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Original Article

In vitro induction of rat liver mitochondrial membrane permeability transition pore opening by solvent extracts of *Momordica charantia* leaves

*[1]Adeola Folasade Ehigie, [1]Leonard Ona Ehigie, [2]Samuel Akintunde Odediran, [1]Olusegun Kayode Afolabi, [1]Olaniyi Temitope Adedosu and [3]Olufunso Olabode Olorunsogo

[1]Department of Biochemistry, Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria.

[2]Department of Pharmacognosy, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.

[3]Department of Biochemistry, University of Ibadan, Oyo State, Nigeria.

*Corresponding Author: Adeola F. Ehigie. E-mail: afehigie@lautech.edu.ng; +234 0803 437 9631

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ABSTRACT: Alteration of mitochondrial functions such as permeability transition (PT), a process associated with the uncoupling of oxidative phosphorylation, has been found to play a vital role in the apoptotic process induced by certain anti-cancer agents. When triggered, PT facilitates the release of mitochondrial apoptogenic proteins which in turn activate the caspase cascade of apoptosis. Thus, this study investigated the *in vitro* effects of varying concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) of different leaf extracts [Crude Water-Soluble Extract (CWSE), Decoction (DE) and Methanol Extract (ME)] of *Momordica charantia (M. charantia),* a purported anti-cancer plant of the family Cucurbitaceae on normal rat liver mitochondria. Opening of mitochondrial membrane permeability transition pore (MMPTP) was spectrophotometrically assayed under succinate-energized condition. Results obtained showed concentration-dependent and significant ($P<0.05$) increases in the extents to which MMPTP opening was induced by the three extract types when compared with the control group. Inductions caused by CWSE and DE increased with increasing concentrations while those caused by ME decreased with increasing concentrations, giving the maximum induction at 1.0 mg/ml (8.1-fold increase) of CWSE and the least induction at 1.0 mg/ml (4.3-fold increase) of ME, respectively. Spermine, a reference inhibitor of MMPTP opening, reversed all observed openings. These results indicate that the tested leaf extracts of *M. charantia* are potent (CWSE being the most potent) MMPTP opening inducers and the pathway by which *M. charantia* causes apoptosis in cancer cells is probably mitochondrial-mediated (intrinsic).

KEYWORDS: Mitochondrial membrane permeability transition pore (MMPTP), *Momordica charantia,* Apoptosis, Spermine.

INTRODUCTION

Mitochondria are fascinating organelles that fulfill multiple functions as diverse as energy production, fatty acid ß-oxidation, reactive oxygen species (ROS) production and detoxification, and cell death regulation. The co-ordination of these functions relies on autonomous mitochondrial processes as well as sustained cross-talk with other organelles and/or the cytosol. Thus, a tight regulation of mitochondrial functions ensures cell homeostasis (Martel *et al.*, 2012). Permeability transition (PT), a phenomenon caused by the opening of a voltage-dependent, high-conductance channel (known as the permeability transition pore located in the inner mitochondrial membrane), is a sudden increase of inner mitochondrial permeability to solutes with molecular mass up to 1.5 kDa. PT is implicated

in apoptosis or necrosis as an important event in the control of cell death or survival (Zoratti and Szabo, 1995; Ling *et al*., 2010). Opening of the mitochondrial membrane permeability transition pore (MMPTP) causes the cell to show the characteristics of apoptosis by promoting the release of apoptotic proteins (Ling *et al*., 2010). Apoptosis is a regulated form of cellular suicide that is tightly controlled by a genetic program (Yaun and Horvtiz, 2004) and it can be induced by a variety of stimuli, including ligation of cell surface receptors, serum deprivation and chemotherapeutic agents, among others (Wyllie *et al*., 1984). Apoptosis dysregulation contributes to half of all human diseases. Excessive apoptosis occurs in neurodegenerative disorders such as Alzheimers, Parkinsons, autoimmune disorders, heart disease and infectious diseases, including AIDS (Singh and Anand, 1994) while failure to initiate apoptosis can induce many diseased states, the most well documented being arthritis and cancer; in which the normal constraints on the life span of a differentiated cell are not present, so the cell is not eliminated (Kerr *et al*., 1972). With the foregoing, apoptosis has been tipped as one of the potent defenses against cancers, since the process eliminates potentially deleterious, mutated cells (Reed, 1999). Apoptosis occurs primarily via two well-recognized pathways in cells (Lockshin and Zakeri, 2004; Danial and Korsmeyer, 2004); the extrinsic, or death receptor-mediated and the intrinsic, or mitochondrial-mediated, pathways (Reed, 2004). The intrinsic apoptotic pathway relies primarily on the permeabilization of mitochondrial membranes, with associated release of apoptogenic mitochondrial proteins, leading to activation of caspase-9 and downstream cleavage of caspases 3, 6, or 7 (Guimaraes and Linden, 2004) and is characterized by alteration in mitochondrial polarization and release of such mitochondrial proteins as cytochrome c, which in turn can trigger caspase activation and ultimately execution of apoptosis (Sun *et al*., 2004). *M. charantia,* Linn. (Family: *Cucurbitaceae*), commonly called bitter melon, is an economically important medicinal plant (Paul and Raychaudhuri, 2010) commonly cultivated in tropical regions of India, China, East Africa and Central and South America. It is herbaceous, climbing or having prostate vines with simple or forked tendrils (Garau *et al*., 2003). The parts of the plant used medicinally include the fruit, leaves, seeds, whole plant and seed oil (Anon, 1999). The whole plant is a known anti-diabetic agent, and is used also for expelling intestinal gas, for treating tumors, wounds, rheumatism, malaria and vaginal discharge, and as an abortifacient (Taylor, 2005; Sofowora, 2006). The fruits and seeds of bitter melon have been reported to possess medicinal properties such as anti–HIV, anti–cancer, anti–inflammatory, anti–leukemic, anti–microbial, anti–tumor and anti–diabetic (Taylor, 2002). The popularity of *M. charantia* in traditional medicine suggests that it contains bioactive agents that will be potentially useful in drug development and such phytochemicals as alkaloids, tannins, flavonoids, saponins and glycosides have been found in its leaf extracts (Bakare *et al.,* 2010). Quite a number of reports (Singh *et al*., 1989; Srivastava *et al.,* 1993; Ng *et al*., 1994; Platel and Srinivasan, 1997; Naseem *et al*., 1998), have shown the efficacy of various parts of the plant (seed, fruit and even the whole plant) extracted in different media, such as benzene, alcohol, petroleum ether, acetone, water and as crude extracts and juices in the treatment of many ailments. Most importantly, some of these studies have shown its efficacy in various cancers including breast cancer, skin tumor, prostatic cancer and human bladder carcinomas (Grover and Yadav 2004). In fact, the efficacy of *M. charantia* against some of the aforementioned cancers has been linked to its ability to induce apoptosis (Kobori *et al*., 2008; Fang *et al*., 2012) in tandem, with the observation that many dietary bioactive agents have been found to induce apoptosis through the intrinsic mitochondrial-mediated pathway (Chen and King, 2005). However, there is paucity of information on the role played by *M. charantia* in the induction of apoptosis via the intrinsic pathway and, by extension, induction of the MMPTP opening. Thus, this study seeks to ascertain if *M. charantia* is a potent inducer of the MMPTP opening, a vital prerequisite for the intrinsic apoptotic pathway.

MATERIALS AND METHODS

Plant

The leaves of *M. charantia* 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. Repeat purchases of *M. charantia* leaves were from the same source, and the plant taxonomist authenticated each sample collected.

Extraction of the crude water-soluble extract (CWSE) of M. charantia Leaves

Fresh leaves of *M. charantia* were washed with distilled water to remove dust and then cut into small pieces. 200 g of leaves was liquidized in 100 ml distilled water for 5–10 min using a blender. The resulting homogenate was centrifuged at 4000 rpm for 30 min and the suspension was filtered by passage through a glass funnel containing sterile cotton wool. The filtrate obtained was lyophilized and stored at 4 °C until further use. The yield was about 1.9% with reference to the fresh leaves.

Extraction of the decoction (DE) of M. charantia leaves

Fresh leaves of *M. charantia* were made into a decoction based on a modification of the method of Cunnick and Takemoto (1993). In brief, fresh leaves were thoroughly washed with distilled water, drained and chopped. 200 g of

the chopped leaves were then boiled in 400 ml of distilled water until the volume became half of the original volume (200 ml). The decoction was then filtered by passage through a separating funnel containing sterile cotton wool. The filtrate obtained was lyophilized and stored at 4 °C until further use. The yield was about 1.7% of the raw material.

Preparation of the Methanol extract (ME) of M. charantia leaves

Leaf extracts were prepared by the method of Alade and Irobi (1993) with minor modifications. Briefly, 200 g of shade-dried leaves of *M. charantia* was ground into powder and soaked in 2 litres 95% methanol for 72 hours with intermittent shaking. At the end of the extraction, the extract was filtered using a separating funnel containing sterile cotton wool and concentrated *in vacuo* at 40°C. The dried residue of the crude extract was stored at 4°C until further use. The yield was about 7.5% of the raw material. In all cases, a portion of the dried extract was weighed and dissolved in distilled water to make appropriate concentrations for the study.

Experimental animals

Twenty five Wistar strain albino male rats (4 months old; approximately 220 g) were obtained and kept at the Faculty of Basic Medical Sciences' Animal House, LAUTECH, Ogbomoso, Nigeria, under light-controlled conditions (12h–light/12h–dark cycle) and in well-ventilated plastic cages. The animals received feeds (Ladokun Pelleted 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 Faculty of Basic Medical Sciences' Animal Care and Use Committee.

Chemicals and reagents

Sodium Carbonate (Na_2CO_3), Sodium Hydroxide (NaOH), Sodium-Potassium Tartarate (Na-K-C_4O_6), Hydrated Copper Sulphate ($CuSO_4.5H_2O$), Calcium Chloride ($CaCl_2$), Potassium Hydroxide (KOH), Ethanol and 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.

Mitochondrial isolation

After overnight fasting, the animals were sacrificed by cervical dislocation and livers were isolated essentially according to the method of Olorunsogo *et al.*, (1984) and as reported by Lapidus and Sokolove (1993). Livers were rapidly excised, trimmed to remove excess tissues and washed in a buffer containing 210 mM Mannitol, 70 mM Sucrose, 5 mM HEPES, 1 M KOH, and 1 mM EGTA, pH 7.4. Thereafter the livers were weighed, chopped and suspended in the same buffer to make a 10% homogenate. The suspension was immediately homogenized on ice using a Porter glass homogenizer. The homogenate was centrifuged in an SM-18B High Speed Refrigerated Centrifuge twice at 2500 rpm for 5 min to remove the nuclear fraction and cellular debris. Supernatants obtained were centrifuged at 13000 rpm for 10 min and the mitochondrial fractions obtained were washed three times at 12000 rpm for 10 min with a washing buffer which contained 210 mM Mannitol, 70mM Sucrose, 5mM HEPES-KOH and 0.5% BSA, pH 7.4. The mitochondrial pellets were suspended in swelling buffer (210 mM Mannitol, 70 mM Sucrose, and 5 mM HEPES-KOH, pH 7.4) and immediately dispensed in 1 ml Eppendorf tubes. Isolated mitochondria were used within 3 hours of isolation.

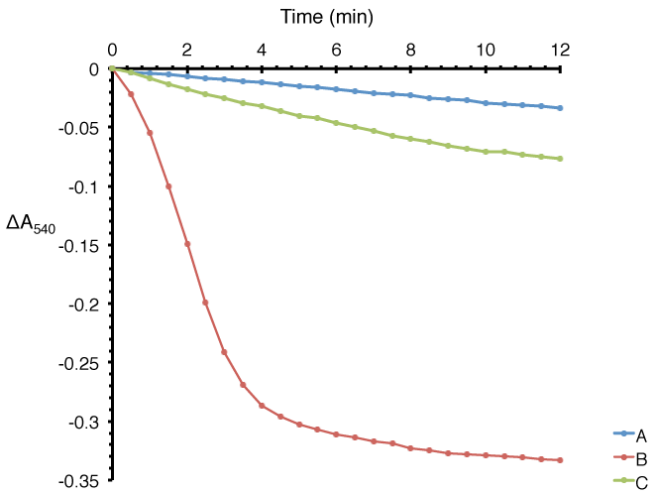

Figure 1: *In vitro* Ca^{2+} - induced opening of rat liver MMPTP and inhibition of the opening by spermine. A: No Triggering (Control); **B**: Triggering Agent (300 μM $CaCl_2$); **C**: Spermine inhibition (300 μM $CaCl_2$ + 4 mM Spermine). Rat liver mitochondria (0.4 mg/ml (protein/ml)) were incubated in a l-cm cuvette in the presence of 80 μM rotenone in swelling buffer (210 mM Mannitol, 70 mM Sucrose, 5 mM HEPES-KOH pH 7.4) for about 3 min at 30 °C prior to the addition of 300 μM $CaCl_2$ (triggering agent). Thirty seconds later, 50 μM of 5 mM sodium succinate was added and mitochondrial permeability was quantified as changes in absorbance at 540 nm. Spermine (4 mM) was added immediately after the addition of rotenone and just before the addition of mitochondria for the determination of spermine inhibition while the addition of 300 μM $CaCl_2$ was omitted in assays without triggering agent. Trace **A** shows a population of intact mitochondria respiring on succinate (not appreciable mitochondrial swelling as shown by little or no change in absorption of light at 540 nm), Trace **B** shows mitochondrial swelling caused by 300 μM $CaCl_2$ and Trace **C** shows spermine inhibition of calcium-induced mitochondrial swelling (The Ca^{2+}–induced changes in absorption of light measured at 540 nm were reversed). The results shown are representative of five such experiments.

Assessment of mitochondrial membrane permeability transition

Permeability transition is detected in isolated mitochondria by the change in the diffraction/absorption of light (measured at 540 nm) that results from matrix swelling (Zoratti and Szabo, 1995). Thus, changes in the volume of isolated liver mitochondria were measured quantitatively at 540 nm in an SM32A spectrophotometer based on the procedure of

Lapidus and Sokolove (1993). Mitochondria (0.4 mg of protein/ml) were pre-incubated in a 1 cm light path glass cuvette in the presence of 80 μM rotenone in swelling buffer containing 210 mM Mannitol, 70 mM Sucrose, 5 mM HEPES-KOH, pH 7.4 for about 3 min at 30°C prior to the addition of 300 μM $CaCl_2$ (triggering agent). Thirty seconds later, 50 μM of 5 mM sodium succinate was added and mitochondrial permeability was quantified as changes in absorbance at 540 nm. 4 mM spermine was added immediately after the addition of rotenone and just before the addition of mitochondria for the determination of spermine inhibition while the addition of 300 μM $CaCl_2$ was omitted in assays without triggering agent. For determination of the extents of opening of mitochondrial membrane permeability transition pore induced by the leaf extracts of *M. charantia* (CWSE, DE and ME), varying concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) of each extract were immediately introduced after the addition of rotenone and just before the addition of mitochondria. Absorbance readings were monitored continuously for 12 minutes (Lapidus and Sokolove, 1993). The 12-minute cut-off was found to be optimal in obtaining a wide range of activities of the triggering agent and extracts on MMPTP opening. The curves tend to level off between 8 and 12 min (e.g., Figure 1). Readings up to the 12th minute gave sufficient absorbance reading without loss of integrity of the mitochondrial samples. Protein concentration was estimated according to the method of Lowry *et al.,* (1951) using bovine serum albumin (BSA) as the standard.

Statistical Analysis

The experiments were repeated 5 times. Since mitochondrial assays allow for multiple testing on a particular isolate, each mitochondrial isolate was used for testing the effect of as many as nine parameters all at a particular concentration (e.g., 0.2 mg/ml) for each experimental run. For each concentration tested, five animals were used. Hence, all parameters were tested on mitochondrial isolates from five separate animals. In all, 25 animals were used for the five different concentrations while the confounding factors of using different animals for different extracts were statistically accounted for. Results were analyzed using student's t test and one-way ANOVA followed by Dunnett's post-hoc test for comparison between control and test groups. All data were expressed as mean ± standard error of the mean. '*p*' values < 0.05 were considered significant. GraphPad Prism 5 and Microsoft Excel 2003 were used for the analyses.

RESULTS

Figure I shows that there were no significant changes in the volumes of intact mitochondria respiring on succinate in the absence of calcium, while the ion induced the opening of mitochondrial membrane permeability transition pore up to about 14.7–fold in the presence of substrate ($CaCl_2$) and rotenone. In Figure 2, spermine, a standard inhibitor of calcium-induced mitochondrial membrane permeability transition pore opening prevented the opening of the pore by about 83%. Figure 3 shows the i*n vitro* induction of rat liver MMPTP opening by varying concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) of the ME of *M. charantia* leaves. The ME induced MMPTP opening in a concentration–dependent manner, albeit, induction decreased with increasing concentrations of extract. Increases in MMPTP's extents of opening varied between 4.3–fold and 5.5–fold when compared with untreated mitochondria (without triggering agent). Maximal induction (5.5–fold) was seen at the least concentration (0.2 mg/ml) and the least induction (4.3–fold) seen at the highest concentration of ME (1.0 mg/ml). The induction seen at 0.4 mg/ml, 0.6 mg/ml and 0.8 mg/ml were 4.8–fold, 4.8–fold and 4.4–fold respectively. Accordingly, swelling was significantly reversed in the presence of 4mM spermine, the reference inhibitor.

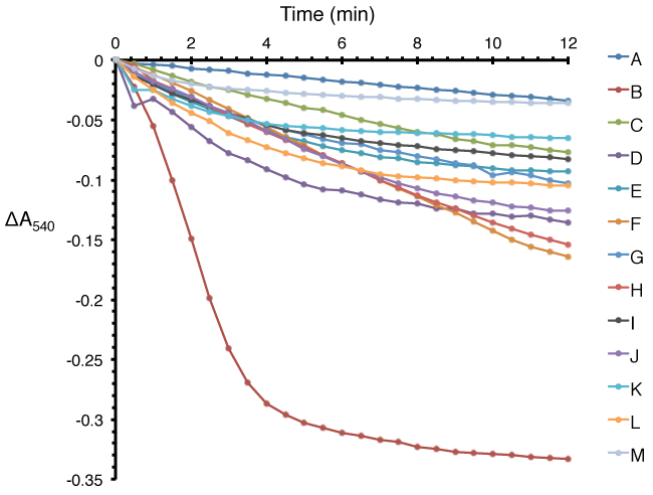

Figure 2: *In vitro* induction of rat liver MMPTP opening by varying concentrations of methanol extract (ME) of *M. Charantia.* **A**: No Triggering (Control); **B**: Triggering Agent (300 μM $CaCl_2$); **C**: Spermine inhibition (300 μM $CaCl_2$ + 4 mM Spermine); **D**: 0.2 mg/ml ME; **E**: 0.2 mg/ml ME + 4 mM Spermine; **F**: 0.4 mg/ml ME; **G**: 0.4 mg/ml ME + 4 mM Spermine; **H**: 0.6 mg/ml ME; **I**: 0.6 mg/ml ME + 4 mM Spermine; **J**: 0.8 mg/ml ME; **K**: 0.8 mg/ml ME + 4 mM Spermine; **L**: 1.0 mg/ml ME; **M**: 1.0 mg/ml ME + 4 mM Spermine. Conditions were same as in Figure 1, except that varying concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) of methanol extract (ME) were immediately introduced after the addition of rotenone in place of spermine, the reference inhibitor and just before the addition of mitochondria for the determination of the effects of the methanol extracts on MMPTP opening. In experiments on spermine inhibiton of extract-induced MMPTP opening, samples were treated with both spermine and the appropriate concentration of DE. Traces **D**, **F**, **H**, **J**, and **L** represent the extent of swelling caused by the varying concentrations of ME (changes in absorbance becomes more negative with decreasing concentrations). The proportional reversal to more positive light absorbance in the presence of spermine (Traces **E**, **G**, **I**, **K**, and **M**) establishes the involvement of MMPTP opening in the observed mitochondrial swelling. The results shown are representative of five such experiments. The data set in **A**, **B**, and **C** are same as shown in Figure 1.

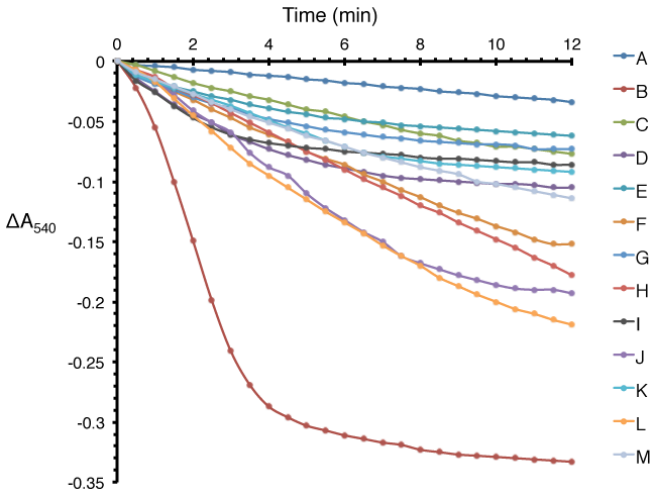

Figure 3: *In vitro* induction of rat liver MMPTP opening by varying concentrations of leaf decoction (DE) of *M. Charantia*. **A**: No Triggering (Control); **B**: Triggering Agent (300 μM $CaCl_2$); **C**: Spermine inhibition (300 μM $CaCl_2$ + 4 mM Spermine); **D**: 0.2 mg/ml DE; **E**: 0.2 mg/ml DE + 4 mM Spermine; **F**: 0.4 mg/ml DE; **G**: 0.4 mg/ml DE + 4 mM Spermine; **H**: 0.6 mg/ml DE; **I**: 0.6 mg/ml DE + 4 mM Spermine; **J**: 0.8 mg/ml DE; **K**: 0.8 mg/ml DE + 4 mM Spermine; **L**: 1.0 mg/ml DE; **M**: 1.0 mg/ml DE + 4 mM Spermine. Conditions were same as in Figure 1, except that varying concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) of methanol extract (DE) were immediately introduced after the addition of rotenone in place of spermine, the reference inhibitor and just before the addition of mitochondria for the determination of the effects of the methanol extracts on MMPTP opening. In experiments on spermine inhibiton of extract-induced MMPTP opening, samples were treated with both spermine and the appropriate concentration of DE. Traces **D**, **F**, **H**, **J**, and **L** represent the extent of swelling caused by the varying concentrations of DE (changes in absorbance becomes more negative with increasing concentrations). The proportional reversal to more positive light absorbance in the presence of spermine (Traces **E**, **G**, **I**, **K**, and **M**) establishes the involvement of MMPTP opening in the observed mitochondrial swelling. The results shown are representative of five such experiments. The data set in **A**, **B**, and **C** are same as shown in Figure 1.

Figure 4 shows the *in vitro* induction of rat liver MMPTP opening by varying concentrations of leaf decoction (DE) of *M. charantia.* Varying concentrations of DE induced MMPTP opening in a concentration-dependent manner, with the induction increasing with increasing concentrations of extract. Fold increases in MMPTP's extents of opening were seen between 4.3 and 7.1 folds. The maximal induction of 7.1–fold was seen at the highest concentration (1.0 mg/ml) and the least induction (4.3–fold) seen at the least concentration of DE (0.2 mg/ml). The inductions seen at 0.4 mg/ml, 0.6 mg/ml and 0.8 mg/ml were 4.8–fold, 5.0–fold and 6.7–fold increases respectively in comparison to the control group. All increases in MMPTP opening caused by DE of *M. charantia* leaves were reversed by spermine. In Figure 5, the *in vitro* induction of rat liver MMPTP opening by varying concentrations of Crude Water-Soluble Extract (CWSE) of *M. charantia* leaves are shown. Varying concentrations of CWSE induced MMPTP in a concentration-dependent manner, with the degree of opening of the pore increasing with increasing concentrations of extract. Fold increases in MMPTP extents of opening were observed to be between 4.6 and 8.1 fold. Maximal induction (8.1–fold) was seen at the highest concentration (1.0 mg/ml) and the least induction (4.6-fold) seen at the least concentration of CWSE (0.2 mg/ml). The inductions seen at 0.4 mg/ml, 0.6 mg/ml and 0.8 mg/ml were 4.8, 5.6 and 6.9–folds respectively in comparison to the control group. All increases in MMPTP opening caused by CWSE of *M. charantia* leaves were reversed by spermine.

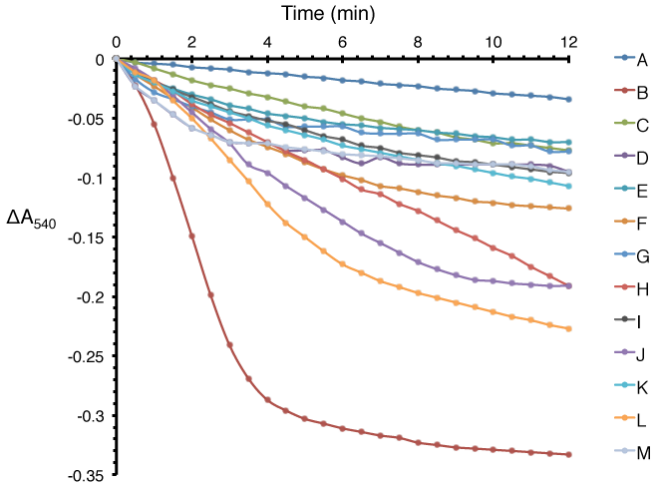

Figure 4: *In vitro* induction of rat liver MMPTP opening by varying concentrations of Crude Water-Soluble Extract (CWSE) of *M. charantia.* **A**: No Triggering (Control); **B**: Triggering Agent (300 μM $CaCl_2$); **C**: Spermine inhibition (300 μM $CaCl_2$ + 4 mM Spermine); **D**: 0.2 mg/ml CWSE; **E**: 0.2 mg/ml CWSE + 4 mM Spermine; **F**: 0.4 mg/ml CWSE; **G**: 0.4 mg/ml CWSE + 4 mM Spermine; **H**: 0.6 mg/ml CWSE; **I**: 0.6 mg/ml CWSE + 4 mM Spermine; **J**: 0.8 mg/ml CWSE; **K**: 0.8 mg/ml CWSE + 4 mM Spermine; **L**: 1.0 mg/ml CWSE; **M**: 1.0 mg/ml CWSE + 4 mM Spermine. Conditions were same as in Figure 1, except that varying concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) of Crude Water-Soluble Extract (CWSE) were immediately introduced after the addition of rotenone in place of spermine, the reference inhibitor and just before the addition of mitochondria for the determination of the effects of the methanol extracts on MMPTP opening. In experiments on spermine inhibiton of extract-induced MMPTP opening, samples were treated with both spermine and the appropriate concentration of DE. Traces **D**, **F**, **H**, **J**, and **L** represent the extent of swelling caused by the varying concentrations of CWSE (changes in absorbance becomes more negative with increasing concentrations). The proportional reversal to more positive light absorbance in the presence of spermine (Traces **E**, **G**, **I**, **K**, and **M**) establishes the involvement of MMPTP opening in the observed mitochondrial swelling. The results shown are representative of five such experiments. The data set in **A**, **B**, and **C** are same as shown in Figure 1.

The effects of the three extract types (ME, DE and CWSE) of *M. charantia* leaves were compared with the control group in Figure 6; there were dose-dependent increases in the extent of *in vitro* rat liver MMPTP induction by the tested leaf extracts of *M. charantia* compared with the control group

(normal control rats). Table 1 summarizes the effects of the three extract types (CWSE, DE and ME) on MPTP opening. The highest induction; that caused by CWSE, being as much as about 55% of that caused by Ca^{2+}, a standard inducer/trigger of the pore opening (Bernandi *et al.*, 1998; Halestrap *et al* ., 1998). Our results thus reveal that the three extract types are potent inducers of the mitochondrial membrane permeability transition pore opening, albeit to varying extents.

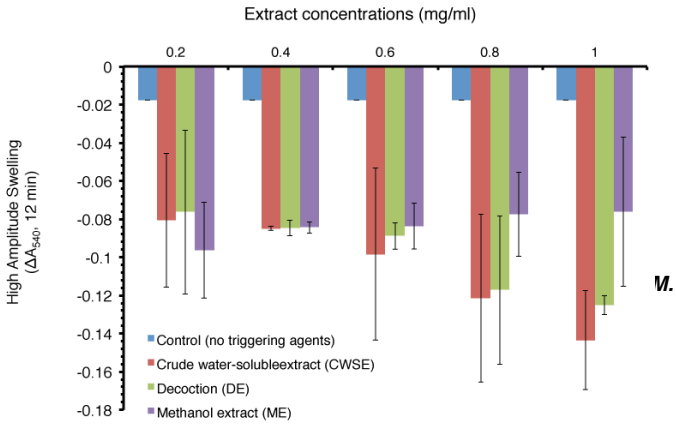

Figure 5: Comparison of the extents of induction of rat liver mitochondrial membrane permeability transition pore opening by the extracts of *M. charantia*. Bar charts shown are means ± S.E.M of data from five separate experiments using different mitochondrial preparations.

DISCUSSION

This study shows the potency of the extracts CWSE, DE and ME of *M. charantia* leaves in the induction of MMPTP opening. Of the three extracts, the CWSE was the most potent as evidenced by its induction of MMPTP opening up to about 8.1-fold increase in comparison with the control group and the highest inductions seen with the other two groups (7.1 and 5.5–folds for DE and ME, respectively). Though extents and patterns of increase of MMPTP opening were varied, the three extracts clearly caused large amplitude swelling of the isolated mitochondria (least induction being about 4-fold increase compared with control group), an indication of their ability to alter the permeability transition of the affected mitochondria (Harworth and Hunter, 1979). MMPTP is a non-selective, high-conductance channel with multiple macromolecular components (Alano *et al.,* 2002), such as Adenine Nucleotide Translocase (ANT) and cyclophilin D (Javadov *et al.*, 2009), which form at sites where the inner and outer membranes of the mitochondrion meet (Crompton, 1999). The opening of the mitochondrial membrane permeability transition pore which could be triggered by Ca^{2+}, P_i (inorganic phosphate), oxidative stress, and inhibited by cyclosporin A, bongkrekic acid, EGTA, adenine nucleotides, and Mg^{2+} (Bernandi *et al.,* 1998; Halestrap *et al.*, 1998) is a critical stage of apoptosis because it results in swelling and ultimate rupture of the outer membrane as well as the redistribution of proapoptotic proteins such as cytochrome c and apoptosis inducing factor (AIF) from the mitochondria to the cytoplasm (Petit *et al.*, 1996; Susin *et al.*, 1996; Skulachev, 1996; Petit *et al.*, 1998). Once released, cytochrome c binds to apoptotic protease activator factor-1 (APAF-1) in the presence of ATP or dATP and forms a complex that processes and activates pro–caspase–3 and pro–caspase–7 (Kroemer, 2002), which ultimately commit the cell to self destruction (apoptosis) (Deckwerth and Johnson, 1993; Newmeyer *et al.,* 1994). Apoptosis is one of the most potent cellular defenses against cancer, because it destroys potentially deleterious and mutated cells (Reed, 1999).

Table 1: Fold increases in MMPTP opening caused by extracts of *M. charantia*.
Values are extract-induced fold increases in MMPTP opening relative to control experiments in which no triggering agents were included. *Values which significantly (P<0.05) decreased when compared with control animals. The changes in absorbance shown here (mean ± S.E.M.) are same as the 25 data points (the 25 values obtained fom time 0–12 min.) used in Figures 2–4 for the varying concentrations of each extract type. They compared the extent of mitochondrial swelling (MMPTP opening) seen at these varying concentrations. ME (Methanolic Extract), DE (Decoction) and CWSE (Crude Water-Soluble Extract).

Groups (n=25)	ΔA_{540}	Fold
No triggering agent	-0.0176 ± 0.002	1.0
Triggering agent	-0.2580 ± 0.045*	14.7
0.2 mg/ml (CWSE)	-0.0808 ± 0.035*	4.6
0.4 mg/ml (CWSE)	-0.0849 ± 0.001*	4.8
0.6 mg/ml (CWSE)	-0.0984 ± 0.045*	5.6
0.8 mg/ml (CWSE)	-0.1214 ± 0.044*	6.9
1.0 mg/ml (CWSE)	-0.1434 ± 0.026*	8.1
0.2 mg/ml (DE)	-0.0763 ± 0.043*	4.3
0.4 mg/ml (DE)	-0.0847 ± 0.004*	4.8
0.6 mg/ml (DE)	-0.0888 ± 0.007*	5.0
0.8 mg/ml (DE)	-0.1172 ± 0.039*	6.7
1.0 mg/ml (DE)	-0.1251 ± 0.005*	7.1
0.2 mg/ml (ME)	-0.0964 ± 0.025*	5.5
0.4 mg/ml (ME)	-0.0844 ± 0.003*	4.8
0.6 mg/ml (ME)	-0.0837 ± 0.012*	4.8
0.8 mg/ml (ME)	-0.0776 ± 0.022*	4.4
1.0 mg/ml (ME)	-0.0762 ± 0.039*	4.3

Two common pathways of apoptosis (the intrinsic or mitochondrial-mediated and the extrinsic or death receptor-mediated pathways) are well-recognized (Alimonti *et al.,* 2003) and the mitochondrial-mediated pathway is the pathway by which many dietary bioactive agents induce

apoptosis, having altered mitochondrial membrane function and/or dissipated the mitochondrial potential (Galati and O'Brien, 2000; Ueda *et al.,* 2001; Morin *et al.,* 2001; Kim *et al.,* 2003; Palozza *et al.,* 2003; Hantz *et al.,* 2005; Martin, 2006). The anti-cancer efficacies of *M. charantia* in various cancers have also been observed (Grover and Yadav, 2004); indeed, several groups of investigators have shown that the mechanism by which *M. charantia* causes cell death in certain cancer cell lines is through apoptosis (Kobori *et al*., 2008; Ray *et al.,* 2010; Li *et al.,* 2012; Fang *et al*., 2012). However, aside from the reports of Odewusi *et al*., (2010) on the *in vivo* induction of MMPTP opening by the leaf decoction of *M. charantia* in male albino rats, reports on the role of the plant in the induction of MMPTP opening are quite few and far between.

Owing to the critical role played by MMPTP opening in apoptotic cell deaths, a well documented means by which *M. charantia* induces cancer cell death (Kobori *et al*., 2008; Ray *et al.,* 2010; Fang *et al*., 2012; Li *et al.,* 2012) and the fact that the MMPTP itself can be considered a potential therapeutic target in cancer chemotherapeutics (Brenner and Grimm, 2006), our interest in the current study is kindled. These results, apart from showing that heating may be a factor in the extent of induction caused by the leaf extracts, also suggest that the types of solvent used in extracting the leaves of the plant equally affect extract potency (i.e., order of potency is: CWSE >DE > ME). The induction observed at 1mg/ml of CWSE translates to about 55% of the induction caused by Ca^{2+}, a known trigger of MMPTP opening (Bernandi *et al.,* 1998; Halestrap *et al*., 1998). This work thus suggests a strong connection between the anti-cancer effects of *Momordica charantia* and its ability to induce mitochondrial-mediated (intrinsic) apoptosis in cancer cells. The extract of *Momordica charantia* best-suited for the induction of MMPTP opening in rat liver is CWSE.

Conclusion

We conclude that the pre-incubation of the tested leaf extracts [Crude Water-Soluble Extract (CWSE), Decoction (DE) and Methanol Extract (ME)] of *M. charantia* with isolated rat liver mitochondria induced large amplitude mitochondrial swelling and that *M. charantia* is likely one of the many bioactive agents which cause cancer cell death by the induction of the intrinsic (mitochondrial-mediated) pathway of apoptosis. Of the three extracts investigated, CWSE is the most potent inducer of MMPTP opening. Further work on the isolation and identification of the active chemicals in the CWSE responsible for the above observation is in progress.

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