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ARTICLE

Fractions of *Adenopus breviflorus* Extract Modulate Calcium-induced Mitochondrial Permeability Transition Pore Opening in Rat Liver

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Abstract

Mitochondrial dysfunction (MD) and impaired apoptotic pathways cause irreversible opening of the Mitochondrial Permeability Transition (MPT) pore, resulting in several pathological conditions e.g. cancer, ageing and neurodegenerative diseases. Many bioactive compounds from plants have been identified as modulators of the MPT pore which makes them possible drugs for the management of MD associated diseases. *Adenopus breviflorus* (*A.breviflorus*) is a tropical medicinal plant used in folkore medicine as an abortifacient and in treating gonorrhoea. In this study, the effects of ethylacetate and methanol fractions of *A.breviflorus* were assessed on rat liver MPT pore and Mitochondrial ATPase (mATPase). The fruit of *A.breviflorus* was extracted with water to obtain the aqueous Extract (AEAB), which was fractionated using vacuum liquid chromatography (VLC) to obtain ethylacetate and methanol fractions of *A.breviflorus* (EFAB, and MFAB). The extent of MPT pore opening and mATPase by EFAB and MFAB were assayed spectrophotometrically. The results obtained showed that EFAB and MFAB have no significant inductive effect on the MPT pore in the absence of Ca^{2+}. However, in the presence of Ca^{2+}, EFAB inhibited calcium-induced MPT pore opening in a non-concentration dependent manner. Maximum inhibition of MPT pore opening was 57.1% at 50 µg/ml. Interestingly, MFAB potentiated calcium ion effect by opening the pore further. Specifically, MFAB opened the MPT pore by 11, 10, 17 and 9% at 50, 150, 250 and 350 µg/ml, respectively. Furthermore, EFAB and MFAB inhibited mATPase activity in rat liver mitochondria at 62.5, 187.5, 312.5 and 437.5 µg/ml by 2.6, 18.8, 37.3, 52.6% and 41.8, 6.8, 24.3, 8.4%, respectively. The ethylacetate and methanol fractions of *Adenopus breviflorus* possess potential phytochemicals that can modulate opening of the mitochondrial permeability transition pore and inhibit mitochondrial ATPase activity in rat liver. These fractions may find use in drug development against diseases where excessive apoptosis takes place.

Keywords: *Adenopus breviflorus*; Ethylacetate; Mitochondrial ATPase; Mitochondrial Permeability; Transition Pore

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1.0 Introduction

The Mitochondrial Permeability Transition (MPT) pore forms between the inner and outer mitochondrial membranes as a large conductance pathway (Halestrap 2009) which opens under conditions of Ca^{2+} overload and high amount of reactive oxygen species (ROS) (Rasola and Bernardi 2014). Oleic acid and inorganic phosphate may also initiate opening of the MPT pore (Rasola and Bernardi, 2014). Although, the exact nature of the pore has not been revealed (Bernardi 2013), it is believed to be made of adenosine nucleotide translocase (ANT), voltage dependent anion channel (VDAC), cyclophilin D (Cyc D) (Crompton, 1999), benzodiazepine receptor (PBR) and Hexokinase II (HK) (Fulda *et al*., 2010). The inner mitochondrial membrane regulates the movement of molecules in and out of the mitochondria and it is only permeable to solutes less than 1.5 KDa. Mitochondrial swelling, uncoupling of oxidative phosphorylation, ATP depletion occurs, whenever the integrity of the inner mitochondrion is compromised. ATP is hydrolysed by mitochondrial ATPase, causing the equilibration of solutes across the inner mitochondrial membrane (Halestrap, 2009; Rao *et al.*, 2014).

Mitochondrial dysfunction has been associated with ageing, cancer and neurodegenerative disorders e.g. Parkinson and Alzheimer's diseases (Fulda, 2010; Hroudova *et al*., 2014; Perez-Hernandez *et al*., 2016). The role of mitochondrial-mediated apoptosis has been well documented in many pathological conditions. The mitochondrion is a store house for proteases that activate various mode of cell death e.g. apoptosis (Fulda, 2016). Generally, apoptosis (programmed cell death) occurs either through the extrinsic or intrinsic (mitochondrial-mediated) pathways (Shawarz *et al*., 2007). Dysfunction in the mitochondrial apoptotic pathway in a cell may lead to cancer (Fulda, 2016), while excessive apoptosis may lead to neurodegenerative diseases and ageing (Hroudova et al., 2014; Sun *et al*., 2016). Mitochondrial Ca^{2+} overload and ROS triggers permeabilization of the mitochondrial membranes in ageing and neurodegenerative diseases (Gorlach *et al*., 2015; Rottenberg and Hoek, 2017; Angelova and Abramov, 2018). This consequently causes mitochondrial swelling, loss of mitochondrial membrane potential, ATP depletion and opening of the mitochondrial membranes. The condition leads to MPT and the release of proapoptotic proteins through the MPT pore from the intermembrane space of the mitochondria. The release of proteins such as cytochrome c, and apoptosis-inducing factor (AIF) from the intermembrane space to the cytosol, compels the cell to die in a programmed fashion. Activation of caspases 9 and 3 lead to cleavage of other proteolytic proteins which results in chromatin condensation, membrane blebbing and consequently, the activation of phagocytes (Wang, 2001).

Generally, there is a drift toward the use of medicinal plants for the management of illnesses and diseases. Plants possess a vast number of phytochemicals e.g. polyphenols, alkaloids, saponins, flavonoids etc. which are basically useful medicines to humans. These phytochemicals may cause opening of the mitochondrial permeability pore in cancer and also retard the processes of ageing in neurodegenerative conditions. Curcumin (Qiu *et al*., 2014) and betulinic (Fulda *et al*., 2010) acid are potent inducers of Mitochondrial Permeability Transition in mitochondria of some cancer cells, while decursin from *Angelica gigas* has been reported to reduce cytochrome c release from the mitochondria and to suppress caspase-3 activities in PC12 cells (Sowndhararajan and Kim, 2017).

Curcumin from *Curcuma domestica* has been suggested from *in vivo* studies to have neuro-protective effects (Chin *et al*., 2013) although, it also has proapoptotic properties. Saponins also have been identified as potent anti-cancer agents (Man *et al*., 2010). *Adenopus breviflorus* (Benth), belong to the family curcumbitaceae. It is a tropical plant, used in herbal medicine in the management of gonorrhoea, stomach disorder in women and as an abortifacient (Elujoba *et al*., 1985). Phytochemicals such as alkaloids, polyphenols and saponins are present in the fruit (Oyedeji *et al*., 2017). The aqueous fruit extract of *Adenopus breviflorus* has been shown to significantly induce opening of the MPT pore in rat liver mitochondria (Oyedeji *et al*., 2017). This study therefore seeks to assess the effects of ethylacetate and methanol fractions of the aqueous fruit extract of *Adenopus breviflorus* on mitochondrial permeability transition, mitochondrial ATPase activity and mitochondrial lipid peroxidation in rat liver mitochondria.

2.0 Materials and Methods

2.1 Extract preparation

The fruit of *A. brreviflorus* were purchased from an open market at Ibadan, Oyo State, Nigeria. The fruit was identified and authenticated at herbarium, Botany Department, University of Ibadan, Nigeria and assigned the reference No. UIH: 22500. The chopped fruit were soaked in distilled water for 48 hours after which, the extract was filtered. The filtrate obtained was concentrated to dryness using a rotary evaporator at 50 ^{o}C to obtain the aqueous extract of *Adenopus breviflorus* (AEAB). Thereafter, 10 g of AEAB was pre-absorbed with 10 g of silica gel and loaded on 180 g of silica gel already packed in a sintered glass. Ethylacetate (100%) and methanol (100%) were poured serially into the sintered glass. The filtrates obtained were concentrated to dryness in a rotary evaporator at 40^{o}C to obtain the ethylacetate fraction (EFAB) and the methanol fraction (MEAB) of AEAB, respectively. The fractions were kept at 4 ^{0}C for experimental use.

2.2 Experimental Animals

Male Wistar rats (150 – 160 g) bought from the Animal House of the College of Medicine, University of Ibadan, were used in this experiment. The rats were kept at temperatures between 28 – 30 ^{o}C in well-ventilated cages. The animals were treated with care according to the rules and protocols of the National Institute of Health (1985). The rats were kept in ventilated cages and fed rat feeds (Ladokun feeds) and water *ad-libitium.* The animals were allowed to get used to the environment for two weeks before the start of the experiment.

2.3 Mitochondria isolation

Isolation of mitochondria from rat liver were prepared according to the method described by Johnson and Lardy (1967), and as modified by Lapidus and Sokolove (1993). The rats were sacrificed and the livers excised. The rat livers were weighed, washed thrice in ice-cold homogenizing Buffer C (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 5 mM HEPES-KOH, (pH 7.4) and minced with a pair of scissors . A 10% suspension liver homogenate was prepared by homogenizing rat liver in ice-cold homogenizing Buffer C in a Potter-Elvehjem glass homogenizer. Centrifugation of rat liver homogenate was carried out in a high speed refrigerated MSE centrifuge at 4 ^{o}C for 5 min at 2,300 rpm. The nuclear fraction and cellular debris sedimented were discarded,

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while the supernant was centrifuged at 13,000 rpm for 10 min to pellet mitochondria. Pelleted mitochondria were washed twice with washing Buffer D (210 mM mannitol, 70 mM sucrose, 0.5% BSA, 5 mM HEPES-KOH, pH 7.4) by spinning at 12,000 rpm for 10 min.

Washed mitochondria pelleted were re-suspended in appropriate volume of MSH buffer (210 mM mannitol, 70 mM sucrose and 5 mM HEPES-KOH, (pH 7.4) dispensed into eppendorf tubes and kept at 4 ^{o}C for use.

2.4 Mitochondrial swelling assay

Swelling of the mitochondria was determined according to the method of Lapidus and Sokolove (1993). The extent of light scattering by the mitochondria was monitored in a Spectrumlab 752s UV/Visible spectrophotometer. Briefly, intact mitochondria (0.4 mg protein/mL) were pre-incubated with 0.8 µm rotenone in MSH buffer for 3 min at 30 ^{o}c. Sodium-succinate (5 mM) was added to the medium 30 secs later. The varying concentration of EFAB or MFAB was added to the assay medium just after the addition of rotenone and then 300 µM $CaCl_2$ was added also. Mitochondrial permeability transition was quantified at 540 nm for 12 min at 30 sec interval. To test the intactness of the mitochondria, absorbance value of mitochondria suspension which was below -0.090 was not acceptable. To check the effect of calcium ion on mitochondria, 4 µM spermine was added to the assay medium before the addition of mitochondrial fraction. To test for effect of the fractions on mitochondria, addition of 300 µM $CaCl_2$ was omitted. Induction fold (I) was calculated as follows

$$I = \mathrm{C} - \frac{\mathrm{E}}{\mathrm{C}} \qquad (1)$$

where E = Change in absorbance of test sample, C = Change in absorbance of control sample

2.5 Assay for Mitochondrial ATPase

Mitochondrial ATPase assay was carried out by modifying the method of Lardy and Wellman (1953). Each assay medium contained 65 mM Tris-HCL (pH 7.4), 0.5 mg protein (mitochondria), 0.5 mM KCl, 1 mM ATP and 25 mM sucrose. The final assay volume was 2 mL. Varying concentration of EFAB and MFAB were added accordingly. The reaction was started by the addition of the ATP and allowed to proceed for 30 minutes with constant shaking at 37^{o}C. One millilitre (1mL) of 10% sodium dodecyl sulphate was added to the contents of each test tube to bring the reaction to a halt. Four millilitres (4 mL) of distilled water was added to each test tube. One millilitre (1 mL) was taken out of each test tube into a clean test tube and 1 mL of 1.25% Ammonium molybdate in 6.5% Sulphuric acid was added to each test tube. One millilitre (1 mL) of 9% ascorbic acid was added and the intensity of the colour developed was measured at 660 nm. All experiment was carried out in triplicate.

2.6 Measurement of Mitochondrial Lipid Peroxidation

Iron-induced mitochondrial lipid peroxidation was determined by slight modification of the method of Ruberto *et al.*, (2000). Mitochondria homogenate was used as the lipid rich medium. Briefly, 0.5 mg/mL of mitochondria and varying concentration of EFAB and MFAB were added to different test tubes and made up to 1 ml with distilled water. Ferrous sulphate (0.07 mM) was used to induce lipid peroxidation. Acetic acid (20%), thiobarbituric acid (TBA) (0.8%) in Sodium dodecyl sulphate (SDS) (1.1%) was added to the assay medium. The mixture in each test tube was vortexed and kept in the water bath for 60 min at 95 ^{o}C. The mixture was allowed to cool and butanol (5 mL) was added to each test tube. The test tubes were extensively vortexed and centrifuged at 4000 rpm for 10 minutes. The reading of the upper organic layer was taken at 532 nm. All experiment was carried out in triplicate.

$$MLP = \left(\mathrm{I} - \frac{\mathrm{E}}{\mathrm{C}}\right) \times 100 \qquad (2)$$

where MLP = Mitochondrial Lipid Peroxidation, E = Absorbance of test, C = Absorbance of the fully oxidized Mitochondria

2.7 Determination of mitochondrial protein

The method of Lowry *et al.*, (1951) was used to quantify mitochondrial protein. Bovine Serum Albumin (BSA) was used as standard.

2.8 Statistical Analysis

MPT graphs are representatives of at least three (3) readings. Data were analysed using One-way ANOVA on software SPSS version 15. $P< 0.05$ was considered as significant. Results were expressed as mean ± standard deviation.

3.0 Results and Discussion

The mitochondrial permeability transition pore forms in between the inner and outer mitochondrial membranes, regulating the influx of solutes in and out of the mitochondrial matrix (Lemasters *et al*., 2009). The MPT pore opens under conditions of high matrix calcium ion concentration. ROS and oleic acid, are also potent inducers of apoptosis (Rasola and Bernardi, 2014). Opening of the mitochondrial membranes lead to the release of proapoptotic proteins e.g. cytochrome c from the mitochondrial intermembrane space to the cytoplasm. Recently, Rao *et al.,* (2014) identified the MPT pore as a crucial target for drug development against several diseases. Some studies however, have identified phytochemicals from plants as potent modulators of the MPT pore (Kipp and Ramirez, 2001; Madreiter-Sokolowski *et al*., 2017).

Figure 1 shows the result of the effect of addition of 300 µM Ca^{2+} on rat liver MPT pore. Mitochondria that has accumulated calcium ion undergo large amplitude swelling reflected as a large decrease in the absorbance of light by mitochondria at 540 nm. The result revealed that in the absence of Ca^{2+}, there was no significant decrease in the absorbance of mitochondria. However, addition of Ca^{2+} significantly $p < 0.05$ induced large amplitude mitochondrial swelling by 15.9 folds and this is seen as a large decrease in the absorbance value. Spermine (0.1 mM), a standard inhibitor of the MPT pore significantly reversed Ca^{2+}-induced MPT pore opening by 86.3%.

This result revealed that, in the absence of Ca^{2+}, mitochondrial membranes were intact as there was no large amplitude swelling of the mitochondria and thus no significant decrease in absorbance. Furthermore, the reversal of Ca^{2+}-induced MPT pore opening by spermine signifies that the integrity of the mitochondrial membranes were not compromised and therefore the mitochondria were suitable for use. Calcium ion is a potent inducer of the MPT pore while polyamines such as spermine and cyclosporine A are good inhibitors of Ca^{2+}-induced MPT pore opening (Lapidus and Sokolove, 1992; Elustondo *et al.*, 2016). High matrix Ca^{2+} concentration and high levels of ROS have been well documented to be critical regulators of cell death and mitochondrial dysfunction in neurodegenerative conditions (Federico *et al*., 2012).

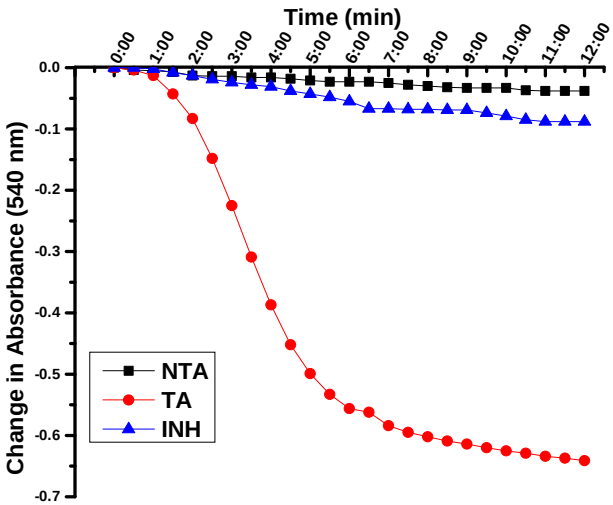

Figure 1: *Inhibition of calcium-induced Mitochondrial Permeability Transition by spermine. NTA: No Triggering Agent, TA: Triggering Agent (Calcium ion), INH: Inhibitor (Spermine)*

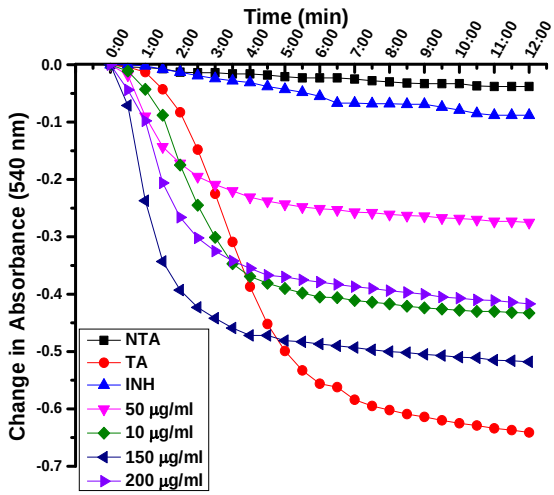

Figure 2: *Effect of EFAB on rat liver MPT pore in the presence of calcium ion. NTA: No Triggering Agent, TA: Triggering Agent (Calcium ion), INH: Inhibitor (Spermine)*

Furthermore, Figure 2 shows the effect of varying concentration of EFAB on MPT pore opening in the presence of Ca^{2+}, the triggering agent of the pore. Whereas calcium ion induced MPT pore opening by 15.9 folds, this result shows that, varying concentration of EFAB reversed calcium-induced MPT pore opening by 57.1, 32.4, 19.2 and 35.0%, respectively. Maximum inhibition (57.1%) was obtained at 50 µg/mL, while the minimum inhibition (19.2%) was obtained at 250 µg/ml. However, the fraction at 150 and 350µg/mL inhibited calcium-induced pore opening by 32.4 and 35% respectively. Although, inhibition of the MPT pore opening was not concentration-dependent. It was also observed that, in the absence of calcium ion, the varying concentration of EFAB had no significant effect on MPT pore opening (Fig. 3).

However, these results have further demonstrated that the bioactive compounds present in the EFAB fraction could not induce MPT pore opening in the absence of Ca^{2+}, nevertheless, these compounds have the potential to inhibit the calcium-induced MPT pore opening. It is therefore likely that the compounds present in EFAB may be interacting with the same protein as calcium ion thereby displacing Ca^{2+} or may be chelators of divalent cations, thereby inhibiting calcium-protein interaction in the pore (Perez-Hernandez, *et al*., 2016). From these results, we suggest that EFAB may be beneficial in the management of pathological conditions where there is excessive cell death as a result of increased opening of the MPT pore due to calcium overload in the mitochondria.

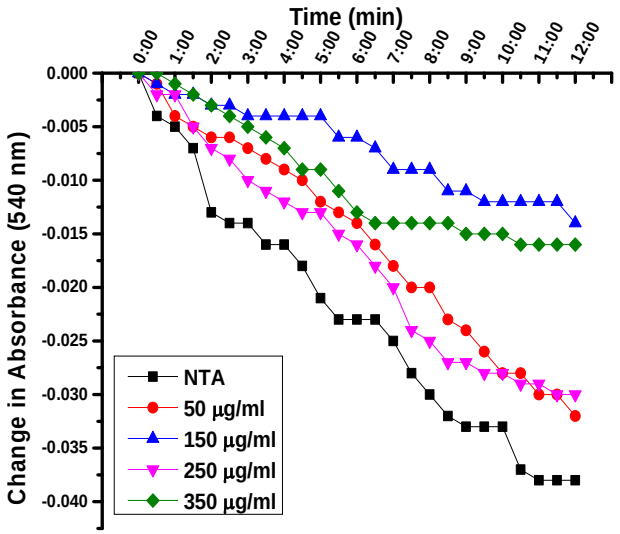

Figure 3: *Effect of EFAB on rat liver MPT pore in the absence of calcium ion. NTA: No Triggering Agent*

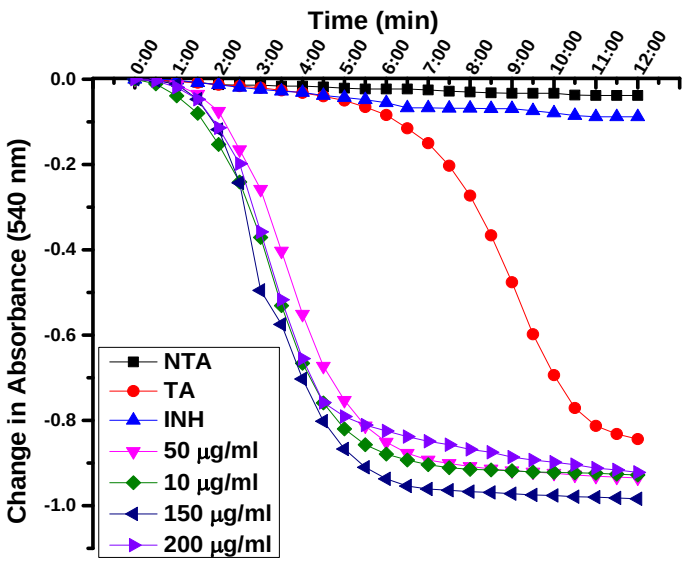

Figure 4: *Effect of MFAB on rat liver MPT pore in the presence of calcium ion. NTA: No Triggering Agent, TA: Triggering Agent (Calcium ion), INH: Inhibitor (Spermine)*

Further study on the effect of the varying concentration of MFAB on MPT pore in the presence of Ca^{2+}, revealed that the varying concentration of MFAB potentiated the extent of Ca^{2+}-induced MPT pore opening significantly at all concentration (Fig. 4).

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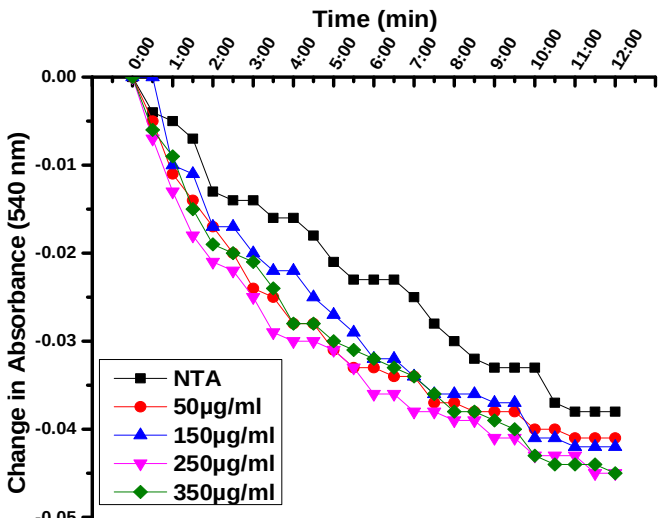

Figure 5: *Effect of MFAB on rat liver MPT pore in the absence of calcium ion. NTA: No Triggering Agent*

This result suggests that the bioactive compounds present in MFAB may be acting synergistically with Ca^{2+}, thereby, potentiating the effect of Ca^{2+} to further induce MPT pore opening more significantly. Interestingly, the varying concentration of MFAB had no inductive effect on MPT pore opening in the absence of Ca^{2+} (Figure 5). We observed that, MFAB showed similar pattern to the result obtained in Figure 3. Furthermore, this result reveals that, in the absence of any pathological condition, MFAB may have no significant effect on the normal physiology of the mitochondria. Identification and structural elucidation of these compounds may be useful in drug development in disease conditions especially in cancer and neurodegenerative disorders.

Table 1: Inhibitory effect of EFAB and MFAB on Mitochondrial ATPase activity

	% Inhibition of Mitochondrial ATPase	
Concentration (µg/mL)	**EFAB**	**MFAB**
Mitochondrial only	0.00	0.00
62.5	2.6 ± 0.07	41.8 ± 0.03*
187.5	18.8 ± 0.001	6.8 ± 0.03
312.5	37.3 ± 0.06	24.3 ± 0.05*
437.5	52.6 ± 0.02*	8.4 ± 0.04

Each value is a mean of 3 determinations ± standard deviation.
**Values are significant at p<0.05 when compared to control*

Aside the production of energy, several metabolic pathways take place in the mitochondria. The organelle is essential both for the survival of a cell and its death (McBride *et al.*, 2006). Energy in the cell is generated by the mitochondria in the form of ATP by the F0F1 ATP synthase. This enzyme has a dual role and can act in a reverse direction as mitochondrial ATPase, hydrolysing ATP under conditions of mitochondrial dysfunction (Zheng and Ramirez, 2000). Table 1 shows the effects of varying concentration of EFAB and MFAB on rat liver mitochondrial ATPase activity.

The EFAB inhibited mitochondrial ATPase activity only at 437.5 µg/mL, while, MFAB significantly inhibited the enzyme at 62.5 and 312.5 µg/mL, respectively. From the study, it may be deduced that in the absence of any pathological condition, the phytochemicals present in EFAB and MFAB may be interacting with the components of the F0F1 ATPase thereby inhibiting ATP hydrolysis and thus enhancing mitochondrial function. Zheng and Ramirez (2000), Kipp and Ramirez (2001), Madreiter-Sokolowski *et al.*, (2017), have reported the inhibitory effects of curcumin, resveratrol, quercetin and epigallocatechin gallate (EGCG) on the mitochondrial ATPase. Also, Calzia *et al.*, (2015) reported the inhibitory potentials of polyphenolics such as quercetin and EGCG on mitochondrial ATPase activity in bovine retina.

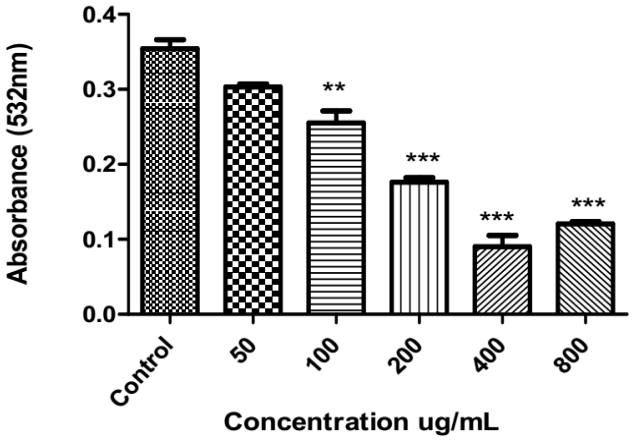

Figure 6: *Inhibitory effect of EFAB on iron-induced mitochondrial lipid peroxidation in rat liver*

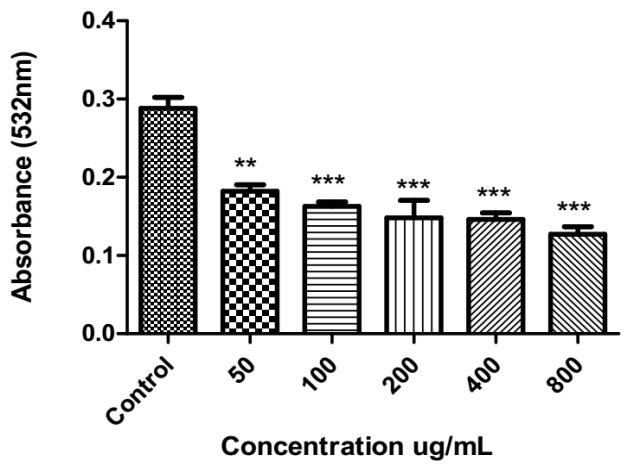

Figure 7: *Inhibitory effect of MFAB on iron-induced mitochondrial lipid peroxidation in rat liver*

The result presented in Figures 6 and 7 revealed that both EFAB and MFAB inhibited iron-induced mitochondrial lipid peroxidation. The varying concentration of EFAB and MFAB significantly ($p < 0.05$) inhibited iron-induced mitochondrial lipid peroxidation when compared with the control at varying concentration. Reactive Oxygen Species are produced by the mitochondria as by-products of mitochondrial respiration.

The inhibitory effects of EFAB and MFAB may be largely dependent on the phytochemicals present in these fractions. Several studies have demonstrated that plants phytochemicals such as flavonoids, phytosterol, polyphenols are potent inhibitors of ROS (Martin, 2006; Khan *et al.*, 2010).

High levels of ROS destroy biomolecules within the cell and the mitochondria, leading to the permeabilization of the mitochondrial membranes, loss of membrane potential and the release of proapoptotic proteases such as cytochrome c and AIF (Rottenberg and Hoek, 2017). Andelova and Abramov (2018), suggests that inhibition of ROS may be a potential target for the treatment of several disorders.

4.0 Conclusion

The findings from this study, suggest therefore that, EFAB and MFAB modulate MPT pore opening, and inhibit mitochondrial ATPase activity and iron-induced mitochondrial lipid peroxidation. We conclude that the phytochemicals present in these fractions may be useful in the development of drugs against mitochondria-related diseases. Consequently, purification and characterization of these active principles will be a positive step in the right direction.

5.0 Acknowledgement

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6.0 Conflict of Interest

Authors declare no conflict of interest.

Authors Contribution

Research conceptualization: OTA, OOO; Research design: OTA, OOO
Research execution: OTA, AIC, OOD
Data analysis and interpretation: OTA, AIC, OOD, OOO
Manuscript Writing: OTA, OOO

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