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# Modulation of Cytochrome *C Release and Opening of Mitochondrial* Permeability Transition Pore by *Calliandra portoricensis* (Benth) Root Bark Methanol Extract

**T. Olubukola Oyebode[1*], O. Tolulope Adebusuyi[1], S. Emmanuel Akintimehin[2] and O. Olufunso Olorunsogo[1]**

*[1]Laboratories for Biomembrane Research and Biotechnology, Department of Biochemistry, College of Medicine, University of Ibadan, Ibadan, Nigeria.*
*[2]Department of Chemical Sciences, Ondo State University of Science and Technology, Nigeria.*

***Authors' contributions***

*This work was carried out in collaboration between all authors. Author OOO designed the study. Author TOO wrote the protocol and the first draft of the manuscript. Authors OTA, SEA and TOO performed the statistical analysis and managed the analyses of the study. Author TOO managed the literature searches. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aim:** Evasion of apoptosis, a form of programmed cell death, is a common feature of most cancer cells. This study evaluated the modulatory effects of different fractions of crude methanol extract of the root bark of *Calliandra portoricensis,* a medicinal plant used in the traditional treatment of prostate tumour on cytochrome C release via opening of the mitochondrial Permeability Transition (mPT) pore in rat liver.
**Methodology:** Opening of the pore, mitochondrial ATPase activity, cytochrome C release and extent of mitochondrial lipid peroxidation were assessed and monitored spectrophotometrically *in vitro.*
**Results:** In the presence and absence of calcium, Vacuum Liquid Chromatography (VLC) Ethylacetate fraction (EFCP) had no effect at all concentrations tested. Varying concentrations (10-

**Corresponding author: E-mail: bukioyebode@gmail.com;*

120 µg/mL) of VLC methanol fraction (MFCP) significantly induced pore opening in the absence of calcium by 2.7,3.9,9.7, 11.2, 12.5, 14.5 and 15.1 folds while spermine reversed this inductive effect. In the presence of calcium, the pore was slightly further opened by MFCP. Furthermore, these fractions enhanced mitochondrial ATPase activity and diminished the extent of $Fe^{2+}$-induced lipid peroxidation in a concentration-dependent manner with significant effect in MFCP. Similarly, cytochrome C release in MFCP was significant when compared to other fractions.

**Conclusion:** Taken together, these results suggest that bioactive agents that possess the potential of interfering with mitochondrial bioenergetics and inducing opening of the pore are present in the most potent methanol fraction of *C. portoricensis,* this would find use in disease conditions where apoptosis needs to be upregulated.

*Keywords: Calliandra portoricensis; mitochondria; mPT pore; cytochrome C; apoptosis.*

## 1. INTRODUCTION

Mitochondria came into focus of contemporary cancer research because of their role in programmed cell death, a physiological process that is evaded in most forms of cancer. Given that the mitochondrial inner membrane is selectively impermeable, the discovery of a nonspecific permeability transition with a threshold of 1.5 kDa suggested the existence of a pore, called the mitochondrial permeability transition (mPT) pore that is responsible for this transition [1]. The mPT pore opening is indeed highly sensitive to $Ca^{2+}$, ADP, inorganic phosphate and chemotherapeutic agents [2]. Inhibition of the mPT pore can be achieved by high concentrations of ATP, NADH, divalent cations and spermine.

Advances in mPT pore structure are still emerging with many suspects but no culprits, however, recent studies reveal that the mPT pore is built around the $F_0F_1$ ATP synthase [3], although other macromolecular components such as Adenine Nucleotide Transferase (ANT) [4], Voltage dependent Anion Channel (VDAC) [5] as well as CyP D [6] have been grouped as activity regulators of the pore.

Although the molecular nature of the mPT pore is not known, its interaction with diverse chemicals reveals that the pore is a potent modulator of mitochondrial-mediated apoptosis. Hence it is pharmacologically relevant in diseases where apoptosis is dysregulated. Examples of compounds that targets the mPT pore include betulinic acid [7] and specific targeting of the $F_0F_1$ ATPase by resveratrol isolated from grape [8].

The importance of the pore is underscored by the release of cytochrome C upon the opening of the pore which eventually results in the activation of caspases and subsequently leading to cell death/ apoptosis. *Calliandra* is a genus of flowering plants in the subfamily *Mimosaceae* under the family *Leguminoseae* (Pea Family). The plant has also been reported to have anticonvulsant [9; 10] antidiarrheal, antispasmodic, antipyretic, antirheumatic and analgesic [11] activities in humans. In addition, *C. portoricensis* has also been reported to exhibit anticholigenic, antacid, antiulcer, molluscidal and ovucidal activities in laboratory animals [11].

Seventeen known compounds were reported for the first time from the genus *Calliandra,* they include gallic acid, methyl gallate, myricitrin, quercitrin, myricetin 3-*O*-â-D-4C1-glucopyranoside, afzelin etc. [12].

Phytochemical screening of the leaves, stem and root extracts of *C. portoricensis* reveal the presence of saponins, glycosides, steroids, fatty acids and digitalis glycosides in the plant [13]. Histopathological studies of acute and chronic effects of *C. portoricensis* leaf extract on the stomach and pancreas of adult Swiss albino mice [14] suggest that chronic administration of *the plant's* leaf extracts may inhibit the proper function of stomach and pancreas. Besides, the leaves and roots of the plant have been shown to have protective effects (antidote) in Wistar rats challenged with snake venom [15]. Epiafzelechin isolated from the stem bark of the plant has displayed antimicrobial effects [16]. *C. portoricensis* has also been shown to possess antisickling property [17]. Besides, *C. portoricensis* is used in combination with *Plumbago zeylanica* in the traditional treatment of prostate enlargement. The active principle of *P. zeylanica* "plumbagin"- a naphtoquinone derivative has been identified and shown to have anticancer and antiproliferative activities in different cancer cell lines even though it is highly toxic [18].

Given the dearth of information on the bioactive agents present in *C. portoricensis,* this research therefore aimed to examine the influence of certain fractions of *C. portoricensis* root bark on cytochrome C release via the induction of the opening of the mPT in rat liver (*in vitro)* and to give an update with respect to the most potent fraction of *C. portoricensis* using Vacuum Liquid Chromatography (VLC) technique for partitioning.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals and Reagents

Cytochrome C, thiobarbituric acid (BDH Chemicals Ltd, Pools, England) sucrose, N-2-hydroxy-ethyl-pipe-arizine-N-2-ethanesulfonic acid (HEPES), rotenone, spermine, Bovine Serum Albumin (BSA), mannitol and all other reagents (EGTA) were purchased from Sigma-Aldrich chemical Co. (St. Louis, MO, USA) and were of the highest purity grade.

### 2.2 Collection and Extraction of Plant Material

Freshly harvested roots *of C. portoricensis* were obtained from Oje market, Ibadan, Oyo State, Nigeria. Samples were identified and authenticated at the Department of Botany, University of Ibadan and a specimen Voucher (number UIH-22466) was deposited in the Herbarium. The roots were washed, the bark peeled and air-dried for three weeks in the laboratory after which they were powdered and weighed. Air-dried, powdered root bark of *C. portoricensis* (7 kg) was soaked with sufficient methanol in all- glass jars at room temperature for 72 h. The filtrate was concentrated under reduced pressure using a rotary evaporator.

### 2.3 Partitioning of crude Methanol extract of *C. portoricensis* (MECP) using Vacuum Liquid Chromatography

Silica gel 60 (0.040–0.063 mm, MERCK) (8 g) was adsorbed to 12 g of MECP. The mixture was air-dried to obtain a powdery form. A sintered funnel for Vacuum Liquid Chromatography (VLC) was packed with 80 g Silica gel (Hopkins and Williams, England). Solvents were added in order of increasing polarity and n-hexane, chloroform, ethylacetate and methanol fractions were obtained in this order. The eluted fractions were concentrated using rotary evaporator under reduced pressure and the resulting concentrated, solvent–free fractions were stored in glass sample bottles and kept in the refrigerator until use.

### 2.4 Experimental Animals

Male Wistar strain albino rats were purchased from the Preclinical Animal House, College of Medicine, University of Ibadan, Nigeria. The rats (70 g and 80 g) were kept in ventilated cages in the Departmental Animal House and had access to food and water *ad libitum*. The rats were allowed to acclimatize for two weeks before experimental work commenced.

### 2.5 Isolation of Rat Liver Mitochondria

Rat liver mitochondria were isolated essentially according to modified method by Johnson and Lardy et al., [19]. Excised livers of sacrificed animals were weighed, washed with homogenizing buffer (210 mM Mannitol, 70 mM Sucrose, 5 mM HEPES-KOH, 1 mM EGTA) and homogenized as a 10% suspension in ice-cold buffer using a Porter Elvehjem glass homogenizer. The nuclear fraction and cellular debris were sedimented by centrifuging the homogenate twice at 2300 rpm for 5 mins. The supernatant obtained was spun at 13,000 rpm for 10 mins to obtain the mitochondrial pellet which was washed twice with washing buffer by spinning at 12,000 rpm for 10 mins. The mitochondrial fraction obtained were immediately resuspended in appropriate volume of MSH (210 mM Mannitol, 70 mM Sucrose, 5mM HEPES-KOH) buffer, and dispensed into Eppendorf tubes, kept at 4℃ and used fresh.

### 2.6 Mitochondrial Protein Estimation

Mitochondrial protein was determined according to the method of Lowry [20] using Bovine Serum albumin (BSA) as standard.

### 2.7 Mitochondrial Swelling Assay

Mitochondrial permeability transition (mPT) was determined according to the method of Lapidus and Sokolove [21]. This was monitored by measuring the changes in absorbance of mitochondria at 540 nm in the presence and absence of calcium ion (triggering agent) in a Spectrumlab 752s UV/Visible spectrophotometer. Mitochondria (0.4 mg protein/ml) were pre-incubated in the presence of .08µM rotenone in a medium containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES-KOH (pH 7.4) for 3 mins at 30℃ prior to the addition of 300 µM $CaCl_2$, while 5 M succinate was added 30 secs later and

mPT was measured at 540 nm for 12 mins at 30 secs interval. The inhibitory effect of spermine on the induction of pore opening was carried out prior to the addition of $CaCl_2$. The inductive effects of fractions were monitored when the fractions were replaced with $CaCl_2$.

## 2.8 Measurement of Mitochondrial ($F_0F_1$) ATPase Activity

Adenosine triphosphatase activity was determined by modification of the method of Olorunsogo and Malomo [22]. Mitochondrial Protein (0.5 mg/mL) was used for this assay while 2,4 dinitrophenol was used as a classical uncoupler. Each test tube (along with duplicates) for the reaction contained 65 mM Tris-HCl buffer pH 7.4, 0.5 mM KCl, 1 mM ATP and 25 mM sucrose in a final reaction volume of 2 mL. Distilled water or 40-120 µg/mL solvent fraction was added accordingly. The reaction was started by the addition of the mitochondrial fraction and incubated for 30 mins with constant shaking at 30℃. Mitochondria were added to the zero-time tube and the reaction was immediately stopped with the addition of 1 mL of 10% Sodium Dodecyl Sulphate (SDS). I mL of the reaction mixture above was kept for phosphate determination following centrifugation.

### 2.8.1 Determination of inorganic phosphate

The concentration of inorganic phosphate released from ATP hydrolysis was performed according to the method of Bassir [23] using Disodium Hydrogen phosphate ($Na_2HPO_4$) as standard. Ammonium molybdate (1.25%) and freshly prepared 9% ascorbic acid were added to 1mL of the reaction mixture and allowed to stand for 30 mins. The intensity of the blue colour was read at 660 nm in a Camspec M105 spectrophotometer.

### 2.8.2 Measurement of mitochondrial lipid peroxidation

Mitochondrial lipid peroxidation was determined by modification of the method of Ruberto et al., [24]. Mitochondria were used as the lipid-rich medium. Briefly, 1 mg/ml mitochondrial protein and varying concentrations of the extract/ fractions were added in a test tube and made up to 1 ml with distilled water. Iron Sulphate (0.07 M) was used to induce lipid peroxidation. Acetic acid (20%), TBA (0.8%) and SDS (1.1%) were added and the resulting mixture was vortexed, heated at 95℃ for 60 mins. On cooling, 5 mL of butanol was added to each test tube, extensively vortexed and centrifuged at 4000 rpm for 10 mins Absorbance of the upper organic layer was measured at 532 nm. All values were based on percentage inhibition using the formula:

(AC– AE/AC) * 100

Where AC is the absorbance of the fully oxidized control and AE is the absorbance in the presence of the extract/fraction.

## 2.9 Assay for Cytochrome C Release

Release of cytochrome C from isolated mitochondria was quantitatively determined by measuring the Soret (ɣ) peak for cytochrome C at 414 nm (ɛ =100 mM/cm according to a method previously established by Appaix et al., [25] using cytochrome C as standard. Isolated mitochondria were pre-incubated in a buffer medium containing 210 mM mannitol, 70 mM sucrose and 5 mM HEPES-KOH (pH7.4) in the presence of 0.8 µM rotenone at 30℃ for about 30 mins in the presence of varying concentrations of the fractions using 24 mM calcium as the standard triggering agent. After the incubation, the mixture was centrifuged at 15000 rpm for 10 mins. The optical density of the supernatant was measured at 414 nm which is the soret peak (ɣ) for cytochrome C.

## 2.10 Statistical Analysis

All data were presented as mean ± standard deviation. Statistical analysis of data was performed using the one-way analysis of variance. $P< 0.05$ was adopted as statistically significant.

# 3. RESULTS AND DISCUSSION

## 3.1 Results

### 3.1.1 Calcium induced permeability transition of normal rat liver mitochondria and spermine reversed the inductive effect

Fig. 1 reveals the inductive effect of calcium-ion on isolated rat liver and its reversal by spermine, a standard inhibitor of mPT pore opening. The data reveals that calcium induced mitochondrial permeability transition (mPT) pore opening in isolated rat liver while spermine inhibited this inductive effect. This indicated that the mPT pore of the control animal was intact and not uncoupled *ab initio*, therefore suitable for further use.

### 3.1.2 Effects of VLC ethylacetate and methanol fractions of *C. portoricensis* root bark on rat liver mitochondrial permeability transition (mPT) pore in the absence of calcium

Having ascertained the integrity of the mitochondria, we determined the effects of the fractions of the extract of *C. portoricensis* on mitochondrial permeability transition in the absence of calcium.

Previous work in our laboratory revealed that using separating funnel technique as a means of partitioning, the ethylacetate fraction of the crude methanol extract (EFCP) was the most potent in opening the mPT pore followed by aqueous fraction [26]. Here we have used the Vacuum Liquid Chromatography (VLC) technique, a more acceptable procedure in a pilot study to obtain chloroform (CFCP), ethylacetate (EFCP) and methanol fraction of *C. portoricensis* (MFCP).

The effects of crude methanol extract of *C. portoricensis (MECP) and* chloroform fraction of *C. portoricensis* (CFCP) had initially been reported [26]. The effects observed on mitochondrial permeability transition were the same with fractions obtained with VLC technique. However, VLC EFCP gave a different activity with respect to opening of the mPT pore. Hence, we present the effects of a new methanol fraction (MFCP) as a result of the VLC procedure and VLC ethylacetate fraction on induction of mPT pore opening.

The VLC ethylacetate fraction (EFCP) had no effect on the mPT pore at all concentrations tested (10-120 µg/mL) as shown in Fig. 2. However, addition of different concentrations of methanol fraction (MFCP) to succinate-energized mitochondria resulted in significant opening of the mPT pore by 2.7, 3.9, 9.7, 11.2, 12.5, 14.5, 15.1 folds at 10, 20, 40, 60, 80, 100, and 120 µg/mL, respectively. The inductive effect (11.2, 12.5, 14.5, 15.1 folds) observed at higher concentrations (≥ 60 µg/mL) were greater than the induction of pore opening caused by calcium alone (12 folds) (Fig. 3).

Overall, the pattern of induction by the varying concentrations of the extract was concentration-dependent.

### 3.1.3 Effects of VLC ethylacetate and methanol fractions of the *C. portoricensis* root bark on rat liver mitochondrial permeability transition (mPT) pore in the presence of calcium

The effects of ethylacetate fraction on mPT pore in the presence of calcium are shown in Fig. 4. Again, EFCP had no effect whatsoever on calcium-induced opening of the mPT pore at all concentrations tested.

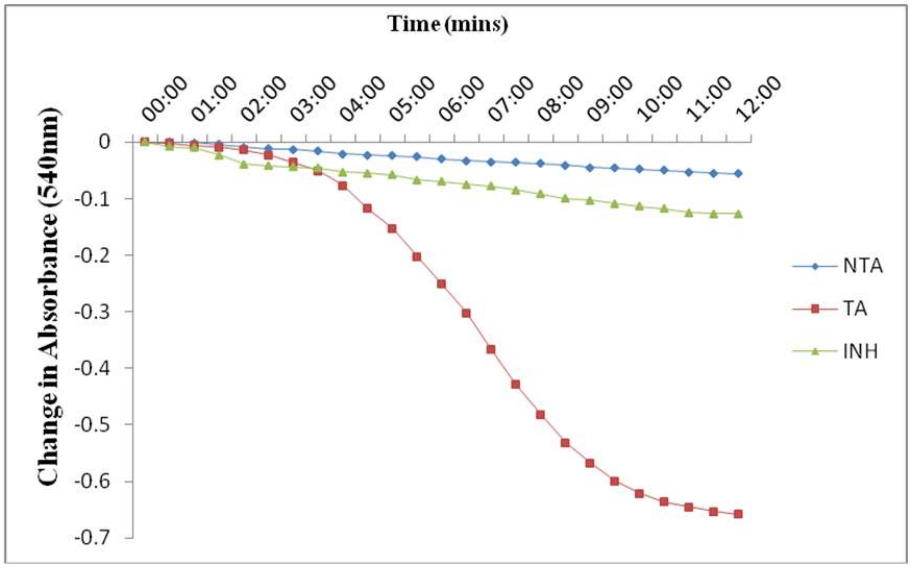


**Fig. 1. Calcium-induced opening of mitochondrial membrane permeability transition pore in normal liver mitochondria respiring in the presence of succinate-rotenone and its inhibition by spermine**

*NTA: No triggering agent, TA: Triggering agent, INH: Inhibitor (spermine)*

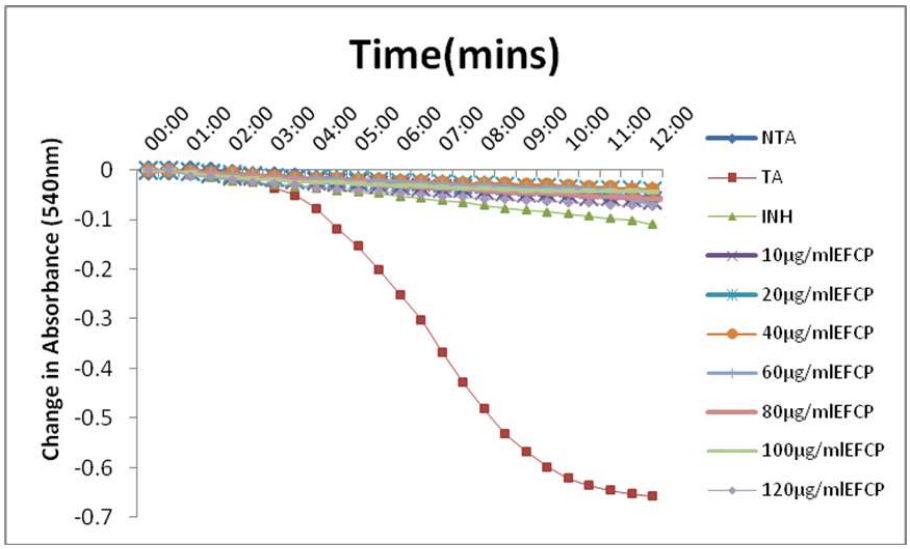


**Fig. 2. Effects of various concentrations of ethylacetate of *C. portoricensis* (EFCP) on the mitochondrial membrane permeability transition pore in the absence of calcium**

*NTA: No triggering agent, TA: Triggering agent, INH: Inhibitor (spermine)*

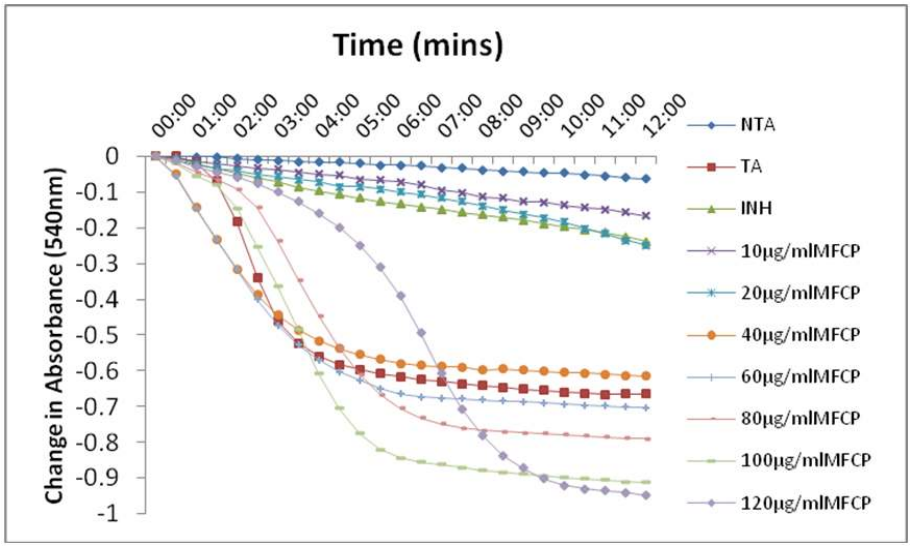


**Fig. 3. Effects of various concentrations of methanol fraction of *C. portoricensis* (MFCP) on the mitochondrial membrane permeability transition pore in the absence of calcium**

*NTA: No triggering agent, TA: Triggering agent, INH: Inhibitor (spermine)*

Fig. 5 shows the effect of MFCP on calcium-induced opening of the mPT pore. According to the results, MFCP potentiated calcium–induced opening of the pore. In this regard, the maximum inductive effect of 13.7 folds was obtained at the highest concentration (120 µg/mL) while a minimum inductive fold of 2.7 was obtained at 10 µg/mL. Calcium alone had an inductive fold of 12 folds. Taken together, of all the solvent fractions, VLC methanol fraction is potent with respect to opening of the mitochondrial permeability transition pore.

#### 3.1.4 Spermine reversed methanol fraction of *C. portoricensis* – induced opening of mitochondrial permeability transition pore in the presence of calcium

To determine the effect of spermine, a standard inhibitor of pore opening on most potent

methanol fraction–induced opening of the mPT pore in the presence of calcium, addition of 4 mM spermine was done immediately following addition of rotenone and just before the addition of mitochondria. As seen from the results (Fig. 6), MFCP induction of mPT pore opening was reversed by spermine in the presence of calcium at all concentrations tested. This is in line with what obtains in calcium-induced opening, which is also reversible by spermine.

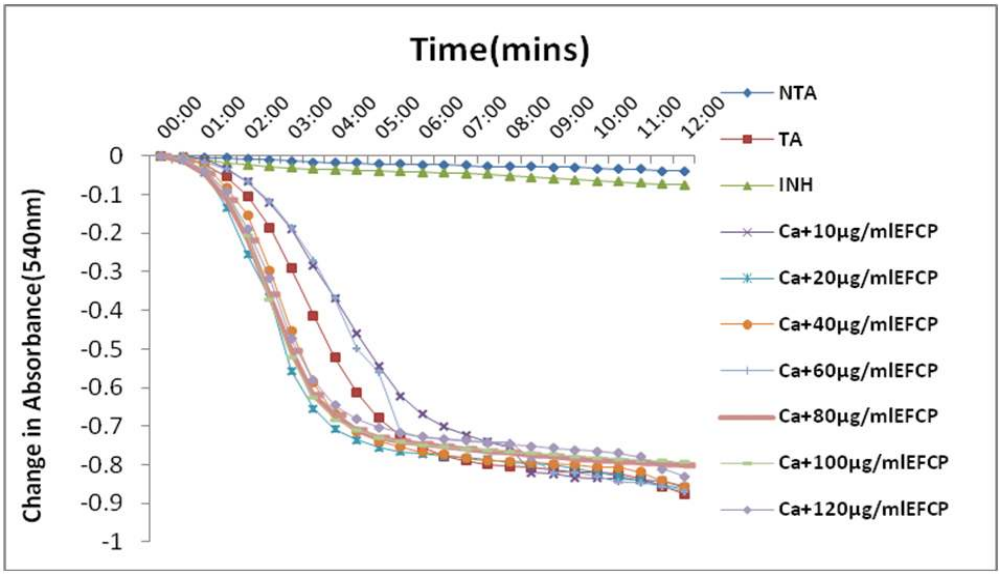


**Fig. 4. Effects of various concentrations of ethylacetate fraction of *C. portoricens*is (EFCP) on calcium-induced opening of mPT pore**

*NTA: No triggering agent, TA: Triggering agent, INH: Inhibitor (spermine)*

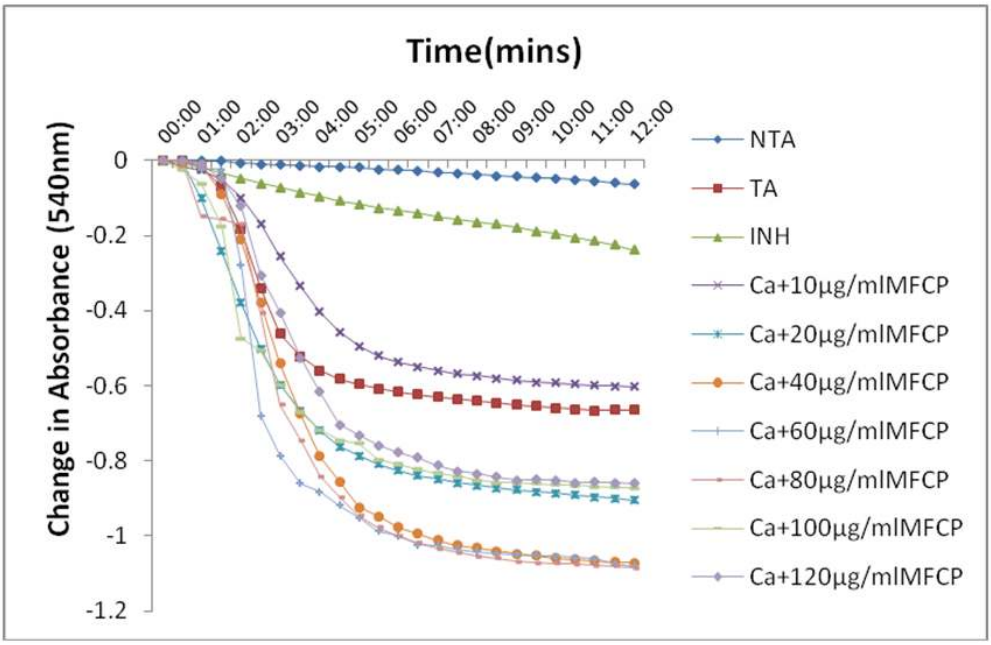


**Fig. 5. Effects of various concentrations of methanol fraction of *C. portoricens*is (MFCP) on calcium-induced opening of mPT pore**

*NTA: No triggering agent, TA: Triggering agent, INH: Inhibitor (spermine)*

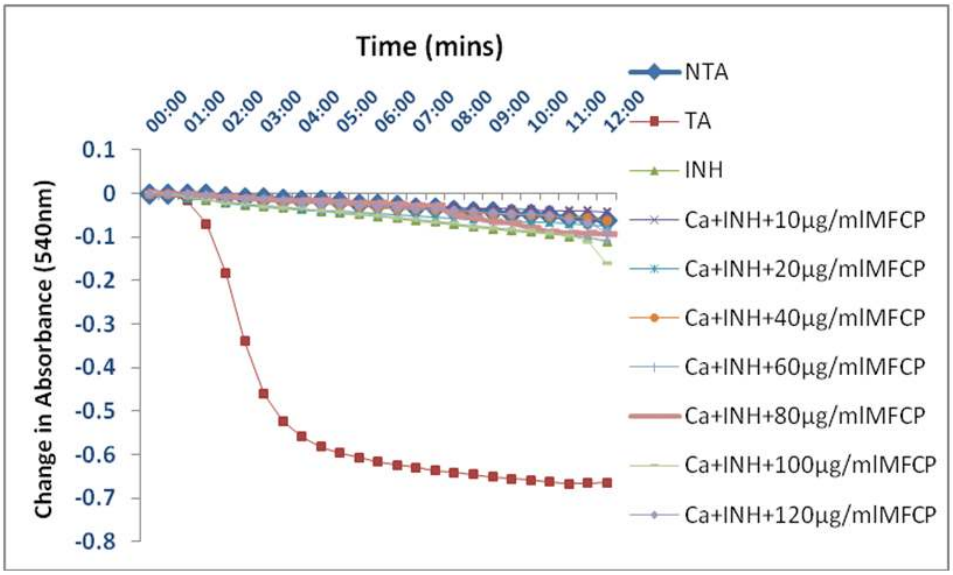


**Fig. 6. Effects of spermine on methanol fraction-induced opening of mitochondrial membrane permeability transition pore in the presence of calcium**

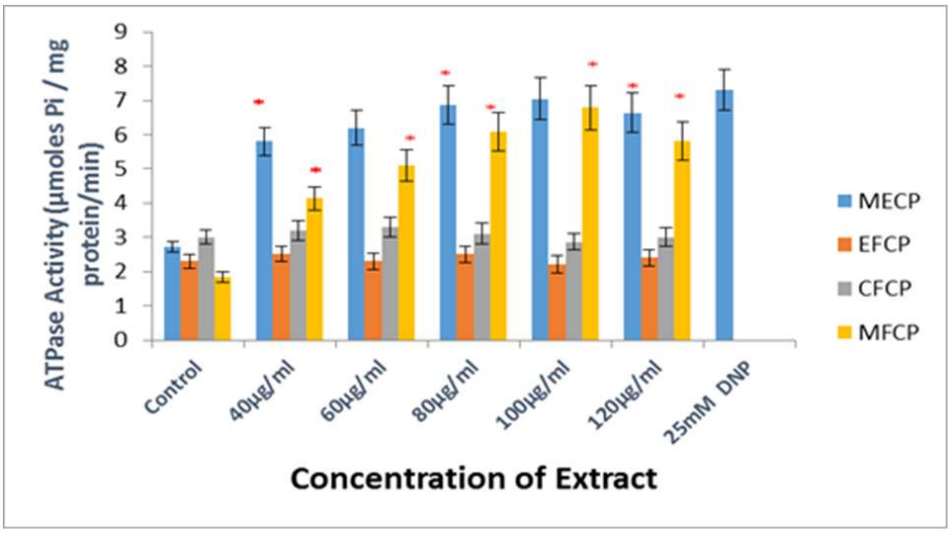


**Fig. 7. Effect of fractions of methanol extract of *Calliandra portoricensis* on mitochondrial ATPase activity**

** means that values are statisticaly significant (p <0.05)*

### 3.1.5 Effects of different fractions of crude methanol extract root bark of *C. portoricensis* on mitochondrial $F_OF_I$ ATPase activity

Mitochondrial ATPase activity was enhanced by the crude methanol extract (MECP) in a concentration -dependent manner. A maximum enhancement of 2.6 folds was observed at 100 µg/mL. This enhancement was close to that of 2, 4-dinitrophenol (DNP), a standard uncoupler of oxidative phosphorylation. Similarly, potent methanol fraction (MFCP) elevated the hydrolysis of ATP to ADP and inorganic phosphate maximally with 3.7 folds at 100 µg/mL. Levels of inorganic phosphate released were also close to that of 2, 4-DNP. Conversely, ethylacetate (EFCP) and chloroform fractions (CFCP) did not show significant enhancement of ATPase activity at all concentrations tested.

### 3.1.6 Solvent fractions of the root bark of *C. portoricensis* inhibit $Fe^{2+}$-induced mitochondrial lipid peroxidation

The effects of fractions of *C. portoricensis* on $Fe^{2+}$-induced mitochondrial lipid peroxidation were carried out according to method described by Ruberto et al., [24]. According to the results, all fractions of *C. portoricensis* inhibited the generation of mitochondrial lipid peroxides (Fig. 8). In this regard, methanol extract (MECP), chloroform (CFCP), ethylacetate fraction (EFCP) and methanol fraction (MFCP) inhibited $Fe^{2+}$ - induced lipid peroxidation maximally at 800 µg/mL with percentage inhibition of 90%, 59%, 41% and 93%, respectively. From the results,

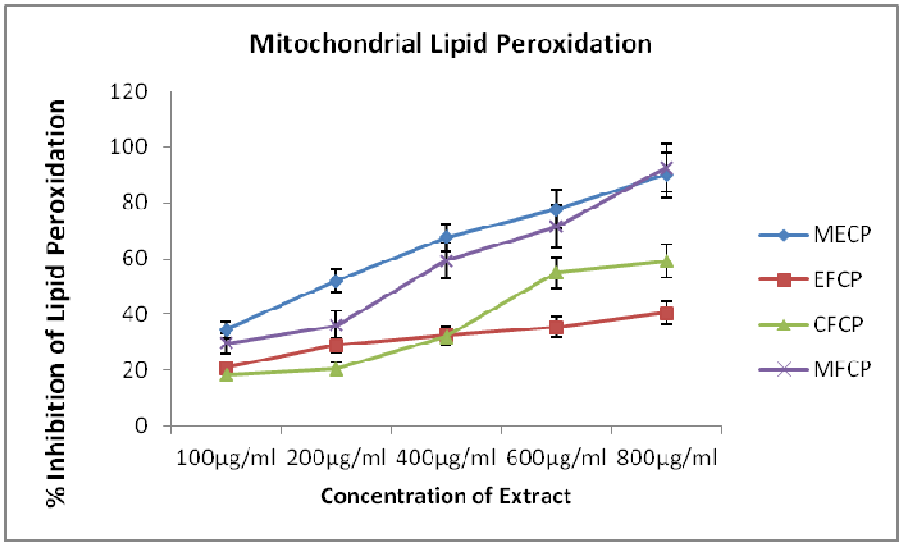


**Fig. 8. Percentage inhibition of $Fe^{2+}$ - induced lipid peroxidation of fractions of methanol extract of *C. portoricensis* root bark**

** means that values are statisticaly significant(P <0.05) Each value is a mean of of four different determinaions ± standard deviation*

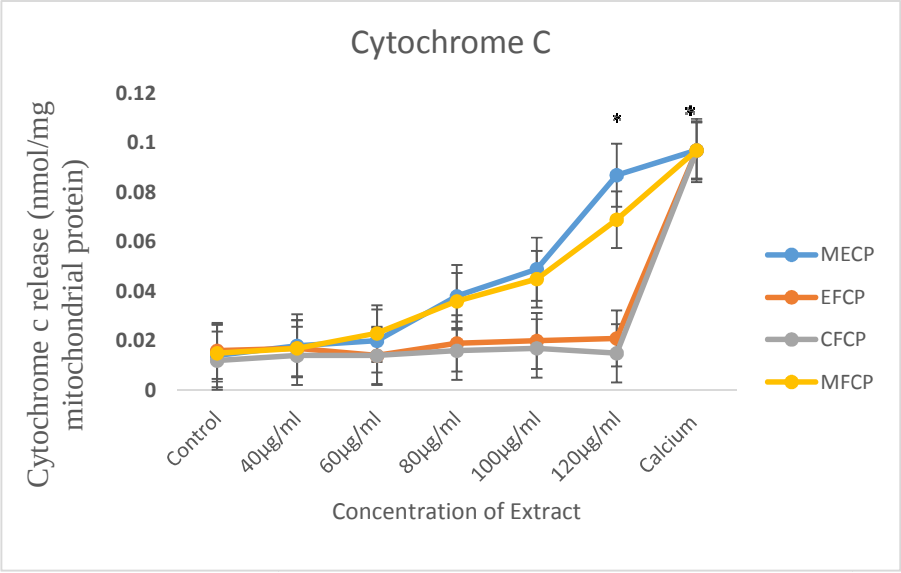


**Fig. 9. Effects of solvent fractions of crude methanol extract of *Calliandra portoricensis* on Cytochrome C release**

*Each value is a mean of 4 determinations ± standard deviation*

MFCP strongly inhibited generation of lipid peroxides as well as MECP. CFCP showed moderate inhibition while EFCP had the least potency. Overall, the effect of the fractions on inhibition of mitochondrial lipid peroxidation was concentration-dependent.

#### 3.1.7 Effect of solvent fractions of *Calliandra portoricensis* on mitochondrial cytochrome C release in rat liver mitochondria

The effects of varying concentrations of the solvent fractions of *C. portoricensis* on cytochrome C release are shown in Fig. 9. As seen from the results, there was significant release of cytochrome C from the mitochondria following exposure to the crude MECP and MFCP while CFCP and EFCP did not show significant difference from the control.

## 3.2 DISCUSSION

Dysregulation of the apoptotic process plays a pivotal role in all forms of diseases. In this regard, excessive apoptosis is one of the hall marks of tissue wastage while downregulated apoptosis is a major feature of all cancer cells. It is now well established that the mitochondrion is a central regulator of mammalian cell apoptosis [27]. The permeabilization of the mitochondrial outer membrane and release of apoptogenic proteins notably cytochrome *C* are undoubtedly central to the events in apoptosis. Mitochondrial permeabilization is due to the opening of the mitochondrial Permeability Transition (mPT) pore formed by a protein complex, a pore whose enduring interest is due to the role it plays in virtually all disease conditions [28].

Research has shown that phytochemicals may trigger apoptosis through the mitochondrial-mediated pathway and other numerous molecular targets [29]. Examples of such chemopreventive agents include plumbagin, a plant-derived naphthoquinone, isolated from *Plumbago zeylanic*a, which is used concurrently with *Calliandra portoricensis* in the traditional treatment of prostate enlargement. Besides, honokiol and oridnonin isolated from *Magnolia officinalis* and *Isodon rubescens* respectively, have been shown to restrain cancer by modulating targets for the mitochondrial apoptotic signalling pathways with the activation of caspases 3 and 8 [30]. The mPT pore is a non-selective, high conductance channel with multiple macromolecular components including cyclophilin D which is the main regulator of the pore. The list of the agent that promotes the opening of the pore is long. Notably, calcium, inorganic phosphate, reactive oxygen species and a variety of oxidant chemicals that induce the onset of mPT. Conversely, inhibition of the pore is achieved by cyclosporine A, bongkrekic acid and Adenine Nucleotides. This study evaluated the potency of fractions of *C. portoricensis* in mediating permeability transition in isolated rat liver mitochondria via the release of cytochrome C.

Vacuum liquid chromatography (VLC) was employed to obtain ethylacetate (EFCP), chloroform (CFCP) and Methanol (MFCP) fractions of the crude MECP. The EFCP had no effect whatsoever on the mPT pore in the absence and presence of calcium.

We have also shown in this study that methanol fraction of *C. portoricensis* (MFCP) in the absence of calcium had an inductive effect on the opening of the pore which was greater than that of calcium at higher concentrations (Fig. 3). The observations that the inductive effects were enhanced even in the presence of calcium, and could be reversed almost totally by spermine, a standard mPT pore inhibitor (Fig. 6), indicate that there was no disruption of mitochondrial membrane integrity while the induction of mPT pore occurred. These findings indicate clearly that the bioactive agents in these fractions are not interacting directly with the membrane but rather acting in a manner to facilitate the opening of the mPT pore possibly by binding to contact sites on the pore where calcium occupies to facilitate induction.

The observation in this study that mitochondrial permeability transition was not affected by EFCP of *C. portoricensis* in the presence and absence of calcium is clearly due to the absence of the active principle in this fraction in comparison to methanol fraction. This showed that the bioactive agents responsible for the opening of the pore are highly polar constituents and hence more soluble in methanol which has a higher polarity index when compared with ethylacetate.

It has been established that opening of the pore results in reduction in efficiency of oxidative phosphorylation which impacts on decrease in energy levels in addition to dissipation of mitochondrial transmembrane electrochemical proton gradient, cessation of ATP synthesis and loss of respiratory substrates as well as

nucleotides from the mitochondrial matrix [31,32].

Currently, researchers are proposing that mPT pore is the mitochondrial ATPase. A group of thought is hypothesizing that the mPT pore forms at the interface between ATP synthase dimers or perhaps tetramers, [32,33,34,35] and there is another hypothesis that the mPT pore arises from the c-ring of the $F_0$ sector of the mitochondrial ATPase [36,37,38] In order to ascertain the involvement of mPT pore in mitochondrial ATP synthase, effects of fractions of *C. portoricensis* on mitochondrial ATPase activity were investigated. This was determined spectrophotometrically by following the rate of release of inorganic phosphate from the ɣ-position of ATP during the ATPase action. The data obtained in this study showed that crude methanol extract (MECP) and methanol fraction (MFCP) of *C. portoricensis* enhanced mitochondrial ATPase activity to varying extents in male Wistar rats. In this regard, the enhancement observed by MECP and MFCP were close to that of 2,4 dinitrophenol (DNP), a classical uncoupler of oxidative phosphorylation. Studies have shown that 2,4 DNP, an ionophore induces mitochondrial dysfunction and its toxicity causes death in humans [39].

These results lay credence to our findings that MECP [26] and MFCP induced opening of the mPT pore in rat liver mitochondria. It is also consistent with reports that mPT pore opening causes uncoupling of oxidative phosphorylation by inhibiting uptake of inorganic phosphate. This converts mitochondria to ATP consumers instead of producers, thus, leading to elevated levels of inorganic phosphate [38]. Our results further provide evidence that the state of the mPT pore is involved in the proper functioning of the mitochondrial ATP synthase.

The finding that EFCP and CFCP did not show significant enhancement of mitochondrial ATPase activity at all concentrations tested is in consonance with our previous reports [26] on their insignificant effect on the opening of the mPT pore in rat liver mitochondria. It seems likely that the bioactive agents responsible for induction of pore opening are the same candidates interacting with the mitochondrial ATP synthase. Thus, this may be another pointer that attests to the view of Alavian and his colleagues [33] that the ATP synthase may be an essential part of the mPT pore.

Given the role of reactive oxygen species as mediators of aging and age-related diseases, it became important to determine if fractions of *C. portoricensis* utilize mitochondrial membrane permeabilization as a probable mechanism of promoting mitochondrial dysfunction.

Incubation of mitochondria in the presence of $FeSO_4$ causes a significant increase in lipid peroxidation due to the fact that some metals have a strong catalytic power to generate highly reactive hydroxyl radicals via the fenton reaction mechanism. The effects of fractions of *C. portoricensis* on iron-induced lipid peroxides were tested using the method of Ruberto [24] using mitochondria as lipid rich source.

Our results revealed that varying concentrations (100-800 µg/mL) of both MECP and MFCP strongly inhibited generation of mitochondrial lipid peroxides in a concentration–dependent manner. The CFCP and EFCP displayed moderate and minimal inhibitory effects respectively, in this regard.

Interestingly, the finding that fractions of *C. portoricensis* inhibited generation of mitochondrial lipid peroxides indicate their protective effect on membrane bilayers by shielding the mitochondrial membranes from free radical induced severe cellular dysfunction. Moreover, it suggests that the mechanism of induction of pore opening is not via generation of reactive oxygen species that could lead to peroxidation of mitochondrial membrane lipids but rather via interaction with components of the pore. Hence, our results rules out mitochondrial membrane peroxidation as a possible mechanism for membrane permeabilization by potent fractions of *C. portoricensis.*

Evidence abound that the release of cytochrome C from the intermembrane space into the cytosol as a result of opening of the pore is a point of no return for apoptosis to take place (31). In the presence of the crude MECP and MFCP, the cytochrome C released from the mitochondria was statistically significant ($p< 0.05$) at the highest concentration tested. This confirms that the opening of the pore occurred when mitochondria were exposed to these fractions and led to the release of cytochrome C which is inevitable for mitochondrial mediated apoptosis to take place.

## 4. CONCLUSION

Finally, the nature of substances responsible for the effects exhibited by the most potent MFCP is still not known. Further work will therefore include elucidation and characterization of the active principle(s) present in MFCP and their effects on molecular markers of apoptosis. This could be relevant in management and treatment of diseases characterized by insufficient apoptosis.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

Ethical approval for this study was obtained from the University of Ibadan Animal Care & Use Research Ethics Committee (ACUREC)

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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