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Toxic Effects of Unripe *Carica papaya* (Linn) Fruit Extract on Healthy Rat Liver Mitochondria

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Abstract

The opening of the mitochondrial Permeability Transition (mPT) pore preceeds the activation of programmed cell death (apoptosis) and its functional status serves as marker of mitochondrial health. Unripe fruits of *Carica papaya* are used in the traditional treatment of several diseases. There is paucity of information on the level of safety of the consumption of the plant. The effects of crude Methanol Extract of *Carica papaya* (MECP) on the status of the mPT pore in healthy rat liver were investigated in this study. Mitochondrial FOF1 ATPase activity, mitochondrial permeability transition and mitochondrial lipid peroxidation as well as the release of cytochrome c were evaluated spectrophotometrically using standard methods. The MECP activated mPT pore opening in the absence of calcium in a concentration-dependent fashion. Specifically, induction folds of 3.1, 6.0, 9.1, 11.9 and 14.3 were recorded at 20, 60,100, 140and 180 µg/ml, respectively. In addition, MECP potentiated calcium-induced pore opening of the mPT pore in a concentration-dependent fashion by 22.5, 24.1, 25.0, 25.1 and 25.5 folds, respectively at 20, 60, 100, 140 and 180 µg/mL. Furthermore, mitochondrial ATPase activity was significantly ($p < 0.001$) stimulated at pH (7.4) while the extent of cytochrome c release increased by 5 and 7 folds, respectively, at the highest concentrations tested. Interestingly, Fe^{2+}-induced mitochondrial lipid peroxidation was inhibited by varying concentrations of MECP. Specifically, significant ($p < 0.001$) reduction in levels of mitochondrial lipid peroxides were observed at 50, 100, 200, 300, 600 µg/ml MECP by 10, 22, 53, 74, 112 %, respectively. These findings indicate that unripe *Carica papaya* fruit extract contains bioactive compounds that cause mitochondrial injury via activation of the mitochondrial permeability transition pore opening in healthy liver cells. Hence, its use in the management of diseases should be approached with caution.

Keywords: Mitochondria; *Carica papaya*; Permeability transition; FOF1 ATPase activity

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Introduction

The use of plants in the traditional treatment of several diseases is well established. Evidence of the potency of plants and plant-based agents in the management of a wide range of diseases is generally accepted [1,2]. Given that a few plant components have been implicated in the incidence of herbal induced-liver injury [3,4], it is pertinent to look into the safe regimen of plants and plant products.

Carica papaya L. is a member of the family *'Caricaceae'* and is indigenous to Africa, Central America, South of Mexico, and India [5,6,7]. Different parts of the world employ various parts of papaya plant for therapeutic purposes [8]. Specifically, *C. papaya* is used to treat intestinal helminthiasis, hypertension, malaria, diabetes mellitus, jaundice and hypercholesterolemia in Nigerian ethnomedicine [7,9]. Papain, the major proteolytic enzyme present in the *C. papaya* fruit, has a lot of applications. Industrially, it is used as meat-tenderizers and in chewing gum production and also a useful component of many beauty application [10,11]. Both ripe and unripe fruits of *C. papaya* are known for their high nutritional value and are therefore incorporated into various salad/juice-containing menu [12,13]. In African ethnomedicine, the papaya fruit is used to induce labor during child birth [14] and also for treatment of digestive ailments and various forms of infectious diseases [15,16]. The abortifacient, laxative as well as wound healing properties of the fruit have also been reported [17,18,19]. In this regard, wounds treated with the unripe fruit extract were found to heal faster under a shorter time-frame than required when compared with ripe fruit extract-treated wound [20]. This is because the unripe fruits contain a higher antioxidant content relative to its ripe counterpart [21]. Other well-known pharmacological properties of *C. papaya* include hypoglycemic, anticancer, antibacterial, gastro-protective, anti-dengue and immunomodulatory effects [15,16,22,23].

Studies have shown that extracts of certain plants may be detrimental to healthy liver cells via induction of opening of the mitochondrial permeability transition pore, an event that has been implicated in cell death and several disease conditions [24,25,26]. The mitochondrial Permeability Transition (mPT) is associated with a sudden change in the permeability of the inner mitochondrial membrane which occurs as a result of opening of the mPT pore. This opening occurs in response to a number of factors including high levels of calcium, inorganic phosphate, oxidative stress and interaction with certain bioactive agents [27,28,29]. The formation of the mPT pore and its irreversible opening is a 'sine qua non' in cell death because it results in the influx of hydrophilic molecules up to a molecular weight of 1,500 Da into the mitochondrial matrix which causes mitochondrial swelling, stimulation of ATP hydrolysis and cytochrome c release [30,31].

Based on experimental studies, different results have been reported on the toxicological effect of *C. papaya* extract. Some authors have shown that *C. papaya* extracts have no adverse effects on the functions of vital organs in rats [32,33]. On the contrary, Kharisma *et al.,* [34] recently reported induction of fatty liver changes by ex-

tract of *C. papaya* unripe fruit. In addition, the anti-fertility effects of *C. papaya* extract have been reported in male and female rats [17, 18, 35]. There is paucity of information on the toxicological effects of the unripe fruits of *C. papaya* on mitochondrial permeability transition pore, a useful index of mitochondrial health. This study aimed to shed light on the effect of crude methanol extract of unripe *C. papaya* on the functional status of the mPT pore in liver of healthy male Wistar rats.

Methods

Reagents and chemicals

Thiobarbituric Acid (TBA), Trichloroacetic acid (TCA), Bovine Serum Albumin (BSA), Cytochrome c, Thiobarbituric acid, Sodium Dodecyl Sulphate, Mannitol, sucrose, N-2-hydroxy-ethyl-pipe-arizine-N-2-ethanesulfonic acid (HEPES), rotenone, spermine, Folin-Ciocalteu reagent and all other reagents were of highest purity grade and were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA).

Experimental animals and ethical study approval

A total of thirty male albino rats (Wistar strain) ranging between 100 g-120 g in weight were obtained from the Department of Physiology, Animal House, University of Ibadan, Ibadan, Nigeria. The animals were allowed to acclimatize for 14 days in cages in the Animal House of the Department of Biochemistry, University of Ibadan in a humidity and temperature-controlled condition where they had access to water and chow at will. All procedures in this study conformed to the guiding principles for research involving animals as recommended by the Declaration of Helsinki and National Institute of Health and related regulations of animal use and care committee. In addition, the study was approved by the University of Ibadan Animal Care and Use Research Ethics Committee and an approval number: UI-ACUREC/019/0381 was assigned to this study on October, 2019.

Preparation of plant material

Fresh unripe mature fruits of *Carica papaya* (120 g) were obtained from a local farmland in Ibadan, Oyo State, Nigeria. The plant material was identified and authenticated at the Department of Botany, Faculty of Science, University of Ibadan, Ibadan, Nigeria and a voucher specimen was deposited in the herbarium (voucher No. UIH-32057). The fruits were washed with distilled water, peeled, seeds removed and the pulp cut into small pieces. They were oven dried and finely powdered with an electric blender. Oven-dried powdered fruits of *C. papaya* were soaked with sufficient methanol in all- glass jars at room temperature for 72h. The filtrate was concentrated under reduced pressure using a rotary evaporator 400C (Stuart Rotavapor) to obtain the crude methanol extract of *C. papaya* (MECP) (15 g) and preserved for further use.

Isolation of low-ionic-strength rat liver mitochondria

Rat liver mitochondria were isolated from livers of male albino rats using the procedure previously described by Johnson and Lardy [36] and as modified by Olorunsogo et al. [37]. After

acclimatization, the animals were sacrificed by cervical dislocation and the livers were excised, weighed and rinsed with homogenizing buffer (210 mM mannitol, 70 mM sucrose, and 5 mM HEPES-KOH at pH 7.4) and 1 mM ethylene glycol tetra-acetic acid to remove blood stains. The incorporation of EDTA in the homogenizing buffer was to chelate calcium that could otherwise compromise the integrity of the mitochondria. Thereafter, they were homogenized as a 10% suspension in ice-cold homogenization buffer. In order to preserve the intactness of the mitochondria, the homogenization was carried out on ice using a glass Teflon homogenizing flask. Subsequently, the resulting homogenate was centrifuged done twice in a MSE angle 13 refrigerated centrifuge at 2300 rpm for 10 minutes twice to remove the cellular debris. The resulting supernatant was centrifuged at 13,000 rpm for 10 minutes to pellet the mitochondria. In order to remove impurities, the isolated mitochondria were washed with washing buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES at pH 7.4 and 0.5 % Bovine serum albumin) at 12,000 rpm for 10 minutes. The mitochondria obtained were immediately resuspended in a small quantity of mannitol, sucrose and HEPES buffer (210 mM mannitol, 70 mM sucrose, and 5 mM HEPES–KOH at pH 7.4) and dispensed into Eppendorf tubes and kept on ice. The mitochondria were used within 4 h of isolation.

Mitochondrial swelling assay

In this study, mitochondrial matrix swelling which is an indication of permeability transition was monitored as a decrease in absorbance at 540 nm. The mPT was determined by the method of Lapidus and Sokolove [38] by measuring changes in absorbance of mitochondrial suspension in the absence and presence of exogenous calcium. Intact mitochondria (0.4 mg/ml) were pre-incubated in the presence of 0.8μM rotenone, an inhibitor of complex1, mitochondrial-sustaining buffer (MSH- containing mannitol, sucrose, HEPES) for 3.5 minutes before the addition of 5 mM sodium succinate, the respiratory substrate. In order to assess Ca^{2+}-induced matrix swelling, mitochondria were pre-incubated in 0.8 μM rotenone in MSH buffer for 3 minutes. Subsequently, Ca^{2+} was then added to the mitochondrial suspension in the cuvette while sodium succinate was added 30 seconds later to make a total volume of 2.5 mL. Spermine, a classical inhibitor of mPT, was used as the standard inhibitor of mitochondrial swelling, and was added prior to mitochondrial pre-incubation with rotenone, the respiratory substrate. Change in absorbance was estimated at 540 nm at 30 seconds interval for 12 minutes in a T70 UV-visible spectrophotometer, PG Instrument Ltd. To determine the effect of MECP on Ca^{2+}-induced mPT pore opening, different concentrations (20-180 μg/ml) were separately pre-incubated with mitochondria for 3 minutes following the addition of Ca^{2+} to the reaction mixture. However, calcium was excluded in the reaction mixture when the direct effect of the extracts on intact mitochondria was d (absence of calcium). Swelling rate was quantified as 540/min/mg. Mitochondrial protein was estimated according to the method described by Lowry *et al.,* [39] using Bovine Serum Albumin

(BSA) as standard.

Determination of mitochondrial ATPase (mATPase) activity and lipid peroxidation

Mitochondrial ATPase activity was determined by the method described by Olorunsogo and Malomo [40]. The estimation of inorganic phosphate released as a result of ATP hydrolysis was measured according to the procedure described by Bassir [41] and as modified by Olorunsogo and Malomo [40].

A modified Thiobarbituric Acid Reactive Species (TBARS) assay method was employed to measure the concentration of lipid peroxides formed from mitochondrial membrane lipid peroxidation using the method of Ruberto *et al.,* [42] when mitochondria were used as the lipid source. Mitochondrial lipid peroxidation was estimated by levels of thiobarbituric acid reactive substances (TBARS).

Percentage inhibition of lipid peroxidation by MECP was estimated from the absorbance value of the fully oxidized control relative to that obtained in presence of extract using the formula (E-C/C) * 100.

Determination of cytochrome c release

The quantification of cytochrome c release from isolated mitochondria treated with or without MECP was estimated using the method of Appaix et al., [43] using the Soret (c) peak from cytochrome c at 414 nm (e = 100 mmol/L/cm]). Summarily, mitochondria (1 mg protein/mL) were pre-incubated in the presence of 0.8 mmol/L rotenone in a medium containing 210 mM mannitol, 70 mM sucrose and 5 mM HEPES–KOH (pH 7.4) for 30 minutes at 270C in the presence of different concentrations of the extract and using 24 mM calcium as the standard triggering agent. The mixture was centrifuged at 15,000 rpm for 10 minutes, immediately after incubtion. The optical density of the supernatant was measured at 414 nm which is the Soret (c) peak for cytochrome c.

Statistical analysis

Data are presented as mean ± standard deviation of triplicate determinations. Statistically significant differences were determined using one-way analysis of variance with GraphPad Prism (version 5.0) and significant differences were set at $p < 0.05$.

Results

Calcium-induced mitochondrial membrane permeability transition pore opening in normal rat liver mitochondria and its reversal by spermine

Over a period of twelve minutes, the mitochondrial suspension (containing 1mg/ml protein) contained succinate as its respiratory substrate. During this time frame, there was no significant change in absorbance of the mitochondria in the presence of rotenone, a standard inhibitor of complex 1 (Figure 1). However, when calcium was added to the mitochondrial suspension, a significant change in absorbance was recorded indicative of mitochondrial swelling and this was however reversed by the addition of spermine, a standard mPT pore desensitizer, by 84 %.

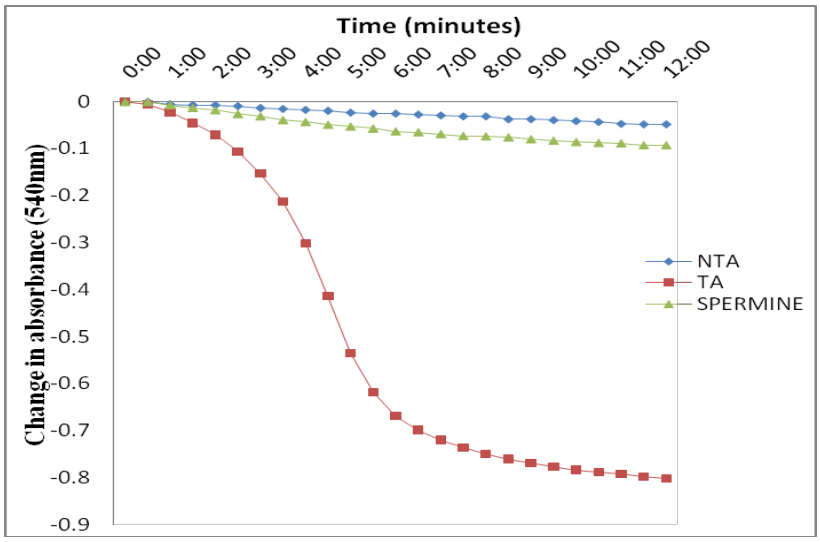

Figure 1. Representative illustration of calcium-induced opening of normal rat liver mitochondrial membrane permeability transition pore and the reversal effect of spermine. NTA = No Triggering Agent, TA = Triggering Agent. Mitochondria were suspended in MSH buffer and energized by succinate and the absorbance measured at 540 nm. Spermine was added as inhibitor.*Typical data of one experiment are shown and similar results were obtained in at least three different preparations

Varying concentrations of crude methanol extract of Carica papaya (MECP) induces opening of intact mitochondria in the absence of calcium

Figure. 2 is the representative profile of effects of various concentrations of MECP on mPT pore in the absence of calcium. According to the data, MECP at 20, 60, 100, 140 and 180 µg/mL induced pore opening by 3.11, 6.00, 9.08, 11.92 and 14.28 folds, respectively, in a concentration-dependent manner when compared to control. Maximum induction (14.28 folds) was observed at 90 µg/mL. Interestingly, spermine, a reference mPT inhibitor, reversed MECP-induced swelling of the mitochondria at all concentrations tested.

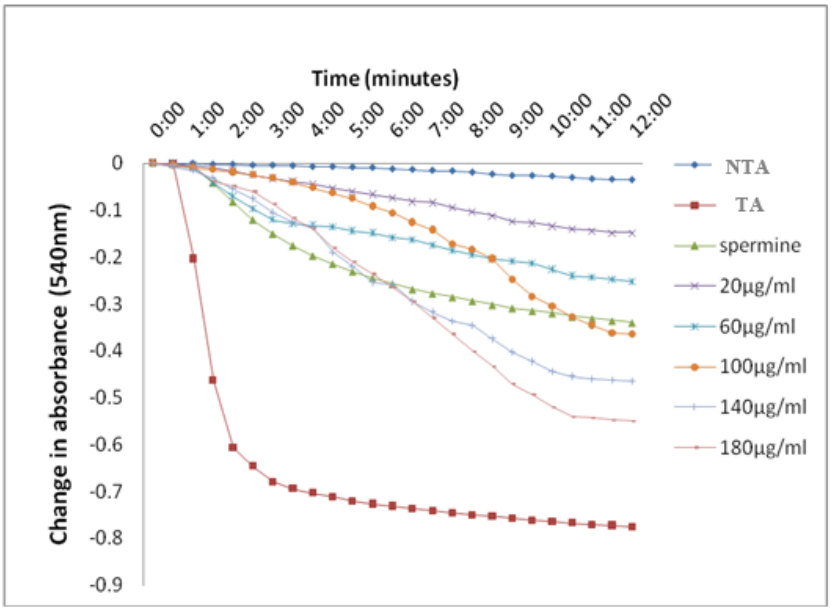

Figure 2. Representative profile of changes in absorbance of mitochondria when varying concentrations of crude methanol extract of *Carica papaya* (MECP) were incubated with intact mitochondria in the absence of calcium NTA: Non Triggering Agent, TA: Triggering Agent (Calcium) *Typical data of one experiment are shown and similar results were obtained in at least three different preparations.

Crude methanol extract of Carica papaya (MECP) potentiates calcium-induced opening of intact mitochondria

Figure 3 is a representative plot of changes in absorbance of mitochondria when various concentrations of MECP were incubated with mitochondria in the presence of calcium. Addition of different concentrations of MECP to intact mitochondria resulted in potentiation of calcium-induced opening of the mPT pore in a concentration-dependent style. Specifically, induction folds of 22.53, 24.06, 24.97, 25.14 and 25.50 were obtained at 20, 60, 100, 140 and 180 µg/ml MECP. It was noted that even at the lowest concentration of 20 µg/mL MECP, there was more induction (22.53 folds) than the highest concentration in the absence of calcium (14. 28 folds).

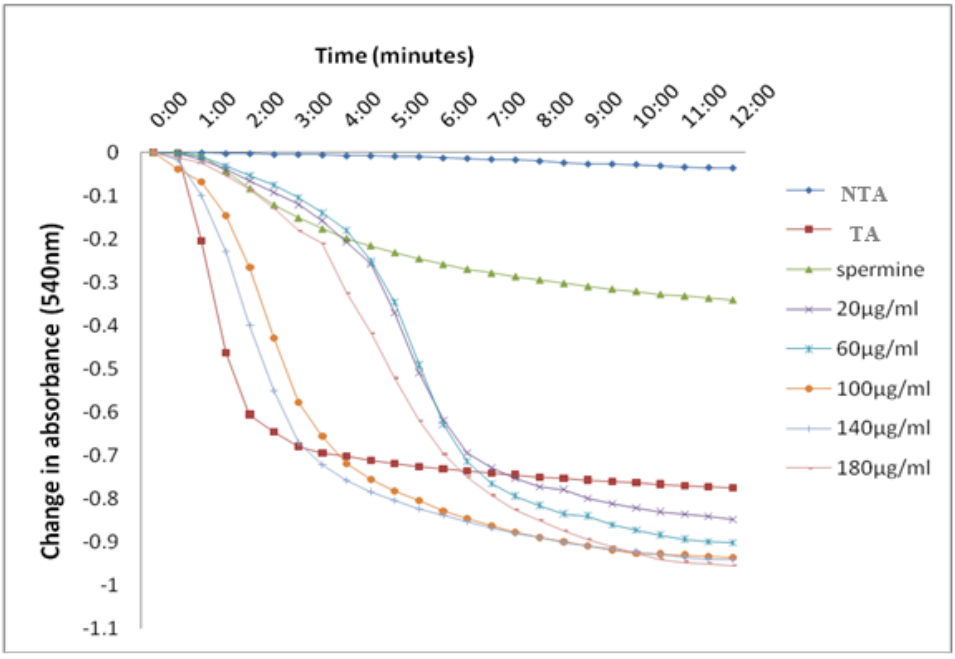

Figure 3. Representative profile of changes in absorbance of mitochondria when crude methanol extract of *Carica papaya* (MECP) were incubated with intact mitochondria in the presence of calcium. NTA: Non-Triggering Agent, TA: Triggering Agent (calcium) *Typical data of one experiment are shown and similar results were obtained in at least three different preparations.

Varying concentrations of MECP activate rat liver mitochondrial ATPase activity and induced cytochrome c release

The effects of varying concentrations of MECP on mitochondrial ATPase activity at physiologic pH and release of cytochrome c are presented in figure 4. The results showed that similar concentrations of MECP that induced opening of the mPT pore (in the absence of calcium) enhanced mitochondrial ATPase activity at pH = 7.4 significantly ($p < 0.01$) up to 5 folds at the highest concentration, which was close to the enhancement effect by 2, 4-DNP, an uncoupler of oxidative phosphorylation (Figure 4a). In addition, opening of the pore by MECP led to the release of cytochrome c into the cytosol (7 folds) at the highest concentration tested (Figure 4b).

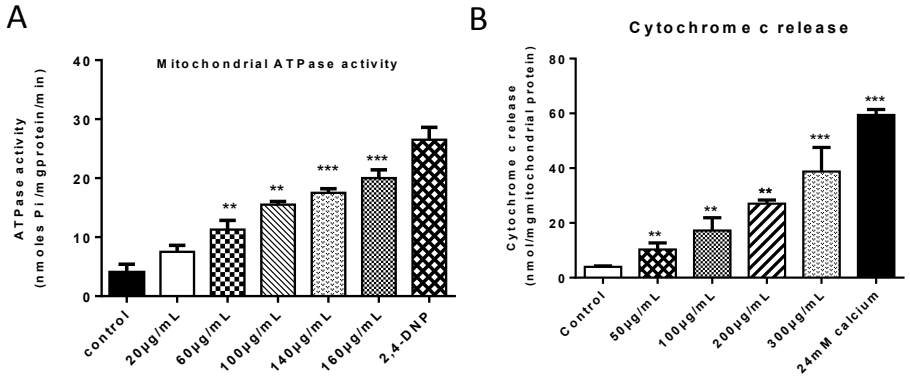

Figure 4. Effects of varying concentrations of MECP on rat liver mitochondrial ATPase activity and extent of cytochrome c release (Figure 4b). Assays were carried out in triplicates. Values are statistically significant at concentration **p < 0.01, ***p < 0.001 compared with control

Inhibition of ferrous-induced mitochondrial lipid peroxidation by crude methanol extract of C. papaya.

Given that mitochondrial membranes are composed of lipids which are susceptible to oxidation, the effect of MECP on Fe2+-induced mitochondrial lipid peroxidation was carried out (Figure 5). Levels of iron-induced mitochondrial lipid peroxides generated were significantly ($p < 0.01$) reduced by increasing concentrations (50, 100, 200, 300, 600 µg/ml) of the MECP by 10, 22, 53, 74, 112 %, respectively.

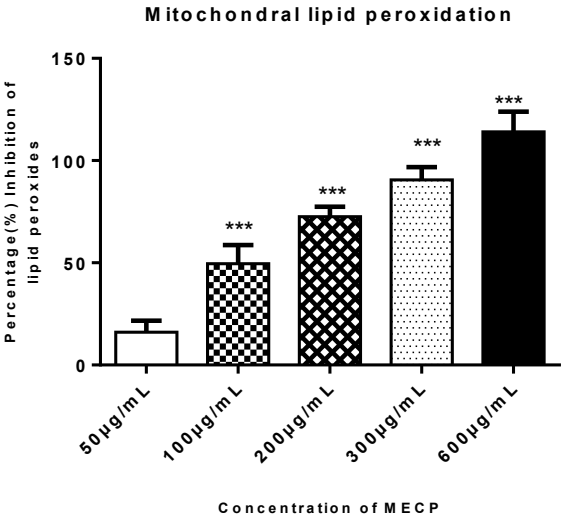

Figure 5. Percentage inhibition of ferrous-induced lipid peroxidation by crude methanol Extract of *Carica papaya*. Assays were carried out in triplicates. Values are statistically significant at concentration **p < 0.01, ***p < 0.001 compared with control

Discussion

The transitional event that causes the release of the peripheral cytochrome c protein from the outer layer of the outer mitochondrial membrane into the cytosol via the opening of the mPT pore is a point of no return in various forms of programmed cell death [44]. Although the exact molecular nature and composition of the mPT pore is still evolving, lately, the mitochondrial ATP synthase has been strongly implicated as the pore [45,46]. The default function of cytochrome c is to serve as an electron carrier in the electron transport chain during oxidative phosphorylation. This function is abated upon the induction of a death signal which triggers the release of this life-supporting protein into the cytosol initiates a cascade of events that culminates in cell death [28,31]. The location of cytochrome c is therefore crucial to its cellular function. We have shown in this study that unripe fruits of *Carica papaya* increases the concentration of cytosolic cytochrome because of the opening of the mitochondrial membrane permeability transition pore in healthy liver cells. This effect appears toxic.

Over the years, a number of bioactive agents have served as functional modulators of molecular targets in diverse diseased conditions. In this regard, the mPT pore represents a potential target for the treatment of a number of diseases associated with dysregulated cell death [47,48,49,50]. Inducers of pore opening are beneficial in triggering cell death in diseases characterized by cell death insufficiency (e.g tumours) while mPT pore inhibitors are useful in preventing pathological loss in vital cells associated with excessive cell death (neurodegenerative diseases). Given the role of mPT pore as a pointer to mitochondrial health, its functional status was assessed when intact mitochondria were treated with unripe crude methanol extract of unripe *C. papaya*. Fresh unripe *Carica papaya* was used in this study because research has shown that degree of ripening influences the bioavailability of plant extracts. This has been attributed to difference in the proportion of phytochemicals present in unripe as opposed to ripe fruits [20]. Although the ripe and unripe fruit as well as other different parts of *C. papaya* have been reported to possess a wide range of biological effects, it is known that unripe papaya fruits have better antioxidant activity than its ripe counterpart [13,21,51].

Mitochondrial swelling assay is often used to measure mitochondrial permeability transition, given that the opening of the pore leads to influx of solutes that causes the mitochondrial matrix to swell as a result of the unfolding of the inner mitochondrial membrane which has a larger surface area than the outer mitochondrial membrane [38]. Our results showed that the integrity of mitochondria used in this study was intact *ab initio* and therefore suitable for further use because calcium-induced swelling was almost completely prevented by spermine, a standard inhibitor.

The findings that MECP induced significant opening of the pore in a concentration-dependent fashion compared to control in the absence of calcium indicated that MECP contains certain bioactive agent (s) that could interact with the mitochondrial membrane including the components of the pore leading to its activation and opening. Conversely, in the presence of calcium, the observation that varying concentrations of MECP further potentiated calcium-induced pore opening confirms the inductive effect of MECP on inner mitochondrial membrane and that the bioactive agents in MECP worked in synergy with calcium, a standard pore inducer. Several publications have reported the involvement of the mitochondrial ATP synthase/ FoF1-ATPase in the formation of the mPT pore [45,46]. Besides, this enzyme is crucial in the synthesis of ATP under aerobic conditions. In order to shed light on the effect of unripe MECP on the basal activity of FoF1-ATPase at physiological pH, the effect of varying concentrations of MECP on mitochondrial ATPase activity was determined. The finding that MECP activated mitochondrial ATPase activity at physiological pH (7.4) which was comparable to that of 2,4 dinitrophenol, a classical uncoupler of oxidative phosphorylation further ascertains the inductive effect of MECP on mPT pore and that decreased ATP synthesis occasioned by increasing levels of inorganic phosphate is involved in

the inductive potential of MECP. Interestingly, inorganic phosphate has been revealed to be one of the inducers of mPT pore opening [52]. This reveals that MECP-stimulated ATPase activity influences and promotes the activation of the mPT pore in healthy liver cells.

Furthermore, the evidence of cytochrome c release by varying concentrations MECP confirms that opening of the mPT pore resulted in swelling of the mitochondrial matrix and enhancement of the mitochondrial ATPase hydrolytic activity.

Given that reactive oxygen species have been implicated as inducers of pore opening by interaction with regulatory components of the pore, we tested if production of mitochondrial lipid peroxides contributes to mPT pore opening by MECP. Interestingly, our data showed that varying concentrations of MECP reduced the levels of ferrous-induced lipid peroxides formed. This observation shows that indeed the bioactive components of MECP are major players directing influencing/interacting with the pore to bring about its activation. This inhibitory effect of MECP on mitochondrial peroxide formation agrees with the earlier reported antioxidant potential of *C. papaya* [13].

We have shown in this study that certain components present in the unripe fruit extract are toxic and may cause liver damage. Although, the fact that the extract opens the mitochondrial permeability transition pore and may cause cell death may be of importance in the management of diseases where cell death needs to be upregulated e.g cancer.

Altogether, these data confirm that unripe extract of *C. papaya* possesses potential hepatotoxic effect on healthy liver cells because of its inductive effect on the mPT pore which leads to release of cytochrome c and this may explain the previous reported toxic effect of *C. papaya* [17, 18,35]. Furthermore, our results corroborate the findings of Khumasi et al., [34] that suggest that unripe *C. papaya* extract causes fatty liver changes in tissues.

This study suggests that induction of mitochondrial injury may be involved in the pathomechanism of fatty liver changes induced by unripe *C. papaya*.

Conclusion

Put together, this study reveals that bioactive components that alter the functional status of the mitochondrial permeability transition pore in isolated rat liver mitochondria leading to release of cytochrome c into the cytosol and reduction in levels of ATP in mitochondria are present in unripe crude methanol extract of C. papaya. Given that C. papaya is widely used for therapeutic purposes, further studies to ascertain its safety is required in order to set a limit for its use in treatment of diseases.

Further work will be carried out to identify the toxic components responsible for *C. papaya's* hepatotoxicity. Interestingly, these components may be useful in selective targeting in several disease conditions that require upregulation of cell death.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal rela-

tionships that could have appeared to influence the work reported in this paper.

Acknowledgment

None.

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