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Inductive effects of fractions of crude water-soluble extract of *Momordica charantia on* rat liver mitochondrial membrane permeability transition pore

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Abstract

Background: The mitochondrial membrane permeability transition (MMPT) pore opening has been implicated as a final cell death pathway in numerous diseases and therefore understanding conditions dictating this opening is crucial for developing targeted therapies. Cells committed to suicide, signal the release of cytochrome c from the inner mitochondrial membrane; a point of no return in the intrinsic/mitochondrial apoptotic pathway. The efficacy of *Momordica charantia* (MC) against certain cancers has been linked to its ability to induce apoptosis; however, the underlying mechanism of the induction is still unknown. This study was designed to evaluate the effect of leaf extract of MC on the opening of MMPT pore in normal Wistar rat liver mitochondria.

Methodology: The leaves of MC were obtain from the Botanical Garden, OAU, Ile-Ife Campus and was cold-extracted in distilled water to obtain the Crude Water-Soluble Extract (CWSE). N-hexane, dichloromethane, ethylacetate, butanol and aqueous fractions were obtained from the CWSE via solvent partitioning. The *in vitro* effects of these fractions on rat liver MMPT pore and ATPase activity at various concentrations (75, 100 and 125 µg/ml) were spectrophotometrically assayed.

Result: At all concentrations, all fractions of CWSE of MC show significant induction of the MMPT pore but the highest induction was observed at 125µg/ml of butanol fraction with a 23.56-fold increase when compared with the control group. In the same vein, the ATPase activities were also significantly enhanced by in vitro treatment with all but the ehtylacetate fraction; peaking at 14.13 mMPi/mg protein/min for the butanol fraction at 125µg/ml in comparison with the control group.

Conclusion: We thus conclude that the fractions of interest derived from the CWSE of MC are both potent inducers of and enhancers of the MMPT pore and mitochondrial ATPase activity respectively, the butanol fraction being the most potent.

Keywords: *Momordica charantia, Crude water soluble extract, ATP, Mitochondrial membrane permeability transition pore, Mitochondrial ATPase activity, Mitochondrial swelling.*

Résumé

Contexte: L'ouverture des pores de la transition de perméabilité membranaire mitochondriale (MMPT) a été impliquée en tant que voie de mort cellulaire finale dans de nombreuses maladies. Par conséquent, la compréhension des conditions dictant cette ouverture est cruciale pour le développement de thérapies ciblées. Les cellules suicidaires signalent la libération du cytochrome c de la membrane mitochondriale interne; un point de non-retour dans la voie apoptotique intrinsèque / mitochondriale. L'efficacité de *Momordica charantia* (MC) contre certains cancers a été liée à sa capacité à induire l'apoptose; néanmoins, le mécanisme sous-jacent de l'induction est encore inconnu. Cette étude visait à évaluer l'effet de l'extrait de feuille de MC sur l'ouverture des pores de MMPT dans les mitochondries de foie de rat Witsar normal.

Méthodologie: Les feuilles de MC ont été obtenues du jardin botanique, UOA, du campus Ile-Ife et ont été extraites à froid dans de l'eau distillée pour obtenir l'extrait brut soluble dans l'eau (CWSE). Le N-hexane, le dichlorométhane, l'acétate d'éthyle, le butanol et des fractions aqueuses ont été obtenus à partir de CWSE via un partage par solvant. Les effets in vitro de ces fractions sur l'activité des pores et de l'ATPase de la MMPT dans le foie de rat à diverses concentrations (75, 100 et 125 µg / ml) ont été analysés par spectrophotométrie.

Résultat: A toutes les concentrations, toutes les fractions de CWSE de MC présentent une induction

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plastic cages. The animals received 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), 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, Cyclosporine, Rotenone, and Sodium Succinate hexahydrate were Products of Sigma-Aldrich Co, USA. All Chemicals were of analytical grade.

Mitochondrial Fraction Isolation

Overnight-fasted animals were sacrificed by cervical dislocation and liver mitochondrial fraction were isolated essentially according to the method of Olorunsogo *et al.*, (1984) and as reported by Lapidus and Sokolove (1993) [9,10]. 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 210mM Mannitol, 70mM Sucrose, 5mM HEPES-KOH and 0.5% BSA, pH 7.4. The mitochondrial pellets were suspended in swelling buffer (210 mMMannitol, 70 mM Sucrose, and 5 mM HEPES-KOH, pH 7.4) and immediately dispensed in 1 ml Eppendorf tubes.

Mitochondrial swelling assay

Mitochondrial permeability transition opening was determined according to the method of Lapidus and Sokolove [10]. This was monitored by measuring the changes in absorbance of mitochondria at 540 nm in the presence and absence of calcium ion (triggering agent) in a Spectrumlab 752s UV/Visible spectrophotometer. Mitochondria (0.4 mg protein/ml) were pre-incubated in the presence of 8μM rotenone in a medium containing 210mM mannitol, 70mM sucrose, 5mM HEPES-KOH (pH 7.4) for 3 minutes at 30°C prior to the addition of 300μM $CaCl_2$, while 50μM sodium succinate was added 30 seconds later and MMPT pore opening was measured at 540 nm for 12 minutess at 30 seconds interval. The inhibitory effect of cyclosporine on the induction of pore opening was carried out prior to the addition of $CaCl_2$. The inductive effects of fractions were monitored when the fractions were replaced with $CaCl_2$.

Determination of ATPase activity

Mitochondrial ATPase assay was done by modifying the method of Lardy and Wellman (1953) [11]. Each test medium contained 65 mM Tris-HCL (pH 7.4), 0.5 mg protein (mitochondria), 0.5 mM KCl, 1 mM ATP and 25 mM sucrose. The final assay volume was 2 mL. Changing concentrations of the MC portions (n-hexane, dichloromethane, ethylacetate, butanol and aqueous fractions) were included as needs be. The reaction was begun by the addition of the ATP and permitted to continue for 30 minutes with consistent shaking at 37°C. One milliliter of 10% sodium dodecyl sulfate was added to the mixture in each test tube to convey the reaction to a stop. After which four milliliter of distilled water was added to each test tube and then one milliliter of the resulting solution removed into fresh test tubes where one milliliter of 1.25% Ammonium molybdate in 6.5% Sulphuric acid was added. One milliliter of 9% ascorbic acid was further added for colour development which was estimated at 660 nm. All analysis was completed in triplicate.

Estimation of Inorganic Phosphate Released

The concentration of inorganic phosphate released following the hydrolysis of ATP was determined according to the method described by Bassir and as modified by Olorunsogo and Malomo [12] using Disodium Hydrogen phosphate (Na2HPO4) as standard. Ammonium molybdate (1.25%) and freshly prepared 9% ascorbic acid were added to 1mL of the reaction mixture and allowed to stand for 30 minutes. The intensity of the blue colour was read at 660 nm in a spectrophotometer.

Statistical Analysis

significative du pore de MMPT, mais l'induction la plus élevée a été observée à 125 µg / ml de fraction de butanol avec une augmentation de 23,56 fois par rapport au groupe témoin. Dans la même veine, les activités ATPase ont également été significativement augmentées par un traitement in vitro avec toutes les fractions sauf la fraction acétate d'éthyle; culminant à 14,13 mMPi / mg de protéine / minute pour la fraction butanol à 125 µg / ml par rapport au groupe témoin.

Conclusion: Nous concluons donc que les fractions d'intérêt dérivées de la CWSE de MC sont à la fois des inducteurs puissants et des amplificateurs de l'activité du pore de MMPT et de l'ATPase mitochondriale, la fraction butanol étant la plus puissante.

Mots-clés: *Momordica charantia, extrait brut soluble dans l'eau, ATP, pore de transition de la perméabilité de la membrane mitochondriale, activité ATPase mitochondriale, gonflement mitochondrial.*

Introduction

The mitochondrial permeability transition pore is a presumed proteinaceous entity in the inner mitochondria membrane. MMPT pore opening has generally been attributed to a structural change in a protein embedded within the membrane, which in certain conditions seems to usually perform a physiological role [1, 2]. Mitochondrial permeability transition has been found to be involved in the regulation of apoptosis, as the mitochondrial pro-apoptotic factors such as cyt. C., AIF and Smac/Diablo, which are normally confined to the mitochondrial matrix are released through it into the cytosol. Once released, Cyt. C binds with Apaf-1 which prompts the activation of caspases in the presence of ATP/dATP [3]. Kerr and colleagues in 1972 raised the possibility that a large percentage of cell loss from tumors was due to apoptosis and this hypothesis has been confirmed by subsequent studies which revealed a high frequency of apoptosis in spontaneously regressing tumors and in tumors treated with cytotoxic anticancer agents [4].

These observations therefore suggest that apoptosis contributed to a high rate of cell loss in malignant tumors and could promote tumor regression [5]. It is now well established that anticancer agents induce apoptosis, and that disruption of apoptotic programs can reduce treatment sensitivity [6]. Sun *et al.* (2004), identified representatives from various classes of chemopreventive agents from *in vitro* studies with sufficient evidence to provide a detailed account of their apoptotic mechanisms. Most of these compounds can activate caspases through intrinsic effector mechanisms that are regulated by Bcl-$_2$ family members (e.g inhibition of Bcl-$_2$ expression or induction of Bax expression) or the mitochondrial permeability transition (e.g dissipation of mitochondrial inner trans membrane potential) [7].

The popularity of *Momordica charantia* in various systems of traditional medicine for several ailments suggests that the plant contains bioactive agents that could be potentially useful in drug development. Several studies using modern techniques have authenticated its use in diabetes and its complications. Most importantly, some of these studies have shown its efficacy in various cancers including breast cancer, skin tumor, prostatic cancer, and Hodgkin's disease [8].

The aim of this study was therefore to assess the inductive effect of fractions obtained from CWSE of MC on MMPT pore opening; a vital pre-requisite for the intrinsic apoptotic pathway. The effects of the fractions were also assessed on rat liver mitochondrial ATPase activity.

Materials and methods

Plant

Fresh leaves of *Momordica charantia* were obtained from medicinal plant garden and other locations within Obafemi Awolowo University Ile-Ife Campus, Osun State. The plant was authenticated by Mr. I. I. Ogunlowo, at the Faculty of Pharmacognosy Herbarium, of the same institution. The plant herbarium no is FPL-1783.

Preparation of extracts

The leaves of MC were weighed, rinsed with clean water and air-dried after which it was blended to macerate. One kilogram of the collected macerate was soaked in 1litre of distilled water and left for 12 hours at room temperature and then sieved using a muslin bag to obtain both the filtrates and the residues. The filtrate obtained was subjected to filtration and then concentrated using Rotatory evaporator at 65°C. The moist extract obtained after concentration was freeze dried using a Freeze drier. The powdery extract was partitioned to obtain n-hexane, dichloromethane, ethylacetate, butanol and aqueous fractions. All fractions were stored at 4°C.

Experimental animal

Twenty five Wistar strain albino male rats (4 months old; 180-200g) were obtained and kept at the Faculty of Basic Medical Sciences' Animal House, Ladoke Akintola University of Technology, Ogbomoso, Nigeria, under light-controlled conditions (12h"light/12h"dark cycle) and in well-ventilated

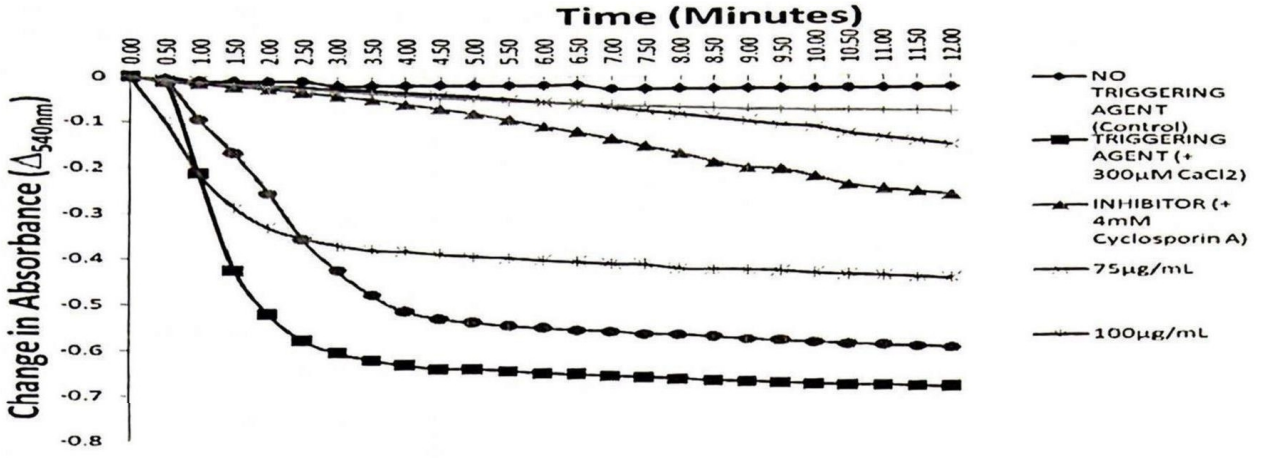

Fig. 3: The effect of ethylacetate fraction of CWSE of *Momordica charantia* on MMPT pore at varying concentration.

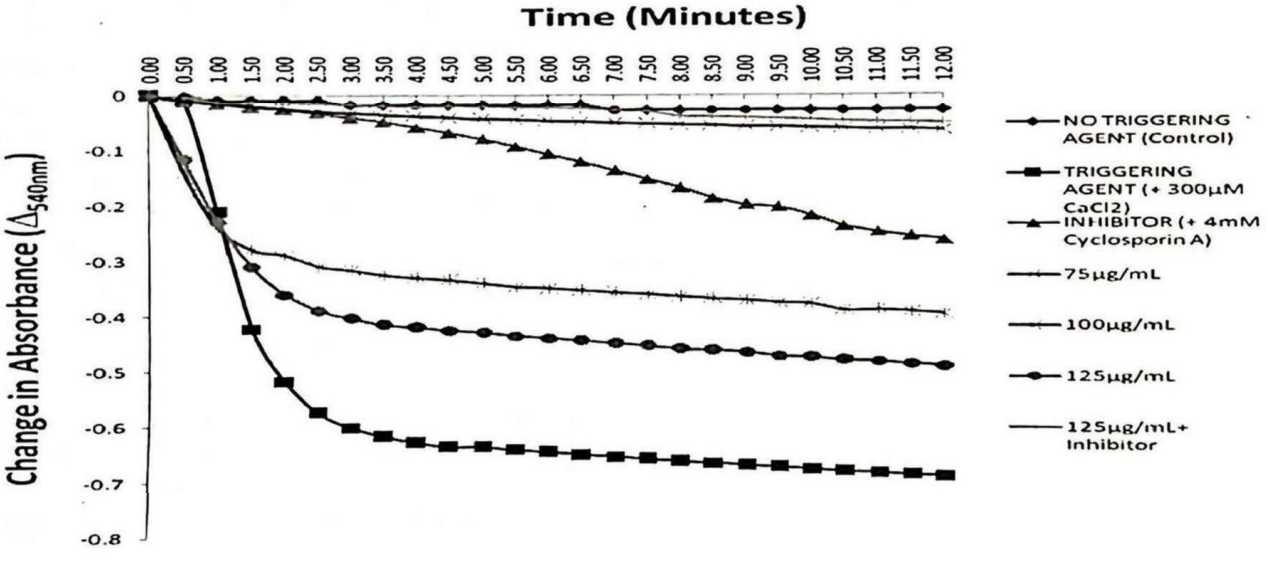

Fig. 4: Effect of the n-hexane fraction of CWSE on MMPT pore opening at varied concentrations.

increase (Δ540nm = -0.597) at highest concentration (125µg/mL) as compared with control while at 75µg/mL and 100µg/mL, Δ540nm of -0.154 and -0.446 which translates to 5.7 and 16.52 fold increases were observed respectively. At all concentrations, ethylacetate fraction of CWSE of MC triggered significant ($P<0.05$) openings of MMPT pore. As shown in figure 4, all concentration of n-hexane fraction of CWSE of MC trigger significant ($P<0.05$) induction such that a 2.37-fold increase was observed at 75µg/ml with a Δ_{540nm} of -0.064, at 100µg/ml, Δ_{540nm} = -0.394 (14.59-fold increase), while at 125µg/ml a Δ_{540nm} of -0.488 which translates to 18.07 fold increase was observed.

As shown in figure 5, all concentrations of dichloromethane fraction of CWSE of MC show significant ($P < 0.05$) induction. Such that a Δ_{540nm} of –0.059 (2.19-fold increase) was observed at 75µg/ml. At 100µg/ml, Δ_{540nm} = –0.299 (11.07-fold increase). The highest induction was observed at 125µg/ml, with Δ_{540nm} of –0.343 which translates to 12.70-fold increase. According to figure 6, The aqueous fraction of CWSE of MC has a concentration-dependent inductive effect on MMPT pore in an increasing order such that at 75µg/ml, (Δ_{540nm} of –0.052a"1.95-fold increase); at 100µg/ml, Δ_{540nm} of –0.536 a"19.85-fold increase) and at 125µg/

The data were statistically evaluated using one way analysis of variance (ANOVA) and student's T-test. All the results were expressed as Mean ± Standard Deviation (SD). The $p < 0.05$ were considered to be statistically significant.

Results

Figure 1 shows no significant changes in the volume of intact mitochondria respiring on succinate in the absence of calcium, while calcium ion induced significant opening of mitochondrial permeability was reversed by cyclosporine, the standard inhibitor of the pore by about 62%. As shown in figure 2, butanol fraction triggers the *in vitro* opening of the MMPT pore in rat liver mitochondria in a concentration-dependent manner such that (75<100<125µg/mL). a 23.5-fold increase (Δ540nm= -0.636) in permeability transition was observed at the highest concentration of 125µg/mL when compared to the control. Δ540nm of -0.065 and -0.481 which translates to 2.41 and 17.81 fold increases respectively were observed at 75µg/mL and 100µg/mL.

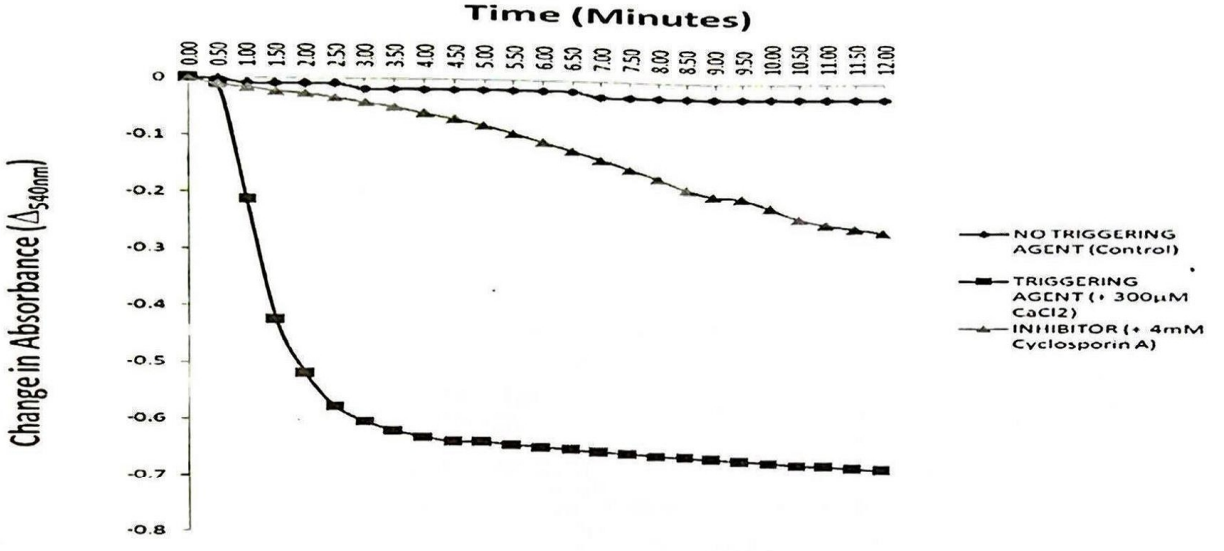

Fig. 1: In vitro induction of the opening of MMPT pore by Ca^{2+} and inhibition by cyclosporine in a male Wistar rat strain.

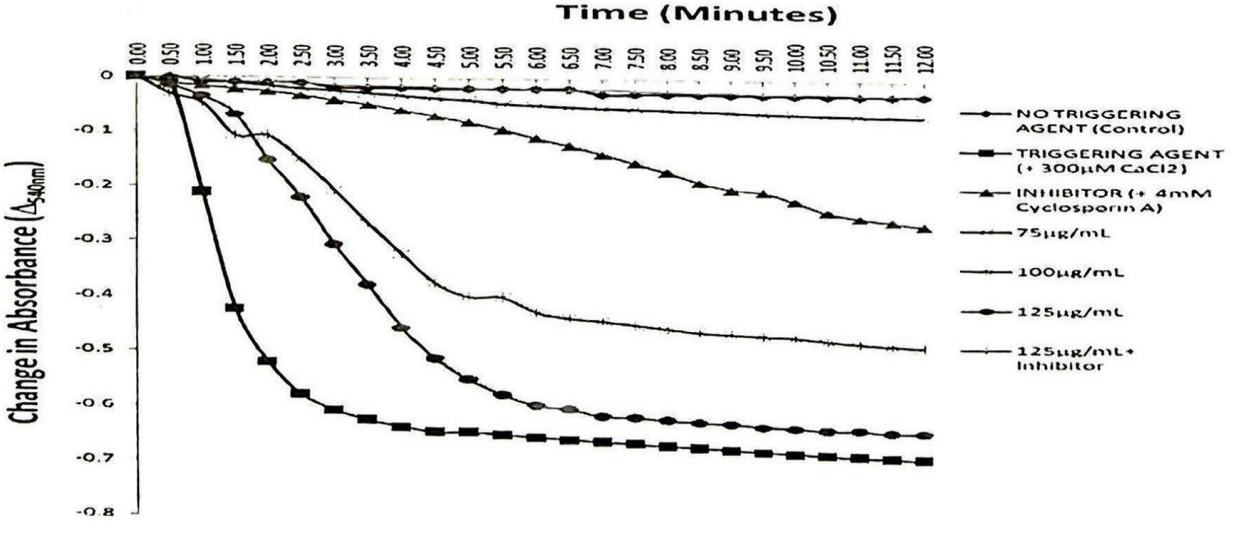

Fig. 2: The effect of butanol fraction of CWSE of *Momordica charantia* on MMPT pore at varying concentration.

transition pore up to about 10 folds in the presence of succinate and rotenone. This observed induction All inductions being significant at (P<0.05). figure 3 also shows a maximal induction of 22.11-fold

ml, a Δ_{540nm} of −0.624=23.11-fold increase). All MMPT pore openings were significant at ($P<0.05$).

Mitochondrial ATPase activities were significantly enhanced by the aqueous, butanol and dichloromethane fractions at all concentrations, however, the n-hexane fraction shows significantly ($P<0.05$) increased ATPase activity only at 125µg/ml while the ethylacetate fraction show significant ($P<0.05$) decreases of ATPase activities at the various tested concentrations (figure 7).

Discussion

Cancer is a genetic disorder characterized by dysregulation of various cellular pathways that orchestrate cell proliferation, differentiation and death. Cancers are caused by carcinogens and mutagens [13]. A cell becomes cancerous when a series of oncogenes and/or onco-suppressors become dysfunctional hence inducing a neoplastic phenotype which causes high proliferation kinetics and loss of cell-cell contact inhibition in the cells [14]. Mitochondria have emerged as an intriguing target for anti-cancer drugs, inherent to vast majority if not all types of tumors. Drugs that concentrate on mitochondria to exert anti-cancer activity has become the center of attention of recent analysis due to their effective clinical potential (which has not been maximized thus far). The exceptional potential of mitochondria as a target for anti-cancer agents has been reinforced by the discouraging finding that even tumors of the same type from individual patients differ in a number of mutations. This is consistent with the idea of personalized therapy, an elusive goal at this stage which is in line with the notion that tumors are unlikely to be treated by agents that focus on only a single gene or a single pathway. This endows the mitochondrion an invariant target present in all tumors, with an exceptional momentum [15].

A critical stage of apoptosis is the opening of the mitochondrial membrane permeability transition pore because the release of cytochrome C into the cytosol finally commits the cell to self destruction [16-18]. Mitochondria are the cells' powerhouse, but also their suicidal weapon store. Many lethal signal transduction pathways converge on mitochondria to cause the permeabilization of the mitochondrial outer membrane, prompting the cytosolic release of pro-apoptotic proteins to the hindrance of the bioenergetic elements of mitochondria. The mitochondrial metabolism in malignant growth cells is deregulated inferable from the utilization of glycolytic intermediates, which are regularly bound for oxidative phosphorylation, in anabolic reactions. Initiation of the cell death mechanism in cancerous cells by repressing tumor-explicit modifications of the mitochondrial digestion or by invigorating mitochondrial membrane permeabilization could consequently be promising restorative methodologies.

Previous observations have shown that bioactive agents that alter mitochondrial membrane function and/or dissipate the mitochondrial potential can induce apoptosis. For example, epigallocatechin galate (EGCG) in green tea, depolarizes mitochondria in numerous human cell lines including prostate and lung cells, leading to apoptosis [19]. The vanilloid curcumin, found in tumeric, and capsaicin, found in chili peppers, can open the MMPT pore and collapse mitochondrial potential, leading to induction of apoptosis [19]. Curcumin, a polyphenol, induces mitochondrial swelling and collapses the MMPT, resulting in apoptosis in numerous cell types [20, 21]. Beta carotene, a carotenoid found in carrot, can induce release of cytochrome c from mitochondria and alter mitochondrial membrane potential in different tumor cell lines derived from leukemia, colon adenocarcinoma, and melanoma cells [22]. Interestingly, prior studies in our laboratory have also confirmed the MMPT pore opening potentials of *Momordica charantia,* first as a decoction and also in different solvent extracts [23, 24].

We found out that of the tested extracts, the crude water soluble extract was the most potent; hence the present study was conceived. The results showed concentration-dependent large amplitude mitochondrial swelling in all fraction-treated groups, suggesting the undisputable essence of MC as an MMPT pore inducer. All fractions obtained had their maximal inductive effect at highest concentration (125µg/mL) such that fold-increases of 23.56, 22.11, 18.07, 12.70 and 23.11 were observed for groups treated with butanol, ethylacetate, n-hexane, dichloromethane and aqueous fractions respectively compared with the control group. These observed large amplitude swellings caused by the fractions of the CWSE of MC suggest a possible role for the medicinal plant in the treatment of ailments arising from apoptosis deregulation.

Opening of the MPTP allows free entry into the mitochondria of any small molecule (<1500 Daltons) including protons. [25]. An important consequence of opening of the MPTP is uncoupling of oxidative phosphorylation [26]. Loss of membrane potential interferes with the production of ATP, the cells main source of energy, because mitochondria must have an electrochemical gradient to provide

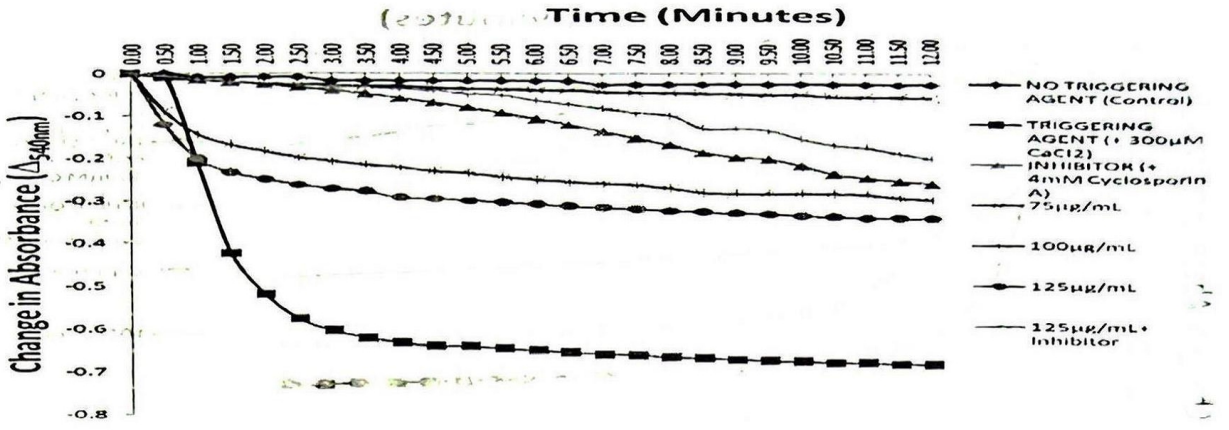

Fig. 5: Effect of the dichloromethane fraction of CWSE on MMPT pore opening at varied concentrations.

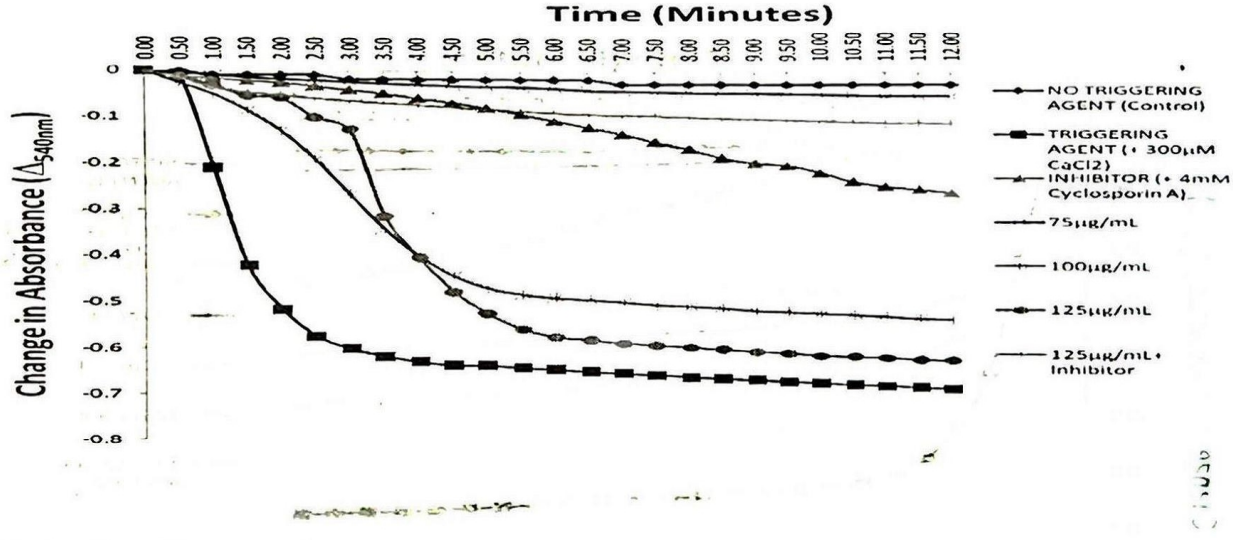

Fig. 6: Effect of the aqueous fraction of CWSE on MMPT pore opening at varied concentrations.

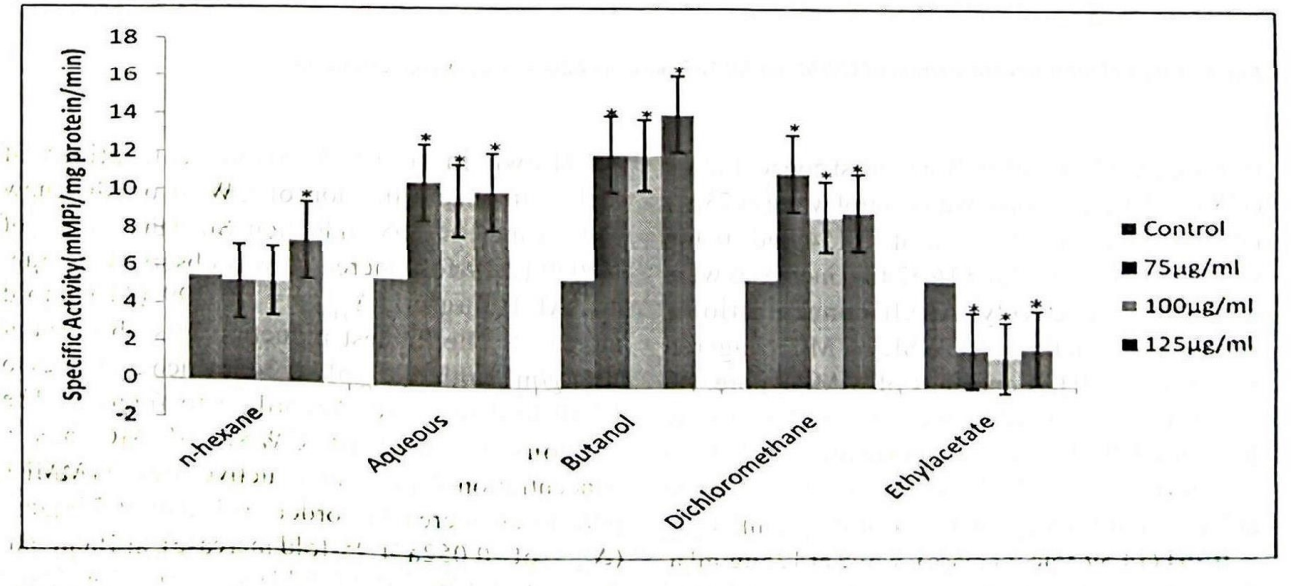

Fig. 7: The comparative effects of different fractions of CWSE of *MC on Mitochondrial* ATPase activity

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the driving force for ATP production. In cell damage resulting from conditions such as neurodegenerative diseases and head injury, opening of mitochondrial permeability transition pore can greatly reduce ATP production, and can cause ATP Synthase (through its reversal) to begin hydrolyzing, rather than producing ATP [27]. Opening of the MPTP leads to permeability transition (PT), a sudden increase of inner mitochondrial permeability to solutes with molecular mass up to 1.5 kDa which is implicated in apoptosis or necrosis as an important event in the control of cell death or survival [28]. This opening generates a colloidal osmotic pressure across the inner mitochondrial membrane which drives water into the matrix and causes swelling. The inner membrane being extensively folded into cristae can expand to compensate but the outer membrane cannot and this ruptures, releasing intermembrane proteins. It is the release of these proteins such as Cytochrome C that enables the mitochondria play a role in apoptosis i.e the release of cytochrome C causes cells to go through apoptosis by activating pro-apoptotic factors [29-31].

In consonance with the induction of the MMPT pore observed in this study, fractions obtained from CWSE of MC except for ethylacetate fraction enhanced ATPase activities, in a non concentration-dependent manner. For example, ATPase activities at the three tested concentrations for butanol fraction were all significantly enhanced with respect to the control group while for the n-Hexane fraction, significant increase in the activity of ATPase was only observed at 125µg/ml. The highest ATPase activity observed across all fractions was at 125µg/ml for butanol fraction.

The observed increases in the activity of the mitochondrial ATPase must have been due to the release of inorganic phosphate (Pi), an indication of the uncoupling of phosphorylation in the mitochondrion, a process which is synonymous with MPT pore opening and mitochondrial swelling. Also, we observed that the CWSE fractions at all concentrations induced opening of the MMPT in a concentration-dependent manner, that is, the inductive effect increased as concentration increased. This may have to do with the assumption that the active components of the plant may be interacting with specific components of the pore such as adenine nucleotide translocase (ANT). Although it is yet to be determined which of the active components exerts the observed effect, there is incontrovertible evidence that exposure to *MC will* possibly elicit opening of the pore and subsequently the release of cytochrome C and activation of the execution caspases. A process which will be useful in the development of drugs which rely on the permeabilization of the mitochondrial membrane in the treatment of diseases caused by dysregulated apoptosis.

Conclusion

Conclusively, fractions obtained from the CWSE of *Momordica charantia* leaves significantly induced large amplitude mitochondrial swelling consequent to the opening of the MMPT pore, and mostly enhanced mitochondrial ATPase activity. An indication that the plant's phytochemicals are potent agents with possible usefulness in the treatment of diseases arising from the down-regulation of apoptosis and responsive to the up-regulation of the same via opening of the MMPT pore.

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