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Effect of Different Solvent Fractions of *Sarcocephalus latifolius* (Smith) Bruce on Rat Liver Mitochondrial Membrane Permeability Transition (mPT) Pore

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ABSTRACT

Mitochondria are an essential pharmacological target for the development of cytotoxic drugs. Many bioactive agents of plant origin have been found to induce mitochondrial-mediated apoptosis via membrane permeability transition (mPT) pore, osmotic swelling and ultimately the release of cytochrome C. *Sarcocephalus latifolius* (SL) is used in folkloric medicine for the treatment of breast cancer, but there are no scientific data to support this claim. The study therefore evaluated the *in vitro* effects of *Sarcocephalus latifolius* (SL) fruits chloroform (CFSL), ethyl acetate (EFSL), and methanol (MFSL) fractions on rat liver mitochondria. Male Wistar rats with average weight of 90 ± 10 g were used for this study. Rat liver mitochondria were isolated by differential centrifugation. mPT pore opening, mitochondria ATPase activity, Fe^{2+}-induced lipid peroxidation, and cytochrome C release were assayed spectrophotometrically at 540 nm, 660 nm, 532 nm, and 414 nm, respectively. In the absence of Ca^{2+}, CFSL, EFSL, and MFSL induced mPT pore opening in a concentration-dependent manner, with CFSL exhibiting the highest activity. Interestingly, CFSL and EFSL inhibited lipid peroxidation, while MFSL induced lipid peroxidation in a concentration-dependent manner with CFSL showing the highest percentage inhibition of 90%. Mitochondrial ATPase activity and cytochrome C release were significantly enhanced by CFSL. These findings suggest that CFSL and EFSL contain certain bioactive agents that can induce mPT pore opening and subsequently result in mitochondrial-mediated apoptosis. This preliminary finding will serve as a template for drug development for ailments that require the up-regulation of apoptosis.

***Keywords*:** *Sarcocephalus latifolius,* Mitochondrial Membrane Permeability Transition, Mitochondrial-mediated apoptosis, Mitochondrial ATPase activity, Lipid peroxidation.

Introduction

Natural compounds as an alternative to conventional approach in the management of diseases, has been an age long practice, which can be traced back to the onset of existence. Some of the compounds are notable for their high antitumor properties and are extracted from medicinal plants.[1] Contemporary medicines despite having experienced remarkable progress in the past few decades, the use of medicinal plants products in the rural areas is still in vogue. Interestingly, the upsurge in the demand for herbal medicines can be attributed to the rise in human population, the insufficient provision, and the high cost of western medicines in developing countries and the consciousness of the damaging side effects of the synthetic drugs when compared to natural products. [2] Plant-based products are sold in many forms including health foods, food supplements, and herbal teas for health maintenance or personal care.[3]

Natural products which comprise the constituents or metabolites of medicinal plants, animals, insects, marine organisms, and microorganisms are significant sources of major bioactive compounds and novel drugs.[1]

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These products have played vital roles in drug discovery and development especially as antitumor agents, hence the search for anticancer agents from medicinal plants, is an interesting feild of scientific research.[1]

Mitochondria, also referred to as "powerhouse of the cell", are double membrane, intracellular organelles and the main sites for aerobic respiration in cells as well as energy-producing structures.[4] They are linked with several ailments, and play significant roles in cell signaling, programmed cell death regulation, and energy metabolism in drug-induced cancer cells death (CCD). Therefore, the mitochondria have been recognized as an essential pharmacological target for the discovery of cytotoxic drugs, and as an important target in the management of cancers,[4] and their dysregulated function has been proven essential for tumorigenesis, tumor growth, and tumor metastasis.[5]

Apoptosis is an essential programmed pathway to cell death and in the near future will be a target for antitumor agents.[6,7] Of the numerous pathways to apoptosis, the intrinsic and extrinsic pathways are activated via caspase-dependent reaction, and this is made possible via the cleavage of hundreds of proteins.[6] An imbalance between cell proliferation and death can result in diseases associated with deregulated apoptosis. Under physiological and pathological conditions, apoptosis plays an important role in cell growth, differentiation, regulation and the upkeep of cell populations and death in multicellular organisms,[8] and has been proven critical in long-lived mammals.[6] Apoptosis is unique and probably the commonest form of programmed cell death, of high biological importance [9] and is therefore associated with several pathological conditions like ischemic damage, autoimmune disorders (e.g. AIDS), heart disease, rheumatoid arthritis and cancer.

Apoptosis is dependent on the caspases (cysteine-dependent aspartic specific proteases) which are a class of cysteine proteins that cleave

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target proteins.[10] It therefore means that caspase activity is essential to successful apoptosis as they led to the cleavage of hundreds of various proteins.[11] Caspases are classified into two: the initiator caspases (caspase-2, 8, 9, and 10) and the executioner caspases (caspase-3, 6, and 7).[10]

The two major apoptotic pathways (extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway) are highly regulated so that apoptosis will only occur if there is a signal from the cell.[6] In the mitochondrial-mediated apoptotic pathway, there is an indirect or direct action on the mitochondria via the permeability pore or the Bcl-2 family which eventually leads to the discharge of apoptotic factors like cytochrome c, AIF, and Smac⁄DIABLO with a consequent activation of the cascade of caspases, nuclear DNA fragmentation and cell death. On the other hand, the death signals⁄ ligands bind to the death receptors, to trigger the activation of both caspase-8 and caspase-3 (the executioner caspase) and other downstream proteases, that stimulate and trigger the destruction of cellular proteins and eventual cell death, in the extrinsic apoptotic pathway.[8,11] Furthermore, dysregulated apoptosis is the basis for a wide variety of diseases, which include infertility, diabetes mellitus, immunodeficiency, and acute and chronic degenerative diseases, cancer and autoimmune disorders.[12]

The mitochondrial outer membrane permeabilization (MOMP) or the mitochondrial membrane permeability transition pore (mPT) is a major component of the mitochondrial-mediated apoptotic pathway.[13-15] Intrinsic pathway, which is also known as mitochondrial-mediated pathway, occurs when the mPT pore opens, with a consequential release of cytochrome c, which ultimately leads to apoptosis. mPT pore opening is a critical event that determines cell survival and is exploited in situations where apoptosis is dysregulated. Furthermore, mPT has therefore turned out to be a significant pharmacological target for the discovery of cytotoxic drugs and in diseases where there is aberrant apoptosis.

Numerous suggestions have been put forward with respect to the molecular components of the mPT pore, but the proteins responsible for pore formation have not been fully recognized, to date.[16] As the quest for the mPT pore components continues, many scientists have put up several proposals over time, but none has successfully identified the pore components. Most of the researchers concluded that "As more work continued on this, there is a growing body of evidence suggesting that induction of the mPT may be a protective mechanism for stimulating apoptosis especially in cancer cells and that targeting the mPT may be a promising strategy for improving anticancer therapies".[14,15]

Sarcocephalus latifolius (SL) also called Pincushion fruit is a semi-climbing evergreen shrub or a tree, belonging to the Rubiaceae family.[17] The plant is native to tropical West Africa; found in the humid rain forests or the savannah woodlands, with flowers that are round heads, fragrant, white or whitish yellow in colour and fruits when matured are of moderate size, edible, and reddish-brown in colour.[17]

Other names of the plant include African cinchona, African peach, African quinine, Country fig, Doundake, Dundaki, or "Marga" (Hausa, Nigeria), Egbesi or Ogbesi (Yoruba, Nigeria) and Ubuluinu or "Nbitinu" (Igbo, Nigeria).[18] Others are Guinea peach, Igbeshi (Sierra Leonean), Liane à fraise or Pêcher africain (French), Nauclea, Negro peach, Peach root, (French), Pin-cushion fruit, Pin-cushion tree, Rata-bakmi (Singhalese), Sierra Leone peach, and Strawberry tree, depending on what part of West Africa it was harvested. The warty, brownish-red and fleshy fruits (Figures 1 and 2) with their sweet apple-tasting flavour are eaten locally and are sold in local markets where their creamy flower heads are bought for preparation as a vegetable.

A previous study on the phytochemical constituents of the crude methanol extract (CMESL) as well as the n-hexane, chloroform, ethyl acetate, and methanol solvent fractions (HFSL, CFSL, EFSL, and MFSL, respectively) of the matured fruits of *S. latifolius* revealed the presence of secondary metabolites like anthraquinones, terpenoids, flavonoids, alkaloids, saponins, tannins and cardiac glycosides.[19] Some of the pharmacological and toxicological effects of *Sarcocephalus latifolius* include anticancer,[18] anti-inflammatory,[20] anti-oxidant,[21] nephroprotective, and hepatoprotective activities, [22] just to mention a few.

Furthermore, the use of extracts of the different parts of SL, for the control of various illnesses especially in Africa is widely documented, but there is little documentation on the use of the fruit extracts. The fruits of *Sarcocephalus latifolius* have been used in traditional medicine in the management of tumors but the possible mechanism of action has not been established. In addition, it is yet to be established whether fractions of *Sarcocephalus latifolius* contain potent phytochemicals that modulate mitochondria-mediated apoptosis.

Finally, the mPT pore opening as mentioned earlier is a critical event that determines cell survival and it is exploited in situations where apoptosis is dysregulated. Programmed cell death (apoptosis) is an established and a very well-regulated mechanism that play a key role in the treatment and management of tumors. Anti-tumor agents can induce apoptosis through various mechanisms, including mPT pore opening.[11]

It is on this background that the present study investigated the effect of solvent fractions of the fruits of *Sarcocephalus latifolius* on rat liver mitochondrial membrane permeability transition.

Materials and Methods

Chemicals and reagents

All reagents and solvents were of analytical grade, and were products of Sigma-Aldrich Chemical Co. St. Louis, USA.

Plant collection and identification

Fresh matured and ripe fruits of *Sarcocephalus latifolius* (Smith) Bruce were harvested from the forests in Eruwa, Iddo LGA of Oyo State in December 2021. Three herbarium were prepared and sent to the Forestry Research Institute of Nigeria (FRIN), Ibadan for taxonomical authentication. The plant material was identified and authenticated, and assigned a voucher number: FHI 110092. The herbarium specimen was deposited in the herbarium, Department of Pharmacognosy, University of Ibadan for future reference.

Figure 1: Matured fruits sighted on the tree just before harvesting at Iddo L.G.A. Oyo State, Nigeria

Figure 2: Post harvested matured fruits stacked after collection

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Preparation of extract and fractions

The fruits of *Sarcocephalus latifolius* (SL) were washed and air-dried at room temperature. The dried fruits were pulverized using a mortar and pestle. The powdered fruits (50 g) was macerated in absolute methanol (500 mL) for 72 h. The crude methanol extract was filtered through a Whatman No.1 filter paper, and then concentrated using a vacuum rotary evaporator (Stuart Rotavapor, UK) at 40ºC. The concentrated extract was further dried over a water bath at 37ºC for about four days to allow for the complete removal of solvent. The crude methanol extract (CMESL) was fractionated into n-hexane (HFSL), chloroform (CFSL), ethyl acetate (EFSL), and the methanol (MFSL) fractions using vacuum liquid chromatography in the order of increasing solvent polarity. The crude extract and solvent fractions (CMESL, HFSL, CFSL, EFSL, and MFSL) were stored in a refrigerator at 4°C until further use.

The percentage yield of the extract and fractions was calculated as follows:

Percentage yield (%) = {(X – Y) / Z} × 100 …… 1

Where X is the weight of extract/fraction + dish, Y is the weight of empty dish and Z is the total weight of the dried powdered plant.

Preparation of low ionic strength rat liver mitochondria

Rat liver mitochondria were prepared following the technique of Johnson and Lardy (1967)[46] as modified by Olorunsogo and Malomo (1985),[23] and Nwaechefu *et al*. (2022).[24] The liver tissue was washed with 10% suspension of ice-cold homogenizing buffer, then homogenized in a Porter-Elvehjem glass homogenizer. The homogenate was centrifuged at 2300 rpm for 5 min in already chilled centrifuge tubes in a high-speed refrigerated MSE centrifuge (Progen Scientific, UK) at 4ºC to eliminate complete/unbroken cells. The supernatant from the sedimentation of the nuclear fraction was centrifuged at 13,000 rpm for 10 min to give the mitochondria pellet. The mitochondria pellet was washed twice by spinning at 12,000 rpm for 10 min. The pellet were immediately suspended in a solution of ice-cold MSH Buffer (pH 7.4), stored in aliquots in Eppendorf micro-tubes and kept cold on ice for immediate use.

Assessment of mPT in rat liver mitochondria

Accretion of Ca^{2+} in mitochondria may result in mitochondrial permeability transition (mPT). The inner mitochondrial membrane (IMM) is highly porous to small (1.5 KDa) solutes.[25] Isolated mitochondria with mPT have great swelling/distension potential that leads to a reduction in absorption at 520 nm. mPT was evaluated experimentally by measuring mitochondria distension which in turn is measured by a reduction in absorbance.

To assess mitochondrial permeability transition, mitochondrial swelling was quantified by the method of Lapidus and Sokolove (1994),[25] and modified by Nwaechefu *et al*. (2022).[24] Firstly, mitochondrial homogenate (0.4 mg/mL) was added to 0.8 µmol of the swelling buffer (pH 7.4) to initiate the reaction. The solution was pre-incubated with $CaCl_2$ at 30ºC. After 30 seconds, the mitochondria was energized by the addition of 50 µM succinate. The absorbance of the resulting medium was measured at 540 nm every 30 seconds for 12 min.

Determination of mitochondrial ATPase activity

The mitochondrial ATPase (mATPase) activity was determined by the technique of Lardy and Wellman (1953).[26] Concentration of the inorganic phosphate (Pi) discharged throughout the reaction was determined by the method decribed by Bassir (1963),[27] with slight modification by Olorunsogo and Malomo (1985).[23] The modification involved using 1 mg/mL instead of 2 mg/mL mitochondrial protein for the assay.

Eleven test tubes were arranged in duplicates in a test tube rack. To each tube, was added 0.25 M sucrose, 5 mM KCl, and 0.1 M Tris. Variable concentrations of the extract and fractions of SL were added to the tubes, and the solutions were made up to 2 mL with distilled water. Thereafter, 10 mM ATP (1 mL) was added to the tubes, and placed in a shaker water bath at 27ºC after careful mixing. At time zero, mitochondria were added and the reaction was stopped instantly by the addition of 1 mL of 10% SDS. The addition of mitochondria was repeated for all remaining test tubes except for the blank. Following the addition of mitochondria, 2, 4-DNP was added to the uncoupler labelled tube, and the mixture was shaken for 30 min. Finally, to stop the reaction, 1 mL of SDS was added to each test tube (except for the zero time) every 30 seconds. The reaction mixture (1 mL) was taken for phosphate determination.

Determination of inorganic phosphate

Distilled water (4 mL) was added to 1 mL of the sample in a test tube. Ammonium molybdate (1 mL of 1.25%), and 1 mL of 9% freshly prepared solution of ascorbic acid were added. The content was mixed thoroughly, and left to stand for 20 min. The procedure was repeated using standard solution of potassium dihydrogen phosphate (0.2 mg/5 mL). The absorbance of the coloured complex formed was measured at 660 nm using Camspec M105 Spectrophotometer. A standard calibration curve of phosphate curve was prepared from which the concentration of inorganic phosphate released was calculated.

Mitochondrial lipid peroxidation (mLPO) assay

Lipid peroxidation of rat liver mitochondria was estimated using an adapted thiobarbituric acid reactive species (TBARS) method as described by Ruberto *et al*. (2000).[28]

Mitochondrial lipid peroxidation (mLPO) was assessed by evaluating the thiobarbituric acid reactive (TBAR) products. An insignificant quantity of malondialdehyde are generated during LPO process and this can react with thiobarbituric acid to produce a pink coloured complex, that absorbs light at 532 nm and is freely extractable into organic solvents such as butan-1-ol. The procedure is usually calibrated using MDA (Malondialdehyde) as a reference standard.

The mitochondrial homogenate (4 mg/mL) were added to varying concentrations of the extract and fractions of SL in test tubes. LPO was induced by the addition of 0.05 mL of 0.07 M ferrous sulphate ($FeSO_4$). The mixture was incubated for 30 min at room temperature. Thereafter, 1.1% SDS containing 1.5 mL of 20% acetic acid (pH 3.5) and 1.5 mL of 0.8% (w/v) thiobarbituric acid were added. The resulting solution was thoroughly mixed using a vortex mixer and then boiled at 95°C for one hour. The solution was left to cool to room temperature. Butanol (3 mL) was 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 mLPO by the test samples was calculated.

Quantification of Cytochrome c released from isolated mitochondria

The Cytochrome c release (CCR) was quantified using a technique described by Appaix *et al*. (2000) with little modifications. Liver mitochondria (LM), at a final concentration of 1 mg/mL was pre-incubated in 2.5 mL of the reaction medium, comprising MSH buffer. In addition, 0.8 µM rotenone (10 µL), varying concentrations of the extract and fractions of SL (20, 60, 100, 140 and 180 µg/mL), 25 µM calcium (50 µL) as a positive control were mixed, and incubated at 25ºC for 3.5 min. Succinate 5 mM (50 µL) was then added to energize the mitochondria, and left to stand for 30 min at 25°C. The mitochondria solutions were then centrifuged at 13000 rpm for 30 min. The supernatant was filtered using a 0.2 µM Millipore membrane. The OD (absorbance) of flawless supernatants was read against blank at 414nm, in a spectrophotometer (Spectrum Lab 776, China). The quantification of cytochrome c released was extrapolated from a standard cytochrome c curve prepared using 1 mg/mL concentration of pure cytochrome c (Sigma-Aldrich, Germany).

Statistical analysis

SPSS Statistics standard version 20 software (SPSS Inc., Chicago) was used for analysis. Data was presented as mean ± standard deviation (SD) of at least three independent measurements. Differences between means were analysed by One-way Analysis of Variance (ANOVA), and Duncan's Multiple Range Test (DMRT). P-value < 0.05 was regarded as statistically significant.

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Results and Discussion

Mitochondria are double membrane organelles found in the matrix or cytosolic compartment of a cell. They are essential for the production of energy and are also associated with numerous types of cellular damage and/or cell death including programmed cell death (apoptosis), necrosis, and other compromised machineries via mitochondrial outer membrane permeabilization (MOMP).[4] It has been documented that the mitochondria mediate the intrinsic apoptotic pathway triggered by the cytotoxic effect of various types of chemotherapeutic agents.[4] Some of the diverse stimuli that can trigger the mitochondrial-mediated/intrinsic apoptotic pathway include high concentration of cytosolic Ca^{2+}, increased production of reactive oxygen species (ROS), low oxygen concentration (hypoxia), and environmental influences like UV exposure.[29,30] In a situation where the cells are unable to adapt to the stress caused by these stimuli, apoptotic cascade is triggered leading to upsurge in apoptotic protein manifestation, and ultimately the release of Cytochrome c (Cyt C). This is a non-negotiable point for apoptosis to occur, and subsequently leads to the extinction of the cells.[31,32]

Although, the factors essential for MOMP continue to be poorly defined, quite a few protein complexes on mitochondrial membranes related to MOMP have been identified. For example, the mitochondrial membrane permeability transition (mPT) is thought to be implicated in the intrinsic apoptotic pathway.[33,34] A modification in the permeability of the inner mitochondrial membrane (IMM) is known as mPT.

Evidence from recent studies has confirmed that the mitochondrion is a central regulator of apoptosis.[35] The novel approaches in apoptosis, have therefore employed avenues to exploit mPT pore opening that triggers apoptosis, as a gene-controlled process, where numerous biochemical features branded as apoptotic genes and proteins are likely targets in the treatment of tumors/cancers.[36] This study utilized strategies that directly induced apoptosis via an interplay of the Bcl-2 family proteins. The bioactive components of the most potent solvent fraction of *Sarcocephalus latifolius* (SL) fruit extract induced apoptosis via the mitochondrial-mediated pathway by enhancing and inducing mPT pore opening. This is in line with recent report that successful apoptosis triggered by mPT pore opening ultimately leads to MOMP, and activation of caspases and protein substrate cleavage, which are major biochemical features in apoptosis.[37] Furthermore, an intricate network of events that determine the fate of the cell between survival and death are known to control the mitochondrial-mediated apoptotic pathway and are centered on mPT or MOMP.[37] For example, it is well documented that there is no mitochondrial-mediated or intrinsic pathway of apoptosis without the proper localization and control of the Bcl-2 family proteins on mitochondria.[38] Several scientific reports also present evidence that purified solvent fractions of *Brysocarpus coccineus*,[39] *Drymaria cordata*,[40] *Alstonia boonei*,[41] and *Calliandria portoricensis*,[42] also induce mitochondrial-mediated apoptotic pathway via mPT pore opening. Furthermore, Gossypol (in cottonseed), Artonin E (a prenylated flavonoid in *Artocarpus elastcus*), Camalexin (a phytoalexin from cruciferous plants), and Quercetin (in onions) exert their antitumor effects via MOMP in apoptotic cancer cell death.[43-45]

In this study, the strategy used for the assessment of mPT pore opening involved the addition of a triggering agent, usually Ca^{2+} (calcium) as an extra-mitochondrial calcium that must first enter the mitochondria to cause the swelling of the organelle. This approach involves spectrophotometric monitoring of the Ca^{2+}-induced opening of the mPT pore.[46,47] The response to the induction of the mPT pore opening with the addition of exogenous calcium establishes mitochondrial intactness, and the calcium-induced pore opening is reversed significantly with spermine, a potent inhibitor of mPT pore opening. In this study, it was observed that the mPT pore of the various assay preparations used was vulnerable to induction by the addition of extra-mitochondrial calcium and in resemblance to that of normal intact mitochondria, the induction of pore opening was reversed up to about 81.7% by the addition of spermine (Figure 3). This demonstrates that the mitochondria used for the various *in vitro* assays were intact, uncoupled and not compromised, hence suitable for further use.

The results obtained in the present investigation agree with previous studies, which established spermine as an inhibitor of the mPT pore.[25] Although, findings from previous studies have shown the effect of fractions of the fruit extract of *Sarcocephalus latifolius* in inducing mPT pore opening, CMESL, CFSL, and EFSL was observed in this study to cause a high degree of enlargement (swelling) of the intact isolated mitochondria in a concentration-dependent manner, (Figures 4 - 9), an indication of their abilities to alter the mitochondrial permeability transition. The effect of the fractions of *Sarcocephalus latifolius* (SL) fruit extract on mPT pore, *in vitro*, is shown in Figures 4 - 11.

Interestingly, the CFSL had the highest mPT pore opening inducing effect causing about 18.9 fold inducing ability, while the EFSL induced mPT pore opening up to 11.3 fold. The MFSL induced pore opening in an irregular pattern. This observation suggests that SL contains bioactive agents that may have interacted with the components of mitochondrial membrane pore leading to the release of apoptogenic proteins like cytochrome c and caspase co-factors that will eventually lead to programmed cell death (apoptosis).

On the other hand, when the uncompromised mitochondria were exposed to CFSL, EFSL and MFSL, in the absence of calcium ion, opened the mPT pore significantly. Moreover, the opening of the mPT pore was reversed by spermine indicating that CFSL, EFSL and MFSL stimulation of mPT pore opening had no damaging effect on membrane integrity. On the addition of a triggering agent (Ca^{2+}) into the assay medium, CFSL, EFSL and MFSL upturned the Ca^{2+}-induced pore opening in a similar fashion as spermine. This observation might be attributed to the existence of some agents in the extract/fractions which may possess calcium-chelating property hence decreasing the Ca^{2+} available to induce pore opening. The reversal of these inductions by SL was concentration-dependent except for the MFSL. The concentration-dependent induction of mPT pore opening by CFSL and EFSL suggests that the fractions contain bioactive constituents that may have interacted with some specific components of the pore, resulting in mPT pore opening and ultimately apoptosis.

Furthermore, the fact that the inductive effect of CFSL and EFSL on mPT pore, increased as the concentration of the fractions increased, but was reversed almost completely by the standard inhibitor, spermine. This is indicative of the fact that there was no form of alteration or disruption of the mitochondrial membrane integrity while the induction of the pore occurred. Moreover, since the effect was concentration-dependent, it may be deduced that the active principle increased with increase in concentration of the fractions used. These observations relating to the inductive ability of SL are similar to the observations with Gossypol on the modulation of mPT pore in exerting their pharmacological effect.[45]

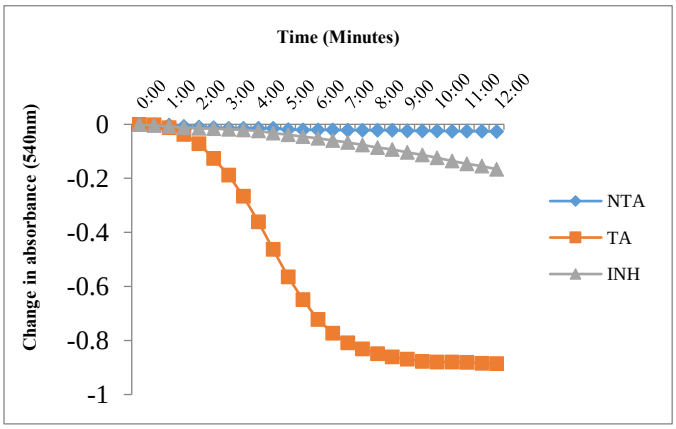

Figure 3: Changes in absorbance of intact mitochondria in the absence and presence of calcium. NTA - No triggering agent (without calcium), (*in vitro*), TA - Triggering agent (with calcium), INH - Inhibitor (spermine).

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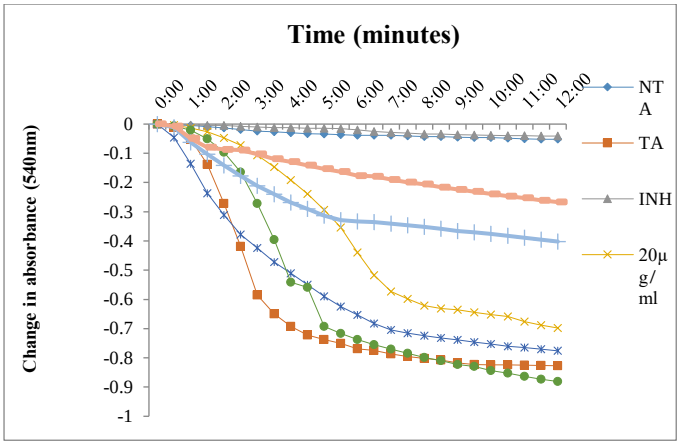

Figure 4: Changes in absorbance of mitochondria due to CMESL in the absence of calcium. NTA - No triggering agent (without calcium), (*in vitro*), TA - Triggering agent (with calcium), INH - Inhibitor (spermine).

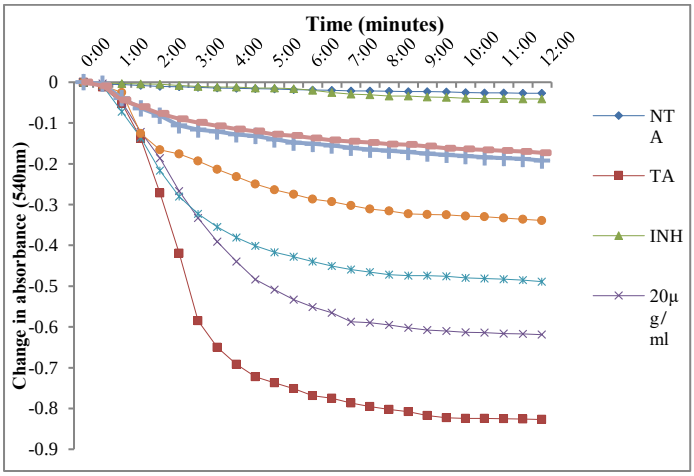

Figure 5: Changes in absorbance of mitochondria due to CMESL in the presence of calcium, where there was a reversal of induction. NTA - No triggering agent (without calcium), (*in vitro*), TA - Triggering agent (with calcium), INH - Inhibitor (spermine).

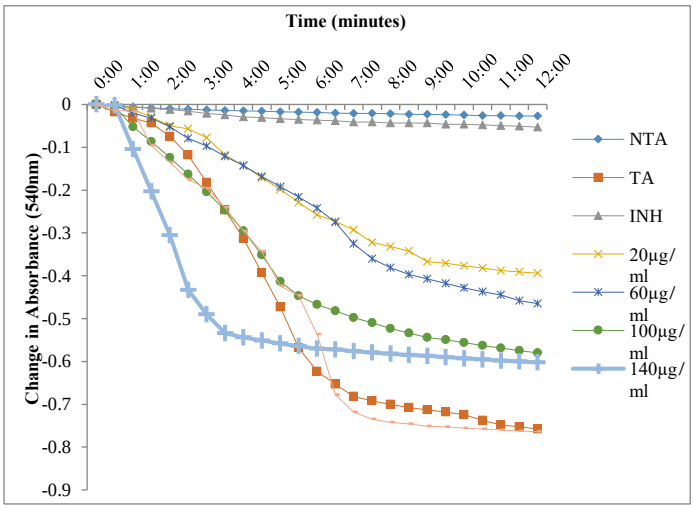

Figure 6: Changes in absorbance of mitochondria due to CFSL in the absence of calcium. NTA - No triggering agent (without calcium), (*in vitro*), TA - Triggering agent (with calcium), INH - Inhibitor (spermine).

From the results as shown in Figure 12, the CFSL, EFSL and MFSL enhanced ATPase activity with CFSL exhibiting the highest activity in a concentration-dependent manner. CFSL caused the release of the highest concentration of Pi compared to that of the EFSL and MFSL at the same concentration, as revealed by the results of ATPase activity. The enhancement of mATPase activity, observed for CFSL was similar to that seen with the mPT pore opening inducer, 2, 4-dinitrophenol (DNP) used in the assay. DNP is a standard uncoupler of oxidative phosphorylation, usually used as a standard reference during investigation involving ATPase activity.[48] The results of the study support the earlier findings that CFSL, EFSL and MFSL induced mPT pore opening in rat liver mitochondria. This also corroborated the findings that the opening of the mPT pore leads to the inhibition of oxidative phosphorylation by the inhibition of Pi (inorganic phosphate) uptake. This in turn automatically counteract the ideal function of the mitochondria as ATP creators, thus increasing Pi level.[49] The ATPase stimulating activity of CFSL indicates the presence of some phytoconstituents in the plant that are able to uncouple electron transport from oxidative phosphorylation, resulting from mPT pore opening. Stimulation of ATPase activity by SL could also affect mitochondrial function and change ATP level, mitochondrial transmembrane potential and accumulation of ROS that have been associated with numerous cellular processes including cellular protection, programmed cell death and ageing. Furthermore, stimulation of ATPase activity by fractions of SL fruit extract could thus enhance its anti-tumor activity and might be a potential therapeutic target for mitochondrial-dependent apoptosis. In conclusion, the notion that mPT pore arises from ATP synthase, unlocks a new field of research in uncovering the role of the mPT pore opening in the pathophysiology of cancer at the molecular level. The influence of the fractions of *Sarcocephalus latifolius* (SL) on mitochondrial F1F0-ATPase (mATPase) activity at pH 7.4 is shown in Figure 12.

Scientific evidence demonstrates the production of ROS by mitochondria as side products of respiration.[50] These free radicals majorly target the phospholipid bilayers of cell membranes. The mitochondrial membrane comprises of phospholipids with about 18% of this as cardiolipin and 90% as unsaturated fatty acyl chains.[50] It is documented that the oxidation of this cardiolipin may in fact be a vital factor in initiating programmed cell death by allowing the discharge of cytochrome c from the IMM, hence enabling MOMP.[51,52] These alterations in cardiolipin causes various changes including the folding of the IMM, alteration of its fluidity and organization, and the function of the respiratory complexes.[53] Bearing in mind the role of ROS in many diseases especially age-related ailments, this study was was aimed at investigating the effect of CFSL, EFSL, and MFSL on mitochondrial membrane peroxidation (mLPO) and the probable mechanism in enhancing mitochondrial-mediated apoptosis. LPO assay involves the incubation of liver homogenates with ferrous sulphate ($FeSO_4$) which induces significant increase in mitochondrial lipid peroxidation (mLPO). This elevation in mLPO is basically because some metals (e.g. Fe^{2+}) have the catalytic potentials in the production of highly reactive OH^- through the Fenton reaction process. The ability of the fractions of *Sarcocephalus latifolius* (SL), CFSL, EFSL, and MFSL to prevent LPO was evaluated using the method described by Ruberto *et al.* (2000)[28] and the result is presented in Figure 13.

The results of this investigation revealed that increasing concentration of CFSL and EFSL led to a corresponding increase in the inhibition of Fe^{2+}-induced mLPO. The fractions showed the highest percentage inhibition of 85.00 ± 1.41% and 74.50 ± 0.71% for CFSL and EFSL, respectively at the highest concentration of 1.6 mg/mL. Interestingly, the reverse was the case with MFSL, where there was no inhibition of mLPO, rather an increase in mLPO in a concentration-dependent manner (Figure 13). This could be attributed to the high level of flavonoids and phenolic compounds in SL. Phenolics and flavonoids are well known for their strong antioxidant activity and they are potent free radical scavengers.[54]

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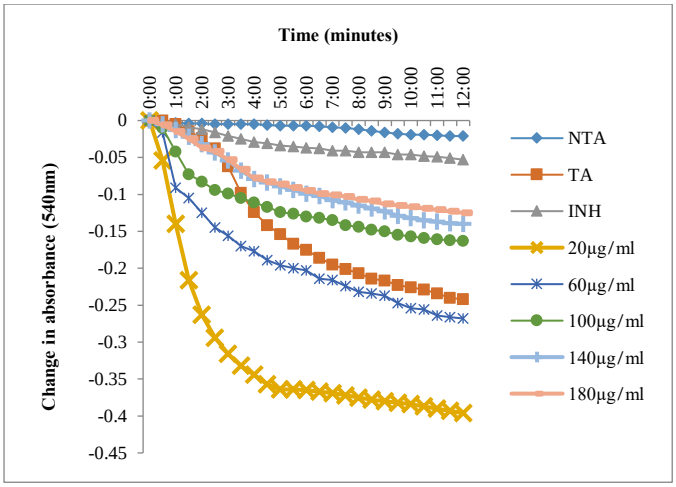

Figure 7: Changes in absorbance of mitochondria due to CFSL in the presence of calcium. There was a reversal of induction. NTA - No triggering agent (without calcium), (*in vitro*), TA - Triggering agent (with calcium), INH - Inhibitor (spermine).

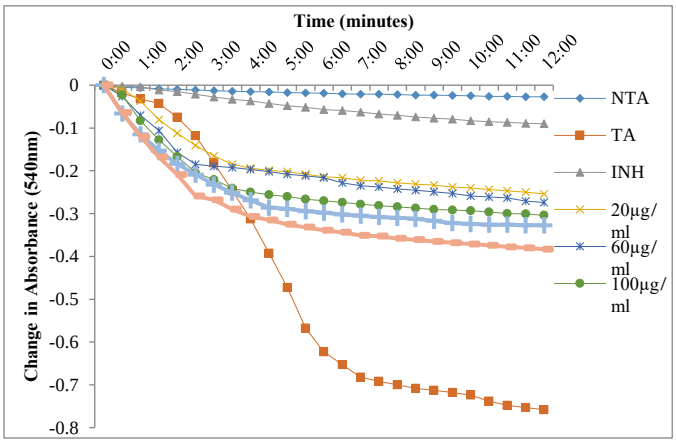

Figure 8: Changes in absorbance of mitochondria due to EFSL in the absence of calcium. NTA - No triggering agent (without calcium), (*in vitro*), TA - Triggering agent (with calcium), INH - Inhibitor (spermine).

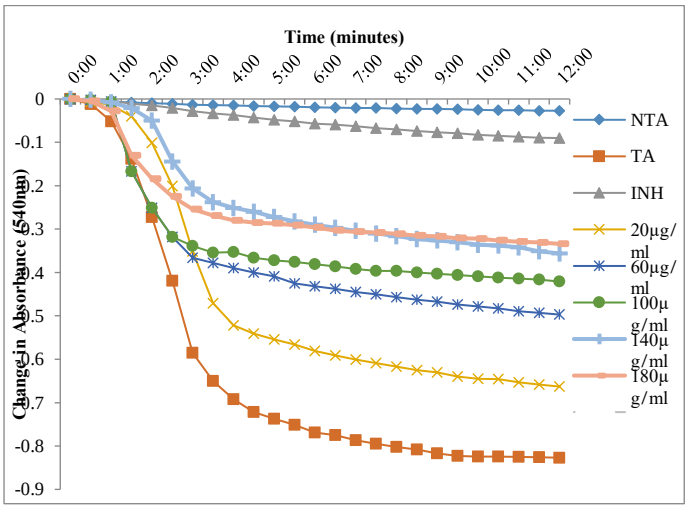

Figure 9: Changes in absorbance of mitochondria due to EFSL in the presence of calcium. There was a reversal in induction. NTA - No triggering agent (without calcium), (*in vitro*), TA - Triggering agent (with calcium), INH - Inhibitor (spermine).

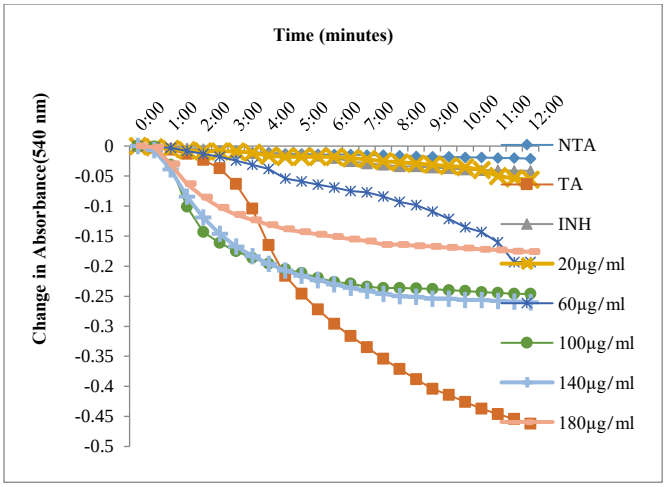

Figure 10: Changes in absorbance of mitochondria due to MFSL in the absence of calcium. NTA - No triggering agent (without calcium), (*in vitro*), TA - Triggering agent (with calcium), INH - Inhibitor (spermine).

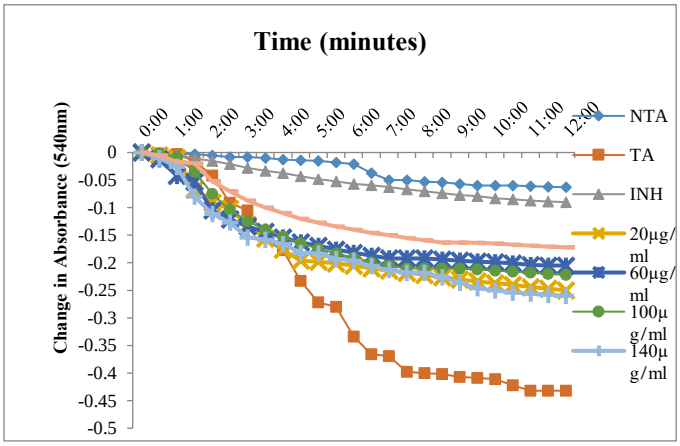

Figure 11: Changes in absorbance of mitochondria due to MFSL in the presence of calcium. NTA - No triggering agent (without calcium), (*in vitro*), TA - Triggering agent (with calcium), INH - Inhibitor (spermine).

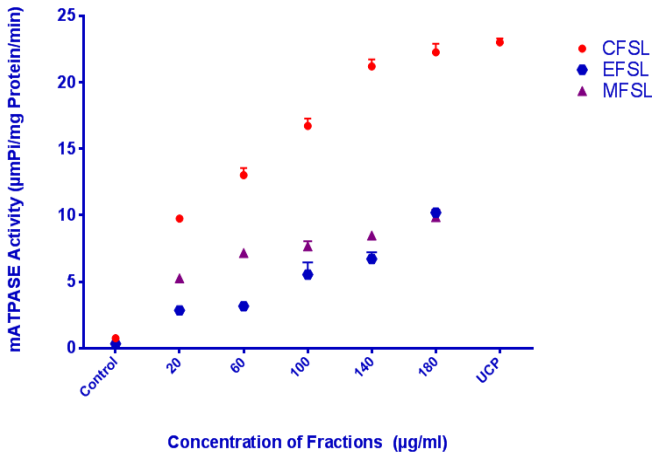

Figure 12: Comparison of the effect of CFSL, EFSL, and MFSL on mitochondrial ATPase activity. Each value is statistically significant at $p<0.05$, compared to the control. CFSL = Chloroform fraction of *Sarcocephalus latifolius* fruits, EFSL = Ethyl acetate fraction of *Sarcocephalus latifolius* fruits, MFSL = Methanol fraction of *Sarcocephalus latifolius* fruits.

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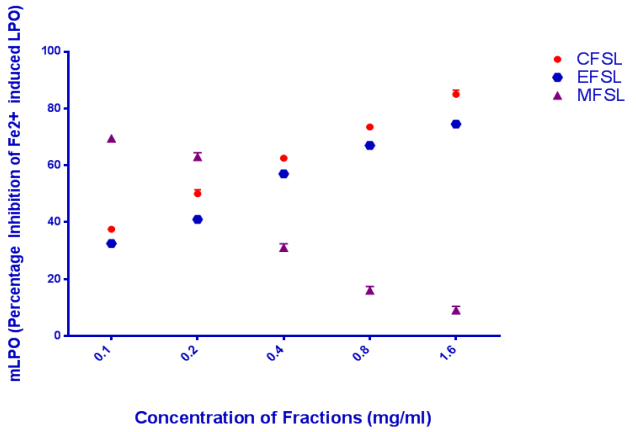

Figure 13: The effect of CFSL, EFSL, and MFSL on Fe^{2+} - induced Lipid Peroxidation, *in vitro.* Each value is statistically significant at $p < 0.05$ compared to the control. CFSL = Chloroform fraction of *Sarcocephalus latifolius* fruits, EFSL = Ethyl acetate fraction of *Sarcocephalus latifolius* fruits, MFSL = Methanol fraction of *Sarcocephalus latifolius* fruits.

These phytochemicals have been shown to interrupt or even terminate mLPO by chelating metal ions.[54] The results from this study suggest that *Sarcocephalus latifolius* fruit may possess protective effect on the membrane bilayer, thus preventing free radical-induced cellular damage. The findings from this study also suggest that SL contains bioactive constituents that may act as free radical scavengers and also propose that the induction of mPT pore opening by SL may not be via the production of ROS, which could cause mitochondrial membrane lipid peroxidation, but possibly through the interaction of the bioactive constituents of SL with the pore components. This rules out mLPO as a likely mechanism for mPT induction by SL fractions. The effect of SL fractions on Fe^{2+}-induced mitochondrial lipid peroxidation (mLPO) in normal rat liver mitochondria is shown in Figure 13.

Cytochrome c (Cyt c) is situated in the mitochondrial IMS, functioning majorly as an electron shuttle in the respiratory chain and interrelates with cardiolipin (CL) in healthy cells.[13,55] In the cytoplasm, Cyt c controls the allosteric stimulation of Apaf 1 that is essential for the proteolytic development and maturation of caspase-9 and caspase-3.[13] These stimulated proteases eventually cause apoptotic cell disassembly. Nonetheless, the released cytochrome c in the cytoplasm has been implicated in some major cell functions (e.g. differentiation); and there are rising opinions that its release from the mitochondrial IMS does not always take place in an all-or-nothing manner and that MOMP may not always lead to apoptosis.[13,55] Furthermore, the release of cytochrome c due to mitochondrial pore opening has been shown to be key in the stimulation of mitochondrial-mediated apoptosis.[52,55]

Figure 14 shows the results of the effect of the chloroform fraction (CFSL), ethyl acetate fraction (EFSL), and methanol fraction (MFSL) on cytochrome c release in isolated intact mitochondria of normal rat liver. There was an increase in the concentration of cytochrome c released with increasing concentration of the fractions (CFSL and EFSL), and this was significantly different from the control ($p \leq 0.05$) at all the concentrations. For MFSL, there was slight increase in the concentration of cytochrome c released with increasing concentration of the fraction. A significant difference ($p \leq 0.05$) from the control was observed only at 180 and 220 µg/mL. Among the three fractions, CFSL had the highest activity in terms of the release of cytochrome c, followed by MFSL, while EFSL had the lowest activity, corresponding to about 29, 25, and 16 folds for CFSL, MFSL, and EFSL, respectively when compared to the control. The increased release of cytochrome c caused by CFSL could be attributed to mPT pore opening. This result conforms to the mPT result where CFSL also had the highest effect.

So far, the study has demonstrated the effects of the fractions of SL (CFSL, EFSL and MFSL) as potent inducers of mPT pore *in vitro* using isolated mitochondria from rat liver. CFSL exhibited the highest activity with respect to stimulation of mATPase activity, inhibition of mLPO, and induction of mPT pore opening. These observations suggest that the bioactive constituents triggering the induction of mPT pore opening might be more in the less polar chloroform fraction compared to the more polar ethyl acetate and methanol fractions. However, the study may be limited by small sample size, lack of *in vivo* validation, potential variability in mitochondrial isolation techniques, and the need for further investigation into the specific mechanisms underlying the observed effects. Therefore, future research should focus on expanding the sample size, *in vivo* studies to validate the *in vitro* findings, standardizing mitochondrial isolation protocols to reduce variability in assay results, exploring the molecular pathways involved in mPT pore modulation by *Sarcocephalus latifolius* fractions, and investigating the potential applications of these findings in clinical settings.

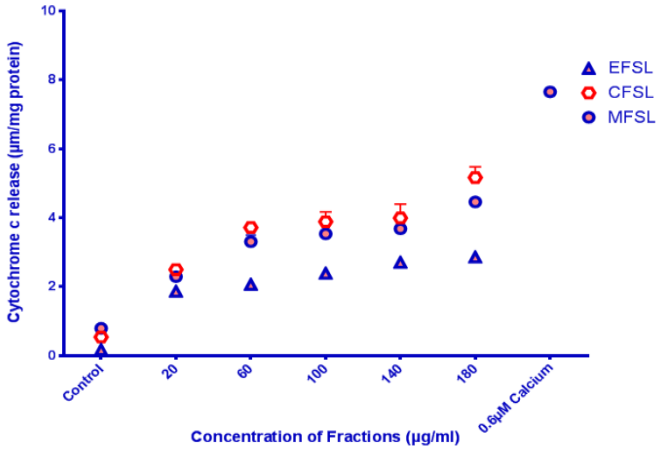

Figure 14: Cytochrome C release (CCR) by CFSL, EFSL and MFSL following mPT induction. n = 6. CFSL = Chloroform fraction of *Sarcocephalus latifolius* fruits, EFSL = Ethyl acetate fraction of *Sarcocephalus latifolius* fruits, MFSL = Methanol fraction of *Sarcocephalus latifolius* fruits.

Conclusion

Sarcocephalous latifolius (SL) is a plant that occupies a notable place in traditional medicine. The findings from the present study suggest that the chloroform, ethyl acetate, and methanol fractions of SL (CFSL, EFSL, and MFSL) contain bioactive compounds that induce mPT pore opening and subsequently induce mitochondrial-mediated apoptosis with CFSL being the most active. Therefore, SL fractions especially CFSL could serve as a potential source of bioactive compounds with beneficial effect in the treatment of ailments that require the up-regulation of apoptosis, including the treatment and management of tumors.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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