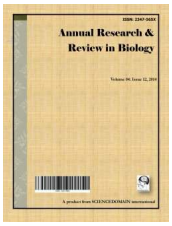

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Induction of Mitochondrial-Mediated Apoptosis by Solvent Fractions of Methanol Extract of *Heliotropium indicum* in Rat Liver Cells

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Authors' contributions

This work was carried out in collaboration between all authors. Author AOO designed the study, wrote the protocol, managed the analysis of the study, performed the statistical analysis and wrote the first draft of the manuscript. Author YDA fed, treated and took the care of the animals and also did the literature searches. Author OOO designed, approved the study and read through the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Mitochondrial Membrane Permeability Transition (MMPT) pore has emerged as a promising target for drug development because the release of cytochrome c upon the opening of the pore is a point of no return for mitochondrial-mediated apoptosis to occur. *Heliotropium indicum* (HI) is as an anti-tumor and wound healing agent in traditional medicine. It is not known whether its mode of action involves the induction of apoptosis via the opening of the MMPT pore.
Methodology: Mitochondria, isolated from male albino rat liver (about 100 g), were exposed to varying concentrations (10, 30, 50, 70, and 90 µg/ml) of solvent fractions of methanol extract of HI i.e Chloroform (CFHI), Ethylacetate (EFHI), Methanol (MFHI) and crude Methanol Extract (MEHI) of HI. Opening of the pore, cytochrome c release, mitochondrial ATPase activity and extent of mitochondrial lipid peroxidation were assessed spectrophotometrically *in vitro*. Activation of caspases 9 and 3 were also assessed using ELISA kits.

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Results: In the absence of Ca^{2+}, CFHI, EFHI, MFHI and MEHI induced the opening of the pore in a concentration-dependent manner with CFHI having the highest induction fold of 26 and MFHI as the lowest having 6.6. All the fractions inhibited lipid peroxidation in a concentration-dependent manner. Also, these fractions induced the release of cytochrome c with CFHI having the highest effect and the least by MFHI. Mitochondrial ATPase activity was enhanced by all the fractions with CFHI having the highest stimulatory effect. Interestingly, intra-peritoneal administration of CFHI and MEHI at 2, 5, 10 and 20 mg/kg body weight for 21 days resulted in significant opening of the pore, the release of cytochrome c and activation of caspases 9 and 3. All these effects were highest with 20 mg/kg body weight.

Conclusion: These findings therefore suggest that Chloroform Fraction of *Heliotropium indicum* is the most potent of all these fractions and therefore contains the bioactive agent that induces mitochondrial-mediated apoptosis in normal liver cells. The fraction will therefore be useful for further studies for drug development in diseases requesting up-regulation of apoptosis.

Keywords: Mitochondria; apoptosis; Heliotropium indicum; MMPT; caspases.

1. INTRODUCTION

It is well established that apoptosis, a form of programmed cell death, is characterized by a number of distinct cellular morphological changes and energy-dependent biochemical processes [1]. It is also well known that the process is the major mechanism through which unwanted cells are eliminated by multicellular organisms to ascertain normal development and cellular homeostasis. The biochemical process of apoptosis may occur by a number of mechanism including; extrinsic or death-receptor pathway, intrinsic or mitochondrial-mediated pathway which involves the opening of Mitochondrial Membrane Permeability Transition (MMPT) pore in situations of intracellular calcium overload or oxidative stress or cellular insult and by the perforin/granzyme pathway [2].

Although the structural and molecular nature of mitochondrial membrane permeability transition is not yet fully elucidated, it has however been established that the inner membrane of the mitochondria is able to respond to certain stimuli such as Ca^{2+} overload, hypoxia and oxidative stress by opening of the MMPT pore [3,4]. On opening of the pore, the release of cytochrome c leads to a point of no return for apoptosis to take place as this step leads to the formation of apoptosome, activation of caspases, the degradation of cellular components and eventually cell death [5,6,7,8]. Several human pathological conditions including many types of cancer, diabetes, neurodegenerative diseases, ischemic damage, autoimmune disorders, neoplasia, AIDS and other viral infections have been linked to too little or too much apoptosis [9].

Although scientific studies have not been able to ascertain the exact component of the MMPT pore which is directly linked to mitochondrial-mediated apoptosis, components such as Voltage Anion Channel (VDAC), Adenine nucleotide translocase (ANT) and cyclophilin D were proposed to be the component of the pore in the inner mitochondrial membrane (IMM). However, results obtained from null experiments confirmed that the absence of VDAC did not actually affect the formation/induction of the pore [10]. Also, there are established facts that some complex proteins present at the outer mitochondrial membrane (OMM) are able to modify / regulate the MMPT pore [11,12].

Basically, the balance between the levels of pro-apoptotic proteins (e.g. BAX and BAK) and anti-apoptotic proteins (e.g. Bcl-2, Bcl-X_L) and BH3-only proteins (e.g. BID, BAD, NOXA, PUMA) decide if apoptosis will take place [11]. This understanding has led researchers to seek for the modulators of Bcl-2 protein family in relation to the mechanism of action of such agents with respect to mitochondrial-mediated apoptosis. The manipulation of MMPT pore and/or mitochondrial-mediated apoptosis has therefore become a strategy for drug development in situations of dysregulated apoptosis [13]. It has been recorded that specific bioactive agents found in medicinal plants are able to modulate the MMPT pore either by inducing or inhibiting it [5].

In this connection, a number of phytochemicals [7] including polyphenols, have been shown to possess chemopreventive and specific therapeutic properties in the development of cancer and diseases that may result from too little apoptosis [14]. There is considerable

evidence that bioactive agents in some plants (sulforaphane in cruciferous vegetables, genistein in soybeans and epigallocatechin gallate in green tea etc.) were able to induce MMPT pore opening. Quite a number of these agents have been identified and they currently under preclinical and clinical trials [7].

Heliotropium indicum, a herb grown throughout Africa, is reported to have antipyretic, wound healing, antileukemic activity [15,16] anti-asthmatic [17], anti-tumoric [18], antioxidant, antibacterial [19]. However, the active principle or bioactive component of this plant has not been described with respect to modulation of mitochondrial-mediated apoptosis.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Mannitol, sucrose, N-2-Hydroxy-ethyl-pipe-arizine-N-2-ethanesulfonic acid (HEPES), rotenone, spermine, Folin-Ciocalteau reagent, Bovine Serum Albumin (BSA), and all other reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and were of the highest purity grade available.

2.2 Plant Material

Fresh leaves of *Heliotropium indicum* were obtained from a local farmland in Ibadan, Oyo State, Nigeria. Botanical identification and authentication was done at the herbarium, Department of Botany, University of Ibadan, Nigeria. The fresh plant was separated from extraneous materials, washed, air-dried for about two weeks and then blended to a powder which was later soaked in methanol for 72 hours. The filtrate obtained was concentrated using rotary evaporator and concentrated to dryness in a water bath at 37℃.

2.3 Partitioning of Crude Methanol Extract of *Heliotropium indicum* (MEHI) Using Vacuum Liquid Chromatography

The column was packed three-quarters full with silica gel 60 (0.040–0.063 mm, MERCK). Silica gel 60 (0.040–0.063 mm, MERCK) 8 g was added to 12g of the methanol extract sample. The gel-sample mixture was stirred until a homogenous mixture was obtained. The mixture was air-dried to obtain a powdery form. The sample was applied to the top of the column with the pump switched on. Solvents were added in order of increasing polarity and n-hexane, chloroform, ethylacetate and methanol fractions were obtained in this order. All these fractions were concentrated to dryness under pressure using rotary evaporator at 40℃, stored in glass sample bottles and kept in the refrigerator until use.

2.4 Experimental Animals

Male Wistar strain albino rats weighing between 100 – 120 g were obtained from the Pre-Clinical Animal House, Faculty of Basic Medical Sciences, College of Medicine, University of Ibadan, Nigeria. The animals were allowed to acclimatize for 14 days in cages in the Animal House, Department of Biochemistry, University of Ibadan before the commencement of the experiments. All animals had access to water and chow *ad libitum* and were kept under standard conditions of temperature and humidity.

Rules guiding animal studies as stipulated by the Ethical Committee of University of Ibadan were followed. These rules are similar to international guidelines on animal handling.

2.5 Isolation of Rat Liver Mitochondria

Rat liver mitochondria were isolated essentially according to the method of Johnson and Lardy [20] as modified by Olorunsogo et al. [21]. Briefly, the animals were sacrificed by cervical dislocation and the livers excised and trimmed to wash excess tissue. The livers were then weighed and washed with homogenizing buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES-KOH, pH 7.4 and 1 mM EGTA), and homogenized as a 10 percent suspension in ice – cold buffer using a Potter Elvehjem glass homogenizer. The resulting homogenate was centrifuged in an MSE refrigerated centrifuged at 2300 rpm for 5 minutes to remove the nuclear debris. This was done twice and the supernatant obtained was centrifuged at 13,000 rpm for 10 minutes to obtain the mitochondrial pellet. The supernatant was discarded while the pellet was washed with washing buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES-KOH, pH 7.4, 0.5 percent BSA) twice at 12,000 rpm for 10 minutes. The mitochondria obtained were immediately re-suspended in an appropriate volume of MSH buffer (210 mM Mannitol, 70 mM Sucrose, 5 mM HEPES-KOH, pH 7.4), and immediately

dispensed into eppendorf tubes and kept on 4℃ ice.

2.6 Mitochondrial Swelling Assay

Mitochondrial Membrane Permeability Transition was monitored by following the changes in absorbance of mitochondria suspension at 540 nm in the presence or absence of calcium (Standard Triggering Agent) in a T70 UV/Visible spectrophotometer essentially according to the method of Lapidus and Sokolove [22]. Briefly, mitochondria (0.4 mg protein/ml) were pre-incubated in the presence or absence of 0.8 µM rotenone in a medium containing 210 mM mannitol, 70 mM sucrose and 5 mM HEPES-KOH (MSH) (pH 7.4) for 3 mins at 27℃ prior to the addition of 120 µM $CaCl_2$. Thirty seconds later, 5 mM succinate was added and mitochondrial permeability transition was quantified at 540 nm for 12 mins at 30 secs interval. The intactness of the mitochondria was determined by addition of 4 mM spermine immediately following the addition of rotenone and just before the addition of mitochondrial fraction. Addition of 300 µM $CaCl_2$ was omitted whenever there was no requirement for calcium-induced opening of the pore. Mitochondria of untreated animals were used for these (*in vitro)* studies.

2.7 Determination of Mitochondrial Protein

Mitochondrial protein was estimated according to the method Lowry et al. [23] using bovine serum albumin (BSA) as standard.

2.8 Assessment of Mitochondrial F_oF_1 ATPase Activity

Adenosine triphosphatase (ATPase) activity was determined by a modification of the method of Olorunsogo and Malomo [24]. Each reaction mixture contained 65 mM Tris-HCl buffer pH 7.4, 0.5 mM KCl 1 mM ATP and 25 mM Sucrose. The reaction mixture was made up to a total volume of 2 ml with distilled water. The reaction was started by the addition of mitochondrial suspension (specific) and was allowed to proceed in a shaker water bath for 30 minutes at 27℃. The reaction was stopped by the addition of 1 ml of a 10 percent of sodium dodecyl sulphate (SDS). The zero time tube was prepared by addition of ATP to the reaction vessel with immediate addition of SDS but for 30 seconds intervals for other reaction vessels.

2.8.1 Estimation of inorganic phosphate released

The concentration of inorganic phosphate released was measured according to the procedure described by Bassir [25] and as modified by Olorunsogo and Bababunmi [21]. 4mls of distilled water was added to the each of the reaction mixture after which 1 ml is pipetted from the reaction vessels into a fresh test tube. 1 ml of 1.25% ammonium molybdate and 1 ml of freshly prepared solution of ascorbic acid were dispensed simultaneously into the fresh test tube. The test tubes were allowed to stand for 30 minutes. The absorbance was read at 660 nm. A water blank was used to set the spectrophotometer at zero.

2.9 Measurement of Mitochondrial Lipid Peroxidation

A modified Thiobarbituric Acid Reactive Species (TBARS) assay method was deployed to measure the lipid peroxide formed from mitochondrial membrane lipid peroxidation using the method of Ruberto et al. [26]. Mitochondria (2 mg/ml protein) and varying concentrations of the extract and fractions were added to each reaction mixture and made up to 1 ml with distilled water; 0.05 ml of $FeSO_4$ (0.07M) was added to induce lipid peroxidation and the mixture incubated for 30 mins. 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% (w/v) thiobarbituric acid in 1.1% sodium dodecyl sulphate were then added and the resulting mixture was vortexed and heated at 95℃ for 60 mins. After cooling, 5.0 ml of butan-1-ol were added to each tube and centrifuged at 4,000 rpm for 10 mins. The absorbance of the organic upper layer was measured at 532 nm. Percentage inhibition of lipid peroxidation by the extract was calculated from the absorbance value of the fully oxidized control and that in the presence of extract using the formula (E-C/C) * 100

2.10 Assay of Cytochrome C

Cytochrome c released from isolated mitochondria was quantitatively determined by measuring the Soret (Y) peak for cytochrome c at 414 nm (ε = 100 $mM^{-1}cm^{-1}$), according to a method previously established by Appaix et al. [27]. Isolated mitochondria were pre-incubated in a buffer medium containing 210 mM mannitol, 70 mM sucrose and 5 mM HEPES-KOH (pH 7.4) in

the presence of 0.8 µM rotenone at 27℃ for about 30 mins. The Soret (Y) peak for cytochrome c at 414 nm was recorded with inclusion of varying concentrations of the fractions of *H. indicum* using 300 µM calcium as the standard triggering agent (TA).

2.11 Assay of Caspases 9 and 3

Analysis of caspases 9 and 3 were carried out using an ELISA kit, a product of Elabscience biotechnology Ltd., Technology Industry Park, WuHan, Peoples Republic of China. A microplate reader (DNM-9602A from China) was used to read the optical density at 450 nm wavelength. After acclimatization, 2, 5, 10 and 20 mg/kg body weight doses of CFHI and MEHI were administered intraperitoneally for two weeks after which the animals were sacrificed and the liver excised. The removed liver was weighed and rinsed with phosphate buffered saline thoroughly until a clear wash was obtained. The washed livers were homogenized on ice and the homogenates were centrifuged at 8,000 rpm for 5 minutes. The supernatant thus obtained were then put in sample bottles and freezed. After freezing for two days, the samples were brought out to thaw. This was done twice after which the homogenates were used for caspases 9 and 3 analysis

2.12 Statistical Analysis of Data

The data were statistically evaluated using one way analysis of variance (ANOVA). All the results were expressed as mean ± standard deviation (SD). The $p < 0.05$ was adopted to be statistically significant.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Calcium-induced mitochondrial membrane permeability transition pore opening in normal rat liver mitochondria and its reversal by Spermine

The intactness and integrity of mitochondria used in this study were ascertained by following the rate of decrease in absorbance of mitochondria in the absence and presence of Ca^{2+} and the reversibility of calcium-induced swelling by spermine, a standard inhibitor of the opening of mitochondrial membrane permeability transition (MMPT) pore in the presence of rotenone over a period of twelve minutes. In this regard, the results depicted in Fig. 1 showed that in the absence of exogenous calcium, there was no significant change in absorbance of mitochondria respiring in the presence of succinate and rotenone over a period of twelve minutes, whereas calcium-induced swelling of the mitochondria was almost completely reversed by spermine, thus indicating that the mitochondria used for this study were intact and suitable for use.

3.1.2 Effects of varying concentrations of crude methanol extract of *H. indicum* (MEHI) on the MMPT pore in the absence and presence of Ca^{2+}

Typical representative profiles of the effects of varying concentrations of crude Methanol extract of *H. indicum* (MEHI) on the MMPT pore in the absence of Ca^{2+} are shown in Fig. 2. The results showed that At concentrations 10, 30 and 50. 70 and 90 µg/ml there was no significant induction of MMPT pore opening when compared with NTA.

Also, as seen from the results in Fig. 3, there were decreases in absorbance of mitochondria respiring on succinate in the presence of calcium although the extent of decrease in absorbance was higher in the presence of lower concentration of the extract while higher concentrations appeared to reverse the calcium-induced opening of the pore. The results indicate the inhibition of Ca^{2+}-induced opening of the MMPT pore by the extract in a concentration-dependent.

3.1.3 Effect of varying concentrations Methanol Fraction of Methanol Extract of *H. indicum* (MFHI) on the MMPT pore in the absence and presence of Ca^{2+}

Fig. 4 depicts the pattern of the effect of MFHI on MMPT pore in the absence of Ca^{2+}. On exposure to varying concentrations of MFHI to succinate-energized mitochondria in the absence of calcium showed insignificant change in absorbance of mitochondria, indicating that MFHI does not have effect on the pore, even at 90 µg/ml. Also, there was insignificant inhibition of Ca^{2+}-induced opening of the pore as observed in Fig. 5. A comparison of the results obtained on addition of varying concentration of MFHI and MEHI showed that MEHI had a higher inductive effect on MMPT pore than MFHI.

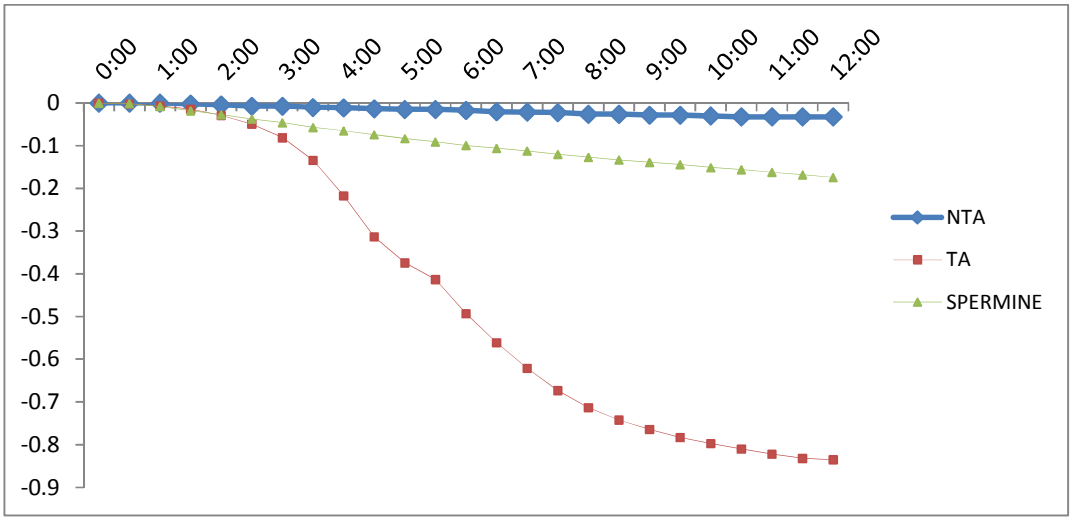

Fig. 1. Calcium-induced mitochondrial membrane permeability transition pore opening in normal rat liver mitochondria and its reversal by Spermine

Abbreviations: NTA: No Triggering Agent; TA: Triggering Agent

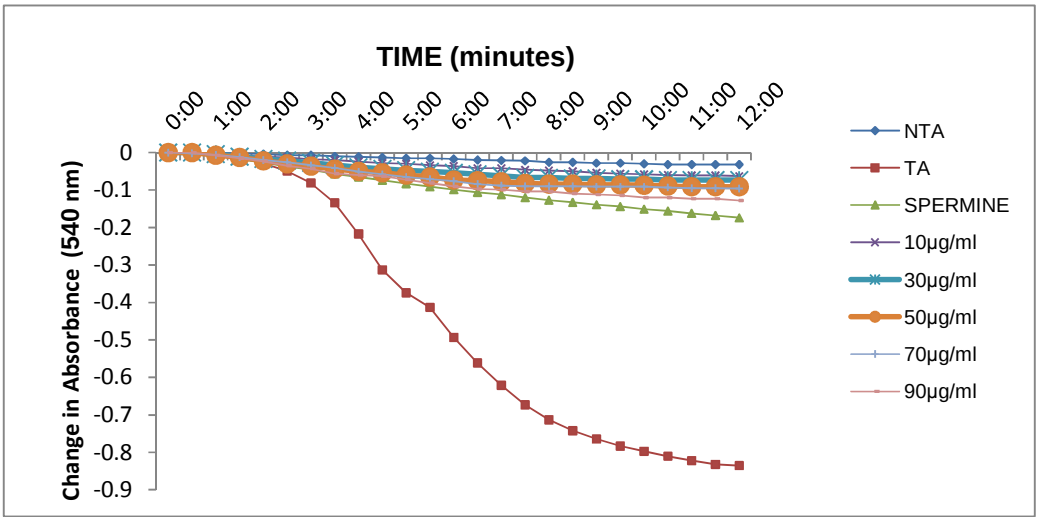

Fig. 2. Effect of varying concentrations of crude methanol extract of *H. indicum* (MEHI) on the MMPT pore in the absence of Ca^{2+}

Abbreviations: NTA: No Triggering Agent; TA: Triggering Agent

3.1.4 Effect of varying concentration of ethylacetate fraction of methanol extract of *H. indicum* (EFHI) on the MMPT pore in the absence and presence of Ca^{2+}

The effect of varying concentrations of EFHI on the MMPT pore in the presence and absence of Ca^{2+} were reported in Figs. 6 and 7, respectively. Mitochondria respiring on succinate in the presence of rotenone were exposed to different concentrations of EFHI in the absence of calcium and the extent of decrease in absorbance of mitochondria were 2.1, 3.8, 6.1, 10.3 and 14.2 folds, respectively, at 10 µg/ml, 30 µg/ml, 50 µg/ml, 70 µg/ml and 90 µg/ml. These results indicate that different concentrations of EFHI were more potent in opening the pore than MEHI. However, there was concentration-dependent reversal of Ca^{2+}-induced opening on exposure of EFHI to succinate-energized mitochondria in the presence of calcium as seen in Fig. 7.

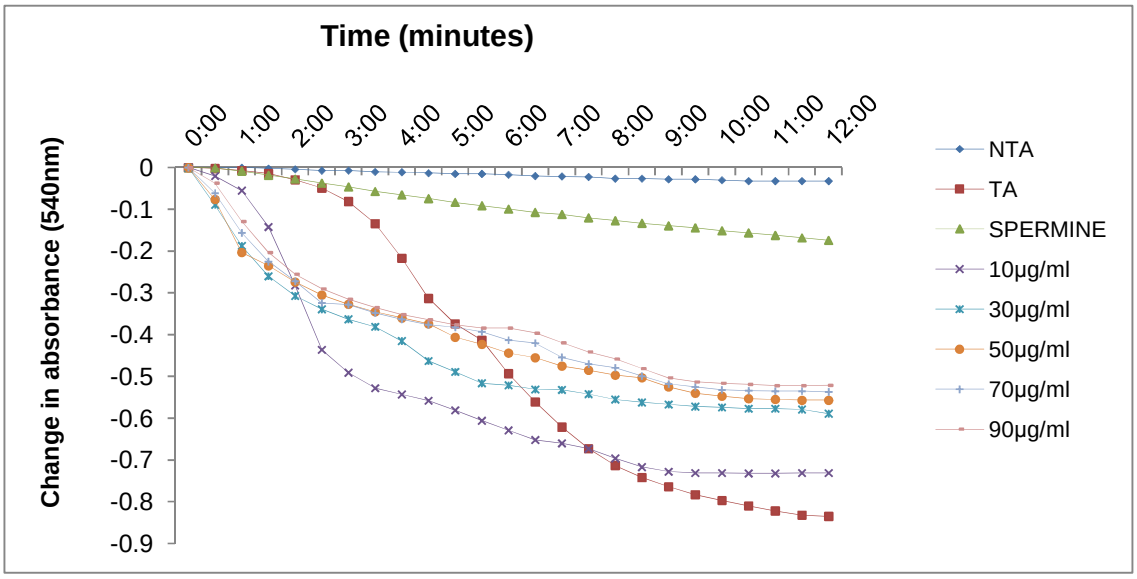

Fig. 3. Effect of varying concentrations of crude methanol extract of H*. indicum* (MEHI) on the MMPT pore in the presence of Ca^{2+}

Abbreviations: NTA: No Triggering Agent; TA: Triggering Agent

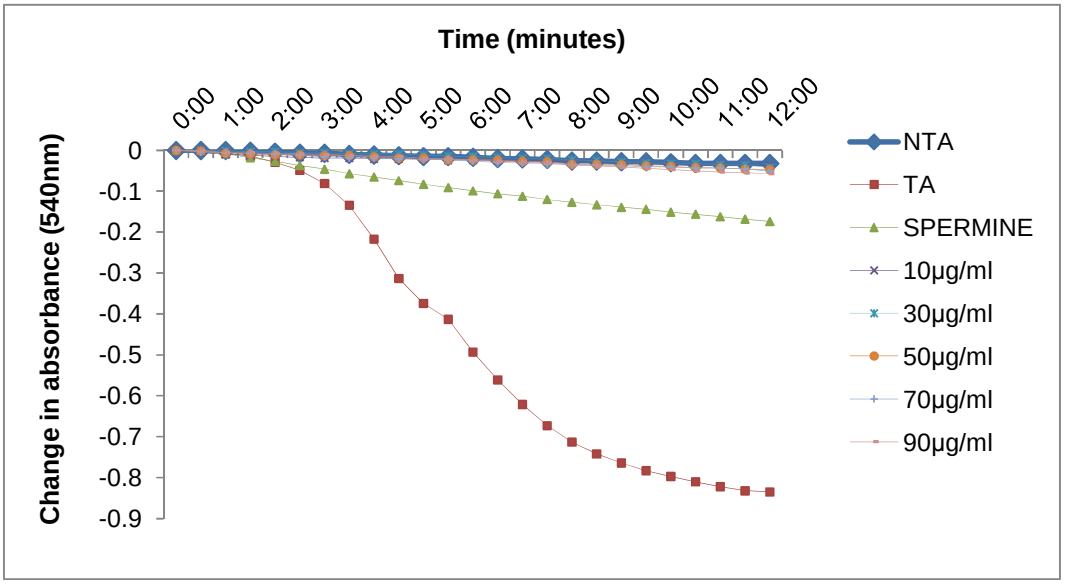

Fig. 4. Effect of varying concentrations methanol fraction of methanol extract of *H. indicum* (MFHI) on the MMPT pore in the absence of Ca^{2+}

Abbreviations: NTA: No Triggering Agent; TA: Triggering Agent

3.1.5 Effect of varying concentrations of chloroform fraction of methanol extract of *H. indicum* (CFHI) on the MMPT pore in the absence and presence of Ca^{2+}

In the absence of Ca^{2+}, exposure of succinate-energized mitochondria to varying concentrations of CFHI on MMPT pore, caused significant decreases in absorbance of mitochondria as shown in Fig. 8. At lower concentrations of this fraction (10 µg/ml and 30 µg/ml) there was no significant decrease in absorbance, indicating that at these concentrations, CFHI did not induce the opening of MMPT pore, but at higher

concentrations (50 µg/ml, 70 µg/ml and 90 µg/ml) there was significant induction of the MMPT pore. The maximum inductive effect was 25.6 folds at the highest concentration – 90 µg/ml; whereas, there was a reversal of Ca^{2+}-induced opening in a concentration-dependent manner in the presence of Ca^{2+} as depicted in Fig. 9. With respect to the opening of Mitochondrial Membrane Permeability Transition Pore (MMPT), chloroform fraction is the most potent.

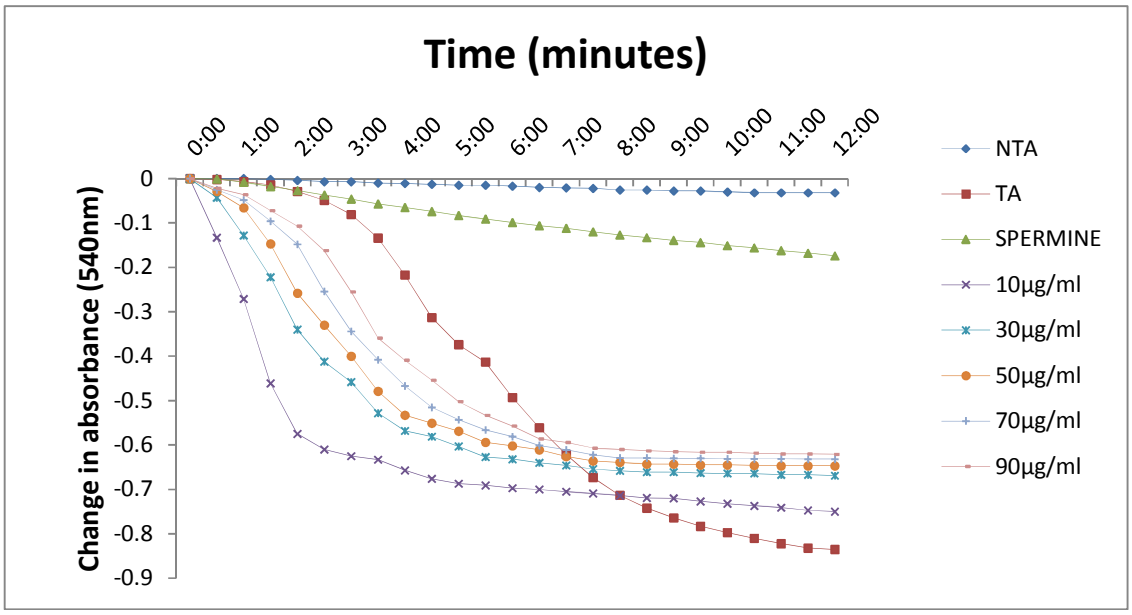

Fig. 5. Effect of varying concentrations of methanol fraction of methanol extract of *H. indicum* (MFHI) on MMPT pore in the presence of Ca^{2+}

Abbreviations: NTA: No Triggering Agent; TA: Triggering Agent

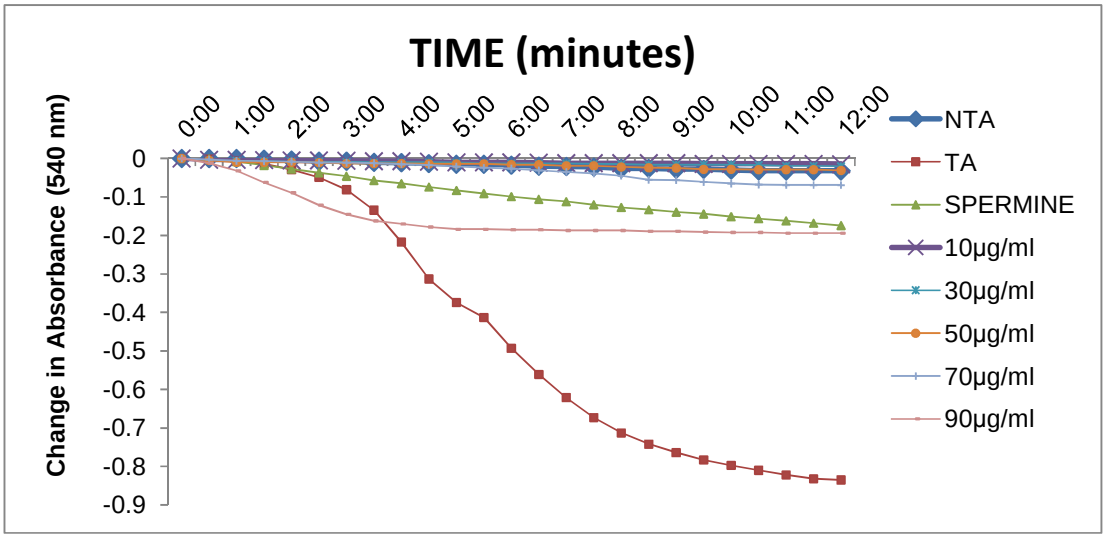

Fig. 6. Effect of varying concentration of ethylacetate fraction of methanol extract of *H. indicum* (EFHI) on the MMPT pore in the absence of Ca^{2+}

Abbreviations: NTA: No Triggering Agent; TA: Triggering Agent

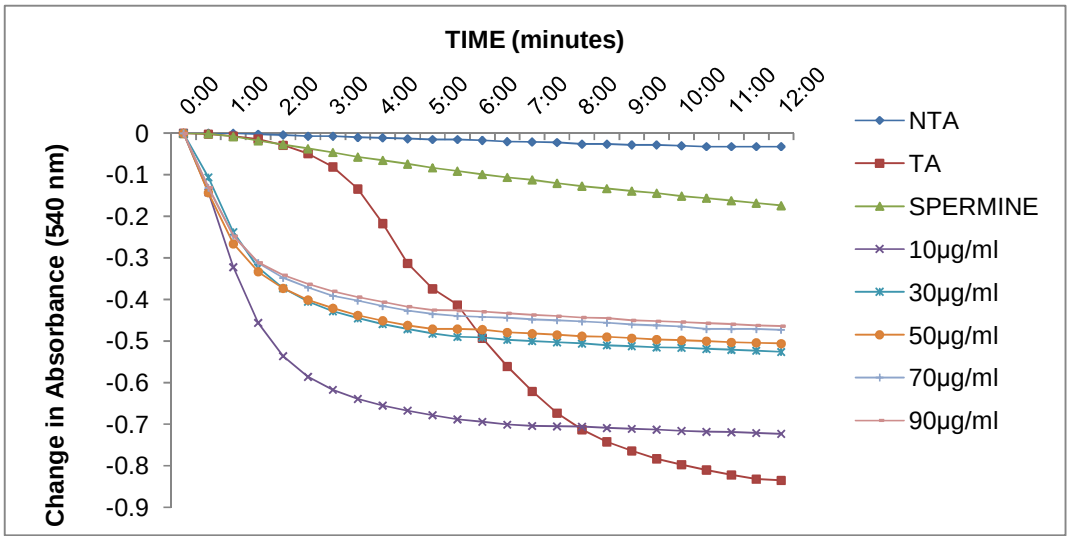

Fig. 7. Effects of various concentration of ethylacetate fraction of methanol extract of *H. indicum* (EFHI) on MMPT pore in the presence of Ca^{2+}

Abbreviations: NTA: No Triggering Agent; TA: Triggering Agent

3.1.6 Effects of different solvent fractions of crude methanol extract of *H. indicum* on mitochondrial F_0F_1 ATPase activity

Table 1 shows the effects of varying concentrations of the solvent fractions of the crude extract on enhancement of F_1F_0 ATPase activity in rat liver mitochondria. The results showed that EFHI and CFHI significantly ($P<0.05$) enhanced ATPase activity in a concentration-dependent manner as compared to MEHI and MFHI that had no significant effect on ATPase activity at all the concentrations tested. From the results obtained, CFHI enhanced the activity of ATPase more than EFHI, while that of MEHI and MFHI were insignificant at various concentration.

3.1.7 Effects of different solvent fractions of crude methanol extract of *H. indicum* on Cytochrome c release in rat liver mitochondria

The amounts of cytochrome c released by mitochondria in the presence of crude extract and its fractions were reported in Fig. 10. The result showed that on addition of varying concentrations of MEHI, MFHI, EFHI and CFHI to MSH-pre-incubated mitochondria respectively, there was concentration-dependent release of cytochrome c. MFHI and MEHI from the results, did not significantly cause the release of cytochrome c on exposure to the mitochondria as compared to the control while EFHI and CFHI significantly did.

3.1.8 Effects of different solvent fractions of *H.indicum* on Fe^{2+}-induced lipid peroxidation in normal rat liver mitochondria

Inhibition effect of varying concentrations by methanol extract and fractions of *H.* indicum of lipid peroxidation induced by ferrous sulphate were reported in Fig. 11. The result showed that in a concentration-dependent manner, all the fractions exhibited inhibitory effect on lipid peroxidation induced by the ferrous sulphate. The percentage inhibition was significantly high in reaction vessels with CFHI and EFHI 88 percent and 72 percent at maximum concentration (800 µg/ml) respectively.

3.1.9 Effects of chloroform fraction (CFHI) and methanol extract (MEHI) of *H. indicum* on caspases 9 and 3

Interestingly, levels of Caspase-9 and Caspase-3 (initiator and executioner caspases; respectively), were significantly up-regulated at increasing dosage of CFHI (2, 5, 10 and 20mg/kg body weight, $p < 0.05$, respectively), whereas there was insignificant increase in the activity of these caspases with increasing dosage of MEHI as shown in Figs. 12 and 13.

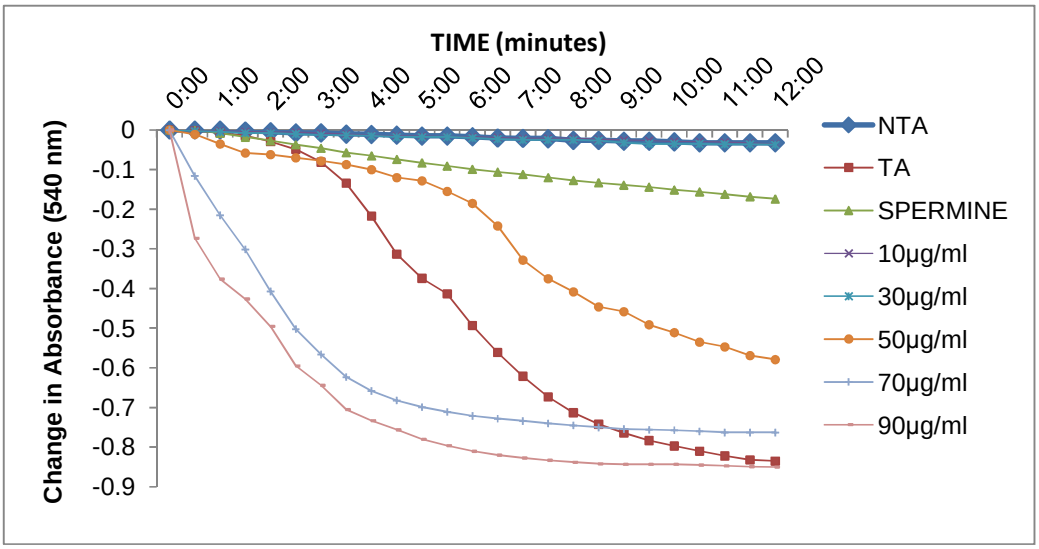

Fig. 8. Effect of varying concentrations of chloroform fraction of methanol extract of *H. indicum* (CFHI) on the MMPT pore in the absence of Ca^{2+}
Abbreviations: NTA: No Triggering Agent; TA: Triggering Agent

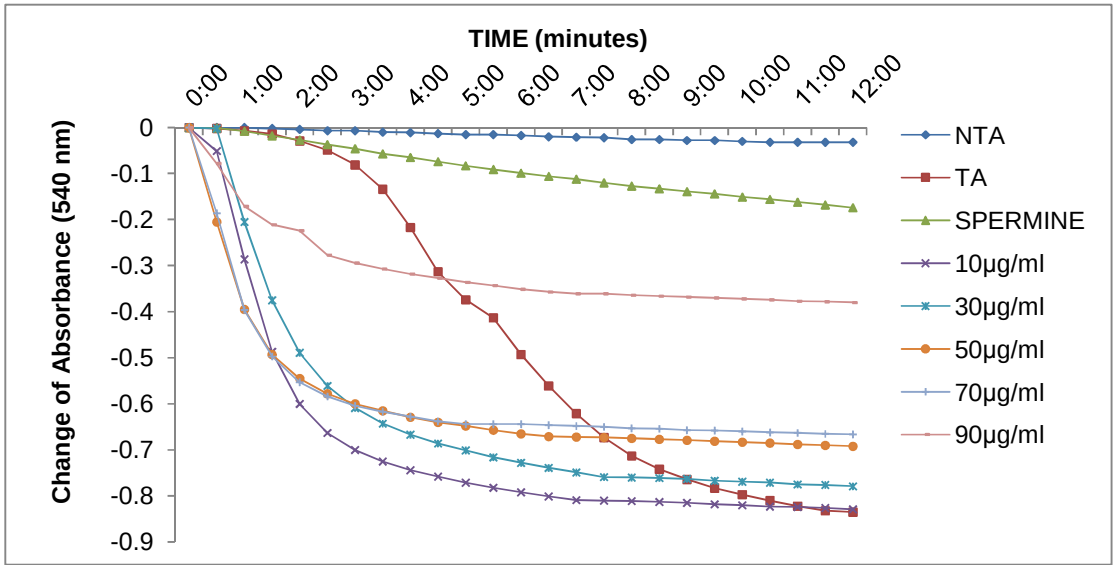

Fig. 9. Effect of varying concentrations of chloroform fraction of methanol extract of *H. indicum* (CFHI) on the MMPT pore in the presence of Ca^{2+}
Abbreviations: NTA: No Triggering Agent; TA: Triggering Agent

3.2 Discussion

Apoptosis or programmed cell death is a crucial component of the development and health of multicellular organisms [28]. Although apoptosis is a structured and likely the most frequent form of programmed cell death, there are other non-apoptotic types of cell death that are also of biological significance [29]. Mitochondrial-mediated apoptosis is initiated by the opening of the mitochondrial membrane permeability transition (MMPT) pore which has recently become a promising target for diseases that originate from too little or too much apoptosis [13]. Several findings have confirmed that there are plant based bioactive agents that are capable of eliciting chemo-protective and therapeutic effects through the induction or inhibition of the

opening of MMPT pore [5]. Bioactive agents such as Robustaside-B and *para*-hydroxyphenol were isolated from *Cnestis ferruginea* D.C. and has also been established to be responsible for the opening of the MMPT pore as reported by Adisa and Olorunsogo [30]. Some other bioactive agents characterized from medicinal plants are quercetin from onion, epigallocatechin galate (ECGC) from green tea, capsaicin in chili pepper, etc [31].

There is however paucity of information on the ability of the *H. indicum* to induce mitochondrial-mediated apoptosis.

On exposure of the intact mitochondria to the methanol extract and its various fractions in the absence of Ca^{2+}, our results clearly show that chloroform fraction has the highest inductive effect as compared to crude extract and ethylacetate fraction. While, the methanol fraction of the plant has no significant effect on the MMPT pore. The order of the inductive potency was CFHI>EFHI>MEHI>MFHI. The observation that CFHI (in the absence of Ca^{2+}) was able to open the pore in similar fashion to Ca^{2+}-enhanced opening suggests that the chloroform fraction of *H. indicum* may contain bioactive agent(s) that may be relevant in cases where mitochondrial-mediated apoptosis needs to be upregulate. It was also observed that the inductive effect increased in a concentration-dependent manner. This also suggests that the bioactive agents responsible for the induction of the pore are largely active at the highest concentrations (90 µg/ml) of the fractions as shown. However, the observation that CFHI and EFHI were able to reverse calcium-induced pore opening suggests that the plant extract may be competing with Ca^{2+} for active site or may be actually chelating Ca^{2+} by reducing its concentration available to interact with the components of the pore. Given that the opening of the MMPT pore leads to a significant increase in the inner membrane permeability, dissipation of mitochondrial membrane potential and most especially interference of ATP production, due consideration should be given to ATP synthase; an enzyme responsible for the production of ATP. Through the years, it is accepted that the assemblage of the pore component allows for the movement of protons from the matrix to the space between the inner and outer membrane of the mitochondrion thereby creating a concentration gradient. This further confirms the reason for the enhancement of ATPase activity once the pore opens, as this is measured by the concentration of inorganic phosphate, *Pi* produced from ATP hydrolysis. There are however, convincing evidences from several laboratories that propose that MMPT pore is a core part of the ATPase [32]. Interestingly, the results show that CFHI and EFHI significantly enhanced the ATPase activity. This confirms that the fractions have bioactive phytochemicals that are able to interact with pore component. This enhancement was concentration-dependent and also affirms that the bioactive component is

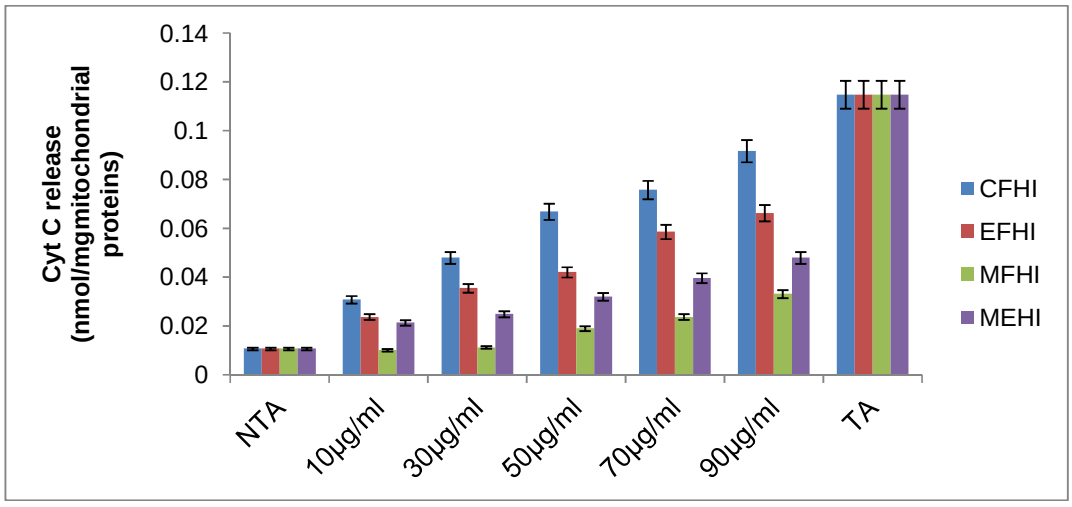

Fig. 10. Effects of varying concentrations of CFHI, EFHI, MFHI and MEHI on Cytochrome C release in normal rat liver mitochondria

Abbreviations: NTA: No Triggering Agent; TA: Triggering Agent, CFHI: Chloroform Fraction of Methanol extract of H. indicum, EFHI: Ethylacetate Fraction of Methanol extract of H. indicum MFHI: Methanol Fraction of Methanol extract of H. indicum, MEHI: Crude Methanol Extract of H. indicum

Table 1. Concentrations of inorganic phosphate released ((µmoles /mg protein/min) by different fractions of methanol extract of *H. indicum* (pH 7.4)

	CFHI	EFHI	MFHI	MEHI
Control	0.60±0.05	0.43±0.03	0.38±0.04	0.30±0.04
10 µg/ml	0.66±0.05*	0.61±0.01*	0.32±0.05	0.35±0.03
30 µg/ml	0.76±0.02*	0.63±0.01*	0.36±0.01	0.37±0.01
50 µg/ml	0.87±0.06*	0.64±0.01*	0.41±0.06	0.39±0.01
70 µg/ml	0.94±0.03*	0.70±0.03*	0.44±0.04	0.40±0.01
90 µg/ml	0.77±0.03*	0.73±0.03*	0.42±0.03	0.62±0.05
2,4-Dinitrophenol	0.94±0.14	1.15±0.12	0.96±0.12	1.04±0.04

Each value is a mean of four determinations± Standard deviation

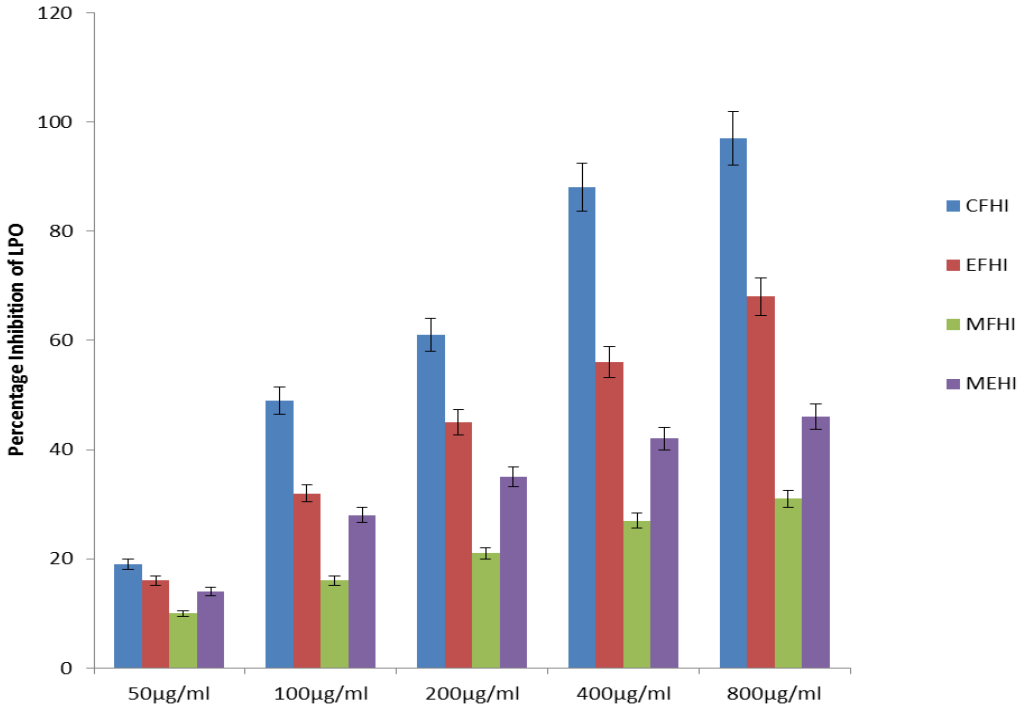

Fig. 11. Effects of varying concentrations of CFHI, EFHI, MFHI and MEHI on Fe^{2+}-induced lipid peroxidation in normal rat liver mitochondria

Abbreviation: CFHI: Chloroform Fraction of Methanol extract of H. indicum, EFHI: Ethylacetate Fraction of Methanol extract of H. indicum, MFHI: Methanol Fraction of Methanol extract of H. indicum MEHI: Crude Methanol Extract of H. indicum

highly effective at higher concentrations of the fractions. An established tag-along principle on mitochondrial-mediated apoptosis is the subsequent release of cytochrome c sequel to the opening of the MMPT pore. Results obtained show that there is release of cytochrome c following the opening of the pore. The amount of cytochrome c released was concentration-dependent in mitochondria exposed to different fractions of *H. indicum.* These results further confirmed that the induction of the MMPT pore was mitochondria-mediated as its release is a 'no-return' point for apoptosis to take place. This result followed the pattern earlier recorded with respect to effect of the extract and fractions of the plant on MMPT pore where CFHI had the highest induction, CFHI also had the highest effect on the quantity of cytochrome c released, confirming that mitochondria-mediated apoptosis could take place. The fact that the percentage inhibition of lipid peroxidation increased on exposure of the peroxide-induced mitochondria to the fractions indicates that the fractions did not induce the opening of the pore by producing

peroxides by causing damage to the lipid bilayer. This is because agents capable of oxidative stress are able to destroy the lipid bilayer of the membrane which can result to the membrane leakage and not opening of the pore, but the fractions were all able to inhibit the peroxide formation on mitochondria pre-incubated with ferrous sulphate. Furthermore this ability of the plant can play a protective physicochemical role on the membrane bilayer against the effect of free radicals that could damage the membranes. The pattern of the percentage inhibition of lipid peroxidation especially for CFHI, EFHI and MEHI were similar to that of the inductive effect of these fractions on intact mitochondria (i.e. concentration-dependent). This further confirms that the mechanism of interaction of bioactive components with mitochondria was not via damage of the lipid bilayer but rather by interaction with the molecules required to form the pore.

As highlighted earlier, apoptosis occur via various pathways. These pathways are initiated by their own signaling molecules such as caspases. For example, the intrinsic, extrinsic

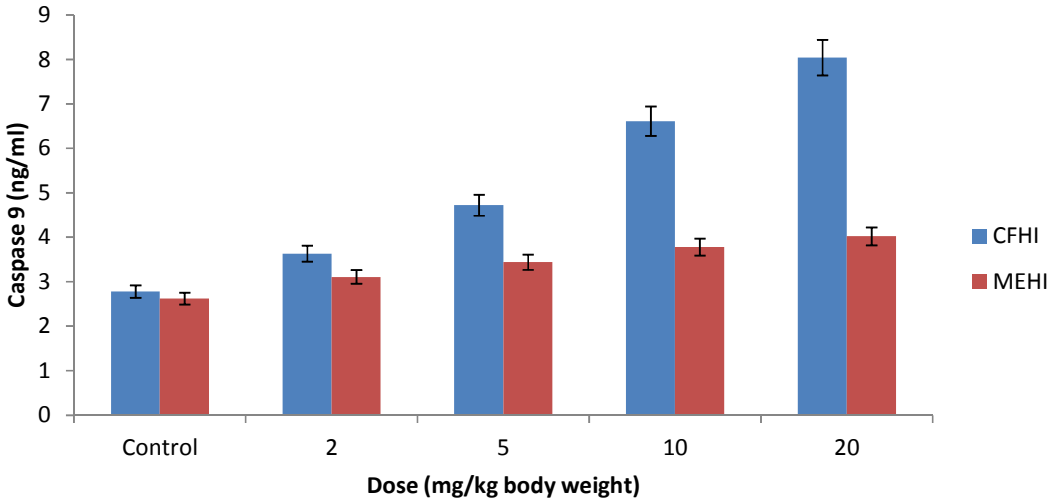

Fig. 12. Effect of varying doses of CFHI and MEHI on level of Caspase 9
CFHI: Chloroform Fraction of Methanol extract of H. indicum; MEHI: Crude Methanol Extract of H. indicum

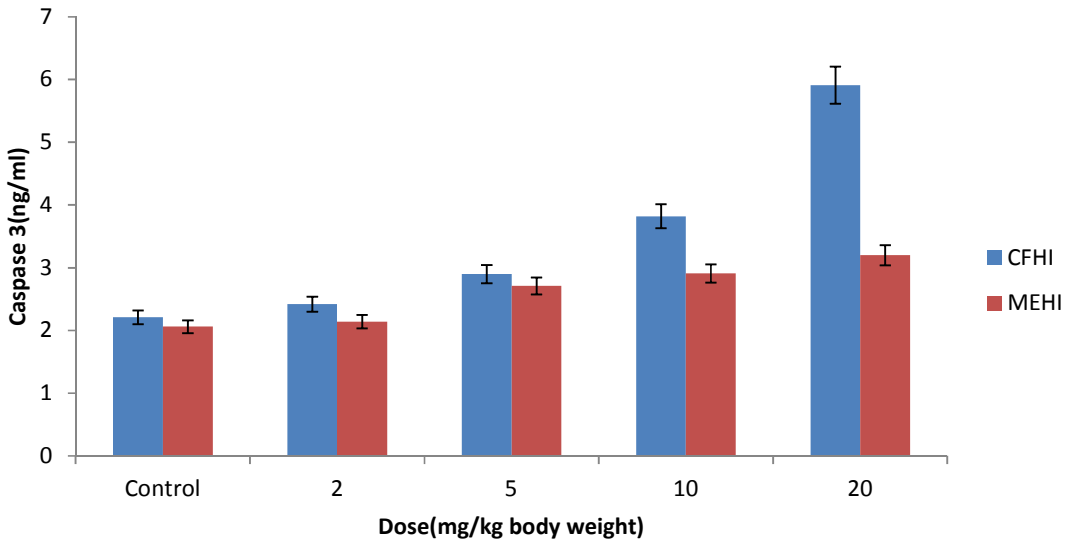

Fig. 13. Effect of varying doses of CFHI and MEHI on level of Caspase 3
CFHI: Chloroform Fraction of Methanol extract of H. indicum; MEHI: Methanol Extract of H. indicum

and perforin/granzyme B pathways can be initiated by caspase-9, caspase-8 and caspase-10, respectively [33]. On assessment of caspases (9 and 3) in wistar rats administered varying doses of MEHI and CFHI, it was observed that the activities of these two enzymes in CFHI administered rats significantly increased in a dose-dependent pattern indicating that CFHI-induced MMPT pore opening is due to activation of the mitochondrial pathway.

4. CONCLUSION

Finally, the nature of substances responsible for the effects shown by CFDC are still unknown. Therefore, there is a need for further work to elucidate and characterize the structure of putative agent(s) present in CFDC and their effect on induction of mitochondrial-mediated apoptosis. This could be relevant in the management and treatment of diseases where there is need for upregulation of apoptosis.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All experimental procedures conform to the guiding principles for research as recommended by the Declaration of Helsinki and National Institute of Health.

DISCLAIMER

Some part of this manuscript was previously presented in the following conference.
Conference name: Fifth Unibadan Conference of Biomedical Research
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Location: Conference center, University of Ibadan. Ibadan, Nigeria
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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Dash PR, Whitley GS, Ayling LJ, Johnstone AP, Cartwright JE. Trophoblast apoptosis is inhibited by hepatocyte growth factor through the Akt and beta-catenin mediated up-regulation of inducible nitric oxide synthase. Cellular Signalling. 2005;17:571-80.
2. Takenori T, Yoshinobu N. Role and pathological significance of apoptosis induced by influenza virus infection. The Open Antimicrobial Agents Journal. 2010;2:22-25.
3. Bernadi P, Krauskopf A, Basso E, Petronilli V, Blachly-Dyson E, Di Lisa F. The mitochondrial permeability transition form *in vitro* artifact to disease target. FEBS J. 2006;273:2077-99.
4. Bernardi P, von Stockum S. The permeability transition pore as Ca^{2+} release channel: New answers to an old question. Cell Calcium. 2012;52:22-7.
5. Martin KR. Targeting apoptosis with dietary bioactive agents. Exper Biol Med (Maywood). 2006;231:117-129.
6. Elmore S. Apoptosis: A review of programmed cell death. Toxicol Pathol. 2007;35(4):495–516.
7. Dong HS, Mi-Kyung K, Hee SK, Hyun HC, Yong SS. Mitochondrial permeability transition pore as a selective target for anti-cancer therapy. Rev on Frontiers in Oncology. 2013;3(Art 41):1-11.
8. Levi CA, Ejere VC, Asogwa CN, Iweh P, Nwatu KU, Levi UE. Apoptosis: Its physiological implication and therapeutic possibilities. Journal of Pharmacy and Biological Sciences. 2014;9(1):38-45.
9. Rathmell JC, Thompson CB. Pathways of apoptosis in lymphocyte development, homeostasis, and disease. Cell. 2002;109: 97-107.
10. Kroemer G, Dallaporta B, Resche-Rigon M. The mitochondrial death/life regulator in apoptosis and necrosis. Annu. Rev. Physiol. 1998;60:619–42.
11. Czabotar PE, Lessene G, Strasser A, Adams JM. Control of apoptosis by the BCL-2 protein family: Implications for physiology and therapy. Nature's Review: Molecular Cell Biology. 2014;15:49-63.
12. Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene. 2007;26:1324–1337.
13. Elustondo PA, Nichols M, Negoda A, Thirumaran A, Zakharian E, Robertson GS, Pavlov EV. Mitochondrial permeability transition pore induction is linked to formation of the complex of ATPase C-subunit, polyhydroxybutyrate and inorganic

phosphate. Cell Death Discov. 2016;2: 16070.
14. Fischer U, Schulze K. Apoptosis-based therapies and drug targets. Cell Death; 2005.
15. Ashutosh M. A study on analgesic activity of *Heliotropium Indicum* L. Stem. IJPRD. 2011;3(10):1-4.
16. Dash GK, Murthy PN. Studies on wound healing activity of *Heliotropium indicum* Linn. leaves on rats. ISRN Pharmacology. 2011;8. Article ID: 847980
17. Boyer PD. The ATP synthase – a splendid molecular machine. Annual Review of Biochemistry. 1997;66:717–749.
18. Dodehe Y, Barthelemy A, Calixte B, Jean DN, Allico JD, Nelly F. *In vitro w*ound healing effect of *n*-butanol fractions from *H. indicum*. Journal of Innovative Trends in Pharmaceutical Sciences. 2011a;2(1):1-7.
19. Dodehe Y, Barthélémy A, Christine L, Metowogo K, N'guessan JD, Allico JD, Annelise L, Nelly F. Isolation of wound healing compounds from *Heliotropium indicum.* Journal of Applied Pharmaceutical Science. 2011b;01(10):102-106.
20. Johnson D, Lardy H. Isolation of liver or kidney mitochondria. Methods Enzymol. 1967;10:94-96.
21. Olorunsogo OO, Bababunmi EA, Bassir O. Uncoupling effect of N-phosphonomethylglycine on at liver mitochondria. Biochem. Pharm. 1979;27: 925-927.
22. Lapidus RG, Sokolove PM. Spermine inhibition of permeability transition of isolated rat liver mitochondria; An investigation of mechanism. Arch Biochem Biophys. 1993;306(1):246-253.
23. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. J Biol Chem. 1951;193:265-275.
24. Olorunsogo OO, Malomo SO. Sensitivity of Oligomycin-inhibited respiration of isolated rat liver mitochondriato perfluidone, a fluorinated arylalkylsulfonamide. Toxicology. 1985;35(3):231-40.
25. Bassir O. Handbook of practical biochemistry. Ibadan University Press, Ibadan, Nigeria. 1963;13.
26. Ruberto G, Baratta M, Deans S, Dorman H. Antioxidant and antimicrobial activity of *Foeniculum vulgare* and *Crithmum maritimum* essential oils. Planta Med. 2000;66:687-693.
27. Appaix F, Minatchy M, Riva-Lavieille C, Olivaires J, Antonnson B, Saks VA. Rapid spectrophotometric method for quantitation of cyctochrome c release from isolated mitochondria or permealized cells revisited. Biochimica et Biophysica Acta. 2000;1457:175-181.
28. Miura M, Zhu H, Rotello R, Hartwieg EA, Yuan J. Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homolog of the C. elegans cell death gene ced-3. Cell. 1993;75(4):653-60.
29. Raff MC, Barres BA, Burne JF, Coles HS, Ishizaki Y. Programmed cell death and the control of cell survival: Lessons from the nervous system. Science. 1993;262(5134): 695-700.
30. Adisa RA, Olorunsogo O. Robustaside B and *para*-hydroxyphenol: Phenolic and antioxidant compounds purified from *Cnestis ferruginea* D.C induced membrane permeability transition in rat liver mitochondria. Molecular Medicine Reports. 2013;8:1493-1498.
31. Bernardi P, Petronilli V. The permeability transition pore as a mitochondrial calcium release channel: A critical appraisal. J. Bioenerg. Biomembr. 1996;28:129–36.
32. Alavian KN, Beutner G, Lazrove E, Scccheti S, Park H, Licznerski P, et al. An uncoupling channel within the c-subunit ring of the FiFO ATP synthase is the mitochondrial permeability transition pore. PNAS. 2014;29:10580-10585.
33. Sadowski-Debbing K, Coy JF, Mier W, Hug H, Los M. Caspases – Their role in apoptosis and other physiological processes as revealed by knock-out studies. Archivum Immunologiae et Therapiae Experimentalis. 2002;50:19–34.

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