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Research Article

Some Solvent Fractions of the Fruits of *Xylopia aethiopica* Enhance Mitochondrial-Mediated Apoptosis in Rat Liver

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Abstract

The mitochondrial membrane Permeability Transition (mPT) pore has emerged as a promising target for drug development in diseases where there is dysregulation of apoptosis. In this study, the effects of various fractions of the methanol extract of *Xylopia aethiopica* (XA), a potent medicinal plant, were investigated on mitochondrial-mediated apoptosis. The methanol extract was partitioned in succession between dichloromethane, ethylacetate and methanol to obtain Dichloromethane (DFXA), Ethylacetate (EFXA) and Methanol (MFXA) fractions of the fruits. The effects of DFXA, the most potent fraction, on mPT pore, mitochondrial ATPase (mATPase), lipid peroxidation, DNA fragmentation, cytochrome c release and caspases 9 and 3 activities were estimated. Varying concentrations of DFXA (20, 60, 100, 140 and 180 μg/ml) significantly induced pore opening in the absence of calcium by 9.92, 12.36, 13.75, 14.92 and 15.47 folds, respectively. Similarly, Ca^{2+}-induced mPT pore opening was further enhanced by 8.3, 9.14, 10.5, 18.53 and 20.5 folds and mATPase activity was significantly elevated by these concentrations of DFXA. In contract, EFXA and MFXA did not have any significant effect at lower concentrations but induced pore opening at 180μg/ml by 1.69 and 6.4 folds, respectively. *In vivo*, DFXA caused the induction of mPT pore in the absence of calcium, activation of the activities of caspases 9 and 3, significant DNA fragmentation in a dose-dependent manner and also ameliorated ferrous-induced mitochondrial membrane lipid peroxidation. These findings reveal that the dichloromethane fraction of *Xylopia aethjopica* contains a bioactive agent capable of inducing mitochondrial-mediated apoptosis and may be useful for drug development in diseases where apoptosis is compromised.

Key Words: *mitochondrial permeability transition, mATPase, cytochrome c, caspases*

INTRODUCTION

Apoptosis, a highly coordinated sequence of events for the execution of programmed cell death, plays a vital role in development and tissue homeostasis (Kerr *et al.,* 1972; Meier *et al.,* 2000). Dysregulated apoptosis has been implicated in a variety of pathological conditions. For example, inadequate apoptosis may contribute to oncogenesis while too much apoptosis may lead to cell loss in neurodegeneration, diabetes mellitus, and HIV/AIDS (Fadeel, 1999). It is now well established that apoptosis may occur via a number of pathways including the extrinsic and the intrinsic or mitochondrial-mediated pathway during which the mitochondrial Permeability Transition (mPT) pore opens and thus allows the release of pro-apoptotic proteins such as cytochrome C (Salvesen and Renatus 2002). The process eventually results in the activation of caspases and then cell death. The opening of mPT pore is therefore a major step in the induction of mitochondrial- mediated apoptosis and this is a point of no return for apoptosis (Lopez and Tait, 2015). In this connection, mPT pore has emerged as a promising target for drug development in diseases where there is dysregulation of apoptosis.

Although, the nature of the exact components of the pore is still under debate, the number of naturally occurring substances that modulate pore opening and mitochondrial-mediated apoptosis keep increasing. For instance, quercetin, hesperidin and curcumin are reported to have potent anti-apoptotic activity (Bounival *et al*., 2009; Huang *et al*., 2018). Other examples include betulinic acid, berberine (Selzer *et al*., 2002; Fulda *et al*., 2010). Some of these compounds are currently under clinical trial for diseases associated with dysregulated apoptosis. We have shown that some solvent fractions of certain medicinal plants modulate mitochondrial-mediated apoptosis (Adisa *et al.,* 2010; Odewusi *et al.,* 2010; Adedosu *et al.,* 2012; Oyebode *et al.,* 2012; Oyebode *et al.,* 2017). Also, we have demonstrated that robustaside B and p-hydrophenol from *Cnestis ferruginea* (Adisa and Olorunsogo, 2013) and artesunate (Anyasor *et al.,* 2009) modulate mitochondrial-mediated apoptosis by interacting with the mPT pore. Several modulators of mitochondrial-mediated apoptosis are currently being investigated and some have been discovered to elicit their chemopreventive effects through the

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induction of mitochondrial membrane permeability transition pore e.g. betulinic acid (Fulda *et al.,* 1997; Selzer *et al.,* 2002), CD 437 (a retinoid) (Marchetti *et al.,* 1999; Belzacq *et al.*, 2001)

Xylopia aethiopica Dunal (Annonacae), a medicinal plant commonly known as Ethiopian or Negro pepper, has been used in Europe, Asia and Africa as a pepper substitute and spice in local cooking. Various parts of the plant have been traditionally employed in therapeutic preparations (Ogunkunle and Ladejobi, 2006). Extracts from *Xylopia aethiopica* have been shown to have antioxidant, antibiotic, anti-pyretic effect, anti-inflammatory, anesthetic, anti-allergic, antiviral and anticancer properties (Del-Rio *et al.,* 1997; Adaramoye *et al.,* 2011; Yusuf *et al.,* 2014). These properties are believed to be mediated by different phytochemicals found in the plant such as flavonoids, terpenoids, fixed oil and volatile aromatic oil (Fleischer, 2003), glycosides saponins, tannins and phytosterols (Ezekwesili *et al.,* 2010; John-Dewole *et al.,* 2012).

In this paper, we investigated the potency of various solvent fractions of *Xylopia aethiopica* on mitochondrial-mediated apoptosis with a view to have insight into the possibility of using the phytochemical component of the plant for drug development in diseases associated with dysregulated apoptosis.

MATERIALS AND METHODS

Chemicals and Reagents: All reagents were of the highest purity grade available and were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Other chemicals and assay kits were of pure form and analytical grade.

Experimental Animals: Male Wistar strain albino rats weighing between 100 – 120g 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 the Animal House, Department of Biochemistry, University of Ibadan, Nigeria 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. For *in vivo* studies, the animals were divided into four groups and were administered varying doses of DFXA for 14 days after which they were sacrificed and their livers used for mitochondrial isolation. Group 1 or control received 10% dimethylsulfoxide (DMSO), while the remaining groups received 25, 50 or 100mgDFXA/kgbodyweight. The animals had free access to water and laboratory *chow* throughout the period of the experiment. 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.

Plant Materials: Fresh fruits of *Xylopia aethiopica* were obtained from Bode market in Ibadan, Oyo State, Nigeria. Botanical identification and authentication were done at the Herbarium, Department of Botany, University of Ibadan, Nigeria. The fruits were washed, air-dried for about six weeks and blended to a powder which was later soaked in 100% methanol for 72 hours. The filtrate obtained was concentrated using a rotary evaporator and concentrated to dryness in a water bath at 40°C.

Partitioning of the Crude Methanol Extract of *Xylopia aethiopica* (MEXA) into various solvent fractions using Vacuum Liquid Chromatography: The column was packed three-quarters full with silica gel 60 (0.040–0.063mm, MERCK). The gel sample mixture was stirred until a homogenous mixture was obtained. The mixture was air-dried to obtain a powdery form and was applied to the top of the column with the pump switched on. Solvents were added in order of increasing polarity and eluted. The n-hexane, dichloromethane, ethylacetate and methanol fractions were obtained in this order. All these fractions were concentrated to dryness under pressure using rotary evaporator at 40°C, stored in glass sample bottles and kept in the refrigerator until use.

Isolation of Rat Liver Mitochondria: Rat liver mitochondria were isolated according to the method described by Johnson and Lardy (1967) and as modified by Olorunsogo *et al* (1979). Briefly, the animals were sacrificed by cervical dislocation and the livers excised and trimmed, to remove excess tissue. The livers were then weighed and washed with homogenizing buffer (210mM mannitol, 70mM sucrose, 5mM HEPESKOH, 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 sedimented nuclear debris. This was repeated 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 resuspended in an appropriate volume of MSH buffer (210mM Mannitol, 70mM Sucrose, 5mM HEPES-KOH, pH 7.4).

Protein determination: Mitochondrial protein was estimated according to the method described by Lowry *et al* (1951) using bovine serum albumin (BSA) as standard.

Measurement of mitochondrial swelling: Mitochondrial swelling assay was carried out according to the method of Lapidus and Sokolove (1993). Intact mitochondria (0.4mglml) were pre-incubated in the presence of 0.8μM rotenone and MSH buffer for 3.5 minutes prior to the addition of 5mM sodium succinate. In order to assess Ca^{2+}-induced swelling, mitochondria were pre-incubated in 0.8μM rotenone and MSH buffer for 3 minutes. Ca^{2+} was then added to the reaction mixture while sodium succinate was added 30 seconds later in a total reaction volume of 2.5ml. Spermine was used as the standard inhibitor of mitochondrial swelling, and was added prior to mitochondrial pre-incubation with rotenone. Change in absorbance was estimated at 540nm at 30 seconds interval for 12 minutes in a T70 UV-visible spectrophotometer, PG Instrument Ltd. To determine the effect of the extracts of *Xylopia aethiopica* on Ca^{2+}-induced mPT pore opening, different concentrations of each of the extract fractions were separately pre-incubated with mitochondria for 3 minutes following the addition of Ca^{2+} to the reaction mixture. But calcium was excluded in the reaction mixture when the direct modulatory effect of the extracts on intact mitochondria was desired. Mitochondrial swelling was measured as decrease in absorbance at 540nm. Permeability transition in the

mitochondria isolated from the treated groups was carried out on the same mitochondrial protein content from these groups and the absorbance monitored accordingly. Mitochondria of untreated animals were used for these (*in vitro)* studies while those exposed to DFXA were used for *in vivo* studies.

Assay for Mitochondrial FoF1 ATPase (mATPase) Activity: mATPase activity was determined by as described by Olorunsogo and Malomo (1985). Each reaction mixture contained 65mM Tris-HCl buffer pH 7.4, 0.5mM KCl, 1mM ATP and 25mM Sucrose (pH 7.4). 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 (of known protein content) and was allowed to proceed in a shaker water bath for 30 minutes at 27oC. The reaction was stopped by the addition of 1ml of a 10 percent of sodium dodecyl sulphate (SDS) and each reaction mixture was then centrifuged at 3000g. The supernatant was kept for phosphate determination. 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.

Estimation of inorganic phosphate released: The concentration of inorganic phosphate released was measured according to the procedure described by Bassir (1963) and as modified by Olorunsogo *et al* (1979). To 300µl of each solution was added 300µl of distilled water in a clean test tube. 1ml of 5% ammonium molybdate and 1ml of 9% of freshly prepared ascorbic acid were immediately added. The contents of each tube were thoroughly mixed and allowed to stand for 20 minutes and the absorbance read at 680nm in a spectrophotometer using a water blank to set the instrument at zero.

Lipid Peroxidation Assay: 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* (2000). Mitochondria (2mg/ml protein) and varying concentrations of the extract and fractions were added to each reaction mixture and made up to 1ml with distilled water; 0.05ml of $FeSO_4$ (0.07M) was added to induce lipid peroxidation and the mixture incubated for 30minutes. 1.5ml of 20% acetic acid (pH 3.5) and 1.5ml 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 95oC for 60 minutes. After cooling, 5.0ml of butan-1-ol were added to each tube and centrifuged at 4,000rpm for 10 minutes. The absorbance of the organic upper layer was measured at 532nm. 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

Assay of lipid peroxidation (*in vivo*)

The malondialdehyde (MDA) content of mitochondria was determined by measuring the formation of thiobarbituric aid reactive substances (TBARS) present in the mitochondria according to the method of Varshney and Kale (1990). An aliquot of 0.4 ml of test sample (mitochondria) 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 80oC. This was immediately cooled on ice to room temperature and centrifuged at 3000 rpm for 10 minutes. The clear supernatant was collected and absorbance was measured against a reference blank of distilled water at 532 nm. The MDA level was calculated using extinction co-efficient of 0.156/µM/cm (Adam – Vizi and Seregi, 1982).

Assay of Cytochrome C: Quantitatively, cytochrome c released from isolated mitochondria was determined by measuring the Soret (ϒ) peak for cytochrome c at 414nm (ε = 100mM-1cm-1), according to the method of Appaix *et al.* (2000). Isolated mitochondria were pre-incubated in a buffer medium containing 210mM mannitol, 70mM sucrose and 5mM HEPES-KOH (pH 7.4) in the presence of 0.8µM rotenone at 27oC for about 30 minutes at 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 minutes. The optical density of the supernatant was measured at 414nm which is the soret (γ) peak for cytochrome c.

Assay of Caspases 9 and 3: Activities of caspases 9 and 3 were measured in liver post mitochondrial fraction 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 450nm wavelength. After acclimatization, 25, 50, 100 and 200mg/kg body weight doses of MEXA and DFXA were administered intraperitoneally for two weeks after which the animals were sacrificed and the livers 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 supernatants thus obtained were then put in sample bottles and frozen. After freezing for two days, the samples were thawed. This was done twice after which the samples were used for caspases 9 and 3 analysis. Mean values of duplicate measurements were presented and caspases 9 and 3 activities were calculated from the standard curve.

Assessment of DNA fragmentation: The extent of fragmented DNA was determined by the method of Wu *et al.,* (2006). Liver (1g) was homogenized with 20ml of TET buffer (5mM Tris, 20Mm EDTA, and 0.2% Triton X 100, pH 8). The mixture was centrifuged at 27,000rpm for 20 minutes and the supernatant was separated from pellet. TE (2ml) was added to the pellet. Diphenylamine (3 mL, DPA) solution, prepared by dissolving 1.5g of DPA in 100mL of acetic acid and 1.5mL of concentrated H_2SO_4 was added to 1mL of supernatant. These were incubated at 37ºC for 16-24 hours and the absorbance read at 620nm

RESULTS

The data presented in figure 1 indicate that over a period of twelve minutes there was no significant change in the absorbance of mitochondria respiring on succinate in the presence of rotenone. On the addition of calcium, there was a large amplitude swelling and the transition pore opened by 14 folds compared to control (no calcium). Interestingly the calcium-induced swelling of the mitochondria was almost completely reversed (99%) by spermine (a standard inhibitor). This shows that the mitochondria used in this study were intact and therefore were suitable for further use.

Figure 2 summaries the effect of varying concentrations of DFXA (20, 60, 100, 140 and 180µg/ml) on mPT pore in the absence of calcium. Here, there was significant induction of opening of the pore by 9.92, 12.36, 13.75, 14.92 and 15.47 folds, respectively. In figure 3 the inductive effect of DFXA on pore opening was also seen on the addition of calcium, where the effect was concentration-dependent, with varying concentrations (20, 60, 100, 140 and 180µg/ml) of the fraction (DFXA), exhibiting significant induction of pore opening by 8.3, 9.14, 10.5, 18.53 and 20.5 fold, respectively.

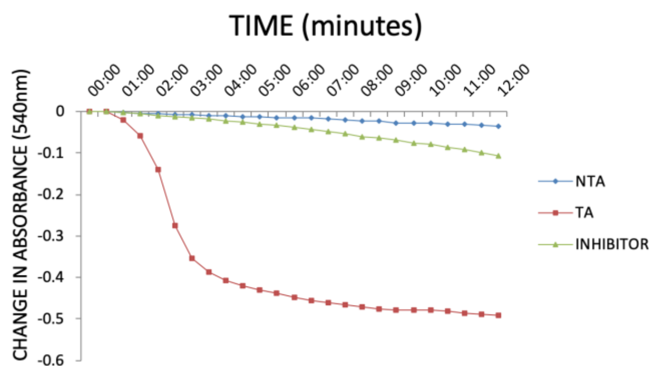

Figure. 1:
Calcium-induced mitochondrial membrane permeability transition pore opening in normal rat liver mitochondria and its reversal by spermine.
NTA: No triggering agent; TA: Triggering agent

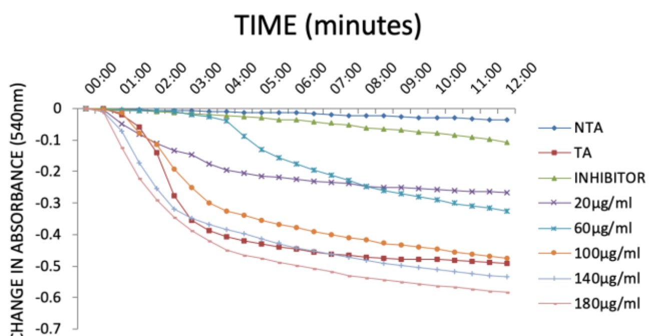

Figure 2:
Effects of varying concentrations of dichloromethane fraction of *Xylopia aethiopica* (DFXA) on the mitochondrial membrane transition pore in the absence of Ca^{2+}.
NTA: No triggering agent; TA: Triggering agent

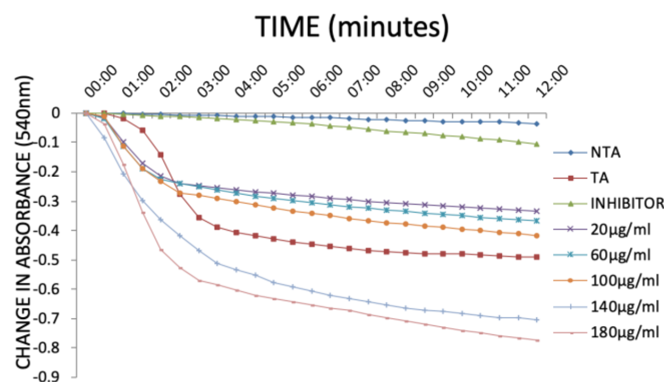

Figure 3:
Effects of varying concentrations of dichloromethane fraction of *Xylopia aethiopica* (DFXA) on the mitochondrial membrane transition pore in the presence of Ca^{2+}.
NTA: No triggering agent; TA: Triggering agent

Figures 4 and 5 show that the ethylacetate fraction (EFXA) and methanol fraction (MFXA) did not have any significant effect at lower concentrations but induced pore opening at highest concentration of 180 µg/ml by 1.69 and 6.4 folds, respectively. Also, methanol fraction (MFXA) induced pore opening in the absence of calcium at varying concentrations (20, 60, 100, 140 and 180µg/ml) by 3.0, 4.9, 5.5, 5.8 and 6.4 folds, respectively. Thus, indicating that the methanol fraction (MFXA) was more potent than the ethylacetate fraction (EFXA) but both were not as potent as the crude methanol extract (ME) and dichloromethane fraction of the plant (DFXA) which is the most effective in inducing the opening of the pore. This fraction was therefore, used for further experiments.

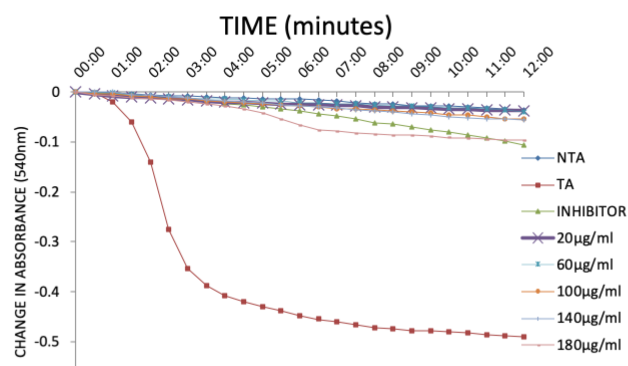

Figure 4:
Effects of varying concentrations of ethylacetate fraction of *Xylopia aethiopica* (EFXA) on the mitochondrial membrane transition pore in the absence of Ca^{2+}.
NTA: No triggering agent; TA: Triggering agent

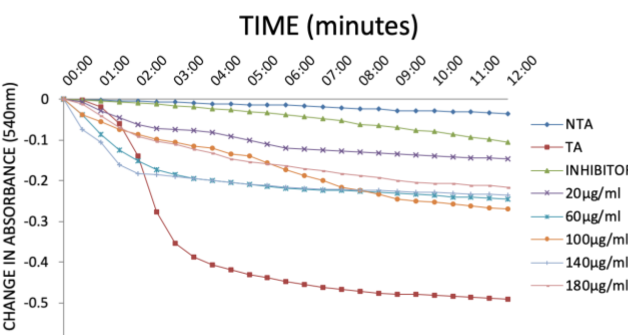

Figure 5:
Effects of varying concentrations of methanol fraction of *Xylopia aethiopica* (MFXA) on the mitochondrial membrane transition pore in the absence of Ca^{2+}.
NTA: No triggering agent; TA: Triggering agent

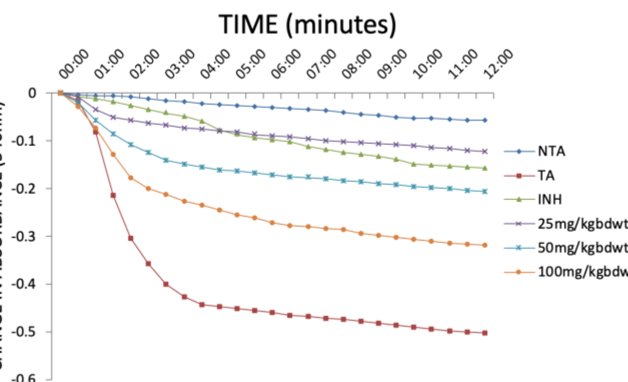

Figure 6:
Representative profile of changes in absorbance over a period of 12 minutes in the absence of calcium of mitochondria isolated from livers of rats previously exposed (ip) to various doses of DFXA for period of 14 days

Figure 6 shows the representative profile of changes in absorbance over a period of 12 minutes of mitochondria isolated from animals previously intraperitoneally administered varying doses of DFXA for 14 days. The assay was carried out in the presence and absence of calcium at all doses. The data obtained show clearly that the mPT was opened at all the doses tested.

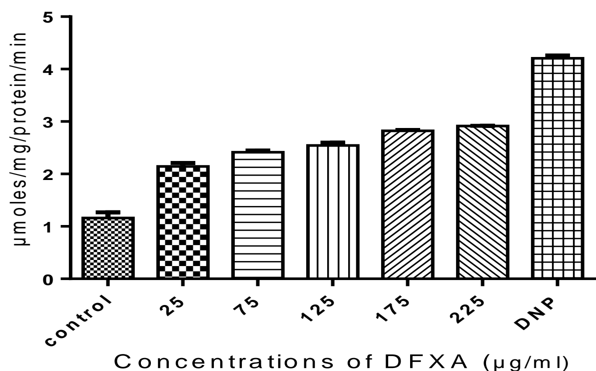

Figure. 7:
Effects of varying concentrations of DFXA on rat liver mitochondrial ATPase (mATPase) activity. DNP- dinitrophenol

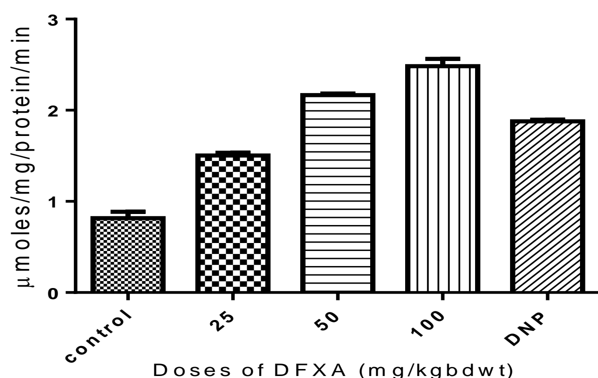

Figure. 8:
Mitochondrial ATPase (mATPase) activity of livers of rats previously exposed to various doses (ip) of DFXA for a period of 14 days. DNP-dinitrophenol

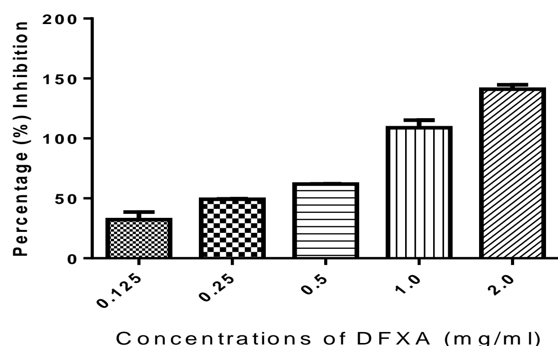

Figure 9:
Percentage inhibition of ferrous-induced lipid peroxidation of normal mitochondria in the presence of varying concentrations of DFXA. Each value is a mean of 4 different determinations (±SD)

Figures 7 and 8 show the effects of varying concentrations and dosages respectively, of DFXA on enhancement of mATPase activity in rat liver mitochondria. The results showed that DFXA significantly ($P<0.05$) enhanced the ATPase activity in a concentration-dependent manner with enhancement of over 100% at 75µg/ml of DFXA while the ATPase activity was also significantly enhanced by about 200% at 50mg/kgbodyweight. Figure 9 shows the inhibitory effects of varying concentrations of DFXA on lipid peroxidation induced by ferrous sulphate. The results showed that the fraction exhibited inhibitory effects of 65 percent at a concentration (500µg/ml).

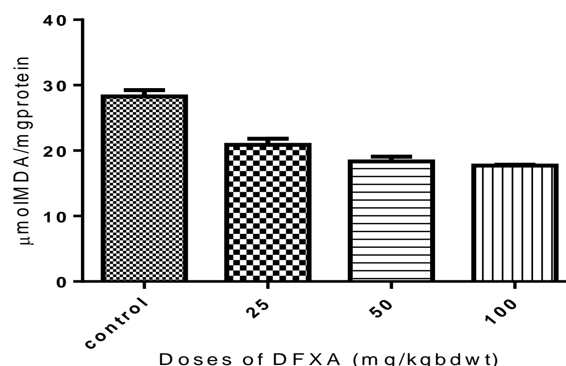

Figure 10:
Degree of lipid peroxidation of mitochondria of animals previously exposed to varying doses (ip) of DFXA for a period of 14 days

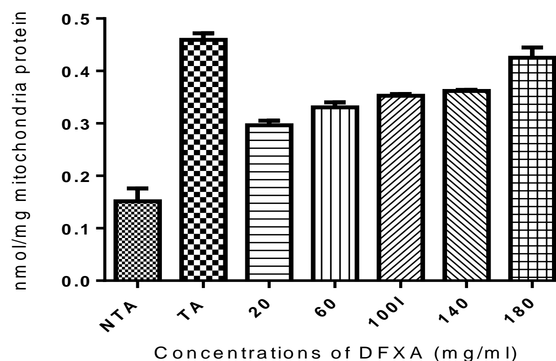

Figure 11:
Pattern of the extent of cytochrome c release in normal rat liver mitochondria in the presence of different concentrations of DFXA. NTA: No triggering agent; TA: Triggering agent

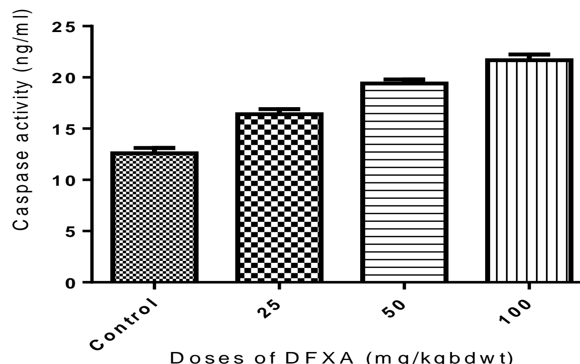

Figure 12:
Caspase 9 activity in post-mitochondrial fraction of livers of animal previously exposed (ip) to various doses of DFXA for a period of 14 days.

Figure 10 shows the inhibitory effects of MEXA on lipid peroxidation *in vivo*. Here, lipid peroxidation was inhibited at all doses tested. Figures 11 and 12 show that the levels of caspase 9 and caspase 3 activities were elevated with increasing dosages of DFXA. For instance, these activities

were maximally activated at the highest doses administered. Figure 13 shows the levels of the release of cytochrome c by mitochondria in the presence of DFXA. The results obtained show that, on addition of varying concentrations DFXA to MSH-pre-incubated mitochondria, there was concentration-dependent release of cytochrome c. Interestingly, the amount of cytochrome c released in the presence of DFXA was concentration-dependent. Figure 14 shows the degree of DNA fragmentation in liver cells caused by the DFXA after 14 days of intraperitoneal administration. The data obtained show that there was DNA fragmentation in the hepatocytes of animals exposed to DFXA in a dose-dependent manner with the maximal effect of about 80% at 100mg/kg.

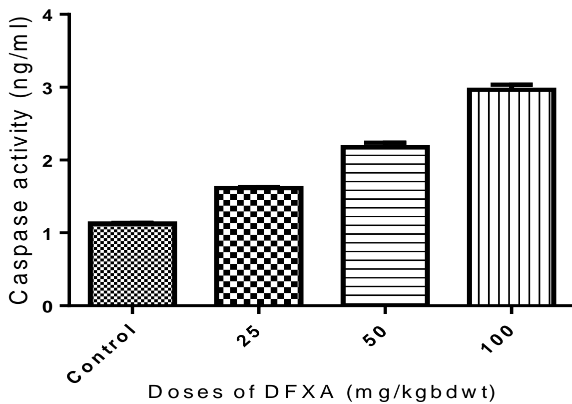

Figure 13:
Caspase 3 activity in post-mitochondrial fraction of livers of animals previously exposed (ip) to various doses of DFXA for a period of 14 days.

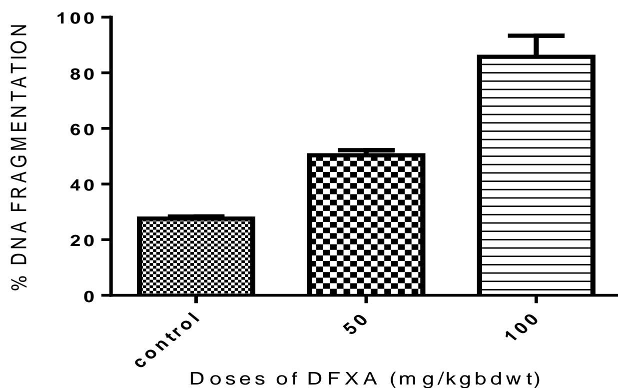

Figure 14:
The extent of DNA fragmentation in the hepatocytes of animals previously exposed to various doses of DFXA for a period of 14 days

DISCUSSION

Mitochondrial permeability transition a consequence of opening of mitochondrial membrane transition pore (mPT), is a cellular catastrophe which initiates bioenergetics collapse and apoptosis. The pore has therefore, been implicated in the pathophysiology of several major human diseases such as Ischemia, diabetes mellitus, neurodegeneration and cancer. Its role in cell death or apoptosis is orchestrated by the highly coordinated sequence of events including the release of cytochrome c, other pro-apoptotic proteins and the eventual activation of caspases which oversee the digest of the entire cell. Opening of the pore therefore represents a major therapeutic target because it can be influenced by several compounds. (Briston *et al.,* 2018).

In this study the modulatory effects of different solvent fractions of the fruits of *Xylopia aethiopica* on mitochondrial-mediated apoptosis were assessed by measuring a number of apoptotic parameters including the opening of mPT pore. This was achieved by determining the susceptibility of the mPT pore to exogenous calcium which causes swelling of the mitochondria or a decrease in light scattering. The decrease in absorbance is normally reversed by spermine or cyclosporin A, a standard inhibitor of the mPT pore opening (Lapidus and Sokolove, 1993; Javadar and Karmazyn, 2007). Our results confirm that the mPT pore opened in normal mitochondria in the presence of exogenous calcium and that spermine reversed this effect of calcium thus indicating that the mitochondria were intact and suitable for further use. Interestingly, there was significant opening of the pore when intact mitochondria were exposed to different concentrations of DXFA in the absence of calcium. This effect was reversed by spermine indicating that these fractions did not destroy the integrity of the lipid bilayer of the mitochondria but rather influenced the opening of the mPT pore. Our results on the amelioration of the ferrous-induced lipid peroxidation by varying concentrations of DFXA supported this conclusion. Similarly, in the presence of exogenous calcium, the opening of the mPT pore was further enhanced by varying concentrations of DFXA suggesting, clearly, that certain bioactive components of the fraction maintained the calcium-induced mPT opening. This observation indicates that the bioactive component in the fraction did not inhibit pore opening and may be useful in the development of chemotherapeutic substances that may find applications in diseases where apoptosis is down-regulated.

It is well established that the opening of the pore usually results in the release of some pro-apoptotic proteins such as cytochrome c from the intermembrane space to the cytosol and this is sometimes referred to as a point of no return for apoptosis to occur. We therefore, assessed *in vitro* the extent of the release of cytochrome c in the presence of varying concentrations of DFXA. Our results show that the degree of cytochrome c release was concentration dependent as seen with the extent of the opening of the mPT pore. These results confirm that the opening of the pore induced by DFXA resulted in cytochrome c release.

It is also well known that opening of the pore results in bioenergetics collapse and lack of ATP synthesis although the mATPase has now been postulated to be responsible for mitochondrial permeability transition. An assessment of mATPase in the presence of varying concentrations of DFXA revealed that the mATPase activity was significantly enhanced in a concentration-dependent manner thus suggesting that the bioenergetic status of the mitochondria was compromised in the presence of DFXA. These results show that *in vitro*, different concentrations of DFXA induced mPT pore opening resulting in the release of the cytochrome c and a concomitant enhancement of mATPase activities without any damage to the mitochondrial bilayers.

Altogether, these findings indicate that certain bioactive components present in DFXA could induce mitochondrial-mediated apoptosis. It was on this premise that we assessed the status of the biomarkers of mitochondrial-mediated apoptosis in the livers of animals previously exposed to varying doses of DFXA. Our initial attempt to assess the biomarkers in animals orally exposed to different doses of DFXA did not yield any

significant result. It was concluded that the bioactive components in DFXA were either biodegraded or not absorbed or assimilated from the gut. It was against this background that the animals were intraperitoneally exposed to varying doses of DFXA for 14 days after which they were sacrificed and their livers used for mitochondrial preparation.

The results obtained from this study show clearly that the mPT pore of animals exposed to different doses of DFXA opened significantly in a dose-dependent manner. These results are similar to our previous observations *in vitro* and they confirm that certain bioactive agents in DFXA have the potency to interact with certain components of the mitochondria or cytosol and thus mediate opening of the pore.

As seen in *in vitro* assessment of the mATPase, the enhancement of the activity of the enzyme in the livers of mitochondria of animals exposed to DFXA for a period of 14 days was highly significant at all the doses administered. Although it is well established that disruption or opening of the mPT pore results in bioenergetics collapse or depletion of ATP which is usually seen in enhancement of mATPase activity, it is not clear whether this effect has any relationship with the postulation of Bernadi, (2018) that ATP synthase acts as the pore. The finding that DFXA inhibited lipid peroxidation at all the doses used in this study confirm that the opening of pore and enhancement of mATPase were not due to any disruption of the mitochondrial membrane bilayer.

It has been established that the release of cytochrome c arising from opening of the pore causes the formation of apoptosome and activation of both the initiator and executioner caspases which bring about the degradation of the cellular components (Acehan *et al.,* 2002). In this study, we assessed the activities of caspases 9 and 3 in livers of animals intraperitoneally exposed at varying does of DFXA. The results obtained reveal that the activities of these enzymes were significantly activated at all the doses tested. The finding confirms that the opening of mPT pore caused the release of cytochrome c which results in the activation of initiator caspase 9 and the activation of executioner caspase 3. An important feature of apoptosis is the degradation of cell components and especially the occurrence of nuclear fragmentation which is seen as DNA fragmentation (Wyllie, 1980). In this study, the degree of DNA fragmentation in the hepatocytes of the animals intraperitoneally exposed to different doses of DFXA was significantly high and was also dose-dependent. This confirms our earlier finding that DFXA contains potent bioactive components that modulate mPT pore opening thereby causing cytochrome c release and the eventual activation of caspases and degradation of cell components.

In particular, these findings reveal that DFXA contains important bioactive components which should be further purified and subjected to structural elucidation or characterization in order to ascertain their role in mitochondrial-mediated apoptosis in cancer cell lines and with a view to subjecting the compound to clinical trial and drug development.

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