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Antidiabetic effects of the heat-killed *Actinomycetales species* in the liver and kidney of diabetic rats

Abstract

Type 1 diabetes mellitus (T1DM) occurs due to the decrease in insulin secretion following the destruction of pancreatic beta cells. This disease is increasing worldwide, especially among children under the age of 5 years, which is usually associated with irreversible complications such as hepatopathy and nephropathy. The present study aimed to investigate the antidiabetic effect of the heat-killed *Actinomycetales species,* including *Gordonia bronchialis (Gb)*, and *Tsukamurella inchonensis (Ti)* in streptozotocin-diabetic rats by oral administration. This experiment was performed in six groups, including healthy control, diabetic control, low-dose Gb (G1), high-dose Gb (G2), low-dose-Ti (T1), and high-dose Ti (T2). Subsequently; the levels of ALT, AST, total protein, albumin, BUN, creatinine, CRP, IL-1β, and IL-2 were measured in the serum samples in the 14th and 21st days. Besides, histopathological lesions were studied in the liver and kidney. Our findings showed that Gb and Ti could alter the examined serum parameters, particularly in the T2

groups. Also, histological examination revealed a remarkable attenuation in the pathological lesions such as focal necrosis, vascular congestion, and hemorrhage in the liver and kidney of the treated rats by Gb and Ti. Here, it is concluded that oral administration of the heat-killed *Actinomycetales species,* particularly with a high dose of *Ti,* could beneficially improve the progression of T1DM and its various complications, which can be used to treat T1DM in the future.

Keywords: Type 1 diabetes mellitus, *Gordonia bronchialis*, *Tsukamurella inchonensis*, hepatopathy, nephropathy

Introduction

Diabetes Mellitus (DM) is not a single disease but a general term that describes a collection of metabolic conditions, which result in high blood glucose levels due to defects in insulin function or secretion or both [1, 2]. Increasing evidence reported that it has affected approximately 285 million individuals globally, and this number is anticipated to increase to 439 million in 2030 [3], which is associated with severe and irreversible complications, such as nephropathy and hepatopathy [4]. Type 1 DM (T1DM) and Type 2 DM (T2DM) are the two primary forms of diabetes [5]. T1DM, formerly known as insulin-dependent diabetes mellitus (IDDM) [2]. The annual incidence of T1DM varies widely in different countries (from less than 1 person in 100,000 in Asia to more than 41 cases in 100,000 people in Europe). Children are newly diagnosed with this disease [5]. This disease is increasing worldwide, especially among children under the age of 5 years [1, 5-7]. Chemokines play a crucial role in both immune system and inflammatory processes, which have been suggested as inducers of β-cell damage in human insulin-dependent diabetes mellitus [1].

Actinomycetales species can switch off pre-existing Th2 preponderance and stimulate Th1-mediated mechanisms. Recently, some aerobic Actinomycetales species, like *Gordonia bronchialis* and *Tsukamurella inchonensis* are capable of exerting subtly different adjuvant or immunomodulatory activities [7, 8]. In this regard, it has been revealed that subcutaneous injection of these killed bacteria improves T2DM and obesity in mice animal models [8]. Also, our previous reports presented the improvement impacts of the heat-killed *Actinomycetales species* in the pancreas [9], testes [10], and intestine [11] of diabetic rats. Thus, in the present study, the beneficial effects of the heat-killed *Actinomycetales species,* including *Gordonia bronchialis (Gb)* and *Tsukamurella inchonensis (Ti),* were investigated in streptozotocin-diabetic rats by oral administration. For this purpose; the liver and kidney biochemical indicators such as ALT, AST, total protein, albumin, blood urea, and creatinine were evaluated in the serum samples. Besides; the C-Reactive Protein (CRP), IL-1β and IL-2 levels were measured and associated with histopathological evaluation of the liver and kidney.

Results

Biochemical findings

Lower levels of serum insulin along with elevated glucose values were detected in control diabetic rats compared to the other treated groups (supplementary file). Interestingly; there were no significant differences ($p > 0.05$) in glucose values between the diabetic rats and the treated groups in a dose-dependent manner. Moreover; lower insulin levels were observed in the diabetic rats, which improved significantly in the treated groups by using the bacteria, especially in Ti-recipient groups (figure supplementary 1).

The marked decreased values of serum albumin and total protein (figure 1A, B) were assessed in the diabetic animals when compared with other groups, which improved beneficially in all

diabetic-treated groups. In albumin measurement, there were notable differences in healthy rats with other experimental groups, and also a marked difference ($p < 0.05$) was noted among the low-dose and high-dose Gb recipient groups on the 14th and 21st sampling days. In total protein data, both low and high-dose Gb and Ti recipient groups showed significant differences ($p < 0.05$) with the healthy and diabetic animals. Notably, the highest levels of both albumin and total protein were observed in the T1, G2, and G2 groups, on the 7th, 14th, and 21st sampling days.

Significantly decreased levels of blood urea and creatinine (figure 1C, D) ($p < 0.05$) were observed in all diabetic-treated groups as compared with the diabetic group. The significant difference in low-dose and high-dose groups was only in urea values on the 14-sampling day.

The activities of AST and ALT diminished in the diabetic rats (figure 1E, F) when compared with the healthy and diabetic-treated rats, particularly in Gb-recipient groups with a dose-dependent manner ($p < 0.05$).

CRP, IL-1β, and IL-2 serum levels

Here, remarkably higher levels of IL-1β and CRP inflammatory cytokines were found within the diabetic rats as compared to healthy animals, which improved in a dose-dependent manner in all diabetic-treated groups (figure 1G, H). On the other hand; considerable lower levels of IL-2 was observed in the diabetic animals when compared with the healthy rats. The serum levels of IL-2 significantly increased and improved in all diabetic-treated groups without a dose-dependent manner between Gb-recipient and Ti-recipient groups.

Histopathological findings

In the liver, healthy control rats presented a normal tissue structure consisting of evenly arranged polyhedral hepatocytes radiating outward from the central vein to the periphery. By contrast; there were severe to moderate pathological changes in the control diabetic group, including cell swelling

and vacuolar degeneration of hepatocytes, particularly around the central veins, dilatation and congestion of sinusoids, congestion in the central veins, focal single-cell necrosis and mild hepatitis. Surprisingly; the livers of the animals in T1, T2, G1 and G2 groups exhibited marked improvements in all of the histopathological features (figure 2), particularly in Ti high dose recipient group in the 21st after treatment with mild hepatocyte degeneration and vascular congestion.

In the kidney, a normal renal parenchymal structure (figure 3), together with well-defined glomeruli and tubules, was observed in the healthy control rats. In contrast; the diabetic animals with no treatment presented severe to moderate pathological changes comprising tubular epithelium degeneration, vacuolization and single-cell necrosis, vascular congestion, focal hemorrhage, focal interstitial nephritis, and atrophy with the congestion of glomeruli. Interestingly; all treated groups showed significant improvements in the renal lesions, mainly at each of both doses of Gb, which presented only mild vascular congestion and tubular hyaline casts.

Discussion

The present results demonstrated a gradual alteration in the serum concentrations of the blood glucose and insulin levels, and the liver and kidney biochemical indicators (studied by total protein/albumin, blood urea/creatinine, and ALT/AST), which were associated with the CRP, IL-1β, and IL-2 levels in the diabetic group. Considerably, these findings are consistent with the present histopathological studies, which included severe hepatic and nephrotic lesions. As expected; these current observations agree with other findings in the diabetic animal models [12, 13]. Of note; most of the present diabetic complications (in the liver and kidney) induced by STZ were remarkably reduced following *Actinomycetales species'* oral administration. Interestingly, *T. inchonensis* and *G. bronchialis* presented more improvement in the liver and kidney, respectively.

Our study indicated that the plasma levels of ALT and AST were remarkably increased in the diabetes animals like previous evidence [13], which provides liver and kidney dysfunctions due to STZ and its cellular damages to the hepatocytes and proximal renal tubules, respectively [12, 13], which were also found in the present study and confirmed further histologically. Elevated activities of these enzymes were found more frequently in diabetic people in comparison with healthy populations [14]. Besides, we found significantly lower serum levels of total protein and albumin associated with higher concentrations of blood urea and creatinine in the diabetic rats, which was proposed previously by other researchers [15] and clarified progressive renal damage in diabetes. The proinflammatory cytokines, including IL-1 and IL-2 may play crucial roles either individually or in combination in the pathogenesis of DM [16]. In insulin-dependent DM, a deficiency in IL-2 production by CD^{4+} T lymphocytes within the IL-2 system has been identified [17]. Unusual levels of serum IL-2 receptor have been found in young children with DM and are unlikely to be an early marker of insulin dependent diabetes mellitus [18]. In a previous study, long-term monitoring of diabetic patients has revealed a marked reduction in the levels of IL-2 [1]. IL-1β is essential for the β-cell lysis in DM, while IL-1 receptor antagonist (IL-1ra) is believed to be of a preventative nature by impeding the actions of IL-1 [19]. In some studies involving patients who recently developed DM, IL-1 production levels were significantly higher than observed in chronic IDDM patients and healthy controls [1]. The plasma concentration of CRP is very low in healthy people, but it increases up to 1000 times after infection and inflammation [20]. Following the innate immune response, phagocytic cells produce inflammatory cytokines like IL-1, which act on liver cells to produce CRP, and circulating CRP have also been determined as the inflammatory response markers [21]. However, Erbagci et al. [22] demonstrated that CRP level was not different

in diabetic patients from those of the control group. The present findings in the alterations of IL-1β, IL-2, and CRP serum levels are consistent with those of earlier studies [1, 23].

T1DM frequently causes by auto-reactive pro-inflammatory CD^{4+} T and CD^{8+} cells [2]. According to our findings and previous studies [7, 8], it seems that both Gb and Ti bacteria may effect on the presentations of cellular immunity by Th1 and Th2 as different pathways of maturation of CD4+ cells, which can enhance protective immune responses. Taken to gather, the present results demonstrated that treatment by both *T. inchonensis* and *G. bronchialis* produces a profound impact on liver and kidney functions in the diabetic condition in a time and dose-dependent manner. Of considerable interest, a similar protective effect of *Actinomycetales species* on liver and kidney structures with obesity and diabetes was proposed by the previous study [8]. Indeed, in the current study, all kidney and liver pathological lesions because of STZ administration, such as cellular degeneration, vascular congestion, hemorrhage, focal necrosis, and inflammatory cell infiltration, were notably attenuated in diabetic-treated rats when compared with the non-treated diabetic group.

Conclusion

According to the present results, it was concluded that orally administering heat-killed *Actinomycetes species,* such as *T. inchonensis* and *G. bronchialis* to diabetic rats could suggest them a promising candidate as pharmacological agents for the treatment of liver and kidney complications associated with diabetes in the future. However, more studies related to other diabetic complications are needed.

Materials and methods

Experimental design

Sixty healthy adult male Wistar rats weighing approximately 245–365g, were obtained and divided equally into six groups (Table 1). In five groups, T1DM was induced by an intraperitoneal (i.p) injection of Streptozotocin (STZ) (Sigma Aldrich Co.-USA) with a dosage of 55 mg/kg. Blood glucose levels were assessed three days later, the time-point when treatments were initiated [9-11]. The treatments were managed according to Table 1 by two different doses (low dose and high dose) of two the heat-killed *Actinomycetales species,* including *G. bronchialis* (Gb) and *T. inchonensis* (Ti), and also normal saline (for the diabetic and healthy control groups) [8-11], which administered orally applying intragastric gavage technique for 14 consecutive days. The animals were monitored daily for 21 days. Blood specimens were collected after anesthesia (by i.p administration of 50 and 8 mg/kg BW of ketamine and xylazine, respectively) on the 7th, 14th and 21st days. Sera were discreet at 750 × g for 15 min for upcoming biochemical and immunological assessments. Besides, five rats in each group were euthanized, and tissue specimens from the liver and kidney were collected for histopathological examination, which were fixed in 10% buffered formalin.

Ethical approval

The experiment was authorized by the Research Ethics Committee, Tabriz University of Medical Sciences, Iran (ethical approval code: 5-4-1171).

Biochemical assays

Serum biochemical indicators assessment

All of the examined biochemical indicators, such as blood glucose levels and serum insulin values, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT), blood urea and creatinine, albumin and total protein were evaluated on the 7th, 14th and 21st sampling days of sampling by commercially available kits following the manufacture ’ s instructions (Pars Azmoon,

Tehran, Iran) and using a spectrophotometer (Photometer 5010, Berlin, Germany). The activities of AST and ALT were evaluated by a modified method of Reitman-Frankel at 340 nm [24]. The measurement of blood urea, and creatinine was performed based on the methods of diacetyl monoxime (546 nm) and Jaffe (500 nm), respectively [24]. Besides, the evaluation of serum albumin and total protein was performed according to the methods of bromocresyl green (546 nm) and biuret (580 nm), respectively [24].

IL-1β, IL-2 and CRP evaluation

The levels of IL-1β, IL-2, and CRP were assessed in the preserved serum samples on the 14th and 21st sampling days using Rat ELISA commercial kits (Koma Biotech, Korea) in accordance with manufacturer's instructions [25].

Histopathological examination

The formalin-fixed tissue samples were underwent standard processing, sectioned, and stained with common hematoxylin and eosin (H&E), and then studied microscopically under a light microscope (CH-3, Olympus, Japan). The tissue sections were examined for pathological changes such as atrophy, necrosis, vascular congestion and hemorrhage [26].

Statistical analysis

The provided data were analyzed using SPSS software (SPSS, version 16 for Windows, USA). More specifically, the ANOVA and non-parametric tests were employed to statistically analyze the serum parameters and pathological lesions across the different groups, respectively, and a $p < 0.05$ was deemed significant.

Abbreviations

DM: Diabetes Mellitus

T1DM: Type 1 DM

T2DM: Type 2 DM

Gb: *Gordonia bronchialis*

Ti: *Tsukamurella inchonensis*

CRP: C- Reactive protein

STZ: Streptozotocin

AST: aspartate aminotransferase

ALT: alanine aminotransferase

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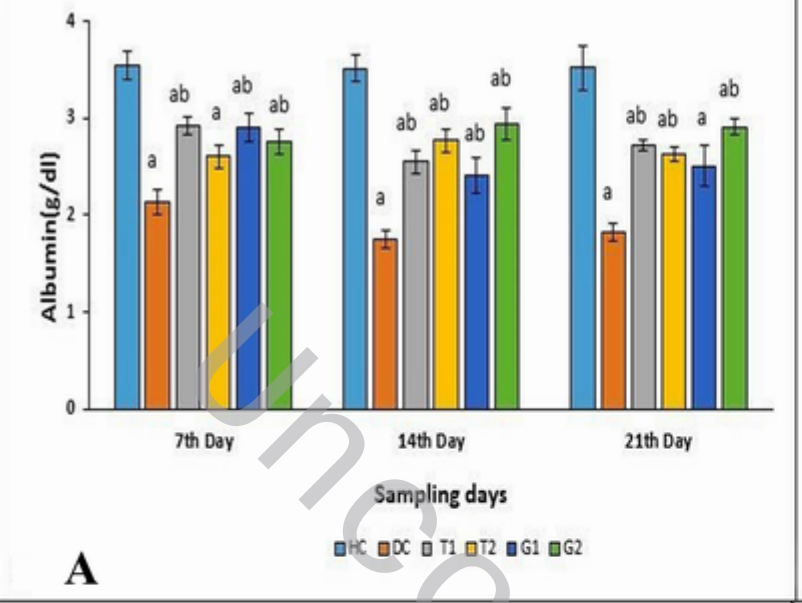
Figure Legends:

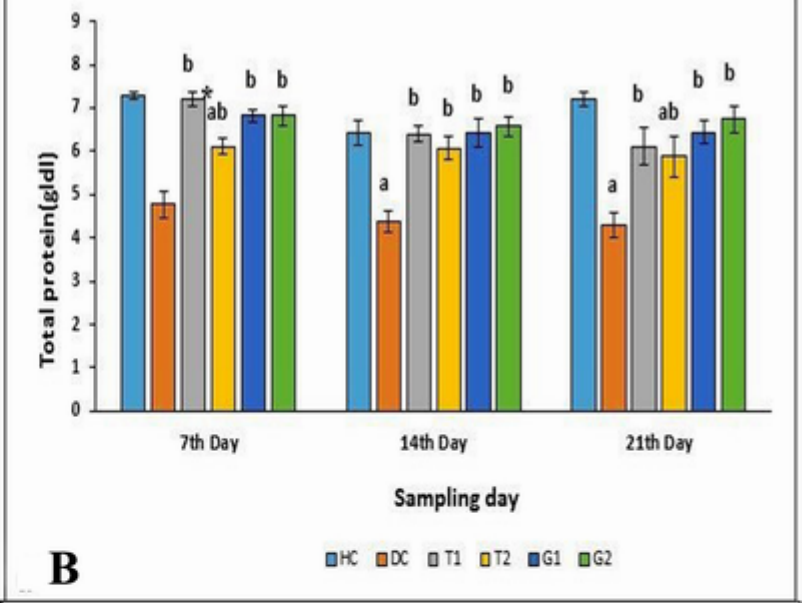
Figure 1. The effects of oral administration of *Actinomycetales species* on the serum levels of albumin (A), total protein (B), urea (C), creatinine (D), AST (E), ALT (F), CRP/Il-2/IL-1β/day 14 (G), and CRP/Il-2/IL-1β/day 21 on STZ-induced diabetes. Data are presented as the mean ± SD. Differences were considered significant with $p < 0.05$. **a**: a significant difference with healthy control (HC); **b**: a significant difference with diabetic control (DC); *: a significant difference between low dose and high dose treated groups.

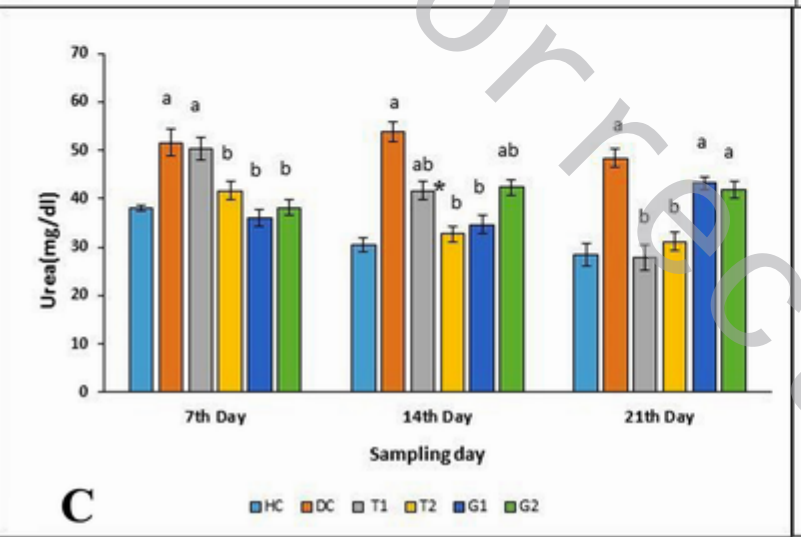
Figure 3. Liver, rat, STZ-induced diabetes. **a**: healthy control with a normal liver structure; **b**: diabetic control with severe cell swelling and hemorrhage (arrows); **c**: high dose Ti-recipient group (T2) with mild cell swelling; **d**: low dose Ti- recipient group (T1) with mild to moderate cell swelling; **e**: low dose Gb- recipient group (G1) with mild to moderate cell swelling and hemorrhage (arrows); **f**: high dose Gb-recipient group (G2) with mild cell swelling and focal hemorrhage (arrow). H&E.

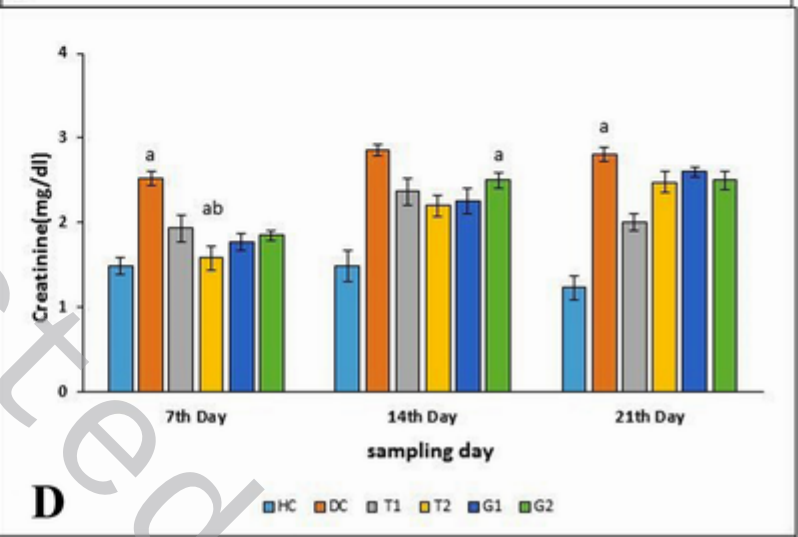
Figure 3. Kidney, rat, STZ-induced diabetes. **a**: healthy control with a normal renal parenchymal structure; **b**: diabetic control showed severe to moderate tubular epithelium degeneration (d), vacuolization and cell necrosis (n), congestion (c) and hemorrhage (h) associated with enhancement of urinary space (us); **c**: low dose Gb-recipient group (G1) with mild congestion; **d**: high dose Gb- recipient group (G2) with mild congestion; **e**: low dose Ti- recipient group (T1) with mild to moderate congestion and cellular degeneration (arrows); **f**: high dose Ti-recipient group (T2) with mild to moderate congestion and cellular degeneration (arrows). H&E.

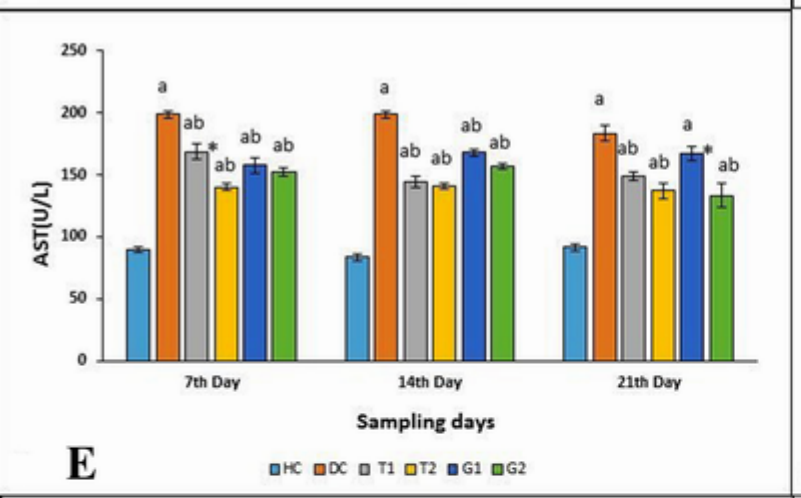
Figure S1. The effects of oral administration of *Actinomycetales species* on the serum levels of glucose (A) and insulin (B) on STZ-induced diabetes. Data are presented as the mean ± SD. Differences were considered significant with $p < 0.05$. **a**: a significant difference with healthy control (HC); **b**: a significant difference with diabetic control (DC); *: a significant difference between low dose and high dose treated groups.

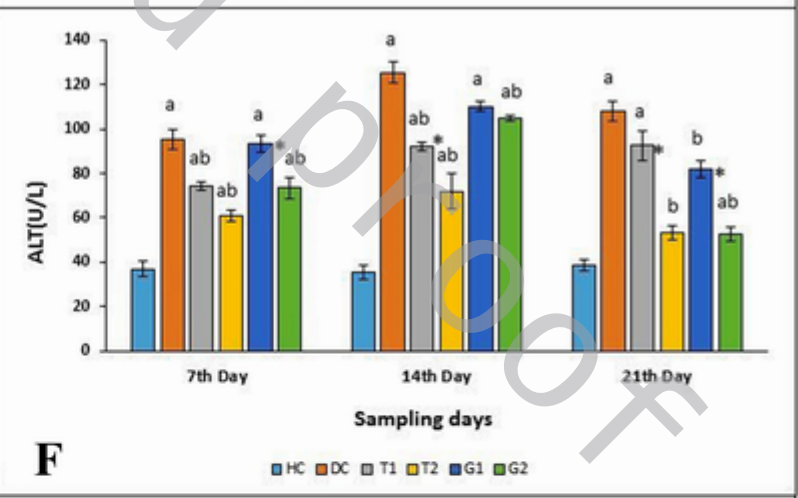


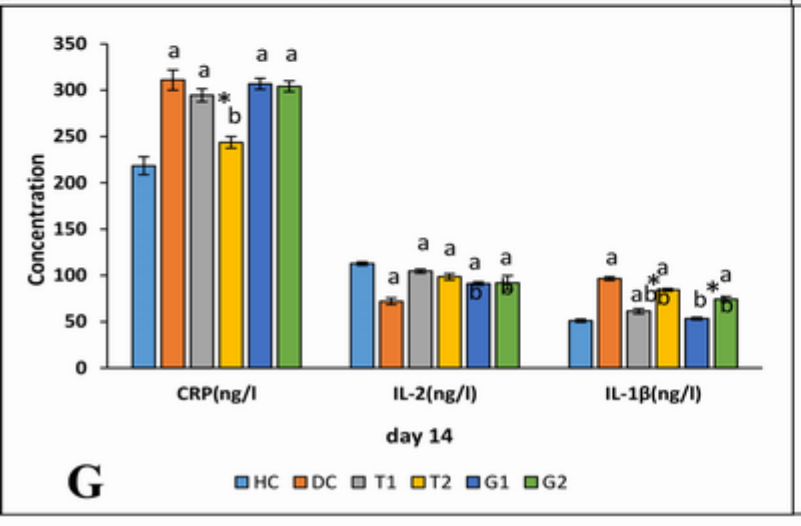


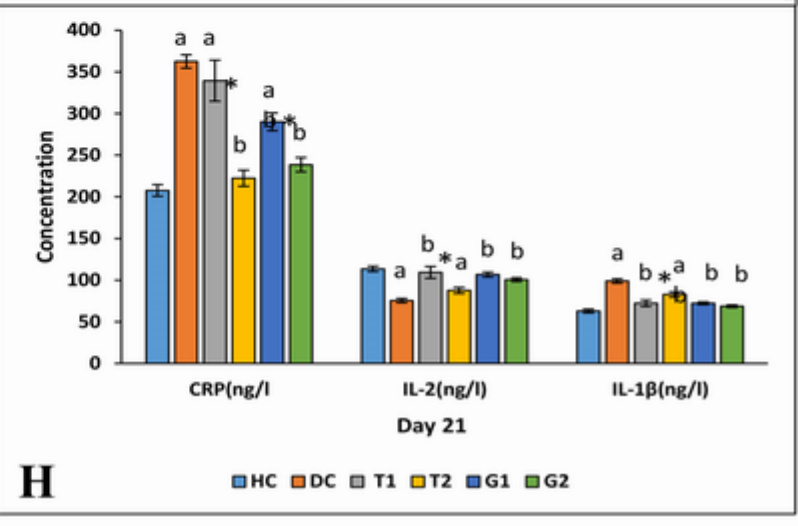


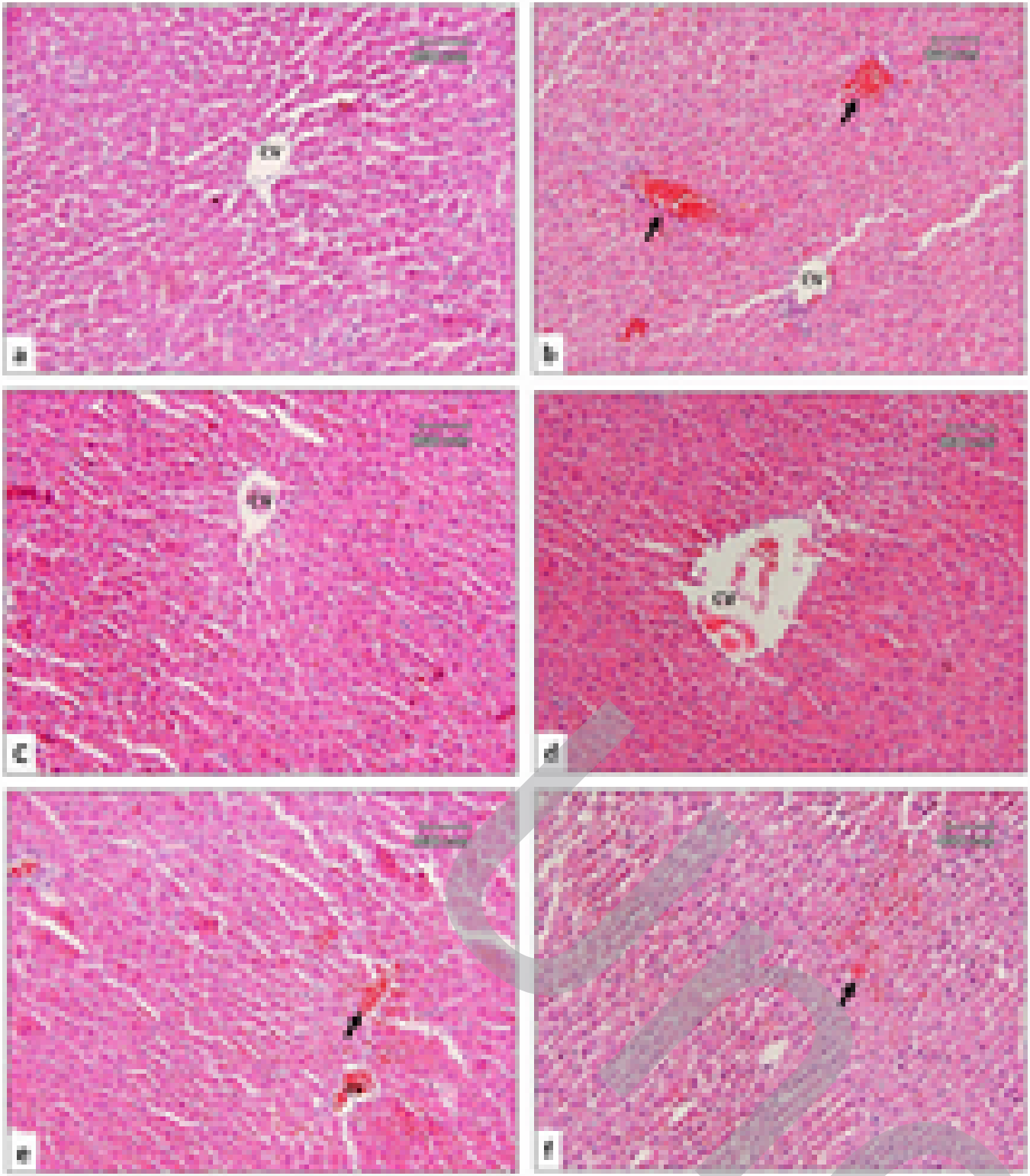








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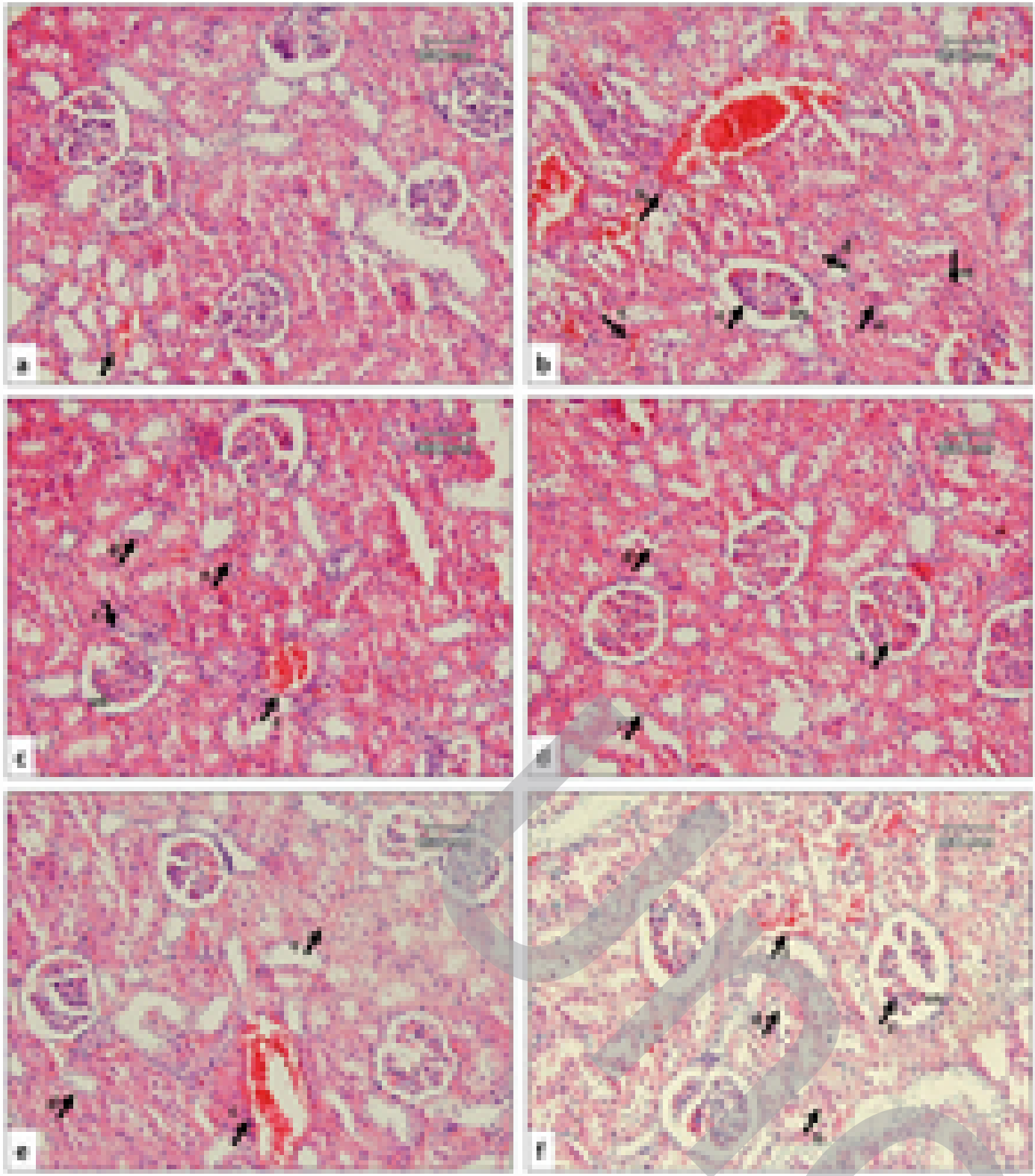
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Table 1. Different treatments were conducted into six groups of 10 rats each in the present study.

groups	Treatment for 14-contineous days
Low dose Gb	Diabetes treated with 10^5 CFU/rat* *G. bronchialis*
High dose Gb	Diabetes treated with 10^7 CFU/rat *G. bronchialis*
Low dose Ti	Diabetes treated with 10^5 CFU/rat *T. inchonensis*
High dose Ti	Diabetes treated with 10^7 CFU/rat *T. inchonensis*
Diabetic control	Diabetes treated with normal saline
Healthy control	No diabetes treated with normal saline

*CFU/rat: Colony Forming Unit).

Wistar rat (male)
n=60

n=10

Normal saline
Healthy control

STz+Normal saline
Diabetic control

STz+Low dose Ti
T1

STz+high dose Ti
T2

STz+Low dose Gb
G1

STz+High dose Gb
G2

Ti: *Tsukamurella inchonensis*

Gb: *Gordonia bronchialis*

Examined parameters

- ALT
- AST
- Total protein
- Albumin
- BUN
- Cratinine
- IL-1β
- CRP
- Liver
- Kidney

Histopathology