**Chloroform Fraction of Methanol Extract of *Funtumia elastica* (Preuss) Stem Bark Induces Mitochondrial-Mediated Cell Death Via Mitochondrial Permeability Transition Pore Opening**

**Abstract**

**Aims:** *Funtumia elastica* is a tropical medicinal plant traditionally used in the treatment of tumor. However, its mechanism of action via Mitochondrial Permeability Transition (mPT) Pore is yet to be unraveled. The mPT pore has become a significant target for drug development because its opening triggers cytochrome c release, ultimately leading to mitochondrial-mediated cell death. This study therefore aimed to explore the effects of the chloroform fraction of the methanol extract of *Funtumia elastica* (CFFE) on mitochondrial-dependent cell death through mPT pore opening.

 **Methodology:** The crude methanol extract (CMFE) was partitioned successively to obtain n-hexane (HFFE), chloroform (CFFE), ethyl acetate (EAFE) and methanol (MFFE) fractions. Two sets of the animals separately and equally divided into four groups were treated as follows; First set: Group I received corn oil(10 ml/kg) while groups II, III and IV received 200 mg/kg of CMFE, CFFE and MFFE, respectively. Second set: Groups I, II, III and IV were treated with corn oil(10 ml/kg), CFFE(50 mg/kg), CFFE(100 mg/kg) and CFFE(200 mg/kg), respectively. Rat liver mitochondria were isolated by differential centrifugation. The effects of the extract and fractions were investigated on mPT pore, mitochondrial ATPase (mATPase)activity, mitochondrial membrane lipid peroxidation(mLPO), Caspases 9 and 3 activation, and, hepatic DNA fragmentation. Histological examination on the liver was assessed. Data were analyzed using ANOVA at α 0.05.

**Results:** Oral administration of CMFE, CFFE, EAFE and MFFE at 200 mg/kg gave induction folds of 2.9, 12.3, 8.3 and 6.3, respectively, related to the control. The CFFE at 50, 100 and 200mg/kg induced pore opening by 3.7, 10.0 and 13.0 folds, respectively, related to the control. The CFFE caused significant enhancement of mATPase activity, caspases 9 and 3 activation, dose-dependent induction of hepatic DNA fragmentation, and decrease in malondialdehyde generation. Histological assessment at lower doses showed normal morphology while mild congestion was observed at the highest dose.

**Conclusion:** These results suggest the presence of phytochemicals in CFFE that can induce mitochondrial-dependent cell death via mPT pore opening. Further work is therefore necessary to characterize and isolate the active principle in CFFE that is responsible for this property.

**Keywords**: Mitochondrial permeability transition pore, *Funtumia elastica*, cell death.

**Introduction**

Mitochondrion is an intracellular organelle that is responsible for various cellular activities including ATP generation [1]. Despite its involvement in cellular energy generation, it has been implicated in programmed cell death. A key player in this event is the mitochondrial permeability transition (mPT) pore which is a protein formed under certain pathological situations such as traumatic brain injury and stroke [2]. The mPT is an increase in the permeability of inner mitochondrial membrane to ions and solutes <1.5 Kda in size. This is mediated by a channel known as mPT pore. The induction of mPT pore opening can lead to impairment of cellular bioenergetics, mitochondrial swelling, release of some proapoptotic proteins such as cytochrome c, ATP hydrolysis and cell death [3,4]. Although the structural and molecular nature of mPT pore is not yet fully elucidated, it has however been established that the inner membrane of the mitochondria is able to respond to certain stimuli such as Ca2+ overload, hypoxia and oxidative stress by opening of the mPT pore [3,4]. On opening of the pore, the release of cytochrome c leads to a point of no return for apoptosis to take place as this step leads to the formation of apoptosome, activation of caspases, the degradation of cellular components and eventually cell death [5,6]. The central signaling pathway of apoptosis includes a set of cysteine proteases called cysteinyl aspartate proteinases (caspases), which are activated by proteolysis and processing cascades triggered by proapoptotic signaling[ 5]. Activation of caspase 9 (initiator caspase) and caspase 3 or 7 (effector caspases) are crucial to the execution of mitochondrial-mediated cell death [7]. Since the mPT pore is associated with cell death, it has become a pharmacological target for the development of drugs that are relevant in diseased conditions involving dysregulated mitochondrial-mediated cell death; such as tumors and cancers [8,9]. Certain compounds and medicinal plants have been reported to exert their chemotherapeutic potential against tumors and cancers by inducing mitochondrial permeability transition (mPT) pore opening [10,11]. *Funtumia elastica* is a tropical medicinal plant traditionally used in west Africa for the treatment of tumors. Decoctions of its bark and leaves have been employed to manage cancers of the skin, throat, stomach, and breast in the Ashanti region of Ghana [12]. Additionally, *Funtumia elastica* has been used to treat other ailments, including asthma, fungal infections, whooping cough, and hemorrhoids. Its documented pharmacological activities include antioxidant [13], anti-inflammatory, antimicrobial [12], antiplasmodial and antileishmanial [14] properties. Previous studies in our laboratory demonstrated that the methanol extract of *Funtumia elastica* induced mPT pore opening, enhanced mitochondrial ATPase activity, facilitated cytochrome c release, and inhibited mitochondrial lipid peroxidation in vitro. This study aims to investigate the effects of oral administration of *Funtumia elastica* on mitochondrial-mediated cell death through mPT pore opening.

**Materials and methods**

**2.1 Chemicals and Reagents**

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

**2.2 Preparation of Funtumia elastica stem bark extract**

 *Funtumia elastica* was obtained from the Botanical Garden, University of Ibadan, authentication was carried out at the Botany Department while the voucher number UIH-22465 was deposited at the herbarium. *Funtumia elastica* stem bark was peeled, air-dried, powdered using a blender and weighed. The dried bark powder (1000 g) was macerated in 2.5 liters of absolute methanol for 72 hours with constant shaking every 24 hours. The extract obtained was filtered and concentrated in a Rotary Pump at 40°C under pressure to obtain crude methanol extract of the stem bark of *Funtumia elastica.* This was further subjected to mild heating on water bath at 40 ºC to obtain a solvent-free extract (CMFE), partitioned between n-hexane, chloroform, ethylacetate and methanol using vacuum liquid chromatography technique and concentrated to dryness to obtain solvent-free n-hexane (HFFE), chloroform (CFFE), ethylacetate (EAFE) and the methanol (MFFE) fractions.

**2.3 Ethical approval and experimental animal care**

The animals used were cared for according to all relevant National regulations and institutional policies on animal care were adhered to. The study was approved by the Animal Care Use and Research Ethics Committee (ACUREC), University of Ibadan, Ibadan, Nigeria, and assigned the certificate number UI–ACUREC⁄038-0521/11.

**2.4 Experimental Animals**

Two sets of male Wistar rats weighing between 100g–120g were acclimatized and kept in standard cages with free access to rat chow and water. All the experiments have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

**2.4.1. First set**

Twenty albino male rats were randomly and equally assigned into four groups. Group 1 received corn oil (10 ml/kg) while groups 2, 3 and 4 were treated with 200 mg/kg CMFE, CFFE, EAFE and MFFE, respectively. The dose was chosen based on literature search and pilot study. The animals were sacrificed after 28 days of oral treatment. The mPT assay was carried out to determine the potencies of the fractions.

**2.4.2. Second set**

Based on the results from the first set, fraction CFFE, which exhibited the highest potency was selected for use in the second set. Twenty albino male rats were equally assigned into four groups. Group 1 received corn oil (10 ml/kg), while groups 2, 3 and 4 were treated with 50, 100 and 200 mg/kg CFFE, respectively. The rats were sacrificed after 28 days of oral treatment. Histological assessment of the liver were carried out.

**2.5 Tissue preparation for histopathology**

Liver was used for histopathology. Ultra-thin sections were stained with Hematoxylin and eosin (H&E) for histological examinations. The histological pictures were taken using an Olympus microscope, Japan.

**2.6 Isolation of rat liver mitochondria**

The isolation was carried out as described by Johnson and Lardy [15], with little modification by Olorunsogo et al. [16]. 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. All experiments with isolated mitochondria were performed within 4hr of the preparation.

**2.7 Mitochondrial swelling assay**

Mitochondrial Permeability Transition (mPT) was monitored following the method of Lapidus and Sokole, [17]. 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 270C prior to the addition of 120µM 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

**2.8 Determination of mitochondrial protein**

This was determined according to the method of Lowry et al. [18], using bovine serum albumin as standard.

**2.9 Assessment of mitochondrial F0F1 ATPase activity**

The F0F1 Adenosine triphosphatase was carried out by a modification of the method of Olorunsogo and Malomo [19], using 2, 4 Dinitrophenol (2,4 DNP) as a standard uncoupling agent. 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 270C. 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.

**2.10 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 [20] and as modified by Olorunsogo and Malomo [19]. 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.

**2.11 Assessment of Lipid Peroxidation**

Malondialdehyde (MDA) content in mitochondria was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) present in the mitochondria according to the method of Varshney and Kale, [21]. The rat liver mitochondria (0.5 mg protein/ml) were incubated with 1.6ml of Tris- KCL buffer to which 0.5ml of 30% TCA was added. Then 0.5ml of 0.75% TBA was added and placed in a water bath for 45minutes at 80ºC. This was then cooled in ice to room temperature and centrifuged at 3000rpm for 10min. The clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532nm.

**2.12 Analyses of caspases 9 and 3**

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 freezed. 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. The Caspases 9 and 3 levels were determined by following the manufacturer’s instruction, using CUSABIO® (Rat Caspase-9 (Casp-9) and Caspase-3(Casp-3) ELISA Kit; Catalog Number (CSB-E08863r). A microplate reader (DNM-9602 A from China) was used to read the optical density at 450 nm wavelength.

**2.13 DNA Fragmentation**

 The percentage hepatic DNA fragmentation was determined according to the method of Wu et al. [22]. Liver was sliced with scissors and homogenized in 10 volumes of Tri-EDTA Triton buffer (TET) pH 8.0. Homogenates were centrifuged at 27,000 × g for 20 min to separate intact chromatin (pellet A) from fragmented (pellet B). Pellet A was suspended in Tris EDTA buffer (TE) pH 8.0. An aliquot (1 ml) of each sample (pellet and supernatant) was placed in separate test tubes and then 1 ml of freshly prepared diphenylamine solution was added to each. Reaction mixture was incubated at 37 °C for 20 h. Absorbance of the mixture was then measured at 620 nm.

Calculation: Quantity of fragmented DNA was estimated by using the formula:

 % fragmented DNA= {B÷(A+B)}X100 Where A is the intact chromatin and B is the fragmented chromatin. Absorbance was read at 620nm.

 **2.14 Statistical analysis of data**

 The data reported on mPT are representative of multiple (≥4) experiments using microsoft excel 2010. All other data were expressed as mean ± SD.

**3.0 Results**

**Determination of intactness of the isolated**

The intactness of the isolated mitochondria was determined by monitoring the swelling of the isolated mitochondria using spectrophotometer, in the absence of calcium, presence of calcium and presence of inhibitor (spermine). As depicted in figure 1, there was no conspicuous swelling of mitochondria in the absence of calcium over a period of twelve minutes. Nevertheless, the inclusion of exogenous calcium brought about large amplitude swelling of the mitochondria which was almost completely reversed by the inhibitor.

**Figure 1**:Calcium-induced mitochondrial permeability transition pore opening in normal rat liver mitochondria and its reversal by spermine. NTA no triggering agent (without calcium), TA triggering agent (calcium), Spermine standard inhibitor of mPT pore opening

**Effects of the extract and fractions of *Futumia elastica* on the mPT Pore**

Figure 2 depicts a representative profile of changes in absorbance of liver mitochondria of rat treated with 200 mg/kg of crude methanol stem bark extract and fractions of *Funtumia elastica.* The data shows the extent of induction of the various fractions. The CMFE, CFFE, EAFE and MFFE gave induction folds of 2.9, 12.3, 8.3 and 6.3, respectively, related to the control.

**Figure 2**: Representative profile showing the effects of the extract and fractions on mPT pore

**CMFE**: (crude methanol extract of *Funtumia elastica*)

**CFFE**: (Chloroform fraction of the methanol extract of *Funtumia elastica*)

**EAFE**: (ethyl acetate fraction of the methanol extract of *Funtumia elastica*)

**MFFE**: (methanol fraction of the methanol extract of *Funtumia elastica*)

**Effects of CFFE on imPT pore opening**

The data in Fig. 3 shows the effects of varying doses of CFFE (50, 100, 200 mg/kg) on imPT pore opening. Treatment with CFFE shows a significant dose-dependent induction of pore opening compared with the control. The CFFE at 50, 100 and 200 mg/kg caused induction folds of 3.7, 10.0 and 13.0, respectively, when related to the control.



**Figure 3**: Representative profile showing the effects of varying doses of CFFE on imPT pore opening after 28 days of oral treatment

**Effects of CFFE on mitochondrial F1F0 ATPase activity**

The effects of CFFE on mitochondrial ATPase is illustrated in Fig. 4. The results indicated a significant dose-dependent enhancement of mitochondrial ATPase activity related to the control. The CFFE at doses 50, 100 and 200 mg/kg caused increase in mitochondrial ATPase activity by 20, 40, 56 %, respectively, when compared to the control.



**Figure 4:** Effects of varying doses of CFFE on mitochondrial ATPase activity

The values are expressed as mean ± SD of four independent replicates. \*P<0.05, \*\*P< 0.01, \*\*\*P<0.001 compared to the control. 2,4-DNP (2,4-Dinitrophenol-classical uncoupler of oxidative phosphorylation)

**Effects of CFFE on mitochondrial Lipid Peroxidation (mLPO)**

The CFFE inhibited mLPO in a dose-dependent manner related to the control, as indicated in figure 5.



**Figure 5:** Effects of varying doses of CFFE on Lipid peroxidation

The values are expressed as mean ± SD of four independent replicates. \*P<0.05, compared to the control

**Effects of CFFE on caspases 9 and 3 levels**

The data in figures 6 and 7 indicated a dose-dependent increase in the levels of caspases 9 (54.4, 78.8, 98.6 ng/ml) and 3 (10.1, 14.0, 22.6 ng/ml), at doses 50, 100 and 200 mg/kg, respectively, related to the control. CFFE activated caspase 9 by 50% 84%, 150% and caspase 3 by 11%, 55%, 144%, at doses 50,100 and 200 mg/kg, respectively, compared to the control.



**Figure 6:** Effects of varying doses of CFFE on caspase 9 activity

The values are expressed as mean ± SD of four independent replicates. \*P<0.05, \*\*P< 0.01, \*\*\*\*P<0.0001 compared to the control



**Figure 7:** Effects of varying doses of CFFE on caspase 3 activity

The values are expressed as mean ± SD of four independent replicates. \*P<0.05, \*\*\*P<0.001 compared to the control.

**Effect of CFFE on Hepatic DNA Fragmentation.**

This is illustrated in figure 8. The results showed increase in percentage hepatic DNA fragmentation by 20, 37 and 50 % at doses 50, 100 and 200mg/kg, respectively, relative to the control.



**Figure 8:** Effects of varying doses of CFFE on hepatic DNA fragmentation

The values are expressed as mean ± SD of four independent replicates. \*P<0.05, compared to the control.

**Histological examination**

The results on the liver section of the control revealed normal morphology and healthy unfiltrated hepatocytes, without any pathological lesion. At doses 50 and 100 mg/kg, the hepatocytes displayed normal morphology, only with mild congestion at 100 mg/kg. At 200 mg/kg, there was mild congestion of the central venules, hepatic steatosis, while the sinusoids still showed very mild infiltration.



**Figure 9:** Representative Photomicrographs of rat liver section

**(Control):** Shows the morphology of liver section of a normal rat. No lesions found, normal hepatocellular outline. **(50mg/kg):** Shows normal central venules without congestion. The hepatocytes with moderate infiltration by inflammatory cells and the sinusoids appear normal and not infiltrated. **(100mg/kg):** Shows central venules with mild congestion, focal area of inflammatory cells aggregate (black arrow), the sinusoids appear normal and not infiltrated. **(200mg/kg):** Shows the central venules with mild congestion, focal area shows the hepatocytes with infiltration (black arrow), hepatic steatosis, the sinusoids show very mild infiltration. Magnification (X 400).

**Discussion**

Mitochondria play a crucial role in controlling cell death [23]. The mPT pore is now recognized as a determinant of cell survival or death [24]. This study investigated the effect of chloroform fraction of methanol extract of *Funtumia elastica* stem bark on mitochondrial-mediated apoptosis using rat liver. The mitochondria used in this study were intact as indicated by the calcium-induced pore opening and its reversal by spermine, a standard inhibitor of pore opening [17,26]. The inclusion of exogenous calcium (standard triggering agent) into the medium caused increase in Ca2+ transport, leading to matrix Ca2+ overload, causing activation and opening of highly conductive mPT pore [26]. Having ascertained the intactness of the mitochondria used in this study, we explored the effects of the crude extract (CMFE) and its fractions (CFFE, EAFE and MFFE) on mPT pore opening. The extract and fractions caused mPT pore opening to varying degree, indicating the presence of phytochemicals in them that can interact with the pore and cause pore opening. However, CFFE was the most potent, suggesting that it contains the bioactive principle in abundance that can cause the induction of pore opening. This is comparable to the discoveries of Olowofolahan et al. [27,28], which demonstrated that chloroform fraction of methanol extract of *Drymaria cordata* is the most potent with respect to induction of mPT pore opening. The dose-dependent study on CFFE showed increase in induction of mPT pore with increase in dosage. This probably suggests its increase in bioavailability at the target site with increase in the dosage.

 Studies have revealed that mPT pore opening instigates the release of mitochondrial factors, dissipation of membrane potential (Δψ), loss of cellular biochemical homeostasis and mitochondrial ATP hydrolysis [29,30]. The enhancement of mATP activity as a result of graded doses of CFFE treatment suggests that CFFE interacted with the mPT pore to instigate the pore opening, effecting subsequent release of some mitochondrial factors, mitochondrial ATP hydrolysis, mitochondrial energetic dysfunction and subsequently, cell death [2,31]. Studies have shown that reactive oxygen species (ROS) trigger peroxidation of mitochondrial membranes leading to cell death. Interestingly, there are compounds that can mitigate lipid peroxidation by inhibiting radical-induced oxidative damage [32]. The decrease in malondialdehyde levels as a result of CFFE treatment suggests the presence of phytochemicals in CFFE that can reduce free radical generation. Furthermore, it also suggests its physicochemical protective potential on membrane against free radical-induced damage.

The increase in levels of caspase 9 by CFFE treatment suggests that it is an inducer of mitochondrial-mediated apoptosis. Also, the increase in caspase 3 levels by CFFE indicated that activated caspase 9 caused cleavage of procaspase 3 to activate caspase 3 for subsequent downstream reaction. Caspases activation have been demonstrated to be crucial to execution of apoptotic process [7]. One of the hallmarks of apoptosis is nuclear DNA fragmentation. The CFFE caused dose-dependent increase in the hepatic DNA fragmentation, suggesting that the activated caspases by CFFE effected the activation of Deoxyribonlease (DNase) and subsequent cleavage of nuclear DNA. On the histological assessment, the CFFE did not really show any significant pathological lesion on the hepatocytes at the dosages used. However, higher doses could have a detrimental effect based on the effect shown at 200 mg/kg in this study.

**Conclusion**: Based on the findings in this study, it can be suggested that chloroform fraction of methanol extract of *Funtumia elastica* is an inducer of mitochondrial-mediated cell death. Further work is still necessary to characterize and isolate the active principle responsible for these pharmacological potentials. This may be useful in situations that require upregulation of apoptosis.

Disclaimer (Artificial intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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