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Research Article

Chenopodium ambrosioides Linn. induces Mitochondrial Permeability Transition Pore Opening and Cytochrome C Release

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Abstract

The role of mitochondrial permeability transition (mPT) pore in cell death is a known event and strategy in drug development for the management and therapy of tumors and cancers. Some medicinal plants exhibit anticancer activity by inducing mPT pore opening. Chenopodium ambrosioides is a medicinal plant used folklorically for inflammatory conditions and infections. Nevertheless, its effect on mPT pore is yet to be verified. Therefore, this study explored the influence of the methanol extract of Chenopodium ambrosioides and its fractions on mPT pore. Methanol extract of Chenopodium ambrosioides (MECA) was fractionated successively to procure chloroform (CFCA), ethylacetate (EFCA) and methanol (MFCA) fractions. Isolated mitochondria from male albino rat liver were exposed to varying concentrations of the extract and fractions. The mPT pore, cytochrome c release (CCR), mitochondrial ATPase (mATPase) and mitochondrial lipid peroxidation (mLPO) were determined using spectrophotometry. The MECA, CFCA, EFCA and MFCA effected mPT pore opening in the order; CFCA (18.8 folds), followed by MECA (9.0), EFCA (5.8) and MFCA (3.0). The CFCA enhanced mATPase activity, caused CCR and inhibited mLPO. The findings above suggest that CFCA is the most effective and likely accommodates phytochemicals that promote mitochondrial-dependent cell death. The fraction will therefore be subjected to further studies to explore the nature of the substance(s) responsible for this pharmacological potential.

Key Words: *Mitochondrial permeability transition pore, Chenopodium ambrosioides, cell death*

INTRODUCTION

Mitochondrial permeability transition (mPT) is an abrupt dissipation in the permeability of the inner mitochondrial membrane. When this happens, there is inflow of water into mitochondrial matrix, bringing about its collapse, causing the efflux of some proteins into the cytoplasm, and subsequent caspase activation and cell death ((Dejean *et al*. 2005; Dejean *et al*., 2006; Salvador-Gallego *et al*., 2016). Several factors including calcium overload, reactive oxygen species, and mPT formation can instigate the execution of cell death. The events include mitochondrial membrane permeabilization, impairment of cellular bioenergetics, cytochrome c release, (Bonora and Pinton, 2014, Galluzzi *et al*., 2018; Giorgi *et al*., 2018), hydrolysis of ATP, and ultimately, cell death (Bonora *et al*., 2015). The mPT pore is now a target for the evolution of medication pertinent in situations pertaining to impaired mitochondrial-dependent cell death; such as tumors and cancers (Morciano *et al*., 2021). Pharmacological therapy, radiotherapy and surgery are widely utilized as remedies for tumors and cancers. However, these interventions are associated with several side effects, and they are not cost effective. Consequently, there is a need for safer and more effective alternative therapies. Natural products are presently being explored in the therapy of diverse diseases due to their accessibility, cost-effectiveness and minimal side effects (Moghadam *et al*., 2020). Some restorative plants excite their chemotherapeutic potentials against cancers/tumors by activating apoptotic machinery via instigation of mPT pore opening (Marroquin *et al*., 2014; Bonora *et al*., 2020; Olowofolahan *et al*., 2020; Olowofolahan *et al*., 2021; Olowofolahan *et al*., 2024). Chenopodium ambrosioides L. is a medicinal plant of Amaranthaceae family. In folkloric medicine, it is used as a remedy for inflammation and infections. Its antioxidant and anti-inflammatory potentials have been substantiated due to the existence of flavonoids (Reyes-Becerril *et al*., 2019; Zohra *et al*., 2019). Its anthelmintic (Giove, 1996), antiviral (Zanon *et al*., 1999), antibacterial (Sousa *et al*., 2012), antifungal (Pandey *et al*., 2013), antipyretic (Bum *et al*., 2011) properties have been documented. Terpenoids have been detected in C. ambrosioides; therefore, it should be taken with caution as terpenoids could be toxic if taken in large amounts (De Pascual *et al*., 1980; Ain *et al*., 2018). This research unravelled the impact of fractions of Chenopodium ambrosioides on mitochondrial-dependent cell death via induction of mPT pore opening as there is dearth of facts on its mPT pore-opening effect.

MATERIALS AND METHODS

Preparation of Chenopodium ambrosioides methanol extract (MECA): Chenopodium ambrosioides was locally obtained on a farmland in Ibadan, authenticated and identified

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at the Department of Botany, University of Ibadan. The leaves were cut, washed, shade-dried, ground and soaked in methanol for 72 hr. It was filtered and concentrated to obtain methanol extract of Chenopodium ambrosioides (MECA). This was further partitioned successively in order of increasing polarity to obtain n-hexane (HFCA), chloroform (CFCA), ethyl acetate (EFCA) and methanol (MFCA) fractions. These were concentrated to dryness, transferred to clean glass bottles and refrigerated.

Experimental Animals: Male Wistar rats (100–105g) were acclimatized in standard cages and ethical standards (1964 Declaration of Helsinki) regarding animal handling and procedures were followed.

Mitochondrial Isolation: Rat liver mitochondria were isolated essentially according to the method of Johnson and Lardy (1967) and as modified by Olorunsogo *et al* (1979). The animals were sacrificed by cervical dislocation and the livers excised and trimmed to wash excess tissue. The livers were then weighed, washed with homogenizing buffer (210 mM Mannitol, 70 mM Sucrose, 5 mM HEPES-KOH, pH 7.4 and 1 mM EGTA), and homogenized as a 10% suspension in ice–cold buffer using a Porter Elvehjem glass homogenizer. The resulting homogenate was centrifuged in an MSE refrigerated centrifuge at 2,300 rpm for 5 min to remove the nuclear debris. This was done twice and the supernatant obtained was centrifuged at 13,000 rpm for 10 min to obtain the mitochondrial pellet. The supernatant was discarded while the pellet was washed twice with the washing buffer (210 mM Mannitol, 70 mM sucrose, 5 mM HEPES-KOH, pH 7.4, 0.5% BSA) at 12,000 rpm for 10 min. The mitochondria obtained were immediately resuspended in an appropriate volume of MSH buffer (210 mM Mannitol, 70 mM sucrose, 5 mM HEPES-KOH, pH 7.4), and immediately dispensed into eppendorf tubes and kept on ice.

Determination of mitochondrial protein: This was done as described by (Lowry *et al*. 1951), using bovine serum albumin (BSA) as standard. 3 mL of reagent D containing 2% Na2CO3 in 0.1 M NaOH, 2% Na–K-Tartrate and 1% CuSO4 (100:1:1) was added to protein samples mixed and allowed to stand at room temperature for 10 min. Folin–Ciocalteau reagent (0.3 mL) was then added to the mixture and then shaken to ensure a thorough mixing. The mixture was left for another 30 min for color development after which the absorbance was read at 750 nm using CamSpec M106 spectrophotometer. The absorbance values obtained were plotted against the respective concentrations of BSA.

Determination of intactness of mitochondria: Mitochondrial Permeability Transition (mPT) was monitored by observing the changes in diffraction of light in the spectrophotometer through absorbance of mitochondria suspension at 540 nm in a T70 UV/Visible spectrophotometer (China) essentially according to the method of (Lapidus and Sokolove 1993). Briefly, mitochondria (1 mg protein/mL) were pre-incubated in the presence of 0.8 µM rotenone (10 µL) in a medium containing 210 mM mannitol, 70 mM sucrose and 5 mM HEPES–KOH (MSH) buffer (pH 7.4) (2,200 µL) for 31/2 min at 27 °C prior to the addition of 5 mM succinate (50 µL). The mPT was spectrophotometrically monitored for 12 min at 30 s interval. The above experiment was repeated with mitochondria (1 mg protein/mL) pre-incubated in the presence of 0.8 µM rotenone (10 µL) in the same medium for 3 min at 27 °C prior to the addition of 24 µM CaCl2 (25 µL). Thirty seconds later, 5 mM succinate (50 µL) was added and mPT was quantified at 540 nm for 12 min at 30 s interval. This experiment was again repeated with mitochondria (1 mg protein/mL) pre-incubated in the presence of 0.8 µM rotenone (10 µL), 4 mM spermine (63 µL) in the same medium for 3 min at 27 °C prior to the addition of 24 µM CaCl2 (25 µl). Thirty seconds later, 5 mM succinate (50 µL) was added and mPT was quantified at 540 nm for 12 min at 30 s interval.

Mitochondrial swelling assay: This was executed following the protocol of Lapidus and Sokole, (1993). Mitochondria (1 mg protein/mL) were pre-incubated in the presence of 0.8 µM rotenone (10 µl) in a medium containing 210 mM mannitol, 70 mM sucrose and 5 mM HEPES–KOH (MSH) (pH 7.4) (2,200 µL) for 3 min at 27 °C prior to the addition of different concentrations (10–70 µg/mL) of MECA, CFCA, EFCA, MFCA. Thirty seconds later, 5 mM succinate (50 µL was added and mPT was quantified at 540 nm for 12 min at 30 s interval.

Mitochondrial F0F1 ATPase: This was assessed according to the protocol of Olorunsogo and Malomo (1985). Each reaction mixture contained 65 mM Tris–HCl buffer (1300 µl) pH 7.4, 0.5 mM KCl (50 µL), 1 mM ATP (40 µL), 25 mM sucrose (50 µL) and varying concentrations (10–70 µg/mL) of the CFCA. The reaction mixture was made up to a total volume of 2 mL with distilled water. Mitochondrial suspension was added to the reaction medium in a shaker water bath and allowed to proceed for 30 min at 27 °C. Aliquot amount (1 mL) of 10 percent sodium dodecyl sulphate (SDS) solution was added to stop the reaction at 30 s intervals. 2, 4 Dinitrophenol (2, 4-DNP) (50 µL) was used as a standard uncoupling agent. Aliquot of each solution (300 µL) was dispensed into fresh test tubes, followed by the addition of 300 µL of distilled water. To each of the test tube, 1 mL of 5% ammonium molybdate and 1 mL of 9% freshly prepared solution of ascorbic acid were added. The tube was well mixed and allowed to stand for 20 min. The absorbance was read at 680 nm. Water blank was used to set the spectrophotometer at zero.

Determination of cytochrome c release (CCR): This was carried out following the Appaix *et al*. method (2000). Mitochondria (1 mg protein/ml) were preincubated in the presence of 0.8 µM rotenone (10 µL) in a medium containing 210 mM mannitol, 70 mM sucrose and 5 mM HEPES–KOH (pH 7.4) (2,200 µL) for 30 min at 27 °C in the presence of different concentrations (10-70 µg/mL) of CFCA using 24 mM CaCl2 (25 µL) as the standard (Triggering Agent). After the incubation, the mixture was centrifuged at 15,000 rpm for 10 min. The optical density of the supernatant was measured at 414 nm which is the soret (γ) peak for cytochrome c.

Mitochondrial Lipid Peroxidation: A modified thiobarbituric acid reactive species (TBARS) assay was used to measure the lipid peroxide formed using mitochondrial membrane as lipid rich media (Ruberto *et al*., 2000). Mitochondria (1mg/mL protein) and varying concentrations (10–70 µg/mL) of CFCA were added to each test tube and made up to 1ml with distilled water; 0.05 mL of FeSO4 (0.07 M) was added to induce lipid peroxidation and the mixture incubated for 30 min. Then, 1.5 mL of 20 % acetic acid (pH

3.5) and 1.5 mL of 0.8 % (w/v) thiobarbituric acid in 1.1% sodium dodecyl sulphate were added and the resulting mixture was vortexed and then heated at 95°C for 60 min. After cooling, 3.0 mL of butan-1-ol were added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm. Percentage inhibition of lipid peroxidation by the extract was calculated as [AC-AE/AC] x 100. Where AC is the absorbance value of the fully oxidized control and AE is the absorbance in the presence of extract.

Statistical Analysis: The data reported on mPT are representative of multiple (≥4) experiments using microsoft excel 2010. All other data were expressed as mean ± SD. Statistical analysis was performed using GraphPad Prism 9 software. Comparison of the variables was made using analysis of variance (ANOVA at α 0.05) followed by Tukey's post-test.

RESULTS

Intactness

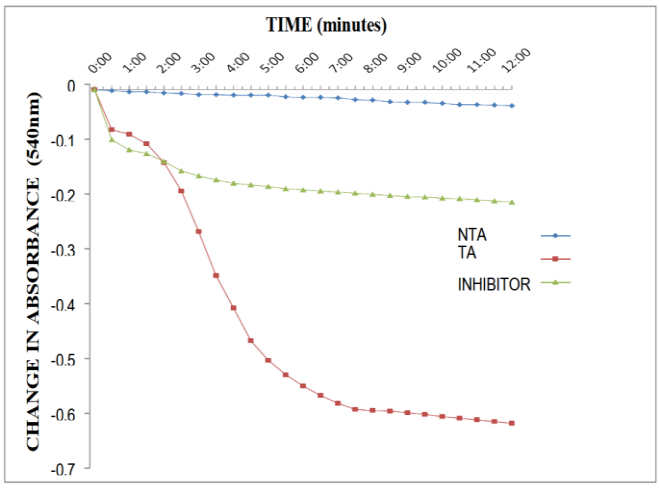

Figure 1:
Calcium-induced mitochondrial permeability transition pore opening in normal rat liver mitochondria and its reversal by spermine.
NTA: Non Triggering Agent (without calcium)
TA: Triggering Agent (with calcium)
INHIBITOR: Inhibitor of mPT pore opening (spermine)

As indicated in figure 1, there was no remarkable change in absorbance of intact mitochondria (Blue line) over a period of twelve min. Nevertheless, when exogenous calcium was introduced into the medium, there was conspicuous induction of mPT pore opening. This was almost completely reversed by spermine, thus indicating that the mitochondria used for this study were intact and suitable for use.

Influence of the Extract and Fractions of Chenopodium ambrosioides on mPT pore

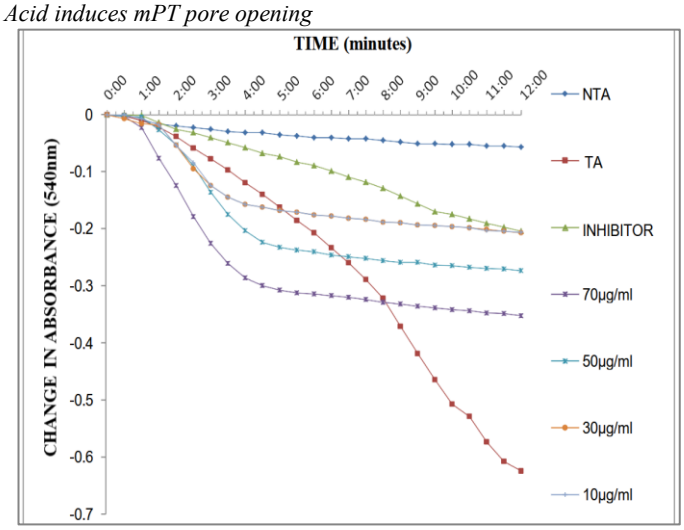

Figure 2:
Representative profile showing the effects of varying concentrations of MECA on mitochondrial permeability transition pore

The methanol extract of Chenopodium ambrosioides (MECA) caused induction of pore opening by 5.0, 5.5, 7.0 and 9.0 folds, at concentrations 10, 30, 50 and 70 µg/ml, respectively, compared with the NTA (Non-Triggering Agent) (Fig. 2). The NTA represents the control in this study.

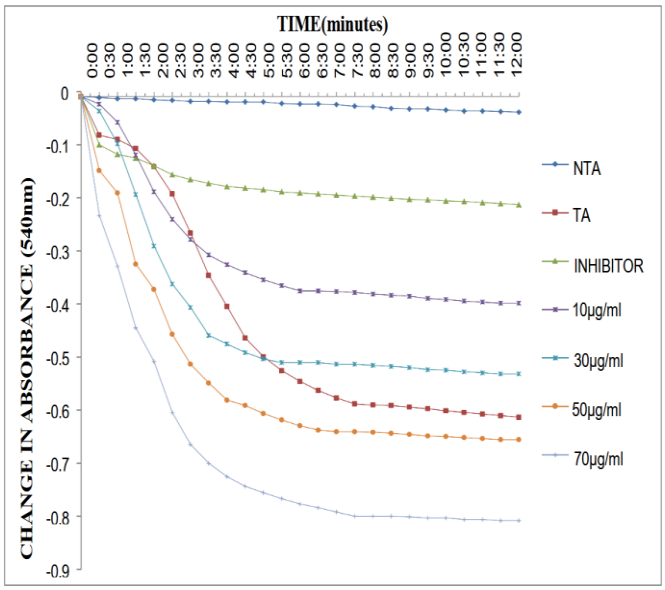

Figure 3:
Representative profile showing the effects of varying concentrations of CFCA on mitochondrial permeability transition pore

The results displayed in figure 3 show the impact of the chloroform fraction of the methanol extract of Chenopodium ambrosioides (CFCA) on mPT pore. Also, there was increase in induction of mPT pore opening by by 7.0, 9.0, 12.0 and 18.8 folds, at concentrations 10, 30, 50 and 70 µg/ml, respectively, related to the NTA.

As indicated in figure 4, ethyl acetate fraction of the methanol extract of Chenopodium ambrosioides (EFCA) effected mPT pore opening by 1.3, 1.5, 4.3 and 5.8 folds, at concentrations 10, 30, 50 and 70 µg/ml, respectively, related to the NTA

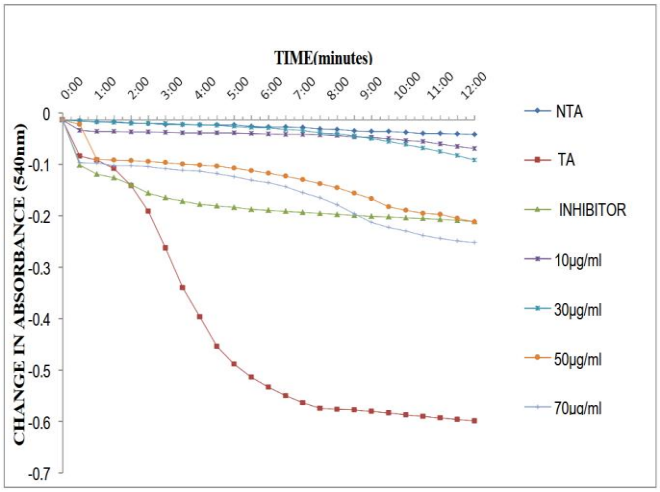

Figure 4:
Representative profile showing the effects of varying concentrations of EFCA on mitochondrial permeability transition pore

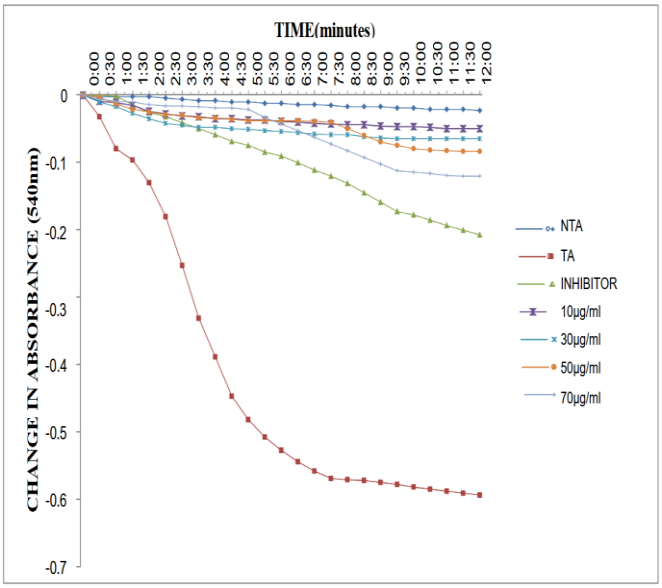

Figure 5:
Representative profile showing the effects of varying concentrations of MFCA on mitochondrial permeability transition pore

Figure 5 illustrated the effects of methanol fraction of the methanol extract of Chenopodium ambrosioides (MFCA) on mPT pore. There was a concentration-dependent induction of pore opening by 1.3, 1.5, 1.8 and 3.0 folds, at concentrations 10, 30, 50 and 70 µg/ml, respectively, in comparison to the NTA.

Effects of Chloroform Fraction of Methanol Extract of Chenopodium ambrosioides (CFCA) on mATPase activity.

Varying concentrations of CFCA significantly enhanced mATPase activity by 0.6, 1.2, 1.5 and 2.2 folds, at concentrations 10, 30, 50 and 70 µg/ml, respectively, related to the control (Fig. 6). The 2,4-Dinitrophenol which serves as a standard uncoupler enhanced mATPase activity by 4 folds increase, when compared to the control.

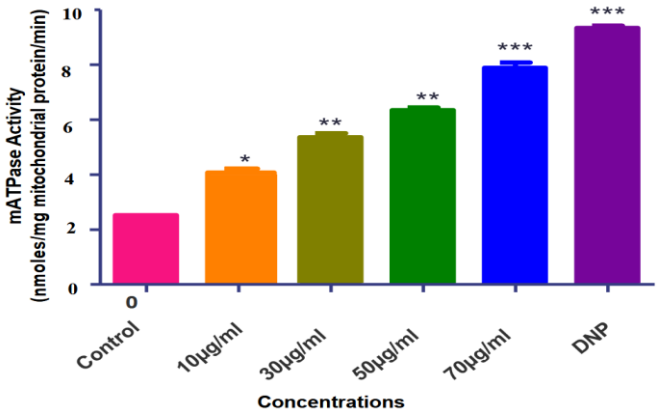

Figure 6:
Effects of varying concentrations of CFCA on mATPase activity
The values are expressed as mean ± SD of five independent replicates, and analyzed using one-way ANOVA method followed by Turkey's post test. *p < 0.05, **p < 0.01, ***p < 0.001, compared to the control.

Effects of Chloroform Fraction of Methanol Extract of Chenopodium ambrosioides (CFCA) on Cytochrome C Release

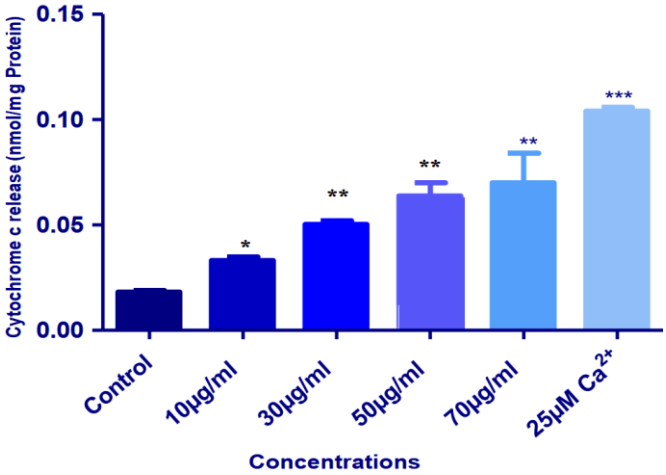

Figure 7: Effects of varying concentrations of CFCA on Cytochrome c release
The values are expressed as mean ± SD of five independent replicates, and analyzed using one-way ANOVA method followed by Turkey's post test. *p < 0.05, **p < 0.01, ***p < 0.001, compared to the control

As indicated in figure 7, CFCA caused significant CCR in a concentration-dependent manner, related to control. At concentrations 10, 30, 50 and 70 µg/ml, the CFCA caused cytochrome c release by 1.5, 2.5, 3.0 and 4.0, folds, respectively, when compared to the control. Calcium, which is a standard triggering agent caused the release of cytochrome c by 5.5 folds, compared to the control.

Effects of Chloroform Fraction of Methanol Extract of Chenopodium ambrosioides (CFCA) on mitochondrial lipid peroxidation

Figure 8 showed a concentration-dependent decrease in malondialdehyde levels effected by CFCA, relative to control. At concentrations 10, 30, 50 and 70 µg/ml, the CFCA decreased MDA levels to 79.0, 65.0, 54.0 and 40.0 % of control.

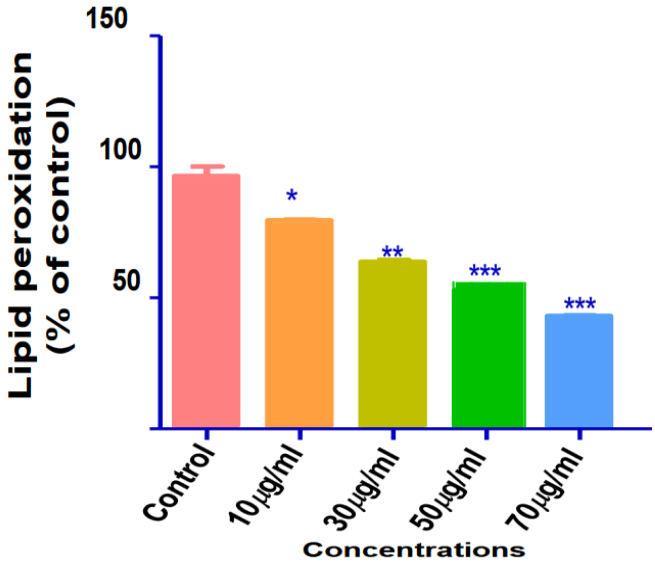

Figure 8:
Effects of varying concentrations of CFCA on mitochondrial Lipid Peroxidation (mLPO)
The values are expressed as mean ± SD of five independent replicates, and analyzed using one-way ANOVA method followed by Turkey's post test. *p < 0.05, **p < 0.01, ***p < 0.001, compared to the control

DISCUSSION

This study demonstrated the influence of Chenopodium ambrosioides on mitochondrial membrane permeability transition pore. The isolated mitochondria in this research were unimpaired, uncompromising and fit for experimental purpose, as indicated by its pore-opening by calcium and reversal by spermine (Inhibitor) (Lapidus and Sokolove,1993; Javadov and Karmazyn, 2007). The isolated mitochondria were incubated with varied concentrations of the methanol extract of Chenopodium ambrosioides (MECA) and its various fractions (CFCA, EFCA and MFCA). The MECA, CFCA, EFCA and MFCA all induced mPT pore opening, suggesting the existence of specific phytonutrients that can associate with the pore constituents and effect induction of mPT pore opening. Nevertheless, since chloroform fraction of the methanol extract of Chenopodium ambrosioides (CFCA) was the most effective with respect to induction of mPT pore opening, this implies that more of the phytochemicals that cause pore opening reside in the CFCA. This is similar to a bio-activity-guided assay carried out on methanol extract of Drymaria cordata which exhibited the chloroform fraction as the most potent regarding pore opening (Olowofolahan *et al*., 2015; Olowofolahan *et al*., 2018). Further studies carried out on the chloroform fraction (CFCA), which is the most potent, showed that it enhanced mitochondrial ATPase activity. This indicates that the CFCA reacted with the pore constituents to cause the pore opening and subsequent release of inorganic phosphate; an inducer of mPT pore opening. The mPT pore now serves as a novel strategy for the development of drugs that can effect induction of mPT pore opening, which could result to cell death. When the pore opens, cytochrome c is released, hydrolysis of ATP results, causing impairment in cellular bioenergetics and subsequently, the cell dies. The cytochrome c relesaed by CFCA suggests that CFCA effected translocation of cytochrome c into the cytosol where it can bind with other factors to execute cell death. (Kroemer *et al*., 2007). This outcome is in consonance with the mPT result as the CFCA effected a concentration-dependent induction of pore opening. However, CFCA inhibited lipid peroxidation, indicating the existence of putative compounds in CFCA that could possibly reduce lipid peroxidation-induced damage (Ruberto *et al*., 2000; Rizzo, 2024). This further implies that the pore opening instigated by CFCA was not through lipid peroxidation as the fraction inhibited ferrous-induced mitochondrial lipid peroxidation. In conclusion, these phytochemicals in CFCA could be further explored, characterized and the active principle(s) isolated. This could be a pertinent remedy in diseased conditions associated with dysregulated mitochondrial-dependent cell death.

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