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RESEARCH ARTICLE

MODULATION OF MITOCHONDRIAL MEDIATED APOPTOSIS BY SOLVENT FRACTIONS OF THE FRUIT EXTRACTS OF *SARCOCEPHALUS LATIFOLIUS* (SMITH) BRUCE

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Abstract

Background and aims: The mitochondrial membrane permeability transition (mPT) pore is a critical event exploited in situations where apoptosis is dysregulated. Bioactive agents of plant origin induce mitochondrial-mediated apoptosis via the opening of the mitochondrial outer membrane permeabilization (MOMP). The fruit of *Sarcocephalus latifolius* is used in folklore medicine for the treatment of tumors and cancer. However, this claim has not been scientifically substantiated.
Methods: In this study, we investigated the inductive effect of the crude methanol extract (CMESL) and a chloroform sub fraction (sCFSL) of *Sarcocephalus latifolius* fruits on mPT, *in vivo*. Thirty-five male wistar rats (90 ± 10 g) were acclimatized, divided into seven groups, and treated with 1% DMSO (control) and 25, 50, and 100 mg/kgbw of each of the fractions for thirty days. Rats were sacrificed and liver mitochondria were isolated by differential centrifugation. The MOMP, DNA fragmentation, p53, Bax and BCl-2 protein expressions, Cytochrome c release, and caspase – 3 and -9 activities were assayed in liver tissue by standard methods. CMESL and sCFSL induced mPT pore.
Results: The sCFSL induced MOMP maximally at 100 mg/kgbw, inhibited LPO (78%), enhanced mitochondrial ATPase activity (27.96± 0.04 µmole/mg Protein/min) than CMESL (17.58± 0.03 µmole/mg Protein/min) at the same dose. Similarly, sCFSL caused DNA fragmentation; (77.33%), enhanced caspases -3 and -9 activation; increased p53 and Bax expression levels, increased Cytochrome c release, and downregulated BCl-2 protein expression, compared to CMESL.
Conclusion: These findings showed that sCFSL contains bioactive agents that can induce mitochondrial-mediated apoptosis, and therefore a potential target to be explored in the management of tumors and cancer.
Keywords: Apoptosis, cancer, MOMP, mPT pore, *Sarcocephalus latifolius*.

INTRODUCTION

The reality of the high incidence of cancer, the increasing trend, and several side effects associated with its management, also make cancer one of the main causes that threaten human life globally[1]. This has been reported in the biennial report 2020–2021 issued by the International Agency for Research on Cancer (IARC) of the World Health Organization[2]. Cancer is a heterogeneous disease described by cell death disorder (CDD). With a global challenge like this, it, therefore, becomes crucial to elucidate its unfathomable pathogenesis and find a lasting management therapy with little or no side effects[2]. The greatest challenge in cancer therapy and management has been how to target the undesired tumor cells for destruction whilst minimizing the effect on the normal traditional surrounding cells. This is seen to be more pronounced when using the traditional methods of cancer therapy (e.g. cytotoxic chemotherapy) as compared to using natural bioactive agents from plants as alternatives. Cytotoxic Chemotherapy destroys malignant cells via the induction of programmed cell death (PCD), not sparing the normal cells[3, 4]. The improved knowledge and a good grasp on apoptosis has been established over the past decades and is today providing novel openings for aiming at the vulnerabilities in tumors and cancer.

Apoptosis, also called programmed cell death (PCD), or Regulated cell death (RCD), is a highly preserved

program and a natural way for eliminating impaired and undesirable cells in the body. Apoptosis is a programmed cell death that occurs in multicellular organisms, with features like shrinkage of the cytoplasm, chromatin condensation (pyknosis), fragmentation of the nuclear (karyorrhexis), and blebbing of the plasma membrane, and terminating this process with the development of minor unabridged vesicles called apoptotic bodies[5, 6]. Because of the alterations in permeability, these unabridged apoptotic bodies are produced, which adjacent cells proficiently engulf and destroy[7]. In most cells in multicellular organisms, apoptosis is synchronized on mitochondria by the Bcl-2 family of proteins. The equilibrium between pro- and anti-apoptotic Bcl-2 family proteins sets inception for mitochondrial apoptosis, an equilibrium that is changed throughout malignance development. Subsequently, evasion of cell death is a recognized malignancy seal[2, 1]. Effective elimination of cancer cells by programmed cell death or apoptosis has been a mainstay and goal of clinical cancer therapy for over 3 decades[8].

Accumulating evidence indicates that RCD/PCD subroutines are the significant biochemical features of tumorigenesis, which may eventually lead to the founding of diverse possible beneficial stratagems. Hitherto, aiming at these subroutines with pharmacological small-molecule compounds (e.g. the bioactive agents in medicinal plants) has been developing as an auspicious therapeutic possibility, which has promptly progressed in various forms of human cancers[2]. Furthermore, more recently, apoptosis is an established mechanism used in the elimination of most tumor cells[9, 10].

Apoptosis is classified as intrinsic (mitochondrial-dependent/ mediated pathway) or extrinsic (death receptor pathway). Noxious substances trigger the mitochondrial-mediated pathway or DNA impairment that causes dysregulation or inequity of intracellular homeostasis and is regulated by distinct signals. This disorder is branded by augmented permeability of the outer mitochondrial membrane (OMM), with subsequent discharge of cytochrome C, which is a point of no return for apoptosis to occur in cells[11]. Permeabilization of both the IMM and OMM has been implicated as the greatest significant footmark in apoptosis-mediated cancer cell death (CCD)[1]. The release of mitochondria outer membrane permeability (MOMP) and cytochrome C causes the development of apoptotic bodies and the activation of caspase-3. The mitochondrial-mediated pathway of apoptosis is predominantly controlled by its influence on mitochondria[11]. Mitochondrial Membrane Permeability Transition (mPT) is the sudden permeabilization of the Inner Mitochondrial Membrane (IMM) in response to a noxious stimulus such as oxidative stress, Ca^{2+} overload, hypoxia, and cytotoxic drugs[11]. The mPT pore opening results in mitochondrial depolarization, swelling, rupture of the OMM, and cell death through apoptosis[11]. The mitochondrial permeability transition pore (mPTP) has been established for regulating apoptosis-mediated CDD[1].

There are rumors that malignant cells seem to be more resistant to PCD than their complement traditional cells but the practicalities of the regulations of PCDs in malignance are very complex and delicate[2]. Furthermore, several positive manipulations of mitochondrial-mediated apoptosis to develop new beneficial methods to solve the issue of selectivity have been on the increase in recent times. Though the precise mechanism fundamental to mPTP-mediated cell death is still indefinable, mPTP-dependent apoptosis mechanism has been well thought out as a significant hold that plays a vital role in the pathogenesis of various forms of tumors[1].

Mitochondria, which are called the ‘power house’ of the cell are organelles found in multicellular organisms, involved in various types of cell damage, biosynthesis, bioenergetics, and signaling functions[13, 1]. Mitochondria have been recognized as an essential pharmacological target for the development of cytotoxic drugs[14, 15] and their dysregulated function has been proven essential for tumorigenesis, tumor growth, and tumor metastasis.

Furthermore, mitochondria play an essential role in PCD (Fig. 1) and arbitrate the mitochondrial-mediated apoptosis program characterized by cytochrome c release, which is regulated by Bcl-2 family proteins governing the MOMP. Bcl-2 family proteins can be divided into two broad categories, including members that function as inhibiting apoptosis (pro-survival/antiapoptotic) and those that trigger apoptosis (pro-apoptotic) functioning as a central force in the stress-signaling networks. It is now known that these Bcl-2 proteins are often dysregulated in many cancers with anti-apoptotic members extremely expressed or their pro-apoptotic counterparts’ downregulated, resulting in increased persistence of malignant cells[13].

***Sarcocephalus latifolius* (Smith) Bruce (African peach)**

(Syn: Nauclea latifilia)

Sarcocephalus latifolius (SL) is one of the top-ranking names in the list of medicinal plants of the world and has been identified with several names depending on its location site in the world[16, 17]. In West Africa, it is identified with different names among nations and tribes. In Nigeria, the Igbo people usually call it "ubulu-inu, or" "Odo-uburu", the Hausas identify it as "Doundake", "Tafashiya" or "tashiyaigia" or "marga", while the Yoruba tribe identifies it as Egbesi/ Ogbesi or Ogbase. Other localities of Nigeria will have a typical vernacular name related to them. Western countries also have English names attached to this plant. These include "pin cushion tree", "African peach", "African Cinchona", "Guinea peach" or "Sierra Leone peach", "Country fig" and "strawberry tree" [17, 18]. The trade name for this plant is Opepe. The French people have also identified the plant as Scille Maritime, Oignon marine, or Medicinal Squill. Its generic name *Sarcocephalus latifolius* is resultant of two Greek words: *Sarco* meaning 'fleshy' and *cephalus* meaning 'headed' with reference to its flowers[19, 20].

Sarcocephalus a genus of humid perennial plants and shrubs has its place in the Rubiaceae family. This plant family has also risen to be one of those often used by

Ethnomedicinal doctors in Sierra Leone and other nearby countries. This evergreen multi-stemmed shrub or minor dispersal tree is a plant that usually develops up to a height of 200 m, and is usually found commonly in the damp temperate tropical forest region or savannah woodlands of West and Central Africa. The flowers of the tree are usually seen from April to June. The fruit is syncarp and fruit ripening takes place from July to September[19]. In Nigeria, one can get fully matured and ripened fruits from October to March, depending on where the fruits are harvested (Figures 2a & b). The fruit, which is not so common in Nigeria, is edible to humans but the Baboons who eat the ripe fruits at ease make the propagation of seeds possible. This plant also offers ecosystem services by way of erosion control. *S. latifolius* is a suitable species for soil conservation and stabilization, offers shade, and acts as a windbreak, and a soil improver, and the leaves of the tree are used as mulch on the farm[21].

There are many uses of SL in traditional medicine[18, 22]. Ailments like stomach pains, fever, and diarrhea, as an antiparasitic and anti-malaria and more recently severe pains, (analgesic effect), due to the significant amount of Tramadol found in the plant[23], have been managed with infusions and decoctions of the bark and leaves of SL in both humans and animals, in the West and South Africa. In Nigeria, *Sarcocephalus latifolius* has also been used traditionally in the management of hypertension[24, 25] and diabetes[26]. Many bioactive agents of plant origin have been found to induce mitochondrial-mediated apoptosis via mPT pore. SL has also been used pharmacologically in the management of many diseases but mitochondrial or its modulatory effects on mitochondrial-mediated apoptosis have not been implicated. Furthermore, the use of the various extracts of these plant parts for the control of various illnesses especially in Africa is widely documented, but there is little or no documentation on the use of the fruit extracts.

The study was therefore carried out to investigate if solvent fractions of SL fruit extract would have an effect on mitochondrial–mediated cell death, and thus serve as a possible drug candidate in an animal model.

MATERIALS AND METHODS

All reagents and organic solvents used for extraction and processing of the plants, (Sigma-Aldrich Chemical) were of a high analytical grade.

Harvesting and the processing of plant

Plant sample preparation

Fresh matured and ripe fruits of *Sarcocephalus latifolius* (Smith) Bruce were harvested in the forests and on the forest paths along Eruwa, Iddo LGA of Oyo State, during the dry season, in December 2022. The fruits were harvested a little at a time. Three herbarium samples of *Sarcocephalus latifolius* whole plants were sent to the Forestry Reserve Institute of Nigeria (FRIN) for taxonomical authentication. The authenticity of the plant was confirmed and a voucher number (FHI 110092), was allocated to the sample. The sample was later deposited in the herbarium in the Pharmacognosy Department, at the University of Ibadan for future reference.

Processing of the harvested plant part to the crude methanol extract and other solvent fractions.

The fruits of *Sarcocephalus latifolius* (SL) were washed, pulverized and air-dried at room temperature. This was pulverized to powder, using a mortar and pestle. The powdered fruits were weighed and soaked in 100% methanol (Sigma, Aldrich Chemical Co. St. Louis, USA), (50g: 500ml) for 72 hours and filtered with Whatman No.1 filter paper, to obtain the "methanol extract" of the fruits of *Sarcocephalus latifolius*. The methanol filtrate was concentrated using a Vacuum Rotary Evaporator (Stuart Rotavapor, UK) at 40ºC to obtain an extract, of a dark brown chocolate mass and left in a water bath at 37^0C for about four days to dry and be void of any solvent. The percentage yield was calculated to give 6.4%. The crude methanol extract (CMESL) was used to obtain N− Hexane, chloroform, ethyl acetate, and the methanol fractions using vacuum liquid chromatography and the different solvents in increasing order of polarity. The crude extract and solvent fractions were stored in a refrigerator at 4°C for further use.

The percentage yield (%) of the CMESL/ solvent fractions was estimated as follows:

% yield of CMESL/ fractions = $\{(X - Y)/Z\} \times 100\%$

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

Further Purification of the chloroform Fraction (CFSL)

This was carried out in a Vacuum Liquid Chromatography (VLC) apparatus, using different solvent systems. This is made up of a sintered glass funnel and a fitted Buckner′s flask with an attached hose rubber that eventually fits into a pump during elution. The process of the purification of the chloroform fraction of the fruit extract of *S. latifolius* (CFSL) was achieved starting with a solvent system containing 100% N- Hexane to 100% methanol. As seen in Table 2, other combinations of solvents were used according to their increased order of polarity.

The procedure for the elution process is stated in Table 2. The gradient elution was achieved using a VLC apparatus with a thinner and longer VLC flask to enhance the purification of the fraction. The sintered glass allows for the observation of the different bands, eluted with care by the careful alteration of the ratios of the solvent systems. Filtrates were collected in glass "Bama" bottles with foil-covered lids to avoid contamination. The filtrates were concentrated separately by evaporation to avoid contamination via the rotary flask evaporator, RE-52A, LAB SCIENCE, England, to give ten (10) different sub-fractions of the chloroform fractions named sCFSL1 – sCFSL10. TLC was also done on each of the samples using nine different mobile phases. The spots on the different plates from each sample, run by the different mobile phases were observed, read, and interpreted under the UV lamp at different frequencies and wavelengths. The best mobile phase was resolved to be the Chloroform

(95%, 19): Methanol (5%, 1) solvent system, and RF values were calculated for each of the spots.

sCFSL 8 eluted with a solvent system of Chloroform (50%): Methanol (50%) was the most potent sub-fraction using mPT pore opening activity as a bio-guided assay to establish this decision.

Experimental animals

An ethical approval certificate was collected from the Animal Care Use and Research Ethics Committee (ACUREC), (Reference number UI – ACUREC/App/10/2017/006) of the University of Ibadan, Nigeria.

About 120 male albino rats (Wistar Strain) with an average weight of 80g bought from the veterinary anatomy department, University of Ibadan, Ibadan were used in the in vivo study. 105, (35) animals were used three times for the main study and fifteen animals were used for the pilot study before the main study. The rats were allowed to acclimatize, divided into seven groups of five rats each, and treated for 30 days. The rats were kept in aired cages with 12 hours of light/dark cycling and were given rat chow and water *ad libitum*. The grouping table is shown in Table 1. The route of administration was intragastric (oral).

Table 1: Grouping and treatment dose of the treated animals.

Group	Treatment	Dose (mg/KgBW)
A (Control)	1% DMSO	1 ml
B	sCFSL	25
C	sCFSL	50
D	sCFSL	100
E	CMESL	25
F	CMESL	50
G	CMESL	100

Low ionic strength rat liver mitochondria were prepared following the technique of Johnson and Lardy (1967) as modified by[27] and described by[28]. The liver tissue was washed with ten percent suspension of tissue in an 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 4^0C to eliminate complete/unbroken cells. This was followed by centrifugation at 13,000 rpm for 10 min of the supernatant obtained from the sedimentation of the nuclear fraction to give the mitochondria pellet. The pellet was washed twice by spinning at 12,000 rpm for 10 min to make it is free from any debris or contaminant, while remaining intact. The mitochondria were instantly suspended in a solution of ice-cold MSH Buffer (pH 7.4), stored in aliquots using Eppendorf micro-tubes already placed on ice for prompt usage.

Assessment of Mitochondrial Permeability Transition (mPT) in rat liver mitochondria

Accretion of Ca^{2+} in mitochondria may result in mitochondrial permeability transition (mPT). The inner mitochondrial membrane (IMM) develops a non-discriminating porousness to small (1.5KDa) solutes[29]. Isolated mitochondria experiencing mPT demonstrate great amplitude swelling/distension that leads to a reduction in absorption at 520nm. mPT was assessed experimentally by quantifying mitochondria distension which in turn is measured by a reduction in absorbance.

To evaluate mPT, mitochondrial distension was quantified by the method of Lapidus and Sokolove (1994), as described by Nwachefu *et al*, 2022. The experiment was initiated by adding mitochondrial protein (0.4 mg/mL) to 0.8 µmol, swelling buffer (pH 7.4). The solution was pre-incubated with $CaCl_2$ at 30^0C. After 30 seconds, the mitochondria was strengthened by adding 50 µM succinate. The absorbance of the subsequent medium was measured at 540 nm each 30 seconds for 12 min.

Determination of mitochondrial atpase activity

The mitochondrial ATPase (mATPase) activity was determined by the technique of[30]. The concentration of the inorganic phosphate (Pi) discharged throughout the reaction was determined by the method of[31], with slight modification by the method of[27]. The alteration involved utilizing 1mg/ml in the place of 2 mg/ml mitochondrial protein for the assay.

Eleven glass test tubes were arranged in duplicates in a test tube rack. To each tube, was included 0.25 M sucrose, 5 mM KCl, and 0.1 M Tris. Variable concentrations of the CMESL and sCFSL were integrated into tubes correspondingly and were made up to 2 mL with distilled H_2O. 10 mM ATP (1 mL) was added to the tubes, and placed in a shaker water bath at 27ºC after careful mixing. At zero-time, mitochondria were added and the response reaction aborted promptly by the adding 1 mL 10% SDS. With the exception of the blank test tube, mitochondria was added to others, 2,4-DNP was added into the uncoupler labeled tube, and the mixture shaken for 30 minutes. Finally, the reaction was stopped on addition of 1 mL SDS to all test tubes (except for zero time) every 30 seconds, and 1mL of the reaction mixture taken for phosphate determination.

Determination of inorganic phosphate

Distilled water (4mL) was incorporated into 1 mL of the sample in a test tube. 1 ml of 1.25% Ammonium molybdate (1 mL of 1.25%) and 1 ml of a 9% freshly prepared solution of ascorbic acid was added. The content was mixed thoroughly and left for twenty minutes. This procedure was repeated using standard solution of potassium dihydrogen phosphate (0.2 mg pi per 5 mL). The strength of the coloured complex formed was read at a (λ) of 660nm using a Camspec M105 Spectrophotometer. A standard calibration curve of phosphate (Pi) was prepared from which the concentration of Pi released was calculated.

ASSESSMENT of mitochondrial Lipid Peroxidation (mLPO) (*In vivo*)

Lipid peroxidation of rat liver mitochondria was estimated utilizing an adapted thiobarbituric acid reactive species (TBARS) procedure by[32].

Mitochondria aliquot (0.4mL) and CMESL or a sub-fraction of CFSL, sCFSL was mixed with Tris-Potassium chloride buffer (1.6mL) in different tubes, to which 30% TCA (0.5mL) was added. Thiobarbituric acid **(**TBA), (0.5mL of 0.75%) was added to the reaction medium and left in a water bath for 45 minutes at 80^0C. The tubes were placed in ice to cool, and

centrifuged at 3000 rpm for ten minutes. Thereafter the clear supernatant carefully extracted using a pipette, was evaluated for absorbance measured against a reference blank of distilled H_2O at 532 nm.

Determination of caspase 3 and caspase 9 activity

The Human CASP3 (Caspase 3) ELISA Kit with Catalog No: E-EL-H001796T and "Human CASP9 (Caspase 9) ELISA Kit, a product of ELabScience Biotechnology Ltd., Technology Industry Park, Wu Han, Peoples Republic of China" was employed for this assay. This kit uses the Sand-ELISA as the method. A microplate reader (DNM-9600A from China) was employed in interpreting the OD at 450nm for the determination of Caspase 3 and Caspase 9 activity respectively. To ensure accuracy in measurements and precision, reagents were not used directly as supplied in terms of the inscribed volume on the reagent bottles but were rightly measured before usage.

Assay of dna fragmentation by diphenylamine (dpa) method

This technique, as defined by[33] was employed to evaluate the endonuclease split products of "apoptosis" in the excised liver from the control and treated rats. DNA is hauled out from tissue homolysate ("homogenate"). The "supernatant" and pellets are together exposed to a "Diphenylamine (DPA)" solution for developing the color complex. The absorbance is then taken spectrophotometrically at 620nm.

Immunochemical Assay for Apoptotic Markers

Immunohistochemistry (IHC) detects antigens (proteins) in cells of a tissue section by taking advantage of the principle of antibodies binding precisely to antigens in tissues. This method was exploited in this study for the detection of various stages of the apoptotic process. These include the detection of the activity of BCl_2 (anti-apoptotic protein), BAX protein (pro-apoptotic protein), p53, and Cytochrome c (playing a role in the rate-limiting step in the mitochondrial-mediated apoptosis).

Preparation of Immunohistochemistry samples

The liver sections from the animals treated with both the CMESL and a subfraction of the chloroform fraction, sCFSL were briefly immersed in 10% phosphate buffer formalin (PBF), then placed in rated alcohol to allow for dehydration and later entrenched in 100% paraffin. Good section units were obtained from these waxed tissues and fixed on glass slides. The antibody DF used in this study was a 1:100 dilution for all the detected antibody markers.

Immunohistochemical Assays

The main antibodies used in this study were Bcl-2, p53, Bax, and Cytochrome c (Elabscience product). The procedures were performed in line with the manufacturer's guidelines. The treated tissue was divided into two microns on the rotary microtome, and placed on the hot plate at 70^0C for about an hour. The sections were then passed through different changes two variations of xylene, three changes of descending ranks of alcohol, and lastly water. The sections were transferred into a boiling "citric acid solution of pH 6.0" for fifteen minutes. Cold water was used to displace the hot citric acid for another five minutes to cool the sections. Peroxidase blocking was completed by just casing them with 3% H_2O_2 for fifteen minutes. These segments were then rinsed with Phosphate Buffer Saline (PBS,) and biotin was used to block endogenous biotin in tissue. After washing with PBS, these sections were incubated with the individual diluted main antibody for 60 minutes.

After this time-lapse, the surplus antibody was properly rinsed off using PBS and a 2^0 antibody (LINK) was applied on the sections for fifteen minutes. Horse Radish Peroxidase (HRP) was applied and used in washing these sections for another fifteen minutes. The leftover of the HRP on the sections was rinsed off using PBS and a working DAB solution was applied to these sections. This gave rise to the observation of a brown reaction almost immediately for an antibody-positive target. Later on, the excess DAB solution and precipitate were washed off with distilled water, and counterstaining of sections was performed using Haematoxylin solution for about two minutes and blued fleetingly. Finally, the tissue segments were dried in alcohol, cleared in xylene and fixed in DPX, and observed under the microscope. Cells with the specific brown color from the DAB reaction, in the cytosol, cell membrane, and the nuclei depending on the antigenic sites were counted as positive and the hematoxylin-stained cells void of any form of brown coloration were scored negatively. All other non-precise binding/ brown relics on cells and connective tissues were ignored. Therefore, positive signals for Bcl-2, p53, Cytochrome c release (CCR), and Bax were represented as brown. The positive staining strength was computed as the ratio of the discolored or stained area to the entire field evaluated using the Image J software.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) of at least three independent measurements (assays). One-way analysis of variance (ANOVA) and Duncan's multiple Range Test (DMRT) was carried out. All statistical analyses were carried out using IBM SPSS Version 20. The p-values of less than 0.05 were adopted as statistically significant. Immunohistochemical plates were analyzed using the Image J scientific application.

RESULTS AND DISCUSSION

A major global health problem that has progressively remained a great concern for decades is cancer. With this emerging universal anxiety, cancer deterrence is one of the most noteworthy public health challenges of this age[1]. The total loss of apoptotic regulation mechanisms has given way to an increase in the survival of tumor or cancer cells. This has also enhanced and improved the buildup of alterations that promote intrusiveness throughout growth development, angiogenesis stimulation, deregulation of cell proliferation, and any form of interference with cell differentiation[34-36]. To date, globally, scientists certainly believe that the dysregulation of apoptosis and mitochondrial dysfunction is a hallmark and therefore a common feature in all forms of

malignancies, with a fallout of the promotion of cell accumulation[1]. There have been persistent disappointments and failures in the modalities available for the management and treatment of tumors[37-38]. Orthodox methods include radiotherapy, chemotherapy, and a combination of the two or a total removal of the affected area by surgery[39]. Even though sometimes effective, there have been quite a number of side effects including the destruction of normal body cells[39-40]. This has led to a search for an approach or treatment of tumors that will be effective, with very little or no side effects while preserving the normal body cells and recording high survival rates of the individuals living with this disease.

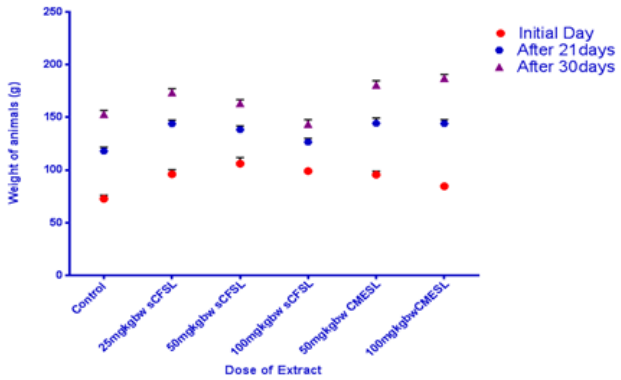


Figure 1: Growth pattern during exposure to the crude extract (CMESL) and a chloroform sub-fraction (sCFSL) for a period of 21 days and 30 days.

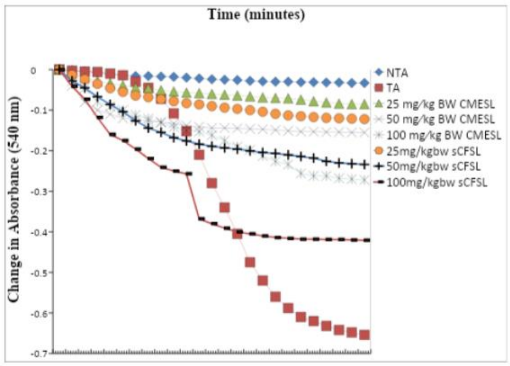


Figure 2a: Changes in absorbance of mitochondria after 21 days of treatment with CMESL and sCSFL, in the absence of calcium.

NTA: Control, TA: "Triggering agent" (Ca^{2+})

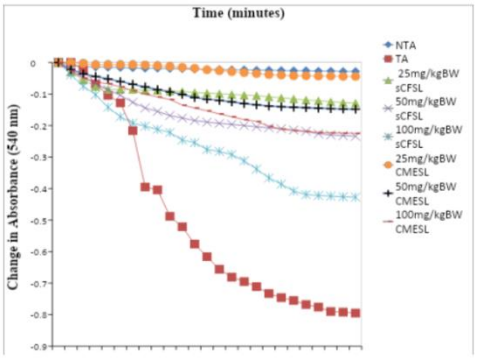
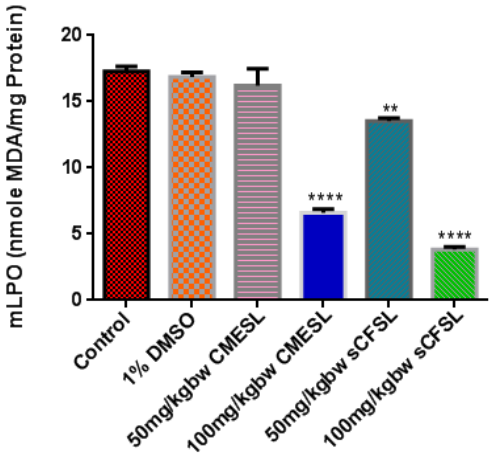


Figure 2b: *In vivo* effects of both CMESL and sCSFL on the induction of mPT after 30 days of treatment, using the same doses.

Induction was in a concentration-dependent manner, with the highest induction at the highest dose of of sCSFL, 100mg/Kg BW.
NTA: Control, TA: "Triggering agent" (Ca^{2+}).



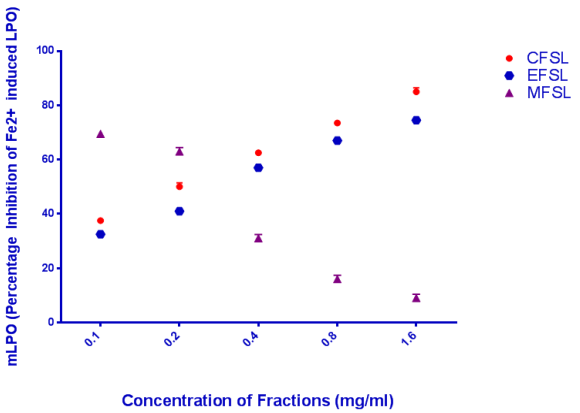


Figure 3a & 3b: Effect of varying doses of CMESL and sCFSL on rat liver mitochondrial LPO (*In vivo*).

The effect of the three fractions, CFSL, EFSL & MFSL on Fe^{2+}- induced Lipid Peroxidation, *in vitro*; -, - or - Each value is statistically significant at $p<0.05$, compared with control using the one-way ANOVA

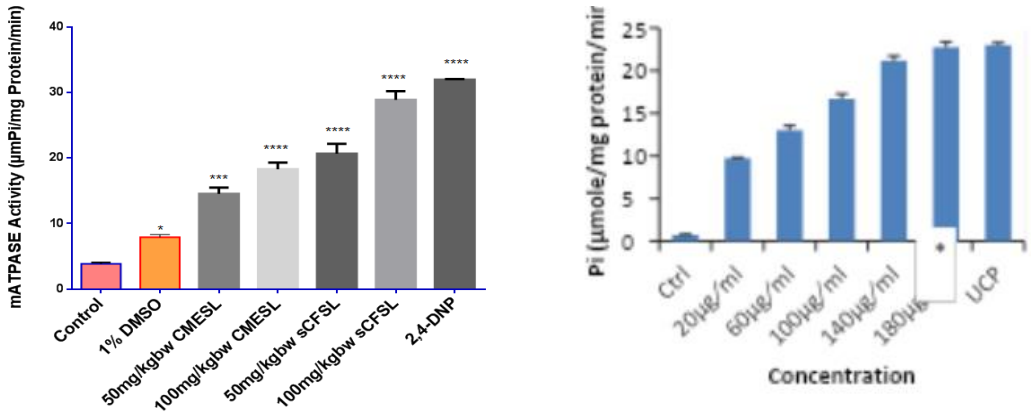


Figure 4a & 4b: Effects of CMESL and sCFSL, on rat liver mitochondrial ATPase activity at pH 7.4, (*In vivo*).
n=6, * significantly different from control at ($p< 0.05$), ***($p<0.001$) and ****($p<0.0001$), and the effect of varying concentrations of chloroform fraction of *Sarcocephalus latifolius* (CFSL), on rat liver mitochondrial ATPase activity at pH 7.4. n=5, * significantly different from control ($p< 0.05$).

Furthermore, mPT has been implicated as the most significant footmark in mitochondrial-mediated apoptosis CCD. Factors such as mitochondrial calcium overload, oxidative stress, and hypoxia are conditions favoring mPT[1]. This leads to the introduction of a nonspecific channel opening with a well-defined diameter in the mitochondrial membrane that permits an unrestricted interchange amid the mitochondrial matrix and the solutes and proteins up to 1.5 kDa in the extramitochondrial cytosol[1]. Additionally, this channel/nonspecific pore is called the mitochondrial permeability pore (mPTP) opening[1]. Several scientific documents have established mPTP in the regulation of the mitochondrial-mediated apoptosis CCD.

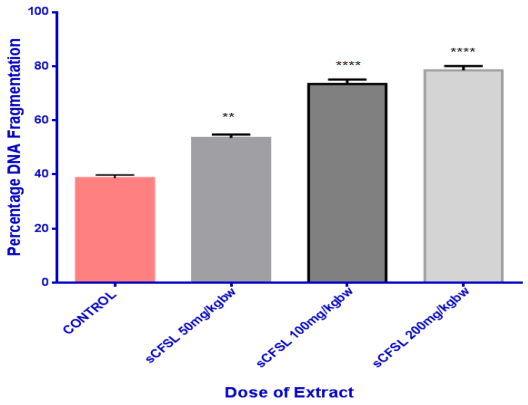


Figure 5: Effect of different doses of sCFSL on percentage DNA fragmentation activities after 30 days of administration.
, values are statistically significant at $p< 0.01$, and ** means, values are statistically significant at $p< 0.0001$, compared with control using the one-way ANOVA.

Results of the study also established that alteration of the balance between pro-death and pro-survival members of the Bcl-2 family could arise in favor of pro-death players by reducing Bcl-2 and Bcl-XL expression levels that are usually extremely expressed at the beginning of many tumors[41]. This is in line with the findings that tumor cells rely on elevated levels of Bcl-2 to counter the continuing upregulation of pro-death BH3-only molecules in reaction to oncogenic pressure[41-42]. Although the definition for apoptosis differs amongst researchers, there is a universal settlement that it is a cell death procedure encompassing caspase stimulation, void of cell inflammation with preservation of organelle (specifically mitochondria and endoplasmic reticulum) integrity. This was why for each assay involving mitochondria, its integrity *ab initio* could not be overemphasized.

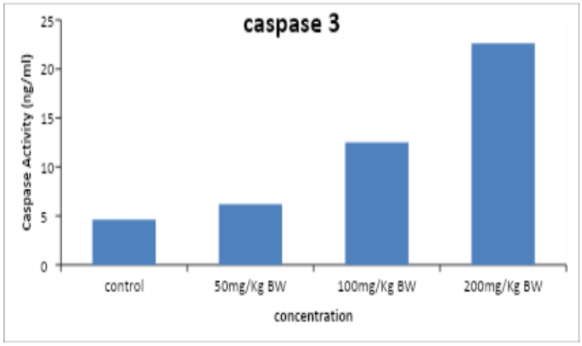


Figure 6: Estimation of the Caspase- 3 activity in rats treated with different doses of sCFSL.

The matured fruits of *Sarcocephalus latifolius* have been used in native medicine in the management of tumors but to date, the possible mechanism of action has not been established. There is also a lack of scientific information or data to support these claims in traditional medicine. Furthermore, with all the side effects attached to the use of the orthodox modalities of treating tumors, there is still a very strong quest for new, healthier discriminating, non-toxic, potent, and inexpensive management for various tumors. Given this fact, there seems to be a shift of attention to the use of bioactive agents from natural sources that specifically target tumor cells while sparing the other cells. This approach of the use of these 'plant-based' or derived compounds will target killing malignant cells while preserving the normal cells, with little or no side effects[43]. All the special pharmacological qualities of novel bioactive compounds put together offer great prospects for novelty in drug discovery[44].

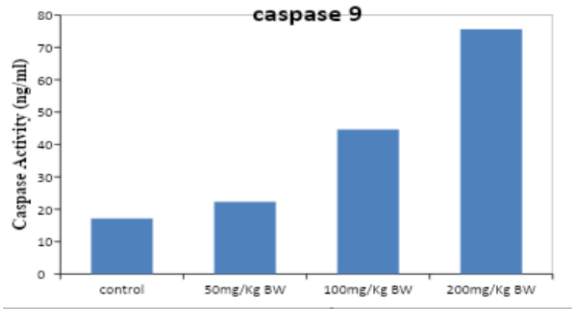


Figure 7: Estimation of the Caspase- 9 activity in rats treated with different doses of sCFSL.

Furthermore, natural products, plants being the major source, are vital constituents of a good quantity of antitumor agents presently utilized in the hospital and therefore vital in the treatment of tumors/cancers[44]. All these stimulated our interest in getting further information on the anti-tumor potentials of *S. latifolius* and its possible mechanism of action via the induction of mitochondrial-mediated apoptosis with emphasis on evasion of the apoptotic cell death signals, one of the hallmarks of tumors and cancers.

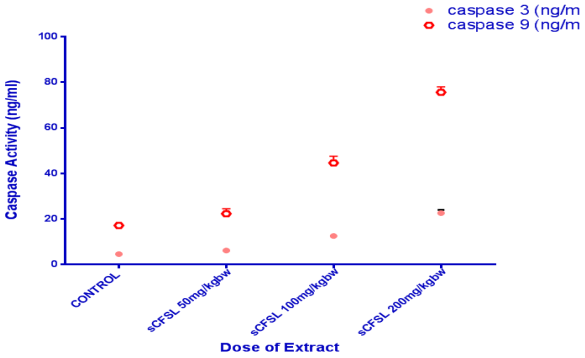


Figure 8: Effect of Different Doses of sCFSL on Caspases 3 and 9 Activities after 30 days of Administration.

-or - Each value is statistically significant at $p<0.05$, compared with the control using the one-way ANOVA.

Several scientific reports from Nigerian scientists also present evidence that purified solvent fractions of *Brysocarpus coccineus*[45], *Drymaria cordata*[46], *Alstonia boonei*[47] and *Calliandria portoricensis*[48], also induce mitochondrial-mediated 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 apoptosis CCD[49-51]. The strategy for the assessment of mPT pore opening used for this study involves 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 this organelle. This approach of the assessment of mPT pore opening involves spectrophotometric monitoring of the Ca^{2+} -induced opening of the mPT pore[28].

Part of the information collected from some of the traditional medicine practitioners, at the onset of this study was the fact that a decoction of the dried powder of the matured fruits of *S. latifolius* is usually ingested or a paste is made out of the pulverized ripe matured fruits and applied on the site of the tumor. Furthermore, studies on the *in vivo* effects of some bioactive compounds existent in therapeutic plants have also been done in recent times. Most of these reports show that these bioactive agents bring about their chemopreventive and healing effects via the stimulation or inhibition of the mPT pore opening[50-52]. Worthy of note is the fact that most of these plant-derived compounds are yet to be fully explored but are prominent compounds that may be the auspicious prospect of tumor treatment. The *in vitro* study investigated earlier showed the chloroform fraction of *S. latifolius* (CFSL) as the most potent for mPT pore opening[53]. The supposed go-between compound triggering the induction of pore opening might be inhabitant in the non-polar fraction. Based on this premise, it became necessary and needful to determine whether the most potent solvent fraction or a partially purified CFSL, sCFSL on its own can induce mPT pore opening, *in vivo* and to understand if the signaling pathways of mitochondrial-mediated apoptosis CCD are involved in the actual mechanism of the interactions sCFSL. A pilot study was carried out using arbitrary high and low doses via Looke's method to establish the LD_{50} of both the crude methanol extract (CMESL) and a sub-fraction of the most potent solvent fraction, sCFSL. From the value of the established LD_{50} the safety doses of CMESL and the sCFSL were calculated. In *in vivo* assessments, the bioavailability of the bioactive component of importance at its target site is very significant and vital, thus a safe dose regimen of the sCFSL was used for the study. Male Wistar rats used were intubated orally via the administration of varying doses of sCFSL, in comparison with CMESL for both 21 and 30 days while the control animal models received 1% DMSO and food pellets, *ad libitum*. At the lapse of this period, mPT was assessed spectrophotometrically using isolated mitochondria from the liver of both the control and the treated animals at 540nm, figure 4a & b. In view of behavioral changes during the period of treatment of the animals, a unique growth chat was recorded, and the summary of the results is shown in Figure 3.

The results from the above study, the influence of the CMESL and sCFSL on mitochondria viz-a-viz mPT pore opening induction *in vivo*, revealed that without the incorporation of a triggering agent, calcium at doses 50 and 100mg/kg, there was induction of the mPT pore with induction percentage/ folds of 14.8% (5.29), 22.5% (8.04) and 23.5% (8.36), 47.8% (15.25) respectively. It was observed (Figures 4a & 4b), that as the period of exposure to the CMESL and sCFSL increased, the degree of induction via mPT pore also increased in a dose-dependent manner, with the highest induction at the highest dose of sCSFL, 100mg/Kg, suggesting that purification might have also enhanced the induction. This is the first time the inductive profile at these dose regimens has been recorded using this medicinal plant, *S. latifolius.*

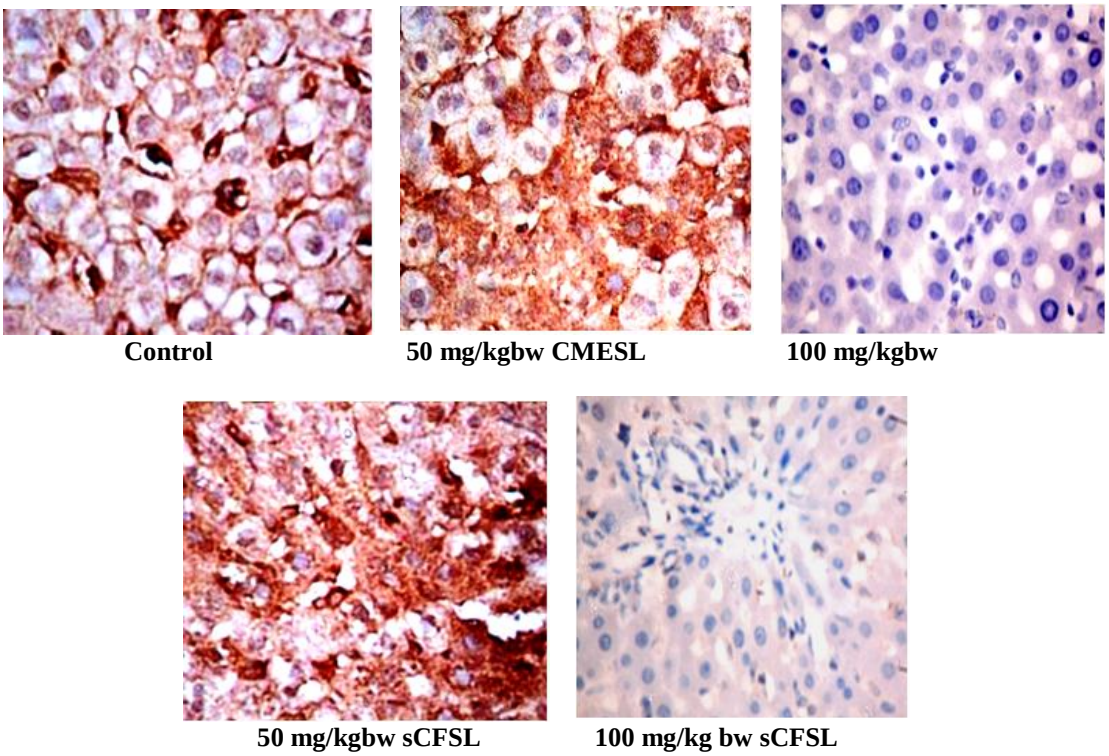


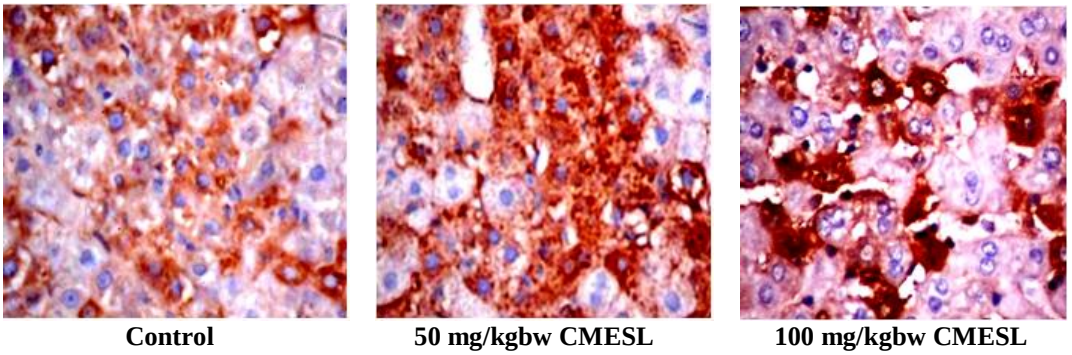
Figure 9: Expression of p53 protein in the liver following exposure to CMESL and sCFSL by immunohistochemical method (X400).

The intensity of expression from plates 1-5, moved from very mild positivity to moderately high positivity to p53

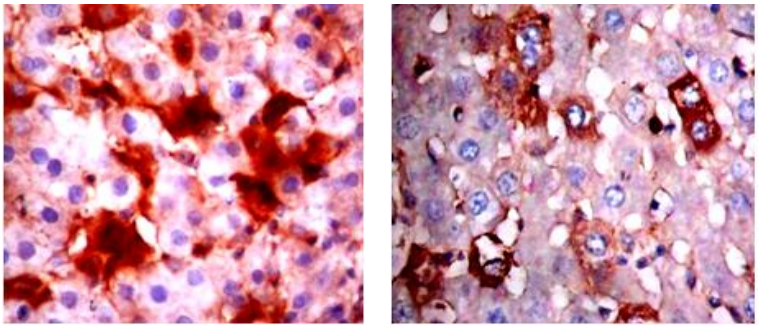
This suggests that at the stated doses, the bioactive constituents of SL were available at the target site to allow interaction with the mPT pore components with the prolonged exposure to treatment hence provoking the observed inductive effects (figure 4a & 4b). On the other hand, with the addition of Ca^{2+}, a triggering agent to the assay medium, there was a potentiation of the calcium-induced pore opening, suggesting that sCSFL, provoked a synergistic effect with calcium in inducing mPT pore opening. Other tests to establish possible mechanisms of induction *in vivo* were also investigated. Figure 5b shows what happens *in vivo* when using different doses of the CMESL and sCFSL to access mitochondrial lipid peroxidation, (mLPO). It was observed that LPO was inhibited in a "dose-dependent manner" and the sCFSL inhibited mLPO better, with an inhibitory capacity of 95.4% at 100mgKg when compared with its CMESL with an inhibitory capacity of 78.9% at the same dose. The results of this study were also in consonance with the *in vitro* results of CFSL, performed earlier in this study to establish CFSL as the most potent fraction from CMESL and its inhibitory effect on lipid peroxidation, concentration-dependent, (Imah-Harry and Olorunsogo, 2024), figure 5a.

Figure 6b represents the influence of varied dosages of CMESL and sCFSL on mitochondrial F1F0-ATPase activity, *in vivo*. The result revealed that there was a significant enhancement of ATPase activity that appears to correspond with increasing dose of treatment, with the highest activity of 27.93 ± 0.240 Pi (µmole/ mg protein/ min) at the highest dose of sCFSL. The *in vivo* effect of oral administration of sCFSL on mitochondrial F1F0-ATPase activity was also in consonance with the *in vitro* results of CFSL in comparison to other solvent fractions, EFSL and MFSL, figure 6a, performed earlier in the study. The enhancement was also "dose-dependent" suggesting that the upsurge in the bioavailability of sCFSL was proportionate with the upsurge in the ATPase activity. This further indicates that purification enhanced the inhibitory capacity of the sCFSL.

As research continues to go deeper into this line of thought, the mitochondria-mediated apoptosis CCD comes as a light into the management of tumors and cancers via mPT. Considering a lot of proof recommending that the stimulation of the mPT might be a defensive approach aimed at triggering cell death, especially in tumor cells and as such, mPT pore may be an auspicious approach for refining antitumor treatments[54]. When cells are under stress, mitochondrial-mediated cell death proceeds, and pro-death Bcl-2 family members Bax or Bak oligomerizes and permeabilizes the mitochondria outer membrane (MOM)[55]. Bax and Bak are capable of penetrating the MOM to trigger cell death by apoptosis. These proteins are also accountable for the recruiting of the organelles responsible for the supply of energy to kill cells and usually remain in normal cells where they adopt a globular α-helical structure, as monomers. Under stress conditions, these proteins are converted to pore-forming molecules via a conformational change and accumulate into oligomeric centers in the MOM. When the pore is permeabilized and cytochrome C (cyt c) is released, caspases are activated[12, 54].



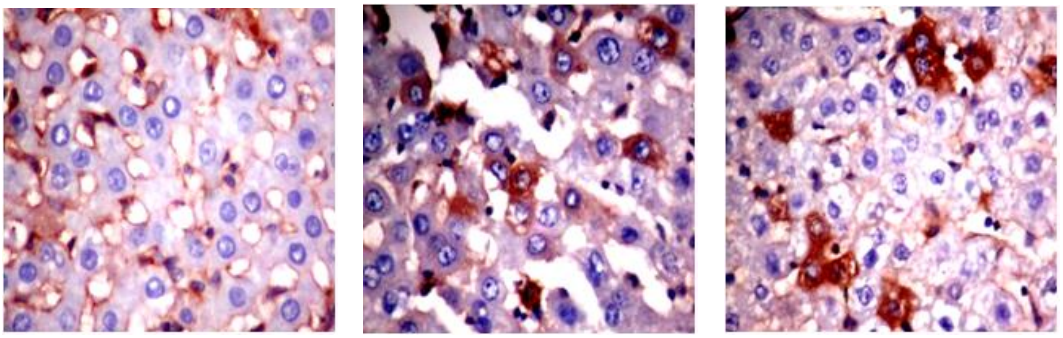
Control **50 mg/kgbw CMESL** **100 mg/kgbw CMESL**



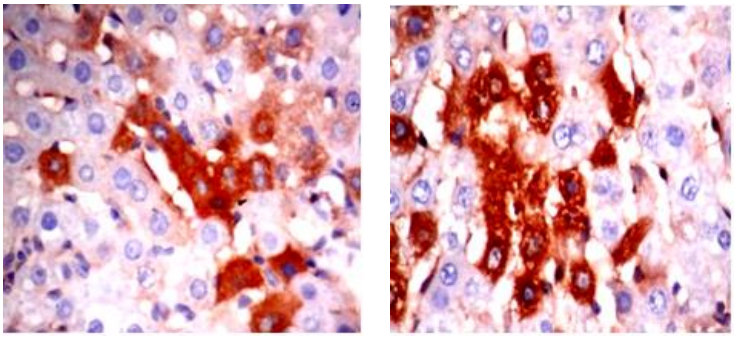
50 mg/kgbw sCFSL **100 mg/kgbw sCFSL**

Figure 10: Expression of bcl-2 protein in the liver following exposure to CMESL and sCFSL by immunohistochemical method (X400).

There was a downregulation of expression to this antibody and it was in a concentration-dependent manner.



Control **50 mg/kgbw CMESL** **100 mg/kgbw CMESL**



50 mg/kgbw sCFSL **100 mg/kgbw sCFSL**

Figure 11: Expression of bax protein in rat liver following exposure to CMESL and sCFSL by immunohistochemical method (X400).

There was an upregulation of the expression of Bax in a concentration-dependent manner.

Finally, a good knowledge of how Bax and Bak regulate mPT all through apoptosis allows for a better proposal of treatments that aim at the Bcl-2-regulated pathway, to the extent that every stage and boundary in the Bax and Bak homo- and heterooligomers are a beneficial goal[55-56]. Because of this, the mPT-mediated damage of the mitochondrial role, release of cytochrome c, and the subsequent accumulation of "ROS" is a justification for apoptosis[47].

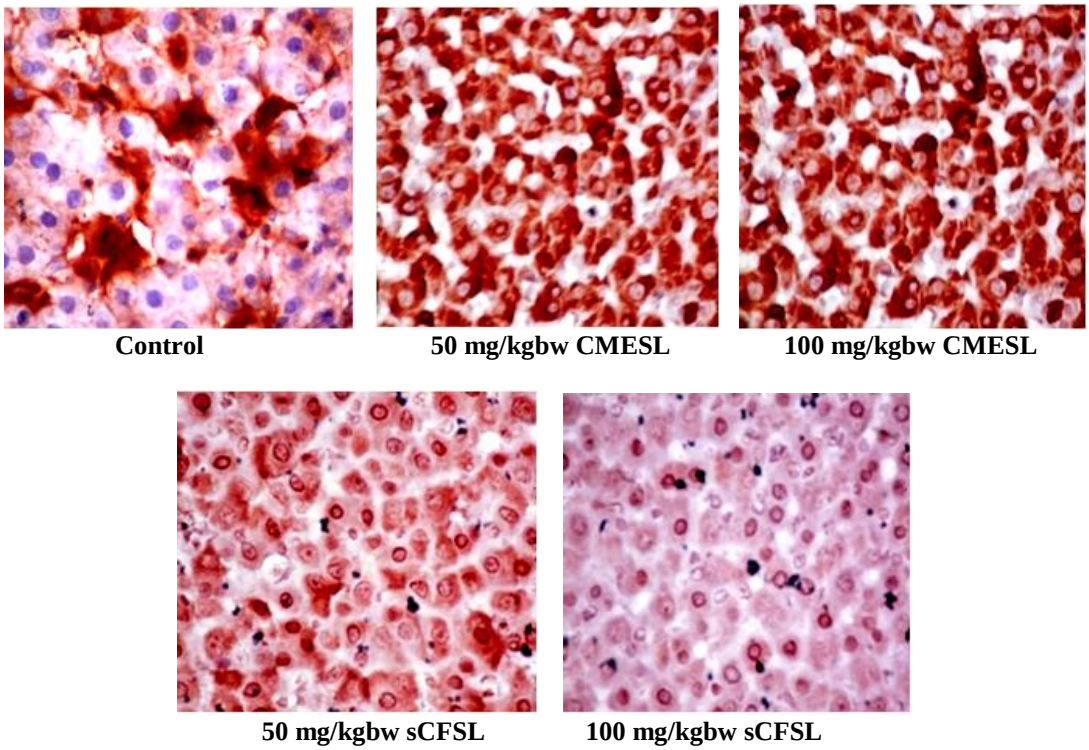


Figure 12: Expression of cytochrome c in the liver following exposure to CMESL and sCFSL by immunohistochemical method (X400).

, values are statistically significant at $p < 0.01$, and * means, values are statistically significant at $p < 0.001$, compared with control using the one-way ANOVA.

The mitochondrial-dependent pathway proceeds via the unleashing of cytochrome c-Apaf-1 combination that leads to the formation of apoptosome, which now becomes a platform to trigger the caspase cascade reaction[12]. This subsequently causes damage to ionic homeostasis, bulge of the mitochondrial matrix, breaking of the OMM, and loss of apoptogenic proteins from the inter mitochondrial space (IMS), enhancing the stimulation and activation of the pro-death pathway, mediated by the cascade of caspases activity and in collaboration with the mitochondrial bioenergetic collapse, apoptosis is inevitable.

One of the hallmarks of the apoptotic process is the activation of a group of unique proteases, called the "cysteine"-dependent aspartate-specific proteases, also known as caspases. These are a family of protease enzymes which perform vital functions in regulating equilibrium/homeostasis in programmed cell death and inflammatory processes[57], thereby playing an important function in determining the cell's fate. They usually occur as sedentary monomeric forerunner enzymes (pro-caspases) and thus must be dimerized for complete activation to facilitate or trigger most of their actions by cleaving (or being bound) to their target proteins[57]. Caspase-3 aims at severing or catalyzing the cleavage of hundreds of cellular protein substrates, mainly related to chromatin condensation and margination, DNA fragmentation, and nuclear collapse[58]. Therefore, protease activity stimulates apoptotic cell death and thus loss of caspase activity will enhance tumor growth. This led to the study of the influence of sCFSL on DNA fragmentation, caspase- 9, and caspase-3 activities. The level of caspases 9 and 3 were determined using ELISA technique. Intriguingly, the results of the study revealed that sCFSL caused an upregulation of the apoptosis terminal factor (caspase-9) and the executor protein (caspase-3), and increased the % DNA fragmentation, all in a dose-dependent manner, as proof of cell death occurrence. The activation of these caspases was noteworthy at 50, 100, and 200mg/kg of sCFSL. The activation of these caspases by sCFSL proposes that sCFSL has the potential to induce mitochondrial-mediated apoptosis. Cytochrome c, which is a point of no return in apoptosis hence, its discharge from the IMS of the mitochondria during the treatment period must have led to the induced activation of caspase-9 and caspase-3 activities[59-60]. Therefore, raised levels of cytochrome c release in cell lysates treated with sCFSL is a suggestion that the breakdown of the MOM via the mPT pore opening was accompanied by the release of the apoptogenic protein, Cytochrome c, which is a compulsory occurrence in the mitochondrial-dependent apoptotic pathway. DNA damage is also one of the topographies of mitochondrial-dependent apoptosis. These events mark very significant events in the sCFSL-induced mitochondrial-mediated apoptosis CCD. The results of this study are also in agreement with the findings of [61] which stated that caspases are indispensable in the execution of the apoptotic process.

Figure 7, illustrates the effects of different doses of sCFSL (50, 100, and 200 mg/kgbw) on hepatic DNA fragmentation and this was meaningfully increased ($p < 0.01$, 0.0001) by 28%, 47%, and 52% respectively, compared to the control. A similar trend was also

observed in the caspase-3 and caspase-9 activities illustrated in figure 8-10. At the stated doses of sCFSL (50, 100, and 200 mg/kgbw) on caspase-3 and caspase-9 activities, there was a significant increase of (26%, 64%, and 80%) and (23%, 62%, and 78%) respectively. All these observed trends were in a dose-dependent manner.

Based on this premise it would not be misleading but wise to say that mPT is a pivotal tool that explains the thin line between the existence or demise of a cell,[62]. Furthermore, with respect to apoptosis and tumor management, there are hypothetical assumptions that the activation of the mPT pore stimulates/triggers PCD in cells hence hindering the proliferation of tumor cells[54]. The results from *in vivo* results of mPT opening effects were in a dose-dependent manner, so it was wise to examine the effects of the apoptotic proteins involved in the mitochondrial-mediated apoptotic pathway. In lieu of this the degree of expression of the pro-death (pro-apoptotic) protein, Bax, and pro-survival (anti-apoptotic) protein, Bcl-2 was examined using cell lysates, by immunohistochemical technique. The levels of the expression of the tumor suppressor gene, p53, and the amount of the apoptogenic protein, cytochrome c released (CCR) was also determined using the immunohistochemical technique.

The results show that sCFSL also inhibited the expression of antiapoptotic proteins (Bcl-2), figure 12, upregulated the pro-apoptotic protein BAX, figure 13 and the tumor suppressor gene, p53, figure 11 and elevated the level of Cyt c released, figure 14, *in vivo*.

On establishing the potency of sCFSL as an inducer of mitochondrial-mediated apoptosis, the study demonstrated the effects of both the CMESL and sCFSL on some apoptotic parameters using immunohistochemical techniques. The results of the study showed that sCFSL caused an elevated immunoreactive expression of CCR, caspase-9, and caspase-3, which are critical to the activation of mitochondrial-mediated apoptosis. Furthermore, alterations in the levels of pro-death Bax and the pro-survival Bcl-2 with the exposure of cell lysates to graded doses of the CMESL and a sub-fraction of the most potent fraction, chloroform (sCFSL), clearly indicate that sCFSL caused a translocation of Bax from the cytoplasm to the mitochondria and perhaps the facilitation of Bax oligomerization, which is a critical event for mPT. Data also showed the upregulation of the expression of p53, a tumor suppressor protein that induces intrinsic apoptosis by inducing the Bax/Bak oligomerization and antagonizing the pro-survival/anti-apoptotic protein, Bcl- 2, also corroborating the effects of sCFSL on cell death. Furthermore, the study revealed a downregulated expression of Bcl-2, a pro-survival apoptotic protein. This could serve to protect the cell against excessive apoptosis, and a therapeutic strategy in combating tumorous/ cancerous cells. All these also support the fact that SL might likely contain bioactive compounds that induce mitochondrial-mediated apoptosis, which can ultimately lead to cell death.

CONCLUSIONS

From this study, it can be concluded that sCFSL must contain bioactive compounds that can efficiently scavenge ROS and therefore could display anti-cancer, anti-tumor, anti-inflammatory, and anti-aging activity. The improvement of the mitochondrial ATPase activity of the *S. latifolius* fruits substantiates its anti-tumor potentials, via the mPT pathway because the released inorganic phosphate is a pore opening inducer. The inhibition of Fe^{2+}- induced mLPO results from this study reveals that *S. latifolius* has the potential to act as scavengers of free radicals and therefore affirms that the induction of mPT pore observed in this study could not be attributed to ROS generation but due to the bioactive compounds present in the matured fruits of *S. latifolius.*

These bioactive compounds also stimulated an elevated level of phosphate ions in the mitochondria, a decrease in the antiapoptotic protein, Bcl-2, and an increased level of pro-apoptotic proteins, p53, and Bax. All these also corroborate the pro-apoptotic properties of *S. latifolius* and must have caused the triggering and stimulation of the apoptotic signaling pathway via the induction of mPT pore opening. The loss of ion homeostasis, mitochondrial swelling, and rupture of the MOM led to elevated levels of apoptogenic proteins, such as cytochrome c. The increased level of cytochrome c released in combination with Apaf-1 with dephosphorylated ATP led to the development of the apoptosome. This now formed a stage to activate the caspase cascade reactions. With an increased activity of pro-caspase-9 to activated caspase-9, which is an initiator caspase, there was a subsequent upsurge in the activation of pro-caspase-3 to activated caspase-3, an executor caspase. Following the activation of caspase-3, there was an onset, total breakdown of nuclear fragmentation, hence increased DNA fragmentation, and at this point, the sCFSL-induced mitochondrial-mediated apoptosis was inevitable.

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AUTHOR'S CONTRIBUTIONS

Imah-Harry JU: formal analysis, conceptualization, manuscript writing. **Olorunsogo OO:** critical review, data organization. Final article was checked and approved by both authors.

DATA AVAILABILITY

The accompanying author can provide the empirical data that were utilized to support the study's conclusions upon request.

CONFLICT OF INTEREST

There are no conflicts of interest in regard to this project.

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