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Original article

Protective effect of ganoderic acid against the streptozotocin induced diabetes, inflammation, hyperlipidemia and microbiota imbalance in diabetic rats

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ABSTRACT

Diabetes mellitus (DM) is a metabolic disorder with numerous symptoms categorized via serves hyperglycemia effect along with altered fat, protein and carbohydrate metabolism mainly resultant from defects in insulin action/secretion or both. The aim of the current experimental study was to comfort the neuroprotective effect of ganoderic acid against the streptozotocin (STZ)-induced type I diabetes mellitus in mice and explore the underlying mechanism. Differentiation of 3T3-L1 preadipocytes effect; hepatic and glucose consumption effect of ganoderic acid was estimated on HepG2 cell lines and peroxisome proliferator-activated receptor (PPAR). FFA content was estimated in adipose and hepatic tissues. Ganoderic acid induced the 3T3-L1 preadipocytes differentiation. The mRNA expression of PPAR was increased in the high glucose-treated group in HepG2 and ganoderic acid treatment down-regulated the mRNA expression of PPAR. Ganoderic acid exhibited the inhibitory effect of α-glucosidase and α-amylase. Ganoderic acid demonstrated the reduced blood glucose and increase insulin level and also reduced the free fatty in hepatic and adipose tissue. Histopathological study showed the enhancement of β-cells in ganoderic acid-treated mice. Finally, their prebiotic effects on gut microbiota were illustrated via enhancing the population of diabetes resistant bacteria and also reducing the quantity of diabetes sensitive bacteria. Ganoderic acid attenuated STZ induced T1DM in mice via inflammatory pathways.

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder with numerous symptoms categorized via serve hyperglycemia along with altered fat, protein and carbohydrate metabolism mainly resultant from defects in insulin action/secretion or both(Reaven and Reaven, 2018). Diabetes is considered as the major health problem worldwide (Mellitus, 1999); and the total number of people suffer from the disease has reached up to 500 million worldwide, while the total number of people suffer from the diabetes is going up to 700 million via 2040 (Poretsky, 2010). DM has appeared as an enormous risk to human health and this might enhance gradually in the next 25 years (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003). Right now available treatments for DM are α-glucosidase and dipeptidyl peptidase-4 inhibitors, thiazolidinedione's, biguanides and sulfonylureas, but these treatments having a limitation with more side effects (Cholesterol Treatment Trialists'; (CTT) Collaborators, 2008). Right now, urgent need of more potential treatment requires for the treatment of DM. Now, researchers now focus on their research on an alternative system of medicine which has a more protective effect with less side effects (Group, 1995). The Chinese olden system medicines are habituated to treating various ailments for an extended history. Several plant-based medicines were tested as anti-hyperglycemic and successfully in clinical trials (Olokoba et al., 2012). Various plant-based drug and medicinal plants show a valuable alternative for the treatment of diabetes mellitus due to their functions in alteration of carbohydrate enzymes and maintaining the glucose homeostasis (Group, 1995).

Type I diabetes mellitus (T1DM) is considered as the autoimmune disease categorized via T-cell mediated autoimmune etiology in a human which destroy the selective insulin oozing pancreatic β-cells via β-cells antigens and leading to insulin

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deficiency (Bastaki, 2005). Even if the exact induction of T1DM expansion is not clear, but it appears to consequence a complex interaction between environmental and genetic factors (Group, 1995). T1DM is the crucial form of diabetes occurred in children, and 95% of childhood diabetes cases having T1DM (American Diabetes Association, 2009). According to the WHO, 70,000 children under the age of 15 years develop the T1DM Worldwide and the incidence of growing T1DM via 3–5% every year. While the available successful treatment is islet transplantation, which affords the hopeful approach for T1DM treatment, but this treatment having limitation such as lack of adequate organs donor, higher cost of transplantation (islet), chance for destruction of transplantation islet, ongoing immune-mediated destruction of islet cells and expansion the side effect from the immunosuppressive drugs. Hence, the search for a cost-effective and novel drug that can avoid or treat the T1DM could be a significant approach to reduce the burden of disease.

Several publish literature suggest that the inflammatory reaction play an important role in the development of T1DM (Al-Goblan et al., 2014). The infiltration of inflammatory reaction into the islets cells and activated the various inflammatory mediators (Kumar et al., 2013a). Activated macrophages and T-cells secrete the various pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interferon-ϒ (IFN-ϒ), which are the important mediators leading to the destruction of β-cell in T1DM (Roggli et al., 2010). Previously published literature suggest that IL-4 avoid the insulitis and T1DM via potentiating the function of T helper 2 cell (Th2) (López-Jaramillo et al., 2014; Ye and McGuinness, 2013). TGF-β can alter the TNF-α, IL-1β and IFN-ϒ expression, NK cells, macrophages and β cells (Al-Goblan et al., 2014; Roggli et al., 2010; Stumvoll et al., 2005).

Ganoderma lucidum have been traditionally used for treating of various types of diseases especially lowering the blood glucose level. *G. lucidum* is a medicinal mushroom-forming white rot fungus (Jin et al., 2016; Kao et al., 2013). Ganoderic acid (GA) is a bioactive secondary metabolite of basidomycetous fungi *Ganoderma lucidum* (Jin et al., 2016; Kao et al., 2013). The polysaccharides and triterpenoids in it are consider as the most important pharmacologically active constituents of *G. lucidum*. Several researchers have suggested the hepato-protective, antihypertensive, hypocholesterolemic, anti-histaminic, antitumor and antiangiogenic nature of triterpenoid ("Ganoderma lucidum," 2010; Jin et al., 2016; Kao et al., 2013; Mau et al., 2002). Moreover, polysaccharides showed the beneficial effect against the free radicals, reduce cell damage induced by mutagens and inflammatory reactions (Jin et al., 2016; Sliva, 2003). Due to nature of anti-oxidant and anti-inflammatory of ganoderic acid, the current experimentally study was designed to scrutinized the anti-diabetic effect of ganoderic acid in Streptozotocin (STZ) induced diabetic mice via inflammatory pathway.

2. Material and method

2.1. Chemical

Ganoderic acid, 4-nitrophenyl α-D-glucopyranoside (pNPG), Streptozotocin (STZ), 3-(4,5-Dimethyl thiazol-2-yl)-5-diphenyl tetrazolium bromide (MTT), dexamethasone, O, and α-glucosidase, 3-isobutyl-1-methylxanthine (IBMX), and insulin was purchased from the Sigma Aldrich. The primer used in the current experimental study was synthesized through Invitrogen Biotechnology (USA). The diagnostic kits such as total cholesterol (TC), triacylglycerols (TGs), aspartate aminotransferase (AST) and alanine aminotransaminase (ALT) were purchased from Shanghai China (from Shanghai Rongsheng Biotech Co., Ltd.).

2.2. Invitro α-glucosidase activity

The invitro α-glucosidase activity was estimated via using the previously reported method of Kumar et al., with minor modification of Ahmed et al., method (Ahmed et al., 2015, 2014a; Kumar et al., 2013c). Briefly, different concentration of ganoderic acid mixed with a 20 μl solution of α-glucosidase enzymes, which contain the phosphate buffer saline and α-glucosidase. The reaction mixture was incubated for 15 min at room temperature and the absorbance was estimated at 430 nm via using the 96 well microtiter plates. The percentage inhibition of α-glucosidase was estimated via using the following formula

$$\text{Inhibition}(\%) = \text{Control}_{\text{Absorbance}} - \text{Test}_{\text{Abosrbance}}\text{X}100/\text{Control}_{\text{Absorbance}}$$

2.3. α-amylase activity

Briefly, acarbose and the ganoderic acid sample was mixed and incubated for 15 min; after that added the 20 mM sodium phosphate and incubated for 5 min and starch was added for making up the volume till 2 ml and reaction mixture again incubated at room temperature for 5 min. The reaction mixture was incubated at a boiling water bath for 10 min, the reaction mixture cool on ice and added the deionized water and estimated the absorbance at 540 nm (Ahmed et al., 2014b; Kumar et al., 2014; Mishra et al., 2013). The percentage inhibition of α-amylase was estimated via using the above discuss formula.

2.4. Cell lines

HepG2 and 3T3-L1 cell lines were received from the Type culture collection, CCTCC, Wuhan, China.

2.5. Cell viability assay

Cell lines such as HepG2 and 3T3-L1 were harvested in DMEM (low and high glucose) with fetal bovine serum (10%). All the cells (2×10^4) were seeded in 96 well plates and incubated for 1 day. The cells were treated with ganoderic acid and glibenclamide at different concentration for 2 days. MTT assay was used for the estimation of the cell viability. Briefly, the test and media were replaced with the MTT and incubated at room temperature for 2–4 h and the absorbance of the samples was estimated at 492 nm (Kumar et al., 2017; Verma et al., 2017).

2.6. 3T3-L1 preadipocyte differentiation

For the estimation of 3T3-L1 preadipocyte differentiation, 6 well plates at a density (2×10^4 cells/mL). The differentiated of the 3T3-L1 cells were performed in the DMEM solution containing insulin (10 μg/mL), IBMX (0.5 mM), dexamethasone (1 μM) and FBS (10%) and incubated for 48 h. After 48 h, the cells were kept in the DMEM containing insulin and FBS. The DMEM media changed to basic medium after every 48 h until the cells were harvested. For adipogenesis, the test samples were mixed to the DIM, DMM and basic medium until the cells harvested. Oil Red O was used for the stained the lipid droplets via using the previous method with minor modification (Hamza et al., 2009; Makihara et al., 2016). MTT viability assay was used for the estimation of the nano cytotoxic effect of 3T3-L1 (Kim and Chen, 2004).

2.7. HepG2 cell

The HepG2 cells were harvested in 6-well plates at the density (1×10^6 cells/well) for 24 h. The cells were grown to confluence and made quiescent in serum culture free DMEM low glucose for 12 h. The cells were maintained at high and low glucose in the absence or presence of ganoderic acid for next 48 h.

2.8. RNA isolation and quantitative RT-PCR

Target gene mRNA levels were normalized to those of β-actin using the $2 - \Delta\Delta CT$ method. The fold change was calculated in comparison with the control group, and the fold change of >1.5 was considered statistically significant. Trizol reagent was used for the isolation of RNA from each well via using the manufacturer's instruction. The complementary cDNA was isolated via using the cDNA synthesis kits. Table 1 showed the sequence of primers used and β-actin used as a standard. Target gene mRNA level was standardized to those of β-actin using the $2 - \Delta\Delta CT$ method and estimated the fold change via comparison to control and fold change >1.5 consider as statistically significant.

2.9. Experimental study

2.9.1. Animal

Swiss Albino mice (20 ± 5 g, body weight, either both sex) was used for the current experimental study. For the current experimental study, the rats were received from the Laboratory Animal Centre of Zhengzhou University. The animal procured from the Institutional Animal House and kept in the standard environmental condition (23 ± 5 °C, 12 h dark and light cycle) and received the standard diet pellet (Rodent food Ltd., China) and water *ad libitum*. The current experimental protocol was approved from the Institutional Animal Ethical Committee clearance of the committee for the purpose of control and supervision of experimental on Animal IAC/ZU/0382.

2.9.2. Experimental animal

After the acclimatization (1 week) the mice were divided into 2 groups as following control group (n = 24) and multiple doses treated STZ-induced DM (n = 72). The control group further divided into two groups. The control group divided as Gp-I normal control and Gp-II normal control received ganoderic acid (20 mg/kg). Multiple low doses of STZ-induced group received the intraperitoneal injection of STZ (40 mg/kg, body weight) for 5 consecutive days. After the 1 week, all group mice blood glucose levels were estimated. Before estimation of the blood glucose level, the mice fasted overnight and a blood sample was collected from the tail vein. Blood glucose meter was used for the estimation of the blood glucose level. The mice having blood glucose level >220 mg/dl is considered as diabetic. The STZ induced mice were further divided into 4 groups and each group contains 18 mice. Gp-III (STZ+ ganoderic acid 10 mg/kg, body weight); Gp-IV (STZ+ ganoderic acid 20 mg/kg, body weight); Gp-V (STZ+ glibenclamide 2.5 mg/kg, body weight) or Gp-VI (Gp-III (STZ only). The mice have received the above-discussed dose via oral gavages once per day for next 2 weeks. At the end of the experimental study, the mice were not received the fed (18 h) and the mice were further used for the oral glucose tolerance test. After that, the mice were sacrificed via cervical dislocation and blood samples and different tissues were immediately collected for further estimation (American Diabetes Association, 2009; Rees and Alcolado, 2005).

2.9.3. Body weight and blood glucose level

Throughout the experimental study, body weight was recorded at regular interval. Body weight loss is defined as the difference between day 1 (the first day of treatment) and the weight before sacrifice. Body weight and blood glucose level were estimated at regular interval. The difference between the body weight and blood glucose level were recorded from the first day of treatment (day 1) and till the end of the experimental study.

2.9.4. Oral glucose tolerance test

For the OGTT, the blood glucose level was estimated after the overnight fasted mice (16 h). The estimation of initial blood glucose level was considered as time 0. Mice have received the oral administration of 2 g/kg of glucose solution. The blood glucose level was estimated at a regular interval (30, 60, 120 and 180 min).

2.9.5. Biochemical analysis

For the estimation of biochemical parameters, the blood samples were collected from the puncturing the retro-orbital of fasting mice. Lipid parameters such as total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL), high density lipoprotein (HDL) and very low density lipoprotein (VLDL); hepatic parameters such as ALT, AST and ALP were estimated via using the commercial assay kits via following the manufacturer's instruction. The plasma insulin level was estimated of non-fasting mice at regular interval via using the reported method of Kumar et al., with minor modification (Kumar et al., 2013a, 2013b).

2.9.6. Estimation of FFA in adipose tissue and liver

The epididymal adipose tissue and liver tissue were collected and FFA was estimated via using the commercially available assay kits via using the manufacturer's instruction.

2.10. Fecal bacterial DNA extraction

TIANamp stool DNA kits were used for the extraction of bacterial DNA of each fecal samples via using the manufacture's instruction. Briefly, gel electrophoresis was used for the estimation of size and integrity of DNA sample. Previously reported method was used for the amplified the rRNA gene from the microbial genome DNA (Kennedy et al., 2014; Nechvatal et al., 2008). The reverse primers S-D-Bact-0785-b-A-18 (5′-TACNVGGGTATCTAATCC-3′) and forwarded primers S-D-Bact-0564-a-S-15 (5′-AYTGGGYDTAAAGNG-3′) were prepared according to the previous reported method with minor modification (Judelson, 2013; Rozen and Skaletsky, 2000). During the PCR reaction, DNA (5–100 ng) sample, $MgCl_2$ (1 mM), Green Master Mix (1·GoTaq) and primer (2 pmol) and mention the thermal cycling condition. Qiaquick 96 kits and quantified via

Table 1
List of primers.

S. No	Gene	Primer sequence	
		Forward	Reverse
1	PPAR-α	5′-CCTCGGTGACTTATCCTGTGGT-3′	5′-GACATCCCGACAGAAAGGCAC-3′
2	β-actin	5′-CACCCAGCACAATGAAGATCAAGAT-3′	CCAGTTTTTAAATCCTGAGTCAAGC-3′

using the PicoGreen dsDNA reagent using the manufacture instructions.

2.11. Histopathological examination

For the histopathological examination, the pancreas tissue and liver tissue was collected and fixed in the 10% solution of formalin and embedded in paraffin. 5 μm portions were cut and stained with the eosin and hematoxylin.

2.12. Statically analysis

All the data presented in the current experimental study in the form of mean ± SD. Post hoc testing was used for the estimation of statistical significance via using the GraphPad Prism. $p < 0.05$ was considered as statistically significant.

3. Result

Scrutinize the FFA released from the liver and adipose tissues

During the diabetes the level of FFA content considerably increased. Fig. 1 showed the effect of ganoderic acid on the epididymal adipose tissue. Fig. 2b exhibited the significant difference between the STZ induced and ganoderic acid treated group rats. FFA content was significantly higher in STZ induced group rats (4785 ± 145.67) and dose-dependent treatment of ganoderic acid reduced the FFA content in adipose tissue (1174.34 ± 129.4) at a dose of 20 mg/kg. A similar effect was observed in the liver tissue (Fig. 2a). STZ induced group mice showed the 171 ± 12.34 FFA content in liver and dose-dependently treatment of ganoderic acid significantly ($P < 0.001$) reduced the FFA content (108.34 ± 7.97) at a dose of 20 mg/kg.

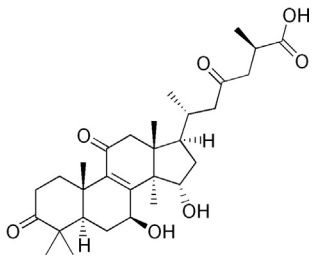


Fig. 1. The structure of ganoderic acid.

3.1. α-Glucosidase and α-amylase inhibitory effect

Table 2 showed the inhibitory effect of ganoderic acid. The gandoeric acid demonstrated the inhibition of α- glucosidase, α-amylase and DPPIV (IC_{50} = 78.45 ± 1.64, 56.23 ± 2.38 and 42.34 ± 1.82 μg/mL), respectively. For the positive control acarbose (well-known α-glucosidase inhibitor) and vildagliptin (well-known DPPIV inhibitor) showed the moderate α-glucosidase and DPPIV inhibitory effect with an IC_{50} value of 28.76 ± 1.35 and 14.37 ± 1.02 μg/mL.

3.2. 3T3-L1 preadipocyte differentiation

Fig. 3a showed the cytotoxic effect of ganoderic acid on the 3T3-L1 Preadipocyte. The high concentration of ganderic acid (12.5, 25 and 50 μg/mL) was used for the current experimental study. The 3T3-L1 preadiopocytes were incubated with ganoderic acid and glibenclamide during the oil-red O staining and adipogenesis was performed at 10 days after the incubation of differentiation. Fig. 3b showed the estimation the absorbance of oil Red O stained lipid droplets. The induced differentiation was estimated via TG content in the cytoplasm, glibenclamide treatment induced showed the significant differentiation as compared with induced control group (Table 3). The differentiation rate of ganoderic increase in a dose-dependent manner. Fig. 3c demonstrated the lipid deposition of different groups. Glucose consumption was significantly increased in the glibenclamide and ganoderic acid group.

3.3. Quantification of PPAR-α

For the confirmation of molecular mechanism of ganoderic acid in HepG2 cells exposed to low and high glucose concentration, for investigating the involvement of PPAR-α in fatty acid metabolism in the liver. Fig. 4 showed that the expression of PPAR-α was increased in high glucose-treated HepG2 cells. The mRNA expression of PPAR-α was significantly increased 3 fold as compared to control group. Ganoderic acid down-regulated the gene expression of PPAR-α as compared to the control group.

Table 2
The effect of ganoderic acid on the enzyme.

S. No	Group	IC 50 (μg/mL)		
		α-amylase	α-glucosidase	Dipeptidyl peptidase IV
1	Ganoderic acid	56.23 ± 2.38	78.45 ± 1.64	42.34 ± 1.82
2	Vildagliptin	–	–	14.37 ± 1.02
3	Acarbose	–	28.76 ± 1.35	–

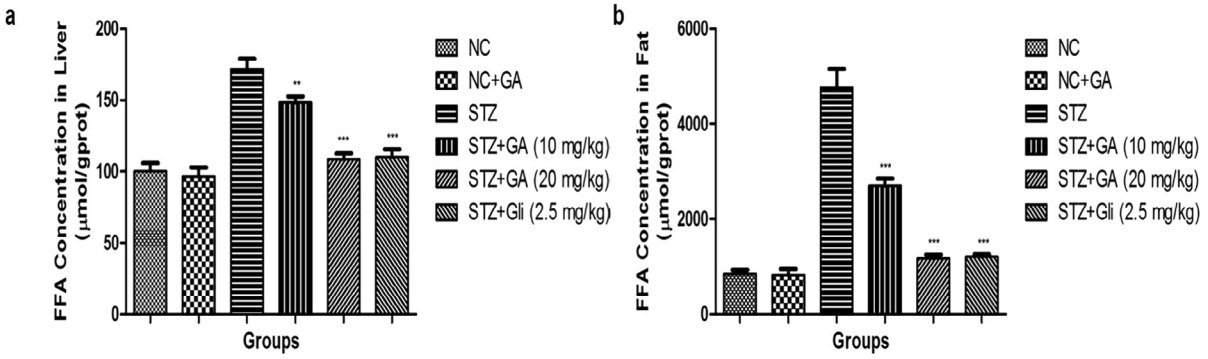


Fig. 2. The effect of ganoderic acid on FFA in liver and epididymal adipose tissues. Statistically significant different are indicated via asterisks and estimated via ANOVA. $^{***}p < 0.001$, $^{**}p < 0.001$, $^{*}p < 0.05$ compared with the model group.

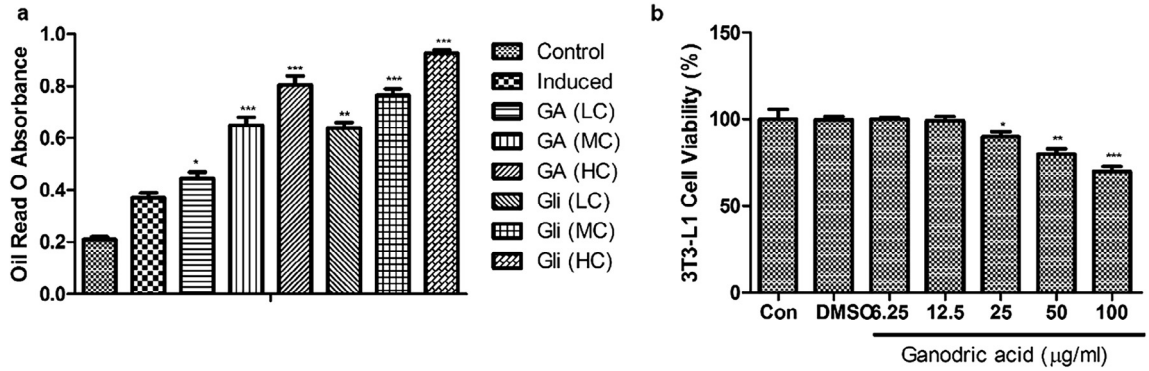


Fig. 3. The effect of ganoderic acid on 3T3-L1 preadipocyte differentiation. (a) MTT. Statistically significant different are indicated via asterisks and estimated via ANOVA. $^{***}p < 0.001$, $^{**}p < 0.001$, $^{*}p < 0.05$ compared with the model group.

Table 3
. Glucose consumption of ganderic acid in 3T3-L1 adipocyte.

S. No	Group	Glucose consumption (mmol/L)
1	Control	9.83 ± 0.63
2	Ganoderic acid (6.25 μg/mL)	11.34 ± 0.72
3	Ganoderic acid (12.5 μg/mL)	11.73 ± 0.34
4	Ganoderic acid (25 μg/mL)	11.87 ± 0.63
5	Ganoderic acid (50 μg/mL)	12.04 ± 0.37
6	Ganoderic acid (100 μg/mL)	12.43 ± 0.84
7	Glibanclamide (1 μg/mL)	12.62 ± 0.67

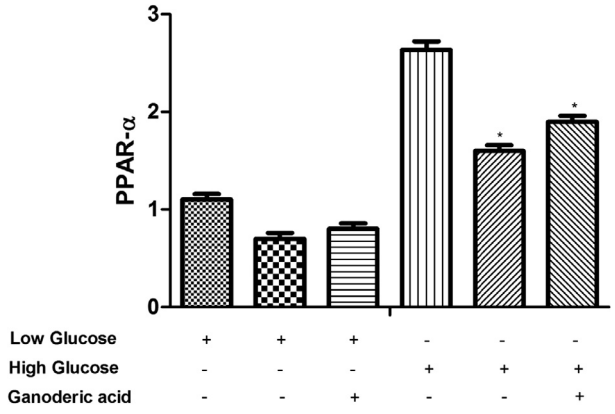


Fig. 4. Ganoderic acid inhibit the hepatic steatosis in STZ-induced T1DM. Statistically significant different are indicated via asterisks and estimated via ANOVA. $^{***}p < 0.001$, $^{**}p < 0.001$, $^{*}p < 0.05$ compared with the model group.

3.4. Ganoderic acid improves glucose tolerance

Glucose tolerance test was performed for the estimation of glucose utilization of rodent. Fig. 5 showed the effect of ganoderic acid on the oral glucose tolerance test. Fig. 5a demonstrated that the ganoderic acid treatment reduced the blood glucose level to about 50% in first 60 min. After that, the ganoderic acid treatment reduced the glucose level after the 1 h almost 75–85%. The results suggest that the ganoderic acid having the better utilization of glucose.

3.5. Renal and liver index

At end of the experimental study, the mice were sacrificed and the liver and renal tissues were removed and collected and separately weighted to estimate the viscera index. Fig. 5b showed the increased renal index (2.67 ± 0.22) of STZ induced group mice and ganoderic acid treatment reduced the renal index (1.31 ± 0.17). A similar result was observed in the glibenclamide group mice (1.41 ± 0.13). A similar trend was observed in the liver index, STZ induced group mice showed the increased liver index (6.92 ± 0.35) and ganoderic acid treatment down-regulated the liver index (4.64 ± 0.73), respectively.

3.6. Effect of ganoderic acid on blood glucose level and body weight

During the diabetes mellitus, blood glucose level was increased in both types of diabetes. A similar result was observed in our experimental study, the blood glucose level of STZ induced mice was increased at end of the experimental study. The level of blood glucose level start increased from the day 1 and remain increased end of the experimental study. Gandoeric acid significantly ($P < 0.001$) reduced the blood glucose level and reduced the incidence of diabetes (Fig. 6a).

Body weight is considering as the important marker for estimation the diabetes mellitus. During the diabetes, the body weight was reduced during the expand of disease. During diabetes, the body weight reduced and the same result was found in the STZ induced group mice and dose dependently treatment of ganoderic acid significantly ($P < 0.001$) improved the body weight of treated group mice (Fig. 6b).

3.7. Effect on biochemical parameters

During the diabetes, plasma insulin level decreased and blood glucose level increased. A similar momentum was observed in the STZ induced group. The plasma insulin level was considerably decreased in STZ induced group mice (22.67 ± 3.23 mUI/mL) and ganoderic treatment significantly ($P < 0.001$) improved the plasma insulin level (51.65 ± 5.32 mUI/mL) and suggest the utilization of glucose via various organ (Fig. 7a). Fig. 7b exhibited the effect of glycated haemoglobin of different group of mice. STZ induced group mice showed the increased level of glycated haemoglobin (4.02 ± 0.32 (A1c) (%) and ganoderic acid treatment showed the (1.73 ± 0.18 (A1c) (%) at a dose of 20 mg/kg, respectively. Fig. 7c and d demonstrated the effect of ganoderic acid on the glucose-6-Phosphatase and hexokinase. Ganoderic acid reduced the level of glucose-6-Phosphatase (28.46 ± 1.34 Unit/mg of tissue) and hexokinase (10.12 ± 0.63Unit/mg of tissue), respectively.

Fig. 8 showed the effect of the ganoderic acid on the hepatic parameters. Fig. 8 a and b showed the reduced level of AST (192.11 ± 4.58 U/l) and ALT (102.34 ± 3.84 U/l) in the STZ induced group mice and ganoderic acid treatment increased the level of AST (100.83 ± 5.45 U/l) and ALT (45 ± 2.83 U/l), respectively.

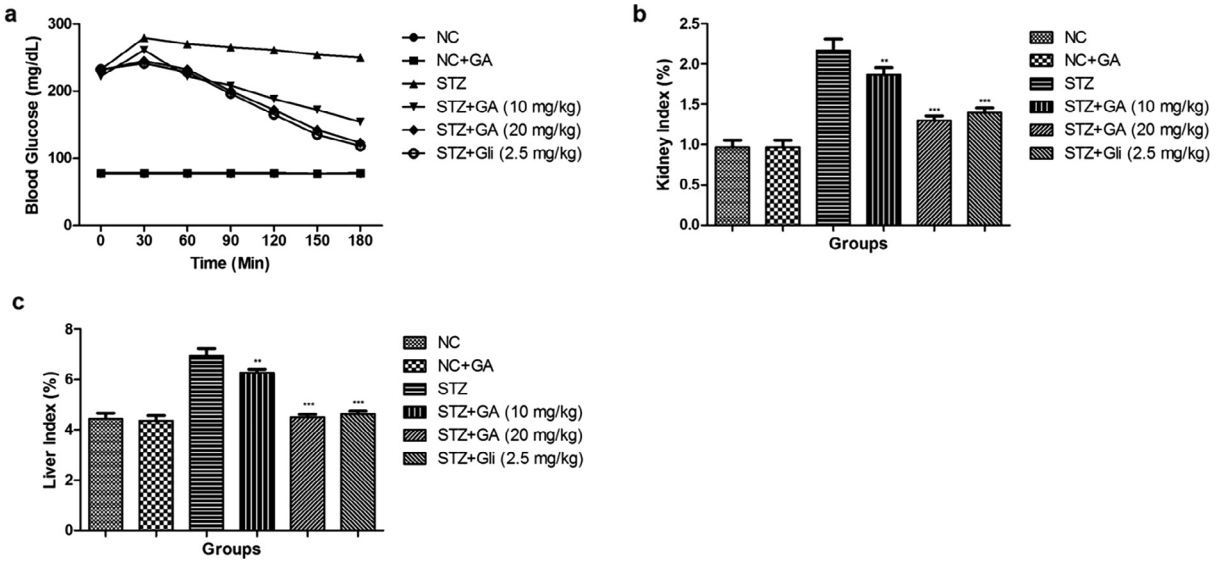


Fig. 5. The effect of ganoderic acid on the oral glucose tolerance test (OGTT). (a) OGTT, (b) kidney index and (c) liver index. Statistically significant different are indicated via asterisks and estimated via ANOVA. $^{***}p < 0.001$, $^{**}p < 0.001$, $^{*}p < 0.05$ compared with the model group.

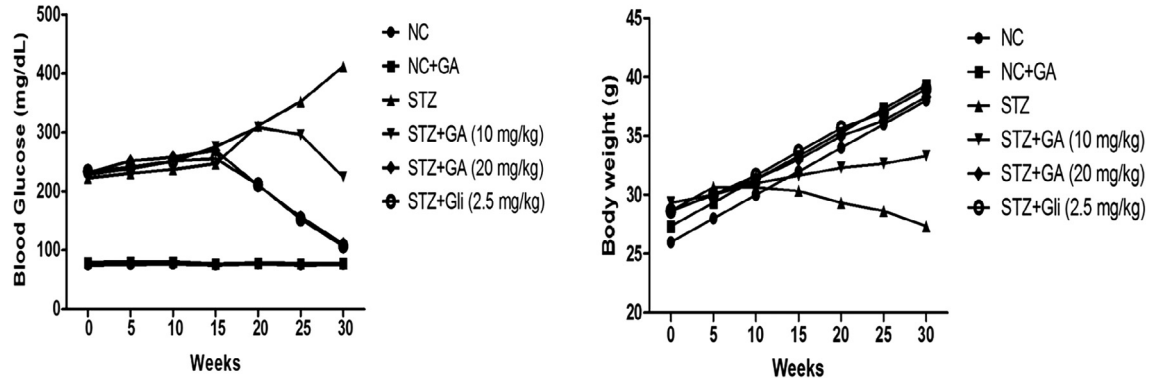


Fig. 6. The effect of ganoderic acid on the blood glucose and body weight of STZ-induced T1DM. (a) blood glucose level and (b) body weight. Statistically significant different are indicated via asterisks and estimated via ANOVA. $^{***}p < 0.001$, $^{**}p < 0.001$, $^{*}p < 0.05$ compared with the model group.

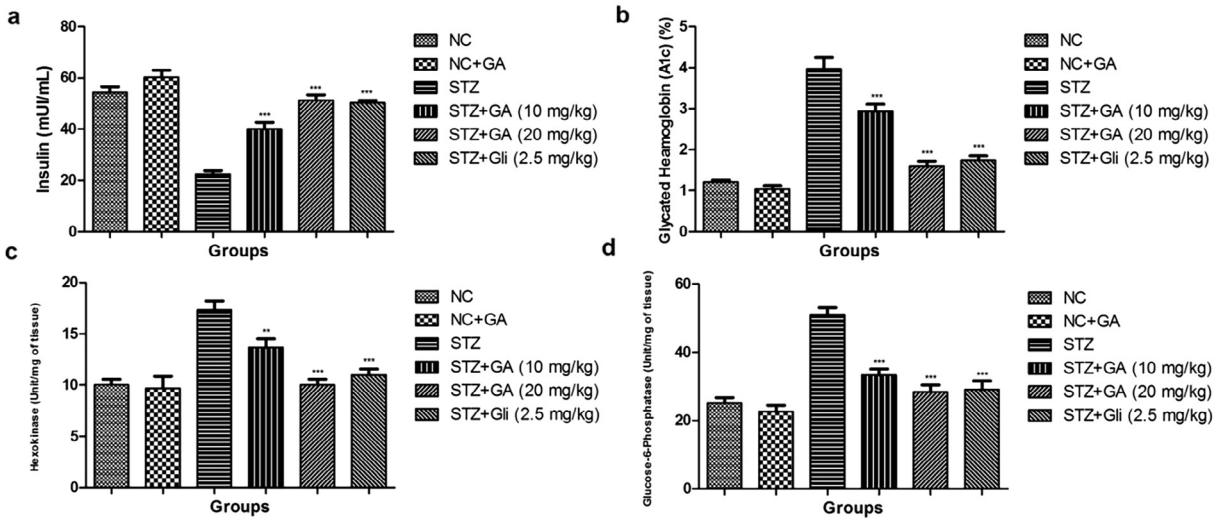


Fig. 7. The effect of ganoderic acid on the biochemical parameters of STZ-induced T1DM. (a) Plasma insulin, (b) glycated haemoglobin, (c) hexokinase and (d) glucose-6-phosphatase. Statistically significant different are indicated via asterisks and estimated via ANOVA. $^{***}p < 0.001$, $^{**}p < 0.001$, $^{*}p < 0.05$ compared with the model group.

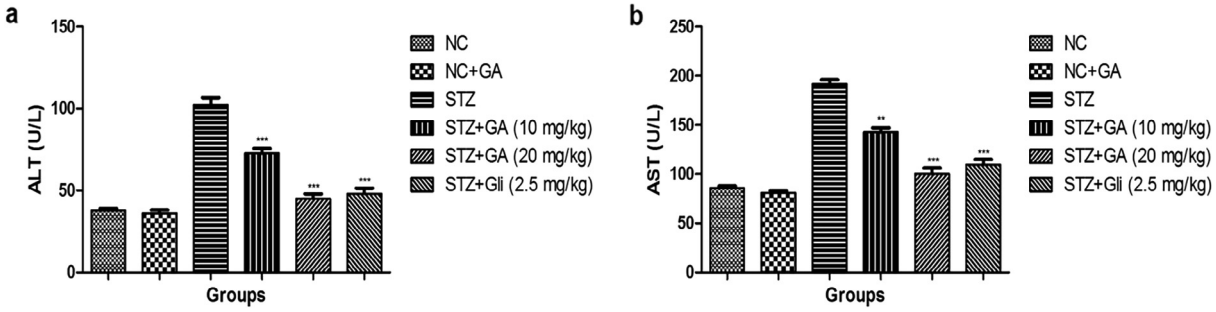


Fig. 8. The effect of ganoderic acid on the hepatic parameters of STZ-induced T1DM. (a) ALT and (b) AST. Statistically significant different are indicated via asterisks and estimated via ANOVA. $^{***}p < 0.001$, $^{**}p < 0.001$, $^{*}p < 0.05$ compared with the model group.

3.8. Effect on pro-inflammatory cytokines

In both types of diabetes mellitus, the incursion of pancreatic islets via immune cells and consequently insulitis (islets inflammation) induces the destruction of β-cell. We scrutinized the anti-diabetic effect of ganoderic acid is associated with down-regulated the islets inflammations. We estimated the array of immune regulatory pro-cytokines and chemokines such as IL-1β, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IFN-γ, MCP-1, TNF-α, MIP-1α, RANTES, and GMCSF. STZ induced mice showed the up-regulation of immune regulatory pro-cytokines and chemokines such as IL-1β, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IFN-γ, MCP-1, TNF-α, MIP-1α, RANTES and GM-CSF and ganoderic acid significantly ($P < 0.001$) down-regulated the immune regulatory cytokines and chemokines (Fig. 9).

STZ induced DM rats showed the increased expression of p-p38, p-ERK, pIkBα and p-P65 and dose dependent treatment of ganoderic acid showed the suppression the expression of p-p38, p-ERK, pIkBα and p-P65 (Fig. 10).

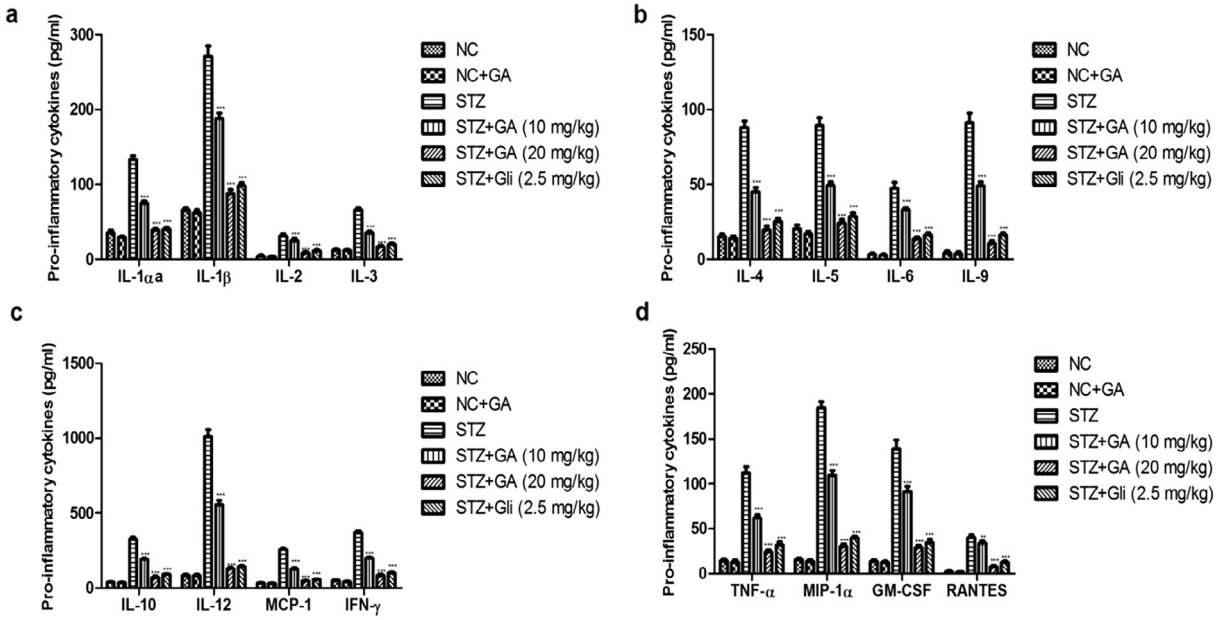


Fig. 9. The effect of ganoderic acid on the pro-inflammatory cytokines of STZ-induced T1DM. Statistically significant different are indicated via asterisks and estimated via ANOVA. $^{***}p < 0.001$, $^{**}p < 0.001$, $^{*}p < 0.05$ compared with the model group.

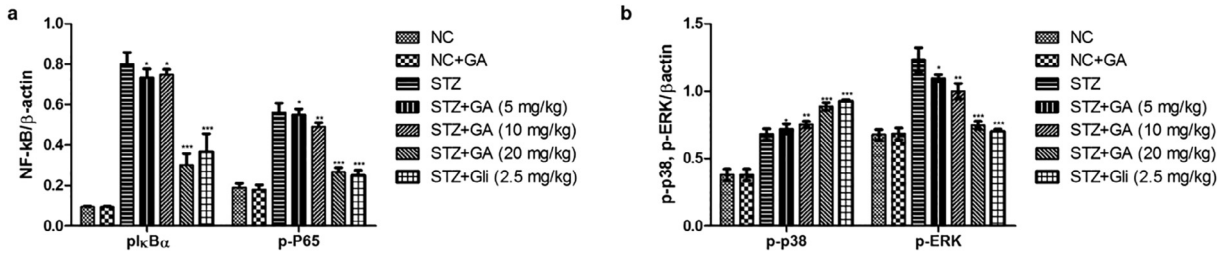


Fig. 10. The effect of ganoderic acid on NF-kB/β-actin and p-p38, p-ERK/βactin in STZ-induced T1DM. Statistically significant different are indicated via asterisks and estimated via ANOVA. $^{***}p < 0.001$, $^{**}p < 0.001$, $^{*}p < 0.05$ compared with the model group.

3.9. Effect of ganoderic acid on gut microbiota

Tables 4 and 5 showed the effect of normal control and ganoderic acid on the gut microbiota. The variations in the 16S rRNA gene sequence reads of fecal content of rats at the phylum and order level. *Bacteroidetes Proteobacteria* and *Firmicutes* bacteria was present in the feces of normal rats and the level of *Bacteroidetes Proteobacteria* and *Firmicutes* increased in the diabetic rats and dose dependent treatment of ganoderic acid reduced the level of *Bacteroidetes Proteobacteria* and *Firmicutes*.

3.10. Ganoderic treatment increase pancreatic islet mass

To estimation if the decreased blood glucose level results from enhanced insulin secretion. The normal control and normal control received ganoderic acid exhibited the almost similar β-cells and STZ induced mice showed the decreased β-cells (0.8 ± 0.03) as compared to normal control and other treated group. Ganoderic acid treatment improved the β-cell mass (1 ± 0.04, 1.6 ± 0.03) at a dose level of 10 and 20 mg/kg. On the other hand, glibenclamide showed the 1.5 ± 0.02 β-cell mass (Fig. 11).

3.11. Effect of ganoderic acid on pancreas histopathology

Fig. 12 showed the effect of ganoderic acid and glibenclamide on the normal and STZ induced type I DM mice. The normal and normal received ganoderic acid (20 mg/kg) showed the unchanged in the pancreas histopathology. STZ induced type I DM mice pancreas histopathology showed the diminution of the Islets of Langerhans size, expansion of necrosis followed via induction of atrophy and fibrosis and decrease the β cells size. Ganoderic acid treatment group rat histopathology showed the alteration the size of Islets of Langerhans, increase the size of β cells and reduce the necrosis reaction in the tissue in a dose-dependent manner. The almost similar histopathology effect was found in the glibenclamide treated group mice histopathology.

4. Discussion

Type I diabetes mellitus (T1DM) is a chronic metabolic disease characterized via T-cell mediated autoimmune damage of pancreatic β-cells. The immune system of body attack to the pancreatic β-cells, ultimately leading to the destruction of cells and insulin deficiency (Drouin et al., 2009; Steppan et al., 2001). In the current experimental investigation, we observed that the ganoderic acid could prevent the onset of hyperglycemia system, some destruction and consequently upheld survival induced via STZ-induced T1DM in mice. Subsequently, ganoderic acid significantly ($P < 0.001$) enhanced the glucose tolerance and reduced the body weight, which was connected with the up-regulated the pancreatic mass, circulating insulin levels, utilization of FFA and down-regulated the α-glucosidase, α-amylase activity (La Greca and MacKey, 2009). The current finding provided evidence that ganoderic acid may be a plant based agent that can provide the protection to the pancreatic islets from the autoimmune-mediated damage and thereby avoid the expansion of T1DM.

Research suggest that treat the postprandial hyperglycemia is an effectual way to treat the diabetes mellitus (type I and II). This is also method to reduce the absorption of glucose in the intestine (Kumar et al., 2013b, 2013a). Studies suggest that intestine is the first organ involved in the glucose homeostasis, where dietary carbohydrates are digested and glucose is start the secretion via retardation of carbohydrate hydrolysing enzymes into the circulation (La Greca and MacKey, 2009; Reaven and Reaven, 2018). Previous suggest the α-Glucosidase is the crucial enzyme in the digestive process, and it situated at the brush border surface membrane of intestinal cells (Kim et al., 2008; Yin et al., 2014). Henceforth, α-glucosidase inhibitors can suppress the glucose absorption,

Table 4
The 16S rRNA gene sequencing of caecal content of rats.

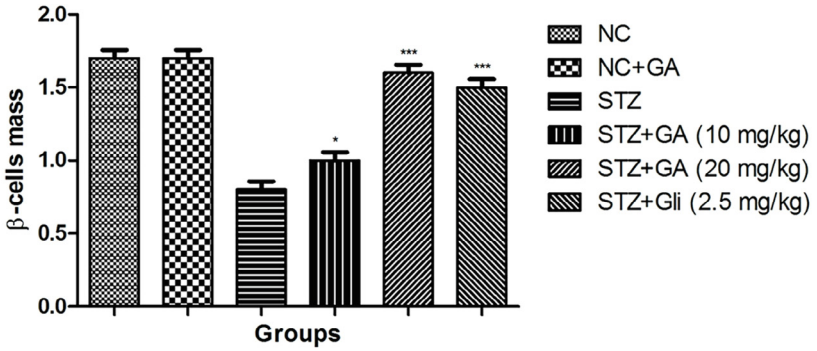
S.No	Genus	Group						
		NC	NC + GA	STZ	STZ + GA (5 mg/kg)	STZ + GA (10 mg/kg)	STZ + GA (20 mg/kg)	STZ + Gli (2.5 mg/kg)
1	*Actinobacteria*	0.4 ± 0.03	0.38 ± 0.02	0.08 ± 0.02	0.13 ± 0.04	0.21 ± 0.02	0.28 ± 0.04	0.35 ± 0.03
2	*Bacteroidetes*	0.25 ± 0.09	0.24 ± 0.09	0.20 ± 0.08	0.25 ± 0.08	0.22 ± 0.06	0.24 ± 0.07	0.23 ± 0.09
3	*Cyanobacteria*	0.6 ± 0.03	0.11 ± 0.02	0.14 ± 0.03	0.18 ± 0.05	0.27 ± 0.09	0.34 ± 0.08	0.3 ± 0.07
4	*Firmicutes*	0.22 ± 0.04	0.21 ± 0.05	0.27 ± 0.09	0.26 ± 0.08	0.24 ± 0.07	0.22 ± 0.09	0.22 ± 0.07
5	*Proteobacteria*	0.35 ± 0.05	0.34 ± 0.08	0.08 ± 0.01	0.1 ± 0.02	0.16 ± 0.02	0.29 ± 0.03	0.28 ± 0.04
6	*Verrucomicrobia*	0.28 ± 0.08	0.27 ± 0.09	0.03 ± 0.01	0.09 ± 0.02	0.13 ± 0.03	0.23 ± 0.04	0.21 ± 0.07

Bars represent means ± SD (n = 5).

Table 5
The 16S rRNA gene sequencing of caecal content of rats.

S.No	Genus	Group						
		NC	NC + GA	STZ	STZ + GA (5 mg/kg)	STZ + GA (10 mg/kg)	STZ + GA (20 mg/kg)	STZ + Gli (2.5 mg/kg)
1	*Bacteroidates*	0.32 ± 0.04	0.32 ± 0.06	0.22 ± 0.04	0.23 ± 0.03	0.25 ± 0.05	0.29 ± 0.02	0.28 ± 0.05
2	*Bifldobacteriales*	0.98 ± 0.1	0.99 ± 0.08	0.04 ± 0.01	0.14 ± 0.01	0.36 ± 0.03	0.53 ± 0.02	0.5 ± 0.04
3	*Burkholderiaies*	0.4 ± 0.03	0.42 ± 0.04	0.11 ± 0.02	0.17 ± 0.06	0.26 ± 0.06	0.37 ± 0.09	0.34 ± 0.04
4	*Campylobacterioles*	0.75 ± 0.06	0.74 ± 0.05	0.21 ± 0.05	0.29 ± 0.04	0.35 ± 0.06	0.48 ± 0.06	0.61 ± 0.09
5	*Clostridiales*	0.24 ± 0.03	0.23 ± 0.04	0.43 ± 0.05	0.4 ± 0.03	0.36 ± 0.06	0.26 ± 0.07	0.29 ± 0.05
6	*Coriobacteriales*	0.20 ± 0.01	0.21 ± 0.02	0.44 ± 0.07	0.4 ± 0.03	0.36 ± 0.05	0.29 ± 0.08	0.32 ± 0.09
7	*Desulfovibrionales*	0.2 ± 0.02	0.21 ± 0.04	0.28 ± 0.04	0.27 ± 0.03	0.26 ± 0.03	0.22 ± 0.09	0.25 ± 0.06
8	*Enterobacteriales*	0.42 ± 0.04	0.4 ± 0.03	0.11 ± 0.06	0.15 ± 0.06	0.19 ± 0.07	0.32 ± 0.04	0.3 ± 0.06
9	*Eryslpelotrichales*	0.64 ± 0.05	0.6 ± 0.04	0.1 ± 0.03	0.21 ± 0.06	0.34 ± 0.06	0.56 ± 0.06	0.5 ± 0.07
10	*Lactibacllales*	0.24 ± 0.04	0.25 ± 0.05	0.18 ± 0.01	0.19 ± 0.04	0.22 ± 0.04	0.25 ± 0.01	0.25 ± 0.03
11	*Selenomonadales*	0.26 ± 0.02	0.23 ± 0.04	0.01 ± 0.01	0.07 ± 0.02	0.12 ± 0.04	0.18 ± 0.01	0.16 ± 0.03
12	*Verrucomicrobiales*	0.43 ± 0.04	0.45 ± 0.08	0.08 ± 0.01	0.19 ± 0.04	0.29 ± 0.07	0.38 ± 0.05	0.36 ± 0.08

Bars represent means ± SD (n = 5).



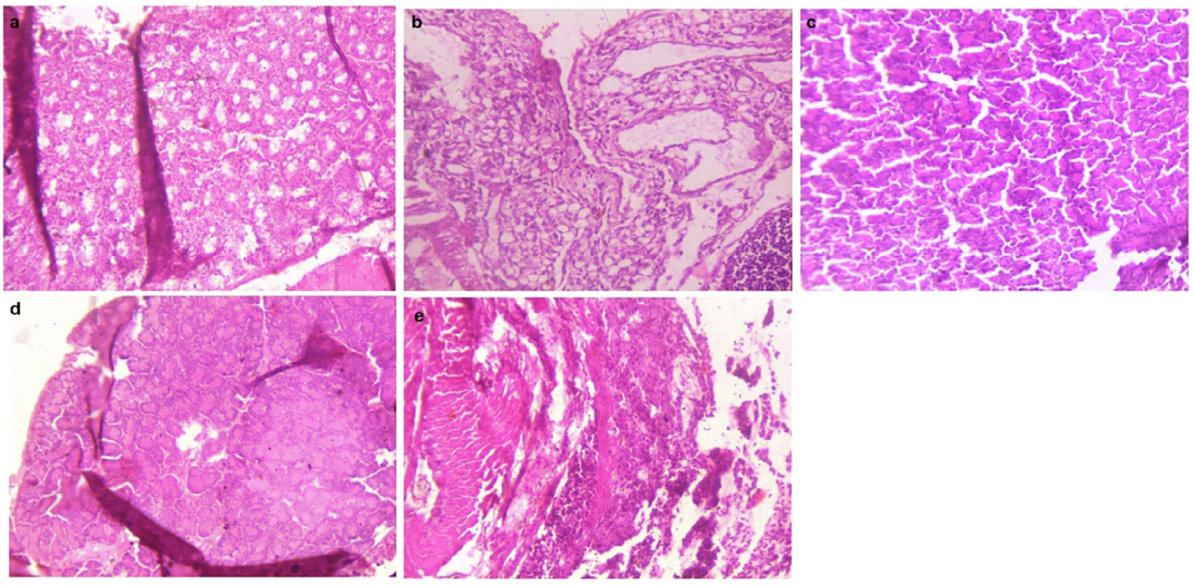


Fig. 11. The effect of ganoderic acid on pancreatic islet mass of STZ-induced T1DM. Islet was calculated via multiplying pancreas weight via relative β-cell area on tissue slides. Statistically significant different are indicated via asterisks and estimated via ANOVA. $^{***}p < 0.001$, $^{**}p < 0.001$, $^{*}p < 0.05$ compared with the model group.

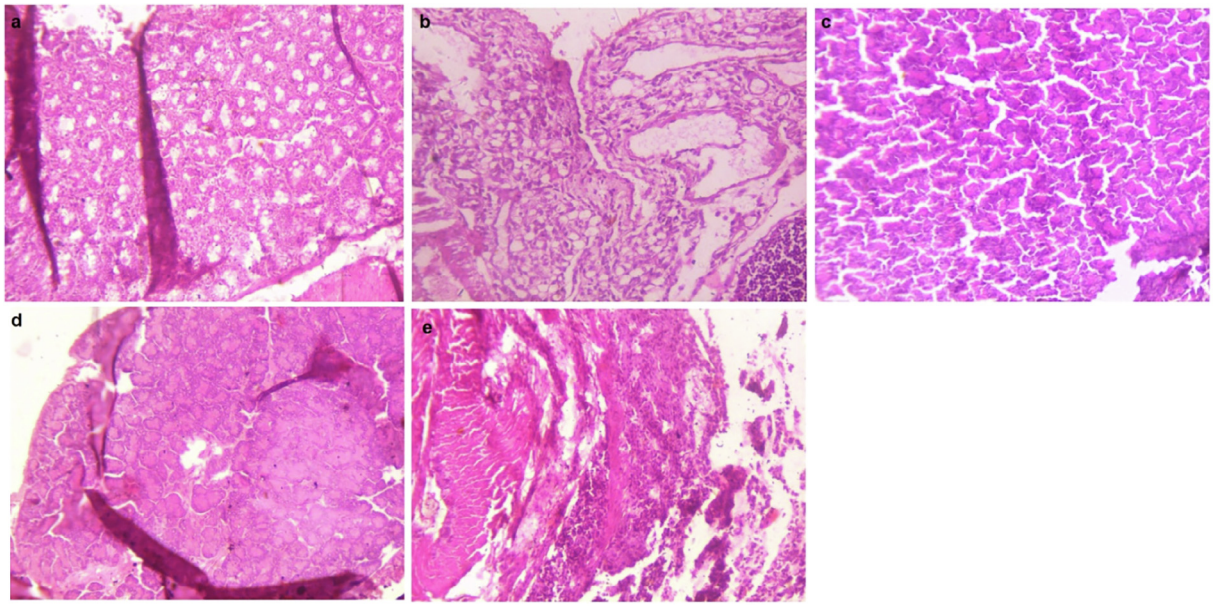


Fig. 12. The effect of the pancreas histopathology of different group of normal and Streptozotocin (STZ) induced type I diabetic mellitus mice. (a) normal control, (b) diabetic control, (c) diabetic control treated with ganoderic acid (10 mg/kg), (d) diabetic control treated with ganoderic acid (20 mg/kg), (e) diabetic control treated with glibenclamide (2.5 mg/kg).

resultant in postprandial hyperglycemia glucose level and decreased diabetes and its complication. Previous studies suggest that adding the acarbose to T1DM patients could reduce HbA1c level and reduce blood glucose concentration in the serum (Kalra, 2014; Kumar et al., 2011a,b; Yin et al., 2014). Its might be also used as adjunctive therapies to insulin treatment for T1DM patients. In our experimental study, ganoderic acid significantly exhibited the α-glucosidase.

No safe models available for visualizing or sampling the human endocrine pancreases, the choice for the researcher are rodent models. The rodent such as mice, inducible diabetic mice, and bio-breading diabetic resistant mice are commonly used for the rodent model for TIDM (Katsarou et al., 2017). Streptozotocin (STZ) is commonly used for the induction of diabetes mellitus (type I and type II) (Furman, 2015; Samuel et al., 2009). STZ (nitrosourea moiety), donor of nitric oxide, which is leading to the destruction of pancreatic β cells (Furman, 2015; Sakata et al., 2012). In the current experimental study, we used the multiple doses of STZ because multiple low doses of STZ induce the development of late progression of hyperglycemia only in the thymus region of mice. When administered the low dose of STZ, it starts the expansion of inflammation in the islets via destroys β-cells within few days. The current model used in the experimental study is similar to the human T1DM.

Several researchers suggest that the blood glucose levels are regulated in several organs (Bordone et al., 2007; Thorens, 2008). The intestine is the 1st major organ involved in the glucose homeostasis, where the carbohydrate consumes in a meal is digested via intestine and secreted glucose into the circulation (La Greca and MacKey, 2009). Previous research suggests that the triterpenoid reduce the intestinal glucose absorption in rodent, which could contribute to body weight gain and postprandial glycemic control (Cudworth, 1978). In the current experimental study, we have observed the same result that gandoeric acid increases the body weight and postprandial glycemic level via providing the protection of pancreatic islets function. Persistently, the mice received the gandoeric acid had significantly ($P < 0.001$) increase the plasma insulin level compared to STZ control group mice. Additionally, ganoderic acid treatment significantly ($P < 0.001$) reduced the blood glucose level and intraperitoneal glucose tolerance, which reflects the β-cells to circulating glucose.

In the current experimental study, we observed that the STZ showed the reduced level of insulin in the circulation and ganoderic acid treatment significantly ($P < 0.001$) increased the exogenous insulin. Moreover, ganoderic acid treatment could enhance oral glucose tolerance and fasting blood glucose level. Ganoderic acid treatment exhibited the increased size of islet and improved the structure of pancreatic β-cell as compared to STZ group, resultant reflect the direct response of pancreatic β-cells into the circulating glucose. Oxidative stress plays an important role in the expansion and commencement of inflammatory reaction, which generally occurs during the various diseases such as diabetes mellitus (Kontogiorgis et al., 2010). Ganoderic acid having antioxidant effects and current finding suggest that the gandoeric acid partly restore the function of β-cells via an antioxidant mechanism. Several researchers suggest that during the T1DM, T cell-mediated autoimmune process start the destruction of pancreatic β-cells, but the molecular mechanism is still unknown and not fully defined (Murakami and Hirano, 2012; Trautmann et al., 2001). Previously published literature suggests that infiltration of immune cells in the islets is considered as the hallmark of T1DM pathogenesis (Al-Goblan et al., 2014; Navarro-González and Mora-Fernández, 2008). Pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β are consider as the central mediators for the inflammatory reaction, and the overexpression of these mediators eases the expansion of diabetes related inflammation, coagulation and endothelial dysfunction (Cudworth, 1978; Morran et al., 2015). Moreover, research suggests that TNF-α induces the activation and accumulation of neutrophil, which boost the immune disorder during the diabetes (Zaccardi et al., 2016). Ganoderic acid significantly ($P < 0.001$) down-regulated the expression of pro-inflammatory cytokines like TNF-α, IL-1β and IL-6 in the renal tissue. On the basis of result, we can conclude that ganoderic acid down-regulated the renal inflammation via attenuating the inflammatory mediators. Additionally, another chemotactic like MCP-1 that activate the macrophages and monocytes and circulates the monocytes into the injury sites. During the T1DM, T-cells, activation the macrophages and T-cells, which further start the production of pro-inflammatory cytokines such as TNF-α, IFN-γ, and IL-1β, these mediators consider as the significant marker to the destruction of β-cell (Day, 2006; Yasushi, 2001). In the current experimental study, we have found that ganoderic acid treatment significantly ($P < 0.001$) decrease the infiltration of immune cell in the pancreatic β-cell. On the basis of the result, we can say that ganoderic acid protects the functional of β-cell via avoiding the destruction of β-cell and infiltration of immune cell. For the confirmation, we further examine the effect of ganoderic acid on the immune system of mice. We observed that ganoderic acid treatment significantly modulated the inflammation-related cytokines, which are considered as the significant marker of the immune system. Ganoderic acid exhibited the low effect on IL-1β, IL-1α, IL-2 to IL-10. But gandoeric acid showed the decreased level of IL-12 in plasma and several researchers suggest that during the TIDM, its play an important role in the expansion of T1DM. Another pro-inflammatory cytokine such as IL-10 is secreted via Th2 cells and circulated through T-cells. Previously published literature suggests that IL-10 administration or gene transfer avoids diabetes and insulitis in the rodent and suggesting that its play an important role in the maintenance of T1DM (Kelly et al., 2015; Zaccardi et al., 2016). During the DITM, the level of IL-10 was considerably increased in STZ control group and ganoderic acid significantly decreased as compared to STZ control. On the basis of the result, we can say that the administration of gandoeric acid reduced the onset of T1DM and insulitis may be due to arousing the alteration of pro-inflammatory cytokines.

At the genus level, *Bacteroidates, Bifldobacteriales, Burkholderiaies, Campylobacterioles, Clostridiales, Coriobacteriales, Desulfovibrionales, Enterobacteriales, Eryslpelotrichales, Lactibacllales, Selenomonadales* and *Verrucomicrobiales* considerably altered in the STZ induced diabetic rats and dose dependently treatment of GA significantly modulated level of bacteria. Previous research developed the correlation between the diabetes and gut microbiota. Patient suffered from the diabetes exhibited the lower abundances in butyrate producing bacteria viz., *Faecalibacterium prausnitzii, intestinalis* and *Roseburia* and increase level of *Streptococcus, Lactobacillus* and *gasseri mutans* (H. and A.R., 2014; Tilg and Moschen, 2014). Children suffered from the diabetes demonstrated the ratio of *Firmicutes* to *Bacteroidetes* and the level of *Actinobacteria, Bacteroides* reduced and on the other hand healthy children exhibited the increased quantity of *Firmicutes* (H. and A.R., 2014; Tilg and Moschen, 2014). During the animal model, *Clostridiaceae* and *Ruminococcaceae* were present in the feces of diabetic rats and *Lachnospiraceae*, *Porphyromonadaceae* and *Prevotellaceae* were found in the feces of diabetic rats. Result showed that the ganoderic acid considerably altered the *Bacteroidates, Bifldobacteriales, Burkholderiaies, Campylobacterioles, Clostridiales, Coriobacteriales, Desulfovibrionales, Enterobacteriales, Eryslpelotrichales, Lactibacllales, Selenomonadales* and *Verrucomicrobiales* bacetria in the feces of STZ induced diabetic rats.

On the basis of result, we can conclude that ganoderic acid exhibited the antidiabetic effect might be due to alteration of immunity, thus guard the islets from immune cell arbitrated damage of pancreatic

β-cells, but the underlying mechanism for action is still unclear. It is well known that oxidative stress plays an important role in the expansion of diabetes and its complication (Cudworth, 1978; Morran et al., 2015). Oxidative stress may play a crucial role in the initiation of chronic inflammation and numerous degenerative diseases such as diabetes, obesity, which is always linked with the reduction of antioxidant level in the number of tissues (Cudworth, 1978; Morran et al., 2015). Our experimental study exhibited the preventive effect of ganoderic acid against insulitis and also protected the islets cells from the immune cell attributed destruction via antioxidant independent mechanisms.

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Further reading

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