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Research Article

Induction of Mitochondrial Membrane Permeability Transition Pore Opening and DNA fragmentation by Solvent Fractions of *Mangifera indica*

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Abstract
The opening of mitochondrial permeability transition (mPT) pore is a crucial step for apoptotic cell death. Certain bioactive agents in medicinal plants elicit their chemo-protective effect against tumor via the induction of the mPT pore opening. This study therefore investigated the effects of different solvent fractions of *Mangifera indica* on mitochondrial-mediated apoptosis via mPT pore opening to ascertain the most potent fraction. Methanol extract of *Mangifera indica* was partitioned successively to obtain n-hexane (NFMI), dichloromethane (DFMI), ethylacetate (EFMI) and methanol (MFMI) fractions. Rat liver and uterine mitochondria were isolated by differential centrifugation. The effects of DFMI, EFMI and MFMI on mPT pore, cytochrome c release, mitochondrial ATPase activity, lipid peroxidation and hepatic and uterine DNA fragmentation were assessed spectrophotometrically while caspases 9 and 3 activities were determined using ELISA technique. The *in vitro* results showed that there was a concentration-dependent induction of mPT pore opening by MFMI and EFMI. However, MFMI was more potent than EFMI while DFMI did not have any effect. Similar pattern of results were recorded on cytochrome c release and mitochondrial ATPase activity. The *in vivo* results on mPT pore also showed MFMI to be the most potent, followed by EFMI while DFMI had no effect. The results on DNA fragmentation and caspases also showed MFMI to be the most potent of the three solvent fractions. The results of this study suggest that MFMI is the most potent containing the bioactive agents that may induce mitochondrial-mediated apoptosis.

Key Words: *Mangifera indica, apoptosis, mitochondrial permeability transition pore*

INTRODUCTION

Apoptosis is one of the potent defence mechanisms by which potentially deleterious and mutated cells are removed from an organism, ensuring proper growth and development, differentiation, metarmorphosis and embryogenesis. (McIlwain*et al*., 2013). Dysregulation of apoptotic signalling has been implicated in various pathologic conditions like cancer, autoimmunity, Alzheimers' disease, etc. (Reed, 2004). Mitochondria have become a vital component of apoptosis execution mediating the intrinsic pathway. This involves movement of some apoptogenic proteins into the cytosol and consequently resulting in cell death (Kallenberger*et al*., 2014). Mitochondrion is composed of outer and inner membrane. Permeabilization of the outer membrane is under tight regulation of Bcl_2 family proteins, controlling the movement of some apoptogenic proteins into the cytosol, thereby activating apoptosis (Crompton, 1999). Induction of mitochondrial permeability transition (mPT) pore can cause mitochondrial swelling, release of cytochrome c and formation of apoptosome which will ultimately lead to cell death. Various experimental evidences have proved that mitochondrial permeabilisation is a major event in the induction of mitochondrial-mediated apoptosis (Wang, 2012). The mPT pore therefore serves as a target for the design of novel drugs which will be needful in diseases where apoptosis needs to be upregulated or downregulated. The application of various medicinal plants with potent bioactive agent have been used in targeting mitochondrial apoptotic machineries through the activation of mPT pore towards efficient and selective treatment of diseases associated with dysregulated apoptosis, such as cancer (Martins, 2006).

Mangifera indica is a species of flowering plants which belongs to the family of Anacardiaceae. It is popularly known as mango. It is folklorically used in the treatment of fibroid, asthma, cough, etc. Its antioxidant, wound healing, immunomodulation, cardiotonic, hypotensive, antidegenerative and antidiabetic properties have been reported (Barreto*et al*., 2008; Shah *et al*., 2010; Gabino *et al*.,2008). Studies have also shown that crude methanol extract of *mangifera indica* (MEMI) is a potent inducer of mPT pore opening (Olowofolahan *et al*., 2018). Though, the chemical nature of the substances responsible for the opening of the pore is still unknown.

In order to elucidate and characterize the structure of putative agent(s) present in MEMI responsible for the induction of mitochondrial-mediated apoptosis, the MEMI was partitioned into fractions and their potencies with respect

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to induction of mPT pore opening were investigated. This study was therefore designed to determine the modulatory effect of various fractions of MEMI on mitochondrial-mediated apoptosis via the the induction of mPT pore opening and thus, identify the most potent fraction for possible subjection to further work on structural elucidation and characterization.

MATERIALS AND METHODS

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.

Plant Material: The leaves of *Mangifera indica*were bought from Bode market in Ibadan, Oyo State, Nigeria and authenticated at Botany department, University of Ibadan, Nigeria, with voucher number UIH 22555.

Preparation of Extract: The leaves of *Mangifera indica* were cut into smaller pieces, washed, shade-dried under laboratory conditions for 4 weeks and pulverized to powder using a grinder. It was then soaked in methanol for 72 hours. The filtrate obtained was concentrated using a vacuum rotary evaporator (N-100, Eyla, Tokyo, Japan) and was later concentrated to dryness using a water bath at 37ºC.

Preparation of the fractions: The crude methanol extract was further partitioned between n-hexane, dichloromethane, ethylacetate and methanol using vacuum liquid chromatography technique. All these fractions were concentrated to dryness under pressure using rotary evaporator at 40$_o$C to obtain the n-hexane (HFMI), dichloromethane (DFMI), ethylacetate (EF) and the methanol (MFDC) fractions.

Experimental Animals: Female Wistar strain albino rats weighing between 90–100g 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. All animal experiments were conducted according to the guidelines of National Institute of Health (NIH publication 85-23, 1985) for laboratory animal care and use.

Animals Groupings, Treatment and Sample collections: Twenty female Wistar rats were divided equally into four groups: Group A was the control group. Group B, C and D were treated with 50mg/kgbdwt DFMI, EFMI and MFMI, respectively as a single dose daily by oral gavage for 28 days. One day after the final exposure, the animals were sacrificed.

Isolation of rat liver mitochondria: Rat liver mitochondria were isolated essentially according to the method of Johnson and Lardy (1967) as modified by Olorunsogo *et al* (1979). 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$_o$C ice.

Isolation of rat uterine mitochondria: The rat uterus mitochondria were isolated essentially according to the method of costa *et al*., (2006) based on differential centrifugation technique. The animals were sacrificed by cervical dislocation. For the extraction of mitochondria, the tissue of uterus was cleaned up from blood and fat, minced and homogenized on ice in 8 ml of isolation buffer containing 70 mM of sucrose, 1 mM of EDTA, and 5 mM of HEPES (pH 7.2). The homogenate is centrifuged for 7 min at 1000 g and temperature of 4$_o$C. The supernatant is separated and centrifuged for 7 min at 12,000g and temperature of 4$_o$C. The pellet was resuspended in isolation buffer (containing no EDTA) and kept on ice (4$_o$C). Protein content was determined by Lowry method.

Mitochondrial Swelling Assay: Mitochondrial membrane permeability transition was monitored by measuring changes in absorbance of mitochondrial suspension in the presence or absence of calcium (the triggering agent) in aT70 UV/visible spectrophotometer essentially according to the method of Lapidus and Sokolove (1993). Mitochondria (0.4mg protein/ml) were preincubated in the presence of 0.8μM rotenone in a medium containing 210mM mannitol, 70mM sucrose and 5mM HEPES-KOH (pH 7.4) for 3mins at 27$_0$C prior to the addition of 120μM $CaCl_2$. Thirty seconds later, 5mM succinate was added and mitochondrial permeability transition quantified at 540nm for 12mins at 30secs interval. To test the intactness of the mitochondria, 4mM spermine was added immediately following the addition of rotenone and just before the addition of mitochondrial fraction**.**

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

Assessments of Mitochondrial F_oF_1 ATPase Activity: FoF1 Adenosine triphosphatase which was determined by a modification of the method of Olorunsogo and Malomo (1985). Each reaction mixture contained 65mM Tris-HCl buffer pH 7.4, 0.5mM KCl 1mM ATP and 25mM sucrose. The reaction mixture was made up to a total volume of 2ml with distilled water. The reaction was started by the addition of mitochondrial suspension and was allowed to proceed for 30

mins at 27$_0$C. The reaction was stopped by the addition of 1 ml of a 10% solution of sodium dodecyl sulphate. The zero time tube was prepared by adding the solution of ATP to the reaction vessel following the addition of sodium dodecyl sulphate. 2,4 Dinitrophenol (2,4-DNP) was used as a standard uncoupling agent.

Estimation of Inorganic phosphate released: The concentration of inorganic phosphate released following the hydrolysis of ATP was determined according to the method described by Bassir (1963) and as modified by Olorunsogo *et al* (1979). 300µl of each solution was dispensed into fresh test tubes, followed by the addition of 300µl of distilled water to each of the test tube. To this was added 1 ml of 5% ammonium molybdate and 1 ml of 9% freshly prepared solution of ascorbic acid. The tube was well mixed and allowed to stand for 20 minutes. The absorbance was read at 680nm. A water blank was used to set the spectrophotometer at zero.

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 (1990). 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$_0$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.

The MDA level was calculated using an extinction coefficient of 0.156 µM^{-1}cm^{-1} (Adam-Vizi and Seregi, 1982).
Lipid peroxidation (nmole MDA/mg protein) = Absorbance × volume of mixture
E532nm × volume of sample × mg protein/ml

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 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.

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.

DNA Fragmentation: The percentage DNA fragmentation was determined according to the method of Wu *et al*., 2005. 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 20 mins 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^{0}C for 20 hours. Absorbance of the mixture was then measured at 620nm.
Quantity of fragmented DNA was estimated by using the formula: % fragmented DNA = B/(A+B) X 100

Statistical Analysis of Data
Statistical analysis was performed using one way analysis of variance (ANOVA). Level of significance was set at $p < 0.05$.

RESULTS

The data presented in figure 1 shows that there was no significant change in volume of intact mitochondria respiring on succinate in the presence of rotenone over a period of 12 minutes. The addition of calcium to the reaction medium caused a significant induction of mPT pore opening which was remarkably reversed by spermine.

The effect of DFMI was depicted in figure 2. As seen from the graph, there was no significant change in the volume of mitochondria over the period of 12 minutes at all the concentrations tested.

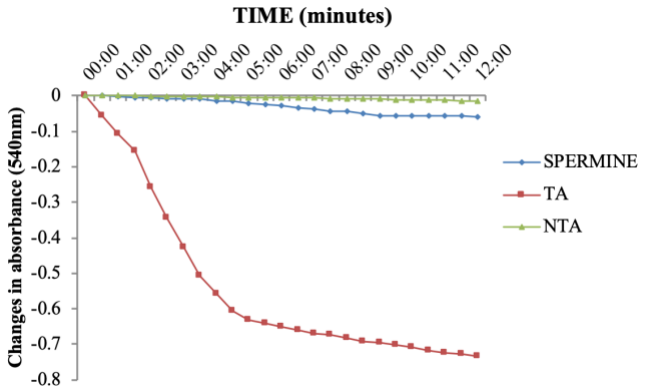

Figure 1:
Calcium-induced mitochondrial membrane permeability transition pore opening and its reversal by spermine

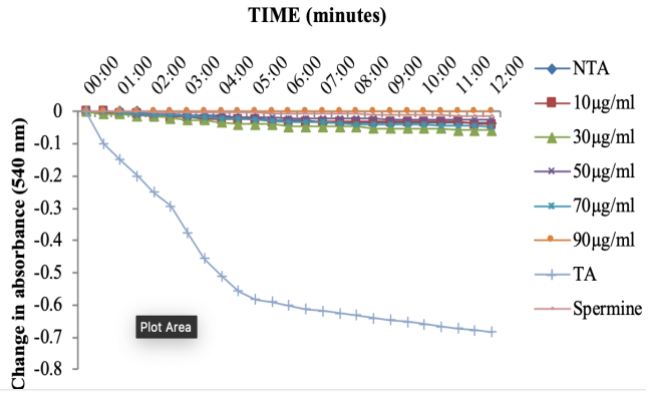

Figure 2:
Effect of varying concentrations of DFMI on rat liver mitochondrial membrane permeability transition pore opening

In the case of EFMI, there was a concentration-dependent increase in the induction of mPT pore opening with the highest

concentration (90µg/ml) having induction folds of 8 when compared with the control as shown in figure 3.

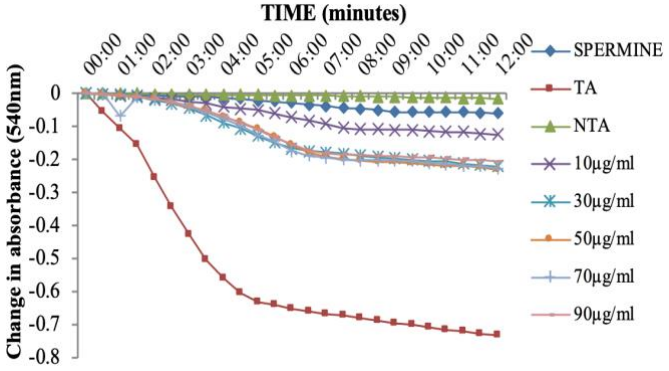

Figure 3:
Effect of varying concentrations of EFMI on rat liver mitochondrial membrane permeability transition pore opening

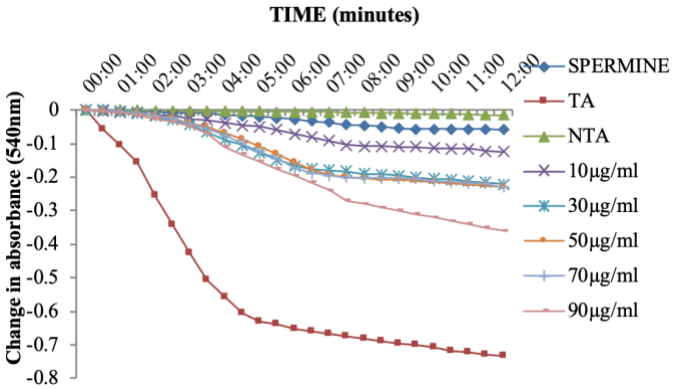

Figure 4:
Effect of varying concentrations of MFMI on rat liver mitochondrial membrane permeability transition pore opening

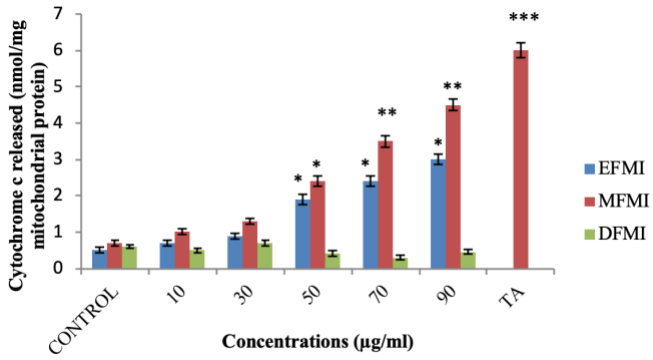

Figure 5:
Effect of varying concentrations of different solvent fractions of *Mangifera indica* on cytochrome c release

Figure 4 shows the effect of MFMI on mPT pore opening. The results also show a concentration-dependent increase in mPT pore opening with the highest concentration having induction folds of 15 when compared with the control.

The effect of varying concentrations of the different solvent fractions of *Mangifera indica* on cytochrome c was illustrated in figure 5. As seen from the figure, DFMI had no significant effect on cytochrome c release while EFMI and MFMI significantly caused cytochrome c. However, MFMI is more potent than EFMI in the release of cytochrome.

Their effects on ATPase activity were presented in figure 6 which also follow similar pattern. MFMI was equally the most potent with respect to enhancement of ATPase activity.

The MFMI being the most potent among the solvent fractions used in this study was investigated for its effect on lipid peroxidation. The results show that MFMI inhibited lipid peroxidation at all the concentrations used in a concentration-dependent manner as indicated in figure 7.

Figure 8 shows the effect of oral administration of different solvent fractions of *Mangifera indica* on rat liver mPT pore opening after 28 days of treatment. From the results MFMI was also the most potent with respect to induction of mPT pore opening.

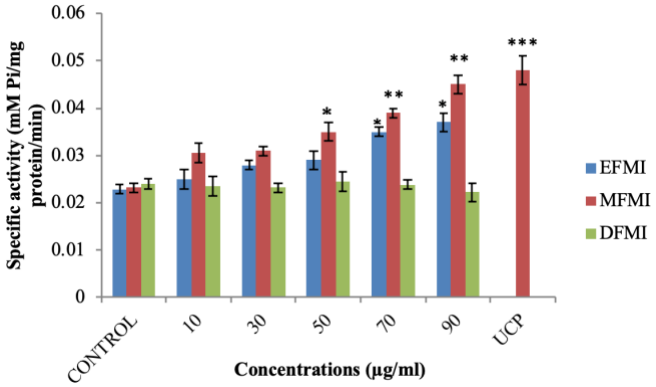

Figure 6:
Effect of varying concentrations of different solvent fractions of *Mangifera indica* on mitochondrial ATPase activity

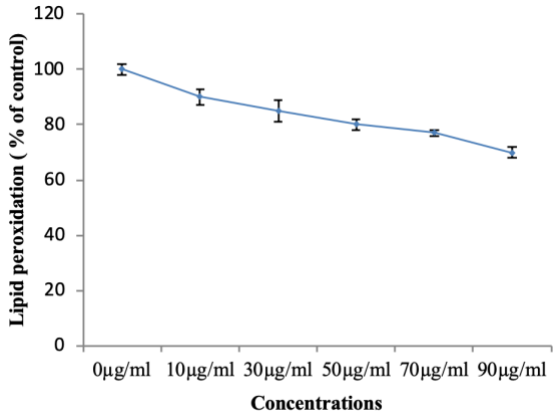

Figure 7:
Effect of varying concentrations of MFMI on Lipid peroxidation

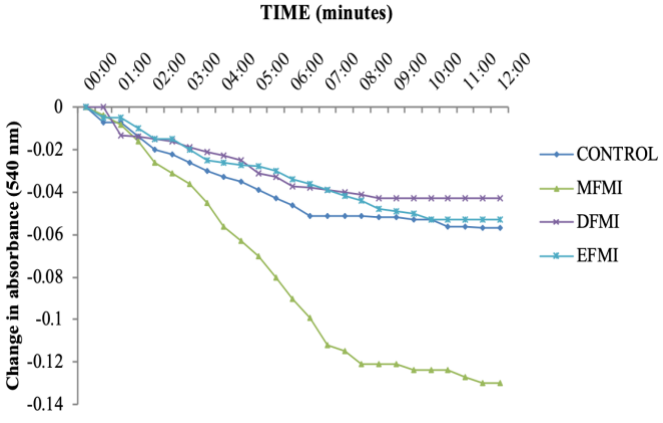

Figure 8:

Effect of oral administration of different solvent fractions of *Mangifera indica* on rat liver mitochondrial membrane permeability transition pore opening after 28 days of treatment

Figure 9 as well shows the results of oral administration of different solvent fractions of *Mangifera indica* on uterine mPT pore opening after 28 days of treatment. The MFMI was also found to be the most potent.

The effects of oral administration of the solvent fractions on caspases 9 and 3 were presented in figures 10 and 11. From the results, EFMI and MFMI significantly caused caspases 9 and 3 activation when compared with the control. However, DFMI did not have any significant effect on the initiator and executioner caspases. With respect to caspase activation, MFMI is more potent than EFMI.

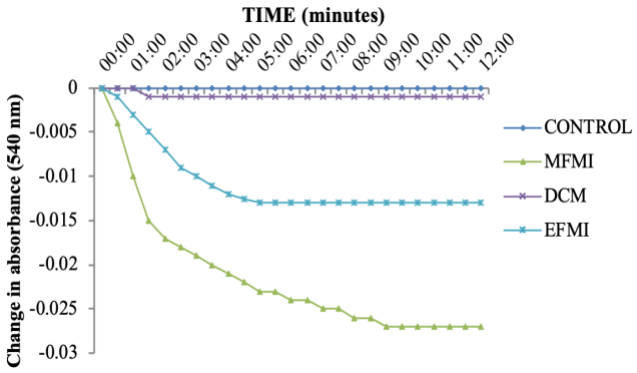

Figure 9:
Effect of oral administration of different solvent fractions of *Mangifera indica* on uterine mitochondrial membrane permeability transition pore opening after 28 days of treatment

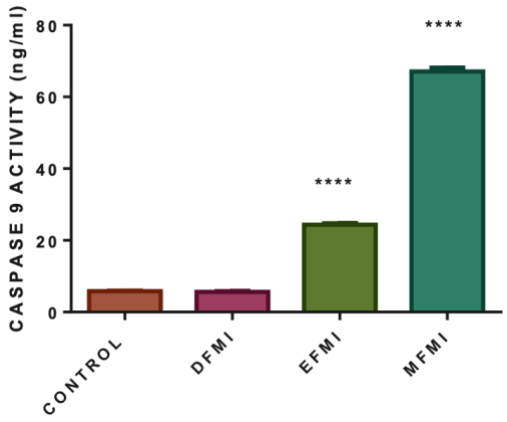

Figure 10:
Effect of oral administration of different solvent fractions of *Mangifera indica* on caspase 9 activity after 28 days

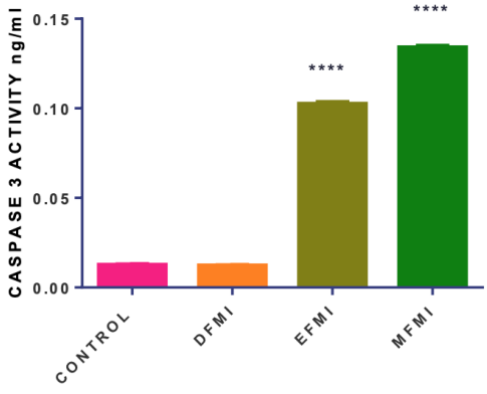

Figure 11:
Effect of oral administration of different solvent fractions of *Mangifera indica* on caspase 3 activity after 28 days

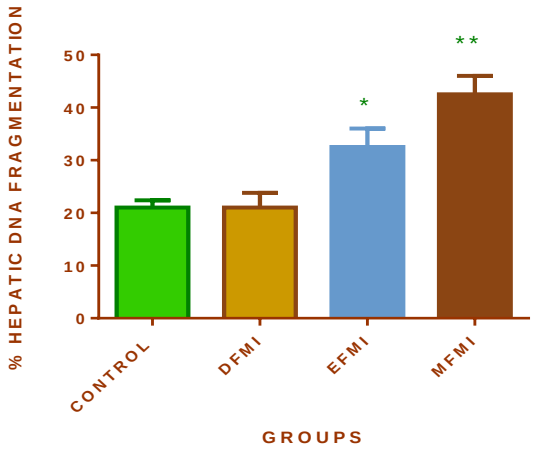

Figure 12:
Effect of oral administration of different solvent fractions of *Mangifera indica* on Hepatic DNA Fragmentation

The data on hepatic and uterine DNA fragmentation showed that both EFMI and MFMI significantly induced DNA fragmentation while DFMI did not show any significant effect when compare with the control group. However, MFMI was more potent than EFMI with respect to both hepatic and uterine DNA fragmentation

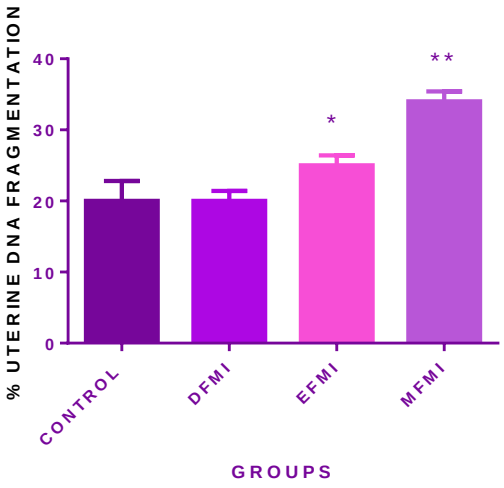

Figure 13:
Effect of oral administration of different solvent fractions of *Mangifera indica* on Uterine DNA Fragmentation

DISCUSSION

Our previous study on methanol extract of *mangifera indica* has shown that it is potent with respect induction of mPT pore opening which could lead to the release of some apoptogenic proteins such as cytochrome c. In this study, we decided to partition the methanol extract into various fractions and determine the most potent with respect to mitochondrial-mediated apoptosis. In this study, the integrity of the mitochondria used for this experiment was first ascertained by monitoring the calcium-induced decrease in light scattering that reflects the mitochondrial swelling accompanying calcium-induced opening of the pore and its reversal by

spermine, a standard inhibitor of pore opening. (Lapidus and Sokolove,1993;
Javadov and Karmazyn, 2007. This shows that the mitochondria used in this study were intact *al binitio* as shown in figure 1. As seen in figure 2, DFMI had no effect on mPT pore .This shows that it does not contain phytochemicals that could interact with the components of the pore and cause the opening of the pore like EFMI and MFMI as shown in figures 3 and 4. The EFMI and MFMI contain the bioactive agents that could induce mPT pore opening, though the active principle is more in the MFMI as it has higher inductive effect than DFMI. This shows that the order of potency of the solvent fractions of *Mangifera indica* with respect to mPT pore opening is MFMI > EFMI >DCMI. This is similar to findings of Olowofolahan *et al*., (2015) where the bioactivity guided assay showed that the chloroform fraction of methanol extract of *Drymaria cordata* (CFDC) was the most potent with respect to induction of mPT pore opening. The release of cytochrome c is a major event in mitochondrial-mediated apoptosis (Green and Kroemer, 2004). The effects of the solvent fractions on cytochrome c release show that MFMI caused the highest release of cytochrome c. This is in consonant with our result on MPT where MFMI is the most potent. This result also confirms the presence of the highest concentration of the putative agent in MFMI as it caused the release of cytochrome c more than EFMI while DFMI had no significant effect. The results on mitochondrial ATPase activity show that MFMI out of all the fractions tested had the highest effect on ATPase activity. This could be due to the presence of some phytochemicals highly concentrated MFMI that interacted with the pore components, caused the opening of the pore and subsequently, the release of cytochrome c. The results from the lipid peroxidation show that MFMI protects against lipid peroxidation-induced damage. Also, it could be inferred that the opening of the mPT pore by MFMI was not as a result of lipid peroxidation but rather by interaction of MFMI with the components of the pore as MFMI inhibited lipid peroxidation. The *in vivo* results on rat liver mPT is also in accordance with the *in vitro* results. This also shows that the fractions especially MFMI which is the most potent was readily available to cause the induction of pore opening. In the uterus, the fractions were able to cause the opening of the pore with MFMI being the most potent. This also suggests that the induction of mPT pore opening in the uterus might be one of the mechanistic approach by which methanol extract of *Mangifera indica* reversed monosodium glutamate-induced uterine hyperplasia (Olowofolahan *et al*., 2018).

Apoptosis could occur via various pathways including the intrinsic, extrinsic and perforin/granzyme B pathways which can be initiated by caspase-9, caspase-8 and caspase-10, respectively (Sadowski-Debbing *et al*., 2002). On assessment of caspases (9 and 3) in wistar rats administered the solvent fractions of *Mangifera indica*, the activities of the two enzymes increased significantly when compared with the control. However, MFMI showed higher potency than EFMI while DFMI showed no significant effect. The results on the caspases confirm that MFMI interacted with the mitochondrial components to cause the release of cytochrome c from the inter membrane space into the cytosol and ultimately lead to caspases activation. One of the hallmark of apoptosis is nuclear DNA fragmentation by a specific nuclease called caspase-activated DNase (CAD). Activation of CAD by the caspases leads to specific cleavage of DNA into internucleosomal fragments of about 180 base pairs. The results on hepatic and uterine DNA fragmentation showed that the activated caspases by EFMI and MFMI lead to the activation of DNase with subsequent cleavage of nuclear DNA. The MFMI being more potent than the EFMI also suggests that the active principle causing the activation of CAD is highly resident in the polar solvent fraction.

The results from this study show that MFMI is the most potent of the fractions used in this study and it is an inducer of apoptosis via mitochondrial-mediated pathway. In conclusion, the nature of substances that is responsible for these pharmacological properties shown by MFMI are still unknown. Therefore, there is a need to elucidate and characterize the structure of putative compound(s) present in MFMI and their effects on induction of mitochondrial-mediated apoptosis. This could be relevant in the management and treatment of diseases where apoptosis needs to be upregulated

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