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**Research Article**

# *Voacanga africana* Leaf Extract Modulates Opening of the Mitochondrial Membrane Permeability Transition Pore in Normal and Diabetic Rat Liver

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**ABSTRACT**

**Objectives:** The opening of the Mitochondrial Membrane Permeability Transition (MMPT) pore as a result of mitochondrial dysfunction may be implicated in diabetes. Many compounds from medicinal plants have shown great potentials in modulating the MMPT pore, hence, this study assessed the effect of the methanol extract of *Voacanga africana* (MEVA) leaves on the MMPT pore; both in normal and diabetic rat liver.

**Methods:** Ten alloxan-induced (150 mg/kg body weight) diabetic and ten normal rats were used for the study. Liver mitochondria of both the normal and diabetic rats were isolated and MMPT was measured spectrophotometrically.

**Results:** In normal rat liver mitochondria, spermine significantly ($p<0.05$) inhibited calcium-induced pore opening. Pre-incubation of normal mitochondria with the MEVA leaves, inhibited calcium-induced pore opening with increasing concentration also. Interestingly, in the absence of calcium ion, there was no significant opening of the MMPT pore. Furthermore, it was observed that alloxan-induced opening of the MMPT pore significantly ($p<0.05$) when compared to the normal control. In the absence of $Ca^{2+}$ however, the MEVA leaves inhibited MMPT pore opening in alloxan-induced diabetic rat liver mitochondria by 3.7, 6.8 and 10.5% at 2.5, 3.5 and 4.5 mg/ml, respectively. However, pre-incubating diabetic rat mitochondria with varying concentration of MEVA leaves in the presence of $Ca^{2+}$ significantly inhibited MMPT pore opening.

**Conclusion:** These findings suggest that the phytochemicals present in the methanol extract of *V. africana* leaves are potent modulators of the MMPT pore and may find use in the treatment of diabetes.

## INTRODUCTION

A number of diseases with growing concern are largely associated with mitochondrial dysfunction.[1,2] Diabetes melitus, an endocrine metabolic disorder and a leading cause of morbidity in the world today has been found to occur as a result of dysfunction in the mitochondrial pathways.[2] Physiologically, diabetes has been characterized by insulin deficiency or as a result of insulin inactivity to bind its receptor on the cell membrane.[3] Diabetes is clinically manifested as hyperglycaemia or impaired glucose tolerance and other disorders,[4] and has led to disruption in the metabolism of carbohydrates, lipids and even proteins. Some studies have shown that hyperglycaemia or high concentration of sugar in the blood is largely associated with elevated levels of reactive oxygen species (ROS) in tissues and organs due to protein glycation, oxidation of glucose.[5,6] Also, it has been shown that mitochondrial dysfunction will promote apoptosis in the β-cells in the pancreas and β- cell death which may develop into diabetes.[7,8] The generation of ROS by the mitochondria has been well documented in the aetiology of diabetes and a host of other ailments. Oxidative stress which is an imbalance between oxidants and antioxidants has been implicated in diabetes and in a broad variety of chronic and acute diseases such as in ageing, cancer and neurodegenerative disorders.[9,10] ROS, high matrix calcium ion concentration, lipid such as ceramide and sphingolipids within the mitochondrion are well established potent inducers permeating the outer mitochondrial membrane, resulting in mitochondrial permeability transition and the irreversible opening of the mitochondrial membrane permeability transition (MMPT) pore.[11] The MMPT pore formed between the inner and outer mitochondrial membrane is believed to be made up of cyclophilin D, Adenine nucleotide translocase, Voltage dependent anion channel, benzodiazepine, hexokinase II[12,13] etc, though the actual structure of the pore has not been fully discovered.

Studies have shown that the activation of Bid, a

member of the Bcl-2 family and a pro-apoptotic protein with other signals generated from within the mitochondrion may result in the permeabilization of the mitochondrial outer and inner membranes.[14] This may cause the release of pro-apoptotic proteins such as Smac / DIABlO, cytochrome C, apoptosis inducing factor (AIF) and a host of other proteins from the intermembrane space into the cytoplasm of the cell. Caspase 9 is activated in the cytoplasm and forms an apoptosome with apoptotic activating factor 1 (Apaf-1) in the presence of dATP.[15] This whole process leads to further activation of downstream caspases, mitochondrial swelling, loss of membrane potential and energy as ATP and eventually cell death with morphological features associated with apoptosis such as the externalization of phosphatidyl serine and blebbing of the cell membrane.[16] The increase in the number of diabetes both in the developing and developed world is on the increase with the attendant side effects on the conventional therapies in the management of diabetes. Currently, there has been no permanent cure to diabetes. However, the burden to search for alternatives from natural sources useful in the treatment and management of diabetes is on the increase.

Bioactive agents from nature such as plants and microbes may be useful in the treatment of diabetes. Plants are large reservoir of nature's bioactive agents and possess potential compounds useful in the treatment of many ailments. These bioactive agents such as flavonoids, alkaloids, phenols, vitamins C, and E have been shown by many studies to be potent scavengers of reactive oxidants[17] which are well associated with the management and treatment of diabetes.[6,18] Compound such as apigenin, curcumin and genisteins from plants are established as very useful in the management of diabetes. Metformin an anti-diabetic drug and MMPT sensitive cyclosporine A (CSA) are also established inhibitors of the MMPT pore in the management of diabetes.[19-21]

*Voacanga africana* is a small tropical tree native to Africa's forests, known as Ako-dodo in Yoruba, hokiyar in Hausa and Pete-pete in Igbo. The plant is very rich in alkaloids especially the bark. The leaves and seeds contain voaphylline, vobtusine and tabersonine.[22,23] The principal indole alkaloids present in the root and stem bark of the plant are voacangine and vobasine.[24] A decoction of the stem bark and root is used in the treatment of mental disorders. Voacangine, as important alkaloid found in the bark of the plant have been reported to have analgesic and local anaesthetic properties.[24] Also, the root decoction is taken orally by women in Senegal to ward off the untoward consequence of premature parturition and painful hernia.[25] Some studies have reported the anti-ulcer, anti-amoebic activities of the extracts of *V. africana* in rats.[26,27] However, the effect of *V. africana* leaf extract on mitochondrial membrane permeability transition has not been studied on diabetes. Therefore this study investigated the effect of the methanol leaf extract of V. africana on liver mitochondria both in the presence and absence of calcium ion in normal and diabetic rats.

## MATERIALS AND METHODS

### Extraction Procedure

Fresh leaves of *V. africana* were harvested and identified at the Forestry Research Institute of Nigeria (FRIN), Ibadan, Oyo State. Two hundred grams of leaves was air-dried, blended and extracted with 2 litres of methanol in a Soxhlet extractor. The methanol was distilled off in a rotary evaporator at 45°C. The crude methanol extract obtained was refrigerated until the start of the experiment.

### Experimental animals

Twenty male Wistar rats used for this study were purchased from the animal house, Physiology Department, University of Ibadan, Ibadan, Nigeria. The animals were kept in ventilated wire mesh cages at room temperature with 12 hours light dark cycle, fed pelleted rat feeds (Ladokun Feeds) and water *ad libitum*.

Ten male Wistar rats were given an intraperitoneal single dose injection of alloxan (150 mg/kg) to induce diabetes while the remaining 10 rats were used as normal, non-diabetic rats. After a 24-hour fast, blood glucose concentrations were measured using 'One Touch' brand blood glucometer and test strip.

### Mitochondria Isolation

Isolation of rat liver mitochondria was carried out by the methods of Johnson and Lardy[28] and Lapidus and Sokolove.[29] The animals were sacrificed by cervical dislocation, the liver excised rapidly, trimmed to remove excess tissue and rinsed in an isolation buffer before it was weighed. A 10% solution of tissue in ice-cold isolation was prepared. The chopped liver was homogenised on ice in a Teflon homogeniser. The homogenised solution was centrifuged at 2300 rpm for 5 min in a MSE cold centrifuge to obtain the nuclear and cellular debris. The supernant obtained was centrifuged at 13000 rpm for 10 minutes. Pelleted mitochondria were washed thrice with the washing buffer and centrifuged at 12,000 rpm. Mitochondria were then suspended in 1 mL of a buffer free of EGTA and Bovine Serum Albumin (BSA) and kept on ice to maintain the integrity of the mitochondrial membranes. Mitochondrial protein content was determined according to the method described by Lowry *et al*.,[30] using BSA as standard.

### Mitochondrial Swelling Assay

Calcium ion is an established inducer of mitochondrial permeability, by disrupting the inner mitochondrial membrane, making it non- selectively permeable to solutes. The mitochondrial swelling assay was carried out by the method described by Lapidus and Sokolove.[29] Mitochondria (400 µg/ml) were incubated in a cuvette containing Mannose, Sucrose and Hepes buffer (swelling buffer pH 7.4) and 0.8 uM rotenone. $Ca^{2+}$ known as the triggering agent was added 3 minutes after mitochondria and 30 seconds before 5 mM Sodium-succinate. The final assay volume was 2500 µL. The cuvette was briefly swirled and quickly transferred into spectrophotometer. Changes in the absorption of light by the liver mitochondria were measured quantitatively ate 540 nm over a period of 12 minutes. The absorbance was taken at 30 seconds interval. The extent of MMPT pore opening inhibition by varying concentration of the MEVA (2.5, 3.5 and 4.5 mg/mL) on calcium-induced MMPT pore was compared to 0.1 mM spermine which was used as a standard inhibitor of the pore both in normal and diabetic rat liver mitochondria. The inductive effect of the varying concentration of the extract on the MMPT pore

opening in the absence of $CaCl_2$ was monitored both in normal and diabetic rat liver mitochondria.

**Statistical analysis**

Data obtained were subjected one way analysis of variance (ANOVA) using SPPS 17. Values were presented as mean ± standard deviation of at least three different experiments. Values were considered significant at $p < 0.05$. Each Figure is a representation of at least three different experiments.

**RESULTS**

Figure 1 shows that calcium ion significantly ($p < 0.05$) induced mitochondrial swelling in the livers of normal rats. There was a rapid and large decrease in absorbance at 540nM in the presence of exogenous $Ca^{2+}$ in normal rat liver mitochondria, showing there was significant increase in swelling of isolated rat liver mitochondria by about 329.9%. In the absence of exogenous $Ca^{2+}$, there was no significant ($p< 0.05$) mitochondrial swelling. However, spermine, a standard inhibitor of the MMPT pore opening significantly ($p < 0.05$) inhibited MMPT pore opening in isolated mitochondria in the presence calcium ion by 64%

Figure 2 shows the inhibitory effects of varying concentration of *Voacanga africana* on mitochondrial permeability transition pore. The result indicated that *V. africana* leaf extract inhibited $Ca^{2+}$-induced large amplitude swelling in rat liver mitochondria. The inhibition of the MMPT pore opening by the varying concentration of the extract increased in a concentration-dependent manner. Specifically, the extract at 2.5, 3.5 and 4.5 mg /mL inhibited MMPT pore opening by 7.5, 10.2 and 24.6%, respectively. It was also observed that spermine inhibited MMPT pore opening (64.2%) in the presence of calcium more significantly ($p < 0.05$) when compared with the varying concentration of the methanol extract of Voacanga africana leaves. Maximum inhibition of the pore by the extract was obtained at 4.5 mg/mL.

Interestingly, it was observed that the methanol extract of *V. africana* leaves in the absence of Ca2+ caused large amplitude swelling that is generally associated with permeability transition. The opening of the pore was concentration dependent with 2.6, 4.1 and 4.6 folds at 2.5, 3.5 and 4. mg/mL, respectively. The induction of the MMPT pore swelling was highest at 4.5 mg/mL concentration (Figure 3).

It was observed that the extract inhibited $Ca^{2+}$ - induced large amplitude swelling by 42.6, 48.4 and 48.8% at 2.5, 3.5, and 4.5 mg/mL, respectively. Furthermore, the extent of inhibition increased with increasing concentration. Maximum inhibition was obtained at the highest concentration - Figure 4. Also, the varying concentrations of the methanol extract induced MMPT pore opening only slightly at the lowest concentration (2.5 mg/mL) by 0.26 folds when compared with the control. However, at 3.5 and 4.5 mg/mL the methanol extract of *V. africana* leaves had no significant effect whatsoever on the induction of the MMPT pore Figure 5.

There was large amplitude swelling of the mitochondria from isolated diabetic rats prior to the addition of $Ca^{2+}$ which further increased in the presence of $Ca^{2+}$ (Figure 6). However, induction of the pore could not be reversed by spermine when compared to the result shown Figure 1.

The varying concentration of the methanol extract of *Voacanga africana* leaves inhibited calcium-induced mitochondrial membrane permeability transition in mitochondria isolated from diabetic rat liver with increase in extract concentration. Specifically, the methanol extract of *V. africana* leaves reversed calcium-induced MMPT in alloxan-induced MMPT pore opening by 3.7, 6.8 and 10.5% at 2.5, 3.5 and 4.5 mg/mL, respectively (Figure 7).

Figure 8 shows that pre-incubation of diabetic rat mitochondria with varying concentration of the extract caused significant reversal of mitochondria swelling. Interestingly, the extract reversed calcium-induced MMPT pore opening with increasing concentration. At 2.5, 3.5 and 4.5mg/mL, the methanol extract of *V. africana* leaves reversed MMPT pore swelling by 24.4, 30.5 and 33.2%, respectively. Spermine could only inhibit the effect of calcium ion by 10.2% only. Inhibition of calcium–induced pore opening by spermine and the varying concentration of the extract was not significant when compared to the result shown in Figure 4.

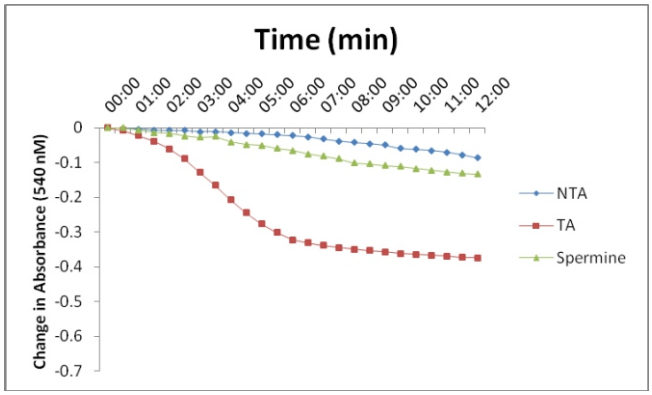


**Figure 1:** Effect of calcium ion and spermine on normal rat liver mitochondria NTA: No Triggering Agent / Control, TA: Triggering Agent (Calcium ion), Spermine: MMPT pore Inhibitor

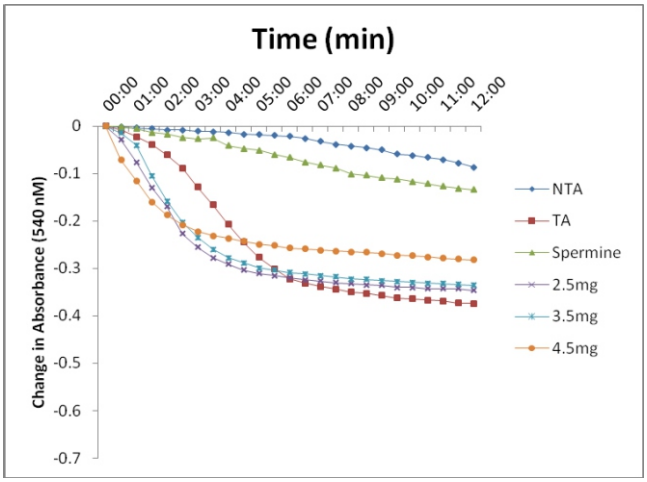


**Figure 2:** Effect of methanol extract of *Vocanga africana* leaves on normal rat liver mitochondria in the presence of calcium ion NTA: No Triggering Agent / Control, TA: Triggering Agent (Calcium ion), Spermine: MMPT pore Inhibitor

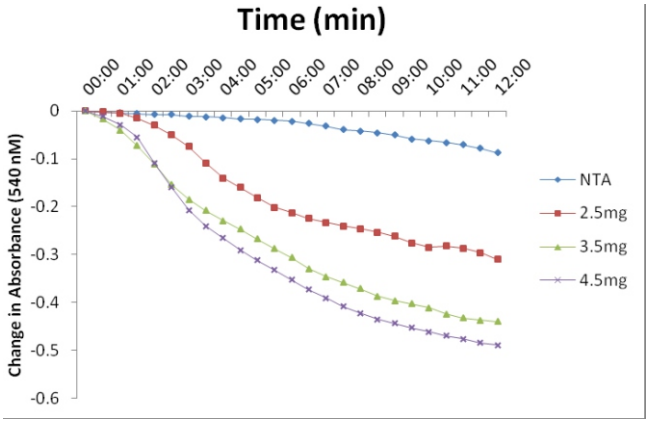


**Figure 3:** Effect of methanol extract of *Vocanga africana* leaves on normal rat liver mitochondria in the absence of calcium ion NTA: No Triggering Agent/Control, TA: Triggering Agent (Calcium ion), Spermine: MMPT pore Inhibitor

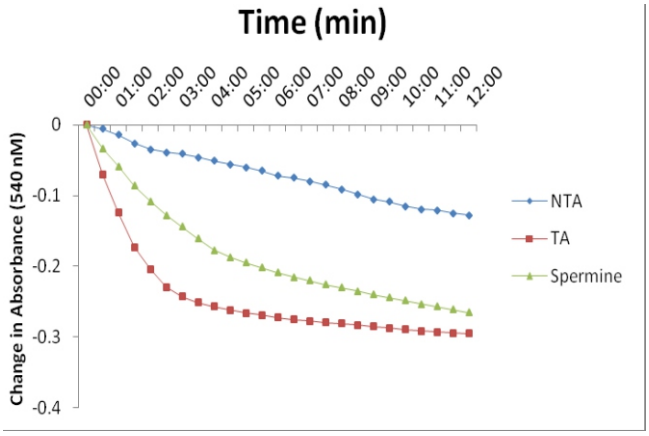


**Figure 6:** Alloxan induces opening of the mitochondrial membrane permeability transition pore in diabetic rat liver NTA: No Triggering Agent / Control, TA: Triggering Agent (Calcium ion), Spermine: MMPT pore Inhibitor

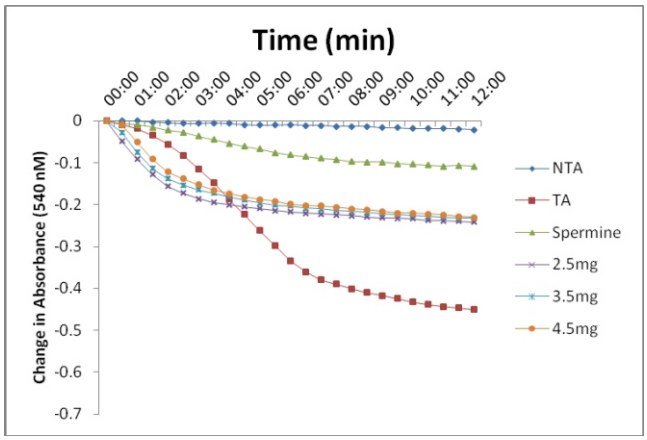


**Figure 4:** Effect of pre-incubating normal rat liver mitochondria with methanol extract of *Vocanga africana* leaves in the presence of calcium ion NTA: No Triggering Agent / Control, TA: Triggering Agent (Calcium ion), Spermine: MMPT pore Inhibitor

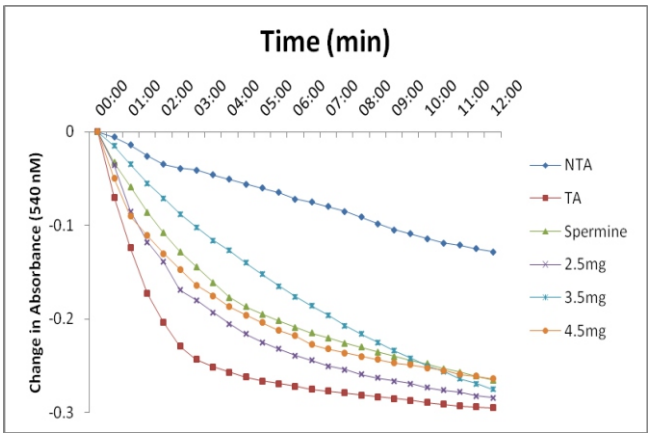


**Figure 7:** Effect of methanol extract of *Vocanga africana* leaves on diabetic rat liver mitochondria in the presence of calcium ion NTA: No Triggering Agent / Control, TA: Triggering Agent (Calcium ion), Spermine: MMPT pore Inhibitor

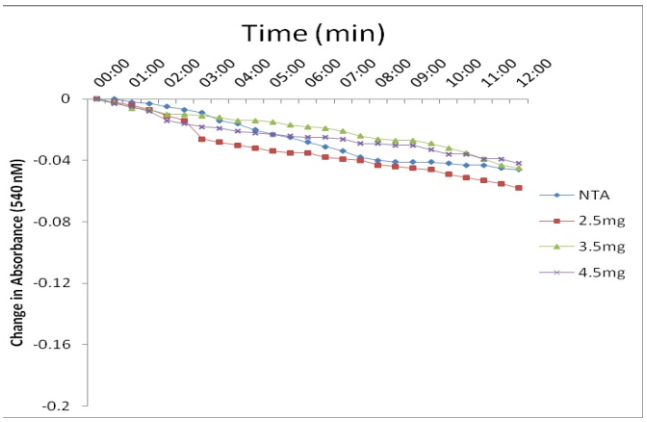


**Figure 5:** Effect of pre-incubating normal rat liver mitochondria with methanol extract of *Vocanga africana* leaves in the absence of calcium ion NTA: No Triggering Agent / Control, TA: Triggering Agent (Calcium ion), Spermine: MMPT pore Inhibitor

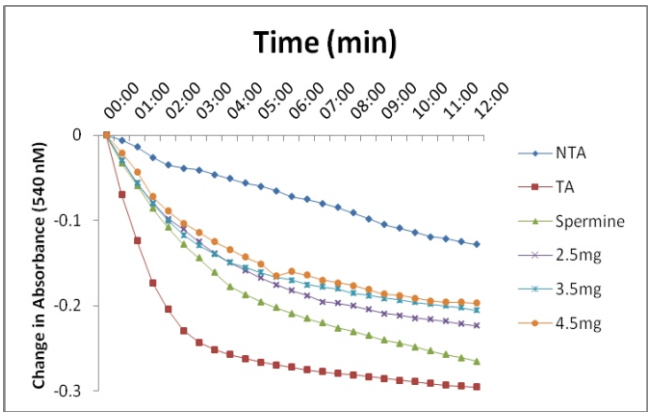


**Figure 8:** Effect of pre-incubating diabetic rat mitochondria with methanol extract of Vocanga africana leaves in the presence of calcium ion NTA: No Triggering Agent / Control, TA: Triggering Agent (Calcium ion), Spermine: MMPT pore Inhibitor

## DISCUSSION

Mitochondrial dysfunction has been identified as a factor that contributes to the aetiology of diabetes mellitus through a number of mechanisms.[31] High levels of reactive oxygen species which may arise from hyperglycemia in diabetes may trigger the permeabilization of the mitochondrial membranes,[14,15,32,33] leading to the uncoupling of oxidative phosphorylation, depletion of ATP, loss of membrane potential and essentially the release of proapoptotic proteins essential of the cell to die in a programmed fashion through the opening of the MMPT pore. ROS have been identified as a major risk factor in the pathophysiology of diabetes. Vitamins C and E have shown very strong antioxidant potential by reducing complications associated with diabetes such as high levels of ROS due to auto-oxidation of glucose.[34] A number of studies also show that antioxidants from natural origin are good scavengers of ROS and thus may be very useful in the management / treatment of diabetes. Phenols and flavonoids such as gallic and epigallocatechin gallate,[35,36] are important phytochemicals which have the potential to modulate opening of the mitochondrial membrane pore in many pathological scenarios.

The findings from this study reveal that although, significant swelling of the MMPT pore was observed upon the addition of calcium ion to isolated mitochondrial from rat liver, this was significantly reversed by spermine, a standard inhibitor of the MMPT pore. The reversal /inhibition of calcium-induced MMPT pore opening by spermine signify that the mitochondrial membranes were intact ab nitio and that the process of oxidative phosphorylation was uninterrupted prior to the addition of calcium ion. This result agrees with several other findings.[29,37] However in contrast to the result obtained in normal rat liver mitochondria, diabetic rat liver mitochondria had undergone large amplitude swelling resulting in further opening of the pore by exogeneous calcium and this was irreversible upon the addition of spermine. This reveals that the intactness of the mitochondrial membrane had been lost *ab nitio* and therefore, membrane permeability had been induced, indicating a pathological condition had ensued.

Although, the methanol leaf extract of *V. africana* reversed calcium-induced MMPT pore opening in isolated mitochondria, the extract at varying concentration induced MMPT pore opening in a concentration – dependent manner in the absence of exogeneous calcium ion. These findings suggest that the methanol leaf extract of *V. africana* may possess phytochemical that may be chelating calcium ion such that calcium ion was not available to bind the proteins that make up the pore and thus could not induce pore opening. Cherrak and colleagues [38] reported that flavonoids have $Fe^{2+}$-chelating properties, thereby serving as antioxidant against ROS produced from $Fe^{2+}$. Also, the extract may contain bioactive compounds which may modulate opening of the mitochondrial membranes under varying conditions. Essentially, the phytochemicals in the extract may be interacting with the proteins that make up the pore under pathological conditions to ensure that the membranes are well protected as compared to a non-pathological scenario. The MMPT pore allows particles which are less than 1500 Da into the mitochondrial matrix. The pore formed between the inner and outer mitochondrial membranes is believed to be formed from the aggregation of proteins such as adenine nucleotide translocase, voltage dependent anion, cyclophilin D, hexokinase and host of other proteins.[12]

Whereas the extent of reversal of calcium-induced MMPT pore opening by varying concentration of *V. africana* leaves was more significant when rat liver mitochondria were pre-incubated with the extract, the percentage inhibition of the MMPT pore opening was lower when mitochondria were not pre-incubated with the extract. Furthermore, it was observed that there was also no significant ($p < 0.05$) opening of the MMPT pore when mitochondria were pre-incubated with the varying concentration of the abstract in the absence of calcium ion. This suggests that pre-incubation of the mitochondria with the methanol extract of *V. africana* leaves may allow certain potent phytochemicals in the extract to have more interaction with the components of the pore, thus, causing a more significant inhibition against calcium ion – a standard inducer of the MMPT pore.

Furthermore, in the absence of exogeneous calcium, diabetic rat liver mitochondria were not intact. Pre-incubating diabetic rat liver mitochondria with varying concentration of the methanol leaf extract of V. africana opened the MMPT pore further when compared with the result obtained earlier when normal rat liver mitochondria were pre-incubated with the extract. It was also observed that the inhibition of the MMPT pore by the MEVA was more significant when compared to spermine the standard MMPT pore inhibitor in Figure 8. This result suggests that the phytochemicals present in the extract are more potent in reversing MMPT pore opening under pathological conditions when compared with spermine. Report from the laboratory of De Marchi[36] identified polyphenols such as quercetin as potent modulators of the MMPT pore. Metformin is also a potent inhibitor of the MMPT pore, its mode of action might be through the inhibition of mitochondrial complex I,[39-41] therefore from our study, we suggest that some of the phytochemicals present in the methanol leaf extract of V. africana may possess similar structure and activity to metformin which may be inhibiting mitochondrial complex 1 proteins and ATP hydrolysis.

It is interesting to note that the extract inhibited pore opening in diabetic rat liver mitochondria in the presence of calcium. Similarly, when diabetic rat liver mitochondria were pre-incubated with varying concentration of MEVA, the extent of the inhibition of the MMPT pore opening was more significant than in the absence of pre-incubation, suggesting pre-incubation may allow more interaction between the bioactive constituents of MEVA and the components of the MMPT pore thus preventing leaky mitochondrial membranes. Metformin and some natural mimics of metformin such as ginsenoside Rd have demonstrated potential to inhibit mitochondrial dysfunction in different pathological scenerios.[21,41]

## CONCLUSION

The search for novel therapies from plants against diseases associated with excessive cell death such as diabetes and neuro disorders is still on going. This study demonstrated that the methanol extract of the leaf of *Voacanga africana* may possess active components which have the potential to stimulate MMPT pore opening under non-pathological conditions. This study has demonstrated that the extract may possess active phyto-constituents that have great potential to

reverse opening of the MMPT pore under pathological conditions such as ageing and diabetes and may find use in the management of the complications associated with diabetes. Further work to identify the nature of these compounds will be required.

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**Conflict of Interest**

The authors of this manuscript have no conflicting interest.

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