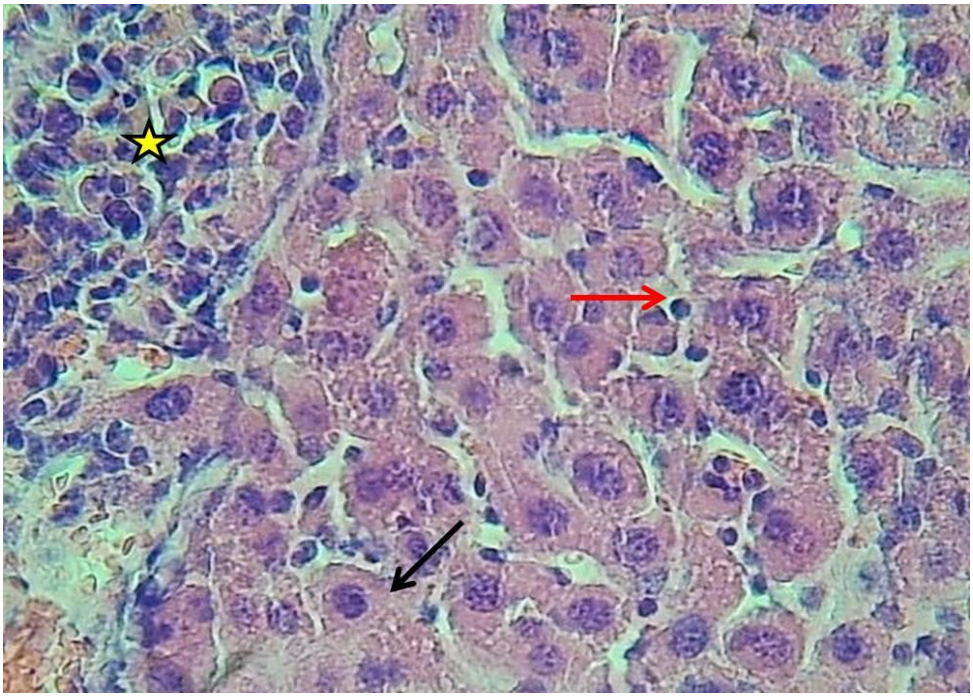

Figure 11: section of liver (G2) shows: marked congestion with dilation of portal vein with marked peri vascular cuffing (Asterisks) & normal hepatocytes (Black arrow) mild hyper cellularity of Kupffer cells (Red arrow) .H&E stain.400x.

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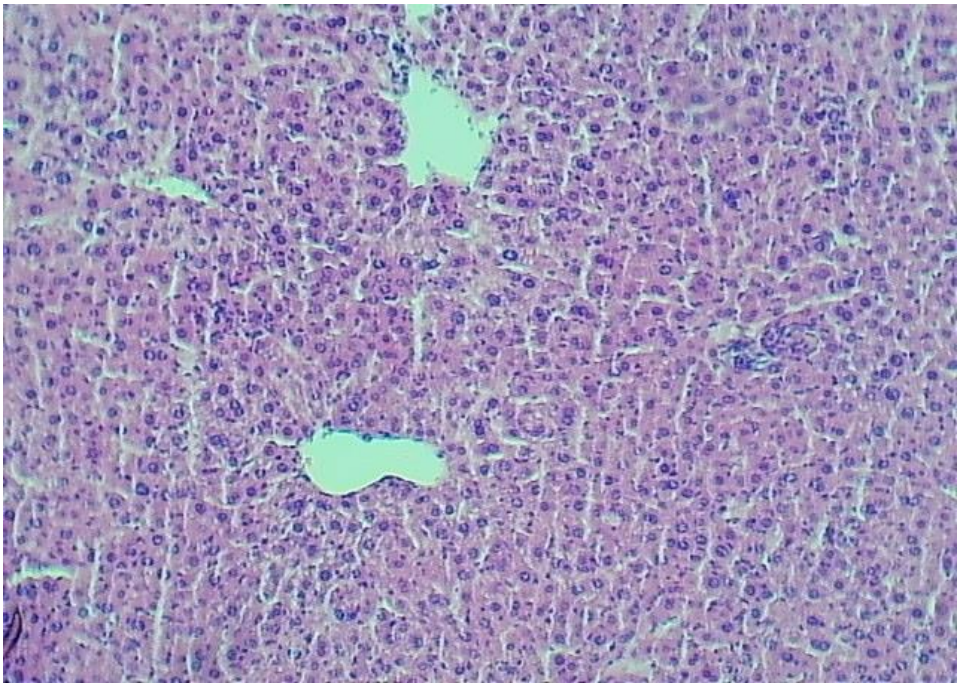

Figure 12: section of liver (G2) shows normal central vein. H&E stain.100x.

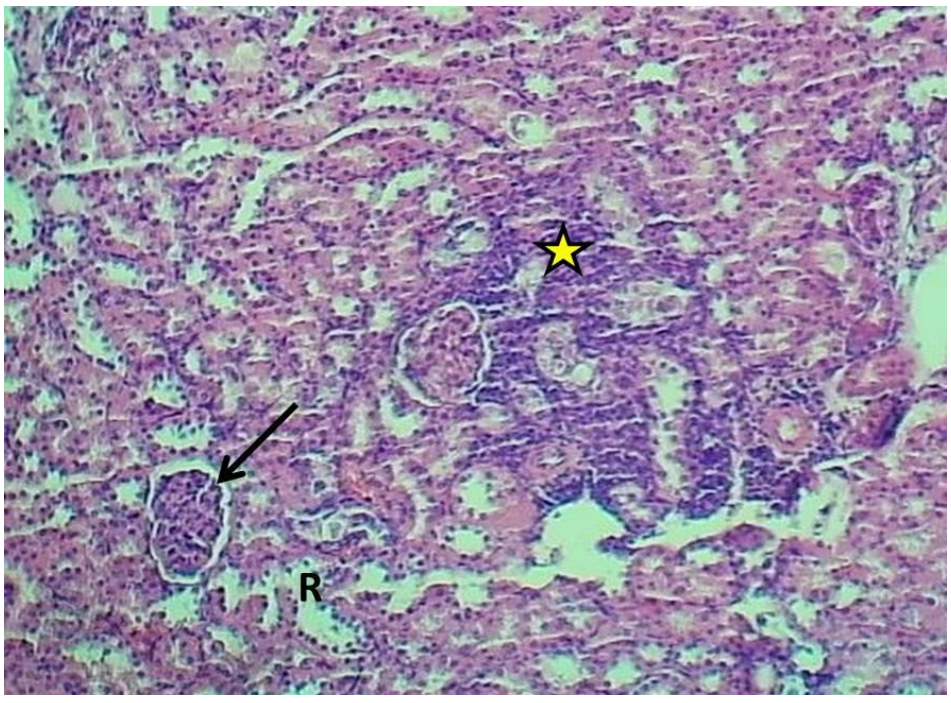

Figure 13: Section of renal cortex (G2) shows: normal glomerulus (Arrow), vascular degeneration of the lining cells of the renal tubules (R) & focal tubular necrosis with aggregation of MNCs (Asterisk). H&E stain.40x

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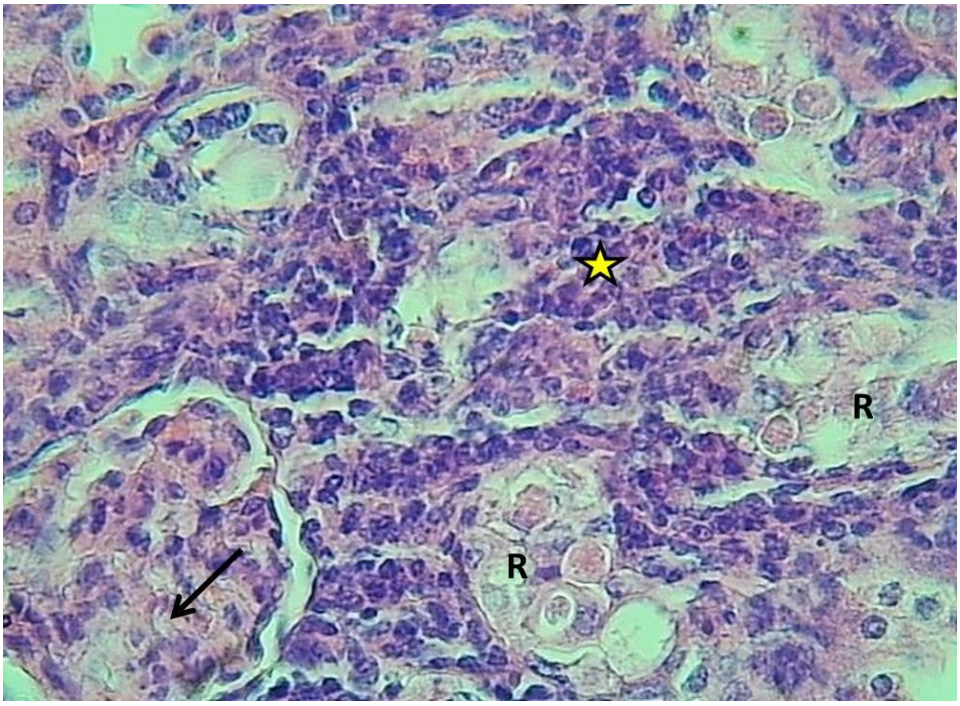

Figure 14: Section of renal cortex (G2) shows glomerular congestion (Arrow), vascular degeneration with necrosis of the lining cells of the renal tubules (R) & focal tubular necrosis with aggregation of MNCs (Asterisk). H&E stain.400x

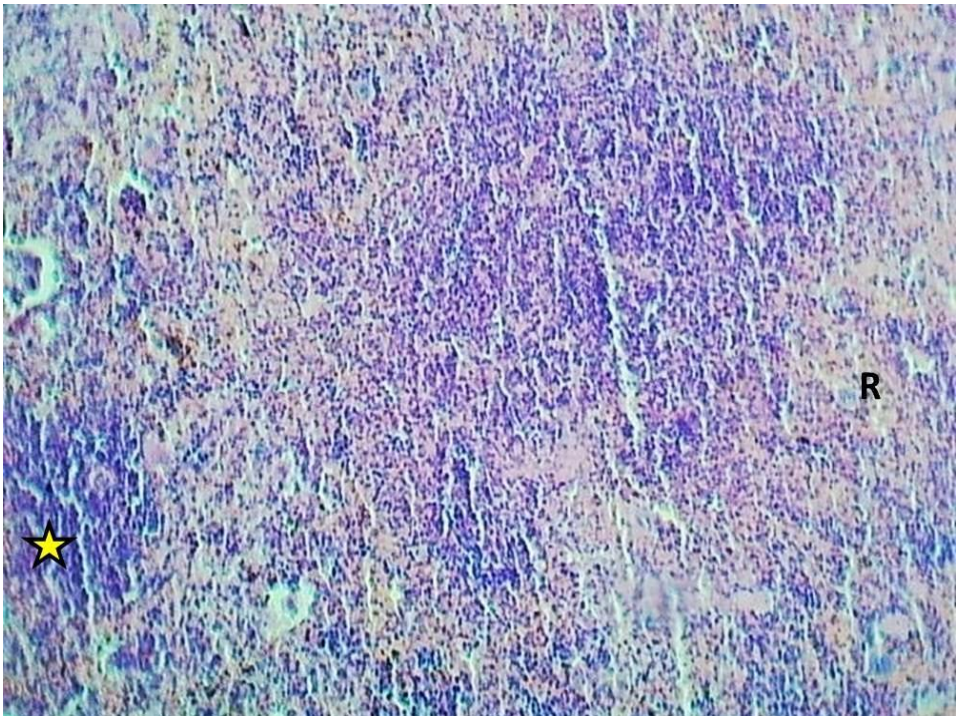

Figure 15: section of spleen (G2) shows: normal lymphoid follicles of white pulp (Asterisk) and normal sinusoid and reticular tissue of red pulp (R). H&E stain.100x.

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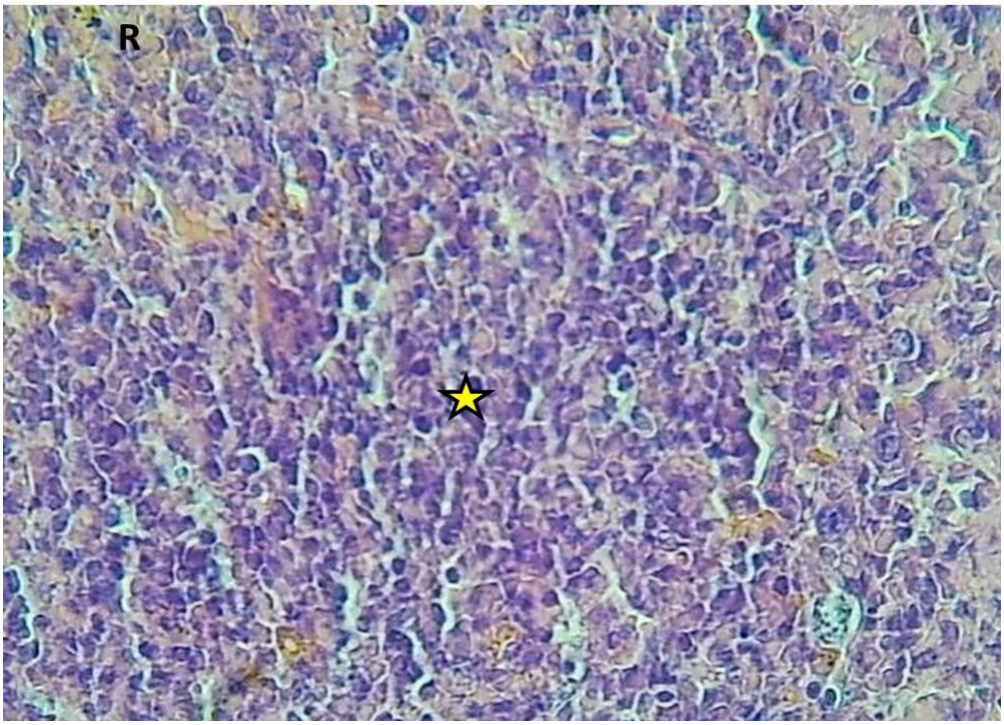

Figure 16: section of spleen (G2) shows: normal lymphoid follicles of white pulp (Asterisk) and normal sinusoid and reticular tissue of red pulp (R). H&E stain.100x.

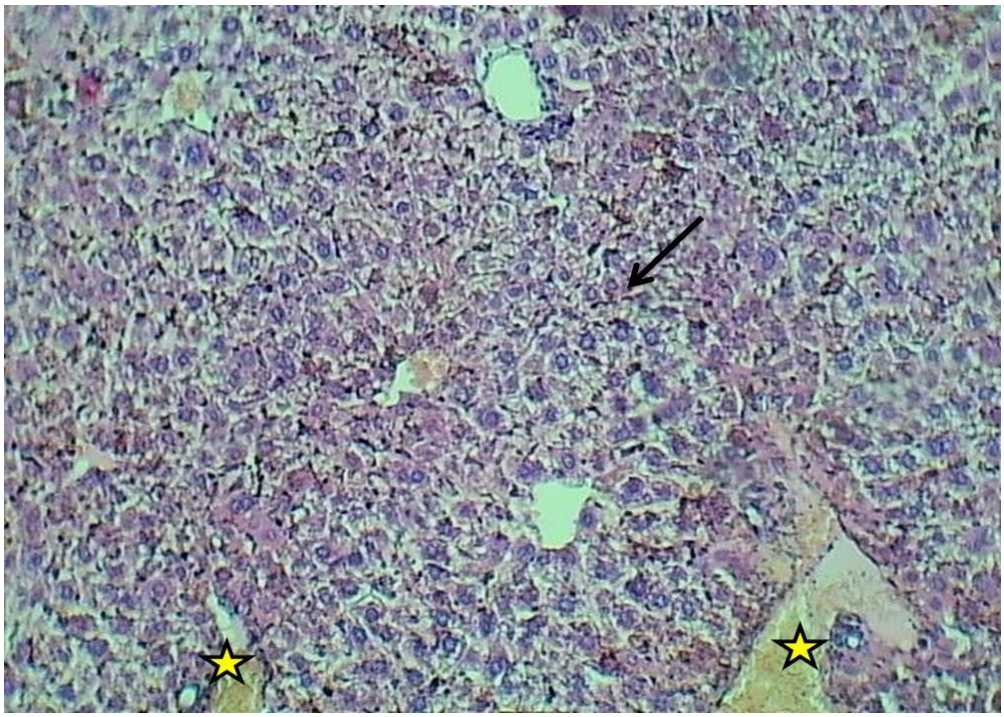

Figure 17: section of liver (G3) shows: marked congestion with dilation of portal vein (Asterisks) & generalized generation & necrosis of hepatocytes (Arrow). H&E stain.100x.

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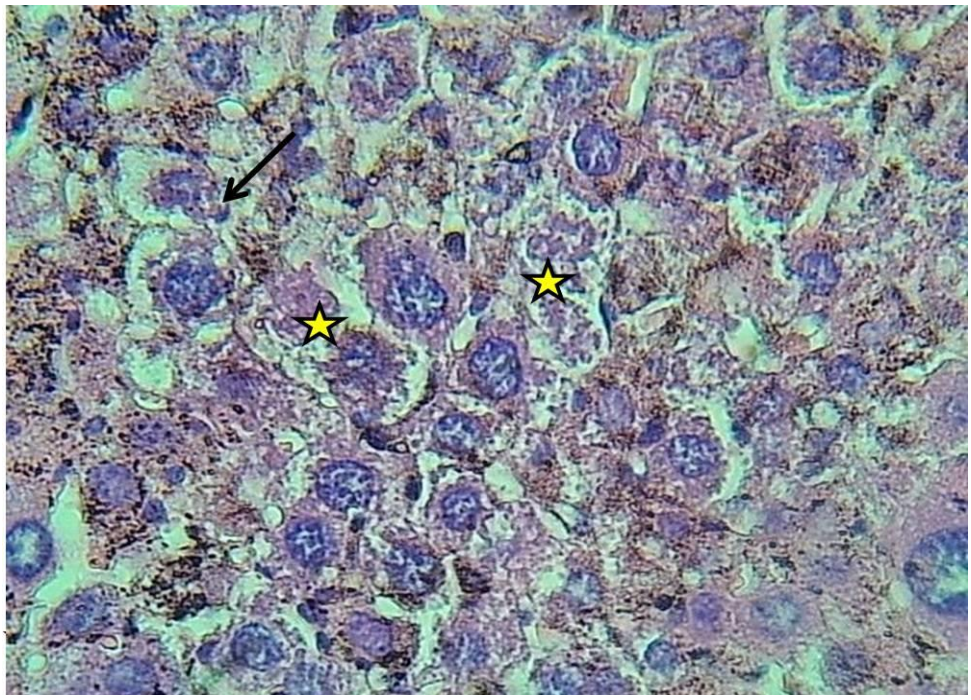

Figure 18: section of liver (G3) shows: marked generalized generation (Arrow) & necrosis of hepatocytes (Asterisk). H&E stain.400x.

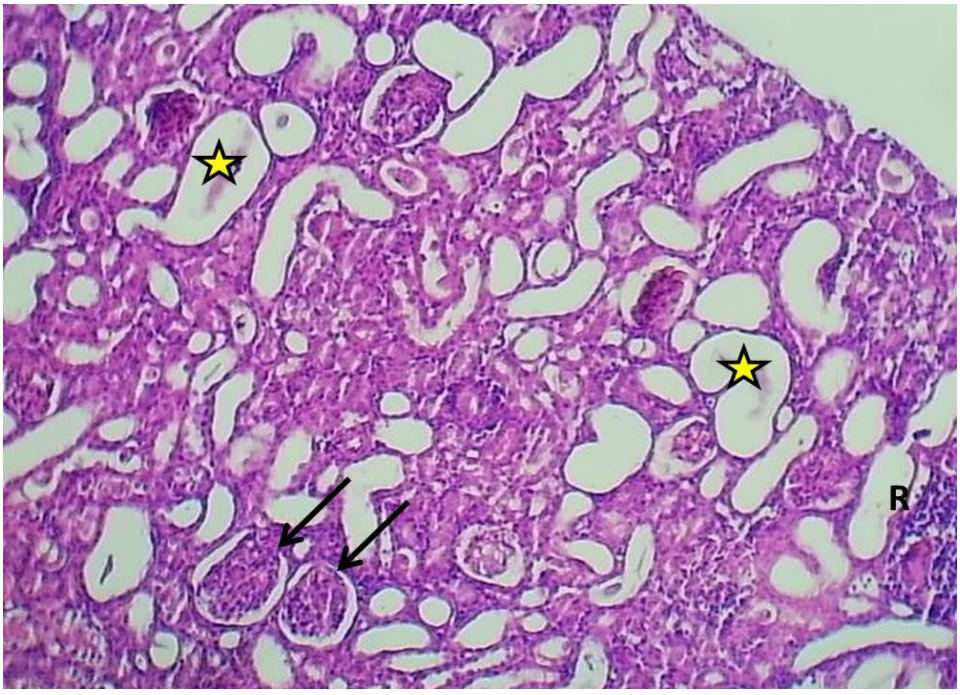

Figure 19: Section of renal cortex (G3) shows: congestion of glomerulus (Arrow), sever tubular dilation with flatting of the lining cells of the renal tubules (Asterisk) & focal tubular necrosis with aggregation of MNCs (R). H&E stain.40x

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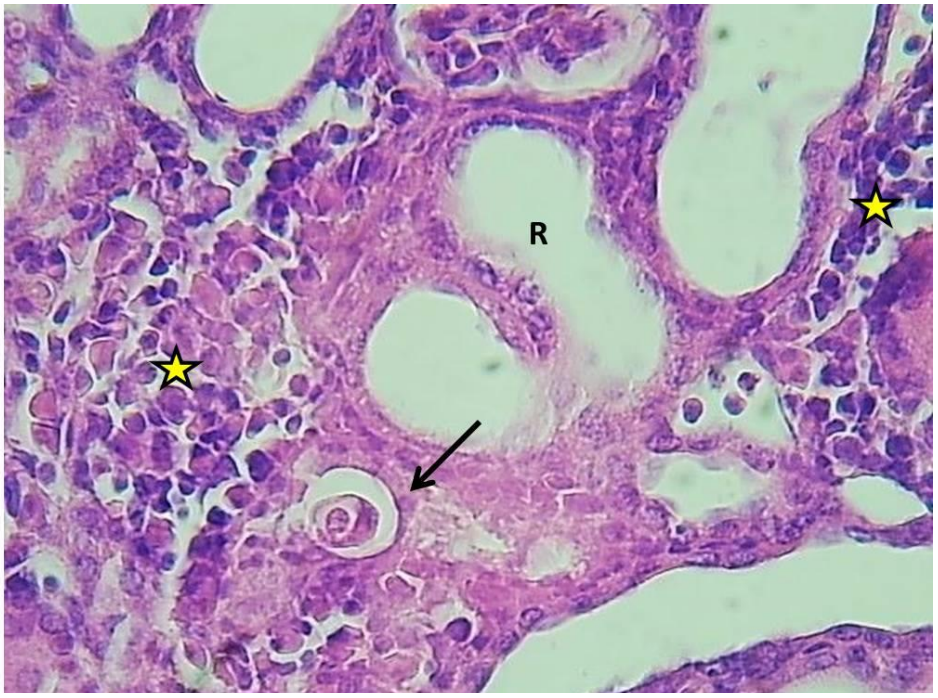

Figure 20: Section of the renal cortex (G3) shows: severe tubular dilation with flatting of the lining cells of the renal tubules (R) & focal tubular necrosis with aggregation of MNCs (Asterisk) & cast formation (arrow). H&E stain.400x

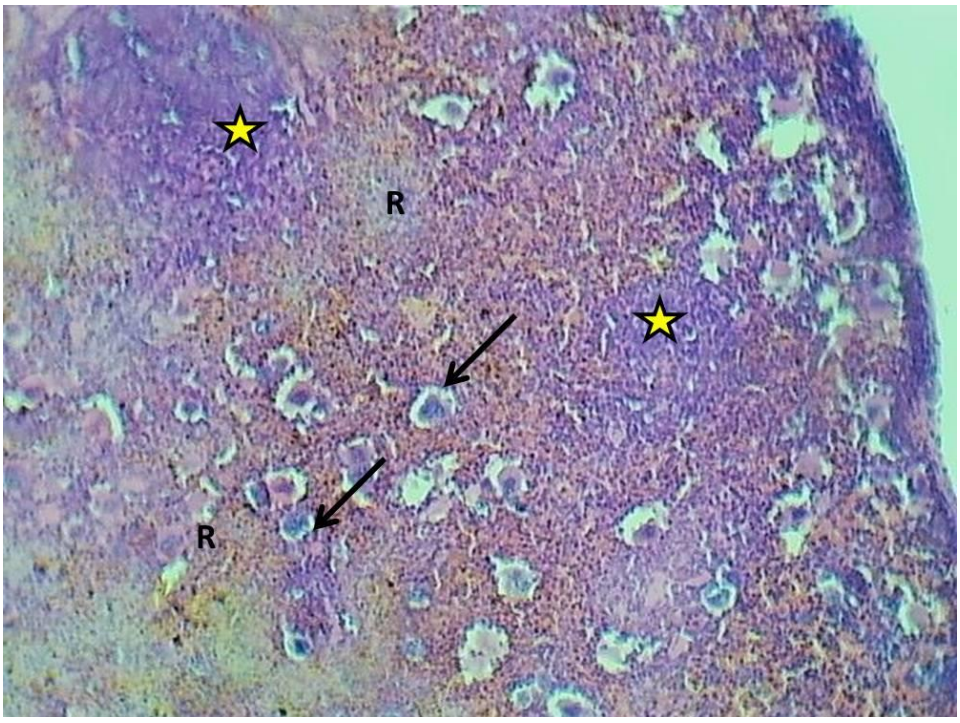

Figure 21: section of spleen (G3) shows: normal lymphoid follicles of white pulp (arrow) and marked severe sinusoidal congestion and distension (R),& marked increased megakaryocytes (Asterisks). H&E stain.40x.

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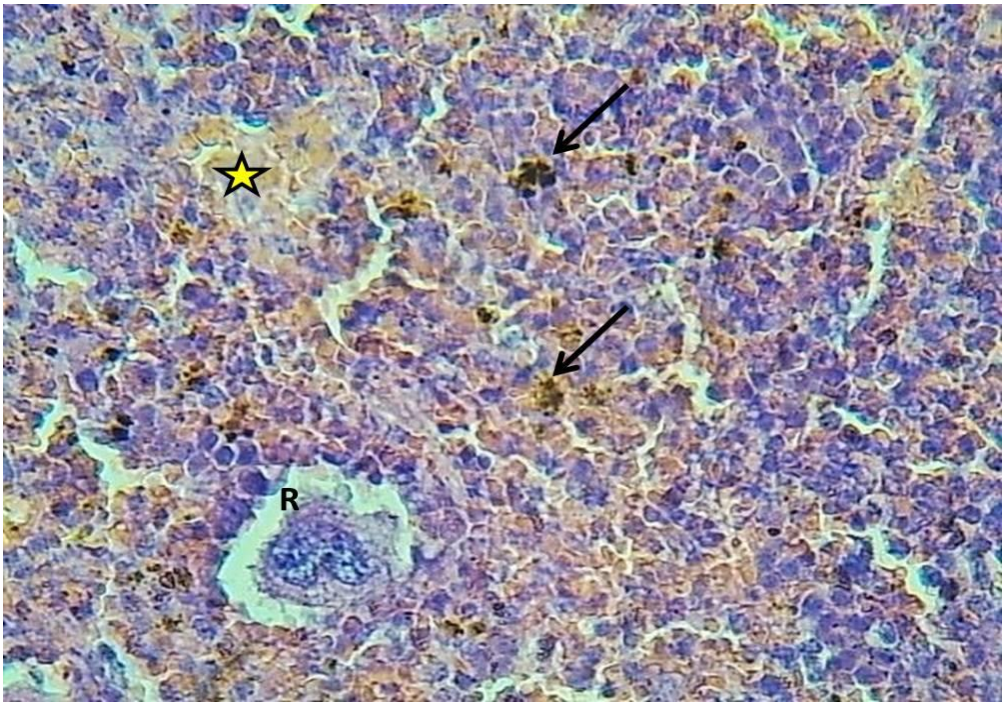

Figure 22: section of spleen (G2) shows: normal lymphoid follicles of white pulp (Asterisks) and normal sinusoid and reticular tissue of red pulp (R)—a marked increase of hemosiderin-laden macrophages(arrows) H&E

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Discussion:

Citrobacter freundii is a foodborne pathogen linked to multiple opportunistic diseases such as pneumonia, hematologic, or newborn infections, and urinary tract infections (21). Out of one hundred fecal samples, the isolation of *C. freundii* yielded positive results (20%). These findings concurred with the percentage of *Citrobacter spp*., found in the Benghazi market (20%) from sheep meat, as reported by (22). The findings are also consistent with those of (23), who recovered 23% of *C. freundii* from 150 samples of raw meat (beef, mutton), as well as carp fish. In contrast to (13), which separated 8 isolates of *C. freundii* from 100 sheep fecal samples. Following PCR analysis to identify the *Citrobacter* isolate, the isolate's 16 s rRNA was examined. As a result, the isolate tested positive for 1500 bp 16S rRNA gene amplification. This outcome was consistent with (8) and (9). PCR analysis was used to identify *C. freundii,* and the results showed that the 16S rRNA gene was present. The results were also consistent with (24), The resulting sequences covered variable regions of the 16S rRNA in bacterial isolates, allowing for the accurate identification of the species of bacteria. The G2(SWCA) antibody titer in this study was larger (1280) than the G1(KWCA) titer (320), and the positive control showed the same titer of G2, which can be attributed to the existence of an anti-*Citrobacter freundii* antibody in the serum. (25) It has been observed that the antibody IgM agglutinates more readily than IgG in an agglutination test. *Citrobacter* species infections stimulate both the local and systemic immune responses because they boost the generation of CD4 + T cells, which are essential for triggering the humoral immune response and causing B cells to produce both IgM and IgG. These immunoglobulins help the bacteria opsonize, which facilitates the phagocytosis-mediated elimination of the organism. (26,27,28). The results of the DTH test for the first and second groups revealed an increase in induration diameter at 24 and 48 hours, followed by a decrease at 72 hours in comparison with the negative control group. This decrease was attributed to monocytic infiltration into the lesion site within the 24–48-hour period. The existence of memory T-cells has been demonstrated to be necessary for this reaction since the response is controlled by the (CD4+ and CD8+) fraction. Th1 (T helper) cells are known to release TNF-β, IL-2, and IFN-γ. For cell-mediated inflammatory responses such as delayed hypersensitivity and macrophage activation, Th1 cells mainly serve as helper cells. (29)

Conclusion

In conclusion, SWCA and KWCA, were observed to promote humoral (Ab titer) and cellular (DTH) immune responses.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication or funding of this manuscript

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