Indian Journal of Biochemistry & Biophysics
Vol. 60, July 2023, pp. 501-515
DOI: 10.56042/ijbb.v60i7.4140

Clitoria ternatea extract-loaded chitosan nanoparticles ameliorate diabetes and oxidative stress in diabetic rats

Maysa A Mobasher[1]*, Shereen A Baioumy[2], Ahmed S Alazzouni[3], Arwa Ishaq A Khayyat[4], Nabil S Awad[5,6], Mohamed A Abdel-Hakeem[6] & Noorah Saleh Al-Sowayan[7]*

[1]Department of Pathology, Biochemistry Division, College of Medicine, Jouf University, Sakaka, 72388, Saudi Arabia

[2]Microbiology and Immunology Department, Faculty of Human Medicine, Zagazig University, Zagazig-31527, Egypt

[3]Zoology and Entomology Department, Faculty of Science, Helwan University, Helwan, Egypt

[4]Biochemistry Department, Science College, King Saud University, PO Box-22452, Saudi Arabia

[5]Department of Genetics, Faculty of Agriculture and Natural Resources, Aswan University, Aswan-81528, Egypt

[6]College of Biotechnology, Misr University for Science and Technology, Giza-12563, Egypt

[7]Department of Biology, College of Science, Qassim University, Buraydah-52377, Saudi Arabia

Received 18 July 2023; *revised 24 July 2023*

Type 2 diabetes mellitus (T2DM) is one of the expanding global health problems and is the most common metabolic disorder characterized by hyperglycemia, which significantly contributes to producing reactive oxygen species (ROS). More than 400 plant species with hypoglycemic activity have been mentioned in the literature. *Clitoria ternatea* (*C. ternatea*), often called Butterfly pea or Asian pigeonwing, is a plant species member of the Fabaceae family. The main goal of this study was to evaluate the methanolic extract of *C. ternatea* (*CT-Mx*)'s and/or chitosan-loaded nanoparticles (CHNPs) antihyperglycemic and antioxidant effects in normal and diabetic rats produced by streptozotocin (STZ). A total of 20 male albino rats had been divided into 4 groups, control non-diabetic (NC), STZ/diabetic control, STZ/diabetic + *CT*-Mx, and STZ/diabetic + *CT*-CHNPs groups. After 28 days, levels of insulin, fasting blood glucose (FBG), aspartate transaminase (AST), alanine transaminase (ALT), superoxide dismutase (SOD), glutathione (GSH), lipid peroxidation, and mRNA gene expression were assessed. Histopathological and immunohistochemical studies were performed for pancreatic tissues. In the STZ/diabetic (Gp2) rats, levels of FBG, AST, ALT, and both CDKN1A and TP53 gene expression were significantly increased. Moreover, the hyperglycemia-induced hepatic oxidative state is evidenced by a significant increment of lipid peroxidation and deterioration in SOD and GSH levels. On the contrary, both the STZ/diabetic + *CT*-Mx and STZ/diabetic + *CT*-CHNPs showed discernible improvement in diabetes-associated complications; however, STZ/diabetic + *CT*-CHNPs (Gp4) rats significantly suppressed the generated oxidative stress and improved antioxidant activity, liver function, and insulin secretion. Also, their pancreatic section exhibited architecture with normal regenerative pancreatic endocrine islets with normal distribution and number of beta cells and suppressing inflammatory and apoptotic gene expression compared to Gp2. Nanocarrier agents showed excellent antihyperglycemic and effects after antioxidative, making it a promising technology for diabetics.

Keywords: Antioxidant Markers, Diabetes mellitus, Gene expression, Liver enzymes, Nano-drug delivery, Streptozotocin

Hyperglycemia is the main symptom of diabetes mellitus (DM), a metabolic condition caused by abnormalities in insulin secretion and/or action[1]. DM has long been regarded as a significant global source of illness and mortality[2]. In diabetics, reactive oxygen species (ROS), created by hyperglycemia, harm cells in various ways and lead to further issues[3]. Significant abnormalities in lipid structure and metabolism have been associated with diabetes[4]. Enhanced malondialdehyde (MDA) levels in diabetics imply that peroxidative damage may contribute to the onset of the disease[3]. However, reduced levels of glutathione (GSH) and superoxide dismutase (SOD) have been linked to causing diabetes complications[3].

In those with diabetes and poor glucose tolerance, there was a substantial difference in the expression of genes implicated in insulin signaling and immune response[5]. FFA, inflammatory cytokines, and ROS have been reported to trigger p53-mediated apoptosis in pancreatic beta cells[6].

Diabetes management without side effects remains a major challenge [7]. Most diabetic patients eventually need to use pharmacotherapeutic drugs at some point. There has been a plethora of hypoglycemic synthetic medicines that can be used to treat diabetes. These

*Correspondence:
E-mail: mmobasher@ju.edu.sa (MAM); nsaoiean@qu.edu.sa (NSA)

synthetic medications have long been the cornerstone of diabetes treatment, efficiently controlling hyperglycemia but causing severe side effects[7].

Many conditions that have proven resistant to conventional treatment can be effectively addressed with herbal medicine[8]. Thus, there is a growing interest in the discovery of natural, and efficient anti-diabetic drugs from medicinal plants to successfully treat DM. This approach has been endorsed by the World Health Organization[9]. There is a lot of hope that phytochemicals found in plants can act as hypoglycemics[10]. Considering the increasing prevalence of numerous fatal diseases among humans, a global trend toward using natural plant ingredients as restorative antioxidants has emerged[11]. Approximately 261 plant species (55 plant families) can be found in Saudi Arabia. The most influential plant families included Fabaceae, Asteraceae, Poaceae, Lamiaceae, Chenopodiaceae, Boraginaceae, and Zygophyllaceae[12].

Clitoria ternatea (C. ternatea), often called Butterfly pea or Asian pigeonwing, is a plant species member of the Fabaceae family that possesses antidiabetic and antioxidant properties[13]. A decoction made from *C. ternatea* flower petals, or the flower itself, is used to make the widely consumed herbal tea[14], or tisane, known as butterfly pea flower tea in Saudi Arabia. Clinical studies addressing the impact of *C. ternatea* flower extract (CTE) on insulin response and antioxidant ability are lacking[15], despite the fact that *C. ternatea* flower extract (CTE) has a high concentration of phytochemicals that have excellent antioxidant, anti-inflammatory, antidiabetic, and antiproliferative/anticancer aspects[13,16-19].

Nanotechnology science has many applications in various sectors due to the advantages of working with nanometric items[20]. The growing success of nanotechnology in the therapeutic field has centred on many ways to treat diabetes, a prevalent metabolic condition[20]. The creation of nanocarrier agents allows the enhanced delivery of various hypoglycemic medicines compared to conventional therapy, resulting in excellent control of elevated blood glucose levels, and making it the most promising technology. Moreover, encapsulating biologically active materials with nanoparticles is a new approach that may face limitations when using natural products as therapeutic agents[21]. Chitosan is a biocompatible and biodegradable natural polysaccharide with numerous medicinal uses. Chitosan nanoparticles (CHNPs) are widely used as a drug delivery technology for encapsulating various pharmaceuticals and botanicals[21]. Also, adding multiple ligands to nanocarriers improves the targeted distribution and protects encapsulated hypoglycemic drugs from degradation, resulting in a longer-lasting blood-glucose-lowering impact. As a result, hypoglycemic nanocarrier agents aim to improve diabetes management while lowering the risk of acute and chronic problems[22].

This study's objective was to assess the antidiabetic and antioxidant properties of chitosan nanoparticles loaded with *C. ternatea* extract on STZ-induced diabetic rats and to detect the advantages of nanotechnology approaches for identifying and treating DM.

Materials and Methods

Plant material and preparation of methanolic extract

Raw materials for this study were gathered from the Aljouf region of Saudi Arabia, where *C. ternatea* grows. The Faculty of Science at Jouf University was responsible for identifying the plant species. The next step was to dry and powder the plant flowers. Using five grams of *C. ternatea* flower powder mixed with 100 mL of methanol, we were able to make a methanolic extract by following the standard Soxhlet procedure. Under vacuum, the solvent was removed, and the resulting extracts were placed in dark glass vials and kept at 4°C[23].

Phytochemical analysis

The extract was phytochemically characterized as previously described in our previous work[24], and the total phenolic and flavonoid compounds (TPCs and TFCs, respectively) were also estimated[24].

Preparation of chitosan nanoparticles loaded *C. ternatea* methanolic extract

CHNPs were prepared by the ionic gelation method[25]. Chitosan solution (0.1 % *w/v*) in acetic acid (1% *v/v*) was prepared and adjusted to pH 4.7. 100 mL of the above solution was stirred vigorously for 30 min and then received 20 mL sodium tripolyphosphate (TPP) aqueous solution (0.1% *w/v*). The formed particles (CHNPs) were collected by centrifugation for 10 min at 10000 rpm. A similar method was used to prepare extract-loaded chitosan nanoparticles, where 100 mg of extract was added to the chitosan solution before adding TPP.

Nanoparticle characterization

The *CT*-CHNPs were characterized for size and morphology using dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern Instruments, UK) and high-resolution transmission electron microscopy (HRTEM; JEOL-JEM- 2100, Japan)[25].

Encapsulation efficiency

Plant extract loading and encapsulation in plant extract nanoparticles were assessed using a Lambda 950 Visible-UV spectrophotometer (PerkinElmer Fremont, CA, USA).

Experimental Animals

In this study, male albino (Sprague-Dawley) rats weighing between 150 and 200 g were used. Rats were obtained from the Nile for Pharmaceuticals & Chemical Industries in Cairo, Egypt. Five rats were housed in each cage and exposed to a regulated light and dark cycle (12:12) and normal room temperature and humidity levels (25°C ± 2°C).

The rats were fed pellets *ad libitum* and had unlimited access to water. The animal studies were authorized by the ethics committee at Helwan University's Faculty of Science (Egypt), which followed their established ethical rules for the Animal Ethics Committee (No. HU-IACUC/Z/AA1017-16). The rats had two days of adaptation before the experiment began.

Induction of Diabetes Mellitus by Streptozotocin

The acclimatized, healthy normoglycemic animals were included in the study. The rats had an overnight fast (8–10 h) before receiving a single intraperitoneal (*i.p.*) injection of freshly manufactured streptozotocin (STZ) in 0.1M citrate buffer (pH 4.45), which was provided by Sigma-Aldrich, St. Louis, MO, USA[26]. STZ is extensively used as an experimental agent capable of inducing diabetes mellitus (DM) because it is an antibiotic that can induce pancreatic-cell destruction. Blood samples from rat tails were used to confirm diabetes 72 h after STZ injection.

FBG levels were assessed during the diabetes induction phase until hyperglycemia stabilized, which was generally a week after STZ injection. FBG levels above 250 mg/dL were a defining characteristic of diabetic rats[26], which were the subjects of the study.

Study design

Animals were grouped into four (five rats each), as follows:

Group 1: Normal control (NC), included non-diabetic control rats.

Group 2: STZ/diabetic control (DC), included STZ-induced diabetic rats as a positive control.

Group 3: STZ/diabetic + *CT*-Mx (included diabetic rats receiving 400 mg/kg BW of *C. ternatea* methanolic extract by oral gavage every day for 28 days) according to our previous work[24].

Group 4: STZ/diabetic + *CT*-CHNPs (included diabetic rats receiving 400 mg/kg BW of *C. ternatea* methanolic extract-loaded chitosan nanoparticles by oral gavage every day for 28 days)[24].

Animals were closely monitored during the experiment, and their body weights (BW) and fasting blood glucose (FBG) were recorded (on days 1, 7, 14, 21, and 28).

Blood samples

After 28 days, rats were sacrificed, and blood collected in sterile tubes. The serum was collected after spinning blood samples at 3000 rpm for five min, then maintained at −80°C until it was needed for the biochemical examination.

Tissue collection

After 28 days, the sacrificed rats' pancreas was rapidly removed, washed with ice-cold saline, and divided into three sections. For gene expression analysis, the first section was kept in Trizol reagent at −80°C. For biochemical analysis, one more portion underwent homogenization in phosphate-buffered saline with a pH of 7.4, centrifuged, and the supernatants were stored at −80°C. The third section was submerged in a 15% formaldehyde solution for histopathological and immune histochemical analysis.

Biochemical analysis

FBG level

By utilizing a suitable strip with an electronic glucometer (On Call Plus Blood Glucose Monitoring System, ACON Laboratories, Inc. San Diego, USA), we were able to estimate FBG from the animal's tail vein in all groups of rats.

Serum insulin level

The serum insulin level was determined at the end of the experiment. For detecting serum insulin levels, an insulin ELISA kit was used for rats (Linco Research Inc.), manufacturer's protocol was applied as a director to the usage of this kit.

Liver enzymes

As mentioned before[27], levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the serum were measured using kits from Biodiagnostic Company (Dokki, Giza, Egypt) (CAT. No. AS 1061(45), and AL 1031 (45), respectively).

Antioxidant Markers

The enzymatic antioxidants superoxide dismutase (SOD) was assayed in the pancreatic tissues according to[28], Glutathione (GSH) was assayed in the pancreatic tissues according to[29], and pancreatic homogenate from both control and experimental animals was tested for malondialdehyde (MDA) levels[30] all according to the manufacturer's instructions of the kits purchased from Biodiagnostic Company (Dokki, Giza, Egypt) (CAT. No. SD 2521, CAT. No. GR 2511, CAT. No. MD 2529), respectively.

Histological examinations

Regularly, pancreatic tissue samples were fixed in 10% formalin, dried in an increasing series of alcohol, cleaned in xylol, and then embedded in paraffin. Tissues were sectioned at a thickness of 4-5 μM, fixed, processed for H&E staining[31], and examined for gross cellular damage using an optical microscope (Optica light microscope (B- 350)).

Immuno-Histochemical Study

NF-κB and insulin antibodies were stained immunohistochemically at dilution of (1:50) and (1:100), respectively, on paraffin-embedded sections (4 μM thick) using an Avidin-Biotin detection system (Ventana, Tucson, AZ, USA), following the manufacturer's instructions[32,33], respectively.

Gene expression analysis

The expression profiles of pancreatic cyclin-dependent kinase inhibitor 1A (CDKN1A), tumor protein p53 (TP53), and fibroblast growth factor 7 (FGF7) mRNA were quantified using real-time PCR (RT-PCR). Reverse transcription was used to create the first strand of cDNA from 1g of RNA[34]. The results of the mRNA expression of the three genes were compared to the expression of B-actin According to[35]. Table 1 lists the primer sequences used in the current study.

Statistical analysis

Results were shown as mean ± SD and are the means of 5 replicates/group. One-way ANOVA was used for statistical analysis. The standard deviation is represented by the bars. Tukey post hoc comparisons between various groups are used when there is a substantial variation in the group means. P values ≤ 0.05 were regarded as statistically significant for all statistical tests, columns with distinct lower-case letters denote statistical ($^{a}P \leq 0.05$ compared with the NC group, $^{b}P \leq 0.05$ compared with the Diabetic control group, $^{c}P < 0.05$ compared with the Diabetic *CT*+ Mx group, $^{d}P \leq 0.05$ compared with Diabetic *CT*+ CHNPs group), Using Excel 365 (Microsoft Corporation, USA) and Minitab version 19, data, were statistically analyzed. To examine the link between the evaluated markers, a Biplot of a principal component analysis was carried out using the ggplot2 package, and the heatmap with RColor Brewer was included in R software.

Table 1 — The primer sequence used for gene expression analysis in the study.

Gene	Primers	Sequence (5′–3′)
CDKN1A	Forward	AGGTGGACCTGGAGACTCTCAG
	Reverse	TCCTCTTGGAGAAGATCAGCCG
TP53	Forward	CCTCAGCATCTTATCCGAGTGG
	Reverse	TGGATGGTGGTACAGTCAGAGC
FGF7	Forward	CTGCCAACTCTGCTCTACAGATC
	Reverse	AGCATCCCAGCAGCCTTTAG
B-actin	Forward	GCACCACACCTTCTACAATG
	Reverse	TGCTTGCTGATCCACATCTG

Results

Characterization of the nanomaterials

The obtained particles showed a hydrodynamic size of 10-20 nm and a mean peak of 17. TEM imaging indicated that the obtained particles have a regular shape with a size average of 20 nm, as seen in (Fig. 1). Furthermore, the prepared particles showed encapsulation efficiency and a loading capacity of 84.00% + 6.40 and 13.21|% + 2.20.

Effect on Body weight (BW)

BW was recorded and results obtained in weeks 2, 3, and 4 for the diabetic rats receiving STZ (Gp2) demonstrated a decrease in BW compared to other groups. Interestingly, by the end of week 3, this reduction was significant compared to groups 3, and 4 ($P < 0.01$, and 0.001), by the end of week 4, this reduction was significant compared to groups 1, 3, and 4 ($P < 0.001$, 0.001, and 0.001), respectively (Fig. 2). This reduction in BW might result from the degradation of structural proteins and fats as there wasn't enough glucose for the body to use for energy. However, rats treated with *CT*-Mx (Gp3) and those treated with *CT*-CHNPs (Gp4) showed a regular pattern of increase in their BW, as seen in (Fig. 2).

Determination of serum FBG and insulin level

FBG was measured at 1, 7, 14, 21, and 28 days. According to our findings, by the end of week 1,

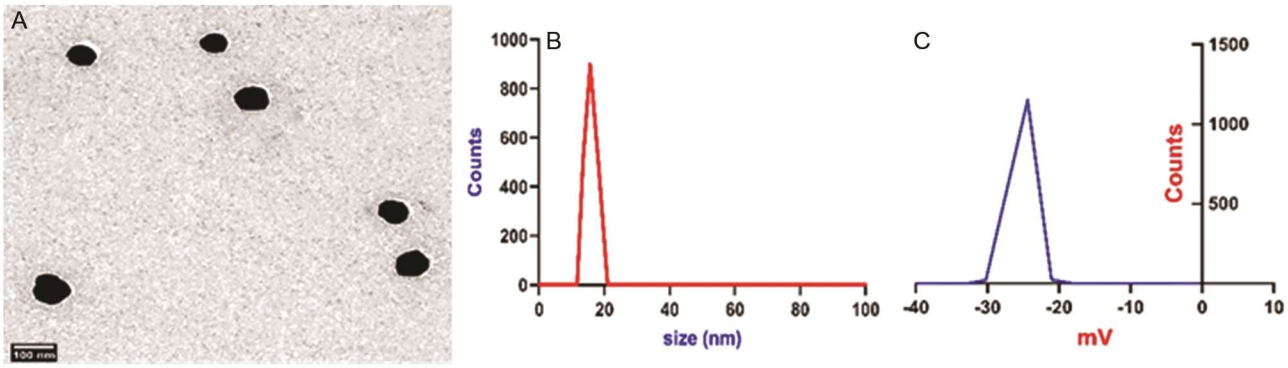

Fig. 1 — (A) TEM image of *CT*-CHNPs; (B) Hydrodynamic size of *CT*-CHNPs; and (C) Zeta potential of *CT*-CHNPs

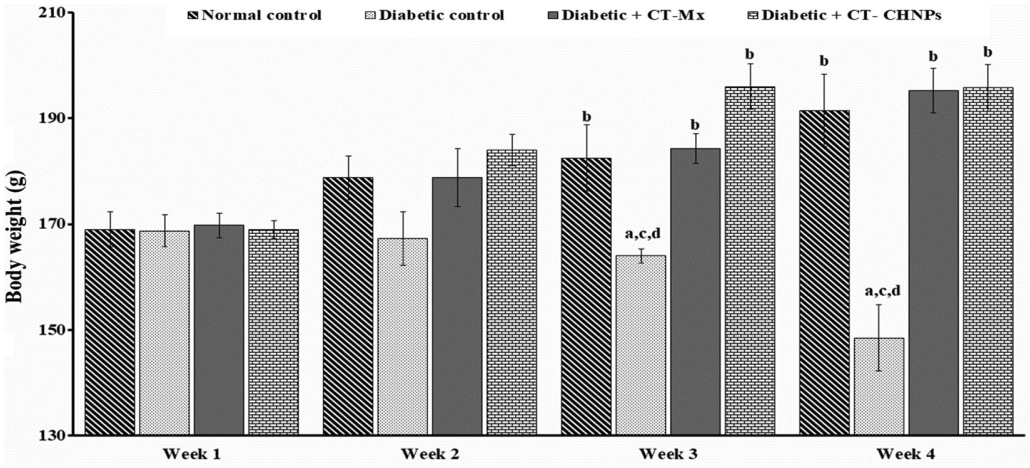

Fig. 2 — Body weight in all groups. Data expressed as mean ± SD, n = 5 for each group

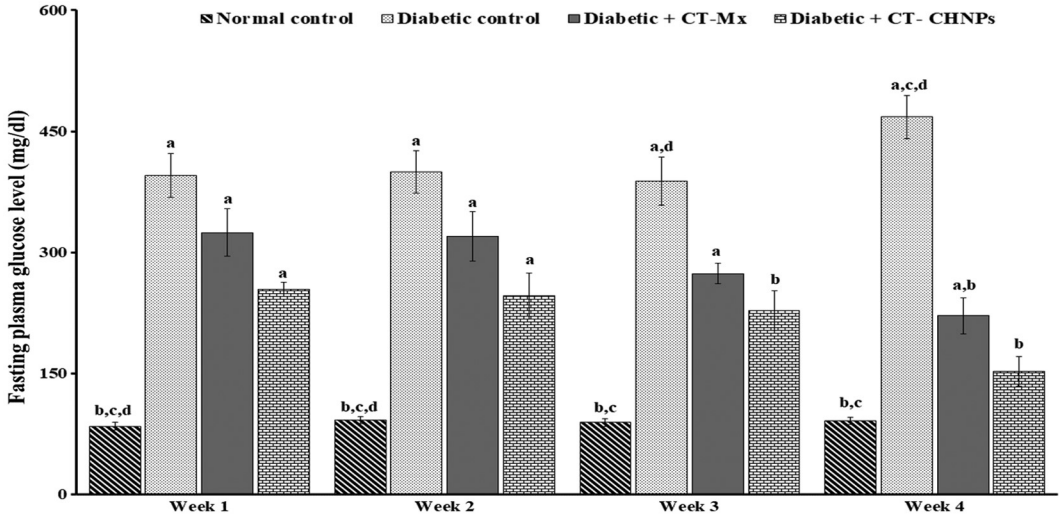

Fig. 3 — Blood glucose levels in the different groups under the study. Data expressed as mean ± SD, n = 5 for each group

diabetic groups 2, 3, and 4 had significant increases in glucose levels when compared to Gp 1 ($P< 0.001$, 0.002, and 0.025), respectively (Fig. 3).

Gp2 had high glucose levels (up to 400 mg/dL) throughout the experimentation, and this increase was highly significant by the end of weeks 2, 3, and 4 when compared to Gp 1 ($P< 0.001$, 0.001, and 0.001), respectively. These outcomes coincided with the drop in insulin levels (Fig. 4) that was highly significant at the end of the experiment compared to groups 1, 3, and

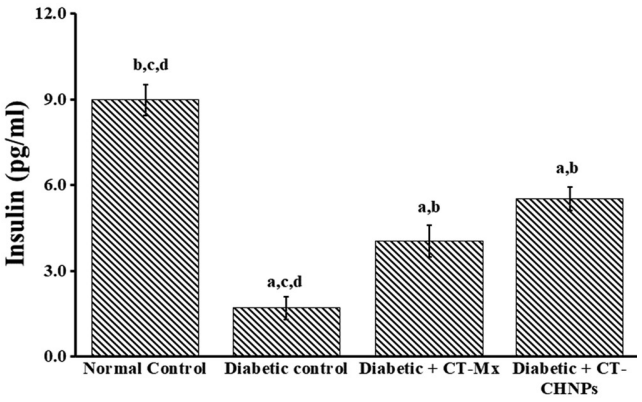

Fig. 4 — Insulin levels in the different groups under the study. Data expressed as mean ± SD, n = 5 for each group

4 (P< 0.001, 0.004, and 0.001), respectively, after week 4.

On the contrary, diabetic rats treated with *CT*-Mx (Gp3) had an increase in FBG during weeks 2 and 3 in comparison to Gp 1 (P =0.005 and 0.003), respectively. While this increase was less significant by the end of week 4 (P = 0.042), respectively, which reflects the effect of the extract. Interestingly, Gp4 demonstrated a dramatic decrease in the FBG without any obvious major variations in Gp1 at the same time by the end of weeks 3 and 4 (P = 0.126 and 0.314), respectively, which reflects the effect of the *CT*-CHNPs in lowering the FBG and this was accompanied by a significant elevation in the insulin level compared to Gp2 (P< 0.001) indicating improvement in the insulin sensitivity, as shown in (Fig. 4).

Moreover, low FBG in Gp4 was highly significant when compared to the increases FBG levels in the Gp2 during weeks 3 and 4 (P = 0.01 and P< 0.001), respectively.

Thus, the results depict the increase in insulin concentration after administration of *CT*-Mx (Gp3) and *CT*- CHNPs (Gp4), and this improvement either by stimulating the beta cells to secrete insulin or by regenerating pancreatic β cells, which was supported by histopathological and immunehistochemical investigations. These results proved that the *C. ternatea* CHNPs could decrease blood sugar levels and have promising effects equivalent to an antidiabetic drug.

Determination of liver function

As shown in Figure 5, in the diabetes group (Gp2), both ALT and AST values were considerably higher than in the control group (Gp1) (P< 0.001 and 0.002), respectively.

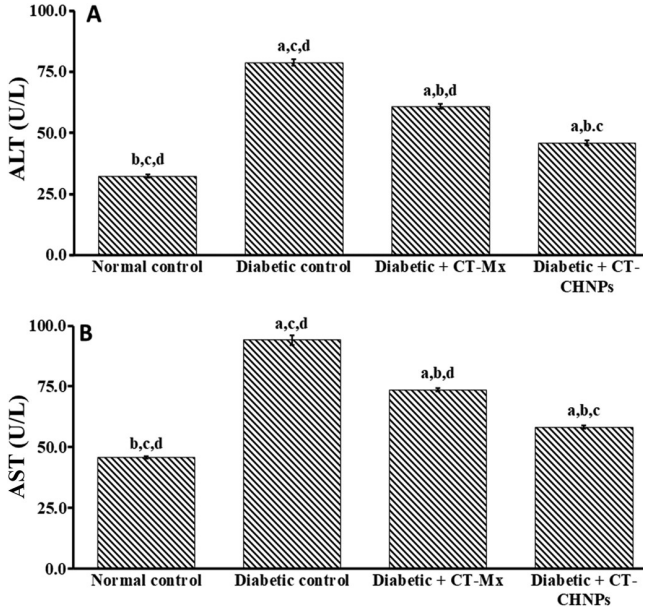

Fig. 5 — (A) Serum ALT (U/L), (B) Serum AST (U/L) levels in all groups. Data expressed as mean ± SD, n = 5 for each group

However, delivery of *CT*-Mx (Gp3) or *CT*-CHNPs (Gp4) to diabetic rats decreased ALT (P< 0.01 and 0.001) and AST levels significantly (P< 0.01 and 0.002) compared to the diabetic group.

These results demonstrated the liver-protective properties of these extracts (*CT*-Mx or *CT*-CHNPs); furthermore, *CT*-CHNPs (Gp4) showed a more effective liver-protective property than *CT*-Mx.

Determination of pancreatic oxidative stress markers

In fact, hyperglycemia increases oxidative stress because it leads to elevated ROS generation, which in turn affects the antioxidant defense mechanisms of the body's cells. Figure 6 showed significantly elevated levels of MDA (P<0.001) in STZ (Gp2)-treated diabetic rats that were accompanied by particularly low SOD (P<0.001) and GSH (P<0.001) levels compared to Gp1.

However, diabetic rats treated with *CT*-CHNPs (Gp4) showed significantly lower levels of the previously elevated MDA levels compared to Gp2 (P<0.001); with a significant improvement of the antioxidant ability reflected by an increase in SOD (P = 0.001) and GSH levels (P<0.001) when compared to group 2 (Fig. 6). While these levels showed non-significant differences in MDA (P = 0.078), and SOD (P = 0.214) when compared to GP1. These findings indicated that *CT*-CHNPs could reduce diabetes-induced liver damage and increase antioxidant enzyme levels in diabetic rats. Diabetic rats treated with *CT*-CHNPs (Gp4) showed

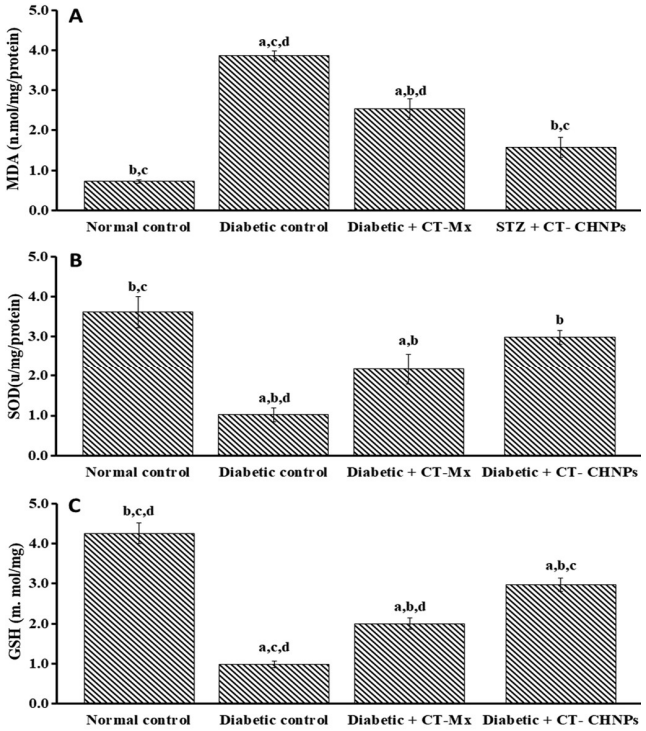

Fig. 6 — (A) pancreatic MDA level, (B) pancreatic SOD activity, (C) pancreatic GSH levels in all groups. Data expressed as mean ± SD, n = 5 for each group.

significant lower levels of MDA levels compared to Gp3 (P = 0.048); with an increase in GSH levels (P = 0.002) and SOD (P = 0.095).

Histopathological Sectioning

In a normal control (GpI), histopathological analysis of the pancreatic exocrine glands revealed normal islets of Langerhans with a normal distribution of beta cells (Fig. 7A & B). In contrast, the diabetic control group showed severe peri pancreatitis with inflammatory and degenerated islets and a reduced number of beta cells (Fig. 7C & D) that mostly occurred through ROS. The pancreas of Gp3 showed vacuolation of islets cells with reduced cell number and mild necrotic tissue area (Fig. 7E & F). The pancreatic section of Gp4 exhibited architecture with normal regenerative pancreatic endocrine islets with normal distribution and number of beta cells compared to Gp2 (Fig. 7G & H). The morphometric study of Islets of Langerhans is represented in (Fig. 8).

Immunohistochemical Study

NF-κB

Evaluation of NF-κB expression in pancreatic tissue from various experimental groups was done. Normal expression was detected in the normal control group (Fig. 9A & B). Meanwhile, strong positive expression was determined in the Gp2 contrasted to the Gp1 (Fig. 9C & D). A moderate expression level was recorded in *CT*-Mx (Gp3) group (Fig. 9E & F). On the other hand, the higher protective effect occurred in *CT*- CHNPs group revealing the lowest expression of NF-κB in the pancreatic tissue (Fig. 9G & H).

Insulin

Evaluation of insulin expression in β-cells in pancreatic islets from various experimental groups was done. Normal marked expression was detected in the normal control group (Fig. 10A & B). Meanwhile, mild expression was determined in the diabetic group (Fig. 10C & D), and moderate expression was recorded in *CT*-Mx (Gp3) group (Fig. 10E & F). On the contrary, marked insulin expression occurred in *CT*-CHNPs group (Fig. 10G & H).

Gene expression analysis

The current investigation used RT-PCR to identify CDKN1A, TP53, and FGF7 mRNA expression level changes as molecular biomarkers. In pancreatic tissues, both *CT*-Mx and *CT*-CHNPs showed antioxidant, inflammatory, and antiapoptotic activities, and the expression levels of these genes were studied compared with Gp1. Both CDKN1A and TP53 genes were increased in response to STZ therapy (Group 2) and showed significant differences compared to groups 1, 3, and 4 for CDKN1A (P< 0.001, 0.003, and 0.001), respectively, and for TP53 (P< 0.001, < 0.001, and < 0.001), respectively. In contrast, the FGF7 gene was considerably down-regulated and showed significant differences compared to groups 1, 3, and 4 (P< 0.001, < 0.001, and < 0.001), respectively (Fig. 11). Treatment with *CT*-Mx (Gp3) after induction of diabetes showed down-regulation of the CDKN1A (P<0.011) and TP53 (P<0.001) genes whilst the FGF7 gene was significantly up-regulated (P< 0.001) when compared to Gp1 (Fig. 11). While treatment with *CT*-CHNPs (Gp4) after induction of diabetes showed significant down-regulation of the CDKN1A and TP53 genes compared to Gp2 (P< 0.001 and < 0.001), respectively, and showed no significant differences when compared to Gp1 for CDKN1A (P< 0.312). Whilst the FGF7 gene was significantly up-regulated. This data revealed that *CT*-CHNPs extract had anti-

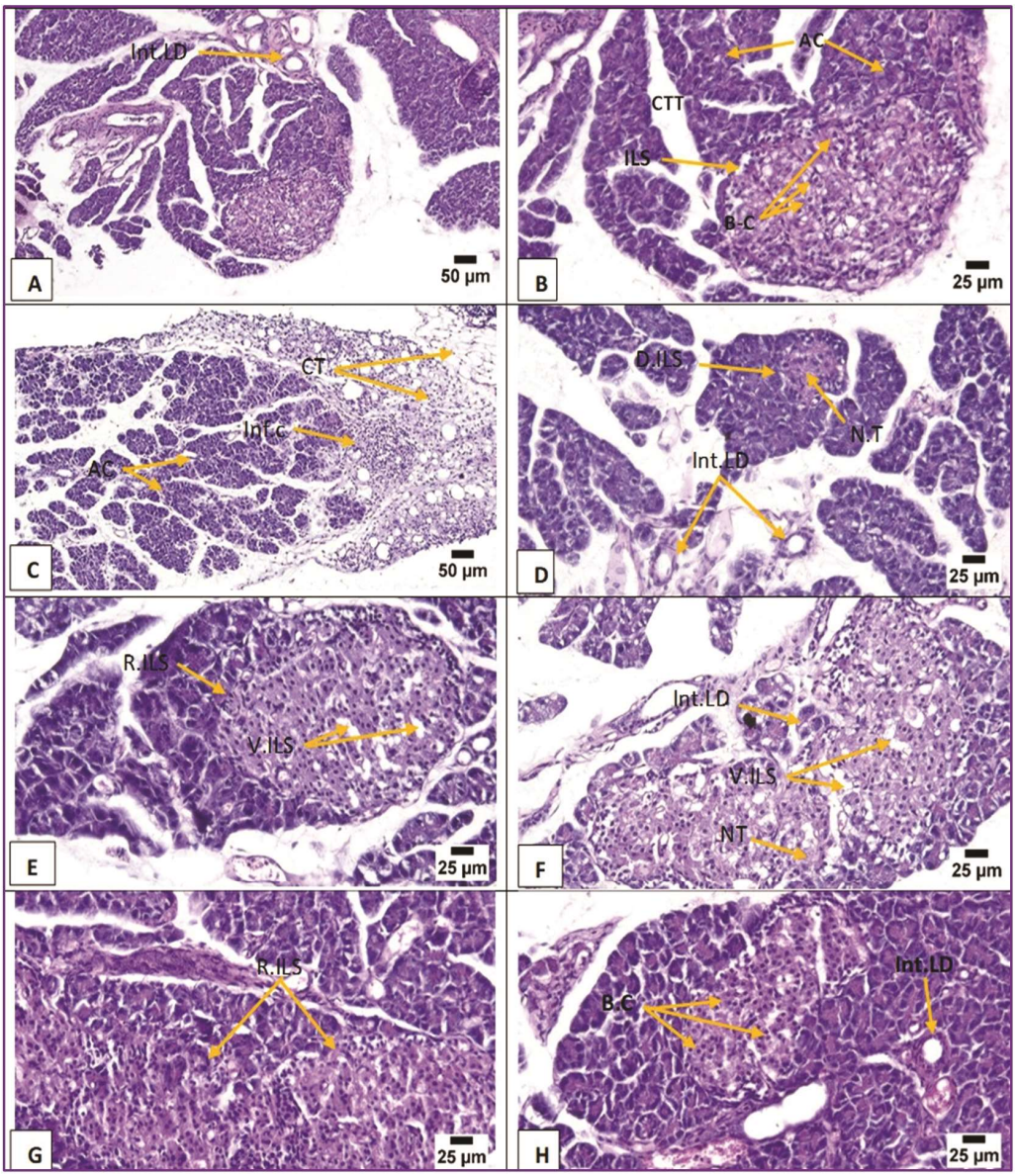

Fig. 7 — Histopathological examinations of pancreatic tissues. (A, B) Photomicrograph of pancreas normal control group showing normal islets of Langerhans (ILS) with a normal distribution of B-Cells (B-C) and normal pancreatic acini (AC) and connective tissue trabecula (CTT) with normal patent interlobular duct (Int. LD) (H&E). (C, D) Photomicrograph of diabetic control group showing severe peri pancreatitis with inflammatory cells (Inf.C) with degenerated islets (D.ILS) showing some necrotic area of islets (NT) and reduced number of beta cells with constricted interlobular duct (Int. LD) (H&E). (E, F) Photomicrograph of the pancreas from diabetic rats treated with *CT*-Mx (Gp3) showing vacuolation of islets cells (V.ILS) with reduced cell number and mild necrotic tissue area (NT) and congested interlobular duct (Int. LD) (H&E). Fig. (G, H) Photomicrograph of the pancreas from diabetic rats treated with *CT*- CHNPs with CT group showing apparently normal regenerative pancreatic endocrine islets (R.ILS) with normal distribution and number of B-cells (BC) and normal patent interlobular duct (Int. LD) all pancreatic cells showed high staining affinity (H&E)

inflammatory and antiapoptotic potentials against the STZ side effects. Gp4 showed significant down-regulation of TP53 genes compared to Gp3 (P = 0.033) and showed no significant differences when compared to Gp1 for CDKN1A (P = 0.139). Whilst the FGF7 gene was significantly up-regulated (P = 0.02).

Relationships among evaluated treatments and parameters

Multivariate analysis revealed that the first two PCs account for 99.8% of the total variation (Fig. 12). PC1 showed higher variation (85.99%) in comparison with PC2 (13.81%). The PC1 divided the assessed treatments into two groups: Groups 1 and 4 were on

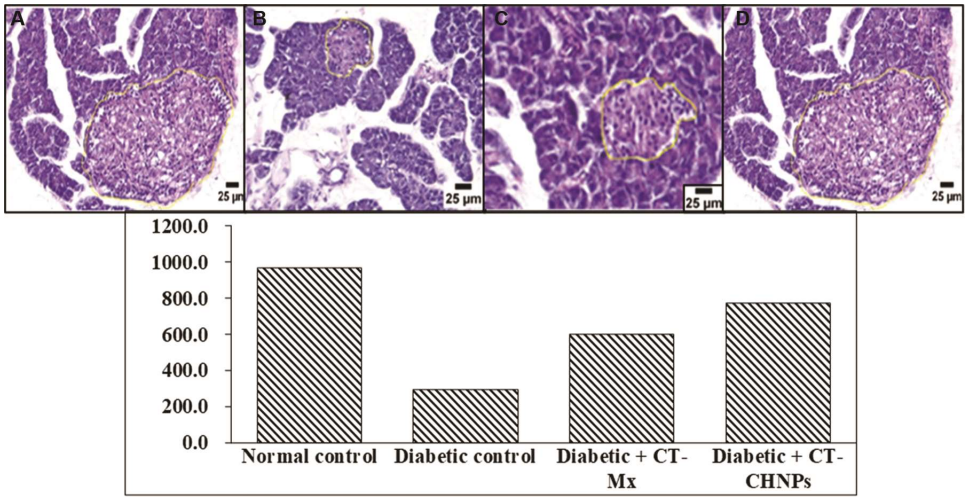

Fig. 8 — Morphometric comparison of the perimeter of the pancreatic islets in the four groups

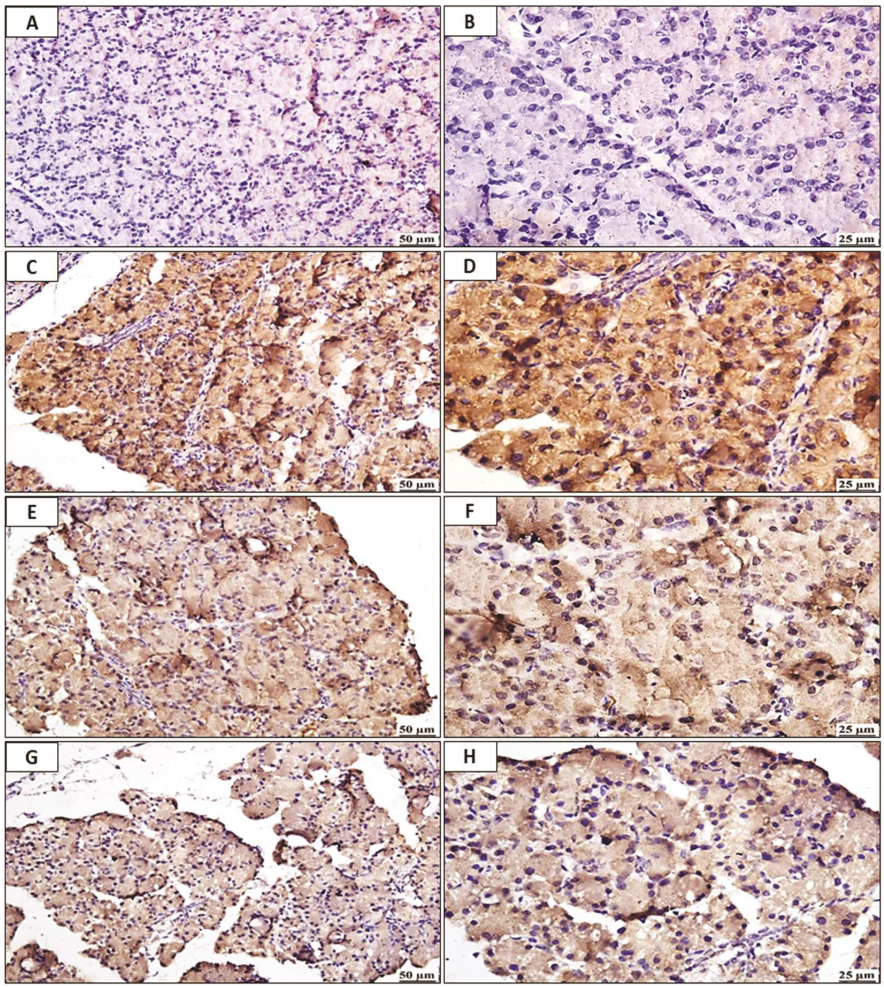

Fig. 9 — NF-κB immunohistochemical examinations of pancreatic tissues. (A, B) Pancreas of the Gp1, showing normal expression of NF-κB. (C, D) The pancreas of the Gp2 (positive group) showing higher expression of NF-κB. (E, F) The pancreas of diabetic + *CT*-Mx (Gp3) rats showing moderate expression of NF-κB. (G, H) The pancreas of diabetic rats treated with *CT*- CHNPs, showing limited expression of NF-κB (immunostaining)

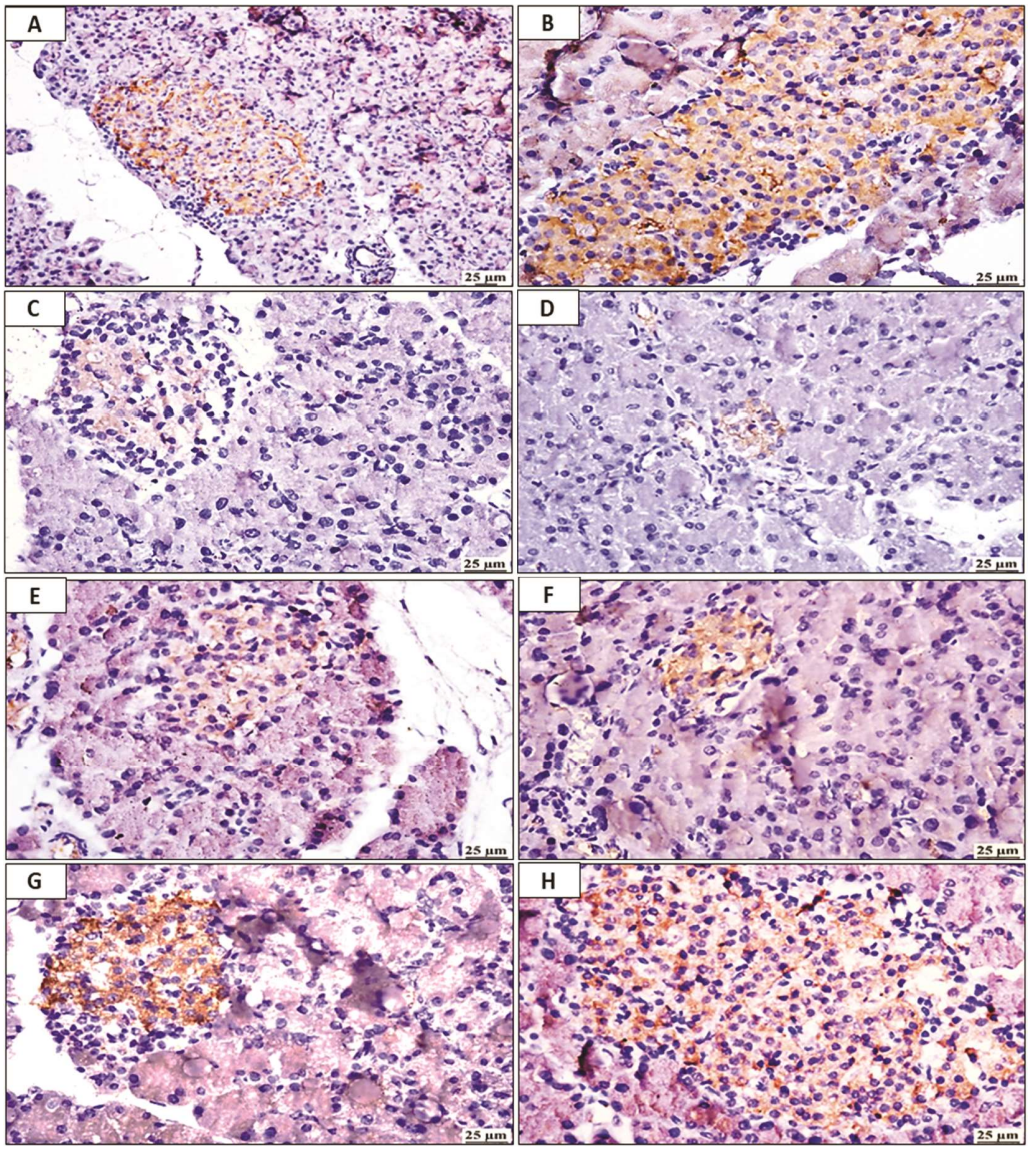

Fig. 10 — Insulin immunohistochemical examinations of pancreatic tissues. (A, B) Pancreas of the Gp1, showing normal expression of insulin in pancreatic islet. (C, D) The pancreas of the Gp2 (positive group) showing weak expression of insulin in pancreatic islets. (E, F) The pancreas of diabetic + *CT*-Mx (Gp3) rats showed moderate expression of insulin in pancreatic islets. (G, H) The pancreas of diabetic rats treated with *CT*- CHNPs, showing higher expression of insulin in pancreatic islet (immunostaining)

the negative side of PC1, while Groups 2 and 3 were on the positive side of PC1. The evaluated parameters: fasting blood glucose level, ALT, AST, MDA, and CDKN1A, were associated with Groups 2 and 3 on the positive side of the PC1. This signifies the high performance of Group 2 and Group 3 in the aforementioned parameters compared to the other treatments. Groups 1 and 4, on the other hand, were on the opposing side and had a worse performance of all these parameters. Otherwise, SOD, GSH, FGF7, and insulin were on the opposite side of Groups 2 and 3, indicating lower performance in these parameters. The FBS level, ALT, AST, MDA, and CDKN1A exhibited robust positive inter-association by adjacent vectors. Likewise, SOD, GSH, FGF7, and insulin revealed robust positive inter-association and negative relationships with the above-mentioned parameters.

The heatmap and hierarchical clustering based on the assessed parameters divided the evaluated Groups into two main clusters. Group 2 was represented in a separate cluster, while the other three groups were clustered in the other one (Fig. 13). Group 2 possessed the highest values for fasting blood glucose

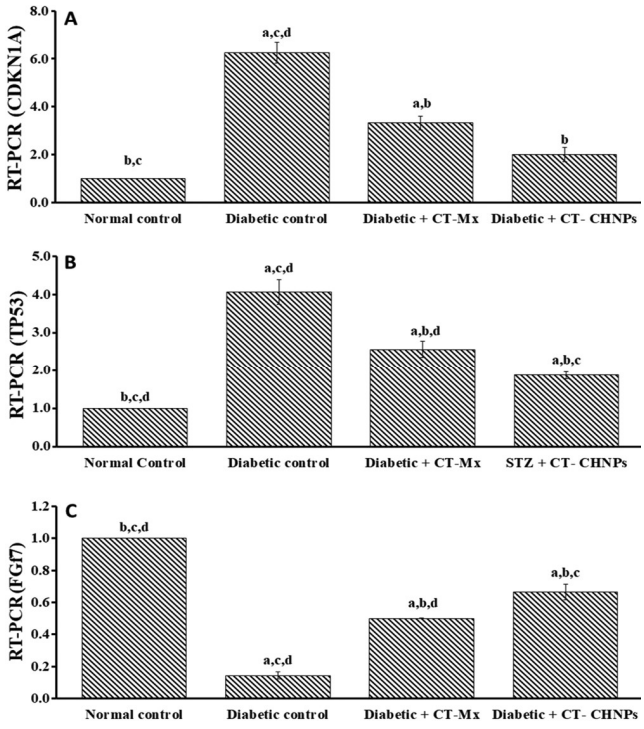

Fig. 11 — Gene expression profiling of the pancreas in rats. (A) CDKN1A; (B) TP53; and (C) FGF7. Data expressed as mean ± SD, n = 5 for each group

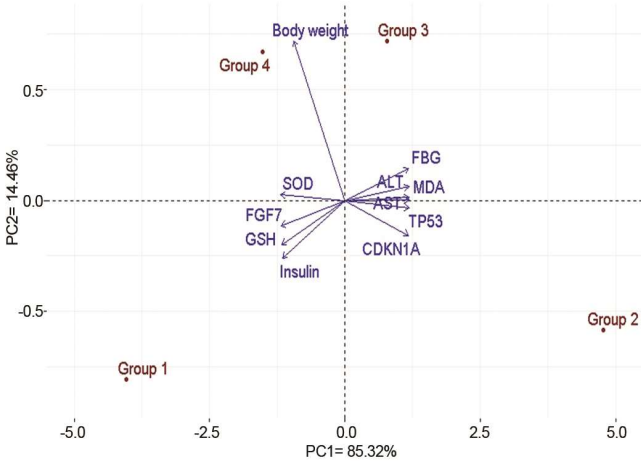

Fig. 12 — Relationships among evaluated treatments and parameters

level, ALT, AST, MDA, and CDKN1A (depicted in blue). SOD, GSH, FGF7, insulin, and body weight, on the other hand, were at their lowest in Group 2 (red values). On the other hand, Group 1 exhibited the highest values for SOD, GSH, FGF7, insulin, and body weight, while the lowest values for fasting blood glucose level, ALT, AST, MDA, and CDKN1A. Group 3 and Group 4 displayed intermediate values for all assessed parameters.

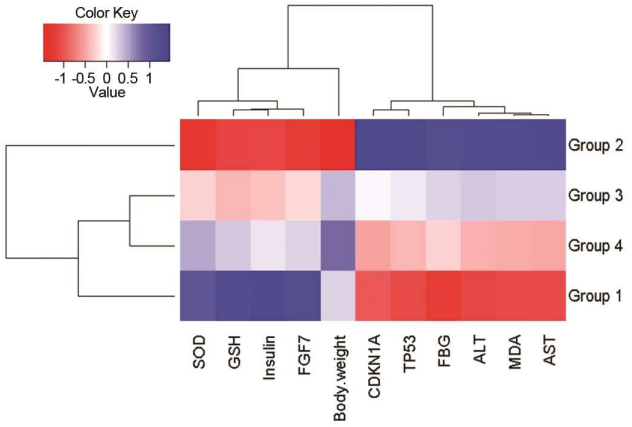

Fig. 13 — Interpretation of the heatmap and hierarchical clustering

Discussion

Chronic hyperglycemia, as well as disturbances in carbohydrate, fat, and protein metabolism, are hallmarks of diabetes mellitus syndrome[36], which is, in turn, associated with a complete or partial lack of insulin action and/or insulin release. Diabetes is brought on by the selective streptozotocin's (STZ) damage of pancreatic β-cells[37].

In the current work, FBG levels were elevated, and serum insulin levels were decreased after intraperitoneal injection of STZ, confirming the successful induction of diabetes in rats that was similar to[38]. Zafar & Naqvi, 2010[39] also found that after STZ was administered, there was a significant high FBG across all groups over time. According to our findings, diabetic rats treated with *CT*-Mx (Gp3) and those treated with *CT*-CHNPs (Gp4) had lower FBG levels and improved insulin secretion.

Chitosan is a cationic polysaccharide that occurs naturally[21]. Due to its unique and beneficial qualities, it has sparked a lot of curiosity as an excipient for the manufacture of nanoparticles. Also, chitosan nanoparticles have been shown to be effective in treating a wide range of diseases[21]. Besides its biodegradability, nontoxicity, and biocompatibility[21], chitosan possesses a number of other desirable qualities. Adding an amino group to chitosan makes it more amenable to various chemical manipulations[40]. Our results showed that the antihyperglycemic action of *CT*-Mx and *CT*-CHNPswas demonstrated *via* (i) a significant decrease of the high level of FBG like other antioxidant herps such as Hypericum perforatum[41], (ii) a significant promotion in insulin secretion. These findings demonstrated the antidiabetic effect of *CT*-CHNPs. Initial research by

Daisy & Rajathi, 2009[42] found that daily dose of *C. ternatea* leaf and flowers (CTL and CTF) aqueous extracts in alloxan-induced diabetic rats whose blood glucose and glycosylated hemoglobin levels were monitored daily for 84 days showed significant reductions in both, indicating that the overall blood glucose level was controlled, likely due to improved insulin secretion. Significantly higher serum insulin levels were observed in CTL and CTF-treated diabetic animals compared to diabetic control animals. CTL and CTF appear to have prompted greater insulin secretion in alloxan-induced diabetic rats. Similar results were seen in alloxan-induced diabetic rats given *C. ternatea* extract twelve h after treatment[43]. This agreed with findings from a study on fresh *C. ternatea* flowers that demonstrated their hypoglycemic and hypolipidemic effects[44]. Moreover, the expression of insulin was evaluated in β-cells in pancreatic islets of different experimental groups by immunohistochemistry. These results showed mild expression in the diabetic group, and moderate expression was recorded in *CT*-Mx group; Interestingly, marked insulin expression was obvious in *CT*-CHNPs treated group. These results are supported by the results of Habibuddin *et al*, 2008[44] who stated that ingestion of butterfly pea flower (*C. ternatea*) ethanol extract orally increases the number of pancreatic beta cells and decreases the level of glycated hemoglobin (HbA1c) in diabetic male Wistar rats (Rattus norvegicus).

According to the current investigation results, TP53 expression was significantly higher in diabetic rats than in controls. Mice with T2DM had higher levels of TP53 expression and produced more pro-inflammatory cytokines, as shown by research by Minamino *et al.,* 2009[45]. In 2015, Homayounfar *et al.* showed that TP53 levels were elevated in peripheral tissues[46]. Study discovered that TP53 expression is elevated in the endothelium of various organs and tissues[47]. These results show that oxidative stress brought on by diabetes causes TP53 expression to rise in many tissues. In addition, multiple mechanisms have been identified by Kung & Murphy, 2016[6] in which p53 activation causes pancreatic dysfunction and reduces insulin production. They also found that p53 is stabilized and activated as a transcription factor under conditions of multiple stresses[6]. In response to stress, the transcription factor p53 is stabilized and activated, which causes the transactivation of genes such as CDKN1A, which induces growth arrest and senescence. Our research revealed that diabetic rats had higher CDKN1A gene expression, which is a sign of oxidative cellular damage[6].

Additionally, hyperinsulinemia and glucose intolerance were also induced by ectopic overexpression of the p53 isoform in mice, and the isoform also up-regulated the expression of a thymidine kinase. These results indicate that p53 can significantly affect growth and development[48].

In contrast, our study discovered that diabetic rats had reduced FGF7 gene expression compared to the group that served as the control. Our findings were similar to what was reported by Moura *et al.,* 2019[49], who discovered that FGF7 levels were significantly suppressed in diabetic skin and that, in contrast to what was seen during wound healing in healthy mice, FGF7 over-expression promotes wound healing in several ways that include increased keratinocyte or fibroblast proliferation and migration. Type 2 diabetes increases ROS and pro-inflammatory cytokines[50]. ROS and its harmful effects are directly eliminated by enzyme-based antioxidants like SOD[50].

In line with Guo *et al.,* 2022[51], who discovered that oral administration of SOD effectively reduced the oxidative stress in diabetic rats, our results showed decreased levels of SOD, GSH, and increased MDA levels reflecting increased oxidants load in diabetic rats, when compared to the control group. GSH, a significant intracellular antioxidant, is essential for limiting the consequences of oxidative stress[52]. Erythrocyte GSH concentration is lower in type 2 diabetic patients, according to several researchers[52]. In 2018 Lutchmansingh *et al*., in their research, confirmed a GSH deficiency in type 2 diabetes and found that these patients had plasma levels of glutathione (GSH) that are lower with absolute synthesis rates than people who don't have diabetes[53].

Talpate *et al.* found that the injection of EECT dramatically decreased the FSG level in a variety of animal models investigated, indicating the efficiency of its antihyperglycemic effects[38]. The effectiveness of *C. ternatea* in various sections as an antidiabetic has already been reported by several researchers[54]. Our results support these reports. It has also been proven that antioxidants are effective at protecting against organ damage brought on by oxidative stress[55]. These findings demonstrate the potential antihyperglycemic and antioxidant action of *C. ternatea*, which may help to lessen diabetes and its consequences. The non-hidden regeneration of pancreatic islets of Langerhans with marked redistribution and activation of B-cells that

showed secretory granules, especially in the *CT*-CHNPs group, were among the factors that contributed to the improvement of glucose levels in the current study.

Moreover, the immunohistochemistry in the present study showed marked expression of NF-κB in the diabetic group while showed moderate and mild expression in both *CT*-Mx (Gp3) and *CT*-CHNPs (Gp4), respectively. This result agrees with the results of P. Xiao *et al*. 2022 who reported that activation of NF-κB is the most important factor in the etiology of diabetes and its consequences[56]. Overexpressing NIK in mice has recently revealed detrimental effects on the survival and function of β-cell in diabetes models. Despite the fact that an accumulation of NIK might momentarily take place under certain circumstances in order to activate the noncanonical pathway, the protein's expression is typically low as a result of the constitutive ubiquitin-mediated degradation of proteins[56].

The possible regenerative activity of *C. ternatea* is attributed to its high antioxidant activity, which scavenges the tissue ROS produced by the injured cells because STZ that was used to artificially induce diabetes in the mice, according to Shende *et al.,* 2012[57]. This is consistent with the research's conclusions and outcomes. Due to its ability to stimulate pancreas regeneration, it may be said that *C. ternatea* extract has the strongest antidiabetic and substantial in-vivo antioxidant activity.

Multiple diabetes-related genes, including CDKN1A, TP53, and FGF7, were modulated in our study's diabetic mice induced by STZ, whereas in the *C. ternatea*-treated groups, CDKN1A and TP53 expressions were decreased, and FGF7 expression was elevated, indicating an improvement in the tissue's antioxidant profile and a reduction in response to stress, particularly when given in nanoparticle form. Similarly, Minelko *et al.,* 2020[58] found that a protein extract from in vivo studies using alloxan-induced diabetic mice showed that the *C. ternatea* flower effectively lowered blood glucose levels. In alloxan-induced diabetic mice, this protein also drastically changed the expression of key genes linked to diabetes, including PPAR, Tcf7l2, Glut2, Capn10, and MCP1. As a result, *C. ternatea* extract might help with diabetes management and could be a promising future target therapy.

Conclusion

C. ternatea methanolic extract has antidiabetic and antioxidant efficacy, as evidenced by the study. This is likely due to the presence of several active chemical compounds, like phenols, and flavonoids, that could ameliorate the side effects associated with diabetic conditions. Both the *C. ternatea* methanolic extract and the novel *C. ternatea* methanolic extract loaded with chitosan nanoparticles showed discernible hypoglycemic activity and improved its associated complications; however, the nano-formula showed noticeably better activity in all tested parameters, including improvement of liver functions and suppression of the oxidative stress generated by diabetes, in addition to suppressing inflammatory and apoptotic gene expression.

Additionally, histological and immunohistochemical analysis showed that *C. ternatea* treatment promotes the regeneration of pancreatic cells that impacts insulin synthesis to control blood glucose levels. Therefore, this might be taken into account for the management of diabetes mellitus. Due to its pharmacological benefits, consequently, we expect that chitosan nanoparticles containing *C. ternatea* methanolic extract could represent a viable future target therapy for diabetes and could be used as safe antidiabetic medicines.

Ethics approval

All experimental protocols followed the criteria for animal experimentation, which was authorized by the Ethical Committee of Helwan University's Faculty of Science (Egypt), (No. HU-IACUC/Z/AA1017-16).

Funding

This work was funded by the Prince Nawaf bin Abdulaziz Chair for Sustainable Development and in collaboration with the Deanship of Scientific Research at Jouf University under grant No (DSR2021-Prince Nawaf bin Abdulaziz Chair-03).

Conflicts of interest

All authors declare no conflict of interest.

References

1 Dilworth L, Facey A & Omoruyi F, Diabetes Mellitus and Its Metabolic Complications: The Role of Adipose Tissues. *Int J Mol Sci*, 22 (2021) 7644.

2 Tomic D, Shaw JE & Magliano DJ, The burden and risks of emerging complications of diabetes mellitus. *Nat Rev Endocrinol*, 18 (2022) 525.

3 Tan Y, Cheong MS & Cheang WS, Roles of Reactive Oxygen Species in Vascular Complications of Diabetes: Therapeutic Properties of Medicinal Plants and Food. *Oxygen*, 2 (2022) 246.

4 Kosmalski M, Śliwińska A & Drzewoski J, Non-Alcoholic Fatty Liver Disease or Type 2 Diabetes Mellitus-The Chicken or the Egg Dilemma. *Biomedicines*, 11 (2023) 1097.

5 Ghalwash AA, Baalash AA, Gaafar NK, Wasfy RE & Noeman SA, The interplay between oat beta glucan, gut microbiota and gut-liver axis in treatment of obesity associated non-alcoholic steatohepatitis and Type II diabetes mellitus. *Indian J Biochem Biophys*, 59 (2022) 14.

6 Kung CP & Murphy ME, The role of the p53 tumor suppressor in metabolism and diabetes. *J Endocrinol*, 231 (2016) R61.

7 Krentz AJ & Bailey CJ, Oral antidiabetic agents: current role in type 2 diabetes mellitus. *Drugs*, 65 (2005) 385.

8 Hasan SM, Khan MI & Umar BU, Effect of ethanolic extract of swietenia mahagoni seeds on experimentally induced diabetes mellitus in rats. *Bangladesh Med Res Counc Bull*, 39 (2013) 6.

9 Singh SK, Kesari AN, Gupta RK, Jaiswal D & Watal G, Assessment of antidiabetic potential of Cynodondactylon extract in streptozotocin diabetic rats. *J Ethnopharmacol*, 114 (2007) 174.

10 Salehi B, Ata A, V Anil Kumar N, Sharopov F, Ramírez-Alarcón K, Ruiz-Ortega A, Abdulmajid Ayatollahi S, Tsouh Fokou PV, Kobarfard F, Amiruddin Zakaria Z, Iriti M, Taheri Y, Martorell M, Sureda A, Setzer WN, Durazzo A, Lucarini M, Santini A, Capasso R, Ostrander EA, Atta-ur-Rahman, Choudhary MI, Cho WC & Sharifi-Rad J, Antidiabetic Potential of Medicinal Plants and Their Active Components. *Biomolecules*, 9 (2019) 551.

11 Baiano A & Del Nobile MA, Antioxidant Compounds from Vegetable Matrices: Biosynthesis, Occurrence, and Extraction Systems. *Crit Rev Food Sci Nutr*, 56 (2016) 2053.

12 Al-Sodany Y, Bazaid S& Mosallam H, Medicinal Plants in Saudi Arabia: I. Sarrwat Mountains at Taif, KSA. *Acad J Plant Sci*, 6 (2013) 134.

13 GOH SE, KWONG PJ, Lee CNG, Jie WNG & Yaw KEE, Antioxidant-rich *Clitoria ternatea* L. flower and its benefits in improving murine reproductive performance. *Food Sci Technol (Campinas),* 42 (2022) e25921.

14 Sofiah S, Aswan A, Yunanto I,Ramayanti C, Amelia PD & Utami AN, Making Herbal Tea from a Mixture of Butterfly Pea Flower (*Clitoria ternatea*) and Ginger Powder (*Zingiber Officinale)* by using Drying Method According to Indonesian National Standards (SNI). *Atlantis Press International B.V.,* 9 (2021) 107.

15 Chusak C, Thilavech T, Henry CJ & Adisakwattana S, Acute effect of Clitoria ternatea flower beverage on glycemic response and antioxidant capacity in healthy subjects: a randomized crossover trial. *BMC Complement Altern Med*, 18 (2018) 6.

16 Leyva-López N, Gutierrez-Grijalva EP, Ambriz-Perez DL & Heredia JB, Flavonoids as Cytokine Modulators: A Possible Therapy for Inflammation-Related Diseases. *Int J Mol Sci*, 17 (2016) 921.

17 Nair V, Bang WY, Schreckinger E, Andarwulan N & Cisneros-Zevallos L, Protective role of ternatin anthocyanins and quercetin glycosides from butterfly pea (*Clitoria ternatea* Leguminosae) blue flower petals against lipopolysaccharide (LPS)-induced inflammation in macrophage cells. *J Agric Food Chem*, 63 (2015) 6355.

18 ALshamrani SM, Safhi FA, Mobasher MA, Saleem RM, Alharthi A, Alshaya DS & Awad NS, Antiproliferative Effect of *Clitoria ternatea* Ethanolic Extract against Colorectal, Breast, and Medullary Thyroid Cancer Cell Lines. *Separations*, 9 (2022) 331.

19 Neda GD, Rabeta MS & Ong MT, Chemical composition and antiproliferative properties of flowers of *Clitoria ternatea* . *Int Food Res J*, 20 (2013) 1229.

20 DiSanto RM, Subramanian V & Gu Z, Recent advances in nanotechnology for diabetes treatment. *Wiley Interdiscip Rev Nanomed Nanobiotechnol*, 7(2015) 548.

21 Verma AK, Singh P, Malik M & Srivastava SK, Synthesis of zinc oxide nanoparticles, its characterization and anti-microbial activity assessment. *Indian J Biochem Biophys*, 59 (2022) 1106.

22 Kesharwani P, Gorain B, Low SY, Tan SA, Ling ECS, Lim YK, Chin CM, Lee PY, Lee CM, Ooi CH, Choudhury H & Pandey M, Nanotechnology based approaches for antidiabetic drugs delivery. *Diabetes Res Clin Pract*, 136 (2018) 52.

23 Sarje S, Bhalerao S, Shinde D, Katariya M & Farooqui S, Phytochemical screening of *cyamopsis tetragonoloba* L. *Extracts*, 5 (2020) 41.

24 Safhi FA, ALshamrani SM, Jalal AS, Awad NS, Sabit H, Abdelgawad FE, Khalil SS, Khodeer DM & Mobasher MA, Asian Pigeonwing Plants (*Clitoria ternatea*) Synergized Mesenchymal Stem Cells by Modulating the Inflammatory Response in Rats with Cisplatin-Induced Acute Kidney Injury. *Pharmaceuticals*, 15 (2022) 1396.

25 Venkatachalam Perumal V, Manickama T, Bang K, Velmurugane P & Ohe B, Antidiabetic potential of bioactive molecules coated chitosan nanoparticles in experimental rats. *Int J Biol Macromol*, 92 (2016) 63.

26 Kenneth KW & Youming H, Streptozocin induced diabetic models in mice and rats. *Curr Protoc Pharmacol*, 5 (2008) 1.

27 Thefeld W, Hoffmeister H, Busch EW, Koller PU & VollmarJ, Reference Values for the Determination of GOT, GPT, and Alka-line Phosphatase in Serum with Optimal Standard Methods. *Dtsch Med Wochenschr*, 99 (1974) 343.

28 Giannopolitis CN & Ries SK, Superoxide Dismutases: I. Occurrence in Higher Plants. *Plant Physiol*, 59 (1977) 309.

29 Mathai CK & Beutler E, Blood Preservatives for the Glutathione Stability Test. *Nature*, 207 (1965) 204.

30 Jiang Z, Hunt J & Wolff S, Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. *Anal Biochem*, 202 (1992) 384.

31 Jensen K, Theory and Practice of Histological Techniques, 6th Edition, *J Neuropathol Exp Neurol*, 67 (2008) 633.

32 Huang H, Liu Y, Daniluk J. Gaiser S, Chu J, Wang H, Li ZS, Logsdon CD & Ji B, Activation of nuclear factor-κB in acinar cells increases the severity of pancreatitis in mice. *Gastroenterology*, 144 (2013) 202.

33 Abunasef SK, Amin HA & Abdel-Hamid GA, A histological and immunohistochemical study of beta cells in streptozotocin diabetic rats treated with caffeine. *Folia Histochem Cytobiol*, 52(2014) 42.

34 Muller PY, Janovjak H, Miserez AR & Dobbie Z, Short technical report processing of gene expression data generated by quantitative real-time RT-PCR. *Biotechniques*, 32 (2002) 1372.

35 Chan YH, Biostatistics102: quantitative data–parametric and non-parametric tests. *Singapore Med J,* 44 (2003) 391.

36 Schoenfelder T, Cirimbelli TM & Citadini-Zanette V, Acute effect of Trema micrantha on serum glucose levels in normal and diabetic rats. *J Ethnopharmacol*, 107 (2006) 456.

37 Gutierrez RM, Gómez YG & Guzman MD, Attenuation of nonenzymatic glycation, hyperglycemia, and hyperlipidemia in streptozotocin-induced diabetic rats by chloroform leaf extract of *Azadirachta indica*. *Pharmacogn Mag*, 7 (2011) 254.

38 Talpate KA, Bhosale UA, Zambare MR & Somani R, Antihyperglycemic and antioxidant activity of *Clitorea ternatea* Linn. on streptozotocin-induced diabetic rats. *Ayu*, 34 (2013) 433.

39 Zafar M & Naeem-ul-hassan Naqvi S, Effects of STZ-Induced diabetes on the relative weights of kidney, liver and pancreas in albino rats: a comparative study. *Int J Morphol.,* 28 (2010) 135.

40 Thakur A & Kathpalia R, Engineered nanoparticles (ENPs): Unexplored potential and limitations. *Indian J Biochem Biophys*, 59 (2022) 1163.

41 Bravo-Osuna I, Schmitz T, Bernkop-Schnürch A, Vauthier C & PonchelG, Elaboration and characterization of thiolated chitosan-coated acrylic nanoparticles. *Int J Pharm*, 316 (2006) 170.

42 Daisy P& Rajathi M, Hypoglycemic Effects of *Clitoria ternatea* Linn. (Fabaceae) in Alloxan-Induced Diabetes in Rats. *Trop J Pharm Res*, 8 (2009) 393.

43 Jeyaraj EJ, Lim YY & Choo WS, Extraction methods of butterfly pea (*Clitoria ternatea*) flower and biological activities of its phytochemicals. *J Food Sci Technol*, 58 (2021) 2054.

44 Habibuddin M, Daghriri HA Humaira T, Al-Qahtani MS & Hefzi AA, Antidiabetic effect of alcoholic extract of *Caralluma sinaica* L. on streptozotocin-induced diabetic rabbits. *J Ethnopharmacol*, 117 (2008) 215.

45 Minamino T, Orimo M, Shimizu I, Kunieda T, Yokoyama M, Ito T, Nojima A, Nabetani A, Oike Y, Matsubara H, Ishikawa F & Komuro,A crucial role for adipose tissue p53 in the regulation of insulin resistance. *Nat Med*, 15 (2009) 1082.

46 Homayounfar R, Jeddi-Tehrani M, Cheraghpour M, Ghorbani A & Zand H, Relationship of p53 accumulation in peripheral tissues of high-fat diet-induced obese rats with decrease in metabolic and oncogenic signaling of insulin. *Gen Comp Endocrinol*, 214 *(*2015) 134.

47 Mobasher MA & Valverde ÁM, Signalling Pathways Involved in Paracetamol-Induced Hepatotoxicity: New Insights on the Role of Protein Tyrosine Phosphatase 1B. *Arch Physiol Biochem*, 120 (2014) 51.

48 Hinault C, Kawamori D, Liew CW, Maier B, Hu J, Keller SR, Mirmira RG, Scrable H& Kulkarni RN, Delta40 Isoform of p53 controls beta-cell proliferation and glucose homeostasis in mice. *Diabetes*, 60 (2011) 1210.

49 Moura J, Sørensen A, Leal EC, Svendsen R, Carvalho L, Willemoes RJ, Jørgensen PT, Jenssen H, Wengel J, Dalgaard LT & Carvalho E, microRNA-155 inhibition restores Fibroblast Growth Factor 7 expression in diabetic skin and decreases wound inflammation. *Sci Rep*, 9 (2019) 5836.

50 Nguyen D, Hsu JW, Jahoor F & Sekhar RV, Effect of increasing glutathione with cysteine and glycine supplementation on mitochondrial fuel oxidation, insulin sensitivity, and body composition in older HIV infected patients. *J Clin Endocrinol Metab*, 99 (2014) 169.

51 Guo J, Liu H, Zhao D, Pan C, Jin X, Hu Y, Gao X, Rao P & Liu S, Glucose-lowering effects of orally administered superoxide dismutase in type 2 diabetic model rats. *NPJ Sci Food*, 6 (2022) 36.

52 Almaeen AH, Alduraywish AA, Mobasher MA, Almadhi OIM, Nafeh HM & El-Metwally TH, Oxidative stress, immunological and cellular hypoxia biomarkers in hepatitis C treatment-naïve and cirrhotic patients. *Arch Med Sci*, 17 (2020) 368.

53 Lutchmansingh FK, Hsu JW, Bennett FI, Badaloo AV, McFarlane-Anderson N, Gordon-Strachan GM, Wright-Pascoe RA, Jahoor F & Boyne MS, Glutathione metabolism in type 2 diabetes and its relationship with microvascular complications and glycemia. *PLoS One*, 13 (2018) e0198626.

54 Sethiya NK, Trivedi A, Patel MB & Mishra SH, Comparative pharmacognostical investigation on four ethanobotanicals traditionally used as Shankhpushpiin India. *J Adv Pharm Technol Res*, 1 (2010) 388.

55 Ramaswamy V, Varghese N & Simon A, An investigation on cytotoxic and antioxidant properties of *Clitorea ternatea*. *Int J Drug Discov*, 3 (2011) 74.

56 Xiao P, Takiishi T, Violato NM, Licata G, Dotta F, Sebastiani G, Marselli L, Singh SP, Sze M, Van Loo G, Dejardin E, Gurzov EN & Cardozo AK. NF-κB-inducing kinase (NIK) is activated in pancreatic β-cells but does not contribute to the development of diabetes. *Cell Death Dis*, 13 (2022) 476.

57 Shende V, Sahane R, Lawar M, Hamdulay N & Langote HAH, Evaluation of anti-compulsive effect of ethanolic extract of *Clitoria ternatea* in mice. *Asian J Pharm Clin Res*, 5 (2012) 120.

58 Minelko M, Gunawan AG, Ali S, Suwanto A & Yanti, Protein extracted from *Clitoria ternatea* modulates genes related to diabetes *in vivo*. *Int Food Res J*, 27 (2020) 610.