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Research Article

Induction of Mitochondrial Membrane Permeability Transition Pore Opening and Cytochrome C Release by Fractions of *Drymaria cordata*

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Abstract

Inducers or inhibitors of opening of the Mitochondrial Membrane Permeability Transition (MMPT) Pore are targets of drug development for conditions arising from dysregulated apoptosis. In this study, the effects of various fractions of the methanol extract of *Drymaria cordata* (DC), a medicinal plant were investigated on (MMPT) pore in the presence and absence of calcium. The defatted methanol extract of the whole plant (MEDC) was partitioned in succession between chloroform, ethylacetate and water. The fractions were concentrated at 40^0C to obtain chloroform (CFDC), ethylacetate (EFDC) and aqueous (AFDC) fractions. Varying concentrations of the fractions in the absence of calcium significantly induced pore opening by 2.5, 4.6,10 and 13 folds for MEDC and 3.6, 13, 15, 18 and 17 folds for CFDC, respectively, while EFDC and AFDC did not have any significant effect at lower concentrations but induced pore opening at 90µg/ml by 3.1 and 1.8 folds, respectively. Also, all the solvent fractions caused the release of cytochrome c in a concentration-dependent manner with CFDC giving the highest release of cytochrome c. In contrast, Ca^{2+}-induced MMPT pore opening was inhibited by MEDC and CFDC. At concentrations of 10, 30, 50, 70 and 90µg/ml, the percentage inhibition was 38, 48, 50, 57 and 65% for MEDC and 7, 14, 15, 28 and 34% for CFDC , respectively, when compared with the effect of spermine, a standard inhibitor. EFDC and AFDC fractions however, did not inhibit pore opening but rather had synergistic effect with calcium. F_IF_O-ATPase activity was enhanced by all the fractions with CFDC having the highest effect. Also, all the fractions ameliorated ferrous-induced mitochondrial membrane lipid peroxidation. These results suggest that the chloroform fraction is the most potent and possibly contains the bioactive agent that may induce mitochondrial-mediated apoptosis..

Keywords: *Drymaria cordata*, Mitochondrial Membrane Permeability Transition Pore

INTRODUCTION

Apoptosis, a form of programmed cell death, is a crucial physiologic process in the development and homeostasis of multicellular organisms (Baehrecke, 2002). Perturbation of this vital process leads to a range of diseases, such as ischemia, cancer, neurodegeneration, and autoimmunity (Saikumar *et al*., 1999). Apoptosis occurs primarily through two well-recognized pathways in cells (Lockshin and Zakeri; Danial and Korsmeyer, 2004). Both effector mechanisms of apoptosis are associated with caspase activation and include the intrinsic, or mitochondrial-mediated, and the extrinsic, or death receptor–mediated, effector mechanism (Reed, 2004). In recent years the central role of mitochondria in apoptotic and necrotic cell death has become apparent, and a major player in this arena is the mitochondrial permeability transition (MPT) pore (Crompton, 1999; Halestrap 1999). The pore is a protein that is formed in the inner membrane of the mitochondria under certain pathological conditions such as traumatic brain injury and stroke. Induction of the permeability transition pore can lead to mitochondrial swelling and cell death through apoptosis or necrosis (Fiskum, 2000). Permeabilization of the outer mitochondrial membrane has been recognized as a major event in the induction of the mitochondrial pathway of apoptosis because opening of the pore causes the release of a range of proapoptotic proteins from the mitochondrial intermembrane space into the cytosol (Yang *et al*.,1997; Lawen, 2003; Green and Kroemer, 2004) This include cytochrome c (Liu *et al*.,1996), AIF (Susin *et al*.,1999), Smac/Diablo (Du *et al*,. 2000), the serine protease HtrA2/Omi and endonuclease G (Suzuki et al., 2001). The release of cytochrome c into the cytosol triggers caspase activation and ultimately apoptosis (Martins, 2006; Xiameng *et al*., ,2014) . The nature of the pore that releases these proteins is still uncertain and the identity of the proteins involved in its formation is highly controversial (Ly *et al*., 2003).

It is clear that the MMPT pore plays an important role in cell death and understanding the molecular mechanisms of MPTP opening may lead to the development of drugs that target the components of the MPT pore.(Stavrovskaya and Kristal, 2005). Research has shown that certain bioactive agents present in medicinal plants elicit their chemoprotective and therapeutic effects through the induction or inhibition of the opening of the pore (Tascilar *et al*., 2006). *Drymaria cordata* (Linn.) Willd (Caryophyllaceae) is a weak spreading herb found widely dispersed in damp places all over the tropics

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of Africa, Asia and the Americas . It is a sprawling herb with procumbent and more or less ascending branched stems, often rooting at the lower nodes, quadrangular, glabrous or papillose especially in the upper internodes, which are slender, generally 2-6 cm long. In tropical Africa, *D. cordata* preparations are used for the treatment of diverse ailments including cold, headache, bronchitis, as poultice on sore (to treat aching, inflamed or painful parts), leprosy, tumors, as fumigant for eye troubles, as cerebral stimulant and antifebrile agent (Burkill, 1985). Extracts of the plant have been reported to possess antitussive (Mukherjee *et al.,* 1997), anti-bacterial (Mukherjee *et al*., 1996), anti-inflammatory anxiolytic (Barua *et al*., 2009), cytotoxic (Sowemimo *et al*., 2009) and analgesic (Adeyemi *et al*., 2008) properties. It is not known whether any of the phytochemical constituents of the plant would modulate intrinsic pathway of apoptosis. This study was designed to determine the modulatory effect of various fractions of the extract of *Drymaria cordatra* on mitochondrial-mediated apoptosis via the release of cytochrome c.

MATERIALS AND METHODS

Mannitol, sucrose, N-2-hydroxy-ethyl-pipe-arizine-N-2-ethanesulfonic acid (HEPES), rotenone, spermine, Folin-Ciocalteu 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

Plant material and experimental animals

Fresh leaves of *Drymaria cordata* were obtained from a local farmland in Ibadan,Oyo State, Nigeria. Botanical identification and authentication was done at the Department of Botany, Faculty of Science, University of Ibadan, Ibadan, Nigeria .The fresh plant was separated from extraneous materials and washed. The leaves were air dried for about two weeks and then blended to powder. Male Wistar strain rats weighing between 100-120g were obtained from the Pre-clinical Animal House, University of Ibadan, Ibadan, Nigeria. The animals were allowed to acclimatize for 14 days in cages in the Animal House of the Department of Biochemistry, University of Ibadan. All animals had access to water and chow *ad libitum* and were kept under standard conditions of temperature and humidity

Solvent Partitioning

Distiled water was added to 90g of dried methanol extract to form a slurry which was partitioned repeatedly with n-hexane using a separating funnel flask until exhaustion. The remaining marc was further partitioned with chloroform until exhaustion. Finally, the marc was partitioned with ethylacetate until exhaustion and the remaining aqueous was collected. All these fractions were concentrated to dryness under pressure using rotary evaporator at 40^{o}C to obtain the n-hexane (HF), chloroform (CF), ethylacetate (EF) and the aqueous (AF) fractions.

Methods

Isolation of rat liver mitochondria

Rat liver mitochondria were isolated essentially according to the method of Johnson and Lardy (1967) and as modified by Olorunsogo *et al* (1979). The animals were sacrificed by cervical dislocation and the livers excised and trimmed to wash excess tissue. The livers were then weighed, washed with homogenising buffer (210mM Mannitol, 70mM Sucrose, 5mM HEPES-KOH, pH 7.4 and 1mM EGTA), and homogenised as a 10% suspension in ice –cold buffer using a Porter Elvehjem glass homogeniser. The resulting homogenate was centrifuged in an MSE refrigerated centrifuge at 2300 rpm for 5 mins to remove the nuclear debris. This was done twice and the supernatant obtained was centrifuged at 13,000 rpm for 10 mins to obtain the mitochondrial pellet. The supernatant was discarded while the pellet was washed twice with the washing buffer (210mMMannitol, 70mM sucrose, 5mM HEPES-KOH, pH 7.4, 0.5% BSA) at 12,000 rpm for 10 mins. The mitochondria obtained were immediately resuspended in an appropriate volume of MSH buffer (210mM Mannitol,70mM sucrose, 5mM HEPES-KOH, pH 7.4), and immediately dispensed into eppendorf tubes and kept on ice.

Mitochondrial swelling assay

Mitochondrial membrane permeability transition was monitored by measuring changes in absorbance of mitochondrial suspension in the presence or absence of calcium (the triggering agent) in aT70 UV/visible spectrophotometer essentially according to the method of Lapidus and Sokolove (1993). Mitochondria (0.4mg protein/ml) were preincubated in the presence of 0.8µM rotenone in a medium containing 210mM mannitol, 70mM sucrose and 5mM HEPES-KOH (pH 7.4) for 3mins at 27^{0}C prior to the addition of 120µM $CaCl_2$. Thirty seconds later, 5mM succinate was added and mitochondrial permeability transition quantified at 540nm for 12mins at 30secs interval. To test the intactness of the mitochondria, 4mM spermine was added immediately following the addition of rotenone and just before the addition of mitochondrial fraction.

Determination of mitochondrial protein

Mitochondrial protein concentration was determined according to the method of Lowry *et al*. (1951) using bovine serum albumin as standard.

Assesment of Mitochondrial FoF_1 ATPase Activity

FoF_1 Adenosine triphosphatase which was determined by the method of Lardy and Wellman (1953) as modified by Olorunsogo and Malomo (1985). Each reaction mixture contained 65mM Tris-HCl buffer pH 7.4, 0.5Mm KCl 1Mm ATP and 25Mm sucrose. The reaction mixture was made up to a total volume of 2ml with distilled water. The reaction was started by the addition of mitochondrial suspension and was allowed to proceed for 30 mins at 27^{0}C. The reaction was stopped by the addition of 1 ml of a 10% solution of sodium dodecyl sulphate. The zero time tube was prepared by adding the solution of ATP to the reaction vessel following the addition of sodium dodecyl sulphate. 2,4 Dinitrophenol (2,4 DNP) was used as a standard uncoupling agent.

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 (1963) and as modified by Olorunsogo and Bababunmi (1979). 300µl of each solution was dispensed into fresh test tubes, followed by the addition of 300µl of distilled water to each of the test tube. To this was added 1 ml of 5% ammonium molybdate and 1 ml of 9%

freshly prepared solution of ascorbic acid. The tube was well mixed and allowed to stand for 20 minutes. The absorbance was read at 680nm.A water blank was used to set the spectrophotometer at zero.

Inhibition of lipid peroxidation

A modified thiobarbituric acid reactive species (TBARS) assay was used to measure the lipid peroxide formed using mitochondria as lipid rich media (Ruberto *et al*., 2000) . Mitochondria (2mg/ml protein) and varying concentrations (100µg/ml – 800µg/ml) of fraction were added to each test tube and made up to 1 ml with distilled water; 0.05 ml of FeSO4 (0.07 M) was added to induce lipid peroxidation and the mixture incubated for 30 min. Then, 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 added and the resulting mixture was vortexed and then heated at 95°C for 60 min. After cooling, 3.0 ml of butan-1-ol were added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532nm .Percentage inhibition of lipid peroxidation by the extract was calculated as [AC-AE/AC] x 100. Where AC is the absorbance value of the fully oxidized control and AE is the absorbance in the presence of extract.

Assay of Cytochrome C release

The quantitative determination of cytochrome c released from isolated mitochondria was performed by measuring the Soret (γ) peak for cytochrome c at 414 nm (ε= 100 mM^{-1} cm^{-1}), according to method of Appaix *et al*., (2000). Mitochondria (1mg protein/ml) were preincubated in the presence of 0.8µM rotenone in a medium containing 210mM mannitol, 70mM sucrose and 5mM HEPES-KOH (pH 7.4) for 30mins at 27^0C in the presence of different concentrations of the fractions, using 24mM calcium as the standard (TA). After the incubation, the mixture was centrifuged at 15,000 rpm for 10 mins . The optical density of the supernatant was measured at 414nm which is the soret (γ) peak for cytochrome c .

Statistical Analysis of Data

Statistical analysis was performed using one way analysis of variance (ANOVA). Level of significance was set at $p < 0.05$ and all the results were expressed as mean ± standard deviation (SD).

RESULTS

The data presented in Figure 1 show that there was no significant change in the volume of intact mitochondria respiring on succinate in the presence of rotenone over a period of twelve minutes. As seen from the figure, addition of calcium caused highly significant increase in MMPT pore opening and were reversed by spermine. Figure 2 shows the effects of various concentrations of MEDC on MMPT pore in the absence of calcium. From the results, 10µg/ml, 30µg/ml, 50µg/ml, 70µg/ml and 90 µg/ml induced pore opening by 2.5, 4.6, 10 and 13 folds, respectively, in a concentration – dependent manner when compared to control. Maximum induction (13 fold) was seen at (90µg/ml) and the least induction fold (2.5) was obtained at (10µg/ml) when compared to the control. In Figure 4 the effects of varying concentrations of CFDC on the pore in the absence of calcium were clearly depicted. Here, different concentrations of the fraction exhibited a significant induction of pore opening by 3.6, 13, 15, 18 and 17 folds, respectively, at 10 µg/ml, 30µg/ml, 50 µg/ml, 70 µg/ml and 90 µg/ml, respectively. Like calcium-induced pore opening, the MEDC and CFDC induction of pore opening was reversed by spermine

Figures 6 and 8 show the effects of EADC and AFDC on MMPT pore in the absence of calcium. At lower concentrations (10µg/ml, 30µg/ml, 50 µg/ml, 70 µg/ml), both fractions had no significant effect on pore opening. However, at 90 µg/ml, both fractions had significant inductive effect of 3.1 and 1.8 folds, respectively.

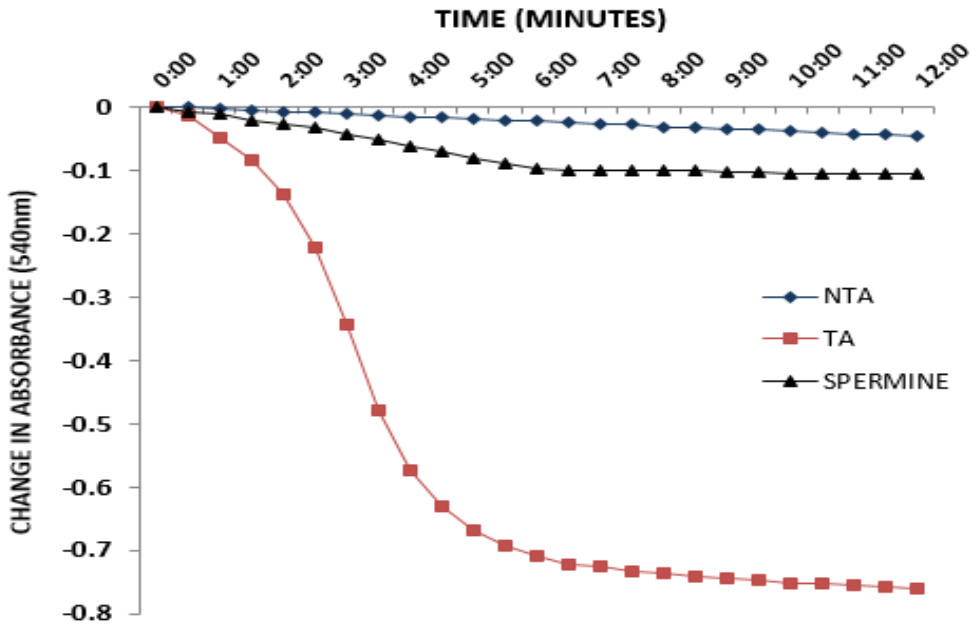

Fig 1:
Calcium-induced mitochondrial membrane permeability transition pore opening in normal rat liver mitochondria and its reversal by spermine. NTA: No triggering agent;
TA : Trigering agen

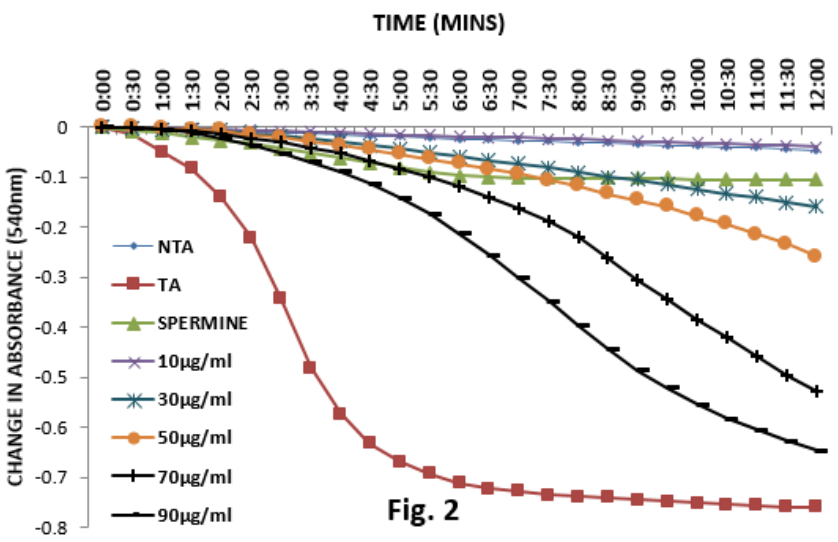

Fig 2 :
Effects of varying concentrations of crude methanol extract of *Drymaria cordata* (MEDC)on rat liver mitochondrial membrane permeability transition pore in the absence of calcium. NTA ; No triggering agent ; TA; Trigerring agent

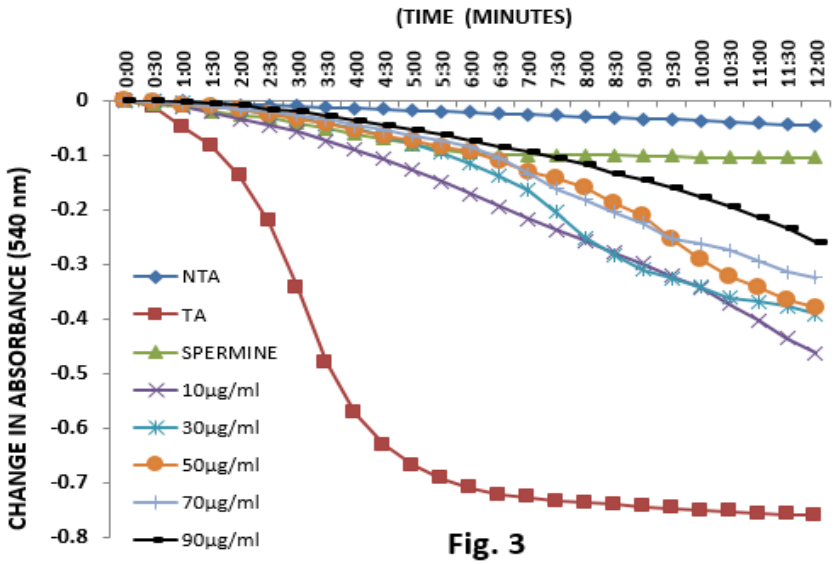

Fig 3:
Effects of varying concentrations of crude methanol extract of *Drymaria cordata* (MEDC) on the mitochondrial membrane permeability transition pore in the presence of calcium.
NTA: No triggering agent ; TA; Triggering agent

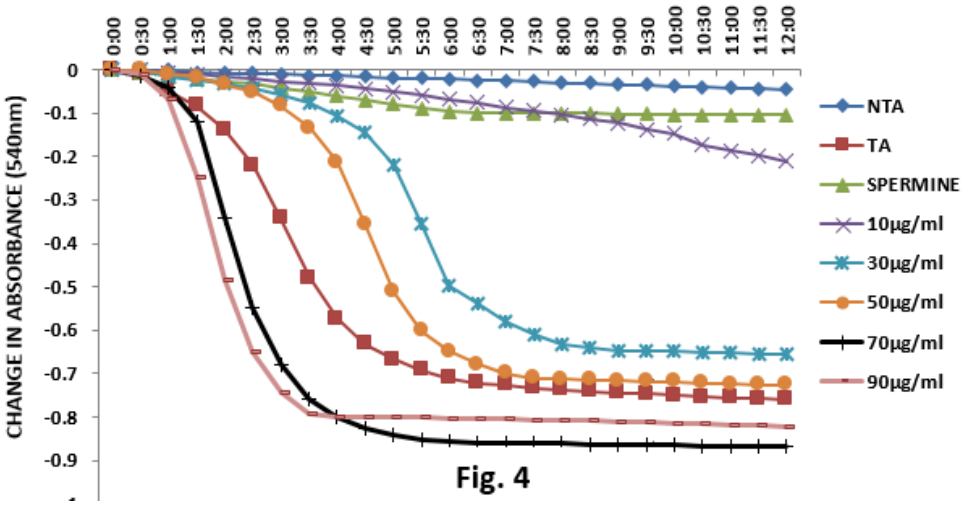

Fig 4: Effects of varying concentrations of chloroform fraction of *Drymaria cordata* (CFDC) on the mitochondrial membrane permeability transition pore in the absence of calcium. NTA: No triggering agent; TA; Triggering agent

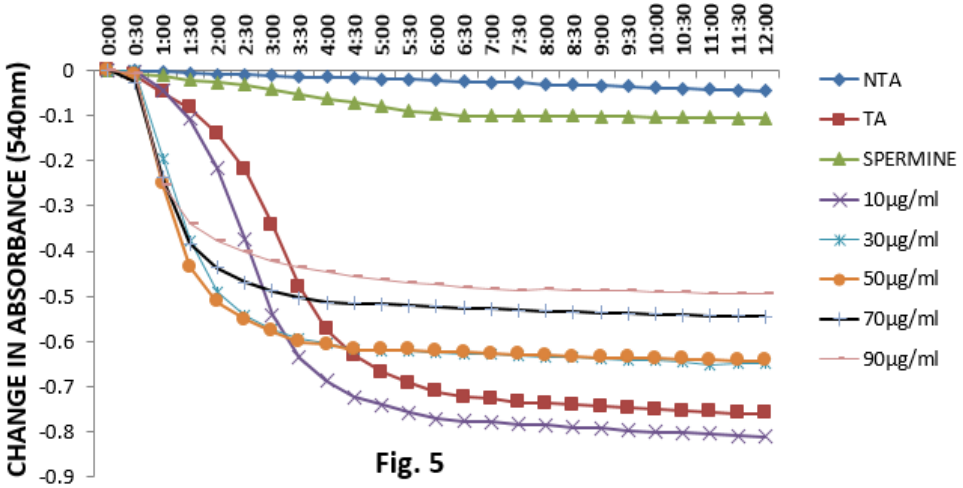

Fig 5 :
Effects of varying concentrations of chloroform fraction of *Drymaria cordata* (CFDC) on the mitochondrial membrane permeability transition pore in the presence of calcium. NTA : No triggering agent; TA: Triggering agent

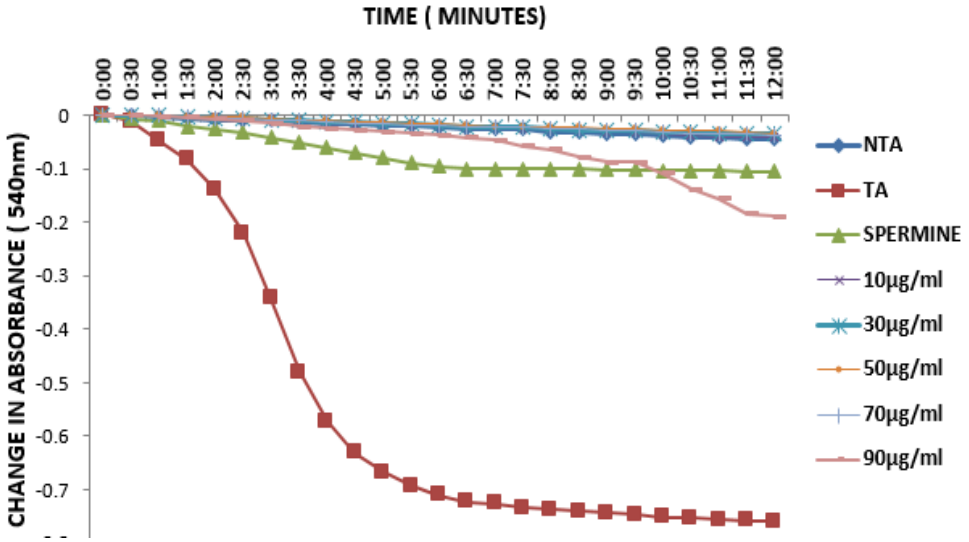

Fig 6: Effects of varying concentrations of ethylacetate fraction of *Drymaria cordata* (EFDC) on the mitochondrial membrane permeability transition pore in the absence of calcium. NTA : No triggering agent; TA: Triggering agent.

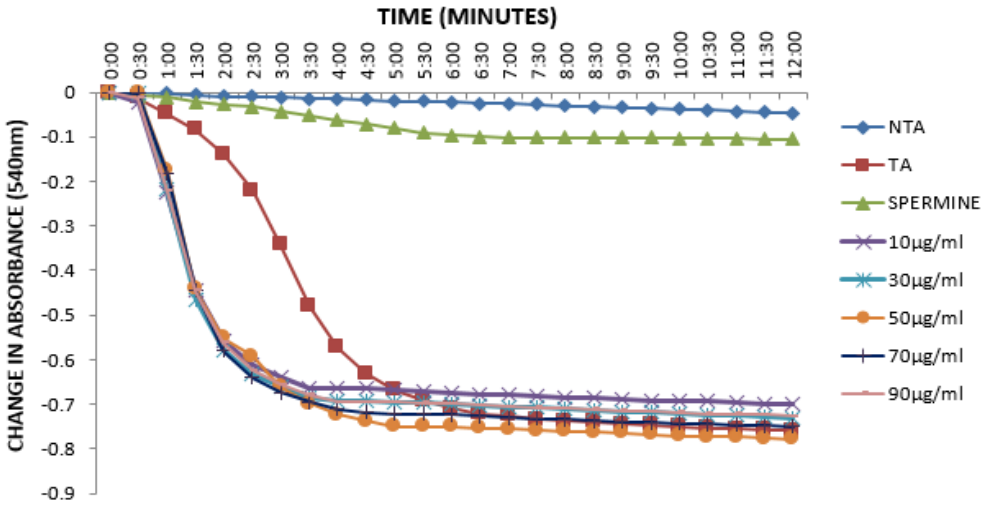

Fig 7: Effects of varying concentrations of ethylacetate fraction of *Drymaria cordata* (EFDC) on the mitochondrial membrane permeability transition pore in the presence of calcium. NTA: No triggering agent; TA: Triggering agent.

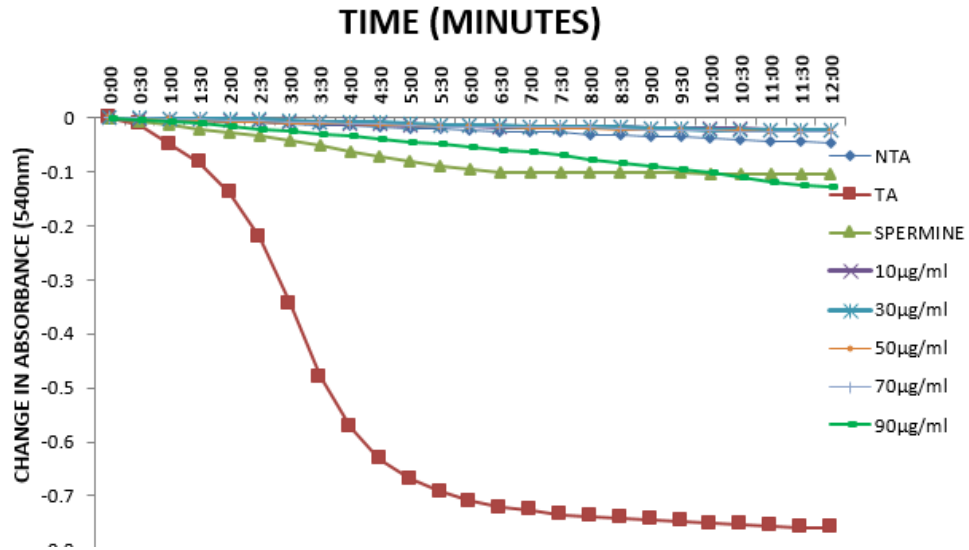

Fig 8: Effects of varying concentrations of aqueous fraction of *Drymaria cordata* (AFDC) on the mitochondrial membrane permeability transition pore in the absence of calcium**.** NTA: No triggering agent; TA: Triggering agent

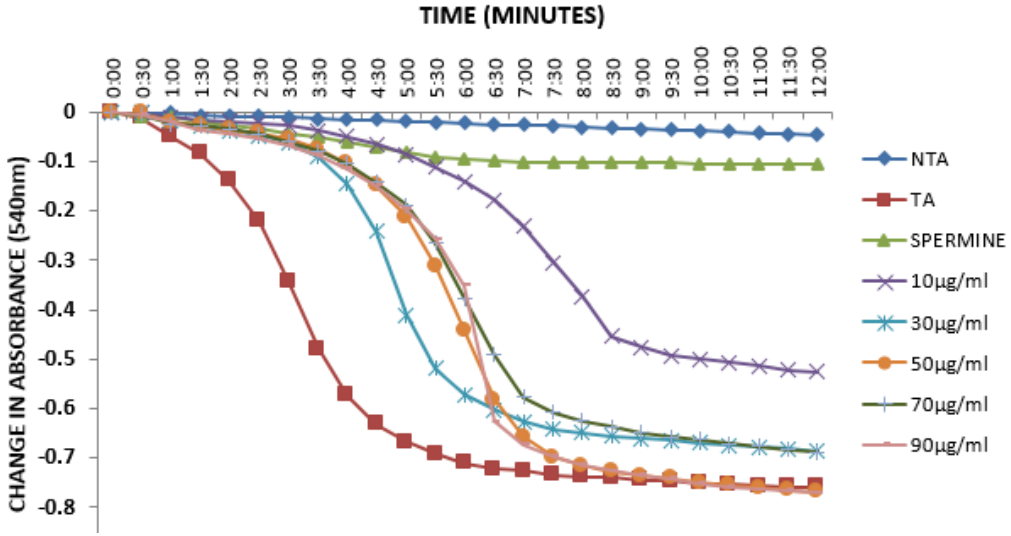

Fig 9: Effects of varying concentrations of aqueous fraction of *Drymaria cordata* (AFDC) on the mitochondrial membrane permeability transition pore in the presence of calcium. NTA: No triggering agent ; TA: Triggering agent.

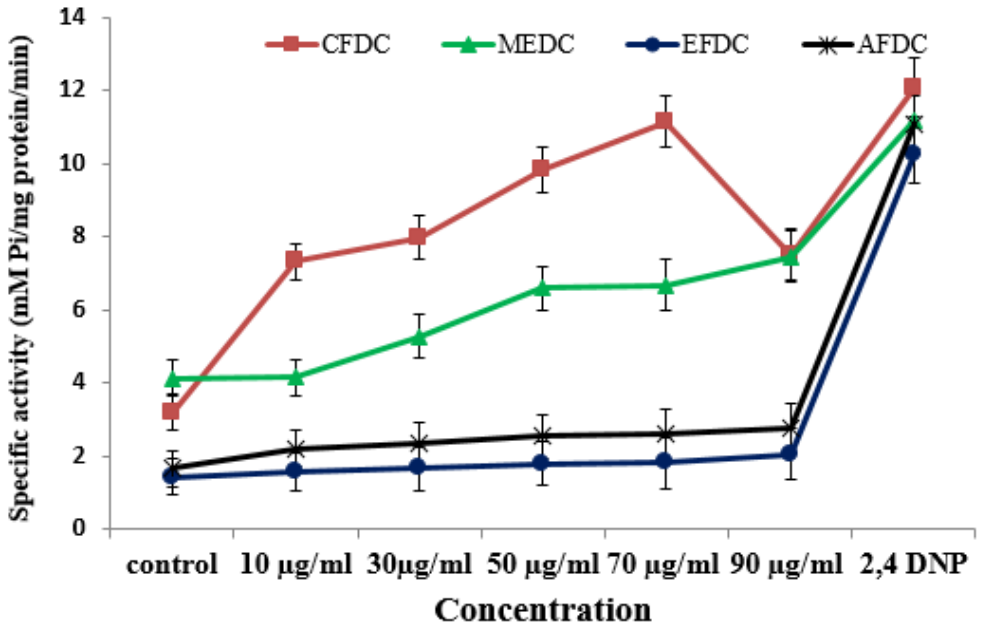

Fig 10 : Effects of different fractions of *drymaria cordata* on specific activity of rat liver mitochondrial F_0F_1 ATPase. Each value is a mean of 4 determinations ± s d

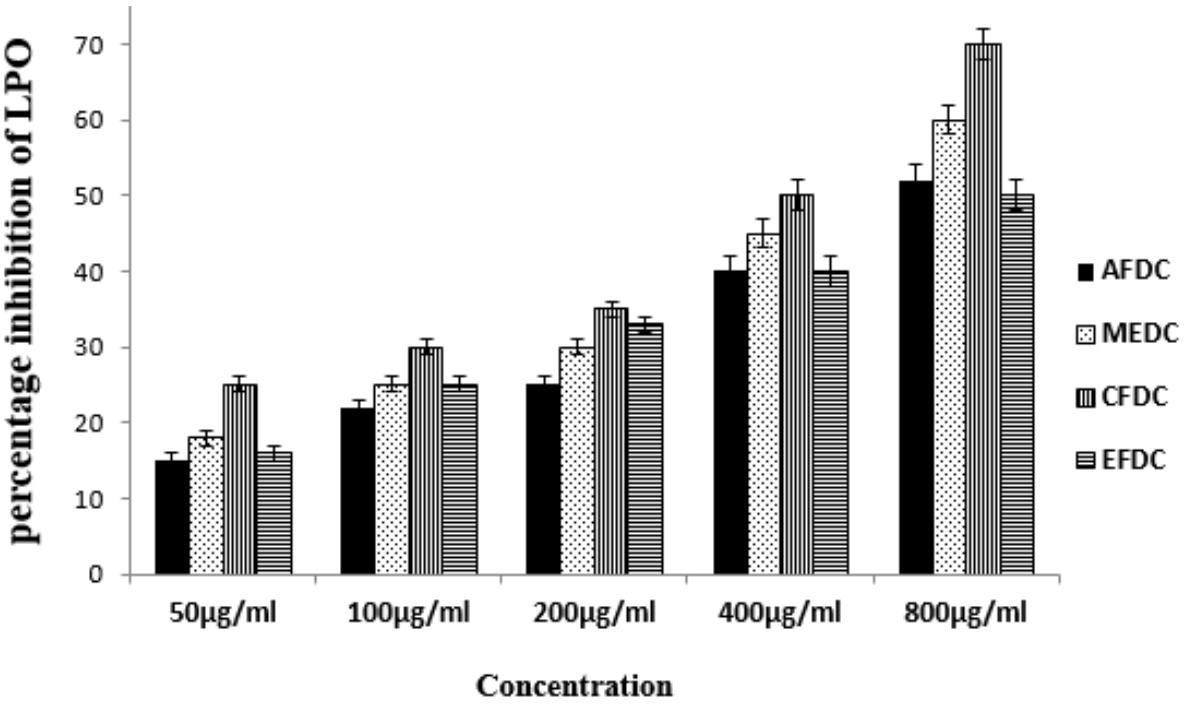

Fig 11: Percentage inhibition of ferrous-induced lipid peroxidation by different fractions of *Drymaria cordata* Each value is a mean of 4 determinations ± s d

The effects of MEDC and CFDC on MMPT pore in the presence of calcium are shown in Figures 3 and 5. The results show that calcium-induced opening was inhibited in a concentration-dependent manner ($P<0.05$). In this regard, MEDC at 10 µg/ml, 30µg/ml, 50 µg/ml, 70 µg/ml and 90 µg/ml inhibited calcium-induced opening respectively by 38, 48, 50, 57 and 65% while CFDC at the same concentrations reduced pore opening by 7, 14, 15, 28 and 34%, respectively. Spermine, a standard inhibitor, showed 81% inhibition. However, similar concentrations of EFDC and AFDC did not inhibit calcium-induced opening as shown in Figures 7 and 9. Figure 10 shows the effects of varying concentrations of the methanol extract and its fractions on mitochondrial F_0F_1 ATPase activity. The data obtained show that MEDC, CFDC, EFDC and AFDC significantly ($P<0.05$) stimulated the ATPase activity to varying degrees. Furthermore, the results show that CFDC had the highestt stimulatory effect followed by MEDC. However, the stimulatory effects shown by EFDC and AFDC were very low and also statistically not significantly different from each other. Inhibition of ferrous-induced lipid peroxidation by fractions of *Drymaria cordata* are shown in Figure 11. The results show that MEDC, CFDC, EFDC and AFDC ameliorated ferrous-induced lipid peroxidation in a concentration-dependent manner..

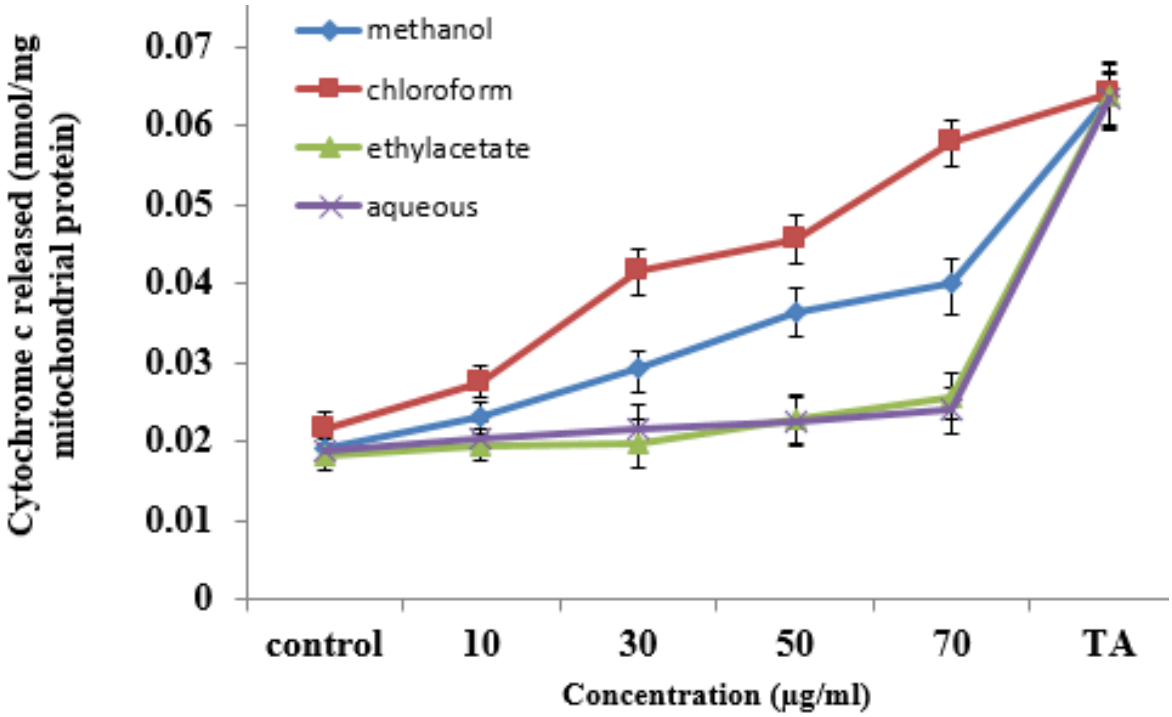

Fig 12: Effects of solvent fractions of crude methanol extract of *Drymaria cordata* on cytochrome c release. Each value is a mean of 4 determinations ± s d

The effects of varying concentrations of the solvent fractions of *Drymaria cordata* on cytochrome c release are shown in Figure 12. As seen from the results, there was significant release of cytochrome c from the mitochondria following exposure to these fractions; the extent of release being CFDC > MEDC while EFDC and AFDC are not significantly different from control.

DISCUSSION

Apoptosis is one of the most potent defense mechanism since it eliminates potentially deleterious, mutated cells (Fischer ,*et al*., 2005). The pathogenesis of many diseases, including cancer, is closely connected with aberrantly regulated apoptotic cell death. Numerous novel approaches are currently being pursued, including gene therapy, antisense strategies, recombinant biology, and classical organic and combinatorial chemistry, to target specific apoptotic regulators (Finley, 2005). An obvious and efficacious approach that is gaining broader acceptance is nutritional modulation via the myriad bioactive components present in functional foods (Faya *et al*., 2014). Experimental evidence indicates that certain bioactive agents present in medicinal plants e.g. epigallocatechin gallate, capsaicin in chili pepper, quercetin in onions, resveratrol in grape, organo-sulfur compounds in garlic, and lycopene in tomatoes among many others, can modulate apoptosis, especially at the stage of the permeabilisation of the mitochondrial membrane (Martins, 2006 ; Faya *et al*., 2014). A combination of *Drymaria cordata* and *Dissotis rotundifolia* is used in the traditional treatment of uterine fibroid, a benign tumor that grows from the muscle layers of the uterus.

In this study, the modulatory effects of extract of *Drymaria cordata* were investigated on mitochondrial-mediated apoptosis by measuring the opening of MMPT pore in isolated mitochondria. This involves the determination of the susceptibility of the pore to exogenous calcium that must first enter the mitochondria. This was done by monitoring the calcium-induced decrease in light scattering that reflects the mitochondrial swelling accompanying calcium-induced opening of the pore and its reversal by spermine, a standard inhibitor of pore opening. (Lapidus and Sokolove,1993; Javadov and Karmazyn, 2007. This showed that the mitochondria used for this study were intact *ab initio* and suitable for the experiment. When intact mitochondria were exposed to MEDC and CFDC in the absence of calcium, there was significant opening of the pore in a concentration-dependent manner compared to NTA. Moreover, the opening of the pore was reversed by spermine showing that MEDC and CFDC induction of pore opening had no deleterious effect on membrane integrity. These findings suggest that MEDC and CFDC may contain certain bioactive agents that could interact with the components of the pore and thereby cause the opening of the pore and apoptosis. Moreover, since the effect was concentration-dependent, it may be deduced that the active principle increased with increase in concentration of the fraction used. Also, the fact that MEDC and CFDC inhibited lipid peroxidation shows that the mechanism of induction of pore opening is not via damage of lipid bilayer by peroxidation. This further shows that the fractions could play a role in protecting the physicochemical properties of membrane bilayers from free radical-induced induced damage. The observation that EFDC and AFDC had no effect on MMPT pore at lower concentrations and little effect at the highest concentration used indicates the presence of low concentration of this active principle in the these fractions compared to MEDC and CFDC. These studies show that the active component responsible for the opening of the pore is highest in the chloroform fraction of the MEDC. Conversely, in the presence of calcium, all the concentrations of MEDC and CFDC caused reversal of calcium-induced pore opening when compared to spermine. This may be due to the presence of certain phytochemicals which have calcium chelating property thereby reducing the concentration of calcium

available to induce pore opening. The EFDC and AFDC had no significant effect on calcium-induced pore opening.

The release of cytochrome c from the matrix as a result of the opening of the pore has been shown to be a point of no return for apoptosis (Green and Kroemer, 2004). In the presence of CFDC, the concentration of cytochrome c released from the mitochondria was highest at all the concentrations used compared to other fractions. The results confirm that the opening of the pore occurred and resulted in the translocation of cytochrome c from the inter membrane space to the cytosol. This *is sine qua non* for apoptosis. This result conforms with the MMPT result where CFDC had the highest effect. However, the stimulatory effect shown by EFDC and AFDC is very low and statistically not significant. Opening of the MMPT pore has been shown to cause the release of mitochondrial factors, the dissipation of membrane potential ($\Delta\psi$), loss of the biochemical homeostasis of the cell and ATP hydrolysis [Kroemer, 2000; Kroemer, 1997].

The modulatory effect of MEDC, CFDC, EFDC, AFDC, on mitochondrial F0F1 ATPase activity was investigated. The highest effect on ATPase activity was observed with CFDC, followed by MEDC. This result confirms that certain phytochemicals present in these fractions were able to interact with the pore and as a result, caused opening of the pore and subsequently, the release of cytochrome c from the mitochondrial inter membrane space. .Also, the results from the lipid peroxidation show that all the fractions of *Drymaria cordata* prevented lipid peroxidation-induced damage. Compounds that inhibit membrane phospholipids peroxidation may be exerting a pharmacological effect in the prevention of radical-induced oxidative pathological events (Ruberto et al., 2000) . Mitochondria exposed to Fe^{2+} in the presence of ascorbate exhibit lipid peroxidation by the decomposition of lipid hydroperoxides to yield alkoxy or peroxyl radicals, leading to the chain reaction of lipid peroxidation (Recknagel et al.,1998. Extracts of *Drymaria cordata* ameliorated mitochondrial lipid peroxidation to varying degrees. This also shows that the induction of opening shown by the solvent fractions was not via lipid peroxidation as the fractions were able to inhibit the lipid peroxidation in a concentration-dependent manner.

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.

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