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Augmentation of Insulin Secretion Induced by Rhizophora Mucronata and Avicennia Marina Extracts in Streptozotocin-Induced Diabetic Rats

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ABSTRACT

Background: *Diabetes mellitus is a chronic metabolic disease and is considered as the third leading cause of death in the developed countries.* ***Objectives:*** *The aim of this study was to assess the antidiabetic, antioxidant and insulin-enhancing potentials of the aqueous extracts of R. mucronata and A. marina or combination of them.* ***Materials and methods:*** *The effects of daily oral administration of aqueous extract from the leaves of R. mucronata, A. marina (400 mg/kg BW for each) and combination of both for 6 weeks on streptozotocin-induced diabetic rats were evaluated considering the blood glucose and insulin levels in the serum. Oxidant/antioxidants status was assessed in the cardiac and muscular tissues. Immunohistochemical expression of insulin in the pancreatic tissue was also assessed.* ***Results:*** *Oral administration of the plants extracts alleviated the diabetes-induced changes in serum glucose, insulin and antioxidants status in the heart and muscles compared to the untreated rats. In addition, these plants enhanced insulin secretion by β-cells of Langerhans as evidence immunohistochemically and biochemically through calculation of HOMA- β.* ***Conclusion:*** *The extract of R. mucronata exhibited a promising antidiabetic, antioxidant and insulin-enhancing effects compared with A. marina extract alone or in combination with R. mucronata.*

Key words: *mangrove, diabetes mellitus, insulin, tissue antioxidants, pancreas-HOMA-IR.*

INTRODUCTION

Diabetes mellitus (DM) is a common worldwide metabolic disorder, which is characterized by hyperinsulinemia, hyperglycemia, hyperlipidemia, and hyperaminoacidemia due to abnormalities in insulin secretion, insulin action, or both. [1] The disease is usually associated with a high level of lipid peroxides, reactive oxygen species (ROS), and a decreased level of the antioxidant enzymes. These enzymes play an important role in scavenging toxic intermediate radicals formed by incomplete oxidation or regulating the production of ROS and the overall tissue antioxidant efficiency. [2]

Noticeable progress has been documented in the management of DM using synthetic medications; however, management of DM and its complication is still an unsolved mystery. Remedies that were developed on the principles of chemical or conventional medication are not very effective and usually have an increased risk of adverse side effects. Additionally, these drugs are typically very expensive, especially for third world countries populations. Consequently, treatment of DM with plant-derived phytochemicals appear to have an extreme potential, as they are easily available and do not require exhaustive pharmaceutical synthesis. [3]

Several studies were conducted to explore the effectiveness of plant extracts on streptozotocin (STZ)-triggered diabetes in the tissue organs of many experimental animals. [1] Many natural products can suppress the enzyme

activities responsible for production of glucose, its absorption, and insulin effectiveness; while, others can modify apoptosis of β-cell and promote insulin action. [4, 5]

Rhizophora mucronata (*R. mucronata*) and *Avicennia marina* (*A. marina)* are two prominent genera of mangrove plants that exist on the earth. [6] Such plants have been used in traditional medicine in the coastal regions of Asian subcontinents for treating health problems such as diabetes, diarrhea, hepatitis, and inflammation. [7] To the best of our knowledge, no previous studies have been conducted on the effect of both *R. mucronata* and *A. marina* on the blood glucose level, the antioxidants status or insulin-secreting cells in the pancreas. Therefore, this study aimed to assess the hypoglycemic, antioxidant and insulin-enhancing efficacy of the aqueous extract of *R. mucronata* and *A. marina* leaves, grown in Saudi Arabia, alone or in combination in albino rats.

MATERIALS AND METHODS

Experimental Animals

One hundred and twenty male Wistar albino rats (200-250 g BW), aged six weeks were purchased from the Animal Experimental Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia. All animals were acclimatized to laboratory conditions for 2 weeks before initiation of the experiment. The animals were fed with normal commercial chow and water *ad libitum*. Throughout the experiments, the animals were handled according to the ethical guidelines for the care of laboratory animals and all experimental procedures were approved by the Animal Care and Use Committee of the King Abdulaziz University.

Plant Extraction

R. mucronata and *A. marina* leaves were collected from Farasan Island, Jizan and Shuaiba area respectively, Saudi Arabia during January 2018. The collected leaves of the studied plants were scientifically identified and authenticated by a plant taxonomist at the Department of Arid Land Agriculture, Faculty of Meteorology, Environment and Arid Land Agriculture, King Abdulaziz University, Jeddah, KSA.

The aqueous extracts of leaves of *R. mucronata* and *A. marina* were prepared according to the methods described by Mohamadi and Havasian. [8] The leaves of both plants were cleaned under running tap water and dried at room temperature then cut into small pieces and finally crushed by an electric grinder to be subjected to extraction. A total quantity of raw powder of both plants used in this experimental study was 1.872 kg of *R. mucronata* and 0.6545 kg of *A. marina*. The yields crude plant extract required in this experiment for a period of 6 weeks was 151.2 g for each plant. The plant extracts were prepared freshly each time and administered orally by stomach tube at a dose of 200 mg/kg BW) of each of the two extract.

Induction of DM

Prior to diabetes induction, the experimental adult male Wistar rats were fasted for 12 hours and diabetes was induced by administering a single intraperitonial injection of freshly prepared STZ (Sigma Chemical Co., St. Louis, MO, USA) at a dose of 60 mg/kg BW in normal physiological saline solution (0.9% NaCl). [9] Three days after STZ injection, the fasting blood glucose (FBG) levels were measured from tail blood samples by using One Touch Ultra Glucometer (Lifescan, Johnson and Johnson, Milpitas, CA, USA). The animals with blood glucose levels ≥ 250 mg/dl were considered diabetic (60 rats) and used for the subsequent experiments. [10]

Experimental Design

The experiment included eight equal groups (n=15 rats each). Group 1 included the control rats that received water and fed with *ad libitum*. Group 2 included STZ-induced untreated diabetic rats. Groups 3 included the diabetic rats treated orally with an aqueous extract of *R. mucronata* leaves. Group 4 included the non-diabetic rats treated orally with an *A.marina* leaf extracts. Group 5 included the non-diabetic rats treated orally with a mixture of aqueous leaf extracts of *R. mucronata* and *A.marina*. Group 6 included the non-diabetic rats received an aqueous extract of *R. mucronata* leaves. Group 7 included the non-diabetic rats received an aqueous extract of *A. marina* leaves. Group 8 included the non-diabetic rats received a mixture of aqueous extract of *R. mucronata* and *A. marina* leaves. The treatments started on the 4th day after STZ injection and continued daily for six weeks.

Measurement of serum glucose and Insulin

Blood was collected from retro-orbital venous plexus of the rats at the 6th week. It was then left for clotting at room temperature and serum was separated by centrifugation at 3000 rpm for 20 min. Serum glucose was evaluated in normal and diabetic rats by an autoanalyzer (Cobas 6000 analyzer series) using diagnostic kits according to the manufacturer's instruction (Roche Cobas Diagnostics, USA). The serum insulin levels were measured using insulin ELISA kits which includes an enzyme immunoassay for the quantitative determination

of insulin in sera of rats (Cat. no. ezrmi-13kelisa, Billerica, MA, USA) according to the method of Thulesen *et al***.** [11]

Homeostasis Model Assessment of Insulin Resistance and pancreatic β-cell function

The homeostasis model assessments of insulin resistance (HOMA-IR) and β-cells function (HOMA-B) were calculated using these equations. [12]

- HOMA-IR= fasting insulin (MIU/L) × fasting glucose (mmol/L) /22.5
- HOMA-B = insulin (MIU/L) × 20 /fasting glucose (mmol/ml) − 3.5

Measurement of Malondialdehyde (MDA) and antioxidants in the tissues

At the end of the experiment, the rats were sacrificed under anesthesia, the heart and muscles of the forelimb were dissected out, rinsed with isotonic saline solution, blot-dried and weighed. After weighing, parts of these tissues were cut into small pieces and minced to order to prepare a homogenate according to the method of Mansouri *et al*. [13] The supernatant obtained was used for measuring the levels of MDA, [14] catalase (CAT), [15] reduced glutathione (GSH) [16] and superoxide dismutase (SOD). [17]

Immunohistochemical study

Parts of the pancreas were fixed in 10% neutral formalin, processed and embedded in paraffin. Sections with 5-µm thickness were cut, and stained with the standard immunohistochemical methods for detection of diabetic insulin biomarkers in pancreatic tissue. [18]

Statistical Analysis

The obtained data in this study were expressed as mean ± standard error (SE). Statistical significance of the difference between groups, with more than two categories, was determined by one-way analysis of variance (ANOVA) followed by Least Significant Difference (LSD) post-hoc test. The statistical software package used for analysis was Statistical Package for Social Sciences (SPSS 24). The values were considered significantly different when the *P* value was ≤ 0.05. [19]

RESULTS

Effects of the plants extracts on serum glucose level

STZ induced-diabetic rats showed increased levels of serum glucose level, compared to the control rats; while, administration of *R. mucronata* or *A. marina* alone or in combination to STZ-induced diabetic rats resulted in a significant decrease in serum glucose levels. The improvement was marked in the diabetic rats treated with *R. mucronata* that have a potential hypoglycemic effect compared with the STZ-induced diabetic rats. Non-diabetic rats that received *R. mucronata, A. marina* extract and mixture of the two plants extracts revealed non-significant changes in serum glucose level when compared with normal rats (Fig.1 A).

Effects of the plants extracts on serum Insulin

There was a highly significant decrease ($p \leq 0.001$) in serum insulin level of STZ-induced diabetic rats compared to the control group. The treatment of diabetic groups with *R. mucronata, A. marina* or their mixture resulted in a significant increase ($p \leq 0.001$; $p \leq 0.05$; $p \leq 0.001$ respectively) in the serum insulin levels compared to untreated diabetic rats (G2). The non-diabetic groups received *R. mucronata, A. marina* or their mixture exhibited non-significant differences in serum insulin levels compared to the control group (Fig. 1B).

Effects of the plants extracts on HOMA- IR and HOMA-β levels

It was observed that induction of diabetes in using STZ resulted in a non-significant increase in HOMA-IR compared to the control group. In addition, treating the diabetic groups with *R. mucronata, A. marina* or their mixture did not significantly reduce HOMA-IR (Fig. 1C).

With regard to HOMA-β, it was noticed that STZ-induced diabetes resulted in a significant increase ($p \leq 0.001$) in HOMA-β compared to the control group. On the other hand, treating the diabetic groups with *R. mucronata, A. marina* or their mixture significantly increased it ($p \leq 0.001$, $p=0.03$, $p=0.01$) respectively compared to the untreated diabetic group (Fig. 1D).

Effects of the plants extracts on heart levels of MDA and antioxidants

Statistical analysis of the heart MDA levels of untreated diabetic group revealed a highly significant increase ($p \leq 0.001$) compared to the control group. The diabetic groups treated with *R. mucronata, A. marina* and their mixture showed a highly significant decrease ($p \leq 0.001$) in the heart MDA levels compared to the untreated diabetic group (G2) with the maximum effect noticed in *R. mucronata*-treated group (Fig. 2A)**.**

In this study, the untreated diabetic rats exhibited a highly significant decrease ($p \leq 0.001$) in the levels of CAT, GSH and SOD in the heart compared to control group. Daily treatment of diabetic rats with *R. mucronata*, *A. marina* and their mixture induced a highly significant increase ($p \leq 0.001$) in the heart levels of CAT, GSH and SOD compared to the diabetic group (Fig. 2 B-D).

Moreover, the levels of CAT, GSH and SOD in the heart were significantly decreased in the diabetic rats received daily gavage of extracts of *R. mucronata*, *A. marina* and their mixture for six weeks compared to the control group (Fig 2 B-D).

Effects of the plants extracts on muscle levels of MDA and antioxidants

It was noticed that the levels of MDA in the diabetic rats was significantly increased ($p \leq 0.001$) compared to the control group. However, daily administration of *R. mucronata*, *A. marina* and their mixture to diabetic rats induced a highly significant decrease ($p \leq 0.001$) in the muscle MDA levels when compared to the diabetic control group (Fig. 3 A).

The untreated diabetic rats showed a highly significant decrease ($p \leq 0.001$) in the levels of CAT, GSH and SOD in the muscles compared to the control group. Nevertheless, the treatment of diabetic groups with *R. mucronata*, *A. marina* and their mixture induced a highly significant increase ($p \leq 0.001$) in the muscle levels of CAT, GSH and SOD compared to the untreated diabetic group (Fig. 3 B-D).

Immunohistochemical assessment of insulin content β-cells

Immunohistochemical expression of insulin in pancreatic tissues was assessed using insulin antibody. It was noticed that there was a strong positive cytoplasmic reaction for insulin in almost all β-cells of islets of Langerhans in the control group. These cells appeared large, rounded with centrally located unstained nuclei and granular brown cytoplasm. Alpha (α) and delta cells (δ-cells) were not stained. Marked reduction was observed in the number of β-cells of islets of Langerhans in the beta cells of STZ-induced diabetic rats (Fig 4).

Surprisingly, all examined sections of pancreas of STZ-induced diabetic rats treated with *R. mucronata* or *A. Marina* revealed moderate positive insulin expression in β-cells compared to the untreated diabetic group. Different sections from pancreatic tissue of STZ-induced diabetic rats treated with the mixture of *R. mucronata and A. Marina* showed strong positive expression of insulin in almost all β-cells; while, few cells showed a moderate positive reaction (Fig 4). Sections of pancreas from un-diabetic rats treated with *R. mucronata* or *A. Marina* revealed strong positive insulin expression in β-cells comparable with the control group (Fig 4).

DISCUSSION

Naturally and experimentally induced diabetes is usually accompanied with increased level of lipid peroxidation and decreased levels of the key antioxidant enzymes. [2] Oxidative stress plays an important role in development and progression of DM due to higher free radical production, damage to cell constituents, and impairment in the antioxidant defense enzymes. [20, 21] Although the antidiabetic and antioxidant effects of *R. mucronata* extracts and *A. marina* extract have been previously described, [10, 22] the antidiabetic and antioxidant effects of the combination of those extracts have not been investigated so far. Therefore, this study aimed to assess the effectiveness of the aqueous fraction of *R. mucronata* and *A. marina* leaves grown in Saudi Arabia alone or in combination as antidiabetic, antioxidants and pancreatic tissue-enhancing agents in albino rats.

Treatment of STZ-induced diabetic rats with a mixture of *R. mucronata* and *A. marina* induced a significant decrease in levels of serum glucose as well as a significant increase in insulin level in comparison to the diabetic control group. The results of the current investigation are in accordance with that of Adhikari *et al*. who reported that, regular administration of extract of *R. mucronata* leaves maintained the normal serum insulin level. [7] The authors added that *R. mucronata* leaves are a rich source of magnesium content and the gas chromatographic study showed the presence of squalene. In the current study, the promising antidiabetic effect of *R. mucronata* extracts may be associated with the insulin mimicking property of its phytochemicals. Such finding coincides the data reported by Ray *et al*. [23]

It was reported that the most extensively used methods for evaluating insulin resistance and insulin secretion in epidemiological studies are HOMA-IR and HOMA-β. [24] In this study, administration of STZ resulted in a non-significant increase in HOMA-IR compared to the control group; while, treating the diabetic groups with *R. mucronata*, *A. marina* or their mixture did not significantly affect it. This was expected, as STZ is a cytotoxic compound especially toxic to the pancreatic β-cells. It enters pancreatic β-cells through glucose transporter type 2 (GLUT2) channels in the cellular plasma membrane, which leads to cellular toxicity and local immune reactions leading to hypoinsulinemia and hyperglycemia in animals. [25] Therefore, this animal model of diabetes is not associated with insulin resistance that was excluded by assessing HOMA-IR. On the other hand, stimulation of

insulin secretion by the plants extracts used in this study was confirmed by calculating HOMA-β. It was evident that, *R. mucronata, A. marina* or their mixture could significantly increase HOMA-β compared to the untreated diabetic group.

In the present study, the activities of antioxidant enzymes CAT, SOD and GSH in heart and muscles tissues were significantly decreased in STZ-induced diabetic rats compared to the normal rats. Treatment of STZ-induced diabetic rats with the aqueous extracts of *R. mucronata* and *A. marina* alone or in combination significantly decreased lipid peroxidation (LPO) as reflected by decreased concentration of MDA. These results were supported by many previous studies. It was reported that diabetes is usually accompanied with increased level of lipid peroxides, ROS, like MDA as well as a decreased levels of the key antioxidant enzymes, CAT, SOD and GR-peroxidase (GSH-Px). [2, 13, 26] Moreover, the protective, ameliorative and enhancing potentials of *R. mucronata* and/or *A. marina* in reducing oxidative stress were attributed to either increasing antioxidants activity or reducing MDA levels in tissues. [27, 28]

The decrease in tissue GSH level could be the result of decreased synthesis or increased degradation by the oxidative stress in diabetes. [29] The reduction in the activities of SOD and CAT in the heart and muscles of diabetic rats may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxides (H2O2). [30] The anti-oxidant effect of the plant extracts, under investigation in this study, was more pronounced in the diabetic rats-treated with *R. mucronata* extract. Such results could be due to the polyphenol-rich compounds in *R. mucronata* extract. [31] Promising antioxidant properties of *R. mucronata* was induced by inhibiting 1,1-diphenyl-2-picrylhydrazyl (DPPH) and radical scavenging that might be due to the high amount of quercetin. The latter is considered as a potent antioxidant that inhibits pro-oxidant enzymes and decreases the oxidative damage in diabetes by improving the antioxidant response. [32] Moreover, Hamzevi *et al.* showed that administration of *A. marina* extract (100 and 300 mg/kg BW) to STZ-induced diabetic rats significantly decreased the level of MDA in liver tissue; while, the activities of the antioxidant enzymes, SOD, GSH, CAT were increased. [28] These findings were also evident in this study.

Immunohistochemical investigations of STZ-induced diabetic rats treated with *R. mucronata*, *A. marina* or mixture of them revealed that, treatment of diabetic rats with the studied herbal extracts induced an excellent insulin-releasing activity induced by *R. mucronata* followed by the mixture of *R. mucronata* and *A. marina* extracts. This may be attributed to the flavonoids compounds in *R. mucronata*, which could play an important role in prevention of β-cell apoptosis, promotion of β–cell propagation beside secretion and enhancement of insulin activity. [6] In addition, Sundarban mangrove *R. mucronata* Lam. leaves were reported to have beneficial antihyperglycemic effect with insulin-mimetic actions in STZ-nicotinamide induced Type 2 diabetic rats. [7]

Among the limitations of this study, the inability to explore the molecular mechanism behind the insulin-secretion enhancing effect induced by *R. mucronata*, *A. marina* and the mixture of them can be mentioned.

CONCLUSION

Oral administration of the plants extracts alleviated the diabetes-induced changes in serum glucose, insulin and antioxidants status in the heart and muscle compared to the untreated rats. In addition, these plants enhanced insulin secretion by β-cells of Langerhans as evidence immunohistochemically and biochemically through calculation HOMA- β. The extract of *R. mucronata* exhibited a promising antidiabetic, antioxidant and insulin-enhancing effects compared with *A. marina* extract alone or in combination with *R. mucronata*. Therefore, these findings are considered of great importance for pharmaceutical industry and scientific research for development of more preventive and effective therapeutic novel plant-derived anti-diabetic drugs.

Conflict of interest

The authors declare no conflicts of interest.

ACKNOWLEDGMENT

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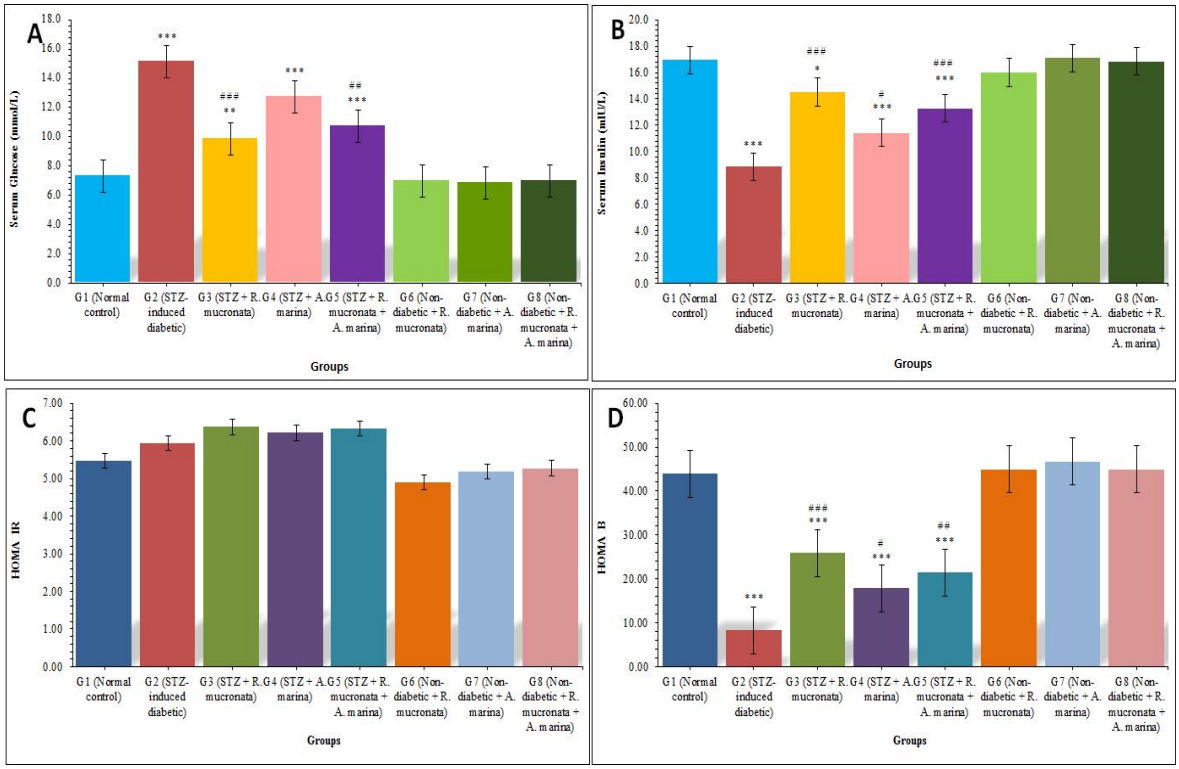

Figure 1: Effect of extracts of *R. mucronata*, *A. marina* and their mixture on serum glucose level (A), serum insulin level (B), HOMA-IR (C), HOMA-B (D) in the studied groups (n=15 each). Results are expressed as mean±SEM. Mean values are significantly different at $p \leq 0.001^{***}$; $p \leq 0.05^{*}$ compared to normal control group. Mean values are significantly different at $p \leq 0.001^{###}$; $p \leq 0.05^{\#}$ compared to STZ-induced diabetic group.

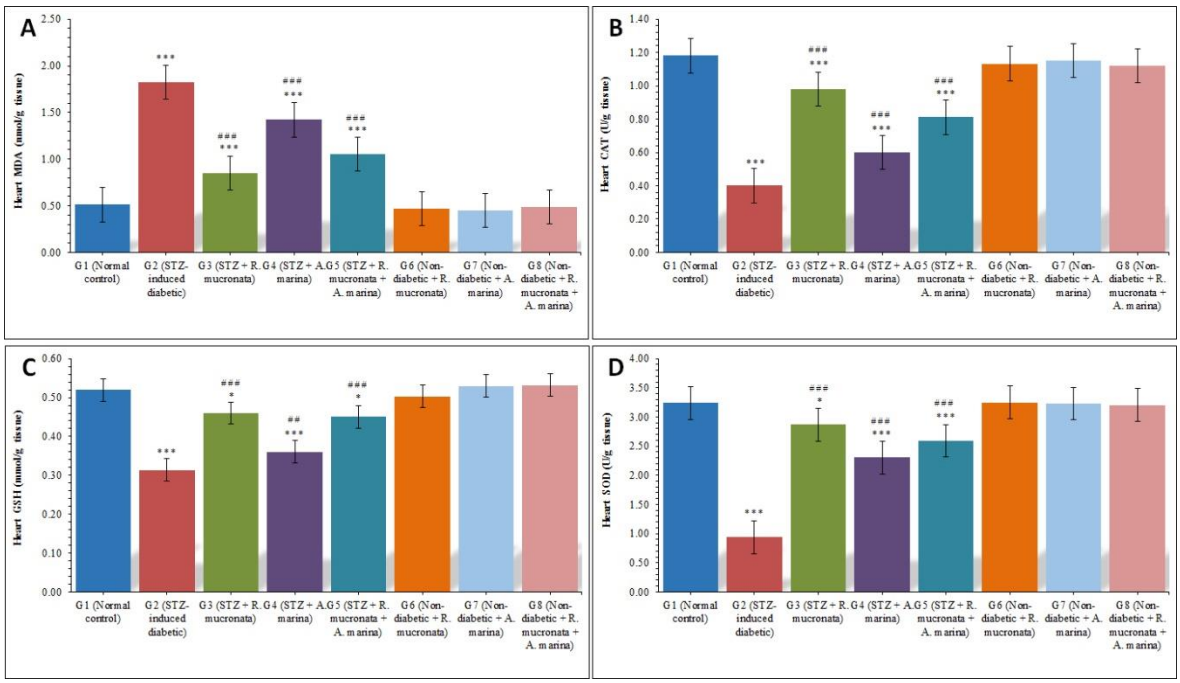

Figure 2: Effect of extracts of *R. mucronata*, *A. marina* and their mixture on the levels of MDA (A), CAT (B), GSH (C) and SOD (D) in the hearts of the studied groups (n=15 each). Results are expressed as mean±SEM. Mean value is significantly different at $p \leq 0.001^{***}$ compared to normal control group. Mean value is significantly different at $p \leq 0.001^{###}$ compared to STZ-induced diabetic group.

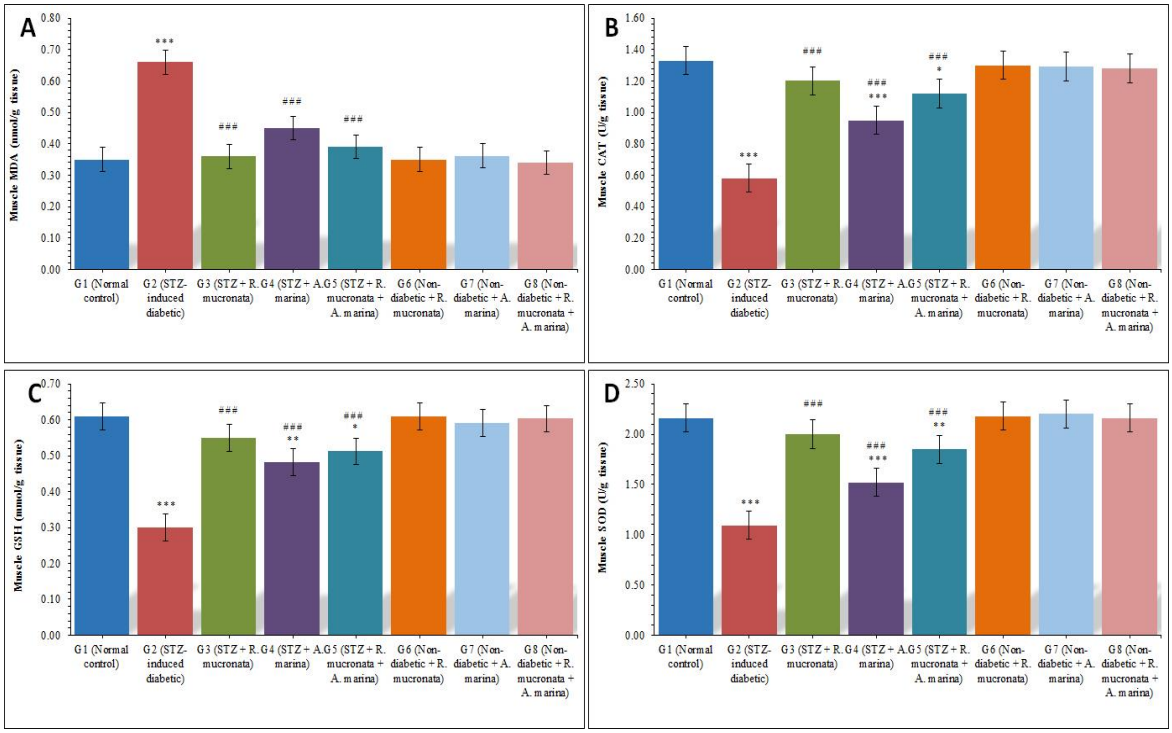

Figure 3: Effect of extracts of *R. mucronata*, *A. marina* and their mixture on the levels of MDA (A), CAT (B), GSH (C) and SOD (D) in the muscles of the studied groups (n=15 each). Results are expressed as mean ± SEM. Mean value is significantly different at $p \leq 0.001^{***}$ compared to normal control group. Mean value is significantly different at $p \leq 0.001^{\#\#\#}$ compared to STZ-induced diabetic group.

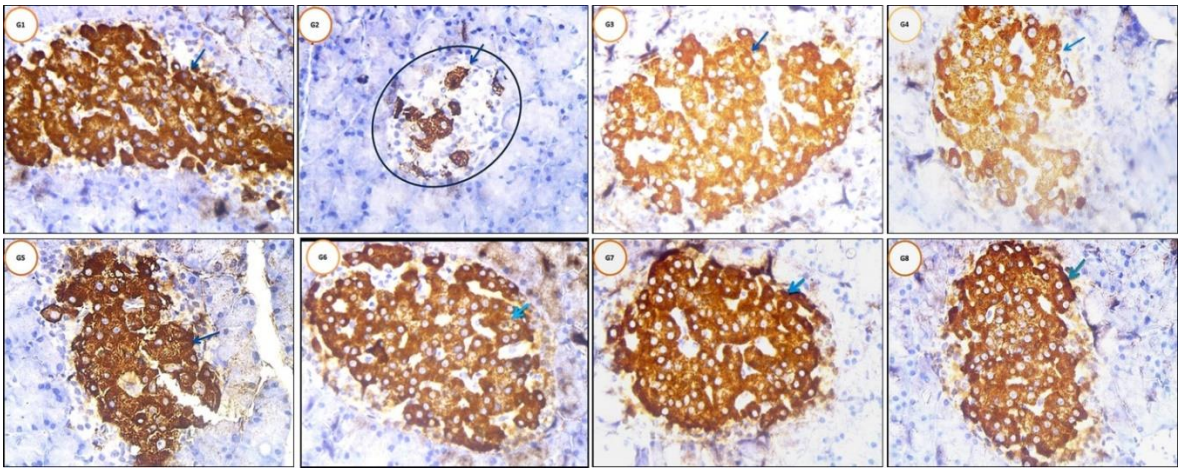

Figure 4: Photomicrographs of rat's pancreas stained immunohistochemically with anti-insulin antibody. There is a strong positive reaction (black arrows) in all β-cells of islets of Langerhans in the control group (G1) and almost all β-cells of diabetic groups treated with *R. mucronata* (G3), *A. marina* (G4) or their mixture (G5) compared to the untreated diabetic group (G2) that show few insulin-stained B-cells. Scale bar=50µ).