EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 6
Issue: 8
73-78
Year: 2019

INHIBITORY EFFECT OF THE METHANOL LEAF EXTRACT OF *MOMORDICA CHARANTIA* ON LIVER MITOCHONDRIAL PERMEABILITY TRANSITION PORE OPENING IN ALLOXAN-INDUCED DIABETIC RATS

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Article Received on 10/06/2019 **Article Revised on 30/06/2019** **Article Accepted on 21/07/2019**

ABSTRACT
Type 2 diabetes mellitus (T2DM), the most common of the three types of diabetes (other types being Type 1 and Gestational diabetes) is a metabolic disorder that occurs when the body cannot properly use glucose, the body's main source of fuel. The plant *Momordica charantia* (MC*)* of the family Cucubitaceae is a popular medicinal plant most useful as an antidiabetic agent, thus, this work reports the inhibitory effect of varying concentrations (0.2, 0.4, 0.6, 0.8 and 1.0mg/mL) of the methanol leaf extract of MC on mitochondrial membrane permeability transition (MMPT) pore opening in liver mitochondria isolated from alloxan-induced diabetic rats. Opening of the MMPT pore, a critical step in the induction of apoptosis was spectrophotometrically assayed under succinate-energized condition using a modified method of Lapidus and Sokolove (1993). The results obtained revealed that there was a concentration-dependent inhibition of the MMPT pore opening in alloxan-induced diabetic rat liver mitochondria treated with the methanol leaf extract of MC *in vitro* such that at 0.2, 0.4, 0.6, 0.8 and 1.0 mg, 59, 54, 51, 45, 43% inhibitions were respectively observed. The degrees of inhibition seen suggest that the methanol extract is an MMPT pore antagonist; especially at high concentrations and that the inhibition of MPTP is likely one of the mechanisms by which MC inhibits apoptosis of pancreatic ß-cells in Type 2 diabetes.

KEYWORDS: Mitochondrial Permeability Transition Pore, Apoptosis, *Momordica charantia*, Alloxan, Diabetes.

INTRODUCTION
Diabetes mellitus (DM) is considered a metabolic and inflammatory disease that affects more than 170 million people around the world. Its increase worldwide is epidemic. Despite a new generation of medications and the advances in clinical treatments, the prevalence of diabetes has risen dramatically. Diabetes is characterized by hyperglycemia, and its control only slowly hinders the progression of the disease's complications, without stopping them. Hyperglycemia triggers several metabolic signaling pathways that lead to inflammation, cytokines secretion, cell death, and, consequently to diabetic complications.[1] Increasing evidence supports that T2DM is modulated through pancreatic β-cell apoptosis. Apoptosis is a complex biological phenomenon characterized by cell shrinkage, chromatic condensation, internucleosomal DNA fragmentation, and disassembly into membrane-encircled vesicles (apoptotic bodies).[2] The induction of the mitochondrial membrane permeability transition (MMPT) pore has been implicated in the cascade of events involved in apoptosis (programmed cell death).[3] MMPT Pore is a voltage-dependent, CsA sensitive, high conductance inner-membrane channel that is critically regulated by a variety of pathophysiological effectors. Recent work indicates that the PTP forms from the F-ATP synthase, which would switch from an energy-conserving to an energy-dissipating device.[4] Formation of the pore causes mitochondrial failure by uncoupling oxidative phosphorylation and accelerating ATP hydrolysis. Pore formation signals cell death (through necrosis), mitochondrial autophagy, and apoptosis (through the rupture of the outer mitochondrial membrane).[5] The principal trigger for pore formation is intramitochondrial Ca^{2+} concentration. Ca^{2+} influx causes the pore to open rapidly while chelating agents such as EDTA or EGTA can reverse this effect. Other divalent cations like Mg^{2+}, Mn^{2+}, Ba^{2+} and Sr^{2+} are competitive inhibitors of Ca^{2+}-induced pore formation.[6] Certain plants have also been identified as potent inhibitors of MMPT pore.[7] MC or

bitter melon, is a vegetable considered to have traditional medicinal use, the fruit is considered as tonic, stomachic, stimulant, emetic, antibilous, laxative and alterative.[8] The plant contains a collection of biologically active phytochemicals including triterpenes, proteins, steroids, alkaloids, saponins, flavonoids and acids due to which the plant possesses anti-fungal, anti-bacterial, anti-parasitic, anti-viral, anti-fertility, anti-tumorous, hypoglycemic and anti-carcinogenic properties. Fruits are used as traditional medication to cure various diseases like: rheumatism, gout, worms, colic, illness of liver and spleen. It is also found useful in the treatment of cancer and diabetes.[9,10,11]

This work aims at elucidating the effect of the methanol extract of MC on MMPT pore in both normal and alloxan-induced diabetic rat.

MATERIALS AND METHODS

Chemicals and Reagents

Sodium Carbonate (Na_2CO_3), Sodium Hydroxide (NaOH), Sodium-Potassium Tartarate ($Na\text{-}K\text{-}C_4O_6$), Hydrated Copper Sulphate ($CuSO_4.5H_2O$), Calcium Chloride ($CaCl_2$), Sodium chloride (NaCl), Alloxan, Potassium Hydroxide (KOH), Methanol were Products of BDH Poole, UK Ltd. and Co., while Folin Ciocalteau Reagent, BSA, Mannitol, Sucrose, HEPES [4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid], EGTA, Spermine, Rotenone, and Sodium Succinate (hexahydrate) were Products of Sigma-Aldrich Co, USA. All chemicals were of analytical grade.

Experimental Animals

Twenty healthy Wistar strain albino male rats (between 120g-150g) were purchased from pathogen-free colonies in the preclinical Animal House, University of Ibadan, Ibadan, Nigeria. They were kept in the Animal house of the Biochemistry Department under light-controlled conditions (12h−light/12h−dark cycle) and in well-ventilated metal cages. The animals received formulated feeds and water *ad libitum*, were allowed to acclimatize over a period of two weeks and cared for in accordance with good laboratory animal care practice prescribed by the University's Committee on Animal care and ethical use.

Animal grouping

The animals were divided into two groups; group 1 and 2, with 10 rats in each group. Group 2 animals were injected with 150mg/kg body weight of alloxan intraperotoneally to induce diabetes while group 1 animals (control) were only kept on their stock diet.

Induction of diabetes

A 10% stock solution of alloxan was prepared by dissolving 1g of alloxan in 10mls of saline. 150mg/kg body weight concentration was used in inducing diabetes. The animals to be induced were fasted for 12hours and their glucose levels were tested after which they were injected with alloxan. Their blood glucose levels were then determined 24, 48 and 72 hours after injection using a ONE TOUCH Brand blood glucose meter. Animals with glucose level of 200mg/dL and above were used in this experiment.

Plant

The leaves of MC were purchased from the Elewe Omo section of the Oje market in Ibadan, Oyo State, Nigeria and authenticated by Prof. Abiodun Ayodele, a Plant Taxonomist of the Department of Botany, University of Ibadan, Nigeria. The voucher number is UIH-22369.

Preparation of methanol extract of *Momordica charantia*

The powdered leaves were extracted using 95% concentrated methanol in a soxhlet extractor apparatus for 24hours. Extraction results in a green solid which was the fraction used for this experimental study.

Liver Mitochondrial Fraction Isolation

The low ionic strength mitochondrion was isolated using a method described by Johnson and Lardy, 1967.[12] The experimental animals were fasted overnight then sacrificed by "cervical dislocation" and quickly dissected. The liver was rapidly excised, trimmed to remove excess tissue and washed in homogenizing buffer (210 mM Mannitol, 70 mM Sucrose, 5 mM HEPES, and 1 mM EGTA, pH 7.4). Thereafter, the liver was weighed, chopped and suspended in the same buffer to make a 10% suspension of tissue in buffer. Immediately the liver was homogenized on ice using a glass Bosch PSB 570-2 Homogenizer. The homogenate was sedimented twice in a MSE centrifuge at 2500rpm for 5 minutes to remove the nuclear fractions and cellular debris, supernatant obtained was centrifuged at 13000rpm for 10 minutes and mitochondrial fraction obtained was washed three times in washing buffer (210 mM Mannitol,70mM Sucrose, 5mM HEPES-KOH and 0.5% BSA, pH 7.4 at 12000rpm for 10 minutes. The mitochondrial pellets were suspended in swelling buffer (210 mM Mannitol, 70 mM Sucrose, and 5 mM HEPESKOH, pH 7.4) and immediately dispensed in 1 ml Eppendorf tubes to make aliquots which were used fresh.

Mitochondrial Swelling Assays

Mitochondrial swelling assays were done using a modified method of Lapidus and Sokolove, 1993.[13] In brief, mitochondria (0.4mg of protein/ml), 8µM rotenone and 300µM $CaCl_2$ were incubated in swelling buffer for about 3minutes at 30°C, prior to the addition of sodium succinate which came 30secs later and mitochondrial permeability was quantified as changes in absorbance at 540 nm. This method was coined as a result of our observing a potentiated triggering of the MMPT pore opening when $CaCl_2$ is directly incubated with the mitochondria rather than added after mitochondria pre-incubation. For spermine inhibition and effect of extracts, spermine and varying concentrations of the methanol extract of MC were each added just after the 3minutes incubation of the mitochondria in the presence

of rotenone and $CaCl_2$ then 5mM sodium succinate was added 30secs after. The resulting mixture was assessed spectrophotometrically at 540 nm.

Statistical analysis: Microsoft Excel, 2010 was used for the analyses.

RESULTS

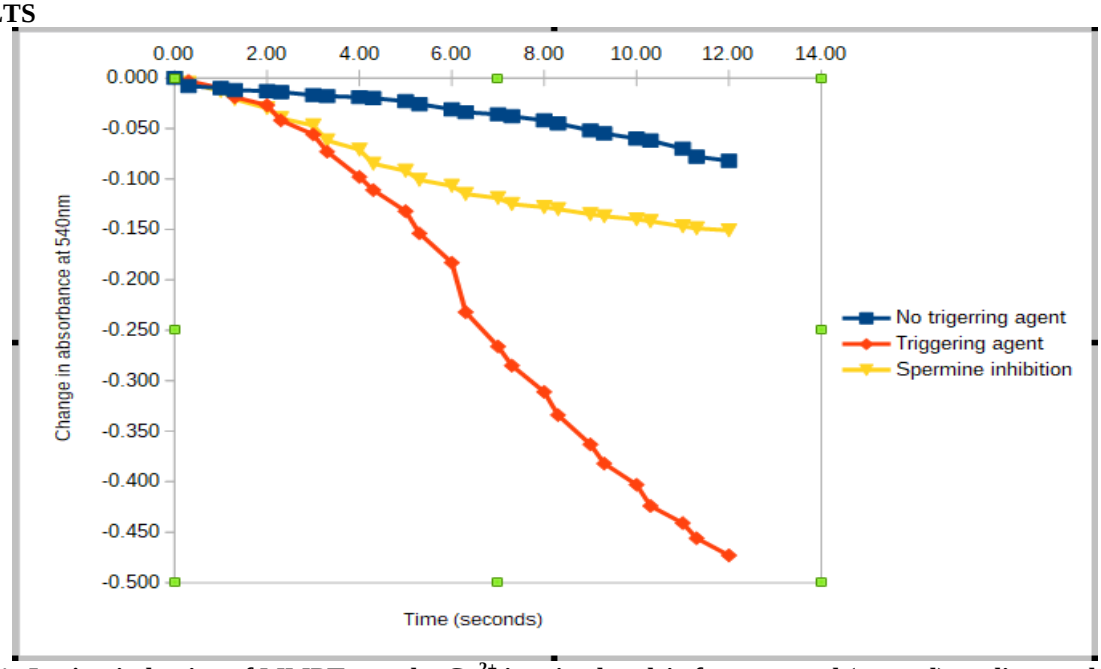

Figure 1: *In vitro* induction of MMPT pore by Ca^{2+} in mitochondria from normal (control) rat liver and reversal with spermine.

Figure 1 above shows a 5.77-fold increase induction in the mitochondria treated with triggering agent ($CaCl_2$) using incubation procedure when compared with the control group (No triggering agent). The observed induction is reversed by spemine, a standard inhibitor of the MMPT pore by 68.1% inhibition.

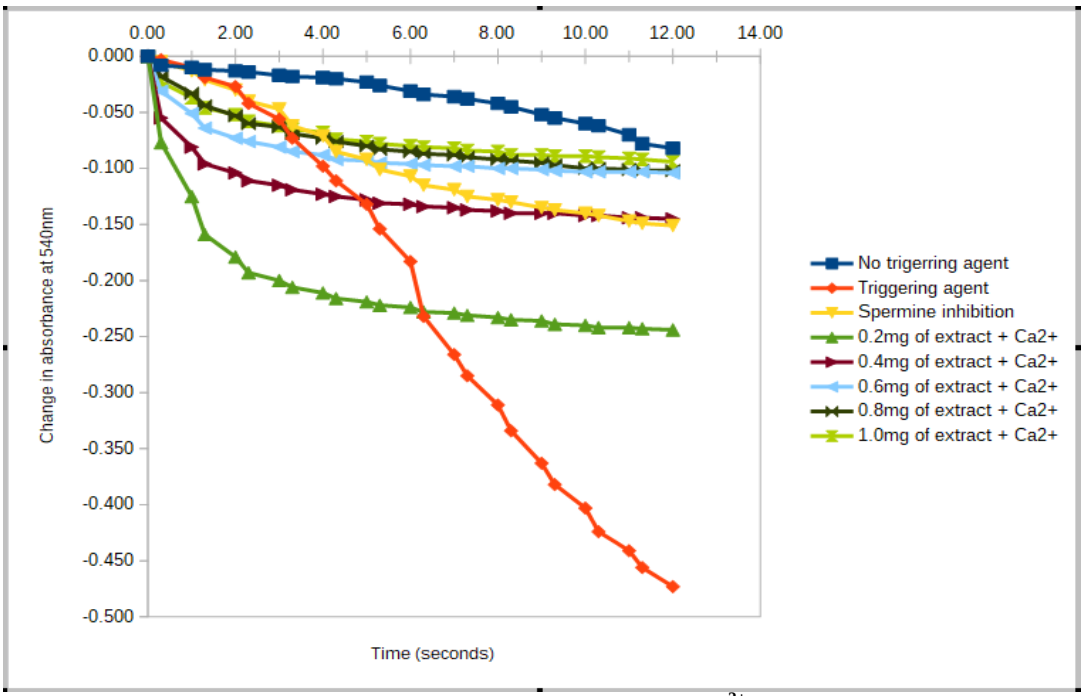

Figure 2: *In vitro* inhibitory effect of the methanol extract of MC on Ca^{2+} - induced MMPT in normal (control) rats.

Varying concentrations of methanol extract reverse the triggering effect of $CaCl_2$ on MMPT pore such that at 0.2, 0.4, 0.6, 0.8 and 1.0 mg, Δ_{540nm} of -0.244, -0.145, -0.104, -0.102, -0.094 *which translate to 48.4, 69.3, 78, 78.4, 80.1 % inhibitions were observed respectively.*

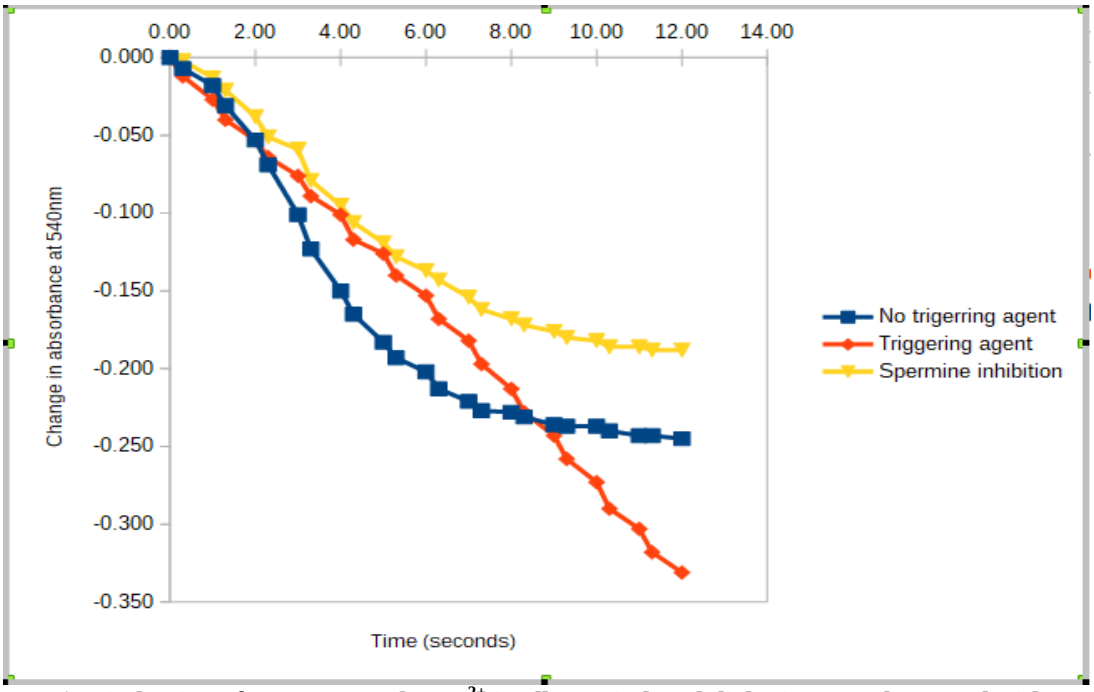

Figure 3: *In vitro* induction of MMPT pore by Ca^{2+} in alloxan-induced diabetic rat and reversal with spermine.

As shown in Figure 3, MMPT pore swelling was observed both in the presence and absence of Ca^{2+} with Δ_{540nm} of -0.331 and -0.245 respectively. Spermine reversed the observed induction by 43% inhibition.

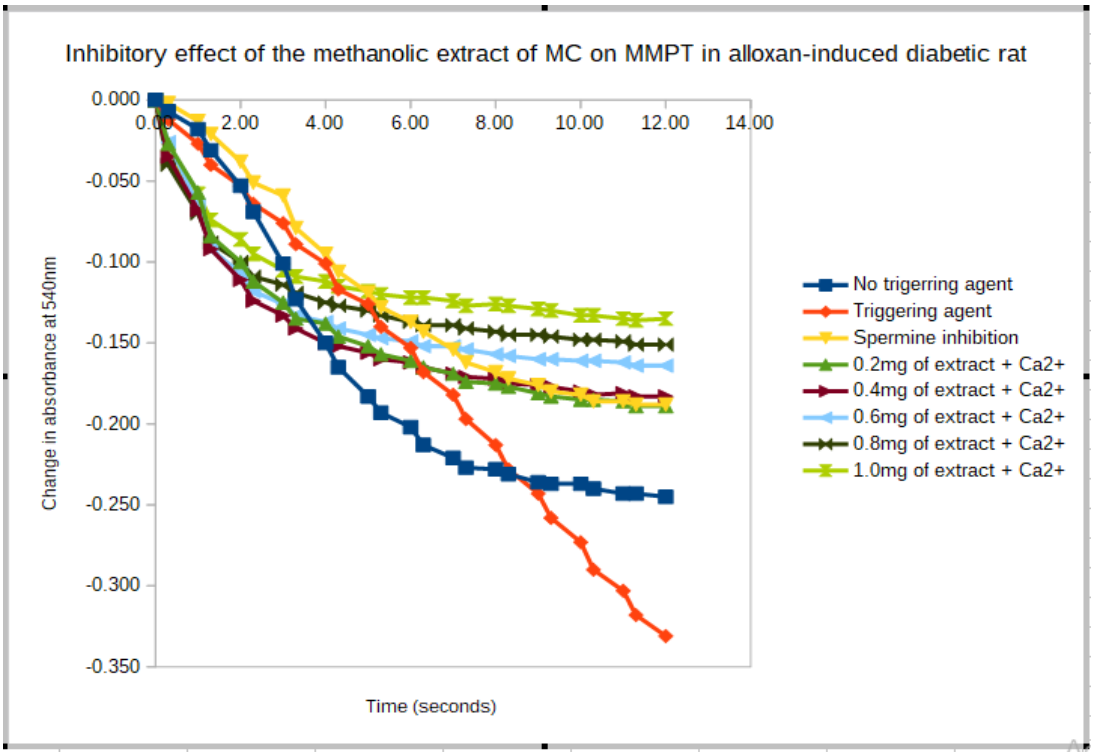

Figure 4: *In vitro* inhibitory effect of the methanol extract of MC on MMPT in alloxan-induced diabetic rat.

Figure 4 shows that methanol extract of MC inhibited the observed swelling in diabetic rat liver mitochondria in a concentration dependent manner following the order of potency; 0.2mg < 0.4mg < 0.6mg < 0.8mg < 1.0mg with Δ_{540nm} being -0.189, -0.183, -0.164, -0.151 and -0.135 respectively translating to 42.9, 44.7, 50.5, 54.4, 59.2 % inhibitions.

DISCUSSION

Several reports have implicated mitochondria as a key player in cell death being a central organelle involved in the signal transduction and amplification of the apoptotic response.[14,15] Mitochondrial dysfunction is an early event preceding nuclear and plasma membrane alterations. It is characterized by an increase in mitochondrial membrane permeability and loss of membrane potential that is regulated by the permeability transition pore complex, an elusive multiprotein channel composed of voltage dependent anion channel, adenine nucleotide translocator, cyclophilin D, peripheral benzodiazepine receptor and probably others.[16] The MMPT pore opening is favored by Ca^{2+} uptake. When respiring mitochondria take up Ca^{2+} in the presence of inorganic phosphate and external adenine nucleotides, the accumulated Ca^{2+} is retained indefinitely but in the absence of adenine nucleotides, the accumulated Ca^{2+} is subsequently released along with other matrix solutes[17]; a process which can result in apoptosis and if unrestrained necrosis. As shown in figure 1, large amplitude opening of the MMPT pore was observed in the opening of the MMPT pore with Δ_{540nm} of -0.473 when compared to the control group (Δ_{540nm} = -0.082). This result is in alignment with the result obtained by Ehigie *et al*., 2018 and Bernardi *et al*., 1992 who both demonstrated in their studies that Ca^{2+} is a potent inducer of MMPT pore.[18,19] The result obtained in figure 2 shows a concentration-dependent inhibitory effect of methanol extract of MC on liver mitochondrial in normal male wistar rats. This result is in alignment with the result reported by Ehigie *et al* (2014), who stated that methanol extract of MC successfully inhibited large amplitude swelling of isolated rat liver mitochondria triggered by Ca^{2+}.[20] In alloxan-diabetic rat, MMPT pore opening was observed not only in the presence of Ca^{2+} the triggering agent but also in its absence; suggestive of an already compromised mitochondrial membrane integrity, a prerequisite to cell death initiation. This observation is in accordance with the report of Halestrap, 1999 and Kristal *et al.*, 1997 which both stated that diabetes can result in > 95% loss of mitochondrial transcriptional capacity and functions which may result in cell death by necrosis.[21,22] It could then be postulated that further *in vitro* treatment of these impaired mitochondria with Ca^{2+} would only result in less pronounced swelling due to the initial loss of mitochondrial integrity via apoptosis and necrosis. The result represented by figure 4 shows that methanol extract of MC inhibited the swelling in diabetic rat liver mitochondria in a concentration dependent manner with percentage inhibitions being as high as 42.9, 44.7, 50.5, 54.4, 59.2% at 0.2, 0.4, 0.6, 0.8, 1.0 mg respectively. Interestingly, the inhibitory effects of methanol extract of MC at higher concentrations (0.4, 0.6, 0.8, 1.0 mg) are more pronounced than the inhibitory effect of spermine (43% inhibition; figure 3), a standard inhibitor of the pore. From the foregoing, this study identifies the methanol extract of MC as a potent inhibitor of the MMPT pore opening typically seen diabetic rats. This may not be unconnected to the mechanism by which *Momorica charantia* treats diabetes.

CONCLUSION

Methanol extract of *Momordica charantia* is a potent inhibitor of MMPT pore of liver mitochondria of diabetic rat and could therefore be useful in ameliorating loss of mitochondrial transcriptional capacity and functions which may result in pancreatic β-cell apoptosis/necrosis normally seen in diabetics.

ACKNOWLEDGEMENT

We acknowledge the support of the technical staff of the Department of Biochemistry, University of Ibadan during the course of this research.

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