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MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION PORE OPENING INDUCTIVE EFFECTS OF VARIOUS FRACTIONS OF DISSOTIS ROTUNDIFOLIA IN ISOLATED RAT LIVER MITOCHONDRIA

[1]Oluokun, V. O., [1]Musa, F.B., [1]Olaleye, O. A., [1]Adebisi, L. A. and [1]Olorunsogo, O. O.

[1]Department of Biochemistry, University of Ibadan, Ibadan, Nigeria.

Abstract : The induction of mitochondrial membrane permeability transition (mPT) pore resulting in cytochrome c release is a vital therapeutic target in apoptosis for eliminating unhealthy or unwanted cells. Dysregulated apoptosis is implicated in various cancers and tumors, making mPT induction a promising approach for developing anti-cancer treatments. This study investigated the effects of different fractions of *Dissotis rotundifolia*, a medicinal plant traditionally used for fibroid treatments, on mPT in rat liver mitochondria. Crude ethanol extract (CEEDr) and fractions: Chloroform (CFDr), Ethylacetate (EAFDr), and Ethanol (EFDr) were assessed for mPT induction, mitochondrial ATPase activity, Cytochrome C Release, and ferrous-induced mitochondrial lipid peroxidation. In the absence of calcium, CFDr showed the highest inductive effect on mPT (22.9-fold increase at 360 µg/mL), followed by CEEDr and EFDr. All fractions enhanced mitochondrial ATPase activities and cytochrome c release while inhibiting ferrous-induced mitochondrial lipid peroxidation in a concentration-dependent manner. The chloroform fraction (CFDr) exhibited the highest potency across all assays, suggesting the presence of pharmacologically relevant phytochemicals that could induce apoptosis. These findings provide a mechanistic basis for the traditional use of *D. rotundifolia* in tumor treatment and highlight its potential as a source of novel anti-cancer compounds targeting the mitochondrial apoptotic pathway.

IndexTerms **– Apoptosis, Bioactive compound, *Dissotis rotundifolia*, Lipid peroxidation, Tumor, Mitochondria membrane**

I. INTRODUCTION

INTRODUCTION

The mitochondrial membrane permeability transition (mPT) pore has emerged as a compelling target for developing therapeutics against diseases characterized by dysregulated apoptosis [1]. The discovery of mPT pore has opened up exciting new avenues for drug discovery and development, particularly leveraging bioactive compounds from medicinal plants that can modulate this pathway.

Extensive research has demonstrated that certain phytochemicals found in plants like turmeric, curcumin, ginger, resveratrol, green tea, broccoli, grapes, tomatoes etc can induce mPT pore opening and apoptosis [1]; [2]; [3]. This provides scientific rationale for investigating the efficacy of medicinal plants against various pathological conditions including tumors, cardiac diseases, cancer, and tissue wasting disorders [4]; [5].

The mitochondrial permeability transition pore is a multi-conductance channel located in the inner membrane of mitochondria [6]. It is a rapid increase in the permeability of the inner mitochondrial membrane (IMM) to ions and solutes with molecular masses up to 1.5 kDa, triggered by harmful stimuli like elevated Ca^{2+} matrix concentration, oxidative stress, and cytotoxic agents [7].

When mPT opens, the outer membrane breaks, leading to the release of intermembrane space proteins like cytochrome c and other factors that are crucial for apoptotic cell demise. Similarly, the inner membrane uncouples oxidative phosphorylation as a result of

being freely permeable to protons and causes the proton-translocating ATPase to reverse direction, actively hydrolyzing ATP instead of synthesizing it. As a result, intracellular ATP levels quickly decrease, disrupting ionic and metabolic homeostasis and activating degradative enzymes such as phospholipases, nucleases, and proteases, which in turn initiate apoptotic cascades [8].

Programmed cell death (Apoptosis), is a crucial defensive mechanism that regulates cell proliferation and maintains tissue homeostasis. Its dysregulation plays a pivotal role in disease progression, including tumorigenesis and chemoresistance [9]; [10]. The mitochondria are central to the intrinsic apoptotic pathway, releasing pro-apoptotic factors that trigger caspase activation and eventual cell demise [11].

Numerous studies have established the ability of medicinal plant extracts and their bioactive components to induce apoptosis via the mitochondrial pathway [12]; [13]. By targeting mitochondrial factors like cytochrome c, these natural compounds can activate the mPT pore and initiate apoptosis, offering therapeutic potential against diseases characterized by dysregulated cell death [5];[14].

Dissotis rotundifolia (*Dr*), also known as Spanish Shawl or pink lady, is a medicinal plant native to tropical West Africa with a long history of traditional use for treating various ailments, including fibroid, dysentery, rheumatism, and venereal diseases [15]; [16]. This plant is rich in bioactive phytochemicals like phenols, flavonoids, tannins, and alkaloids, which have demonstrated antioxidant, anti-inflammatory and anticancer activities [17], [18]. However, the mechanisms underlying its therapeutic effects, particularly against conditions like uterine fibroids, remain poorly understood.

Given the crucial role of apoptosis dysregulation in uterine fibroid pathogenesis [19]; [20], elucidating the potential of *D. rotundifolia* extracts to modulate mitochondrial-mediated apoptosis could provide valuable insights into its efficacy against this condition. This study aims to examine various fractions of *D. rotundifolia* leaves to identify potent inducers of mPT pore opening and mitochondrial-mediated cell death in isolated rat liver mitochondria, thus validating its traditional use in fibroid treatment and contributing to the discovery of novel anti-tumor agents targeting apoptotic pathways.

II. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Mannitol, sucrose, N-2-hydroxy-ethyl-pipe-arizine-N-2- ethanesulfonic acid (HEPES), rotenone, spermine, Folin Ciocalteu reagent, bovine serum albumin (BSA), and all other reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and were of the highest purity grade

2.2 Plant material

Fresh *Dissotis rotundifolia (Dr)* leaves were procured at a local farm land in Ibadan, Oyo State, Nigeria. These leaves were verified and authenticated at the Department of Botany, University of Ibadan, Nigeria, with the certification number UIH: 22644. The Dr leaves after being air-dried for a duration of four weeks at room temperature, subsequently were powdered using a blender. The powdered Dr leaves were then carefully preserved in a clean glass jar to facilitate the extraction process. In order to prepare the crude ethanol extract of *D. rotundifolia*, 10 litres of ethanol were added to 1.5 kg of powdered D. rotundifolia leaves in a conical flask. After 72 hours the mixture was filtered through sterile Whatman No. 1 filter paper. The resulting green filtrate (extract) was then concentrated at 40ºC using a rotary evaporator until it became a dark brown mass. The crude extract was finally dried completely using a water bath.

2.3 Vaccum Liquid Chromatography (VLC) Partitioning of Crude Extract of *D. rotundifolia*

A Buchner sintered glass that had been pre-washed was cleansed using concentrated Sulphuric acid (H_2SO_4). This was done to ensure that the glass was not contaminated. The chromatography column was filled to about 75% with silica gel 60 (0.400 – 0.063, MERCK). The setup involved connection of conical Buchner flask to a vacuum pump. To ensure proper packing of the column, the vacuum pump was activated, and the solvent n-hexane was introduced. The sample slurry, 8 grams of silica gel 60 (0.400 - 0.063, MERCK) in combination with 10 grams of ethanol extract was made and the two ingredients were mixed well until a uniform mixture was achieved. The mixture was then left to dry in the air until it reached a powdery consistency. The gel-extract sample was applied to the top of the column with the pump on and n-hexane, chloroform, ethylacetate and ethanol were added in order of increasing polarity to elute the various fractions. The solvent fractions were concentrated via rotary evaporator at 40ºC in order to obtain a solvent-free fractions (CFDr, EAFDr, and EFDr, respectively). All fractions free of solvent was refrigerated till when it was needed.

2.4 Experimental Animals

Female wistar strain albino rats with weights between 100 and 120 grams were obtained from the Preclinical Animal House (PAH) situated in the College of Medicine at the University of Ibadan, Nigeria. These rats were housed in well-ventilated plastic cages, maintained at a temperature of 25 ± 2 degrees Celsius, and exposed to a 12-hour light/dark cycle. They were provided with unlimited access to food and water. Before commencing the experimental work, the animals underwent a two-week acclimatization period. Ethical approval associated with careful handling of the animals was procure from the Animal Care Use and Research Ethics Committee (ACUREC) at the University of Ibadan, Nigeria.

2.5 Vaccum Liquid Chromatography (VLC) Partitioning of Crude Extract of *D. rotundifolia*

Isolated mitochondria from rat liver were carried out following [21] procedure, with modifications by [22] and this involved the technique of variance centrifugation. The experimental animal was cervically dislocated, the liver was promptly removed and carefully trimmed to eliminate any unnecessary tissue. The liver was placed on blotting paper and its weight was measured. Subsequently, homogenizing buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES-KOH, pH 7.4 and 1 mM EGTA), was used for multiple washes until achieving a clear wash and the liver was censored into small pieces using scissors. Subsequently, the liver was homogenized into a 10 percent suspension in ice-cold buffer using a Potter-Elvehjem glass homogenizer. The resulting homogenate was then centrifuged twice at 2300 rpm for 5 minutes in an MSE refrigerated centrifuge to eliminate nuclear fragments. The supernatant obtained was centrifuged at 13,000 rpm for 10 minutes to pellet the mitochondria. The supernatant from this was discarded while the pellet was washed twice at 12,000 rpm for 10 minutes with washing buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES-KOH, pH 7.4, 0.5 percent BSA). The isolated mitochondria were promptly resuspended in a suitable volume of MSH buffer (210 mM Mannitol, 70 mM Sucrose, 5 mM HEPES-KOH, pH 7.4), then immediately aliquoted into Eppendorf tubes and stored at 4°C. Similar procedure was explored for the isolated mitochondria used for mitochondrial ATPase with the exception that 0.25M sucrose buffer was used throughout the isolation process.

2.6 Determination of Mitochondrial Protein

The protein content in the mitochondria was quantified using the method developed by [23]. Throughout the procedure, Bovine Serum Albumin (BSA) was utilized as the reference standard.

2.7 Vacuum Liquid Chromatography (VLC) Partitioning of Crude Extract of *D. rotundifolia*

The practice employed here relies on the observation that the refractive index of the mitochondrion alters as it expands due to the permeability of its membrane. As a result, it scatters less light, causing a decline in absorbance at a wavelength of 540nm when measured using a spectrophotometer. This wavelength selection was widely adopted in numerous studies involving isolated mitochondria, as outlined by [24]. Primarily, to assess the integrity of the mitochondria, mitochondria (0.4 mg protein/ml) were pre-incubated for 3 and ½ minutes at 27°C in a medium containing 210 mM mannitol, 70 mM sucrose, and 5 mM HEPES-KOH (pH 7.4) with 0.8 µM rotenone. Sodium succinate (5mM) was added to energize the reaction medium immediately after 3.5 minutes of mitochondria incubation. Mitochondrial permeability was measured by observing changes in absorbance at 540 nm over a period of 12 minutes at 30-second intervals. Subsequently, calcium-induced mitochondrial permeability was assessed by pre-incubation of the mitochondria in rotenone and MSH buffer for a duration of 3 mins. CaCl2 (12mM) was added and after 30 secs, the 5mM sodium succinate was included. The reversal of the mitochondrial permeability was assessed by the addition of 4mM spermine into the medium containing MSH buffer, rotenone and mitochondria. Three minutes later, CaCl2 was added, followed by the addition of sodium succinate 30 seconds afterward, and the absorbance was measured. The effects of the crude extract and fractions of D. rotundifolia on mitochondrial pore opening were assessed by adding different concentrations of the extract and fractions to the reaction medium. Changes in the permeability of the mitochondria were monitored by measuring absorbance at 540 nm using a Camspec M106 spectrophotometer over a period of 12 minutes.

2.8 Assessing the Activity of Mitochondrial ATPase

The modification of the original method developed by [25] were employed for the assessment of the enzyme (mATPase) activity. [22] later worked on this and modified the procedure to suit similar purpose. In each sample's reaction mixture (in triplicates), a solution containing 1 mM ATP, 25 mM sucrose, 0.5 mM KCl, 65 mM, Tris-HCl buffer (pH of 7.4) and the extract fractions were present. This solution was then diluted with distilled water to achieve a final volume of 2mL. To the uncoupler test tubes, 25 µM 2, 4-dinitrophenol, was introduced into the reaction mixtures following the addition of ATP (1 mM). To the zero-time tube, addition of 1ml of 10% sodium dodecyl sulphate (SDS) firstly was made afore the mitochondria. The activity was terminated immediately after the introduction of ATP by the inclusion of SDS in the medium (zero-time tube). Thereafter, the reaction was initiated by introducing the mitochondrial suspension (with the exception of Zero-time tube and the ATP-only tube) to each reaction mixture at regular intervals of 30 seconds or 1 minute with a continuous shaken in water bath at 27 ºC for a period of 30-mins. Termination of the reaction was achieved by adding SDS (1mL) with the exception of the zero time tube every 30 seconds or 1 minute. The concentration of released phosphate was then measured by adding 1 mL of 1.25% ammonium molybdate, followed by 1 mL of freshly prepared 9% ascorbate, to 1 mL of the reaction medium, which had been diluted with 4 mL of distilled water. This was allowed to remain undisturbed for a duration of 30 minutes and absorbance measured at 660nm wavelength via M106 Camspec spectrophotometer. A phosphate standard curve was obtained by mixing a standard solution of 1mM potassium dihydrogen phosphate with ammonium molybdate and ascorbate. The intensity of the blue colouration was determined via same wavelength. The standard phosphate curve was plotted and the released inorganic phosphate concentration per milligram protein per minutes, was estimated.

2.9 Quantification of Cytochrome c release

After permeabilizing mitochondrial membranes, isolated mitochondria released cytochrome c was quantified. This was done using a modified version of the [26] method, which quantified cytochrome c using spectrophotometry. Intact isolated mitochondria were pre-incubated in the presence of 0.8 µM rotenone in a buffer medium containing 210 mM mannitol, 70 mM sucrose and 5 mM HEPES-KOH (pH 7.4), different concentrations of extract-fractions and 5mM sodium succinate for 30 mins at 27ºC. The control test tube contains no extract-fractions, while the inclusion of 12mM $CaCl_2$ was used as a standard triggering agent. After incubation, absorbance at 414nm for the Soret (ϒ) peak, indicating the release of cytochrome c of the reaction mixtures were measured after centrifugation at 13,000 rpm for 30 minutes.

2.10 Assessing the Activity of Mitochondrial ATPase

The modification of the original method developed by [25] were employed for the assessment of the enzyme (mATPase) activity. [22] later worked on this and modified the procedure to suit similar purpose. In each sample's reaction mixture (in triplicates), a solution containing 1 mM ATP, 25 mM sucrose, 0.5 mM KCl, 65 mM, Tris-HCl buffer (pH of 7.4) and the extract fractions were present. This solution was then diluted with distilled water to achieve a final volume of 2mL. To the uncoupler test tubes, 25 µM 2, 4-dinitrophenol, was introduced into the reaction mixtures following the addition of ATP (1 mM). To the zero-time tube, addition of 1ml of 10% sodium dodecyl sulphate (SDS) firstly was made afore the mitochondria. The activity was terminated immediately after the introduction of ATP by the inclusion of SDS in the medium (zero-time tube). Thereafter, the reaction was initiated by introducing the mitochondrial suspension (with the exception of Zero-time tube and the ATP-only tube) to each reaction mixture at regular intervals of 30 seconds or 1 minute with a continuous shaken in water bath at 27 ºC for a period of 30-mins. Termination of the reaction was achieved by adding SDS (1mL) with the exception of the zero time tube every 30 seconds or 1 minute. The concentration of released phosphate was then measured by adding 1 mL of 1.25% ammonium molybdate, followed by 1 mL of freshly prepared 9% ascorbate, to 1 mL of the reaction medium, which had been diluted with 4 mL of distilled water. This was allowed to remain undisturbed for a duration of 30 minutes and absorbance measured at 660nm wavelength via M106 Camspec spectrophotometer. A phosphate standard curve was obtained by mixing a standard solution of 1mM potassium dihydrogen phosphate with ammonium molybdate and ascorbate. The intensity of the blue colouration was determined via same wavelength. The standard phosphate curve was plotted and the released inorganic phosphate concentration per milligram protein per minutes, was estimated.

2.11 Lipid Peroxidation Evaluation

[27] study approach was employed to examine and evaluate lipid peroxidation. Lipid peroxide quantification was performed using a modified thiobarbituric acid reactive substances (TBARS) assay, with mitochondria serving as the lipid-rich medium. Rat liver mitochondria (2mg/mL) were mixed with the extract-fractions at varying concentrations (0.5 to 8.0 mg/mL). The induction of lipid peroxidation was carried out by adjusting the resulting mixture to 1.0 mL using distilled water, followed by the inclusion of Fe_2SO_4 (0.05mL). The incubation was then carried out at 370C for 0.5 hour. Subsequently, in SDS (1.1 %), a mixture of 1.5mL of TBA (0.8 %) and 1.5mL of acetic acid (20 %) were added, and the resulting combination was thoroughly mixed using a vortex mixer. Over a duration of 1 hour at 95^0C, the mixture was boiled, chilled and mixed well in 5mL of butanol. After 10 minutes of centrifugation at 3000 rpm, the organic top layer's absorbance at 532 nm was determined and the expression shown below was used to calculate the percentage inhibition of the lipid peroxidation:

% lipid peroxidation inhibition = $\frac{(A_o - A_1)}{A_1} * 100$.

Where: A_o = the absorbance of the control;
A_1 = the absorbance of the sample.

2.12 Statistical Analysis

Data obtained in this study were analysed using descriptive statistics. Three similar determinations were used as a representative profile for the mitochondrial permeability transition assays. Data are presented as the mean ± standard deviation (SD) from at least three independent measurements. The data were analysed using Graph Pad prism. The test of significance was done via One-way analysis of variance. The adaptation of p-values below 0.05 was used to establish statistical significance.

III. RESULTS AND DISCUSSION

3.1 Results

Investigation of Spermine and Calcium Effects on Mitochondrial Membrane Permeability Transition Pore in Rat Liver

The data for this experiment was presented in Figure 1. The results demonstrated a minimal and statistically insignificant alteration in the absorbance of mitochondria that were undergoing respiration when exposed to rotenone 12 minutes. However, the mitochondria exhibited substantia increase in swelling upon introduction of exogenous calcium to the assay. The whole process was reversed significantly when a standard inhibitor (spermine) of the mPT pore opening was added. The aforementioned result indicates that the introduction of external calcium triggered the opening of the mPT pore, while a standard inhibitor (spermine) causes a reversal of the process significantly. Hence, this demonstrates that the mitochondrial integrity is preserved, affirming their suitability for subsequent experiments.

Assessment of the Impact of Crude Ethanol Extract of *Dissotis rotundifolia* (CEEDr) Leaves on Mitochondrial Permeability Transition Pore in Rat Liver, Both with and without Calcium

Figure 2 clearly shows that CEEDr causes the mPT pore to open by 2.1, 5.3, 15.1, and 20.5-folds at different concentrations (120 µg/mL, 200 µg/mL, 280 µg/mL, and 360 µg/mL, respectively). This shows that the induction was concentration-dependent, with maximum induction noticed at 360 μg/mL while lowest induction was seen at 120 μg/mL relative to control (NTA). The result depicted in Figure 3 demonstrates that CEEDr, exhibited inhibition of pore opening induced by calcium. Inhibitory effects of 13.2, 30.9, 33.1 and 45.0% was observed with CEEDr, indicating that the highest concentration inhibited maximally calcium-induced mPT pore opening.

Investigation of the Effects of Chloroform fraction of *Dissotis rotundifolia* (CFDr) Leaves on the Mitochondrial Permeability Transition Pore in Rat Liver, Both with and without Calcium

The influence of CFDr on mPT pore at varying concentrations of 120 µg/mL, 200 µg/mL, 280 µg/mL and 360 µg/mL, respectively in the absence of calcium was depicted in Figure 4. The results showed a significant increase in pore opening by 4.7,

22.8, 22.9, and 20.3-folds, respectively. The various concentrations significantly induced pore opening, with the highest induction at 240 µg/mL and the lowest at 120 µg/mL. Conversely, the calcium-induced pore opening was significantly inhibited in a manner dependent on concentration by the influence of CFDr by 17.4, 19.1, 22.5 and 33.8%, respectively (Figure 5).

Effects of Ethylacetate fraction of *Dissotis rotundifolia* (EAFDr) Leaves on Mitochondrial Permeability Transition Pore in Rat Liver in the Absence and Presence of Calcium

The result in Figure 6, depicts the consequence of EAFDr on the pore of the mPT without calcium. The data obtained revealed that 120 µg/mL (0.6-fold) and 200 µg/mL (0.7-fold) of EAFDr, did not cause a significant increase in the opening of the pore. However, at higher concentrations of 280 µg/mL and 360 µg/mL, a modest increase of 4.4 and 5.0-fold, respectively, was observed. In contrast, Figure 7 demonstrates that the inhibition of calcium-induced pore opening by EAFDr increased in a concentration-dependent manner (0.7, 17.5, 18.8 and 30.3%).

Assessment of the Effects of Ethanol fraction of *Dissotis rotundifolia* (EFDr) Leaves on Mitochondrial Permeability Transition Pore in Rat Liver in the Absence and Presence of Calcium

Figure 8, depicted, influence of EFDr on the opening of the mPT pore in the absence of calcium. The data clearly shows that at the different concentrations of 120 µg/mL, 200 µg/mL, 280 µg/mL and 360 µg/mL, the mPT pore opening increased significantly by 8.4, 16.9, 17.6 and 18.9-folds, respectively, in comparison to the control. The pore opening induction were concentration-dependent. However, Figure 9 display similar effects as that of the previous fractions by significantly inhibiting the induction of pore opening by calcium in a concentration-dependent approach by 1.5, 41.1, 58.1 and 60.1%, respectively. The different solvent fractions of D. rotundifolia significantly induced mPT pore opening, except for EAFDr. Overall, the CFDr shows high potency with regards to mitochondrial membrane permeability transition pore opening, making it most potent in all the fractions tested. Additionally, the data shown in Figures 3, 5, 7, and 9 reveal that all the solvent fractions demonstrated the inhibition of calcium-induced pore opening in a concentration-dependent manner.

Assessment of the Activity of Mitochondrial ATPase by different Solvent fractions of *Dissotis rotundifolia* Leaves

Figure10 depict the enhancement of ATPase activity by solvent fractions of D. rotundifolia. The result obtained shows that different fractions of *D. rotundifolia* enhanced ATPase activity in a concentration-dependent approach. Maximum enhancement was noticed at 450 µg/mL of all the fractions, with CFDr indicating highest enhancement of the ATPase activity followed by EFDr and EAFDr having the lowest effect on ATPase activity. Compared with a standard uncoupler of oxidative phosphorylation 2,4-dinitrophenol, the CFDr exhibited significant ($p<0.01$) effectiveness in terms of increased levels of inorganic phosphate released during ATP hydrolysis.

It can be deduced, that the different concentrations of Chloroform and ethanol fractions exhibited a concentration-dependent enhancement of ATPase activity in comparison to the control. The chloroform fraction of D. rotundifolia was found to possess maximum ATPase activity of the mitochondria. However, the ethyl acetate fraction was observed to be less potent in enhancing the enzyme activity. This finding justifies the Chloroform fraction significant inductive effect on the opening of the mPT pore.

Influence of various fractions of *Dissotis rotundifolia* Leaves on the release of Cytochrome c

The data shown in Figure 11 illustrates the release of cytochrome c from rat liver mitochondria induced by different solvent fractions of D. rotundifolia leaves. It is evident from the results that CFDr, EFDr and EAFDr caused an increase of cytochrome c discharge relative to control. At maximum concentration of 360µg/ml, CFDr increased cytochrome c released by 0.27-fold, EFDr by 0.25-fold and EAFDr by 0.13-fold, respectively. The CFDr, EFDr and EAFDr displayed a concentration-dependent effect on the discharge of cytochrome c. The solvent factions of *D. rotundifolia* enhanced cytochrome c release, with CFDr exhibiting the highest potency in promoting cytochrome c release. These findings align with the effects observed in the mPT and mATPase assays, suggesting a potential favouring of cell death. Over all, the CFDr demonstrated the highest potency, supporting its suitability for further *in vivo* studies.

Impact of Solvent fractions of *Dissotis rotundifolia* on Fe^{2+}-Induced Mitochondrial Lipid Peroxidation (mLPO)

Figure12, presents the result of Fe^{2+}-induced lipid peroxidation establishing the impact of various solvent fractions of D. rotundifolia on the process. As observed in the figure, the different fractions of *Dr* exhibited inhibition of mitochondrial lipid peroxide formation. The highest concentration (8.0mg/mL) demonstrated the maximum inhibitory effects with CFDr inhibiting by 86.75%, EFDr by 83.44% and EAFDr by 49.77%. The Chloroform fraction exhibited the highest inhibitory capacity and ethyl acetate fraction shows the least inhibitory effects. The Fe^{2+}-induced lipid peroxidation was significantly ($p < 0.05$) inhibited in a concentration-dependent pattern. The results suggest that CFDr, EFDr and EAFDr exhibited inhibitory effects on Fe^{2+}-induced mitochondrial lipid peroxidation, with CFDr demonstrating the highest potency.

3.2 Discussion

Research, have demonstrated a correlation between phytochemical compounds found in medicinal plants and the apoptosis induction via mitochondrial permeability transition pore opening [5], [13], [16], [28], [29]. The opening of the mitochondrial membrane permeability transition pore, which causes a sudden alteration in the inner mitochondrial membrane, leads to the uncoupling of oxidative phosphorylation, depletion of ATP, disturbance of ionic and metabolic balance, and the release of cytochrome c and other factors crucial in apoptotic cell demise ([8]. Occurs as an important phenomenon in conditions of dysfunctional cell death. Several Dietary compounds obtained from medicinal plants have been implicated to cause the induction of apoptosis via modulation of mPT. Plant-derived compounds, such as those from the Graviola fruit tree, have been demonstrated to inhibit BCL-2 proteins, increase BAX levels, and promote apoptosis. Curcumin also enhances mitochondrial capacity to undergo mitochondrial membrane permeabilization, resulting in increased cytochrome c release, which triggers caspase activation and

initiates the apoptotic process [30]. Many of these compounds derived from plants do not harm normal cells, unlike chemotherapy drugs that are toxic to both healthy and cancerous cells.

Based on the possible targets of mPT in inducing apoptosis, the inductive effects of solvent fractions of *Dissotis rotundifolia* leaves on mitochondrial membrane permeability transition pore in healthy rats were explored. *Dissotis rotundifolia*, is used folklorically in the treatments of conjunctivitis, cough, diarrhea, dysentery, fever, gonorrhea, headache, rheumatism, painful swellings, toothache and tuberculosis [30]. Interestingly, it is also used in the treatment and management of uterine fibroids [31]. The CEEDr, CFDr, EAFDr and EFDr were used in this study to ascertained the fraction with the potency that could support the antitumour claim of the plant traditionally via the induction of mPT pore in isolated mitochondria in normal rats.

Research has demonstrated that reversing the activity of F1F0 ATP synthase results in the accumulation of inorganic phosphate (Pi). This, in turn, acts as an inducer for mitochondrial permeability transition (mPT) [32]. According to the result of this study, all the fractions of the *Dr* enhanced the ATPase activity with variable potency. The potency varies depending on the concentration. This finding confirms the earlier finding that the extract has a concentration-dependent effect on mPT pore opening. Furthermore, it was observed that the order of potency for this enhancement was CFDr>EFDr>EAFDr, with CFDr showing the highest level of improvement. The CFDr showed remarkable efficacy in enhancing the release of inorganic phosphate during ATP hydrolysis. The observation that the CFDr, EFDr and EAFDr enhanced mitochondrial ATPase activity aligns with the research studies indicating the vital involvement of the mitochondrial ATP synthase in supporting the normal functionality of mPT pore [33];[34].

[35] revealed that the signaling pathways responsible for transforming the ATP synthase into an energy-dissipating mechanism significantly influence the cellular outcome, dictating whether cells will survive or undergo cell death. These pathways are utilised by cancer cells to strengthen their ability to resist apoptosis. The minimal effect of EAFDr on the enhancement of mitochondrial ATPase activity in rat liver was consistent with its negligible influence on the opening of the mPT pore. This could probably be due to bioactive agent fraction composition. This indicates that the agent responsible for triggering the mPT pore opening is also involved in the increase of ATPase function, thus supporting the claim of the F ATP synthase's association with the mPT pore.
Studies have indicated that the point at which apoptosis becomes irreversible is marked by the discharge of cytochrome c from the matrix through pore openings [36]; [37]. Consequently, the research investigated CFDr, EFDr and EAFDr impacts on the cytochrome c release. The most potent fraction which aid the promotion of discharge of cytochrome c from mitochondria was CFDr. This is an indication that CFDr may probably possess certain bioactive compounds causing the highest concentration of cytochrome c to be relocated into the cytosol from intermembrane space. This finding also supports the observation of the CFDr possessing the highest stimulatory effects in inducing the mPT pore opening and the release of inorganic phosphate, which are physiological targets for apoptosis in cases of dysregulated cell death.

Furthermore, the CFDr, EFDr and EAFDr were investigated on ferrous induced lipid peroxidation. According to the literature, reactive oxygen species (ROS) are partially reduced molecules containing oxygen and are classified as free radicals. ROS can produce highly toxic hydroxyl radicals when reduced iron (Fe^{2+}) is present, and an imbalance in ROS generation rates results in oxidative stress. This, in turn, leads to the production of free radicals that can damage DNA, proteins, and lipids [38]. Lipid peroxidation is a crucial event in all biological species. It is acknowledged as the primary molecular process involved in causing oxidative damage to cellular structures and in the development of toxicity that ultimately results in the demise of cells [39]. The buildup of ROS is widely recognised as a major factor promoting the activation of the opening of mitochondrial permeability transition pore [40]. Antioxidants are necessary to mitigate some of the oxidative process and regulate the degree of lipid peroxidation [41]. In order to ascertained that the CFDr, EFDr and EAFDr do not induce the opening of the pore due to reactive oxygen species, $FeSO^{4-}$induced lipid peroxidation assay was conducted using mitochondria as a lipid-rich medium.

The result showed that CFDr, EFDr and EAFDr inhibited lipid peroxidation, and this effect was found to depend on the concentrations used. The order of the effect was CFDr>EFDr>EAFDr which suggests that all the fractions of the extract possess lipid peroxidation inhibition potency with only chloroform fraction (CFDr) exhibited the highest inhibitory capacity. The occurrence that fractions of *Dissotis rotundifolia* leaves were able to prevent the production of mitochondrial lipid peroxides was an interesting observation. This suggests that CFDr, EFDr and EAFDr offer protection to the membrane bilayers of mitochondria, safeguarding them from the negative effects of free radicals and preventing severe cellular dysfunction [42] It can further be implied from the study that the process of the opening of the pore may not be linked to reactive oxygen species triggering effect, instead, this phenomenon is likely attributed to the specific interactions involving pore elements. Consequently, the study negates the potentiality of peroxidation of mitochondrial membrane as a basis for the membrane permeability caused by CFDr, EFDr and EAFDr [43]. The findings of [44] also provide evidence to support the outcome of this study. They found that regular consumption of the methanol extract from a medicinal plant, as part of herbal remedies, can serve as a lipid peroxidation inhibitor, decreasing the risk of diseases associated with disrupted mitochondrial function and excessive oxidative stress.

It can be inferred from this study that *Dissotis rotundifolia* contains certain phytochemicals that could upsurge mitochondrial ATPase activity by increasing the accumulation of inorganic phosphate and also relate with the components of the mitochondrial membrane pore causing the induction of its permeability, thereby resulting in the release of cytochrome c and cell death. Notably, CFDr exhibited the strongest biochemical effects in inducing the opening of the mitochondrial membrane permeability pore. It is therefore very vital to determine the essential bioactive components present in CFDr which could be likely responsible for the cell death effects observed.

This study examined the effects of various solvent fractions from the medicinal plant *Dissotis rotundifolia* on the opening of the mitochondrial membrane permeability transition (mPT) pore, a critical event in the intrinsic apoptotic pathway. Among the fractions

examined, the chloroform fraction (CFDr) exhibited the most potent effects in inducing mPT pore opening, mitochondrial ATPase activity, and cytochrome c release in isolated rat liver mitochondria. Notably, CFDr also demonstrated the highest inhibitory capacity against ferrous-induced mitochondrial lipid peroxidation, suggesting a protective effect against oxidative stress. These findings offer a mechanistic understanding of how D. rotundifolia may influence apoptosis, which could contribute to its traditional use in the treatment of conditions involving dysregulated cell death, such as fibroids. However, further research is warranted to identify the specific bioactive compounds responsible for these effects and to evaluate the *in vivo* efficacy and safety profiles of the most potent fraction, CFDr. Conclusively, this study highlights the therapeutic potential of *D. rotundifolia* and supports the continued exploration of medicinal plants as sources of novel apoptosis-modulating agents.

IV. CONCLUSION AND RECOMMENDATION

This study examined the effects of various solvent fractions from *Dissotis rotundifolia* on mitochondrial membrane permeability transition (mPT) pore opening, a critical event in the intrinsic apoptotic pathway. The chloroform fraction (CFDr) exhibited the most potent effects in inducing mPT pore opening, enhancing mitochondrial ATPase activity, and promoting cytochrome c release in isolated rat liver mitochondria. Notably, CFDr also demonstrated the highest inhibitory capacity against ferrous-induced mitochondrial lipid peroxidation. These findings offer a mechanistic understanding of *D. rotundifolia's* potential influence on apoptosis, supporting its traditional use in treating conditions involving dysregulated cell death, such as fibroids. Further research is warranted to identify specific bioactive compounds and evaluate the *in vivo* efficacy and safety of CFDr.

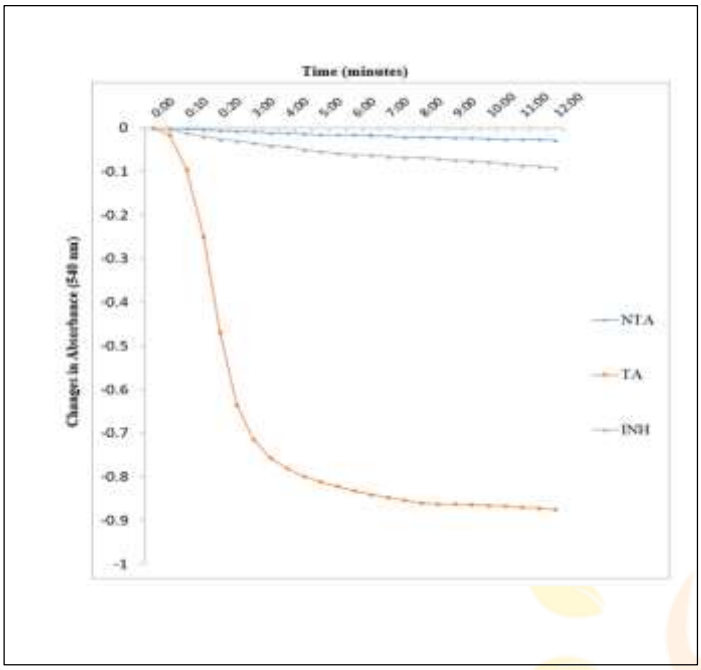

Figure 1: Representative profile of changes in absorbance of mitochondria by calcium and spermine in normal rat liver
NTA: Non triggering agent (without calcium)
TA: Triggering agent (with calcium)
INH: Inhibitor (spermine)

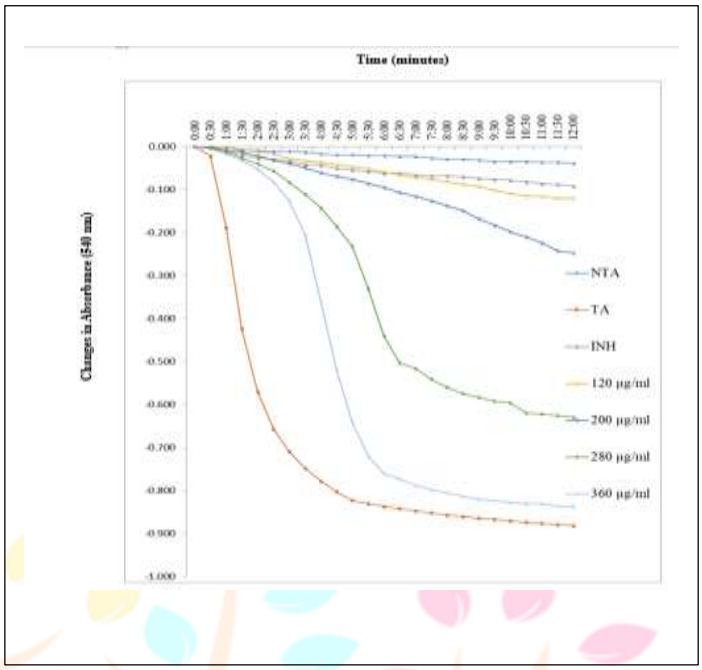

Figure 2: Representative profile of changes in absorbance of mitochondria by varying concentrations of Crude extract of *Dissotis rotundifolia (CEEDr)* in the absence of Calcium

NTA: Non triggering agent TA: Triggering agent

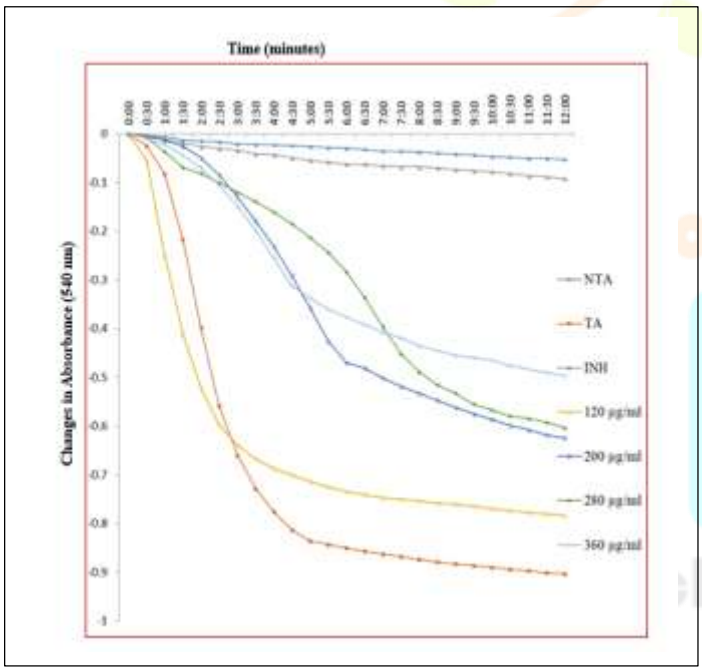

Figure 3: Change in Absorbance of Mitochondria with varying CEEDr Concentrations in the presence of Calcium

NTA: Non triggering agent TA: Triggering agent

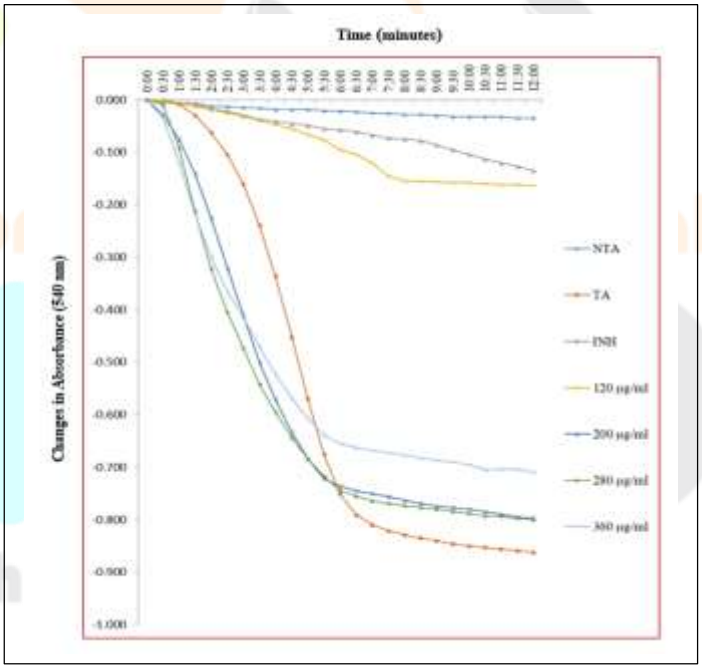

Figure 4: Change in Absorbance of Mitochondria with varying Concentrations of Chloroform Fraction of Dr (CFDr) in the absence Calcium

NTA: Non triggering agent TA: Triggering agent

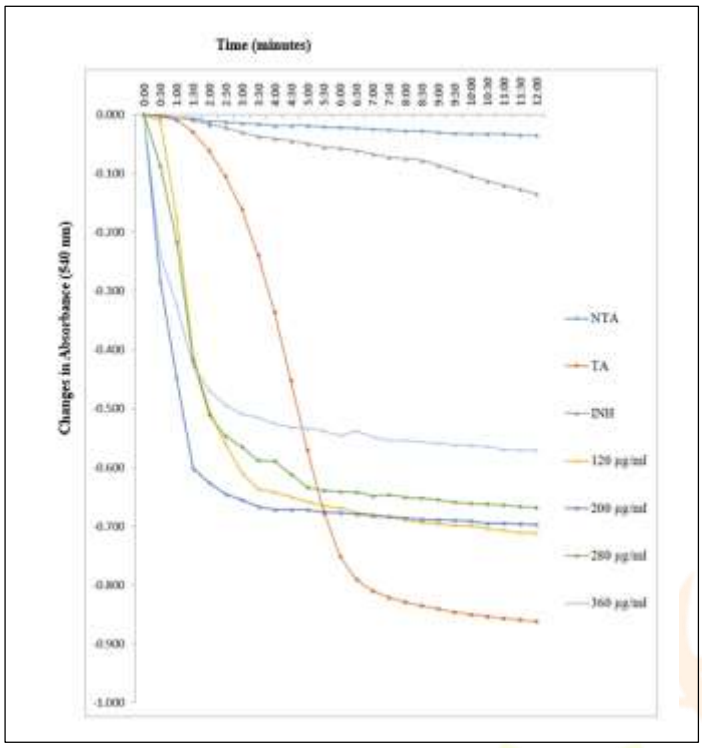

Figure 5: Change in Absorbance of Mitochondria with varying CFDr Concentrations in a calcium medium

NTA: Non triggering agent TA: Triggering agent

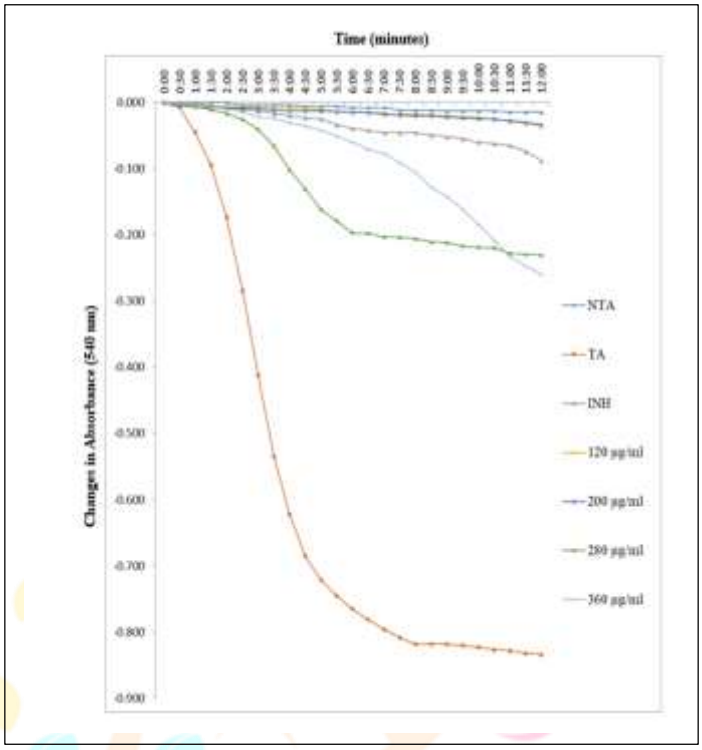

Figure 6 Change in Absorbance of Mitochondria with varying Ethylacetate Fraction Concentrations of Dr (EAFDr) in the absence of Calcium

NTA: Non triggering agent TA: Triggering agent

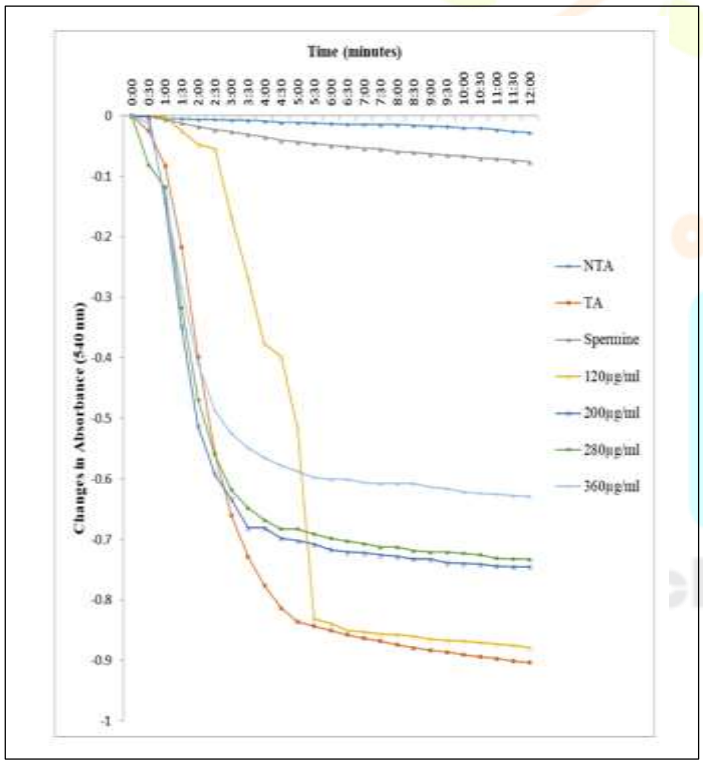

Figure 7: Change in Absorbance of Mitochondria with varying EAFDr Concentrations in the presence of Calcium

NTA: Non triggering agent TA: Triggering agent

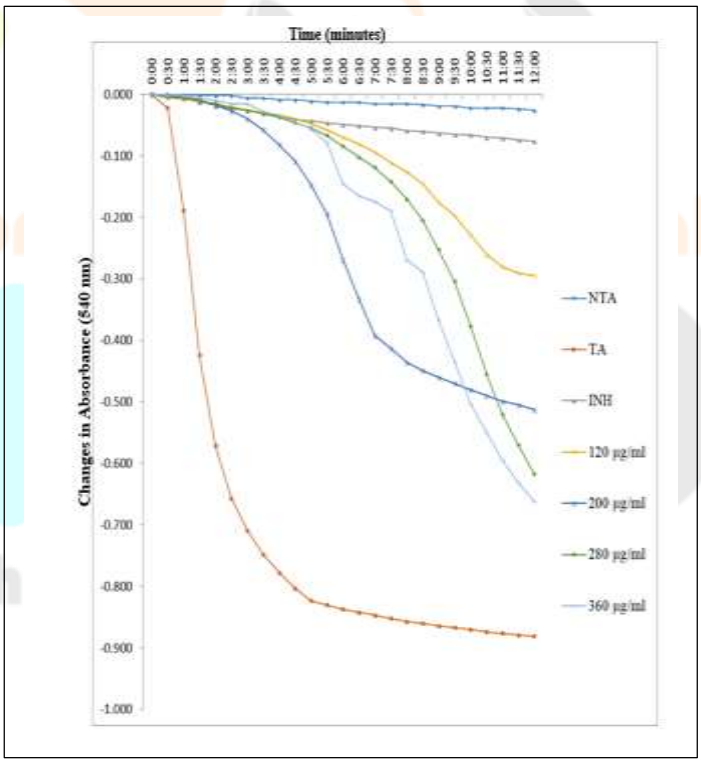

Figure 8: Change in Absorbance of Mitochondria with varying Ethanol Fraction Concentrations of Dr (EFDr) in the absence of Calcium

NTA: Non triggering agent TA: Triggering agent

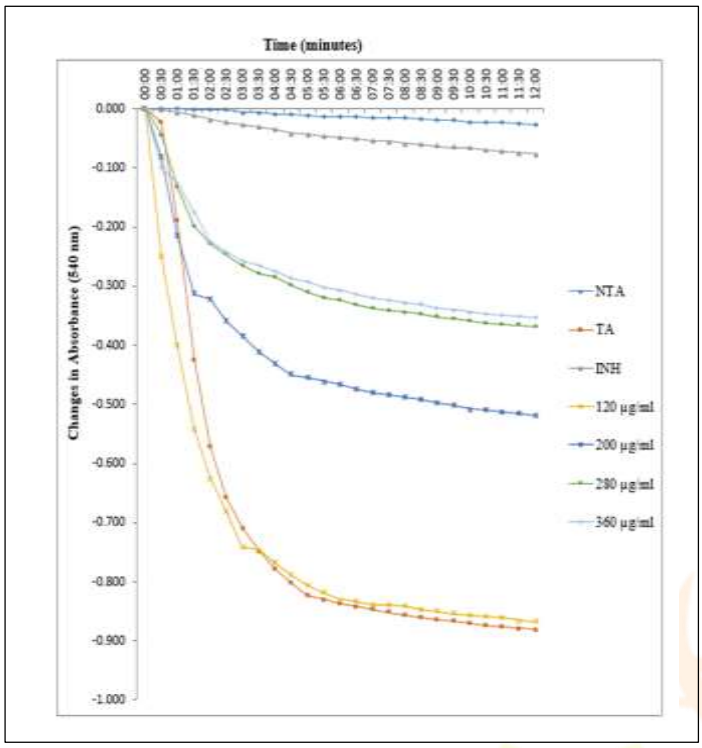

Figure 9: Absorbance change of Mitochondria with varying EFDr in a medium of Calcium

NTA: Non triggering agent TA: Triggering agent

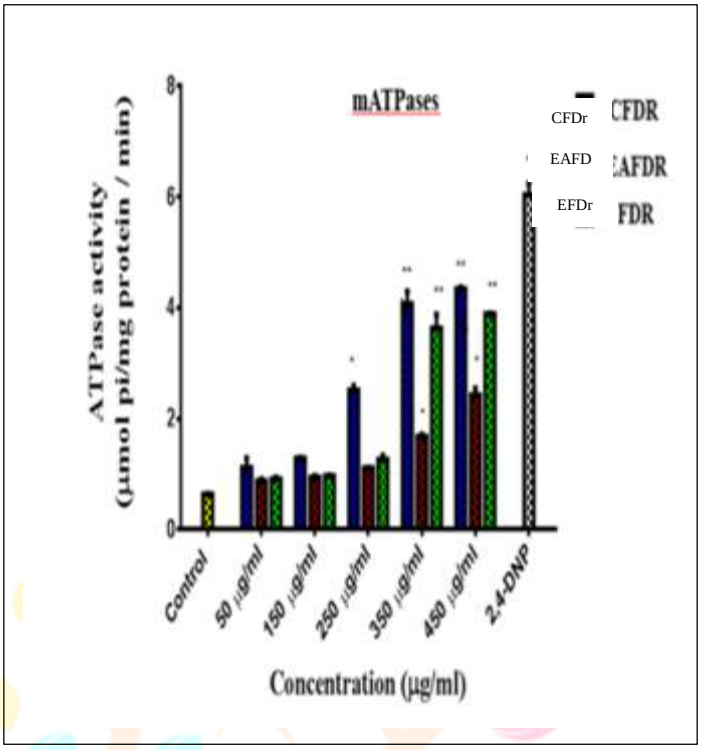

Figure 10: Impacts of Solvent fractions of Dr on mATPase activity of Rat Liver Mitochondria *in vitro*

Results were expressed as mean ± S.E.
Significant difference * = p< 0.05, ** = p< 0.01 (Test Group vs Control)

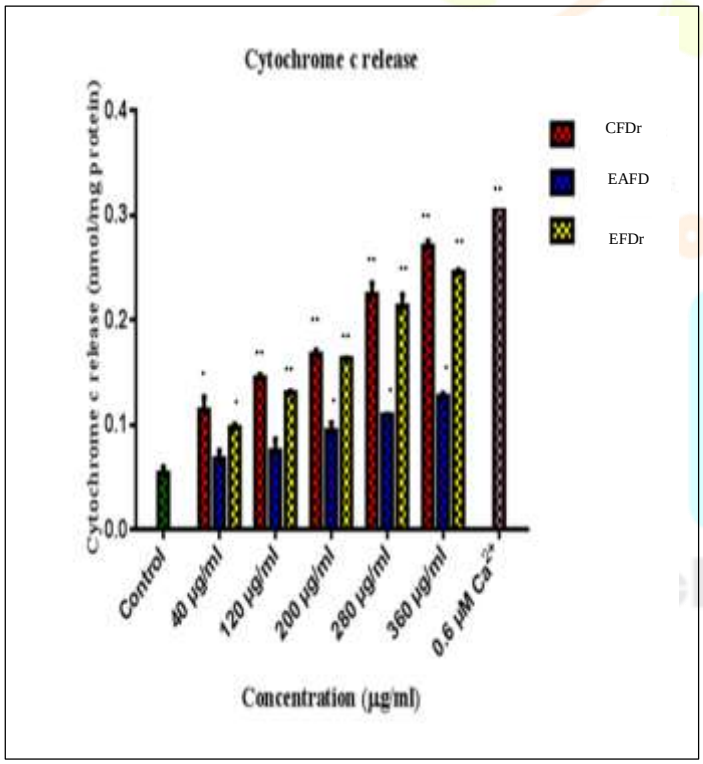

Figure 11: *In vitro* Assessment of Different Dr Solvent fractions on the discharge of cytochrome c

Results were expressed as mean ± S.E.
Significant difference** = p< 0.01, * = p< 0.05, (Test Group vs Control)

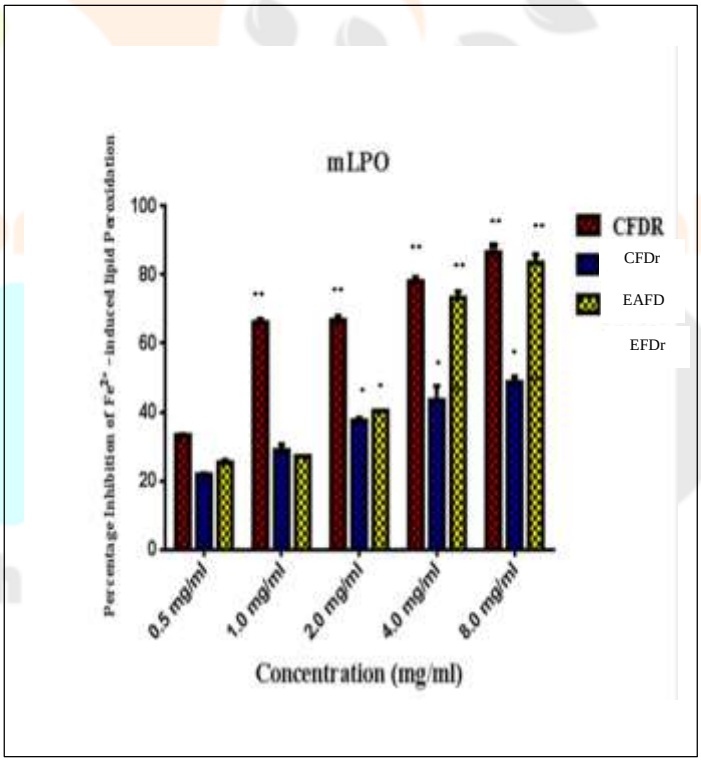

Figure 12: Investigation of the Impacts of Dr Solvent fractions on Fe^{2+} - Mitochondrial Induced Lipid Peroxidation *in vitro*

Results were expressed as mean ± S.E.
Significant difference: ** = p < 0.01, * = p < 0.05(Other concentrations vs 0.50 mg/ml)

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