



Investigating the Modulatory Effect of Methanol Extract of *Daniellia oliveri* (ROLFE) Leaves on Mitochondrial Membrane Permeability Transition (MPT) Pore

Jonah Achem^{1*}, Cosmos Ifeanyi Onyiba¹, Mobolaji T. Akinwole¹,
Jemimah M. Malgwi¹, Omosola L. Bolarin¹ and Olufunso O. Olorunsogo¹

¹Laboratories for Biomembrane Research and Biotechnology, Department of Biochemistry,
College of Medicine, University of Ibadan, Ibadan, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Author JA designed the study, wrote the protocol. Author CIO performed the statistical analysis and wrote the first draft of the manuscript. Authors JA and CIO managed the analyses of the study and reviewed the manuscript. Authors MTA, JMM and OLA managed the literature searches and provided technical assistance. Author OOO supervised the study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJBCRR/2020/v29i930223

Editor(s):

(1) Dr. Chunying Li, Georgia State University, USA.

Reviewers:

(1) Saturnino Carmela, University of Basilicata, Italy.

(2) Wafaa Mohamed Haggag, National Research Centre, Egypt.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/62315>

Original Research Article

Received 10 August 2020
Accepted 14 October 2020
Published 12 November 2020

ABSTRACT

Background: Mitochondrial-mediated cell death begins with opening of mitochondrial membrane permeability transition (mPT) pore and medicinal plants contain phytochemicals that modulate the mPT pore.

Hypothesis and Purpose: We investigated the modulatory effects of crude methanol extract of *Daniellia oliveri* leaves (CMDO) on mPT pore *in vitro*.

Study Design and Methods: Phytochemical screening and antioxidant activities of crude methanol extract of *Daniellia oliveri* leaves (CMDO) were evaluated according to standard procedures. CMDO was partitioned into chloroform fraction (CFDO), ethyl acetate fraction (EFDO) and methanol fraction (MFDO) by Vacuum liquid chromatography (VLC). Effects of CMDO, CFDO, EFDO and MFDO on mPT pore were assessed by spectrophotometry. Effects of the most potent fraction on mitochondrial

*Corresponding author: E-mail: achemjonah20@gmail.com;

ATPase, Fe-induced lipid peroxidation and cytochrome c release were assessed by spectrophotometry. CMDO was subjected to GC-MS analysis to identify the bioactive compounds present.

Results: CMDO contains phytochemicals and showed appreciable total flavonoid content (0.483±0.02 QE mg/100g), total phenolic content (0.886±0.12 GAE mg/100g), total antioxidant capacity (0.039±0.001 AE mg/100 g), ferric antioxidant reducing power (IC₅₀=350 µg/ml) and 2, 2-diphenyl-1 picrylhydrazyl (DPPH) radical scavenging activity (IC₅₀=166 µg/ml). The maximum induction of mPT pore opening in the absence and presence of calcium, respectively, were as follows: CMDO (10.11 folds, 5.18 folds), CFDO (19.9 folds, 16.3 folds), EFDO (7.5 folds, 23.2 folds), MFDO (22.2 folds, 31.3 folds). The most potent mPT pore-opening fraction (MFDO) enhanced mitochondrial ATPase activity, inhibited Fe-induced lipid peroxidation and caused cytochrome c release. GC-MS analysis of CMDO revealed the presence of bioactive compounds including methyl propanamide, Dibutyl phthalate, saturated and unsaturated fatty acids.

Conclusion: Methanol fraction (MFDO) of CMDO most potently induced mPT pore opening via enhancement of mitochondrial ATPase activity, which was substantiated by the release of cytochrome c (*in vitro*). This includes MFDO as a candidate pharmacologic remedy for diseases associated with insufficient apoptosis.

Keywords: *Daniellia oliveri*; Mitochondria; mPT pore; F₀F₁ ATPase; Cytochrome c; apoptosis.

1. INTRODUCTION

Mitochondria are indispensable organelle in the cell and have been implicated in a plethora of pathologies as well as in the initiation cell death [1, 2]. Mitochondrial-mediated cell death begins with permeabilization of the mitochondrial inner and outer membrane [3]. This permeabilization is mainly through opening of the mitochondrial membrane permeability transition (mPT) pore, a pore formed from the inner mitochondrial membrane (IMM) to the outer mitochondrial membrane (OMM) [4]. The opening of mPT pore increases permeability of IMM, dissipates mitochondrial membrane potential, uncouples respiratory chain, halts ATP production, causes mitochondrial swelling, ruptures OMM and releases certain “killer proteins” from the intermembrane space into the cytosol, which initiates cell death [5].

In spite of the fact that the molecular nature of mPT pore has not been fully elucidated, it is well established that the pore opening can be triggered by different agents including Ca²⁺, ADP, inorganic phosphate, Reactive oxygen species (ROS) and several chemotherapeutics (Jennifer and Jeffery, 2015). On the other hand, potent inhibitors of mPT pore opening includes spermine, ATP, NADH, Mg²⁺ and low pH [6]. The mPT pore has now become a potential pharmacologic target for development of drugs against diseases associated with dysregulated apoptosis. Research have shown that certain medicinal plants such as *Adenopus breviflorus* [7], *Alstonia boonei* [8] and *Calliandra*

portoricensis [9] induces the opening of mPT pore in rat models.

Daniellia oliveri (Rolfe) is a plant commonly found in Africa and the Amazon region. In the Yoruba speaking region of Nigeria, it is called “iya”, whereas in the Hausa and Igbo speaking region, it is called “maje” and “ozabwa”, respectively [10]. The bark extract is applied traditionally to keep gastro-intestinal parasite in check [11]. Extracts of *Daniellia oliveri* leaves have been reported to exhibit antidiarrheal [12], antityphoid [13] and antioxidant [14] activities. Decoction of *Sarcocephalus latifolius* and *Daniellia oliveri* roots is used as herbal remedy for attenuation of hyperglycaemia [15]. There is also evidence that extract of *Daniellia oliveri* exhibits antitumor activities in breast, prostate and colon cancer cell lines [16].

Although we have previously investigated the modulatory effect of solvent fractions of *D. oliveri* stem bark on mPT in rat liver [17], we hypothesized that the leaves of *D. oliveri* may be rich in bioactive agents that could modulate the mPT pore. Moreover, there is little information about the modulatory effect of *D. oliveri* on mPT pore, in view of determining its candidacy for pharmacologic intervention against diseases associated with dysregulated cell death. Therefore, we aimed to investigate the modulatory effects of crude methanol extract of *D. oliveri* leaves (CMDO) alongside its solvent fractions on mPT pore in rat liver *in vitro*. Further, we determined the effect of most potent solvent fraction of CMDO on mitochondrial adenosine

triphosphatase (ATPase) activity, Fe-induced lipid peroxidation and Cytochrome c release *in vitro*.

2. MATERIALS AND METHODS

2.1 Collection and Crude Extraction of Plant Material

Freshly harvested leaves of *D. oliveri* were obtained from Bode Herbal Market, Ibadan, Oyo state, Nigeria. The leaves samples were identified and authenticated at the Department of Botany, University of Ibadan and a specimen number (UIH - 22383) was assigned and deposited at the Herbarium. The leaves were air-dried for four weeks in the laboratory and were milled into powder and weighed. The powdered leaves of *D. oliveri* (7 kg) were soaked with sufficient methanol in a large glass jar at room temperature for 72 hours. The mixture was filtered using a sterile Whatmann No 1 filter paper and the filtrate was concentrated under reduced pressure using a rotary evaporator. Methanol was used for the crude extraction due to its amphipathic nature. Other solvents such as n-hexane, chloroform, ethyl acetate and methanol were used to partition the crude methanol extract using vacuum liquid chromatography (VLC) technique in the order of increasing polarity.

2.2 Phytochemical Screening of CMDO

Phytochemical screening was done on the crude methanol extract of *D. oliveri* leaves (CMDO) following the procedures described by Trease and Evans [18].

2.3 Estimation of Total Flavonoid Content (TFC) and Total Phenolic Content (TPC) in CMDO

Total flavonoid content and total phenolic content of CMDO were estimated following the method described by Aryouet-Grand *et al.* [19] and Singleton *et al.* [20], respectively.

2.4 Evaluation of Antioxidant Activities of CMDO

DPPH (2, 2-diphenyl-1 picrylhydrazyl) radical scavenging activity of CMDO was determined by a modification of the protocol established by Kedare and Singh [21]. Ferric antioxidant reducing power (FRAP) of CMDO was estimated according to the method described by Tundis *et*

al. [22]. Total antioxidant capacity (TAC) of CMDO was estimated by the method described by Prieto *et al.* [23]. IC50s (Concentration at which 50% of radicals are inhibited) were extrapolated from plotted charts.

2.5 Partitioning of CMDO Using Vacuum Liquid Chromatography (VLC)

Silica gel 60 (0.04 – 0.063 mm MERCK) 12 g were adsorbed to 20 g of CMDO. The mixture was air-dried to obtain powder. A sintered funnel for vacuum liquid chromatography (VLC) was packed with 8 g Silica gel (Hopkins and Williams, England). Solvents were added in the order of increasing polarity and n-hexane, chloroform (CFDO), ethyl acetate (EFDO) and methanol (MFDO) fractions were obtained. 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 preserved in a refrigerator (4°C).

2.6 Isolation of Mitochondria

Mitochondria were isolated from rat liver according to the modified method by Johnson and Lardy [24]. In brief, excised rat liver was homogenized in an isolation buffer. The homogenate was centrifuged in a refrigerated centrifuge at 2,500 rpm twice for five minutes to pellet the nuclear fraction and cellular debris. The supernatant obtained was spun at 13 000 rpm for 10 minutes to obtain the mitochondrial pellet which was washed twice with a washing buffer by spinning at 12,000 rpm for 10 minutes. The mitochondria obtained were immediately resuspended in an appropriate volume of suspension buffer and dispensed into Eppendorf tubes and kept at 4°C for fresh use.

2.7 Estimation of Mitochondrial Protein

Mitochondrial protein was estimated according to Lowry method [25] using bovine serum albumin (BSA) as standard.

2.8 Mitochondrial Swelling Assay

Mitochondrial permeability transition (mPT) was determined according to Lapidus and Sokolove [26].

2.9 Mitochondrial ATPase Activity

Adenosine triphosphatase (ATPase) activity was determined by modification of the method

described by Olorunsogo and Malomo [27]. In brief, 65mM Tris-HCl buffer (pH7.4), 0.5 mM KCl, 1 mM ATP, 25 mM sucrose, distilled water and the solvent fraction were added accordingly to test tubes in triplicates. Mitochondria were added to each test tube (making up a final reaction volume of 2 mL) at 30secs interval and incubated for 30 minutes with constant shaking at 30°C. Reaction in the Zero-time tubes were stopped with addition of 1 mL of 10% Sodium Dodecyl Sulphate (SDS) immediately after addition of the mitochondria. 2, 4-dinitrophenol was added to one of the tubes and this served as a classical uncoupler (standard). After the incubation time elapsed, 1 mL of the reaction mixture in each tube was pipetted into new tubes for inorganic phosphate determination.

2.10 Inorganic Phosphate Determination

Inorganic phosphate released from the hydrolysis of ATP was estimated according to the method described by Bassir [28].

2.11 Mitochondrial Lipid Peroxidation Estimation

Mitochondrial lipid peroxidation was estimated by modification of the method described by Ruberto et al. [29]. In brief, freshly isolated mitochondria were added to test tubes in triplicates and varying concentrations of the solvent fraction were added. The mixtures were made up to 1 mL with distilled water. 0.07 Iron Sulphate (FeSO₄) was added to induce peroxidation on the mitochondrial membrane. 20% Acetic acid, 0.8% Thiobarbituric acid and 1.1% SDS were also added and the mixture was vortexed and heated at 95°C for 1 hour. After cooling, 5 mL of butanol was added to each test tube and all were thoroughly vortexed and then centrifuged at 4000 rpm for 10 minutes. Absorbance of the upper organic phase was measured at 532 nm. Results were expressed as percentage inhibition of Fe-induced lipid peroxidation using the simple arithmetic equation (Eq. (1)):

$$\% \text{ inhibition of Fe - induced lipid peroxidation} = \frac{A_1 - A_2}{A_1} \times 100 \quad (1)$$

Where A1 is absorbance of fully oxidized control and A2 is absorbance of test tubes containing various concentrations of the solvent fraction.

2.12 Cytochrome c Release Estimation

Release of cytochrome c from isolated mitochondria was estimated by

spectrophotometry at 414 nm according to the method described by Appaix et al. [30].

2.13 Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

GC-MS analysis of crude methanol extract of *D. oliveri* leaves was done with Agilent technologies 7890 GC model. The model of the detector is Agilent technologies 5975 MSD (Mass Spect. Detector). The principle behind GC-MS analysis is separation techniques. The mobile phase is the carrier gas (Helium, 99.99% purity), while the stationary phase is the column. The column model is HP5 MS, with length of 30 m, internal diameter of 0.320 mm and thickness of 0.25 µm. The oven temperature program is has an initial temperature of 80°C, which is held for 1 minute. The temperature increases by 10° per minute to the final temperature of 240°C and is held for 6 minutes. The injection volume is 1 microlitre and heater or detector temperature is 250°C. The sample is put in a vial bottle and placed in an auto injector sample compartment. The automatic injector injects the sample into the liner. The mobile phase pushes the sample from the liner into the column where separation takes place into different components at different retention time. The MS interpret the spectrum MZ (mass to charge ratio) with molar mass and structures. The separated compounds were analyzed by GC-MS and retention times for all compounds were determined. The compounds were identified based on comparison of their mass spectra with those of the internal (computer) library W9N11.L.

2.14 Statistical Analysis

Data were expressed as mean±standard deviation and treated with analysis of variance (ANOVA). Multiple comparisons were done using Tukey's HSD test at 5% of the probability. Statistical analysis was done using MS-Excel 2010 and Graphpad prism version 6.0

3. RESULTS

3.1 Phytochemical Screening and Antioxidant Activities of CMDO

Phytochemical screening of CMDO shows the presence of various phytochemicals and an appreciable amount of flavonoids and phenolics (Table 1). The total flavonoid content (TFC) and total phenolic content (TPC) substantiated the

appreciable total antioxidant capacity (TAC) exhibited by CMDO (Table 2). DPPH radical and Ferric radical (FRAP) were inhibited by CMDO (IC₅₀=166 µg/ml and IC₅₀=350 µg/ml, respectively), thus showing antioxidative potentials (Fig. 1).

3.2 Modulation of MPT Pore by CMDO, CFDO, EFDO and MFDO

Varying concentrations of CMDO, CFDO, EFDO and MFDO showed significant inductions of mPT pore opening in absence of Ca²⁺ and a reversal of calcium-induced pore opening at higher concentrations in presence of Ca²⁺ when compared to NTA (no triggering agent), which served as control. CMDO had a maximum inductive effect of 10.11 folds at 240 µg/ml in

absence of calcium and a maximum inductive effect of 5.18 folds at 240 µg/ml in presence of calcium (Fig. 2). CFDO had a maximum inductive effect of 19.9 folds at 240 µg/ml in absence of calcium and maximum inductive effect of 16.3.18 folds at 160 µg/ml in presence of calcium (Fig. 3). EFDO had a maximum inductive effect of 7.5 folds at 240 µg/ml in absence of calcium and maximum inductive effect of 23.2 folds at 80 µg/ml in presence of calcium (Fig. 4). MFDO had a maximum inductive effect of 22.2 folds at 320 µg/ml in absence of calcium and maximum inductive effect of 31.3 folds at 80 µg/ml in presence of calcium (Fig. 5). MFDO exhibited the highest inductive effect both in absence and presence of Ca²⁺ and this was concentration-dependent. This data shows a representative result of experiments repeated at least 3 times.

Table 1. Phytochemicals in methanol extract of D.oliveri leaves (CMDO)

| Phytochemicals | CMDO |
|--------------------|------|
| Flavonoids | ++ |
| Alkaloids | +++ |
| Phenolics | +++ |
| Terpenes | ++ |
| Saponins | - |
| Tannins | +++ |
| Cardiac glycosides | +++ |
| Anthraquinones | ++ |
| Phlobatannins | +++ |

+ Mildly present; ++Moderately present; +++Strongly present

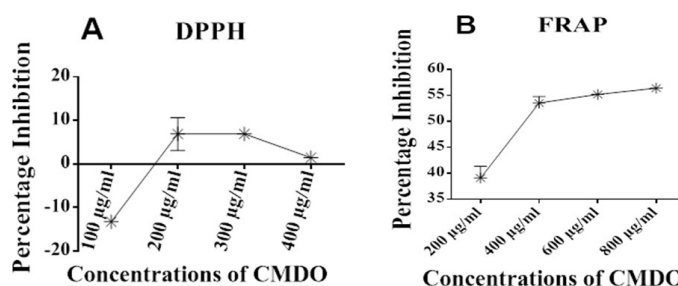


Fig. 1. Percentage inhibition of DPPH (A) and ferric radicals (B) by crude methanol extract of Daniellia oliveri leaves (CMDO). Asterisks (*) represent mean of triplicate values while error bars indicate standard deviations.

Table 2. Total flavonoid content (TFC), Total phenolic content (TPC) and Total antioxidant capacity (TAC) of CMDO

| Assays | Concentration |
|----------------------------|------------------------|
| Total flavonoid content | 0.483±0.02 QE mg/100 g |
| Total phenolic content | 0.886±0.12 GAE mg/100g |
| Total antioxidant capacity | 0.039±0.001 AE mg/100g |

Data is mean±standard deviation of triplicate values.
 QE – Quercetin equivalent; GAE – Gallic acid equivalent;
 AE – Ascorbic acid equivalent

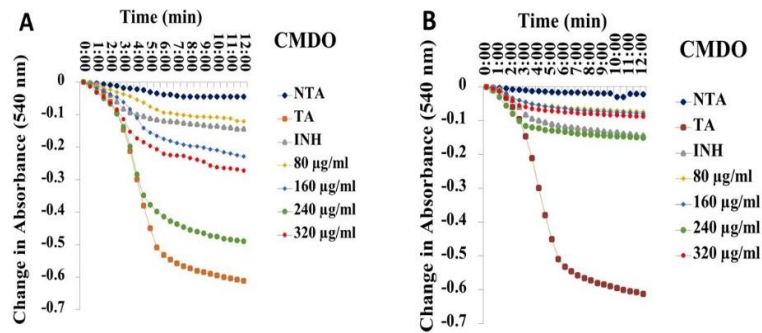


Fig. 2. Effects of various concentrations of CMDO on mPT pore in rat liver mitochondria in absence (A) and presence (B) of calcium (Ca^{2+}). NTA: No triggering agent; TA: Triggering agent (Ca^{2+}); INH: Inhibitor (Spermine)

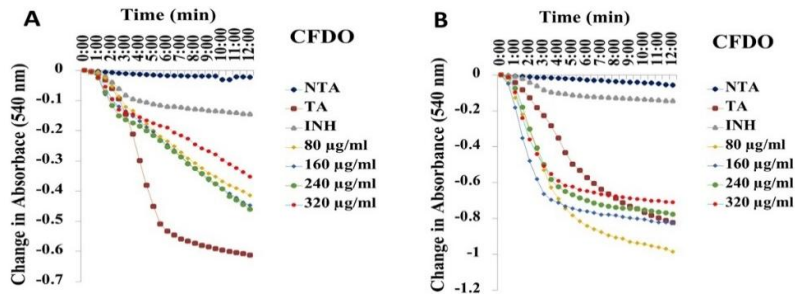


Fig. 3. Effects of various concentrations of CFDO on mPT pore in rat liver mitochondria in absence (A) and presence (B) of calcium (Ca^{2+}). NTA: No triggering agent; TA: Triggering agent (Ca^{2+}); INH: Inhibitor (Spermine)

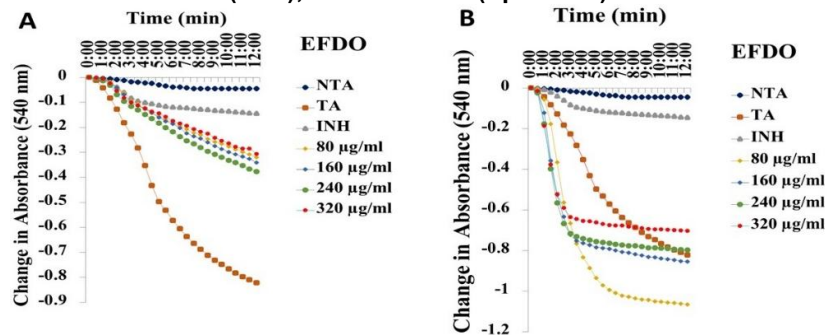


Fig. 4. Effects of various concentrations of EFDO on mPT pore in rat liver mitochondria in absence (A) and presence (B) of calcium (Ca^{2+}). NTA: No triggering agent; TA: Triggering agent (Ca^{2+}); INH: Inhibitor (Spermine)

3.3 Most Potent MPT Pore-Opening Fraction (MFDO) Enhanced Mitochondrial ATPase Activity, Inhibited Fe-Induced Lipid Peroxidation and Caused Cytochrome C Release

MFDO significantly caused a concentration-dependent enhancement of mitochondrial ATPase activity as well as release of cytochrome c when compared to control (Fig. 6). Maximum enhancement of ATPase activity and cytochrome c release was recorded at 600 µg/ml and 320 µg/ml, respectively. The significant increases in percentage inhibition of Fe-induced lipid peroxidation caused by MFDO was concentration-dependent and was maximum at 8 mg/ml (Fig. 7).

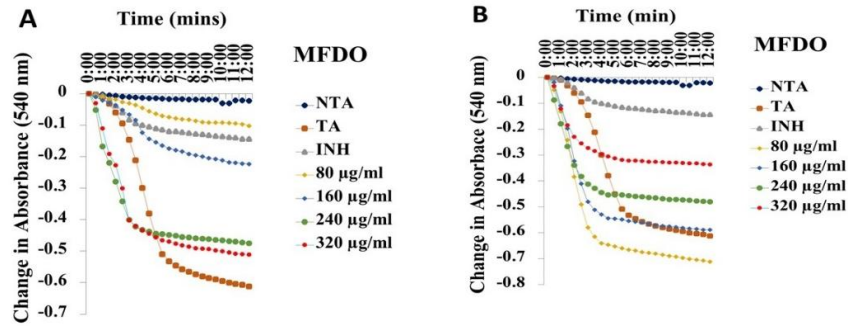


Fig. 5. MFDO demonstrated the highest inductive effect on mPT pore opening in rat liver mitochondria in absence (A) and presence (B) of calcium (Ca^{2+}). NTA: No triggering agent; TA: Triggering agent (Ca^{2+}); INH: Inhibitor (Spermine)

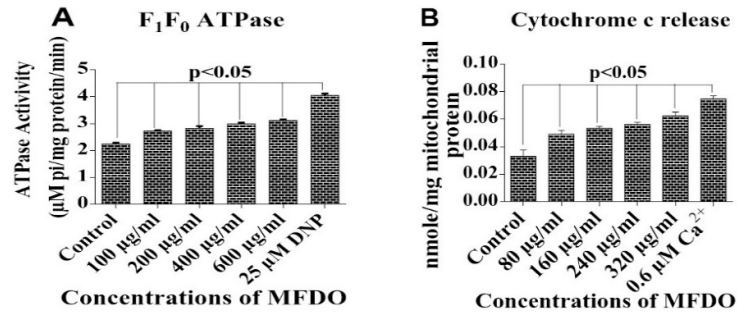


Fig. 6. MFDO enhanced F_0F_1 ATPase Activity (A) and caused the release of Cytochrome c (B) from rat liver mitochondria. Bars represent mean of triplicate values while error bars indicate standard deviations. $p < 0.05$ connotes significant difference with respect to control

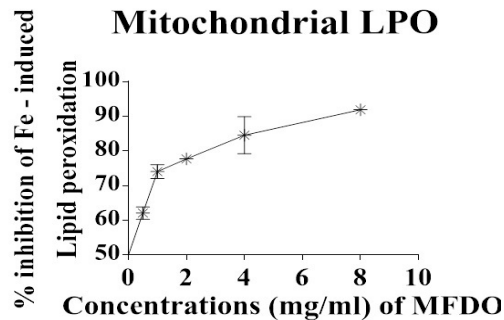


Fig. 7. Methanol fraction of *D. oliveri* leaves (MFDO) inhibited Fe-induced mitochondrial lipid peroxidation (LPO). Asterisks (*) represent mean of triplicate values while error bars indicate standard deviations

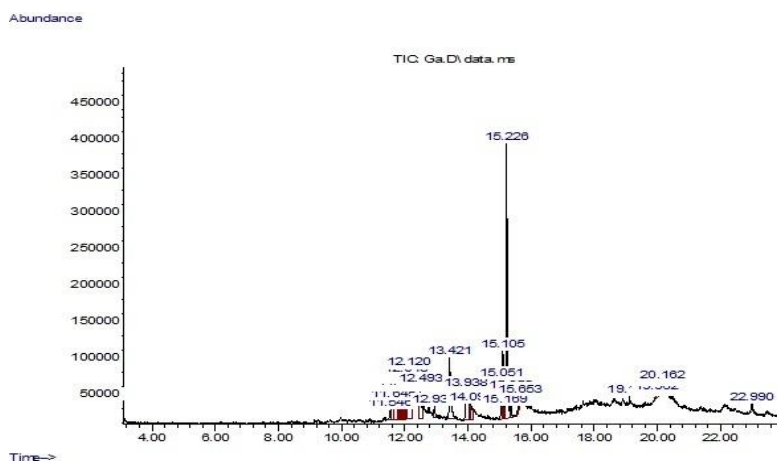
3.4 GC-MS Analysis of CMDO

The GC-MS analysis produced a mass spectra that reveals the presence of several bioactive compounds in CMDO (Fig. 8). Comparison of the mass spectra constituents with those in internal

(computer) library W9N11.L resulted in identification of various phytochemicals including 2-methyl propanamide, Tris (2-aminoethyl) amine, Propanamide, 3-isopropyl-Piperidine, Dibutyl phthalate, Hexadecanoic acid and several other unsaturated fatty acids (Table 3).

Table 3. GC-MS analysis of crude methanol extract of *D. oliveri* leaves (CMDO)

| S/N | Name of Compound | Molecular weight | RT (min) | Abundance (%) |
|-----|---|------------------|----------|---------------|
| 1 | 2-methylpropanamide | 87 | 11.54 | 0.72 |
| 2 | Butyl[1-(2,2-dimethylhydrazino)ethyl]-diazene | 172 | 11.64 | 2.64 |
| 3 | Methyl-3,5-di-o- methyl- α -D-xylofuranoside | 190 | 11.76 | 5.06 |
| 4 | 4,6-Di-O-methyl- α -d-galactose | 208 | 11.80 | 2.67 |
| 5 | Methyl-2-O- methyl α -D-xylofuranoside | 180 | 11.89 | 2.41 |
| 6 | 3-Azabicyclo[3.2.2]nonane | 125 | 12.49 | 4.35 |
| 7 | N-(3-aminopropyl)-1,4-Butanediamine | 145 | 12.94 | 1.04 |
| 8 | 1,4-Butanediamine, N-(3-aminopropyl) | 145 | 12.95 | 1.04 |
| 9 | Methyl ester-Hexadecanoic acid | 270 | 13.42 | 6.37 |
| 10 | Tris(2-aminoethyl)amine | 146 | 14.06 | 1.04 |
| 11 | Propanamide | 73 | 14.09 | 1.43 |
| 12 | 9,12-octadecadienoic acid | 280 | 15.05 | 2.21 |
| 13 | Methyl ester-7-Octadecenoic acid | 296 | 15.10 | 4.94 |
| 14 | Methylpent-4- enylamine | 99 | 15.17 | 0.72 |
| 15 | Docosylpentyl ether | 392 | 15.23 | 18.01 |
| 16 | 16-methyl-Heptadecanoic acid | 298 | 15.35 | 1.94 |
| 17 | 2-Oxo-3-methyl-cis-perhydro-1,3-benzoxazine | 169 | 15.63 | 1.30 |
| 18 | Phenylephrine | 167 | 15.65 | 0.85 |
| 19 | Dibutyl phthalate | 278 | 19.12 | 0.51 |
| 20 | Octatriacontyl pentafluoropropionate | 700 | 19.95 | 0.15 |
| 21 | 2H-Azepin-2-one hexahydro-7-methyl | 127 | 19.96 | 0.12 |
| 21 | 3-isopropyl-Piperidine | 127 | 20.02 | 0.40 |
| 23 | Pterin-6-carboxylic acid | 207 | 22.99 | 0.91 |

**Fig. 8. GC- MS chromatogram of crude methanol extract of *D. oliveri* leaves (CMDO)**

4. DISCUSSION

Phytochemicals are secondary plant metabolites that are of great medicinal value to humans and are constantly employed in treatment of various ailments [31]. In this study, phytochemical analysis of crude methanol extract of *D. oliveri* leaves (CMDO) revealed the presence of flavonoids, terpenes, steroids, alkaloids, phenolics, anthraquinones, tannins, cardiac glycosides and phlobatannins. Although

saponins was absent in CMDO, Our previous study on crude ethanol extract of *D. oliveri* stem bark showed the presence of saponins, in addition to other phytochemicals [17], thus suggesting a dependence of phytochemical detection and extraction on the extracting solvent and parts of *D. oliveri* used.

A significant number of secondary metabolites in medicinal plants act as potent antioxidants. Therefore, this study investigated the free-radical

scavenging ability of CMDO through DPPH, FRAP and TAC assays. Many plant extracts have been reported to scavenge DPPH radicals *in vitro* [32, 33, 34]. It is evident in this study that CMDO showed an appreciable (but non-concentration dependent) inhibition of DPPH radicals and even a more appreciable (concentration-dependent) antioxidant activity against ferric radicals. The reductions in DPPH radical scavenging activity at higher concentrations suggest the presence of other phytochemicals in CMDO that antagonizes its antioxidative potential.

In addition, CMDO also showed an appreciable total antioxidant capacity (TAC), total flavonoid content (TFC) and total phenolic content (TPC). Suggestively, the total antioxidative properties of CMDO can be attributed to the TFC and TPC. The antioxidative potentials reported in this study validates the reports of Muanda *et al.* [14] on the phytochemical constituents and antioxidative capacity of *Daniellia oliveri* leaves, root bark and stem bark.

The *in vitro* apoptotic-modulating potential of CMDO and its solvent fractions (CFDO, EFDO and MFDO) were assessed based on their effects on mPT pore. Results obtained showed that CMDO caused significant induction of mPT pore in absence of calcium but had no significant induction in presence of calcium. This suggests that CMDO may contain certain levels of metal-chelating (anti-nutrient) phytochemicals (such as oxalate and phytate), which may prevent calcium-induced pore opening.

The chloroform (CFDO) and ethyl acetate (EFDO) fractions of CMDO both caused a significant induction of mPT pore opening in absence and presence of calcium; however, the inductive effects in absence of calcium were not concentration-dependent. This suggests that these solvent fractions may contain certain principles that can induce pore opening but, at the same time, reverse calcium-induced pore opening at higher concentrations.

The methanol fraction of *D. oliveri* leaves (MFDO) showed a concentration-dependent induction of mPT pore opening in absence and presence of calcium at a higher magnitude when compared to the other solvent fractions. In spite of its reversal effect on calcium-induced pore opening at higher concentrations, compared to the other solvent fractions, MFDO was the most potent inducer of mPT pore opening. This

suggests that MFDO may contain certain bioactive phytochemicals that may be relevant in combating diseases associated with very little cell death such as cancer. Having established MFDO as the most potent inducer of mPT pore opening, we further evaluated its effects on ATPase activity, lipid peroxidation and Cytochrome c release *in vitro*, in view of establishing its mechanism of mPT pore opening.

Our result shows that MFDO inhibited Fe²⁺-induced lipid peroxidation on rat liver mitochondrial membrane, thus corroborating its aforementioned antioxidative potentials. This also suggests that mPT pore opening by MFDO was not due to ROS generation.

F₀F₁ ATP Synthase was recently proposed to be the major component of mPT pore [5, 35]. Increase in F₀F₁ ATPase activity, which culminates in cessation of ATP synthesis, hydrolysis of ATP and concomitant elevations of inorganic phosphate level, is one of the recently proposed mechanisms of mPT pore opening [36]. Our results reveal that MFDO enhanced ATPase activity, thus suggesting that MFDO contains bioactive agents that interact with mPT pore components.

Release of cytochrome c from the inter membrane space into the cytosol as a result of the opening of mPT pore is a point of no return for apoptosis to take place [37]. Our results show that MFDO caused the release of cytochrome c from the mitochondria (*in vitro*) in a concentration-dependent manner, thus substantiating its earlier induction of mPT pore opening.

GC-MS assay performed on CMDO revealed the presence of many characteristic phytochemicals and several saturated and unsaturated fatty acids. The chromatogram of the GC-MS indicated that this extract contained propanamide, Dibutyl phthalate, 9,12-ctadecadienoic acid, oleic acid and stearic acid. Previous studies have demonstrated the influence of these compounds on cancer cells. Phenols have been shown to generate reactive oxygen species (ROS) under cell culture conditions and trigger apoptosis in cancer cells [38]. Polyphenols have also been demonstrated to exhibit inhibitory effects on carcinogenesis and mutagenesis when consumed in diets rich in fruits and vegetables [39]. Results from several studies on unsaturated fatty acids have indicated their anti-cancer properties [40]. Stearic acid has

been reported to exhibit inhibitory effects against HOG-1 cervical cancer cells [41]. Oleic acid has been found to inhibit cell growth in different tumour cell lines in a calcium signaling-dependent manner [42]. Linoleic acid has been shown to exhibit inhibitory effects against rat hepatoma (AH-109A) cell line [43]. Palmitic acid, which was identified in CMDO, was shown to be selectively cytotoxic to MOLT-4 (Human leukemic) cell line, probably by inhibiting DNA topoisomerase I [44]. Dibutyl phthalate, which is considered as a carcinogenic agent due to its property to induced cell proliferation and invasiveness in breast cancer cells [45], was also identified in CMDO. Moreover, this compound was equally found to induce apoptosis in neural cells [46].

5. CONCLUSION

Our findings indicate that methanol fraction of *D. oliveri* (MFDO) is the most potent inducer of mPT pore opening among the other fractions investigated. As a mechanism, MFDO induced mPT pore opening via enhancement of mitochondrial ATPase activity to cause the release of cytochrome c from the intermembrane space. This therefore includes MFDO as a plausible candidate pharmacologic remedy for diseases associated with insufficient apoptosis. GC-MS profile of the crude methanol extract (CMDO) revealed a number of anti-cancer compounds such as fatty acids and phenols. These bioactive compounds with anticancer properties are of great interest because of their great structural diversity and usual exhibition of low toxicity. In this regard, further research is required to identify and characterize the bioactive principle(s) in MFDO. In addition, *in vivo* experiments are required to validate the induction of mPT pore opening by MFDO. Information from these future studies could be of potential use in treatment of diseases associated with insufficient apoptosis.

ACKNOWLEDGMENTS

We thank the deputy registrar of the Dominican University, Ibadan, Oyo State, Nigeria, Rev. Fr. Samuel Onyiba, for his funding support. We also thank Amaeze Onyinyechi Gift for the proofreading assistance.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Koopman WJ, Willems PH, Smeitink JA. Monogenic mitochondrial disorders. *N Engl J Med*. 2012;366:1132–1141.
2. Sorrentino V, Menzies KJ, Auwerx J. Repairing mitochondrial dysfunction in disease. *Annu. Rev. Pharmacol. Toxicol*. 2018;58:353–389.
3. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. *Science*. 2004;305: 626-9.
4. Adams JM. Ways of dying: Multiple pathways to apoptosis. *Genes Dev*. 2003;17(20):2481-95.
5. Elustondo PA, Nichols M, Negoda A, Thirumaran A, Zakharian E, Robertson GS, Pavlov EV. Mitochondrial permeability transition pore induction is linked to formation of the complex of ATPase C-subunit, polyhydroxybutyrate and inorganic polyphosphate. *Cell Death Discovery*. 2016;2:16070.
6. Jennifer Q, Kwong 1. Jeffery DM. Physiological and Pathological Roles of the Mitochondrial Permeability Transition Pore in the Heart. *Cell Metabolism* 21, February 3, Elsevier Inc. cmet.2014.12.001; 2015.
7. Oyediji TA, Akintehinse T, Avan ED, Soremekun OO, Solomon OE, Olorunsogo OO. Extracts of *Adenopus breviflorus* induce opening of Rat Liver Mitochondrial Membrane Permeability Transition Pore. *Biokemistri*. 2017;29(4):140-145.
8. Olanlokun OJ, Oyeboode TO, Olorunsogo OO. Effects of Vacuum Liquid Chromatography (Chloroform) Fraction of the Stem Bark of *Alstonia boonei* on Mitochondrial Membrane Permeability Transition Pore. *J Basic Clin Pharma*. 2017;8:221-225.
9. Oyeboode OT, Akinyelu OA, Oamen EA, Olorunsogo OO. Methanol fraction of *Calliandra portoricensis* root bark activates caspases via alteration in mitochondrial viability *in vivo*. *J Herbmed Pharmacol*. 2018;7(4):251-258.
10. Keay RWJ, Onochie CFA, Stanfield DP. *Nigerian Trees Vol. II* Department of Forest Research, Ibadan. 1964;65–66.
11. Djoueche CM, Azebaze AB, Dongmo AB. Investigation of Plants used for the Ethnoveterinary Control of Gastrointestinal Parasites in Bénoué Region, Cameroon, *Tropicicultura* 29 (4), 205-211.
12. Ahmadu, A.A., Zezi, A.U., Yaro, A.H., 2007. Antidiarrheal activity of the leaf

- extracts of *Daniellia oliveri* Hutch and Dalz (Fabaceae) and *Ficus sycomorus* Miq (Moraceae), *Afr J Tradit Complement Altern Med*. 2011;4(4):542-528.
13. Musa AD, Yusuf GO, Ojogbane EB, Nwodo OFC. Screening of Eight Plants Used In Folkloric Medicine for the Treatment of Typhoid Fever, *J Chem Pharm Res*. 2010;2(4):7-15.
 14. Muanda F, Koné D, Dicko A, Soulimani R, Younos C. Phytochemical composition and antioxidant capacity of three malian medicinal plant parts. Evidence-based complementary and alternative medicine: CAM, 20 674320; 2011.
 15. Iwueke AV, Nwodo OFC. Antihyperglycaemic effect of aqueous extract of *Daniellia oliveri* and *Sarcocephalus latifolius* roots on key carbohydrate metabolic enzymes and glycogen in experimental diabetes, *Biokemistri*. 2008;20(2):63-70.
 16. Kareem SH. Cytotoxic Activity of Verbenaceae (*Daniellia oliveri*) & Solanaceae (*Capsicum frutescens*) on Breast Cancer, Prostate Cancer, and Colon Cancer cells". ETD Collection for Tennessee State University. Paper AAI1492332; 2011.
 17. Achem J, Oyebode OT, Akinwale M, Bolarin O, Malgwi JM, Olorunsogo OO. Solvent Fractions of *Daniellia oliveri* ((Rolfé) Stem Bark Modulate Rat Liver Mitochondrial Permeability Transition Pore. *Arch. Bas. App. Med*. 2020;8(2020):27–34.
 18. Trease G, Evans W. *A Textbook of Pharmacognosy*, 13th ed. Bailliere Tinnall Ltd, London; 1989.
 19. Arvouet-Grand A, Vennat B, Pourrat A, Legret P. Standardisation d'un extrait de propolis et identification des principaux constituants. *J. Pharma. Belg*. 1994;49:462-468.
 20. Singleton VL, Orthofer R, Lamuela-raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol*. 1999;299: 152-178.
 21. Kedare SB, Singh RP. Genesis and development of DPPH method of antioxidant assay. *J. Food Sci. Technol*. 2011;48:412–422.
 22. Tundis R., Menichini, F., Bonesi, M., 2013. "Antioxidant and hypoglycaemic activities and their relationship to phytochemicals in *Capsicum annum* cultivars during fruit development," *LWT—Food Science and Technology*, 53 (1) 370–377.
 23. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E," *Analytical Biochemistry*. 1999;269(2):337–341.
 24. Johnson D, Lardy H. Isolation of liver or kidney mitochondria. *Methods Enzymol*. 1967;10:94-6.
 25. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;193(1):265-75.
 26. Lapidus RG, Sokolove PM. Inhibition by spermine of the inner membrane permeability transition of isolated rat heart mitochondria. *FEBS Lett*. 1992;313(3):314-8.
 27. Olorunsogo OO, Malomo SO. Sensitivity of oligomycin-inhibited respiration of isolated rat liver mitochondria to perfluidone, a fluorinated arylalkyl-sulfonamide. *Toxicology*. 1985;35(3):231-40.
 28. Bassir O. *Handbook of practical biochemistry*. Ibadan University press, Ibadan, Nigeria. 1963;13.
 29. Ruberto G, Baratta M, Deans S, Dorman H. Antioxidant and antimicrobial activity of *Foeniculum vulgare* and *Crithmum maritimum* essential oils. *Planta. Med*. 2020;66:687-693.
 30. Appaix F, Minatchy NM, Riva-Lavieille C, Olivares AB, Saks VA. Rapid spectrophotometric method of quantification of cytochrome c release from isolated mitochondria or permeabilized cells revisited. *Biochimica et Biophysica Acta (BBA)- Bioenergetics*. 2000;1457:175-181.
 31. Zoremsiami J, Jagetia GC. The Phytochemical and thin layer chromatography profile of ethnomedicinal plant *Helicia Nilagirica* (Bedd). *Int J Pharmacog Chinese Med*. 2018;2(2):000131.
 32. Lalrinzuali K, Vabeiryureilai M, Jagetia GC, Lalawmpuii PC. Free radical scavenging and antioxidant potential of different extracts of *Oroxylum indicum* in vitro. *Adv. Biomed. Pharm*. 2015;2(3):120–130.
 33. Shantabi L, Jagetia GC, Ali MA, Singh TT, Devi SV. Antioxidant potential of *Croton caudatus* leaf extract in vitro. *Transl. Med. Biotechnol*. 2014;2(6):1–15.

34. Tejero J, Gayoso S, Caro I. Comparative analysis of the antioxidant and free-radical scavenging activities of different water-soluble extracts of green, black and oolong tea samples. *Food Nutr. Sci.* 2014;5(22):2157–2166.
35. Alavian KN, Beutner G, Lazrove E, Sacchetti S, Park HA, Licznerski P, Li H, Nabili P, Hockensmith K, Graham M. An uncoupling channel within the c-subunit ring of the F1FO ATP synthase is the mitochondrial permeability transition pore. *Proc. Natl. Acad. Sci. USA.* 2014;111:10580–10585.
36. Rasola, A., Bernardi, P., 2011. The mitochondrial permeability transition in Ca⁽²⁺⁾-dependent apoptosis and necrosis. *Cell. Calcium.* 50, 222 -233.
37. Elena-Real, C.A., Díaz-Quintana, A., González-Arzola, K., Velázquez-Campoy, A., Orzáez, M., López-Rivas, .A, Gil-Caballero, D., Rosa, M.Á., Díaz-Moreno, I.,. Cytochrome c speeds up caspase cascade activation by blocking 14-3-3ε-dependent Apaf-1 inhibition. *Cell Death.* 2018;9(3):365.
38. Xue, Z.H., Li, J.M., Cheng, A.Q., Yu, W.C., Zhang, Z.J., Kou, X.H., Zhou, .F.J. Structure identification of triterpene from the mushroom *Pleurotuseryngii* with inhibitory effects against breast cancer. *Plant Foods HumNutr.* 2015;70:291–296.
39. Surh Y. Molecular mechanisms of chemopreventive effects of selected dietary and medicinal phenolic substances. *Mutation Research.* 1999;428:305–327.
40. Sakai, M., Kakutani, S., Horikawa, C., Tokuda, H., Kawashima, H., Shibata, H. Arachidonic acid and cancer risk: a systematic review of observational studies. *BMC cancer.* 2012;12(1):606.
41. Gleeson, R.P., Ayub, M., Wright, J.T., Wood, C.B., Habib, N.A., Soutter, W.P. Fatty acid control of growth of human cervical and endometrial cancer cells. *British journal of cancer.* 1990;61(4):500.
42. Carrillo, C., Cavia, M., Alonso-Torre, S.R. Antitumor effect of oleic acid; mechanisms of action. A review. *Nutricion hospitalaria.* 2012;27(6):1860-5.
43. Hayashi, Y., Fukushima, S., Hirata, T., Kishimoto, S., Katsuki, T., Nakano, M. Anticancer Activity of Free γ-Linolenic Acid on AH-109A Rat Hepatoma Cells and the Effect of Serum Albumin on Anticancer Activity of γ-Linolenic Acid in Vitro. *Journal of pharmacobio-dynamics.* 1990;13(11):705-711.
44. Harada ., Yamashita, U., Kurihara, H., Fukushi, E., Kawabata, J., Kamei, Y. Antitumor activity of palmitic acid found as a selective cytotoxic substance in a marine red alga. *Anticancer research.* 2002;22(5):2587-2590.
45. Hsieh, T.H., Tsai, C.F, Hsu, C.Y. Phthalates induce proliferation and invasiveness of estrogen receptor-negative breast cancer through the AhR/HDAC6/c-Myc signaling pathway. *FASEB J,* 2012;**26**:778–87.
46. Wójtowicz, A.K., Szychowski, K.A., Wnuk, A., Kajta, M. Dibutyl Phthalate (DBP)-induced apoptosis and neurotoxicity are mediated via the Aryl Hydrocarbon Receptor (AhR) but not by Estrogen Receptor Alpha (ERα), Estrogen Receptor Beta (ERβ), or Peroxisome Proliferator-Activated Receptor Gamma (PPARγ) in Mouse C. *Neurotox Res.* 2017;31:77–89.

© 2020 Achem et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:

The peer review history for this paper can be accessed here:
<http://www.sdiarticle4.com/review-history/62315>