



INDUCTION OF APOPTOSIS IN RAT LIVER CELLS VIA CASPASE ACTIVATION BY CHLOROFORM FRACTION OF METHANOL EXTRACT OF *DRYMARIA CORDATA*

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ABSTRACT

Modulators of mitochondrial Permeability Transition (mPT) pore opening are targets of drug development for conditions arising from dysregulated apoptosis. *Drymaria cordata* (DC) is a medicinal plant used locally in the treatment of tumors, but there is paucity of scientific information to justify these folkloric claims. In this study, the effects of chloroform fraction of the methanol extract of *Drymaria cordata* (CFDC), was investigated on mitochondrial-mediated apoptosis via induction of pore opening. The methanol extract (MEDC) was partitioned successively to obtain n-hexane (NFDC), chloroform (CFDC), ethylacetate (EFDC) and methanol (MFDC) fractions. The animals were equally divided into four groups. Groups I, II, III and IV were treated orally with distilled water, CFDC(50mg/kgbdwt), CFDC(100mg/kgbdwt) and CFDC(200mg/kgbdwt), respectively. Rat liver mitochondria were isolated by differential centrifugation. The effects of CFDC on mPT pore, mitochondrial ATPase activity, mitochondrial membrane lipid peroxidation, cytochrome C release, extent of DNA fragmentation, Caspases 9 and 3 activation and expressions of anti-apoptotic Bcl-2 protein were investigated. Data were analysed using ANOVA at $\alpha_{0.05}$. Oral administration of CFDC at 50, 100 and 200mg/kgbdwt caused the induction of mPT pore opening by 25, 38 and 50%, respectively, and hepatic DNA fragmentation by 20%, 37% and 60%, respectively. CFDC showed significant enhancement of mitochondrial ATPase activity, activation of caspase 9 & 3 and positive immunohistochemical expression of cytochrome c and downregulation of anti-apoptotic Bcl-2 expression. These results suggest that CFDC contains bioactive agent that can induce mitochondrial-dependent cell death which might be relevant in situations where apoptosis needs to be upregulated.

KEY WORDS: *Drymaria cordata*, Mitochondrial Membrane Permeability Transition Pore (MMPT), Apoptosis, caspase.

1. INTRODUCTION

The induction of the mitochondrial membrane permeability transition (MMPT) pore has been implicated in the cascade of events involved in apoptosis (programmed cell death). The intrinsic or mitochondrial-mediated pathway of apoptosis initiated by DNA damage and endoplasmic reticulum stress, leads to permeabilizations of the mitochondrial outer membrane.^[1] However, permeabilization of the mitochondrial outer membrane allows the passage of molecules <1.5 Kda in size.^[2,3,4]

Mitochondrial mPT (MMPT) pore opening disrupts the proton gradient of the inter-membrane space configured by the electron transport chain of the inner mitochondria membrane, thus, uncoupling oxidative phosphorylation, rupture of the outer mitochondrial membrane, release of apoptogenic proteins such as cytochrome C, apoptosis-inducing factor, endonuclease G and small mitochondrial dependent activator of caspase (SMAC).^[5,6] The apoptosome, a complex of cytochrome C and apoptosis

protease activating factor-1 activates caspase 9 by cleavage of its zymogen (pro-caspase 9). Caspase 9 further activates downstream effector or execution caspases (caspase 3 and 7).^[7]

The release of cytochrome c into the cytosol triggers caspase activation and ultimately apoptosis.^[8] 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.^[9]

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.^[10] 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.^[11] Certain bioactive agents especially antioxidants and some essential oils can modulate apoptosis at mitochondrial level. Examples

include Genistein, Benzoyl isothiocyanate (BEITC), Indole-3-carbinol, Beta carotene, among others. Some of these agents have been identified and they are currently under preclinical and clinical trials.^[12]

Drymaria cordata (Linn.) Willd (Caryophyllaceae) is a weak spreading herb found widely dispersed in damp places all over the tropics of Africa, Asia and the Americas. *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.^[13] Extracts of the plant have been reported to possess antitussive^[14], anti-bacterial^[15], anti-inflammatory anxiolytic^[16], cytotoxic^[17] and analgesic^[18] properties. It has been demonstrated in our laboratory that different fractions of *drymaria cordata* caused the induction of mmp pore opening and release of cytochrome c and among all the fractions, chloroform fraction was the most potent.^[19] These findings have therefore ignited our interest to further investigate the potential of the most potent fraction (CFDC) on the modulation of mitochondrial-mediated apoptosis via caspases and some other proapoptotic biomarkers *in vivo*.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Mannitol, sucrose, N-2-Hydroxy-ethyl-piperazine- 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 available.

2.2 Collection of fresh *Drymaria cordata*

The whole plant of *Drymaria cordata* were freshly harvested and obtained from Department of Botany, University of Ibadan, Nigeria. Samples were authenticated and identified at the Herbarium, Department of Botany, University of Ibadan, Ibadan, Oyo State and a specimen Voucher No.UIH-22555 was deposited in the Herbarium. The plants were washed, air-dried for three weeks in the laboratory after which they were powdered with industrial machine and weighed.

2.2.1 Preparation of Crude Methanol Extract and Chloroform Fraction of *Drymaria cordata*

6-kilogramme air-dried, whole plant of *Drymaria cordata* were extracted with sufficient methanol (Sigma Aldrich Chemical Co. St Louis USA) in all- glass jars at room temperature for seventy-two hours. The filtrate was decanted, filtered and concentrated under reduced pressure using a rotary evaporator (Stuart). The crude methanol extract was heated over a water bath at 40°C to obtain a solvent free extract. The crude methanol extract was further partitioned between n-hexane, chloroform, ethylacetate and methanol using vacuum liquid chromatography technique. All these fractions were concentrated to dryness under pressure using rotary

evaporator at 40°C to obtain the n-hexane (HFDC), chloroform (CFDC), ethylacetate (EFDC) and the methanol (MFDC) fractions.

2.3 Experimental Animals

Male Wister strain albino rats weighing between 100g – 120g were purchased from the Preclinical Animal House of the College of medicine, University of Ibadan, Ibadan, Nigeria. All the animals were allowed two weeks period of acclimatization in the Animal House of the Department of Biochemistry, University of Ibadan. The animals were placed under a 12hr light/dark cycle and fed commercial pelletized rat chow and water *ad libitum* throughout the experimental period. Rules guiding animal studies as stipulated by the Ethical Committee of University of Ibadan were strictly followed. These rules are similar to international guidelines on animal handling.

2.4 Animals Groupings, Treatment and Sample collections:

Forty male Wister rats were divided equally into four groups: Group A was the control group. Group B, C and D were treated with 50, 100 and 200mg/kgbdwt CFDC respectively as a single dose daily by oral gavage for 28 days. One day after the final exposure, the animals were sacrificed. Blood was collected by cardiac puncture into plain sample bottles. Serum was prepared by centrifugation (3000rpm for 20 min) and used for the analysis.

2.5 Tissue Preparation for Histopathology

Liver was used for histopathology. They were quickly removed and trimmed and were placed in 10% formalin for about five days for proper fixation, dehydrated by ascending grades of isopropyl alcohol for an hour. The dehydrated organs were cleared in xylene and transferred into two changes of liquid paraffin wax. The tissue sections were stained in Ehrlich's hematoxylin for eight minutes, washed in tap water and dipped in acid alcohol to remove excess stain. These were counter stained in 10% aqueous eosin, incubated and mounted for photomicrography. The Histological pictures were taken with a Digital Microscope, VJ-2005 DN MODEL BIO-MICROSCOPE®.

2.6 Sample Preparation and Analysis of Caspases 9 & 3 using Elisa Technique

The rat liver was excised, 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 frozen. After freezing for two days, the samples were brought out to thaw. This was done twice after which the samples were used for caspases 9 and 3 analysis, respectively.

2.6.1 Analysis 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. This kit uses Sandwich-ELISA as the method. A microplate reader (DNM-9602A from China) was used to read the optical density at 450nm wavelength.

2.7 Immunohistochemistry Study

Immunohistochemistry (IHC) detects antigens (e.g. proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues.

2.7.1 Preparation of Immunohistochemistry Samples

Briefly, liver sections were immersed in 10% phosphate buffer formalin, dehydrated in graded alcohol and embedded in paraffin. Fine sections were obtained and mounted on glass slides. The method used in this study is Avidin Biotin Complex (ABC) method also referred to the Avidin biotin Immunoperoxidase method. The antibody dilution factor used were 1:100 dilution for all the antibody markers.

2.7.2 Immunohistochemical assays

Primary antibodies used in this study were Bcl-2, cytochrome c, caspases-3 and 9 (Elabscience product). Immunohistochemical staining was performed according to the manufacturer instructions. The processed tissue were sectioned at 2microns on the rotary microtome and placed on the hot plate at 70 degree for at least 1hour. Sections were brought down to water by passing the on 2 changes of xylene, then 3 changes of descending grades of alcohol and finally to water. Antigen retrieval was performed on the sections by heating them on a citric acid solution of PH 6.0 using the Microwave at power 100 for 15minutes. The sections were equilibrated gradually with cool water to displace the hot citric acid for at least 5min for the section to cool. Peroxidase blocking were done on the sections by simply covering section with 3% hydrogen peroxide (H₂O₂) for 15min. Sections were washed with PBS and protein blocking were performed using avidin for 15min. Sections were washed with PBS and endogenous biotin in tissue were blocked using biotin for 15min. After washing with PBS sections were incubated with the respective diluted primary antibody e.g antibody diluted 1:100 for 60 min.

Excess antibody were washed off with PBS and a secondary antibody (LINK) were applied on section for 15min. Sections were washed and the (LABEL) which is the horseradish peroxidase (HRP) were applied on the sections for 15min. A working DAB solution is made up by mixing 1 drop (20microns) of the DAB chromogen to 1ml of the DAB substrate. This working solution is applied on sections after washing off the HRP with PBS for at least 5min. The brown reactions begins to appear at this moment especially for a positive target. Excess DAB solution and precipitate are washed off with water. Sections are counterstained with Haematoxylin solution

for at least 2min and blued briefly. Sections are dehydrated in alcohol, cleared in xylene and mounted in DPX. Cells with specific brown colours in the cytoplasm, cell membrane or nuclei depending on the antigenic sites are considered to be positive. The haematoxylin stained cells without any form of brown colours are scored negative. Non specific binding/brown artifacts on cells and connective tissue are disregarded. Positive signals for Bcl-2, Cytochrome c, Caspases-9 & 3 proteins were represented by a brown. The positive staining intensity was calculated as the ratio of the stained area to the total field assessed using image J software.

2.8 DNA Fragmentation

The percentage DNA fragmentation was determined according to the method of Wu *et al.*,^[20] Liver and uterus tissues were sliced with scissors and homogenized in 10 volumes of Tri-EDTA Triton buffer (TET) pH 8.0. Homogenates were centrifuged at 27000g for 20mins to separate intact chromatin (pellet A) from fragmented (pellet B). Pellet A was suspended in Tris-EDTA buffer of pH 8.0. 1ml aliquot of each sample (pellet and supernatant) was placed in separate test tubes and then 1ml of freshly prepared diphenylamine solution was added to each. Reaction mixture was incubated at 37°C for 20 hours. Absorbance of the mixture was then measured at 620nm

CALCULATION

Quantity of fragmented DNA was estimated by using the formula:

$$\% \text{ fragmented DNA} = B/(A+B) \times 100$$

2.9 Isolation of rat liver mitochondria

Rat liver mitochondria were isolated essentially according to the method of Johnson and Lardy,^[21] as modified by Olorunsogo *et al.*,^[22] 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 (210mM mannitol, 70mM sucrose, 5mM HEPES-KOH, pH 7.4 and 1mM 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 2300rpm for 5minutes to remove the nuclear debris. This was done twice and the supernatant obtained was centrifuged at 13,000rpm for 10minutes to obtain the mitochondrial pellet. The supernatant was discarded while the pellet was washed with washing buffer (210mM mannitol, 70mM sucrose, 5mM HEPES-KOH, pH 7.4, 0.5 percent BSA) twice at 12,000rpm for 10minutes. The mitochondria obtained were immediately re-suspended 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 4°C ice.

2.10 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.^[23] Mitochondria (0.4mg protein/ml) were preincubated in the presence of 0.8 μ M rotenone in a medium containing 210mM mannitol, 70mM sucrose and 5mM HEPES-KOH (pH 7.4) for 3mins at 27°C prior to the addition of 120 μ M CaCl₂. 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.

2.11 Determination of mitochondrial protein

Mitochondrial protein concentration was determined according to the method of Lowry *et al.*^[24] using bovine serum albumin as standard.

2.12 Assessments of Mitochondrial FoF1 ATPase Activity

FoF1 Adenosine triphosphatase which was determined by a modification of the method of Olorunsogo and Malomo.^[25] 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°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.

2.13 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,^[26] and as modified by Olorunsogo *et al.*,^[22] 300 μ l of each solution was dispensed into fresh test tubes, followed by the addition of 300 μ l 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.

2.14 ASSESSMENT OF LIPID PEROXIDATION

Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) present in the test sample according to the method of Varshney and Kale.^[27] Under acidic conditions, malondialdehyde (MDA) produced from the peroxidation of fatty acids reacts with the chromogenic reagent 2-thiobarbituric acid to yield a pink coloured complex with maximum absorbance at 532 nm.

An aliquot of 0.4 ml of the test sample was mixed with 1.6 ml of Tris-KCl buffer to which 0.5 ml of 30% TCA was added. Then 0.5 ml of 0.75% TBA was added and placed in a water bath for 45 minutes at 80°C. This was then cooled in ice to room temperature and centrifuged at 3000 rpm for 10 min. The clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532 nm.

CALCULATION

The MDA level was calculated using an extinction coefficient of 0.156 μ M⁻¹cm⁻¹ (Adam-Vizi and Seregi, 1982).

Lipid peroxidation (nmole MDA/mg protein) =

$$\frac{\text{Absorbance} \times \text{volume of mixture}}{E_{532\text{nm}} \times \text{volume of sample} \times \text{mg protein/ml}}$$

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 $\pm$ standard deviation (SD).

3.0 RESULTS

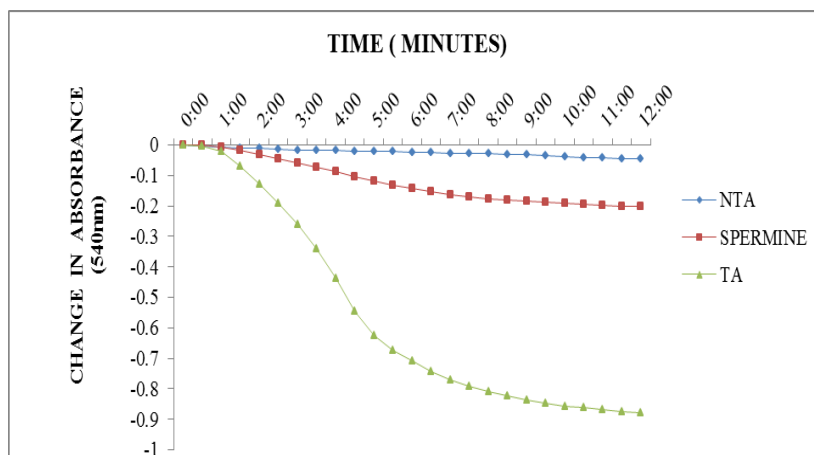


Fig 1: Calcium-induced mitochondrial membrane permeability transition pore opening in normal rat liver mitochondria and its reversal by spermine. NTA: No triggering agent; TA: Triggering agent.

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.

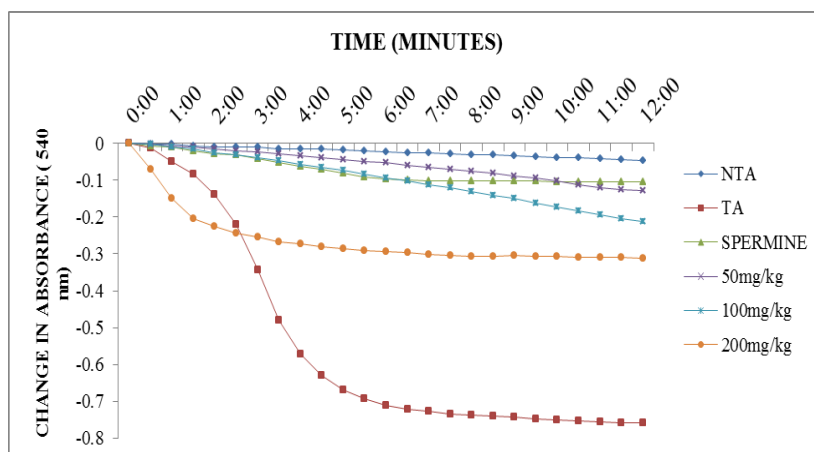


Fig 2: Effects of oral administration of chloroform fraction of *Drymaria cordata* on rat liver MMPT pore for 14 days.

Figure 2 shows the effects of various dosages of CFDC on MMPT pore after 14 days of treatment. From the results. At 50mg/kgbdwt, there was no significant induction of pore opening 100 and 200mg/kgbdwt induced pore opening by 3.3 and 5.5 folds, respectively when compared with the control.

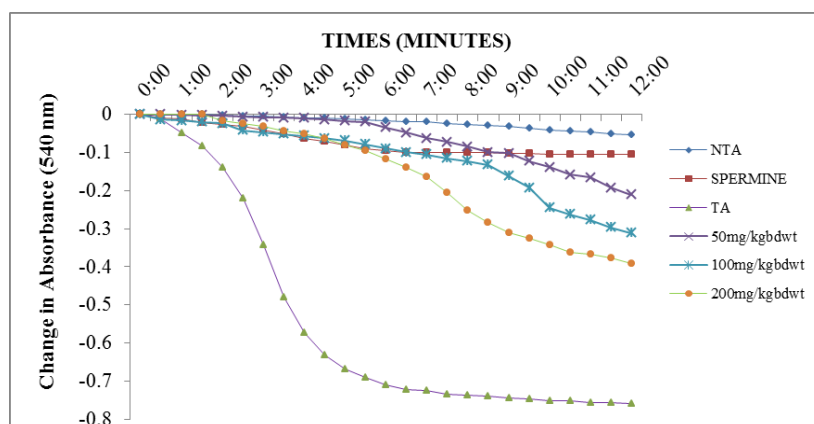


Fig 3: Effects of oral administration of chloroform fraction of *Drymaria cordata* on rat liver MMPT pore for 28 days.

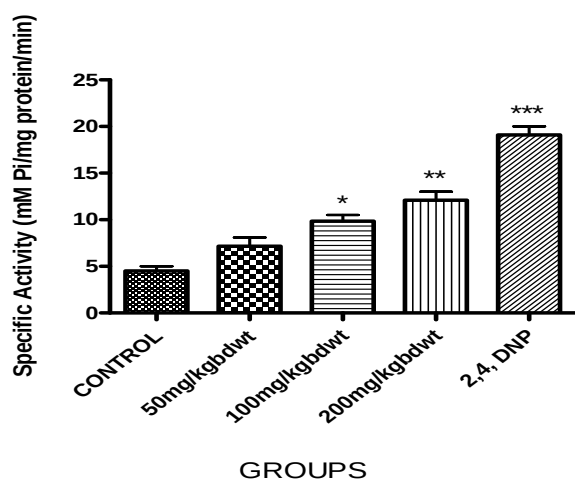


Fig 4: Effects of oral administration of CFDC on mitochondrial ATPase activity *in vivo*.

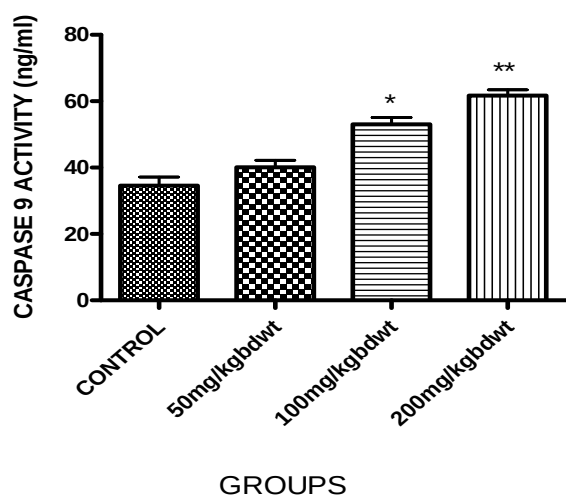


Fig 5: Effect of oral administration of CFDC on caspase 9 activation.

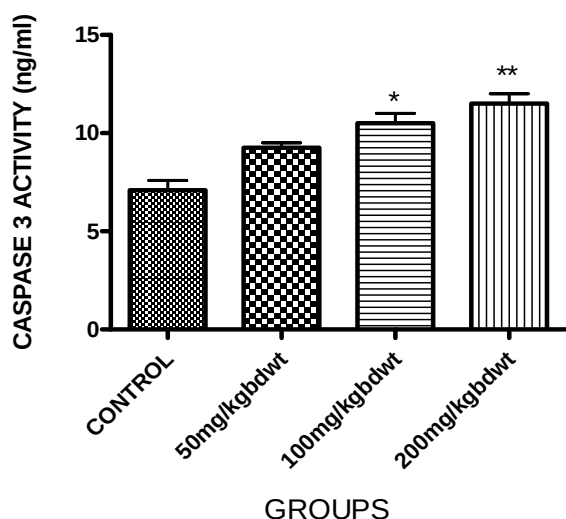


Fig 6: Effect of oral administration of CFDC on caspase 3 activation.

After exposure to the CFDC treatment for 28 days, as depicted by figure 3, 50, 100 and 200mg/kgbw significantly caused induction of pore opening by 2.7, 5.1 and 7.7 folds, respectively. Figure 4 shows the effects of varying dosages of CFDC on mitochondrial F₀F₁ ATPase activity. The data obtained show that CFDC caused enhancement of mitochondrial ATPase activity by 25, 38 and 63%, at 50, 100 and 200mg/kgbw, respectively, and it is significant ($P < 0.05$) at 100 and 200mg/kgbw when compared with the control group. Effects of oral administration of CFDC on caspases 9 and 3 are shown by figures 5 and 6. CFDC caused activation of caspase 9 by 5%, 37%, 63% and caspase 3 by 33%, 53%, 67% at dosage 50, 100 and 200mg/kgbw, respectively and it is significant ($P < 0.05$) at 100 and 200mg/kgbw. DNA fragmentation is one of the hallmarks of apoptosis. The effect of oral administration of CFDC on hepatic DNA fragmentation was illustrated in figure 7.

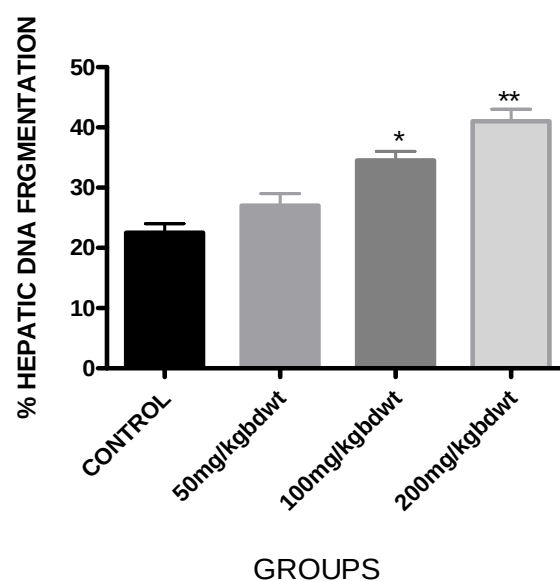


Fig: 7 Effect of oral administration of CFDC on Hepatic DNA Fragmentation.

The results show that there was induction of hepatic DNA fragmentation by 20%, 37% and 60% at dosages 50, 100 and 200mg/kgbw, respectively, when compared with the control. The results were statistically significant ($P < 0.05$) at 100 and 200mg/kgbw.

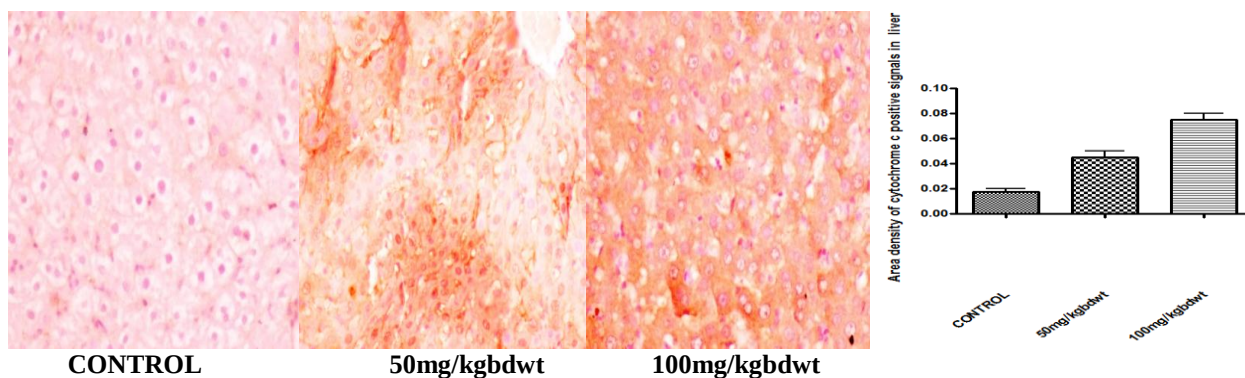


Fig 8: Photomicrograph of rat liver showing the immunohistochemical effect of oral administration of CFDC on cytochrome c release (Mag x400).

Figure 8 shows the immunohistochemical effect of oral administration of CFDC on cytochrome c release. Section from the control showed weak expression for cytochrome c release while the sections from 50 and 100mg/kgbdwt showed strong positive immunoreactivity for cytochrome c release. The expression was more in the group treated with 100mg/kgbdwt CFDC.

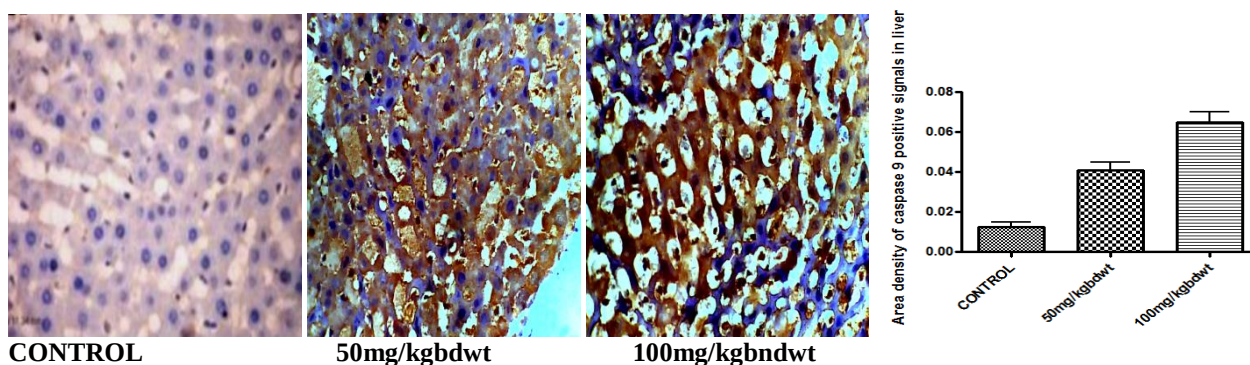


Fig 9: Photomicrograph of rat liver showing the effect of oral administration of CFDC on caspase 9 activation (Mag x400).

Similar pattern of results were obtained in the immunohistochemical analysis for caspases 9 and 3. There was weak expression for caspase 9 activity in the control group, while the 50mg/kgbdwt showed a mild to moderate expression and the 100mg/kgbdwt showed strong positive immunoreactivity for caspase 9 activity as depicted by figure 9. The effect of oral administration of CFDC on caspase 3 activation was shown in figure 10.

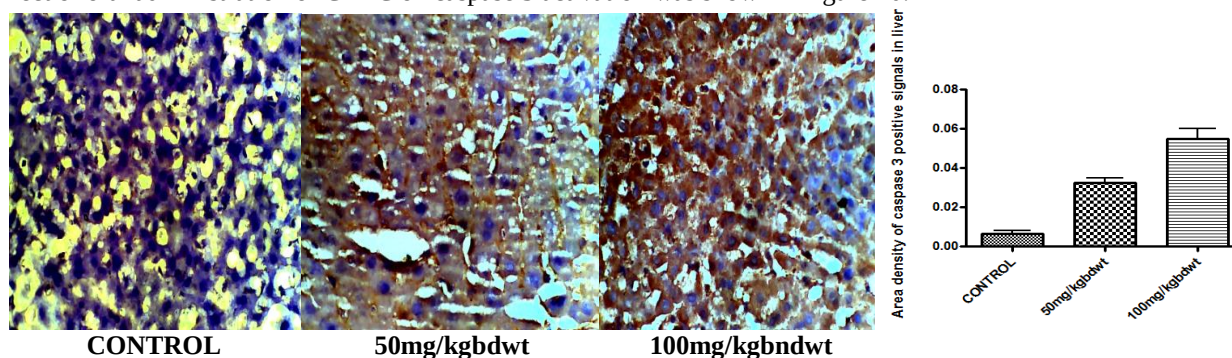


Fig 10: Photomicrograph of rat liver showing the effect of oral administration of CFDC on caspase 3 activation (Mag x400).

There was almost negative immunoreactivity for caspase 3 in the control group, while the 50mg/kgbdwt showed a mild expression and the 100mg/kgbdwt showed moderate to strong positive immunoreactivity for caspase 3 when compared with the control.

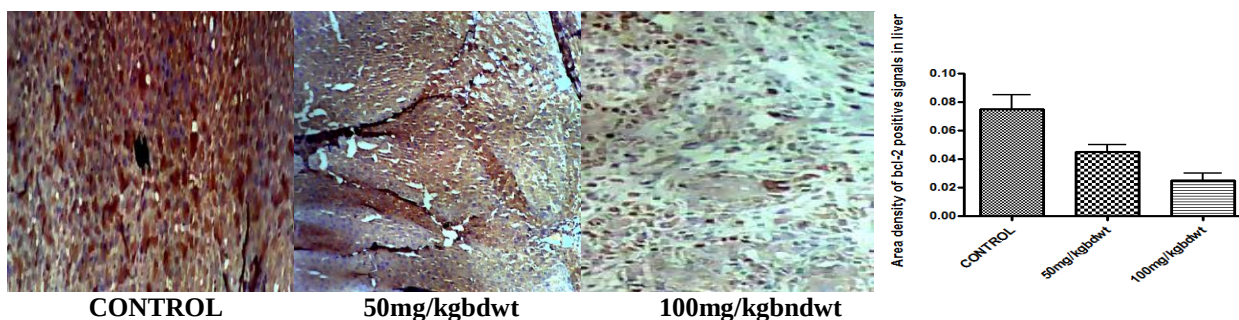


Fig: 11 Photomicrograph of rat liver showing the effect of oral administration of CFDC on Bcl-2 protein expression in the rat liver (Mag x400).

The Bcl-2 is an antiapoptotic protein. The results as pictured by figures 11 showed a downregulation of Bcl-2 protein expression from the control to the treated groups. There was strong positive immunoreactivity for Bcl-2 protein expression in the liver and uterus of the control group, while the expression was mild to moderate in the 50mg/kgbdwt-treated group and there was weak to almost negative expression for Bcl-2 protein in the 100mg/kgbdwt-treated group.

DISCUSSION

Apoptosis is one of the most potent defence mechanism by which potentially deleterious and mutated cells are eliminated from an organism, ensuring proper growth and development, maintenance of tissue homeostasis, differentiation in multicellular organisms, metamorphosis and embryogenesis.^[28] Dysregulation of apoptotic signalling has been implicated in various pathologic conditions including cancer, autoimmunity, persistent infections (failure to eradicate infected cells, neurodegenerative diseases like Alzheimers' disease, Parkinson's disease, AIDS (depletion of T lymphocytes), and ischaemia (stroke, myocardial infarction).^[29] The complex role of mitochondria in apoptosis came into focus when biochemical studies identified several mitochondrial proteins that are able to activate cellular apoptotic programs and the emerging interactions between mitochondrial membrane permeability transition pore components.^[30] Mitochondria play a central role in initiation of the intrinsic pathway of apoptosis by releasing mitochondrial proteins, which normally reside in the intermembrane space, into the cytosol. This triggers assembly of the apoptosome and activation of procaspase-9, leading to a cascade of events which ultimately leads to cell death.^[31] Several novel approaches are currently being pursued in order to target specific apoptotic regulators.^[32] Currently, the use of nutritional modulation via the myriad bioactive components present in functional foods is gaining broader acceptance.^[33] Certain bioactive agents especially antioxidants and some essential oils can modulate apoptosis at mitochondrial level. Examples include Genistein, Benzoyl isothiocyanate (BEITC), Indole-3-carbinol, Beta carotene, among many others.^[34] Based on our previous findings in the laboratory that the chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) is a potent inducer of MMPT pore

opening *in vitro*^[19], we therefore investigated the modulatory effect of oral administration of CFDC on mitochondrial-mediated apoptosis via the release of cytochrome c and some other apoptotic biomarkers. 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.^[23] This showed that the mitochondria of the control animal used in this study were intact *ab initio* and suitable for the experiment.

Findings from our previous *in vitro* study showed that chloroform fraction of methanol extract of *drymaria cordata* (CFDC) is the most potent with respect to MMPT pore opening. This also suggests that the bioactive agents responsible for the opening of the pore are highly resident in the non polar fraction. These results necessitated our curiosity to find out if the chloroform fraction would induce pore opening *in vivo* and if the signaling pathways of mitochondrial-mediated apoptosis are involved in the real mechanism of interaction of CFDC. Owing to the relevance of bioavailability of the compound of interest at the target site and to determine the safety dose regimen for the potent chloroform fraction of *D. cordata*, albino rats were orally administered different doses of CFDC for 14 and 28 days while control animals received the vehicle. (5% DMSO distilled water). The mitochondrial permeability transition was assessed spectrophotometrically in the animal subjects at 540nm. The effects of various dosages of CFDC on MMPT pore after 14 days of treatment showed that at 50mg/kgbdwt, there was no significant induction of pore opening while 100 and 200mg/kgbdwt induced pore opening by 3.3 and 5.5 folds, respectively when compared with the control. After exposure to the CFDC treatment for 28 days, 50, 100 and 200mg/kgbdwt significantly caused induction of pore opening by 2.7, 5.1 and 7.7 folds, respectively. The increase in induction fold after 28 days of treatment might be due to increase in bioavailability of the CFDC at the target site and prolong exposure to treatment. The induction of rat liver MMPT pore opening by CFDC *in vivo* is also in consonance with the *in vitro* study. These results suggest that chloroform fraction of methanol

extract of *Drymaria cordata* was available at the target site to interact with the components of the MMPT pore in order to elicit inductive effect. Also, the induction was dosage-dependent showing that the induction of MMPT pore opening increased with increase in dosage of administration.

Currently, researchers are proposing that mPT pore is the mitochondrial ATPase. A group of thought is hypothesizing that the mPT pore forms at the interface between ATP synthase dimers or perhaps tetramers,^[35,36,37] and there is another hypothesis that the mPT pore arises from the c-ring of the F₀ sector of the mitochondrial ATPase.^[38,39] Although, both points of view are supported by experimental results and still have some weak points, these studies provide compelling evidence that the ATP synthase is required for proper MMPT pore function. Therefore, in order to ascertain the involvement of MMPT pore in mitochondrial ATP synthase, effects of CFDC on mitochondrial F₀F₁ ATPase activity was investigated using 2,4 dinitrophenol (DNP), a classical uncoupler of oxidative phosphorylation as a standard. This was determined spectrophotometrically by following the rate of release of inorganic phosphate from the γ -position of ATP during the ATPase action. The in vivo effect of oral administration of CFDC on mitochondrial F₀F₁ ATPase activity was also in consonant with our in vi vitro results as CFDC caused enhancement of mitochondrial ATPase activity by 25, 38 and 63%, at 50, 100 and 200mg/kgbdwt, respectively, and it is significant ($P < 0.05$) at 100 and 200mg/kgbdwt when compared with the control group. The enhancement was also dosage-dependent showing that increase in the bioavailability of the fraction commensurate with increase in the ATPase activity.

Caspases are crucial for the initiation, propagation and execution of apoptosis. They normally exist as proenzymes, which can be activated through recruitment into activating complexes and by proteolytic cleavage by other caspases or proteases. Perturbation of organelles such as nuclei, endoplasmic reticulum and mitochondria results in the activation of caspases. Upon apoptotic permeabilization of the mitochondrial outer membrane, mitochondrial procaspases 9 would be released from the intermembrane space to the cytosol and become activated.^[40,41] Inappropriate activation of the executioner caspases 3 is prevented by their production as inactive procaspase dimers that must be cleaved by initiator caspases. Once activated, a single executioner caspase can cleave and activate other executioner caspases, leading to an accelerated feedback loop of caspase activation.^[42] In this study, the level of caspases 3 & 9 activities were measured using Enzyme Linked Immunosorbent Assay (ELISA). The data showed CFDC caused upregulation of the caspases 9 (initiator) and 3 (executioner) activity in a dose-dependent manner. The activation was significant at 100 and 200mg/kgbdwt of CFDC. The activation of caspase

9 activity by CFDC suggests that CFDC is a potent inducer of mitochondrial-mediated apoptosis. The increase in caspases activity correlated with increase in doses of the fraction. This is in agreement with the findings of McIlwain *et al.*,^[43] which confirm that caspases are central to the execution of the apoptotic process.

A variety of stress stimuli including growth factor withdrawal, heat shock, permeabilisation of mitochondrial membrane and DNA damage activate the apoptosis intrinsic or mitochondrial pathway. The formation of distinct DNA fragments of oligonucleosomal size (180-200 bp lengths) is a biochemical hallmark of apoptosis in many cells. In this study, the percentage hepatic and uterine DNA fragmentation was determined using diphenylamine (DPA) method^[44], after the exposure of the rats to varying doses of CFDC for 28 days. The data show that there was induction of hepatic DNA fragmentation by 20%, 37% and 60% and uterine DNA fragmentation by 15%, 50% and 75% at dosage 50, 100 and 200mg/kgbdwt, respectively, when compared with the control. The results were statistically significant ($P < 0.05$) at 100 and 200mg/kgbdwt.

Internal stimuli such as irreparable genetic damage, hypoxia, extremely high concentrations of cytosolic Ca²⁺ and severe oxidative stress are some triggers of the initiation of the intrinsic mitochondrial pathway.^[45] Regardless of the stimuli, this pathway is the result of increased mitochondrial permeability and the release of pro-apoptotic molecules such as cytochrome- c into the cytoplasm.^[46] This pathway is closely regulated by a group of proteins belonging to the Bcl-2 family, named after the BCL2 gene originally observed at the chromosomal breakpoint of the translocation of chromosome 18 to 14 in follicular non-Hodgkin lymphoma.^[47] There are two main groups of the Bcl-2 proteins, namely the pro-apoptotic proteins (e.g. Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk) and the anti-apoptotic proteins (e.g. Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1) (Reed, 1997). While the anti-apoptotic proteins regulate apoptosis by blocking the mitochondrial release of cytochrome-c, the pro-apoptotic proteins act by promoting such release. It is not the absolute quantity but rather the balance between the pro- and anti-apoptotic proteins that determines whether apoptosis would be initiated.^[48] Other apoptotic factors that are released from the mitochondrial intermembrane space into the cytoplasm include apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase (Smac), direct IAP Binding protein with Low pI (DIABLO) and Omi/high temperature requirement protein A (HtrA2) (Kroemer *et al.*, 2007). Cytoplasmic release of cytochrome c activates caspase 3 via the formation of a complex known as apoptosome which is made up of cytochrome c, Apaf-1 and caspase 9.^[49] On the other hand, Smac/DIABLO or Omi/HtrA2 promotes caspase activation by binding to inhibitor of apoptosis

proteins (IAPs) which subsequently leads to disruption in the interaction of IAPs with caspase-3 or -9.^[50] Having established the potency of CFDC as an inducer of mitochondrial-mediated apoptosis, we also demonstrated the effect of CFDC on some apoptotic parameters using immunohistochemistry technique. The data showed that CFDC caused an elevated immunoreactive expression of cytochrome c, caspase 9 and caspase 3, which are required for the activation of mitochondrial-mediated apoptosis. However, there was downregulation of the activity of anti apoptotic Bcl-2 protein in a dosage-dependent manner. Upregulation of Bcl-2 protein has been implicated in many cancers. They are critical for their ability to bind to the pro-apoptotic Bcl-2 family members and thereby exert their antiapoptotic function.^[51] Overexpression of Bcl-2 or Bcl-XL potentially inhibits apoptosis in response to many cytotoxic insults, among others by suppressing the generation of ROS, stabilizing $\Delta\psi$, preventing MMPT and consequently blocking the release of cytochrome c. Their down regulation could be a therapeutic strategy in combating cancerous cells.

In these findings, it can be concluded that chloroform fraction of methanol extract of *drymaria cordata* is a potent inducer of mitochondrial-mediated apoptosis. Though, the nature of substances responsible for the effects shown by CFDC are still unknown, but work is in progress in order 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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