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By Roua J. Mohammed

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The effect of prodigiosin extract from *Serratia rubidea* against *Citrobacter freundii* infection in mice

Roua J. Mohammed

Department. Of Microbiology, College of Veterinary Medicine, University of Baghdad, Iraq

ruaa.jassem1103a@covm.uobaghdad.edu.iq

ABSTRACT

This study was prepared due to a lack of studies on the immune modulatory effects of prodigiosin in mice against bacterial infection and the increased demand for natural resources. This study was prepared due to a lack of studies on the immune modulatory effects of prodigiosin in mice against bacterial infection and the increased demand for natural resources. This study aimed to extract prodigiosin from *Serratia rubidaea* against *Citrobacter freundii* in mice by evaluating its immunomodulatory activity and histological alterations. A total of twenty-four Swiss mice were divided up into four groups of six mice each. *C. freundii* (1×10^6 cfu\ml) was administered orally to groups (2,3 and 4) as an infectious dosage, and one milliliter was administered to the first group as a negative control. Following 24 hours from *C. freundii* infection, the G3 and G4 groups were given crude prodigiosin extract in the following dosage amounts: the third and fourth groups received (500 and 1000μg/ml I.P.) respectively. ELISA test was performed to assess IgM, IL-6, and IL-10 on days 3,7 and 14. IgM and IL-6 findings

demonstrated a significant increase in G2 and G4 with differences (P<0.05), however, G3 showed a significant decrease when compared to the negative control. The results of IL-10 concentration revealed that G2 and G4 had significant decreases with differences (P<0.05), with G3 having the highest titer. In conclusion, the study's findings showed that while prodigiosin's high concentration can boost the immune system and help laboratory animals resist bacterial infection, its low concentration acts as an immune suppressant.

Keyword: Prodigiosin, IL6,IL10,IgM, immunomodulator

Introduction

Citrobacter species are opportunistic invasive bacterium and possible zoonotic pathogens that can cause encephalitis, septicemia, as well as respiratory tract infections in both humans and animals (Yimer and Asseged,2018, Liu et al.,2018, Ryan et al.,2004). Due to their accessibility and availability, natural active substances with bacteriostatic characteristics are now being extracted. When it comes to addressing the problem of bacterial resistance, they are seen to be promising alternatives to traditional antibiotics. *Serratia rubidaea* are members of the large Enterobacteriaceae family of bacteria that produce prodigiosin or PG, a dark red pigment (Pereira and de Carvalho,2024). Prodigiosin has a tripyrrole ring structure (Zhou et al.,2016). It functions as a bioactive compound with anticancer (Lazic et al.,2022), antimalarial (Papireddy et al.,2011), antibacterial and immunomodulatory activity (Yip et al.,2021, Gohil et al., 2020, Yip et al.,2021). Previous studies on prodigiosin have focused on vitro studies that inhibit the growth of a wide spectrum of Gram-positive and Gram-negative bacteria using disc diffusion (Yip et al.,2021, Gohil et al., 2020).

The in vivo results of certain studies suggest that prodigiosin administered orally improves the intestinal microbiota of mice and propose that prodigiosin is a promising candidate medication to treat intestinal inflammation since it enhances the intestinal microbiota of mice and is not harmful to their internal organs (Li et al.,2021). According to other studies, prodigiosin and undecyl prodigiosin altered inflammatory signals and potentially reduced the risk of atherosclerotic plaque in mice (Cuevas et al.,2020). T cells may be impacted by the immunomodulatory actions of PG (Oppi et al., 2019). Furthermore, by promoting leukocyte

recruitment and polarizing T cells, PG was able to decrease the levels of circulating IL-2 and TNF, two important variables in regulating the chronic immune response (Ait-Oufella et al., 2011). To clarify prodigiosin's potential biological function in microbial infection during the assessment of immune modulatory effect and histopathological changes for that, the aim of this research study the effect of prodigiosin extract from *Serratia rubidea* against *Citrobacter freundii* infection 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\649,24\3\2024) during this study.

Isolation and identification

S.rubidea was isolated from one hundred fecal cattle samples in Baghdad, each sample was inoculated onto the chrome agar, MacConkey agar, and Nutrient agar, then incubated at 37 °C for 24-48 hrs. (Quinn et al.,2011). The Serratia isolates were identified at the level of species using the traditional morphological, and biochemical tests, vitek2 compact system, and PCR (Ahmed and al-Samarraae, 2021). *Citrobacter freundii* was obtained from the University of Baghdad's College of Veterinary Medicine's microbiology department accession No. OR766039.

Preparation of crud prodigiosin extract

Prodigiosin preparation was extracted by (Maurya et al.2020). The *Serratia rubidea* was cultured on ten nutrient agar plates and they were incubated at room temperature (28 °C) for 2 days. Observe the colonies growing in confluence with the bright red color. From all plates, only the colonies with the bright red color were harvested in sterile saline and collected in centrifuge tubes. The cells were centrifuged at 3000 rpm for 20 min. The suspension was then digested in a glass test tube by adding 1N(NaOH) twice the volume of the suspension and then put in a water bath at 100 °C for 1 hour, the pigment was removed from the suspension by adding the equal

volume of ethanol, the tubes were centrifuged for 20 min at 3000 rpm. To obtain a color solution free of turbidity, petroleum ether was added and thoroughly mixed with the colored solution. The mixture was then vigorously shaken and blended using a vortex mixer. For layer separation, the tubes were allowed to settle for 10 minutes. In an evaporating dish, the top layer was collected. By heating a water bath to 100°C, an acquired solvent was evaporated till dry residue was left. Put the leftovers in 5 ml of acidified ethanol. Crud prodigiosin concentration is estimated using spectrophotometer absorbance fixed at 535 nm (Maurya et al.2020).

Experimental design

Twenty-four healthy Swiss mice of both sexes aged between 7-8 weeks, weighted 13-17g were divided into 4 groups (each group 6 mice). Groups (3 and 4) were inoculation with an infectious dose of *Citrobacter freundii* (1×10^6 cfu\ml orally (Roua,2021). 2nd group positive control inoculation with infectious dose *Citrobacter freundii* (1×10^6 cfu\ml orally) (Roua,2021). 1st group negative control was injected with (1ml PBS). After 24 hours post-infection with *C.freundii*, the G3 and G4 groups administered crud prodigiosin extract as follows: the 4th group administered (1000 μg/ml I.P) .3rd group administered (500 μg/ml I.P). Blood samples were collected on days 3,7 and 14 from all mice groups, and sera were separated for estimating IL-6, IL-10, and IgM concentration by ELISA kits (Elabscience, China, for mice). Two mice from each group were used for histopathological changes at day 7, specimens were taken from the liver, kidney, spleen, and intestine. The tissues were fixed in a 10% formalin solution immediately after removal (Luna,1968).

Statistical Analysis:

Data were subjected to analysis using SAS (Statistical Analysis System - version 9.1). Two-way ANOVA with interaction and Least significant differences (LSD) were performed to assess significant differences among means. Results expressed as mean ± standard error. $P < 0.05$ is considered statistically significant (SAS,2010).

Results

Isolation and identification

Thirty isolates of *S.rubidea* out of one hundred fecal samples from cattle and the colonies of *S.rubidea* were observed in red colonies on Nutrient Fig (1). While dark pink to red on MacConkey agar and Chrome agar.

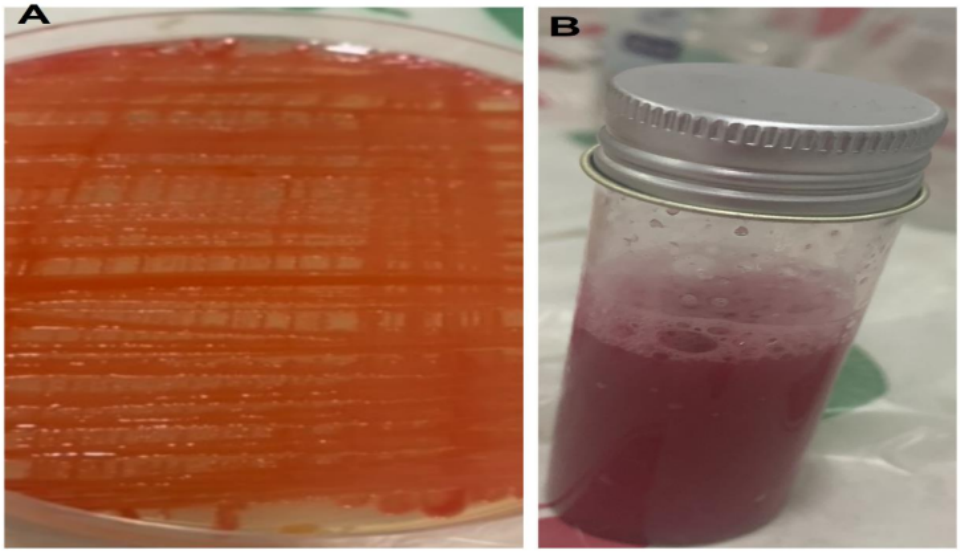


Fig (1): *Serratia rubidaea* (A) colonies on N.A. (B) crud PG

To confirm the identification of *S.rubidea* vitek 2 compact system was done and gave 99% Probability to *Serratia rubidae*. The isolate of *Serratia rubidea* was registered by GenBank under Accession No. (OR757107.1). This isolate was used for crud prodigiosin extract, the prodigiosin production was high at 28 ^{0}C for 48h yielding a final concentration of 130 mg\L, and two concentrations were prepared (1000 and 500 μg/ml).

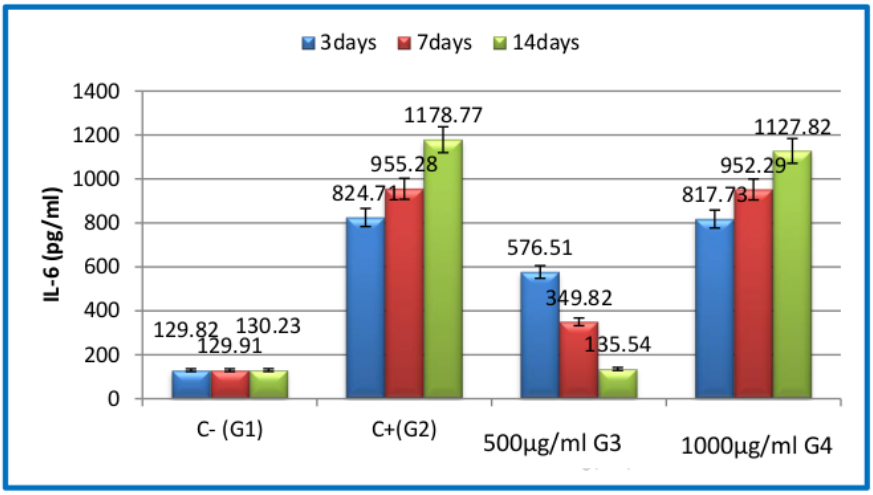
Immune response to prodigiosin

IL-6 concentration for mice

The IL-6 concentration results for groups G2 and G4 showed a significant increase on days 7 and 14, with significant differences ($P<0.05$) compared to the negative control group. G3 showed a moderate increase at day 7, relative to G1 (the control group); G2 (Positive control) showed the

highest IL-6 concentration at days 3 and 7, with 824.71pg\ml and 955.28 pg/ml, followed by G4 at days 3 and 7, with 817.73 pg/ml at day 7 and 952 pg/ml , as indicated in table (1).

Table (1): IL-6

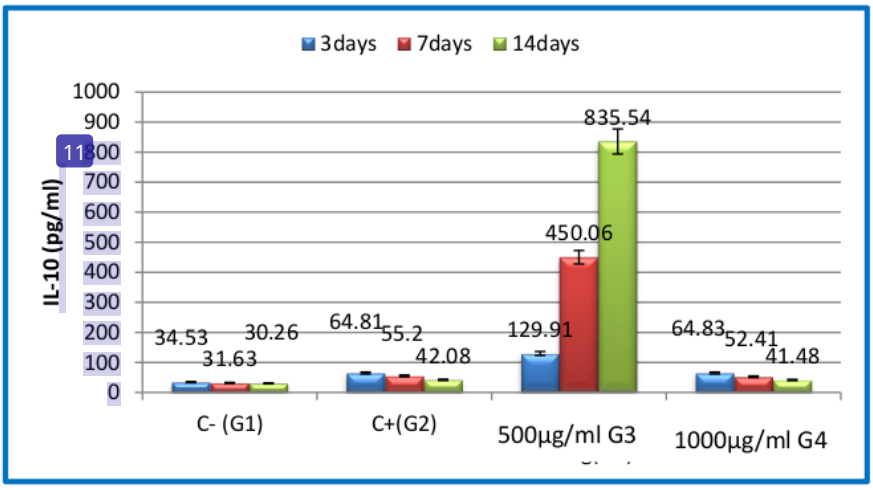


concentration administered with different doses by ELISA test

Il-10 concentration for mice

Comparing the IL-10concentration data for groups G2 and G4 to the negative control group on days 3, 7, and 14 revealed no significant variations. Table 2 shows that, in comparison to the other groups, G4 had the lowest level of IL-10 concentration on days 3, 7, and 14 (64.83, 52.41, and 41.48 pg\ml), followed by G2 which also had the lowest concentration of IL-10 on the same days (64.81, 55.20, and 42.08 pg\ml). G3 had the highest IL-10 concentration on days 3, 7, and 14 (129.91, 450.06, and 835.54pg\ml) table 2.

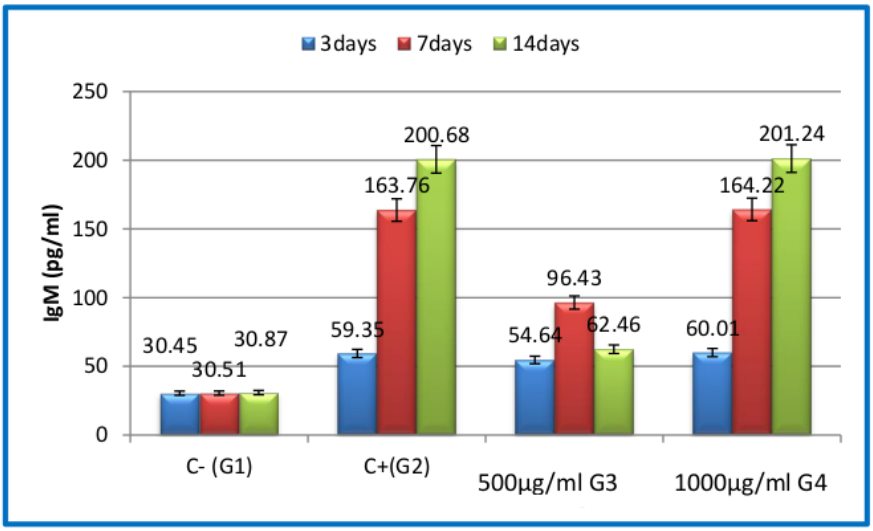
Table (2): IL-10 concentration administered with different doses by ELISA test



IgM concentration in mice

Results revealed that the differences among groups within each period were significant (P<0.05). The concentration of IgM at all periods in the G4 (60.01 pg/ml) and G2(59.35 pg/ml) did not differ significantly but they were significantly (P<0.05) higher than other groups. Concerning the differences among periods within each group, results showed that the IgM in G2 and G4 increased significantly with advanced period while the G3 showed significant decreasing (62.46 pg/ml) at 14 days as compared with 7 days (95.43 pg/ml). On the other hand, the differences among periods in G1 were not significant table 3.

Table (3): IgM concentration in the administered groups with different doses by ELISA test



Histopathological changes

Following a 7-day histological examination revealed that all groups under investigation had varying histopathological alterations. Group 2: the liver shows marked portal fibrosis with marked bridge formation, proliferation of choanocytes, and degeneration with necrosis of hepatocytes Fig (2). Also, the liver shows marked vascular degeneration of hepatocytes, aggregation of MNCs, and portal congestion fig (3). The liver of G2 shows marked deterioration with damage to the cytoarchitecture of the hepatic fig (4). kidney shows marked focal nephritis characterized by local necrosis of renal tubules with aggregation of MNcs fig (5), kidney marked interstitial nephritis with necrosis of renal tissue and aggregation of MNCs and tubular dilation fig (6). spleen shows marked sinusoidal congestion with distention, and slight atrophy of lymphoid follicles with marked irregular shapes (7). enteritis marked thickening of villi with marked hyperplasia of lining cells with severe infiltration of mononuclear leukocytes fig (8). Group 4: liver marked disarrangement of hepatic cords with sinusoidal dilation and degeneration of hepatocytes fig (9), also marked disarrangement of hepatic cords with atrophy of hepatocytes and sinusoidal congestion fig (10). kidney marked nephritis with severe tissue damage and aggregation of MNCs, degeneration with necrosis of lining cells of renal tubules, degeneration of podocytes of glomerulus fig (11). Spleen showed marked lymphoid hyperplasia within follicles of splenic white pulp and congestion with dilation of splenic sinuses with proliferation of megakaryocytes fig (12). The intestine showed hypernulcation of enterocytes fig (13). Group 3: liver severe per central cellular swelling with necrosis of hepatocytes (Red arrows) and per central vein (V) aggregation of MNCs fig (14). kidney marked dilation of bowman capsule with tubular dilation with accumulation of non-cellular eosinophilic substance fig (15). spleen shows splenic sinus dilation with the proliferation of megakaryocytes fig (16). The intestine shows hyperplasia of lining cells of intestinal glands involving paneth cells (17).

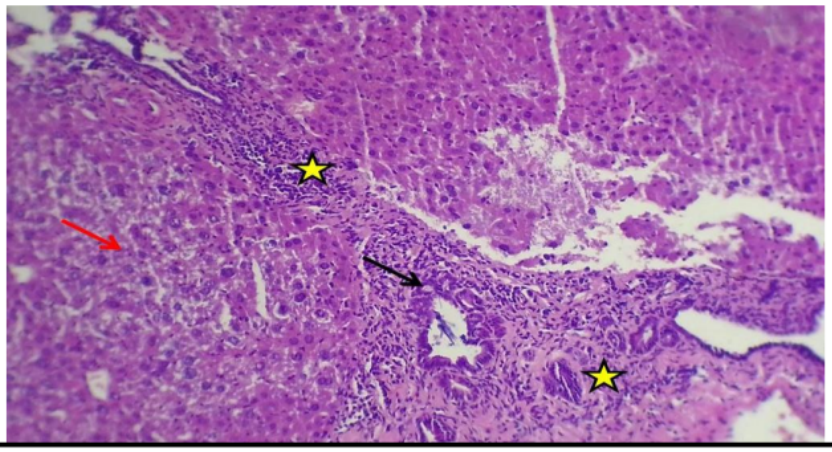
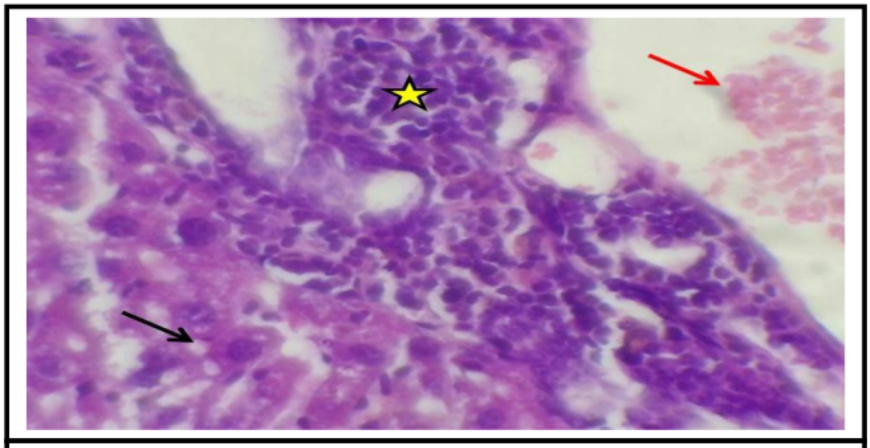
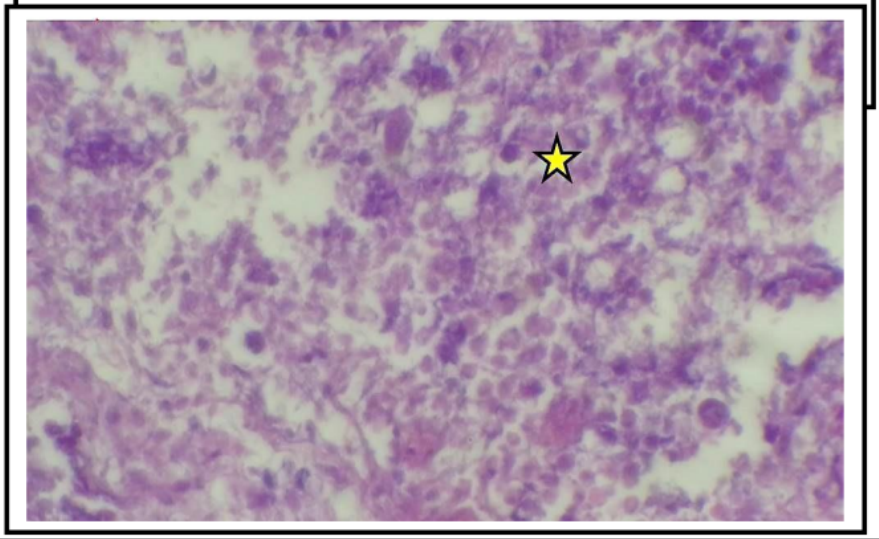


Fig (2): G2 the liver shows marked portal fibrosis with marked bridge formation (Asterisks) proliferation of cholangiocytes (Black arrow) & degeneration with necrosis of hepatocytes (Red arrow)





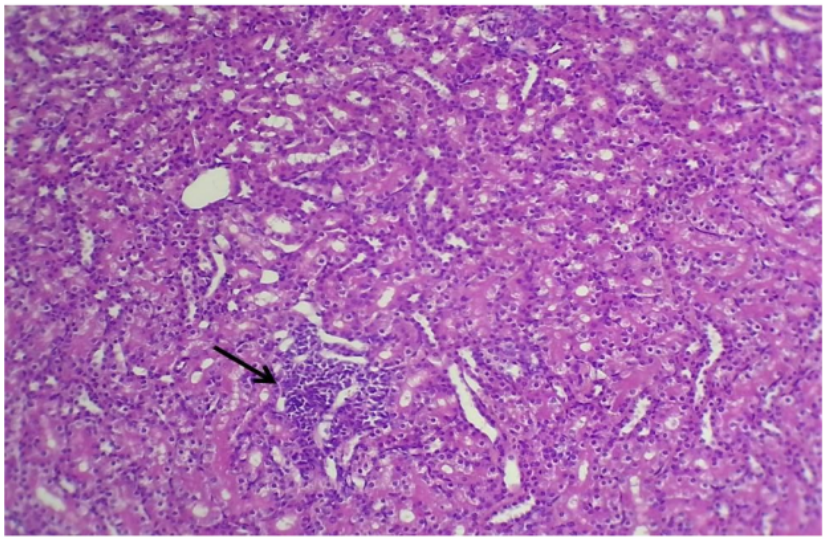


Fig (5) G2: kidney shows marked focal nephritis characterized by local necrosis of renal tubules with aggregation of MNcs. H&E stain.100x

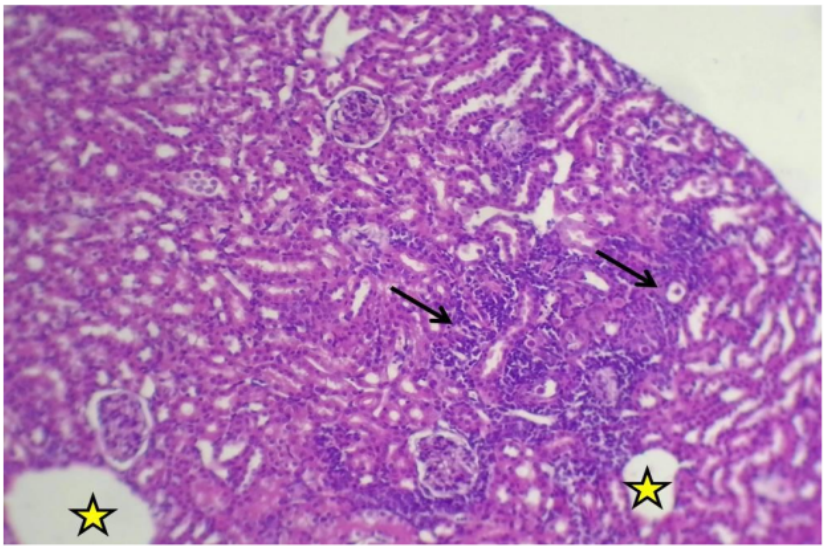


Fig (6): G2 kidney marked interstitial nephritis with necrosis of renal tissue and aggregation of MNCs (Arrows) and tubular dilation (Asterisks).H&E.100x

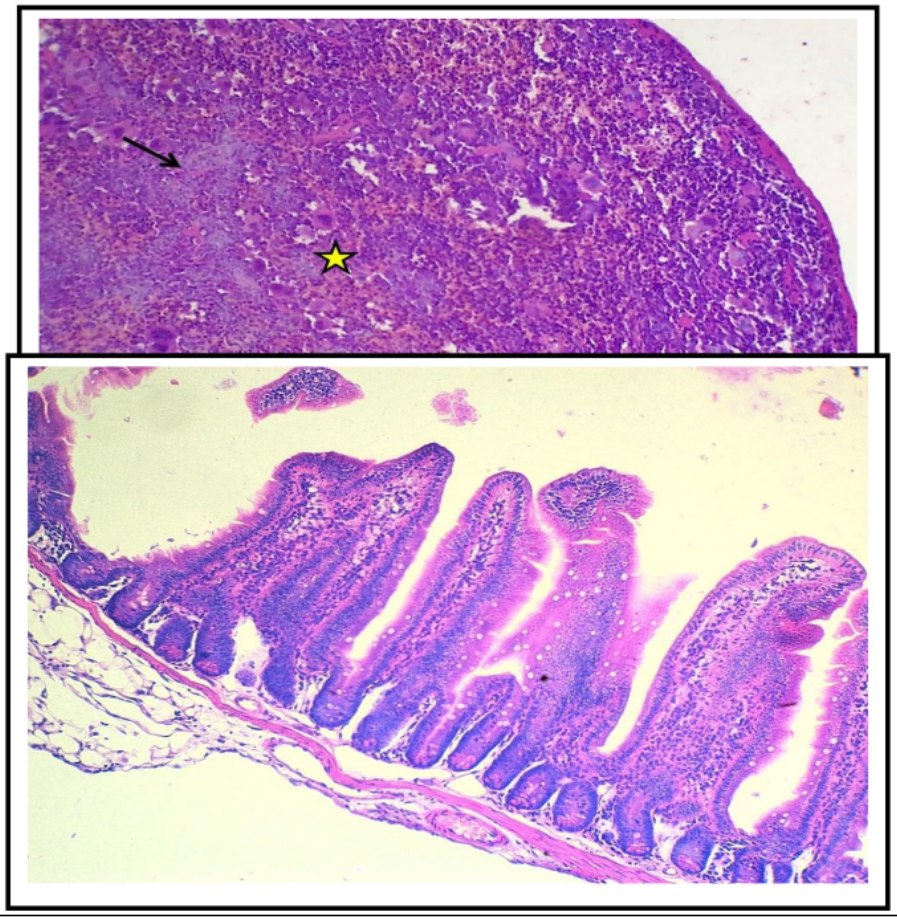


Fig (8) :G2 enteritis marked thickening of villi with marked hyperplasia of lining cells with sever infiltration of mononuclear leukocytes .H&E.100x

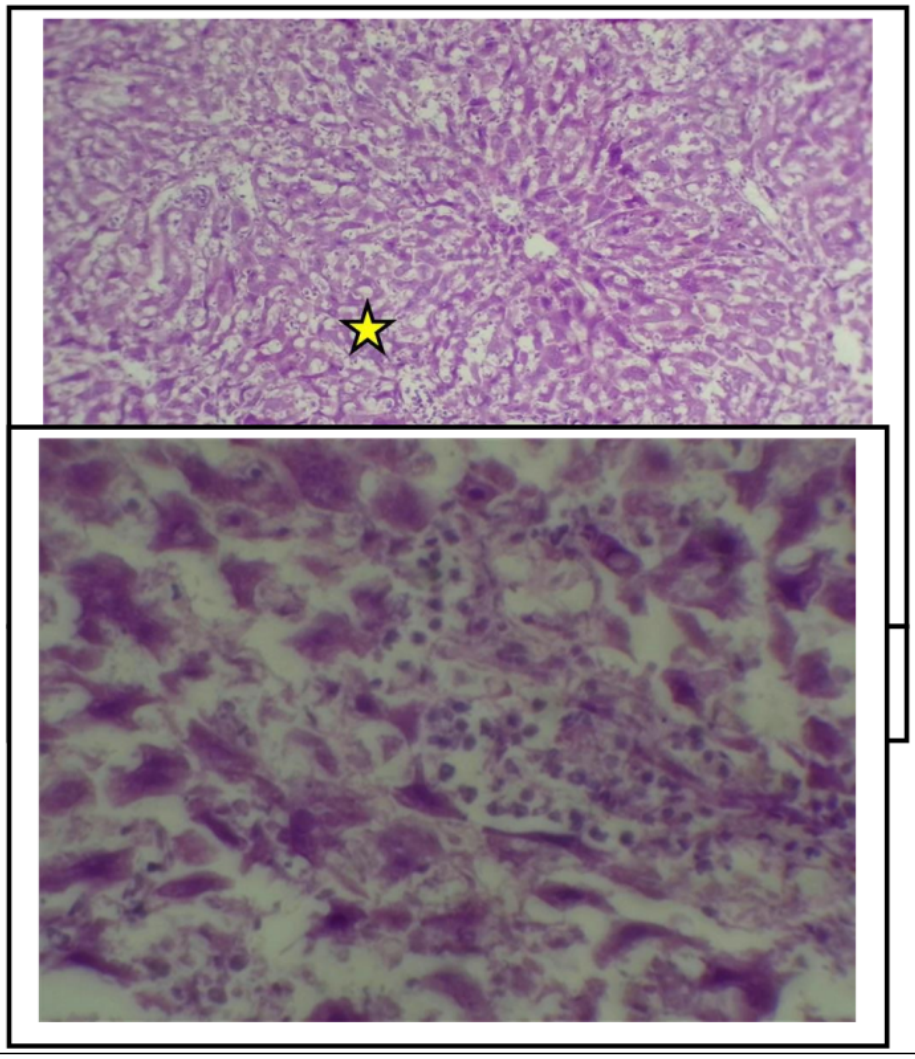


Fig (10): G4 liver marked disarrangement of hepatic cords with atrophy of hepatocytes and sinusoidal congestion (asterisk).H&E. 100x.

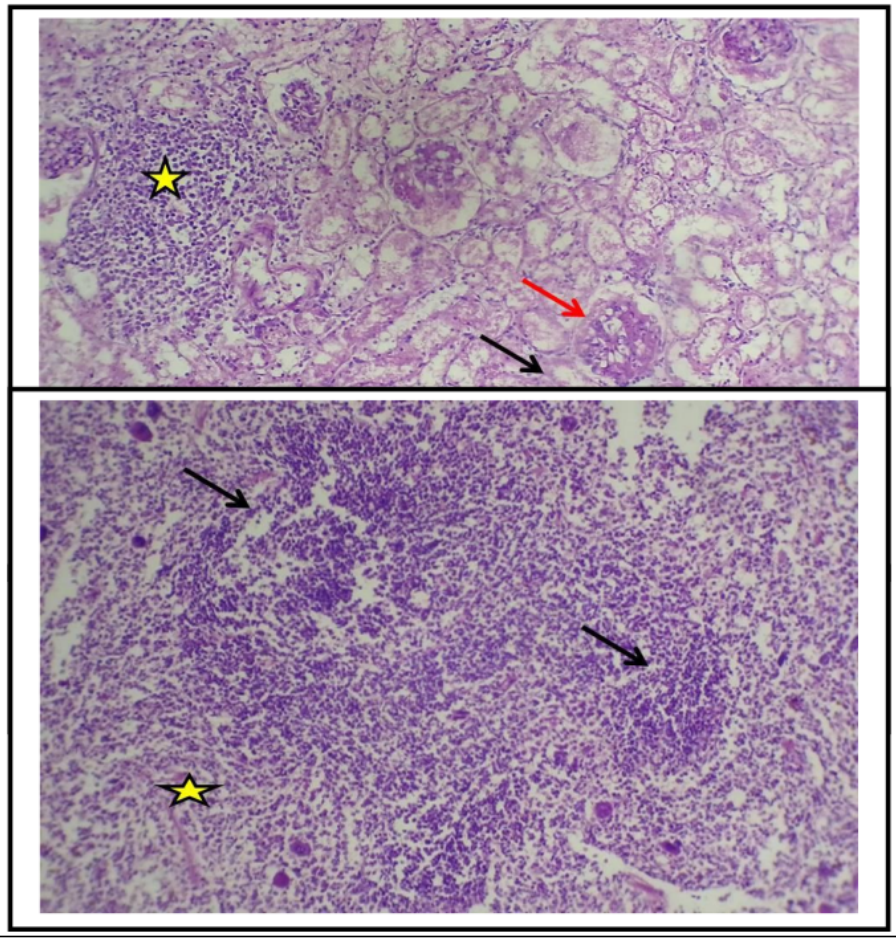


Fig (12): G4 spleen showed marked lymphoid hyperplasia within follicles of splenic white pulp (Arrows) and congestion with dilation of splenic sinuses with proliferation of megakaryocytes (Asterisk).H&E.

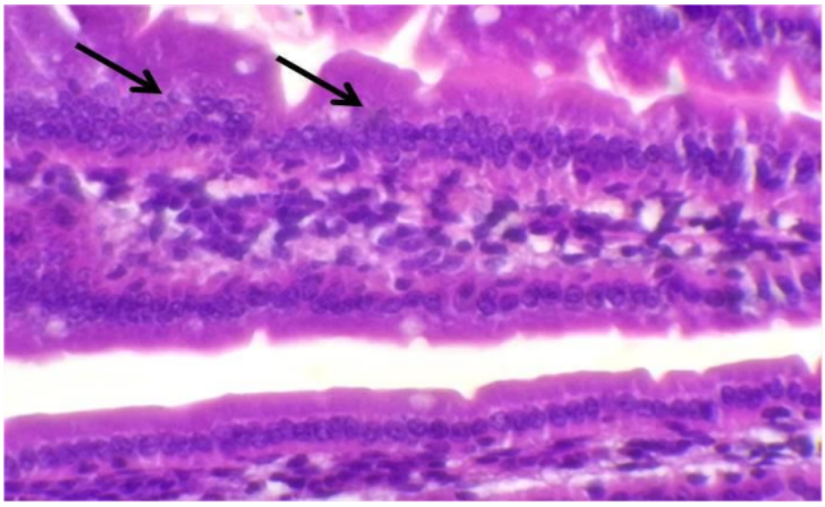


Fig (13):G4 Intestine hypernulcation of enterocytes (Arrows).H&E.

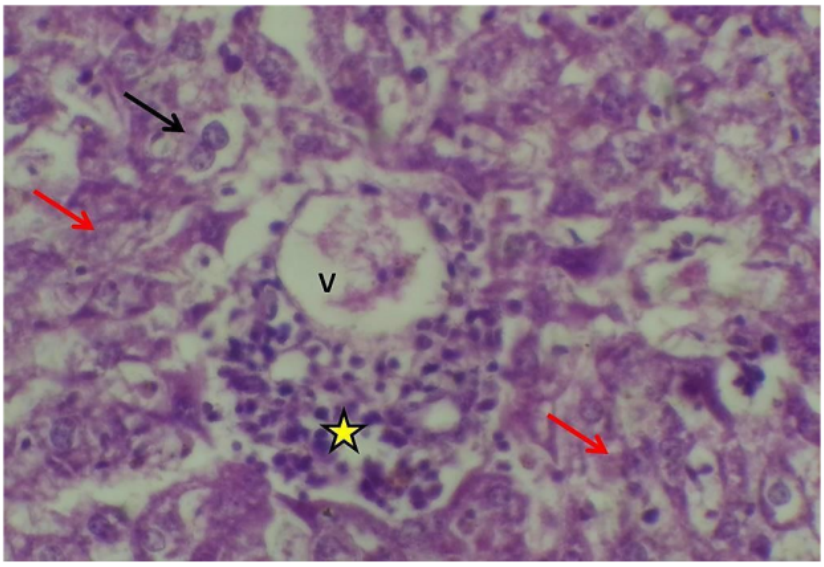


Fig (14) G3 liver severe per central cellular swelling (Black arrow) with necrosis of hepatocytes (Red arrows) and per central vein (V) aggregation of MNCs (Asterisk).H&E.400x

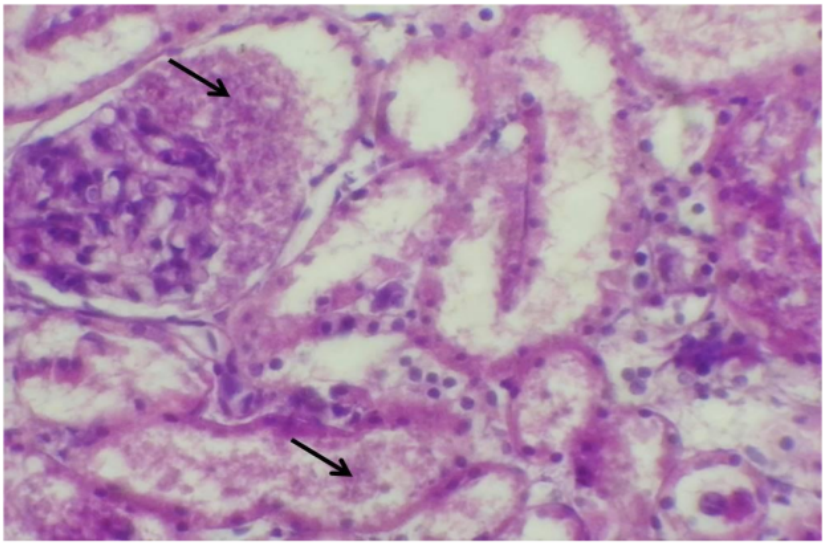


Fig (15): G3 kidney marked dilation of bowman capsule with tubular dilation with accumulation of non-cellular eosinophilic substance (Arrows).H&E. 400x.

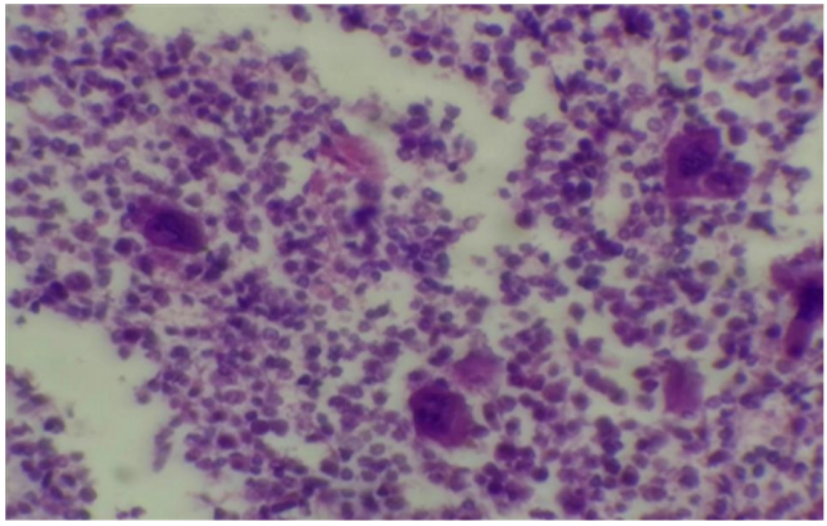


Fig (16): G3 spleen shows splenic sinuses dilation with proliferation of megakaryocytes .H&E. 400x.

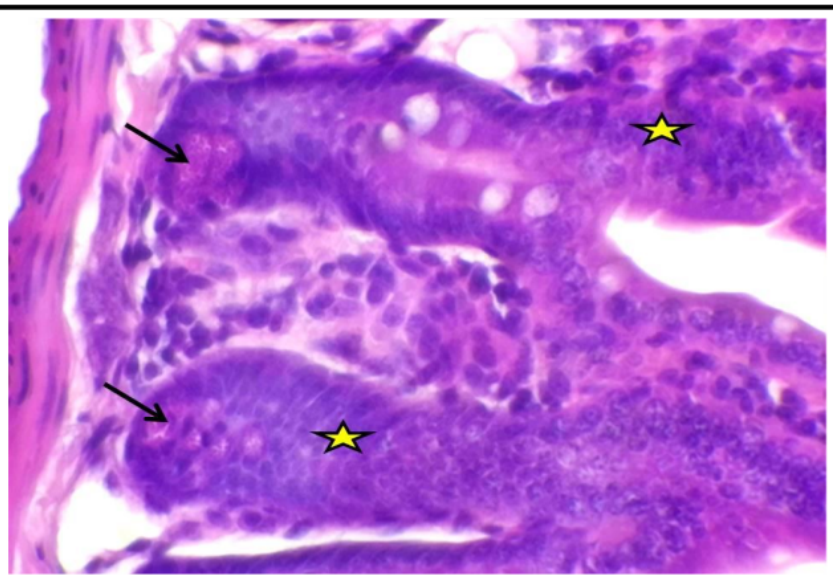


Fig (17): G3 intestine shows hyperplasia of lining cells of intestinal glands (Asterisks) involve Paneth cells (Arrows) .H&E. 400x.

Discussion

A strain of *S.rubidaea* that produced red pigment was identified in this investigation. The isolated strain Accession No. (OR757107.1) was used to extract prodigiosin from the bacterium and it was found that the crude prodigiosin conc. had 130 mg/ml. These findings concur with those of Song et al. (2006), who discovered that 120 mg/ml of prodigiosin conc. was isolated from Serratia ssp. This study looked at the immunological characteristics linked to PG in the context of bacterial infection because PG is a powerful immunomodulator that affects lymphoreticular cells, including B cells, T cells, and macrophages. The results of IL-6 concentration give the highest significant increase in G2(824.71pg\ml and 955.28 pg/ml) and G4(817.73 pg/ml and 952 pg/ml) at days 3 and 7. while, G3 showed a decrease in IL-6 concentration all day compared to other groups, as well as the result of IgM concentration in G2 and G4 increased significantly with advanced period while the G3 showed significant decreasing (62.46 pg/ml) at 14 days as compared with 7 days (95.43 pg/ml). The results may return to activate the immune system and, at high concentrations, function as a mitogen, numerous studies have demonstrated that biosurfactants can promote cell differentiation and the cellular immune response rather than just cell proliferation. Additionally, they observed that biosurfactants inhibited macrophage cells' capacity to create pro-inflammatory cytokines and stimulated lymphocytes' production of anti-inflammatory cytokines, thereby acting as an anti-inflammatory (Stipcevic et al.,2013, Tang et al.,2010 and Park and Kim, 2009). This is consistent with (Al-wazni, 2018).), who viewed this as an effort to show how some biological components might boost the immune system's effectiveness. Research has demonstrated that prodigiosin family chemicals can specifically control T lymphocyte proliferation (Lee et al.,2000, Lee et al.,1998 and Magae et al.,1997) and macrophage responsiveness to inflammatory stimuli (Huh et al.,2007). Prodigiosin exhibits potent antimicrobial properties against a variety of microorganisms, including oxacillin-resistant S. aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Streptococcus pyogenes, Acinetobacter sp., and Escherichia coli. All of these results support prodigiosin's anti-inflammatory and immune-boosting properties (Lapenda et al.,2014). The results of the G3 decrease in the concentration of IL-6 and IgM may be due to low

concentration and prodigiosin has been discovered to have immunosuppressive effects on immunological responses, both humoral and cellular, it prevents T lymphocytes, natural killer cells, macrophages, and lymphocytes from proliferating (Huh 2008, Han 1998). The results of IL-10 concentration showed a significant decrease in G2 and G4, while a significant increase in G3, these results revealed that pigmentation can regulate the production of cytokines to have immunomodulatory effects: it promoted the expression of IL-10 while downregulating TNF-α and IL-6 expression (Verinaud et al.,2015). These results of the histopathological study demonstrated the presence of severe histopathological effects in the specimens of mice caused by their infection with *C. freundii*, compared to the administered group, The histological sections matched the findings of Milano et al. (1997), who reported that the presence of lipopolysaccharide receptors on the surfaces of various cells, whether free or enveloping, proteins bound to LPS binding protein represent the germ and these receptors. The histopathological examination of infected to gram-negative bacteria is represented by tissue necrosis and the breakdown of blood vessels which in turn leads to death through its association with immune system cells as it works to induce them to release inflammatory mediators (proinflammatory mediators), including cytokines, which can cause physiological and pathological changes (Luchi, and Morrison,(2000).

Conclusion:

Prodigiosin is a significant medical and therapeutic chemical. The study's findings showed that while prodigiosin's high concentration can operate to boost the immune system and help laboratory animals resist bacterial infection, its low concentration acts as an immune suppressant.

Acknowledgement

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Conflict of interest

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

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