



Can adjuvant supplements of *Costus speciosus* nanoparticles improve metformin control of hyperglycemia, oxidative stress, and apoptotic changes in Langerhans islets in a rat model of type 2 diabetes?

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Article History

Received: 27 March 2020

Reviewed: 29/March/2020 to 08/May/2020

Accepted: 09 May 2020

E-publication: 16 May 2020

P-Publication: July - August 2020

Citation

Safia M. Bahshwan, Samar O. Abdullah Rabah, Haifa S. Almukadi, Duaa M. Bakhshwin. Can adjuvant supplements of *Costus speciosus* nanoparticles improve metformin control of hyperglycemia, oxidative stress, and apoptotic changes in Langerhans islets in a rat model of type 2 diabetes?. *Medical Science*, 2020, 24(104), 1904-1914

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General Note



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ABSTRACT

Introduction: Type 2 diabetes is one of the chronic and serious conditions that occur commonly. Metformin (MT) is an essential drug used to control symptoms of type 2 diabetes. It is usually taken in combination with other medications to lower blood sugar levels. Nanoparticles of the plant *Costus speciosus* (NPC) decreased the concentrations of blood glucose in the sera of experimental animals with diabetes type 2. **Aim:** This study aimed to examine the potential additive antidiabetic impact of oral NPC and their preliminary mechanism of action when added in combination with MT in type 2 diabetic rats, compared to MT monotherapy. **Methods:** Type 2 diabetes was induced in 30 male Wistar rats using high-fat diet and streptozotocin. The rats were separated into 5 groups and treated orally with MT, NPC, or MT and NPC. **Results:** The results showed that NPC reduced blood sugar levels, increased insulin secretion, decreased lipid peroxidation, downregulated gene expression of caspase-3, and improved the pathology of pancreatic beta cells. The use of the NPC and MT combination showed a marked improvement in body weight and insulin secretion, and a distinct reduction in oxidative stress and beta-cell damage. **Conclusion:** The use of NPC with MT is an effective treatment regimen in managing unfavourable symptoms of experimentally induced diabetes type 2.

Keywords: Nanoparticles *Costus speciosus*, type 2 diabetes, metformin, streptozotocin, insulin, oxidative stress, caspase-3

1. INTRODUCTION

Type 2 diabetes mellitus is considered one of the progressive disorders frequently occurred in modern times. Recent reports showed an increase in its prevalence in the Arab Gulf countries, including the Kingdom of Saudi Arabia (KSA) (Alhyas et al., 2012; Alqurashi et al., 2011; Robert et al., 2018; Saquib et al., 2017). It was showed previously that the incidence of diabetes in the KSA was around 11-14% among middle aged women and men, and 50% among citizens over 65 years of age (Mokdad, 2015). Moreover, one million of Saudi Arabian citizens were expected to be in the pre-diabetes stage (Mokdad, 2015). Type 2 diabetes differs from type 1 diabetes since it is associated with metabolic syndrome. This type of diabetes is characterized by the incidence of resistance of some cells and tissues in the body to the effect of insulin, and accompanied by a decrease in insulin secretion from the beta cells of the pancreas, which leads to high blood glucose concentrations (Hameed et al., 2015).

Metformin (MT), a biguanide agent, is one of the most extensively used drugs in controlling type 2 diabetes (Chaudhury et al., 2017; Marín-Peñalver et al., 2016; Zhou et al., 2018), and in managing symptoms of pre-diabetic patients (Hostalek et al., 2015). MT has a unique mechanism of action that is far away from the beta cells and their insulin secretion (Rena et al., 2017). Type 2 diabetic patients treated with MT were protected against severe hypoglycemia, a life-threatening side effect that usually occurs with other oral antidiabetic medications and with insulin injections as well (Nasri and Rafieian-Kopaei, 2014). Unfortunately, in many cases, MT monotherapy was not as efficient for adjusting blood glucose and hemoglobin A1c (HB A1c) levels. As a result, MT is usually combined with insulin subcutaneous injections or other oral antidiabetic medications (Cavaiola and Pettus, 2000). Using medicinal herbs as therapeutic agents is a global interest since they are believed to have lower side effects than synthetic drugs. Lower cost of the natural herbs is one of their advantages as well. Nevertheless, attainment of high doses of pure natural compounds is a huge obstacle for users (Josephine Ozioma and Antoinette Nwamaka Chinwe, 2019; Karimi et al., 2015). The nanotechnology technique has recently opened up a new era for the use of small amounts of natural extracts in the treatment of diseases, due to their improved solubility and bioavailability (Watkins et al., 2015). For instance, nanoparticles of *Costus speciosus* (NPC) are effective in decreasing blood sugar concentrations in type 2 diabetes induced in rats (Alamoudi et al., 2014).

The current study aimed to examine the combined effect of NPC and MT on controlling blood levels of glucose and on affecting some biomarkers of programmed cell death and oxidative stress in diabetes type 2 produced in rats.

2. MATERIALS AND METHODS

Chemicals

Glucophage tablets (500 mg MT, Merck Santa, France) were used. NPC was kindly provided by Dr. Khediri MM, Physical Chemistry Department, Laith Faculty of Applied Sciences, Umm Al-Qura University, Saudi Arabia. Streptozotocin (STZ) was purchased from Sigma Aldrich, USA ($\geq 98\%$ HPLC).

Animals

The research was conducted on 30 Wistar-type male rats of 220-300 g body weights. The rats were ordered from Mansour Center for Research and Consulting, Jeddah, Saudi Arabia and kept for a week in the laboratories of the King Fahd Center for Research under

standard conditions of pressure, temperature, and humidity as an adaptation period before starting the experiment. The rats were free to drink and eat. The guidelines of the King Abdulaziz University Code of Ethics, the International standards, and the Institutional Animal Care and Use Committee were followed strictly to deal with experimental animals (Nih et al., 2011). The ethical approval of KAU no (182-060-480).

Preparation of STZ solution

STZ was prepared immediately before use with a cold buffer containing 0.05 M citrate solution (pH 4.5) according to the previously reported methods (Al-Thobaiti and Zeid, 2019).

Study design

The experiment was conducted over eight consecutive weeks. Rats were randomly divided into five groups, each containing six rats. Control group rats (group 1) were injected with 0.05 M citrate solution, pH balanced to 4.5, into the intraperitoneal cavity (i.p.). Type 2 diabetes was induced in the other four groups of rats (group 2-5) by feeding them on high-fat diet (HFD) (58% fat) for two weeks, then they were given a single dose of STZ (45 mg/kg, i.p.), (Zhang et al., 2008). Rats in the second group (group 2) did not take any treatment, while rats in the third group (group 3) were given oral MT as a monotherapy (200 mg/kg) (Li et al., 2014), following the induction of diabetes. In the fourth and fifth groups (group 4 and group 5), rats were given oral NPC monotherapy (250 mg/kg) (Bahshwan et al., 2019) and NPC and MT combined therapy, respectively.

Determination of body weight, samples collection, and preservation

After the completion of the study, the final body weights of the rats in all groups were measured. Then, the rats were anesthetized using ether. Blood samples were collected, and serum samples were separated by centrifugation at 3,000 rpm and kept at -80 °C freezer. Pancreatic samples were collected and preserved in 10% of neutral buffered formalin, to be used in histopathological examination and immunohistochemistry.

Determination of fasting serum glucose

Fasting (12 h) serum glucose levels were determined colorimetrically, using the Reactivos GPL kit (Barcelona, Spain) and following its protocol. Basically, glucose is oxidized in the presence of glucose oxidase and forms hydrogen peroxide, which the latter interacts with phenol in the presence of praxidase forming a red color quinone. The red color is measured at 505 nm, which is proportional to the concentration of glucose in the sample.

Determination of fasting serum insulin

The levels of insulin in the fasting sera were determined by the use of a solid phase enzyme-linked immunosorbent assay (ELISA), Immunospec kit (Canada), based on the kits' procedure. In brief, the reaction depends on the use of two anti-insulin antibodies, one is used as a coat for the microplate while the other is labelled with horse radish peroxidase. If insulin is present in the serum, it will be sandwiched between the two antibodies. After 60 min incubation, 3,3',5,5'-Tetramethylbenzidine (TMB) substrate is added leading to the formation of blue color. The reaction is stopped by the addition of the stop solution forming yellow color that is read at 450 nm. The color intensity corresponds to the insulin content in the sample.

Determination of serum malondialdehyde (MDA)

Serum MDA levels were determined colorimetrically, using the Biodiagnostic kit (Egypt) and following its procedure. Briefly, thiobarbituric acid interacts with MDA under acidic conditions. After heating at 95 °C for 30 min, a pink colored adduct is formed and then measured at 534 nm (Satoh, 1978).

Determination of serum reduced glutathione (GSH)

GSH levels in the sera were determined colorimetrically using the Biodiagnostic kit (Egypt) and following its protocol. The assay depends on the reduction of 5, 5' dithio (2-nitrobenzoic acid) with GSH forming a yellow adduct which is measured 405 nm (Beutler et al., 1963).

Haematoxylin and eosin (H&E) staining of pancreas

Thin films (3 to 5 µm) of pancreatic tissues were cut from formalin-preserved pancreas and stained with H & E stain. The slides were then examined under the light microscope for any pathological alterations and photographed by a blind pathologist.

Caspase-3 immunohistochemical staining of pancreas

The immunoperoxidase (PAP, peroxidase/antiperoxidase) reaction was performed using the caspase-3 antibody of Lab Vision (Fremont, Canada, catalog no. RB1197R7). The slides were then examined under the light microscope and photographed by a blind pathologist.

Statistical analysis

Graph Pad Prism version 5 was utilized for presenting and analysing the results of this study. Data were demonstrated by whiskers min to max plot. One-way analysis of variance (ANOVA) was performed to verify the significant difference between groups. Tukey post hoc was then used to assess the significant difference between the treatment groups. The level of significance was accepted at $p \leq 0.05$.

3. RESULTS

Effect of combining MT with NPC on the weight of the diabetic rats

Induction of type 2 diabetes distinctly decreased the body weight of rats in group 2 relative to the control group (group 1) ($p \leq 0.001$). The reduction in the body weight of diabetic rats was not improved to a normal level by any of the treatments used in the other groups. However, rats of group 5 that were treated with both MT and NPC revealed a marked rise in their body weights relative to the diabetic group ($p \leq 0.05$), and that increase was not detected in rats treated with the monotherapies of MT and NPC (group 3 & 4) (Fig. 1).

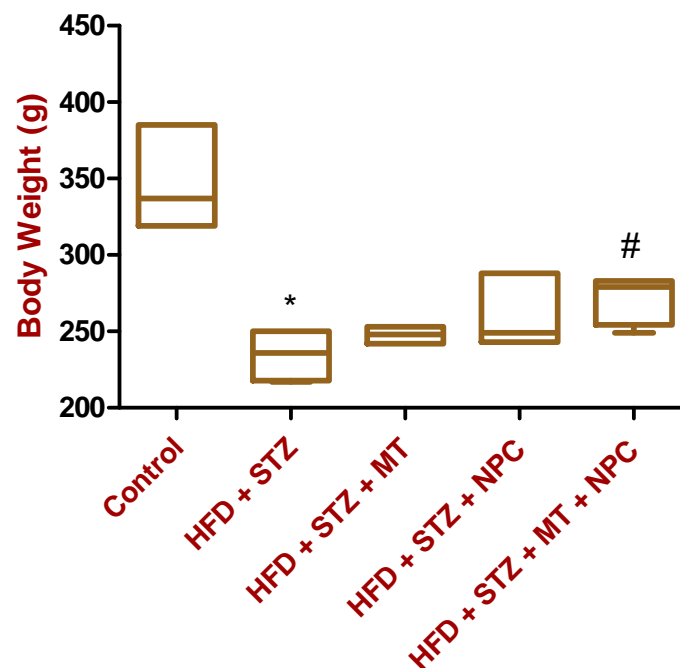


Figure 1 Effect of combining MT with NPC on the body weight of type 2 diabetic rats. Animals in the four groups of treatments and the control group were weighed before and after the experiment. Their final weights in grams (g) are demonstrated by whiskers min to max plot. Every rectangle represents the arithmetic average of all values obtained between the 25th and 75th quartiles of each group. The horizontal lines inside the rectangles represent the median estimate of the values. ANOVA followed by Tukey test were used to assess the significant differences between groups. Significant difference relative to control rats ($p \leq 0.001$) is demonstrated by asterisk symbol (*), and relative to HFD + STZ rats ($p \leq 0.05$) is demonstrated by pound symbol (#).

Effect of combining MT with NPC on the fasting serum glucose levels of the diabetic rats

The levels of glucose in the fasting sera of the diabetic rats (group 2) were significantly increased comparing to the control rats (group 1) ($p \leq 0.001$). A marked reduction in the glucose amounts, which was not less than control values, was detected in the fasting sera of animals in all the three treated groups (group 3-5) relative to the diabetic group ($p \leq 0.001$) (Fig. 2).

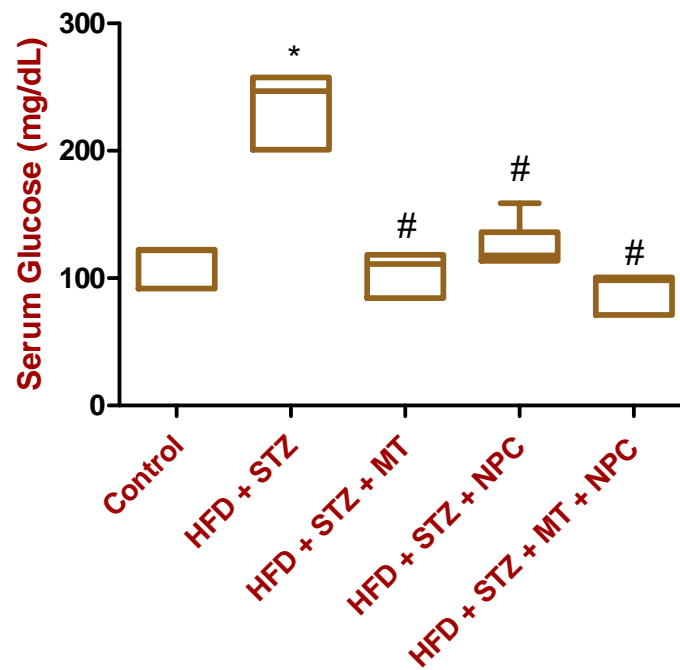


Figure 2 Effect of combining MT with NPC on the fasting serum glucose levels of type 2 diabetic rats. Fasting serum glucose levels of animals in all the groups were measured at the end of the experiment and expressed as mg/dL. Data are demonstrated by whiskers min to max plot. Every rectangle represents the arithmetic average of all values obtained between the 25th and 75th quartiles of each group. The horizontal lines inside the rectangles represent the median estimate of the values. ANOVA followed by Tukey test were used to assess the significant differences between groups. Significant difference relative to control rats ($p \leq 0.001$) is demonstrated by asterisk symbol (*), and relative to HFD + STZ rats ($p \leq 0.001$) is demonstrated by pound symbol (#).

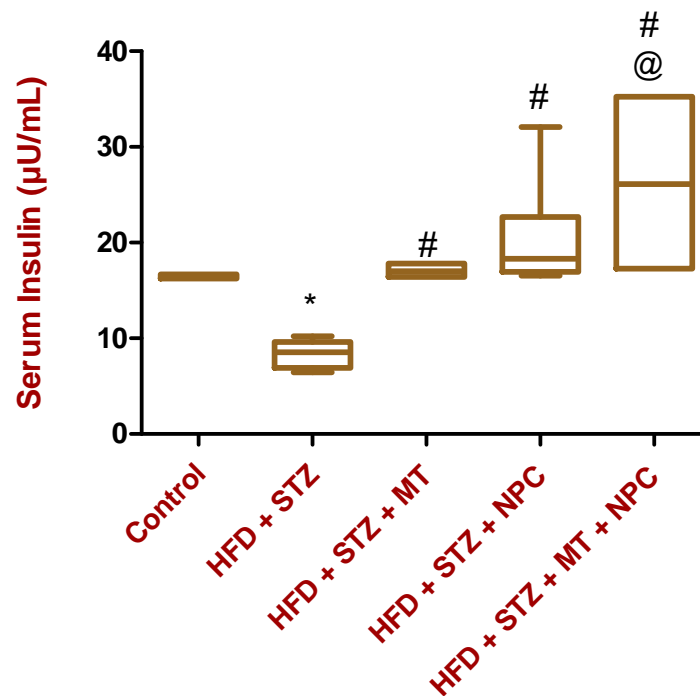


Figure 3 Effect of combining MT with NPC on the fasting serum insulin levels of type 2 diabetic rats. Fasting serum insulin levels of all animals in each group were measured at the end of experiment and expressed as $\mu\text{U/mL}$. Data are demonstrated by whiskers min to max plot. Every rectangle represents the arithmetic average of all values obtained between the 25th and 75th quartiles of each group. The horizontal lines inside the rectangles represent the median estimate of the values. ANOVA followed by Tukey test were used to assess the significant differences between groups. Significant difference relative to control rats ($p \leq 0.05$) is demonstrated by asterisk symbol (*), and relative to HFD + STZ rats ($p \leq 0.05$) is demonstrated by pound symbol (#), while relative to HFD + STZ + MT rats ($p \leq 0.05$) is presented by at symbol (@).

Effect of combining MT with NPC on the fasting serum insulin levels of the diabetic rats

Induction of diabetes distinctly decreased the fasting serum insulin levels of the rats in group 2 compared to the control rats (group 1) ($p \leq 0.05$). Rats treated with MT, NPC and with their combination (group 3-5) presented a marked increase in levels of their serum insulin compared to the diabetic group ($p \leq 0.05$). Moreover, the increase in serum insulin levels of rats in group 5, that were treated with the combination therapy of MT and NPC, was statistically significant than the levels of insulin in the sera of group 3 rats, which were treated with a monotherapy of MT ($p \leq 0.05$) (Fig. 3).

Effect of combining MT with NPC on the serum MDA levels of the diabetic rats

Induction of type 2 diabetes in rats (group 2) markedly elevated their serum MDA levels relative to the control group (group 1) ($p \leq 0.001$). Rats treated with MT, NPC and their combination (group 3-5) revealed a marked decrease in their serum MDA amounts compared to the diabetic animals in group 2 ($p \leq 0.001$). The serum MDA levels of diabetic group treated with both MT and NPC were significantly less than the MDA levels of the MT group (group 3) ($p \leq 0.01$) (Fig. 4).

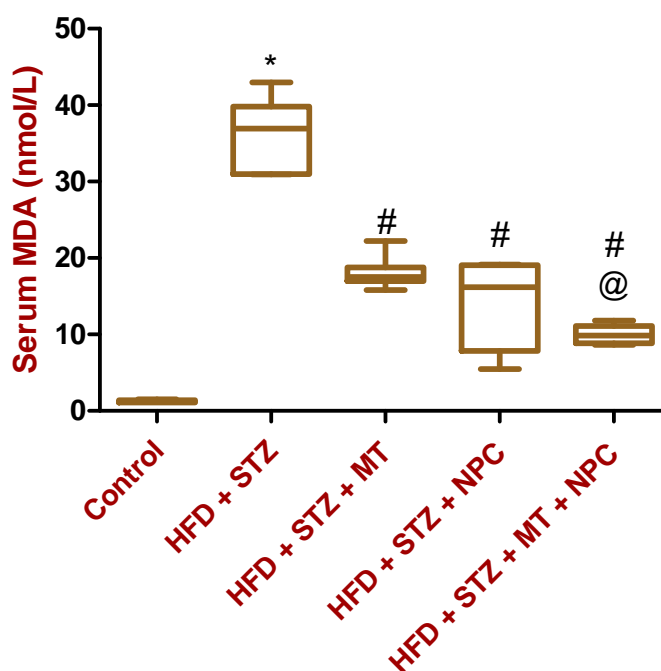


Figure 4 Effect of combining MT with NPC on the serum MDA levels of type 2 diabetic rats. Serum MDA levels of the animals in all the groups were measured at the end of the experiment and expressed as nmol/L. Data are demonstrated by whiskers min to max plot. Every rectangle represents the arithmetic average of all values obtained between the 25th and 75th quartiles of each group. The horizontal lines inside the rectangles represent the median estimate of the values. ANOVA followed by Tukey test were used to assess the significant differences between groups. Significant difference relative to control rats ($p \leq 0.001$) is demonstrated by asterisk symbol (*), and relative to HFD + STZ rats ($p \leq 0.001$) is demonstrated by pound symbol (#), while relative to HFD + STZ + MT rats ($p \leq 0.01$) is presented by at symbol (@).

Effect of combining MT with NPC on the serum GSH levels of the diabetic rats

Serum GSH levels of the diabetic rats (group 2) were decreased in a significant manner comparing to the levels of the control group (group 1) ($p \leq 0.001$). Rats treated with the combination of MT and NPC (group 5) revealed a significant elevation in their serum GSH concentrations relative to the diabetic group ($p \leq 0.001$), and to the MT group ($p \leq 0.001$). In contrast, when treating diabetic rats either with MT (group 3) or NPC (group 4), no increase in serum GSH levels was noticed compared to the diabetic group (group 2) (Fig. 5).

Effect of combining MT with NPC on diabetes-induced islets of Langerhans pathological changes in rats

The induction of diabetes in rats (group 2) caused the development of small-sized Langerhans islands expressing a smaller density of normal beta cells relative to the control group (group 1). Abnormal beta cells lacking nuclei were also detected. Treatment with MT caused a relative improvement in the pathological changes of Langerhans islands, as they appeared to be larger in comparison to the diabetic group, while the number of beta cells remained low, and some were without nuclei as well. NPC treatment improved the pathological condition of the Langerhans islands and beta cells compared to the diabetic group. Ultimately, treatment of rats

with MT and NPC together improved the size of Langerhans islets and increased the density of beta cells having normal nuclei compared to the groups of non-treated and MT-treated diabetic rats (Fig. 6).

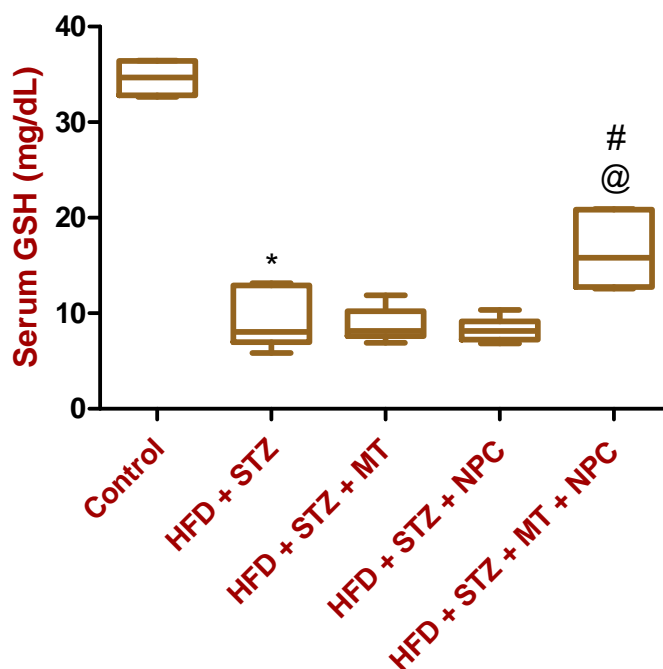


Figure 5 Effect of combining MT with NPC on the serum GSH levels of diabetic rats. Serum GSH levels of all animal groups were measured by the end of the experiment and expressed as mg/dL. Every rectangle represents the arithmetic average of all values obtained between the 25th and 75th quartiles of each group. The horizontal lines inside the rectangles represent the median estimate of the values. ANOVA followed by Tukey test were used to assess the significant differences between groups. Significant difference relative to control rats ($p \leq 0.001$) is demonstrated by asterisk symbol (*), and relative to HFD + STZ rats ($p \leq 0.001$) is demonstrated by pound symbol (#), while relative to HFD + STZ + MT rats ($p \leq 0.001$) is presented by at symbol (@).

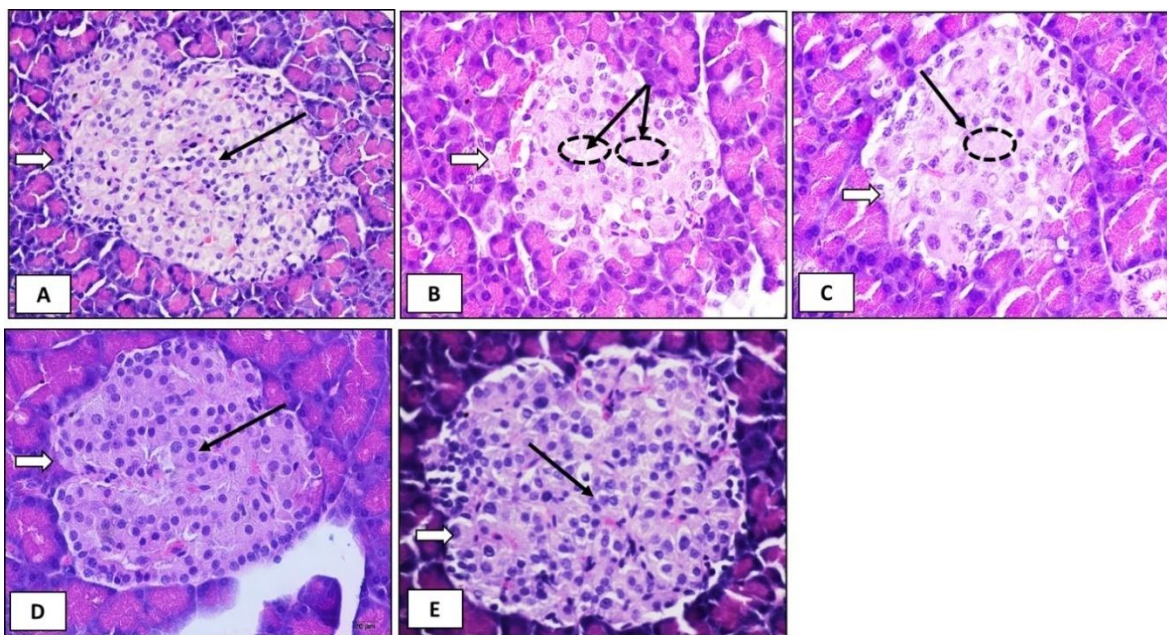


Figure 6 Effect of combining MT with NPC on type 2 diabetes-induced islets of Langerhans pathological changes in rats. Sections of rat pancreas pictured under the microscope (600X H & E) displaying islets of Langerhans (white arrows) from: A: control, B: HFD + STZ, C: HFD + STZ + MT, D: HFD + STZ + NPC, and E: HFD + STZ + MT + NPC groups. The diabetic group showed small-sized islets with a decrease in the centrally located beta-cell density, loss of their nuclei, and hyaline degeneration compared to the normal islets in the control rats. Mild improvement in islets' architecture was detected in MT group. Islets of NPC group were found to have considerable size and preserved cell density of centrally located beta cells. Evidence of preservation of normal morphology was observed in the MT + NPC treated group. Islets of Langerhans are represented by white arrows, while the nuclei of beta cells are marked by black arrows.

Effect of combining MT with NPC on diabetes-induced caspase-3 immunoexpression in islets of Langerhans in rats

An examination of the immunostained pancreatic sections by caspase-3 antibody showed that diabetes induction in group 2 caused an increase in the expression of caspase-3, a key marker for programmed cell death (Lossi et al., 2018), compared to the control group (group 1). However, treatment with MT, NPC, and both (group 3-5) caused a noticed reduction in the expression of caspase-3 compared to the diabetic group (Fig. 7).

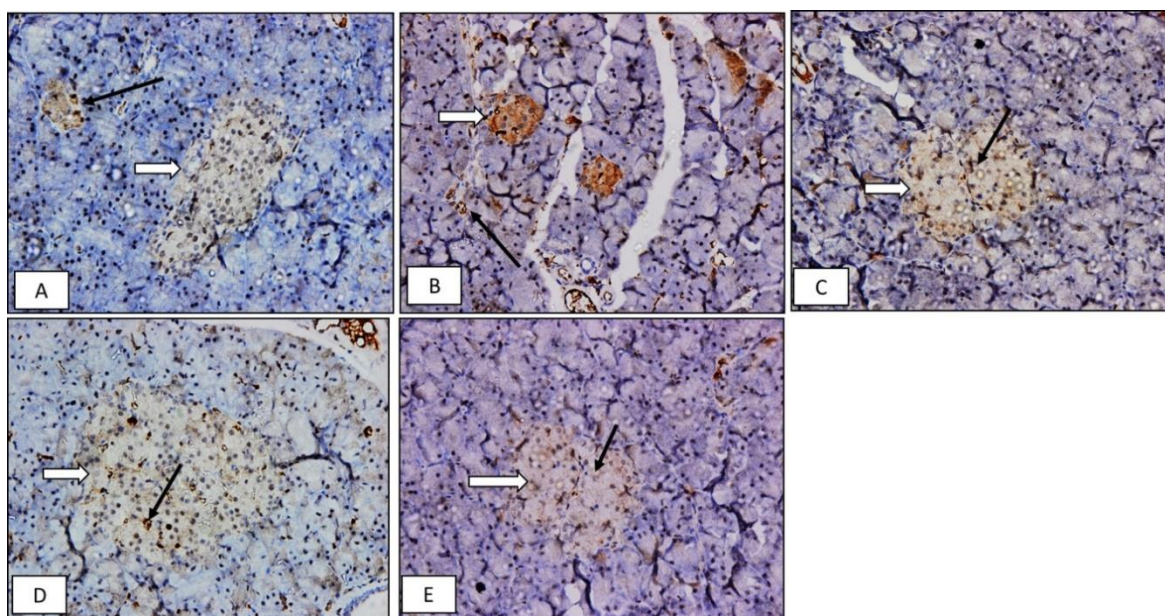


Figure 7 Effect of combining MT with NPC on HFD and STZ-induced caspase-3 immunoexpression in islets of Langerhans in rats. Pictured rat pancreatic sections stained with caspase-3 antibody (400X) showing islets of Langerhans (white arrows) from: A: control, B: HFD + STZ, C: HFD + STZ + MT, D: HFD + STZ + NPC, and E: HFD + STZ + MT + NPC groups. Minimal caspase-3 immunoexpression was detected in the control group. The diabetic group showed a marked increase in caspase-3 immunoexpression. MT, NPC, and MT + NPC groups showed mild caspase-3 immunoexpression. Black arrows show Caspase-3 immunoexpression.

4. DISCUSSION

In the present study, an established method was used to produce diabetes (type 2) in animals (Marciniak et al., 2014). Basically, rats were provided high-fat dietary supplements for two weeks followed by a single STZ injection (Li et al., 2004; Öztürk, 2016; Srinivasan et al., 2005). This well-known diabetic model causes metabolic criteria that are similar to ones occur when type 2 diabetes develops in humans (Srinivasan et al., 2005). An initial increase in bodyweight and/or obesity because of the high-fat diet, then a decreased production of insulin from beta cells, and/or an insulin resistance were shown to be the last events causing constant high blood sugar levels. A single dose of STZ, an alkylating agent, was used as a final step in initiating type 2 diabetes in rats as a disease model that is closely related to individuals (Abdulmalek and Balbaa, 2019; Stamler et al., 1992). When STZ is used in small doses, parts of beta cells are destroyed. The remaining living cells can perform their normal function but the quantity of secreted insulin is small, causing a decrease in body weight and diabetes type 2 (Aybar et al., 2001; Eliza et al., 2009; Gomes et al., 1995; Wang-fischer and Garyantes, 2018). The results of this study indicated that the HFD/STZ diabetic model was characterized by high glucose levels, low insulin levels, and a decrease in the final bodyweights of rats. The reduction in the bodyweight of an animal was a powerful indication for the development of the disease and the presence of insulin resistance, which directs the body to use proteins as an energy source instead of carbohydrates (Ahangarpour et al., 2017).

Following the induction of diabetes, rats in each group took different treatments. They were either treated with MT (group 3), NPC (group 4), or a combination of MT and NPC (group 5). The goal of the study was to find out whether NPC could potentiate the antidiabetic effect of MT. A slight rise in rat's body weights, but not exceeding the control group range, was detected in the group of combined treatments (group 5) relative to the diabetic group. This result was consistent with previously published data on costunolide, one of *Costus speciosus* active compounds, which improved the body weights of STZ-treated rats, probably because of enhanced insulin production from beta cells (Eliza et al., 2009), by inhibiting the nitric oxide synthase enzyme expression (Fukuda et al., 2001; Gunawardana et al., 2008). That increase was not noticed when rats were either treated with MT or NPC only. Nevertheless, further studies have to be performed to verify the effect of the combination therapy on the increase in body weight. The levels of insulin were substantially high in all the three groups that were given treatments relative to the diabetic rats (group 2). Furthermore,

giving the rats the two compounds together led to a significant increase in the serum insulin concentrations comparing to the MT monotherapy group. According to the pathological examination in the current study, healthy pancreatic beta cells were detected in rats treated with NPC alone and with MT and NPC together. Additionally, fasting serum glucose levels of rats were reduced significantly with the combined treatments. MT reduces blood glucose level through multiple mechanisms, such as inhibiting glucose production in the liver, reducing glucose absorption by the intestine, enhancing tissue sensitivity to insulin, and increasing insulin release from the pancreas (DeFronzo et al., 1991; Ilahi et al., 2012). These results were comparable with previously published data concluding that NPC lowered the levels of blood sugar of rats induced by STZ to have diabetes type 2 (Alamoudi et al., 2014).

Elevated blood glucose is accompanied by a state of oxidative stress, which is characterized by an increased production of reactive oxygen species (ROS) and an acute shortage of superoxide dismutase and catalase antioxidants enzymes (Evans et al., 2003; Jain et al., 2006). It was suggested that ROS development was a direct result of hyperglycemia (Brownlee, 2001). The results of the current research revealed an elevation in the products of lipid peroxidation, MDA, and a reduction in the serum GSH of diabetic group. The ability of multiple treatment regimens to reduce MDA was also shown. In addition, treatment with MT and NPC together caused a significant reduction in MDA compared to the MT monotherapy. The combination of MT and NPC was the only treatment regimen that was capable of causing an increase in the amount of the antioxidant molecule, GSH. GSH is a free radical scavenger that counteracts damages induced by free radicals, by reducing hydroperoxides in the presence of glutathione peroxidase enzyme (Öztürk, 2016). The reduction in GSH content in diabetic rats clarifies the mechanism of diabetes-induced oxidative stress. In agreement with our results, *Costus speciosus* extract returned the GSH content in the diabetic animals to the control level, proposing increased defence against the oxidative stress condition in this model (Revathy et al., 2014).

At small doses, STZ causes beta cells destruction and apoptosis, while at elevated doses, it triggers beta cells necrosis (Raza and John, 2012). This study showed that diabetes induced by a small dose of STZ/HFD increased the gene expression of caspase-3, which reflects the programmed death of pancreatic beta cells (Lossi et al., 2018). This is consistent with a previous study that showed the occurrence of programmed cell death as one of the mechanisms of STZ/HFD-induced type 2 diabetes (He et al., 2015). The treatment with MT, NPC, and their combination caused a reduction in the level of gene expression of caspase-3 in pancreatic beta cells. In contrast to our study, a previous research revealed that treatment with MT did not affect the ratio of cleaved caspase-3-positive beta cells in HFD model that was induced in C57Bl/6J male mice (Tajima et al., 2017). Ultimately, it was recommended that alternative medicines may block pancreatic beta-cell programmed cell death which occurs during diabetes course (Oh, 2015).

5. CONCLUSION

The findings of this work showed that NPC has a comparable antidiabetic effect as MT on managing type 2 diabetes, induced by STZ/HFD, in rats. Both MT and NPC have antioxidant and anti-apoptotic effects. Besides, the impact of administering the two drugs is highly effective compared to MT monotherapy in terms of improved body weight, insulin secretion, antioxidant effect, as well as protecting against the pathological consequences on the pancreatic beta cells.

List of Abbreviations

MT: Metformin; NPC: Nanoparticles *Costus speciosus*; HB A1c: Haemoglobin A1c; STZ: Streptozocin; MDA: Malondialdehyde; GSH: Reduced glutathione; HFD: High-fat diet.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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