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BIOCHEMICAL AND MOLECULAR STUDY ON THE EFFECT OF *MURRAYA KOENIGII (CURRY)* AND *MORINGA OLEIFERA* ON CARDIAC MITOCHONDRIAL DYSFUNCTION IN DIABETIC RATS

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ABSTRACT

In the present study, the biochemical and molecular study effect of ***Murraya Koenigii (curry)*** and ***Moringa oleifera*** aquous extract of leaves on cardiac mitochondrial dysfunction in experimentally induced diabetes mellitus in rats by Streptozotocin (STZ) was evaluated. sixty male albino rats **weighting 150-200 g** and divided into four equal groups of 15 rats each. Group 1:(Control group): received normal diet only. Group 2:(Diabetic rats group): rats received a single dose of Streptozotocin (STZ) (50- mg/kg-b.wt i.p). Group 3: (Diabetic rats + *Murraya Koenigii (curry)* aquous extract of leaves at dose of 200mg/kg/ twice daily, orally for four weeks after diabetes induction. Group 4:(Diabetic rats+ *Moringa oleifera* aquous extract of leaves at dose of 200mg/kg/ twice daily orally for four weeks after diabetes induction. Heart tissue was collected at the end of experiment. The obtained results revealed that, a significant increase in MDA, NO, and cytochrome C gene with marked decrease in CAT, GSH Px, SOD1 gene, PGC 1α gene and ATp1A1 gene in diabetic control rats. Treatment with *Murraya Koenigii (curry)* and *Moringa oleifera* were able to mitigate diabetic abnormalities through significant decreasing MDA, NO and cytochrome C gene but higher than normal control and significant increasing CAT, GSH Px, SOD1 gene, PGC 1α gene and ATpase but less than normal control.. These results suggest that *Murraya Koenigii (curry)* and *Moringa oleifera* act as antioxidative, anti apoptic and improve mitochondrial dysfunction so provide agood protection to the heart.

KEYWORDS: *Murraya Koenigii (curry)*, *Moringa oleifera*, STZ, Diabetes, mitochondria, antioxidant enzymes, SOD1 gene, PGC 1α gene, ATpase gene and cytochrome c gene.

INTRODUCTION

Hyperglycemia increases oxidative stress, which contributes to the impairment of the main processes that fail during diabetes, insulin action and insulin secretion. In addition, antioxidant mechanisms are diminished in diabetic patients, which may further augment oxidative stress **(Maritim *etal*, 2003).** H2O2 at slightly higher concentrations can induce the release of cytochrome *c* and apoptosis-inducing factor (AIF) from mitochondria into the cytosol where they trigger the activation of caspase, leading to cell death by apoptosis **(Fisher, 2006).** In multiple different cardiac-derived cells exposed to high glucose, it was demonstrated that mitochondria were fragmented and that cell death was increased. **(Yu *etal*, 2008).** Several studies have addressed the possible participation of dietary antioxidants, such as vitamins, in ameliorating the diabetic state and retarding the development of diabetes complications **(Cuerda *etal*, 2011)**. Mitochondrial dysfunction has recently been identified as a common metabolic defect associated with diabetes, obesity, and its metabolic complications (**Morino etal, 2006).** Although mitochondrial endocrine dysfunction frequently occurs in the context of multisystem disease, some mitochondrial disorders are characterized by isolated endocrine involvement. Furthermore, additional monogenic mitochondrial endocrine diseases are anticipated to be revealed by the application of genome-wide next-generation sequencing approaches in the future. Understanding the mitochondrial basis of endocrine disturbance is key to developing innovative therapies for patients with mitochondrial diseases **(Jasmine *etal*, 2017**).

Curry leaves essential oil corrects pathological and hyperglycemia abnormalities in the diabetic rats due to its antioxidant effect and restorative potential of redox homeostasis mechanism, excellent recovery of pancreatic tissue can be explained by positive effect of curry leaves oil on the production of insulin because of regenerative effect of the exocrine pancreatic cells **(Amin *et al*., 2013).** The major constituent responsible for the aroma and flavor has been reported as pinene, sabinene, caryophyllene, cadinol and cadinene. The leaves have a slightly pungent, bitter and feebly acidic taste, **(Kale *et al*., 2014).**

Moringa can also be used to stabilize sugar levels and can stabilize arterial tension **(Bukar *et al*., 2010; Kasolo *et al*., 2010).** The leaves have also been found to possess antitumour,,

antinflammatory, antiulcer, antispasmodic, diuretic, antihypertensive and antioxidant properties **(Bukar *et.* al., 2010*).***

MATERIALS AND METHODS

Experimental animals

Sixty white male albino rats weighting 150-200 gm were used in the experimental investigation of this study. The rats were obtained from the Laboratory Animals Research Center, Faculty of Veterinary Medicine, Benha University. Rats Animals were housed in separate metal cages, exposed to good ventilation, humidity and to a 12 hr light/dark cycle. Fresh and clean drinking water was supplied ad-libtium. Constant supplies of standard pellet diet, fresh and clean drinking water were supplied ad-libitum.The animals were left for 15 days for acclimatization prior to the beginning of the experiment, and kept at constant environmental and nutritional conditions throughout the period of the experiment.

Chemicals and material used

- **STZ:(Streptozotozin)** was purchased from Sigma Aldrish.
- ***Murraya koenigii (curry)*** was purchased from Saudi Arabia.
- ***Moringa Oleifera*** was obtained from school of agriculture in Mansoura.

Diabetes induction

Rats were fasted for 18 hrs. The experimental induction of diabetes in male rats was induced by a single intraperetinoel (i.p) injection of **50** mg/kg body weight of Streptozotocin (STZ) freshly dissolved in citrate buffer, pH 4.5. Serum glucose estimations were undertaken periodically (days 0, 3, and 7). After week, STZ-treated rats were fasted for 12 h and blood samples were collected from the tail vein for glucose determination. Only those rats in diabetic group with blood glucose level higher than **250 mg/dl** were considered diabetic **(Ramanathan et al., 1999).**

NOTE: you must avoid light during preparation of STZ and you should make fresh preparation of STZ for each ten rats to keep its effect.

Aquous extract of curry leaves

For each leaf extract, 100 gm of shade dried leaves were ground in an electrical grinder and dissolved in 500 ml distilled water. The mixture was left for 24 hrs with a magnetic stirrer at room temperature. The next day the mixture was strained out in a fine sieve and the crude

extract was air evaporated for 3 days. The concentrated leaf extract of plant was then orally administered to the rats in 200 mg/kg body weight) using a syringe **(Gohil *et al*., 2010**, **Maha *etal*, 2013)**.

Aquous extract of moringa leaves

For each leaf extract, 100 gm of shade dried leaves were ground in an electrical grinder and dissolved in 500 ml distilled water at 40-60^{o} C for 48 h. The mixture was left for 24 hrs with a magnetic stirrer at room temperature. The next day the mixture was strained out in a fine sieve and the crude extract was air evaporated for 3 days. The concentrated leaf extract of plant was then orally administered to the rats in 200 mg/kg body weight) using a syringe according to **(Dolly *etal*, 2013)**.

***NOTE*:** We prepare the aquous extract of curry and moringa every week during four weeks to be fresh and keep its effects.

Experimental groups

Group 1 (Normal Control)

composed of 15 male rats, rats were administered distilled water orally using a feeding cannula for study period. 0.01 M citrate buffer, pH 4.5, was injected intraperitoneally to mimic the STZ injections.

Group 2: (Diabetic Control)

Composed of 15 male rats. Diabetes was induced by a single STZ injection (50 mg/kg body wt, i.p., dissolved in 0.01 M citrate buffer, pH 4.5).

Group 3: (diabetic treated with curry)

Composed of 15 male rats, received aqueous extract of curry leaves at a dose **of 200 mg/kg/twice daily. for four weeks.**

Group 4: (diabetic treated with Moringa)

Composed of 15 male rats, received aqueous extract of *Moringa oleifera* leaves at a dose **of 200 mg/kg/ twice daily for four weeks.**

Tissue samples

Heart was washed with ice-cold saline solution (0.9% NaCl), weighed and stored at -80 ºC for the biochemical study of (MDA, NO, CAT, GSH Px) and molecular assay of. (SOD1gene, PGC1α gene, ATPase gene, cytochrome c).

Tissue preparation

The tissues were homogenized with 0.1 M phosphate buffer saline at pH 7.4, to give a final concentration of 20% w/v for the biochemical and molecular assay.

Biochemical analysis

Heart L-MDA concentration was determined according to the method adapted by (**Mesbah et al., 2004). NO** was determined by ELISA kit of my biosource company **Cat.No: MBS010567.** Catalase was determined according to the method described by (**Aebi, 1984**). Glutathione peroxidase activity in heart homogenate was determined according to the method described by **Paglia and Valentine; (1967).**

Molecular investigation

Pure RNA was extracted using total RNA Purification Kit following the manufacturer protocol **(Thermo Scientific, Fermentas, catalog number K0731). cDNA synthesis by Reverse transcription kits (Thermo Scientific, Fermentas, catalog number EP0451). Quantification of RNA using Nanodrop by** The Q5000 **(Uv-Vis spectrophotometer Q5000/USA)** automatically performs all necessary measurements and calculations. The quantities critical threshold (Ct) of target gene were normalized with quantities (Ct) of house keeping gene (*ß-actin*) by used the $2^{-\Delta\Delta Ct}$ method **(Livak and Schmittgen, 2001).**

Statistical analysis

All the data were expressed as means ±S.E. The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS, 18.0 software, 2011 and the individual comparisons were obtained by Duncan's multiple range test (DMRT). Values were considered statistically significant *when* $p<0.05$.

RESULTS

Effect of treatment with curry and moringa on some biochemical parameters and molecular genes towards cardiac mitochondrial dysfunction in STZ-induced diabetic rats.

The obtained results in table (1and 2) revealed that, a significant increase in MDA, NO, and cytochrome C gene with marked decrease in CAT, GSH Px, SOD1 gene, PGC 1α gene and ATpase gene in diabetic control rats. Treatment with *Murraya Koenigii (curry)* and *Moringa oleifera* were able to mitigate diabetic abnormalities through significant decreasing MDA, NO and cytochrome C gene but higher than normal control and significant increasing CAT, GSH Px, SOD1 gene, PGC 1α gene and ATpase but less than normal control.

Table (1): Effect of Curry and Moringa on MDA, NO, CAT, GSH P_X parameters in heart tissues of STZ-induced diabetic rats after 4 weeks of treatment. (Mean±SE).

Groups	MDA (nM/g tissue)	NO (uM/g tissue)	CAT (IU/g tissue)	GSH (IU/g tissue)
G1	28.67± 1.45 ^{d}	14.80± 0.73 ^{d}	6.15± 0.14 ^{a}	32.34± 1.49 ^{a}
G2	64.93± 1.33 ^{a}	71.10 ± 1.31 ^{a}	1.04± 0.05 ^{d}	11.47± 1.19 ^{d}
G3	38.50± 1.21^{c}	28.17± 1.05 ^{c}	3.00± 0.10 ^{b}	25.90± 1.39 ^{b}
G4	50.13± 0.82^{b}	40.17± 1.04 ^{b}	2.14± 0.08 ^{c}	17.64± 1.39 ^{c}

G1=normal control, G2=Diabetic control,G3=Diabetic+curry,G4=Diabetic+Moringa.

Means within the same column carrying different superscript letters are significantly different ($P \leq 0.05$).

Table (2): Changes in relative expression of *Sod1*, PGC1α, ATP 1A1, cytochrome c genes in heart tissues of STZ-induced diabetic rats following treatment by curry and moringa for 4 weeks. (Mean±SE).

Groups	*Sod1*	PGC1α	ATP 1A1	cytochrome c
G1	1.00 ± 0.02 ^{a}	1.00 ± 0.02 ^{a}	1.00 ± 0.02 ^{a}	1.00 ± 0.02 ^{d}
G2	0.02± 0.002 ^{d}	0.14 ± 0.005 ^{d}	0.04± 0.005 ^{d}	4.41 ± 0.12 ^{a}
G3	0.42± 0.03 ^{b}	0.59 ± 0.03 ^{b}	0.51 ± 0.03 ^{b}	1.96 ± 0.09 ^{c}
G4	0.19 ± 0.01 ^{c}	0.35± 0.01 ^{c}	0.24 ± 0.01 ^{c}	3.27 ± 0.11 ^{b}

G1=normal control,G2=Diabetic control,G3=Diabetic+curry,G4=Diabetic+Moringa.

Means within the same column carrying different superscript letters are significantly different ($P \leq 0.05$).

DISCUSSION

In this study, rats treated with STZ showed a significant increase in MDA and this is due to increased oxidative stress caused by STZ induced diabetes.

Rashida etal, 2010 showed Significantly increased levels of serum malonaldehyde in type 2 diabetics with myocardial infarction. Lines of evidence show that MDA is a stable end product of free radicals induced by lipid peroxidation. Lipid peroxidation can generate large

amounts of electrophilic and oxidizing reactive species which can lead to a variety of DNA and tissue damage (**Douki *etal*, 1998)**. Thus MDA serves as a reliable marker for the assessment of free radical induced damage to tissues. In diabetic patients a major factor that is responsible for enhanced free radical generation is hyperglycemia through auto-oxidation of glucose; it may be an important risk factor for cardiovascular disease.(**Wei etal,1998**).

Our results revealed that asignificant increase in nitric oxide (NO) in diabetic rats. Some research articles reported increased NO levels in diabetes patients (**Maejima etal, 2001)** whereas others reported the opposite (**Tessari *etal*, 2010).** It was indicated that hyperglycaemia is responsible in generation of high levels of NO from HUVEC cells through induction of iNOS and eNOS gene expression (**Ramu etal, 2015)**. When nitric oxide (NO•), nitroxyl anion (NO-) and nitrosonium cation (NO+) further exceeds the antioxidant capacity, it results in nitrosative stress (**Bentz *etal*, 2004**).

It was shown that Hydroethanolic extract of *M. oleifera* flower significantly suppressed the secretion and expression of NO and inducible NO synthase (iNOS) (**Woan *etal*, 2015).**

Our results revealed that asignificant decrease in catalase and GSH p_x in diabetic rats. This is due to decreased antioxidant levels. Some research showed that diabetes is one such condition where a decrease in antioxidant levels has been reported. (**Shinde etal, 2011**). During oxidative stress, there is an imbalance between ROS, RNS and the antioxidant system. Also during oxidative stress the GSH: GSSG ratio is decreased (**Circu and Aw, 2012**). Also, It was shown that STZ diabetes in G2 rats showed also an increase in lipid peroxidation and IL-6 and decreased catalase, SOD, and GSH activity (**Abdulrahman and Haddad, 2015**). It was observed that curry leaf powder had the property to increase the antioxidant levels of SOD, GSH-Px, Vitamin E and Vitamin C which, if consumed regularly can delay complications associated with diabetes mellitus(**Sucheta. and Kavitha., 2013).** It was demonstrated that *Moringa. Oleifera* has significant antioxidant activities from both in vivo as well as in vitro studies suggests that the regular intake of its leaves through diet can protect normal as well as diabetic patients against oxidative damage as it can significantly increase SOD,CAT and GST while decrease LPO content (**Dolly etal, 2013**)**.** The leaves of moringa contain quercetin-3-O-glucoside and kaempferol-3-O-glucoside which play a role in antioxidant defence by scavenging free radicals and reducing oxidative stress (**Siddhuraju and Becker, 2003).** Fractional studies of moringa leaf extract (MOE) showed it is composed

of flavonoids, hyperosid, rutosid, terpenoids, oleanoic acid and β-sitosterol which attributed to its antioxidant properties (**Marrufo etal, 2013**).

Also, our results showed asignificant decrease in expression of sod1 gene in diabetic rats. It has been hypothesized that mutations in the SOD1 gene may impair antioxidant enzyme activity thereby leading to accumulation of toxic superoxide anions (**Gurney *etal*, 1994).** It was also found that the SOD1 gene polymorphism influences SOD activity (**Ghattas, and. Abo-Elmatty, 2012).** Decreased activity of the antioxidant enzymes and depletion of total antioxidant capacity may increase the susceptibility of diabetic patients to oxidative injury (**Rahbani *etal*, 1999).** The increase in oxidative stress is genotoxic to the cell. H2O2 can react with metal ions such as iron and produce highly reactive hydroxyl radicals that target DNA.ROS-mediated DNA damage can be a therapeutic target in cancer cells as it signals nucleases to cause DNA strand breaks (**Sreelatha and Padma, 2011).**

Also, our results revealed asignificant decrease in relative expression of PGC1α in diabetic rats.Similar study showed that PGC-1α–responsive genes are down-regulated in obese Caucasians with impaired glucose tolerance and type 2 diabetes (**Mootha *etal*, 2003**). Peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC-1 alpha) plays an important role in regulating mitochondrial biogenesis and myocardial metabolism, it was indicated that PGC-1 alpha plays an important role in regulating expression of myocardial mitochondrial antioxidants SOD2 and thioredoxin and in protecting hearts against transverse aortic constriction induced myocardial oxidative stress, hypertrophy, and dysfunction (**Lu *etal*, 2010).**

Also, it our results revealed asignificant decrease in relative expression of ATPase gene in diabetic rats. some research article reported that diabetic cardiomyopathy has been associated with changes in enzymatic activities in the cardiac sarcolemma, with decreased Na, K-ATPase activity havingbeen observed in the hearts of animals with experimentally induced diabetes**.** Diabetic impairment of Na, K-ATPase activity could be due to altered enzyme kinetics and/or altered subunit expression **(Ng *etal*, 1993)**. The activity of membrane-bound enzymes may also be influenced by membrane environmental factors such as lipid content. Alterations of membrane environment have been observed in various diseases. There is also evidence to suggest that membrane environment may have some effect on insulin sensitivity and insulin-stimulated glucose uptake **(Storlien *etal*, 1991**) and Na, K-ATPase activity (**Pan *etal*, 1994**). Membrane environment can also be modified by diet and a number of therapeutic

diets are used It was observed Altered lipid environment and protein expression were the most likely explanations for the decrease in Na, K-ATPase activity observed in cardiac membranes (**Gerbi**1 ***etal*, 1997**). Na/K ATPase has been implicated in the development of complications and adaptive changes in diabetes (**Greene and Lattimer, 1987**) In experimental diabetes, changes in Na /K-ATPase activity have been reported in the heart, peripheral nerve, kidney and intestine. The magnitude and direction of the changes depend on the duration of diabetes and the organ involved((**Barada *etal*, 1994).** Moreover, diverse studies suggest that mitochondrial oxidative function was compromised in diabetic and prediabetic humans as evidenced by reduced levels of fatty acid oxidation, insulin-stimulated ATP synthesis, and expression of genes involved in oxidative phosphorylation (OXPHOS) (**Rato *etal*, 2014**). With respect to OXPHOS, activity was suggested that mitochondrial diabetes may also affect the complex V (**Parks and Drake, 1982)** and it is interesting to mention that, in diabetic patients' muscle, blue native gel electrophoresis revealed a striking decrease in complex I, III, and IV containing supercomplexes. In addition, impairment of pyruvate dehydrogenase complex on the citric acid cycle and glucokinase activity during diabetes has been reported (**Antoun *etal*, 2016)**.

In this present study, there is increase in cytochrome c in diabetic rats. lines of evidence showed that diabetes promoted a significant increase in hydroxyl radical production which correlated with lipid peroxidation (LPO) levels. Besides, hyperglycemia significantly increased mitochondrial BAX protein expression, cytosolic cytochrome c levels, and caspase-3 activity leading to an increase in apoptotic index(**Daniel etal, 2010)**. **Cytochrome c (cyt c)** is a key molecule in the mitochondrion as it is a water-soluble component of the electron transport chain. When cyt c is released during apoptosis, the electron transport chain, ATP synthesis and mitochondrial membrane potential is affected. In the electron transport chain, cyt c is responsible for transferring electrons from cytochrome c reductase (complex III) to cytochrome c oxidase (complex IV). During this process water is formed by the reduction of oxygen. Due to the release of cyt c, it affects this step in the electron transport chain thus leading to the formation of ROS. An increase in ROS results in oxidative stress, oxidative damage to lipids (lipid peroxidation), protein and DNA ultimately leading to apoptosis **(Wang, 2001).** When cellular DNA is damaged or there is an increase in reactive oxygen species, the cell induces apoptosis via the mitochondria **(Fan *etal*, 2005)**. A pro-apoptotic molecule from the Bcl-2 family (Bax, Bad, Bid), which localises in the cytoplasm, translocates to the mitochondrial outer membrane and binds to voltage-dependent anion

channel (VDAC) and influences its activity. The VDAC protein then forms a subunit of the mitochondrial permeability transition pore (MPTP). When there is mitochondrial swelling, changes in ion channels and depolarisation of the mitochondrial membrane, it results in the opening of MPTP releasing cyt c into the cytoplasm and together with ATP, Apaf-1 and procaspase-9 forms an apoptosome **(Hengartner, 2000).**

So, the G3,G4 treated with curry and moringa respectively can ameliorate the bad effect caused by oxidative stress to avoid complication of diabetes to improve mitochondrial function of heart.this is due to Curry leaves protein antioxidant is an effective antioxidant in preventing membrane damage and associated functions mediated by reactive oxygen species. It can be further developed as an effective bioprotective antioxidant agent to cellular components (**Mylarappa *etal*, 2016**) and the leaves of moringa contain quercetin-3-O-glucoside and kaempferol-3-O-glucoside which play a role in antioxidant defence by scavenging free radicals and reducing oxidative stress (**Siddhuraju and Becker, 2003**). Recently, it was reported that galangin (natural flavonoid) in moringa could maintain liver mitochondrial function in diabetic rats through oxidative stress reduction and both antioxidant enzymes and respiratory complexes activities enhancement (**Aloud *etal*, 2017).** Therefore, the likely role of mitochondrial ROS in diabetes has led to efforts for developing effective antioxidant compounds targeted to mitochondria. It is exhibited at higher concentrations of phenolic compounds in moringa such as quercetin, galangin, taxifolin, catechin, and prenylated flavonoid have been shown to affect mitochondrial energetic processes (**Elingold *etal*, 2008)**. In addition, it has been shown that mitochondria are a plausible main target of flavonoids mediating preventive actions against stress and mitochondrial dysfunction-associated pathologies (**Lagoa *etal*, 2011).**

CONCLUSION

The present study demonstrated that aquous extract of leaves of curry and moringa provided an effective treatment against insulin resistance in STZ-induced diabetic rats and ameliorate the bad effects caused by oxidative stress and improve function of mitochondria due to antioxidant properties.

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