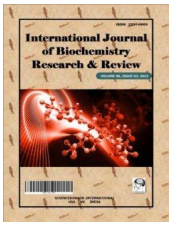

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Inhibitory Activity of Ethanol Extract of *Manihot esculenta* on Mitochondrial Membrane Permeability Transition Pore and Caspase 3 in Type 2 Diabetes Mellitus

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Authors' contributions

This work was carried out in collaboration between all authors. Authors EIOA, OOT and OOO designed the study. Authors EIOA and OOO wrote the protocol. Authors UCB and OOO supervised the work. Authors EIOA, EUM and AOA carried out all laboratory work and performed the statistical analysis. Authors EUM and AOA managed the literature searches. Author EIOA managed the analyses of the study and wrote the first draft of the manuscript. Authors OOT, UCB and OOO edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

The modulatory effect of different concentrations of ethanol extract of matured leaves of *M. esculenta*, Crantz at 200, 600, 1000 and 1400 µg/ml was investigated *in vitro* on mitochondrial membrane permeability transition pore in liver, kidney and heart of diabetic animals in the absence and presence of 20 µM exogenous Ca^{2+}. The extract at these concentrations had no significant ($p< 0.05$) effect on mitochondrial membrane permeability transition (MPT) pore of the three organs in the absence of Ca^{2+}. However, in the presence of Ca^{2+}, the extract exhibited significant ($p< 0.05$) inhibition of mitochondrial membrane transition pore opening: liver by 62.66%, 42.47%, 22.44% and 17.63% at 1400, 1000, 600, and 200 µg/ml, respectively. In the heart, inhibition of MMPT pore opening was by 92.86%, 71.43%, 64.29% and 57.14% at 200, 600, 1400, and 1000 µg/ml, respectively. In the kidney, the extract also inhibited mitochondrial membrane transition pore opening in a concentration-dependent manner by 92.65%, 91.18%, 89.71% and 72.06% at 1400, 1000, 600, and 200 µg/ml, respectively. Caspase-3 activity *in vitro* was also reduced with increasing extract concentrations; thus confirming that the extract inhibits MMPT pore opening in the presence of Ca^{2+}. The extract may be able to protect these organs against damage, resulting from Ca^{2+} overload that may trigger cell death, and as such it may be useful in the management of diseases related to tissue wastage such as cardiomyopathy and nephropathy which are associated with Type 2 diabetes mellitus.

Keywords: Type 2 diabetes; high-energy diet; low-dose streptozotocin; MMPT; Caspase-3.

1. INTRODUCTION

Multicellular organisms ensure optimum tissue homeostasis to maintain harmony among the processes of cell division and growth, differentiation, aging and death by activating a cell suicide process known as apoptosis. This program is an evolutionary conserved cell suicide program whose execution falls under the regulation of finely controlled signalling pathways through the interplay of a group of putative proteins known as the Bcl-2 family. For apoptosis to occur, these proteins modulate mitochondrial outer membrane permeabilization thus controlling the inevitable release of cytochrome c which is sequestered in the intermembrane space of mitochondria [1]. Upon receipt of an apoptotic stimulus, Bax or Bak oligomerize leading to the formation of a high-conductance, non-specific pore across the inner and outer mitochondrial membrane thus allowing the release of the key players of apoptosis initiation into the cytosol [2]. These pro-apoptotic factors in turn may activate the cysteine-dependent aspartate-directed proteases known as caspases or act in a caspase-independent fashion to bring about cell suicide [3].

Major and minor phytonutrients have found usefulness in herbal medicine. They are usually secondary plant metabolites that have the potential to mediate biological processes in such a manner that the antioxidant systems of the body can respond to their challenge to ameliorate or offset the risk of causation of chronic diseases in humans [4,5]. However, there is paucity of scientific evidences to support the hypoglycemic and antioxidative effects of leaf extracts of *Manihot esculenta* Crantz. Several food crops, vegetables, spices, marine and tropical medicinal herbs have been tested in an effort to identify new chemical entities that can serve as potent antioxidants [6]. Traditional medicines, which originate from natural products, are useful anti-diabetic drugs that improve the immunity of the body. They prove to be very effective for diabetes treatment when they are single used and they show measurable effects such as reducing blood sugar, as with western medicines. Thus they are promising for finding active compounds which can be developed into new anti-diabetic drugs [7].

Interestingly, ethno-botanical medicine often offers vast, relatively untapped sources of such drug candidates from natural products. Reactive oxygen species (ROS) formation is an unavoidable process that occurs naturally [8], and capable of leading to amplified increases in oxidative stress and enhanced ROS-mediated cell damage. ROS and free radicals have been implicated in a variety of diabetic complications such as nephropathy [9], retinopathy [10], cardiomyopathy, ischemia reperfusion injury and chronic tubulointerstitial injury [11].

Type 2 diabetes mellitus is a multifactorial disease which is characterised by

hyperglycaemia and abnormal lipoprotein levels [12]. These traits are indicated to cause cell membrane damage and high preponderance of ROS. Generation of free radicals accompanying cellular metabolism in obesity, dyslipidemia, hypertension, and insulin resistance with their underlying biochemical mechanisms, the involvement of certain environmental factors, including lifestyles are risk factors for the pathogenesis of T2DM [13]. It is estimated that > 100, 000 people die annually in India, and between 40, 000–99, 999 people die in Nigeria from diabetes-related complications. The 5th edition of the Diabetes Atlas's new figures revealed that the number of people living with diabetes in the world would likely rise from 366 million in 2011 to 552 million by 2030 [14]. India continues to be at the fore-front of diabetes incidence in the world with Gujarat state having high prevalence of the dreaded disease, while more than 5 million diabetics have been statistically identified in Africa and they are expected to increase to 15 million by 2025.

This lifestyle disease potentiates continuous and increased propagation of free radical-mediated oxidative stress thus elongating the chain of damage [15,16].

Common names for *Manihot esculenta* Crantz include cassava, yuca, tapioca, manioc, Brazilian arrowroot, kappa, gbaguda, paki, ege. The leaves serve as vegetable in many African cultures and are a good source of plant nutrients [17]. The levels of cyanogenic glycosides – linamarin (95%) and lotaustralin (5%) – in the leaves and roots of cassava make the food crop potentially toxic [18, 19]. Poorly processed cassava has been shown to cause goitre and tropical ataxic neuropathy as a result of exposure to chronic, low cyanide levels [1,20,21]. A paralytic disorder, konzo [22], as well as death [23] are popular outcomes of acute cyanide poisoning. In order to render cassava- based foods safe, it is advisable that the roots and leaves be thoroughly processed. Linamarin-free roots are now available in transgenic cassavas [24].

This study was conducted determine the *in vitro* effect of varying concentrations of the ethanol extract of *Manihot esculenta* on mitochondrial membrane permeabilization in diabetic rat kidney, heart and liver both in the absence and presence of exogenous Ca^{2+}; and the effect on Caspase 3 activation, *in vitro*.

2. MATERIALS AND METHODS

2.1 Plant Material

Dried matured *Manihot esculenta* leaves were powdered (500 g) and soaked overnight in 95 % ethanol (1: 3 w/v) in a Soxhlet extractor. The suspension filtered and residue treated same manner again for 48 hours. Continuous hot extraction was done until the final drop of the extract became colorless and the extract was then concentrated *in vacuo* at 60ºC using a rotary evaporator. The yield was 14.5 g of extract, which was kept on the bench until further use.

2.2 Experimental Animals

Twenty-four Sprague-Dawley rats (180–250 g) were used for in vivo experiment. All animals were maintained under standard conditions and control groups were rodent normal pellet diet *ad libitum*, while test groups manipulated for T2DM were fed on high fat diet. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the National Institute of Pharmaceutical Education and Research, Punjab, India. The animals were fasted for 12 hours before sacrifice by decapitation but were allowed access to water. Experiments were conducted thrice.

High fat diet – HFD (58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal) was given to animal groups 2–6 *ad libitum*, for the initial period of 2 weeks, and continuously throughout the treatment period that lasted 4 weeks (1 week during which low-dose STZ was given i.p. —35 mg/kg) [25] and 21 days of extract intubation.

2.3 Preparation of Mitochondria

2.3.1 Isolation of rat liver mitochondria

The principle is based on isolation of low ionic strength mitochondria [26,27]. Rat liver was excised and trimmed after the animal was sacrificed by cervical dislocation under the influence of diethyl ether. The liver was weighed and washed several times in isolation buffer containing 210 mM mannitol, 70 mM sucrose and 1 mM EGTA in 5 mM HEPES -HCl, pH 7. 4 until a clear washing was obtained. A 50% suspension was prepared by homogenising the liver in a glass-Teflon homogeniser. The whole

process was carried out on ice to preserve the integrity of the mitochondria. The homogenised liver was centrifuged at 2300x g for 5 minutes in a SIGMA-6K15 refrigerated centrifuge so as to sediment the nuclear fraction and cell debris 45by low speed centrifugation. The supernatant obtained was centrifuged at the same speed to remove unbroken cells. The supernatant was centrifuged at 13000x g for 10 minutes to pellet the mitochondria. The brown mitochondria pellet was washed by re-suspending in isolation buffer containing 0.5% BSA and centrifuged at 12000x g for 10 minutes. The washing was done twice. The mitochondria were then re-introduced in a buffer containing 210 mM mannitol, 70 mM sucrose, and 5 mM HEPES -HCl, pH 7.4; and dispensed in Eppendorf tubes kept on ice in aliquots.

2.3.2 Isolation of rat kidney mitochondria

Rat kidney mitochondria were isolated according to the method described by Schneider & Hogeboom [28] with slight modifications. Briefly, the kidneys were excised and placed in the ice-cold medium made up of 210 mM mannitol, 70 mM sucrose and 1 mM EGTA in 5 mM HEPES-HCl, pH 7.4. The kidneys were minced in homogenizing buffer (1:10 w/v) and subjected to 3 Dounce strokes with a glass-Teflon homogeniser. The homogenates was diluted to 10 ml per kidney and centrifuged at 900x g for 5 minutes and kept at 4ºC. The supernatant was decanted and further centrifuged at 4500x g for 5 minutes the pellet was suspended in 20 ml of the ice-cold isolation buffer containing 0.5% BSA and centrifuged at 4500x g for 10 minutes. The brown mitochondrial pellet was suspended in 1 ml of medium containing 210 mM mannitol, 70 mM sucrose, and 5 mM HEPES- HCl, pH 7.4.

2.3.3 Isolation of rat heart mitochondria

Rat heart mitochondria were isolated by mincing and homogenising it in isolation medium (210 mM mannitol, 70 mM sucrose, 1 mM EGTA in 5 mM HEPES- HCl, pH 7.4) (1: 10 w/v) using glass-Teflon homogeniser [29] and centrifuged at 700x g for 10 minutes. The resulting supernatant was centrifuged at 8000x g for 10 minutes and the ensuing mitochondria were washed in isolation buffer containing 0.5% BSA at 7000x g for 10 minutes, and the pellets were suspended in 350 µl of isolation buffer without BSA.

Mitochondrial protein estimation was done by the method of Lowry et al. [30] expressed as bovine serum albumin equivalent (BSA equiv.) and the fractions were analysed immediately or within 4 hours of isolation, while the cytosolic fraction was kept frozen at -80ºC until further use.

2.4 Treatment of Mitochondria

Mitochondria that have accumulated calcium can be induced to undergo permeabilization by which pores non-selectively open on the inner membrane to small (1500 Daltons) solutes [31]. Isolated mitochondria undergoing permeability transition (MPT) show colloidosmotic i.e. large amplitude swelling which results in a decreased photometric absorption at 540 nm. Several experimental parameters have been used to study the MPT in isolated mitochondria but in this study, the permeability transition was assessed by measuring the swelling of mitochondria by monitoring the associated decrease in light scattering. The principle behind this method is that, when mitochondria swell, their refractive index changes and thus less light is passed across the cuvette which results in a decrease in the light absorbance measured at the isobestic point from the cytochromes (520 nm or 540 nm). 1 mg/ml organ (liver, kidney and heart) mitochondria were incubated in the assay medium (210 mM mannitol, 70 mM sucrose in 5 mM 2HEPES- HCl, pH 7.4) at 30ºC in the presence of 0.8 µM rotenone [in order to inhibit Complex I (NADH dehydrogenase) thus to experimentally eliminate confounding factors of convergence of electrons in the electron transport system as the system is fed only through Complex II (Succinate dehydrogenas)] [32]. Various concentrations of the extract (200, 600, 1000 and 1400 µg/ml) were added to the mitochondria 3 minutes before 20 µM $CaCl_2$ was added and 30 seconds afterwards, which the assay was energised by 25 mM Sodium Succinate. Mitochondria swelling was measured by continuous time scan at 540 nm over 12 minutes with Hitachi U–3010 Spectrophotometer.

2.5 Statistical Analysis

This was done with the aid of SigmaStat 3.5 using Analysis of Variance (ANOVA). Data were reported as Mean ± Standard Deviation.

3. RESULTS

Exogenous calcium induced the opening of MPT in the kidney of the diabetic control whereas in the absence of Ca^{2+}, there was no significant

swelling. There was significant inhibition of MPT in the absence of exogenous calcium in kidney mitochondria of diabetic rat treated with varying concentration of ethanol extract of *Manihot esculenta*; with 200 µg/ml showing the least inhibition effect (Fig. 1). The varying concentrations of ethanol extract of matured leaves of *M. esculenta* significantly inhibited the kidney MPT of the diabetic control in the presence of calcium. The inhibition was found to be concentration dependent with the high concentrations (1000 and 1400 µg/ml) giving the greatest inhibition (Fig. 2). In the heart, there was a significant decrease in the absorbance at 540 nm in the presence of exogenous calcium ($p<0.05$), although a low amplitude swelling of the mitochondria with mean difference (-0.0136±0.009) compared with intact mitochondria (-0.033±0.017) i.e. in the absence of exogenous calcium. The varying concentrations of the extract inhibited mitochondria permeabilization in the heart (Figs. 3 and 4). The results obtained from the assessment of permeability transition in diabetic rat liver mitochondria showed a significant decrease in the absorbance at 540 nm in the presence of exogenous calcium ($p<0.05$) i.e. large amplitude swelling of the mitochondria with mean difference (-0.307±0.126) compared with intact mitochondria (-0.024±0.007). The varying concentrations of the extract of significantly inhibited the MPT in the liver of diabetic rats in the absence of calcium. The inhibition was found to be concentration-dependent (1400 µg/ml< 200 µg/ml < 600 µg/ml< 1000 µg/ml), while 1400 µg/ml gave the least inhibition (Fig. 5). When 200, 600 and 1000 µg/ml extract were incubated with exogenous calcium in the diabetic group, they had high induction effects in a time-dependent manner, while 1400 µg/ml had inhibitory effect compared to spermine in a concentration-dependent manner (Fig. 6). Table 1 summarises the effect of the extract on Caspase 3 activity, *in vitro*. Increases in the activities of caspase 3 were observed in organs of diabetic rats. Howbeit, the extract significantly reduced caspase 3 activities in a concentration-dependent manner, with the least activity observed at 1400 µg/ml. Across the selected organs, 1400 µg/ml was found to be most potent in the heart, followed by the kidney and least potent in the liver.

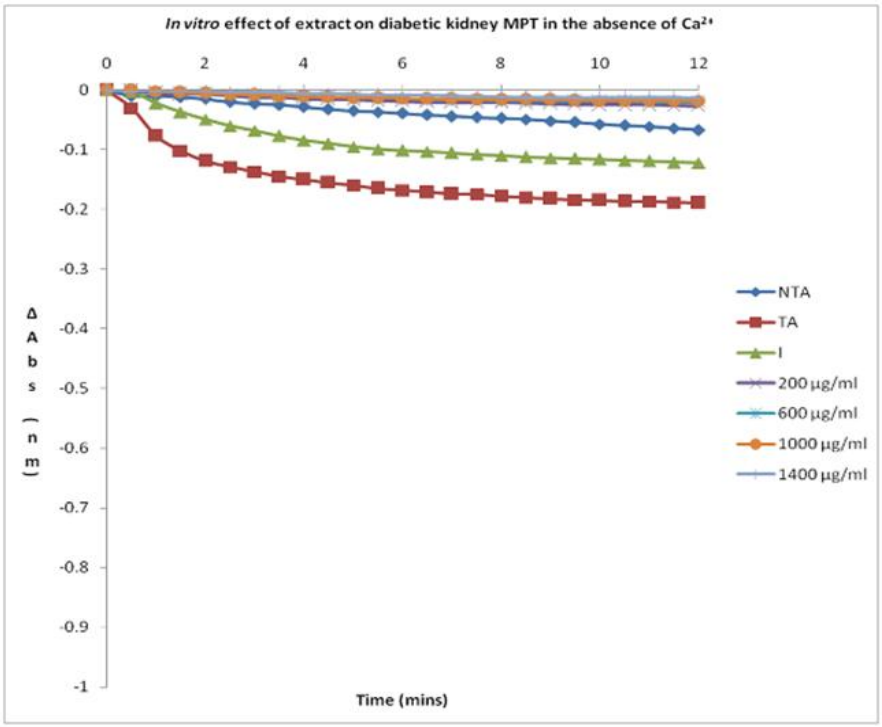

Fig. 1. *In vitro* effect of extract on diabetic kidney MPT in the absence of calcium

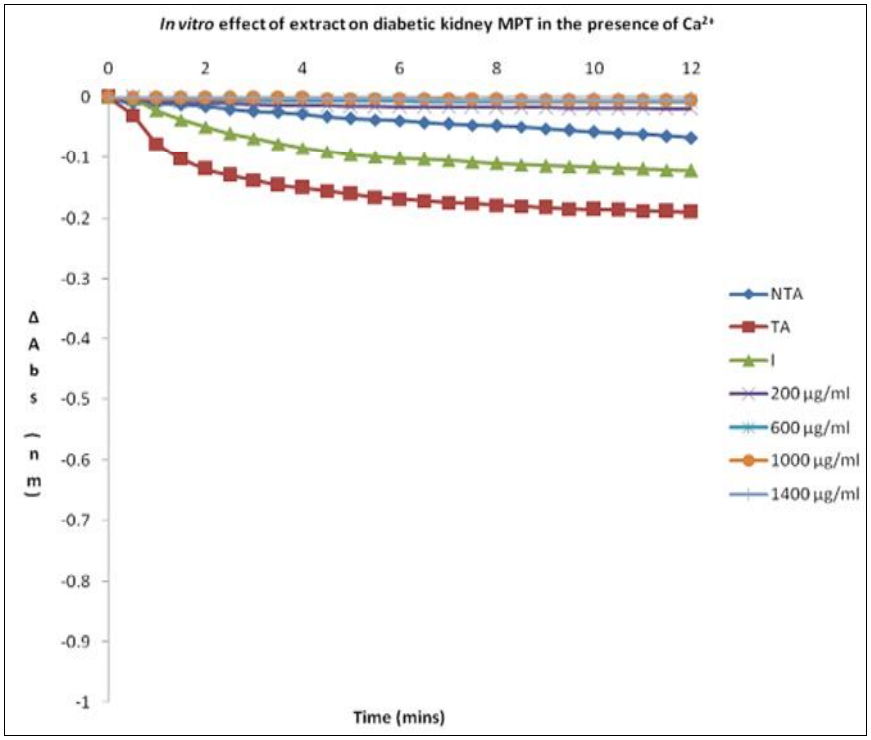

Fig. 2. *In vitro* effect of extract on diabetic kidney MPT in the presence of calcium

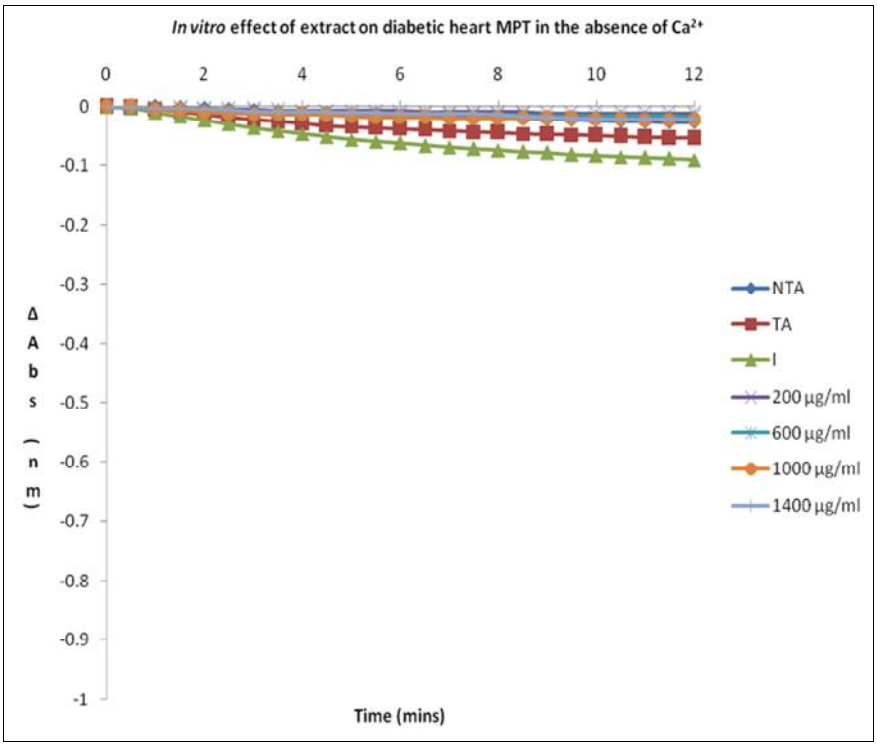

Fig. 3. *In vitro* effect of extract on diabetic heart MPT in the absence of calcium

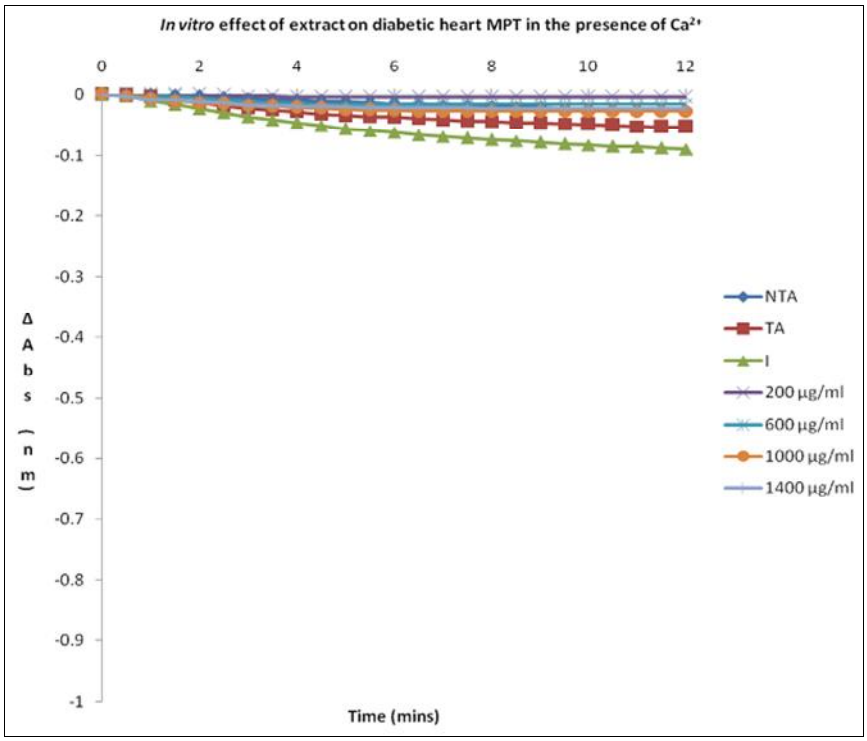

Fig. 4. *In vitro* effect of extract on diabetic heart MPT in the presence of calcium

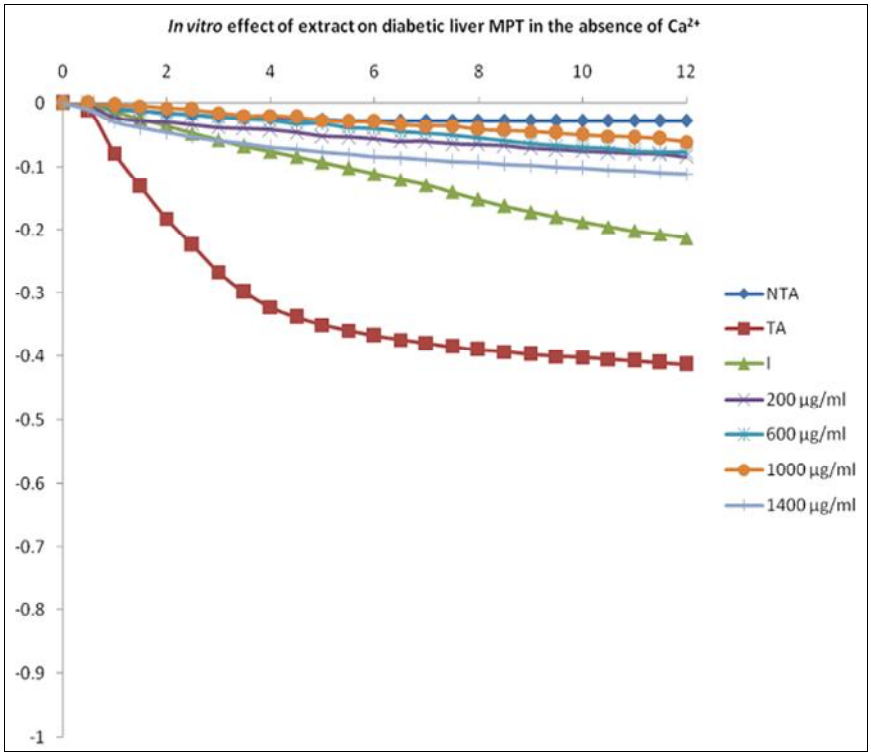

Fig. 5. *In vitro* effect of extract on diabetic liver MPT in the absence of calcium

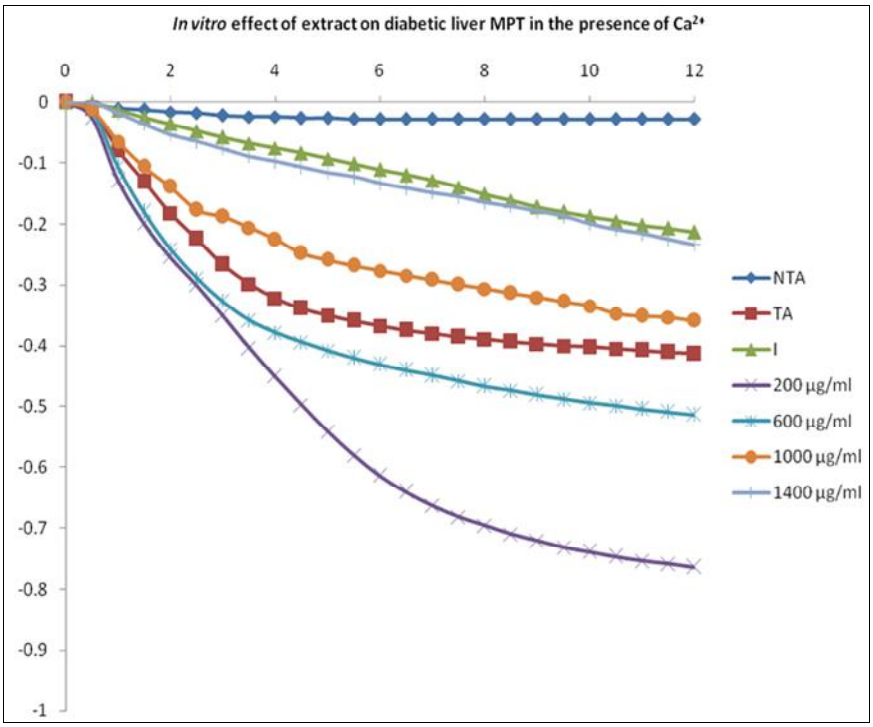

Fig. 6. *In vitro* effect of extract on diabetic liver MPT in the presence of calcium

Table 1. Effect of extract on caspase 3 activity, *in vitro* (U/ mg protein)

Extract (µg/ml)	0	200	600	1000	1400
Kidney normal	2.24±0.22	1.11±0.34*[c]	0.89±0.02* [c]	0.67±0.01* [c]	0.67±0.02* [c]
Kidney diabetic	3.10±0.74*	2.25±1.53[a]	1.55±1.06[a]	0.97±0.76* [a]	0.71±0.14* [a]
Heart normal	1.40±0.44	0.83±0.26*	0.54±0.23*	0.43±0.08*	0.54±0.37*
Heart diabetic	2.19±0.20*	1.43±0.84[a]	0.83±0.47*[a b]	0.83±0.37*[a b]	0.58±0.16*[a b]
Liver normal	2.80±0.31*	2.61±0.84*	2.49±0.42*	2.22±0.24*	1.73±0.25*
Liver diabetic	4.40±2.17	3.79±1.11	2.89±1.93*	2.10±0.46*	2.02±1.02*

4. DISCUSSION

Both in the presence of calcium, the extract significantly inhibited mitochondrial membrane permeability transition pore opening (MPT) in the selected organs of diabetic rats as compared to results obtained in the absence of Ca^{2+}. This indicates that the extract was able to inhibit large amplitude swelling caused by exogenous calcium alone. It should be pointed out that spermine is not a good candidate inhibitor of kidney mitochondrial MPT due to inconsistencies in inhibitory effect. Cyclosporine A is considered a better candidate for kidney mitochondrial MPT [33]. The extract reduced caspase-3 activity with increasing extract concentrations, *in vitro*. Increases in the activities of caspase 3 were observed in organs of untreated diabetic rats compared to those pre-incubated with various concentrations of the extract. Howbeit, the extract significantly reduced caspase 3 activities in a concentration-dependent manner, with the least activity observed at 1400 µg/ml. Across the selected organs, 1400 µg/ml was found to be most potent in the heart, followed by the kidney and least potent in the liver.

5. CONCLUSION

It is clear that apoptosis occurs under both physiological and pathological conditions, such as diabetes [34]. Elevated caspase activity in the kidney, heart and liver of intact mitochondria of diabetic rats was thus predictable. The extract, with increasing concentration showed potency in attenuating the processes leading to the activation of caspase 3 in the kidney and heart. The extent to which the extract inhibited caspase

3 activation in the liver was not as high as observed in the kidney and heart. The results confirmed that the extract inhibits MMPT pore opening in the presence of Ca^{2+}. The inhibition of MPT by the extract invariably led to significant decreases in the activity of caspase 3, which is a downstream factor necessary for apoptotic cell death to occur. Thus, the ethanol extract of *Manihot esculenta* Crantz may be able to protect these organs against damage, resulting from Ca^{2+} overload that may trigger cell death, and as such it may be useful in the management of diseases related to tissue wastage such as cardiomyopathy and nephropathy which are associated with Type 2 diabetes mellitus.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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