AFRICAN JOURNAL OF MEDICINE and medical sciences

VOLUME 41 Second Biomedical Conference Supplement DECEMBER 2012

ISSN 1116-4077

Afr J. Med. med. Sci. (2012) 41, Suppl. 157-169

Modulation of opening of rat Liver mitochondrial membrane permeability transition pore by different fractions of the leaves of *Cnestis ferruginea*. D.C.

RA Adisa, TD Alabi and OO Olorunsogo

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

Abstract

Background: Increased attention is now directed towards the search for novel naturally occurring anticancer agents that can induce mitochondrial membrane permeability transition (MMPT) pore opening and cell death as a chemotherapeutic mechanism to combat cancer incidence.

Aim: The inductive effects of partially purified fractions of leaves of *Cnestis ferruginea*- on rat liver MMPT pore opening was investigated.

Method: De-fatted methanol extract of leaves of *Cnestis ferruginea* was partitioned between water, chloroform, ethylacetate, or butanol separately in succession. The extract solutions were concentrated at 40°C to obtain water (WF), chloroform (CF), ethylacetate (EF) and butanol (BF) fractions. The effects of these fractions (0.2 – 1.4 mg/ml) on MMPT pore opening or mitochondrial swelling in the presence and absence of calcium were evaluated The effects of these fractions on the rat liver mitochondrial F_0F_1-ATPase activity were also assessed.

Results: Ca^{2+}-induced MMPT pore opening was inhibited by 1mg/ml each of MECF, CF, BF, WF and EF by 75.0%, 83.0%, 88.0%, 68.0%, and 71.0%, respectively and compared with the effect of spermine, a standard inhibitor. However, in the absence of Ca^{2+}, the fractions significantly induced MMPT pore opening in intact mitochondria by 7.0, 5.7, 0.7, 4.8, 10.9 folds, respectively. In normal rat liver mitochondria, F_1F_0-ATPase activity was stimulated maximally by MECF, CF, EF, BF and WF by 4.7, 12.7, 1.6, 3.6 and 1.5 folds, respectively, thus indicating that the chloroform fraction is the most potent and therefore contains the active principle in the plant.

Conclusion: The present study revealed that the leaves of *Cnestis ferruginea* contain bioactive substances that induced mitochondrial membrane permeability transition and activated the specific activity of F_0F_1 ATPase. Thus, suggesting strongly that these bioactive agents may serve as a useful chemotherapeutic strategy in cancer therapy.

Correspondence: RA Adisa, Laboratories for Biomembrane Research and Biotechnology, Department of Biochemistry, College of Medicine, University of Ibadan, Ibadan, Nigeria.

Keywords: *Cnestis ferruginea, mitochondrial membrane permeability transition, F_0F_1 ATPase, cancer.*

Résumé

Contexte: Une attention accrue est désormais orientée vers la recherche de nouveaux agents anticancéreux d'origine naturelle qui peuvent induire l'ouverture des pores de transition de perméabilité de la membrane mitochondriale (TPMM) et la mort de la cellule comme un mécanisme chimiothérapique pour lutter contre l'incidence du cancer.

Objectif: Les effets inductifs des fractions partiellement purifiées de feuilles de Cnestis ferruginea sur l'ouverture des pores TPMM sur le foie du rat ont été examinés.

Méthode: L'extrait méthanolique Dégrassé des feuilles de Cnestis ferruginea a été partagé entre l'eau, le chloroforme, l'acétate d'éthyle ou le butanol séparément dans la succession. Les solutions de l'extrait ont été concentrées à 40°C pour obtenir de l'eau (WF), des fractions de chloroforme (FC), d'acétate d'éthyle (EF) et de butanol (BF). Les effets de ces fractions (0,2 - 1,4 mg / ml) sur l'ouverture des pores TPMM ou sur le gonflement mitochondrial en présence et en absence de calcium ont été évalués. Les effets de ces fractions sur l'activité F_0F_1-ATPase mitochondriale du foie du rat ont également été évalués.

Résultats: l'ouverture des pores TPMM induite par la Ca^{2+} est inhibée par 1mg/ml de MECF, CF, BF, WF et EF chacun de 75,0%, 83,0%, 88,0%, 68,0%, et 71,0%, respectivement, comparé à l'effet de spermine , un inhibiteur standard. Cependant, en l'absence de Ca^{2+}, les fractions de façon sensible induisent l'ouverture des pores TPMM dans les mitochondries intactes de 7,0, 5,7, 0,7, 4,8, 10,9 fois respectivement. Dans les mitochondries normales du foie du rat, l'activité F_1F_0-ATPase est stimulée au maximum par MECF, FC, FE, BF et WF de 4,7, 12,7, 1,6, 3,6 et 1,5 fois, respectivement, ce qui indique que la fraction chloroforme est plus puissante et contient ainsi le principe actif dans la plante.

Conclusion: La présente étude a montré que les feuilles de Cnestis ferruginea contiennent des substances bioactives qui induisent la transition de perméabilité de la membrane mitochondriale et a initié

l'activité spécifique du F_0F_1 ATPase. Ce qui montre fortement que ces agents bioactifs peuvent constituer une stratégie chimiothérapeutique utile dans le traitement dù cancer.

Introduction

Apoptosis (programmed cell death) is utilized by cells for eliminating unwanted, aged, dysfunctional cells and regulating tissue homeostasis [1]. Apoptotic pathway could be achieved through the extrinsic or death receptor and intrinsic or mitochondrial-mediated pathways [2]. Abnormal regulation of apoptosis progresses to pathological conditions or diseased states including cancer. In recent years, the central role of mitochondria in apoptotic and necrotic cell death has become apparent, and the mitochondrial permeability transition has been implicated in these phenomena [3]. Mitochondrial permeability transition occurs as a result of calcium overload and oxidative stress resulting in the opening of the mitochondrial membrane permeability transition (MMPT) pore. Consequently, uncoupling of oxidative phosphorylation, ATP hydrolysis by the F_0F_1 ATPase, cytochrome c release and loss of matrix solutes inevitably lead to apoptosis if unaverted as in reperfusion injury [3,4] and neurodegenerative disorders [5] etc. This implies that mitochondria coordinate the late stage of cell death [6]. Mitochondrial permeability transition is also a critical event in pathological cell death induced by ischeamia/reperfusion, intoxication with xenobiotics, neurodegenerative diseases, or viral infection [7, 8]. Prolific research efforts are presently geared towards elucidation of the fundamental mechanisms that regulate apoptosis and associated mediators that trigger or inhibit cell death. The outcomes of these efforts would inadvertently provide a solid foundation for therapeutic strategies for combating cancer.

Many naturally occurring bioactive agents such as phenolic compounds [9], flavonoids [10], and alkaloids [11] have been demonstrated as potential chemopreventive agents in many kinds of cancers. Interestingly, polyphenols have been shown to induce apoptosis in many cell types, and broadly antioxidants have been reported to provide protection against many different types of apoptosis [12]. These compounds have been targeted at the mechanisms of apoptosis which include regulatory pathways such as Bcl-2 family of proteins, induction of mitochondrial swelling and dissipation of membrane potential, modulation of caspase activation and, induction of cytochrome c release [13, 14].

Adenosine triphosphate synthase (EC 3.6.3.4) is the enzyme that catalyses the synthesis of adenosine triphosphate (ATP) from ADP and inorganic phosphate by using the electrochemical force harnessed during electron transfer in the mitochondria. This energy is often formed by movement of protons down an electrochemical gradient from the matrix into the intermembrane space in mitochondria. This enzyme is of crucial importance in almost all organisms because ATP is the common energy currency of all cells [15]. The dissipation of mitochondrial inner transmembrane potential normally utilized for oxidative phosphorylation often triggers the intrinsic pathway of cell death [16]. Many chemopreventive agents have been shown to induce apoptosis by directly inhibiting mitochondrial respiration through the mitochondrial inner transmembrane potential [17, 18]. This scenario encourages the hydrolysis of ATP rather than its synthesis.

Cnestis ferruginea (D.C) (*Connaraceae*) is a medicinal plant with pharmacological activities such as antibacterial, antifungal [19] and anticonvulsant [20]. Phytochemical screening revealed the presence of flavonoids, combined anthraquinones, saponins, steroidal glycosides [21] and a novel isoflavone glycoside, afrormosin-7-O-beta-D-galactoside [22]. Methanol extracts of leaves of *C ferruginea* have been demonstrated to inhibit haemoglobin glycosylation *in vitro* [23] and are recently shown to possess hypoglycaemic activity in streptozotocin-induced diabetic rats and mice [24]. These leaves have also been demonstrated to be a rich source of antioxidants [25].

Medicinal plants are recognized sources of drug candidates against many kinds of diseases. The modulation of dietary intake using the variety of bioactive components present in functional foods is gaining broader acceptance [26]. Phytochemicals such as polyphenols, curcumin, resveratrol, catechin [27] have been shown to induce apoptosis in many cell types, and antioxidants have been reported to provide protection against many different types of apoptosis and cancer [12]. There is a paucity of information on the pharmacological properties and constituents of leaves of *C ferruginea* (D.C). However, Adisa *et al.*, [23] revealed that these leaves are rich in polyphenolic compounds. These findings have ignited our interest to further investigate the potential of the leaf constituents to modulate mitochondrial membrane permeability transition pore opening in rat liver. The study also investigated the effects of the partially purified fractions of the leaves on mitochondrial ATPase activity *in vitro* in order to explain the possible mechanism of action of the leaf extract and fractions in modulation of programmed cell death.

Materials and methods

Collection and Extraction procedure

Fresh Leaves of *C ferruginea* were collected and authenticated at Forestry Research Institute of Nigeria (FRIN), Ibadan and voucher specimens were deposited (Voucher No. 106524). The leaves were shade-dried for one week on laboratory trays. The dried leaves were powdered and weighed. The dried, powdered leaves were de-fatted with n-hexane, extracted with 95% methanol in a soxhlet extractor for 12 hours and concentrated on rotary evaporator (40°C) to obtain the methanol extract of *C ferruginea* (MECF).

The methanol extract was dissolved in distilled water, fractionated and concentrated in succession with chloroform, ethylacetate and butanol to obtain fractions CF, EF, BF and WF (water), respectively.

Management of animals

Male Wistar strain albino rats (n = 20) weighing between 180 – 200g were purchased from the Preclinical Animal House of the College of Medicine, University of Ibadan, Ibadan, Nigeria. All the animals were allowed two weeks period of acclimatization in the Animal House of the Department of Biochemistry, University of Ibadan. The animals were placed under a 12hr light/dark cycle and fed commercial pelletized rat chow and water *ad libitum* throughout the experimental period.

Preparation of Low ionic strength mitochondria

Low ionic strength mitochondria were prepared from rat liver by standard differential centrifugation as modified by Schneider [28] according to the method described by Johnson and Lardy [29]. Briefly, the animals were sacrificed by cervical dislocation and the livers were quickly excised, trimmed and washed with buffer C (210mM Mannitol, 70mM Sucrose, 5mM Hepes-KOH pH 7.4, 1mM EGTA). The tissues were blotted, weighed and minced and a 10% suspension of it in ice-cold buffer C was then homogenized on ice with a Potter Elvehjem all-glass Teflon homogenizer. The homogenate was centrifuged at 2,300rpm for 5 minutes in a MSE centrifuge at 4°C. The supernatant was further spun at the same speed to remove the remaining nuclear debris. The mitochondria were pelleted by spinning the supernatant obtained at 13000rpm for 10 minutes. The post mitochondrial fraction was discarded and the mitochondria were washed twice with buffer D (210mM Mannitol 70mM Sucrose, 5mM Hepes-KOH pH 7.4, 0.5% BSA). The mitochondria were re-suspended in MSH (210mM Mannitol, 70mM Sucrose, 5mM Hepes-KOH pH 7.4) and aliquoted on ice into pre-cooled Eppendorf tubes. Mitochondrial protein content was determined using the procedure described by Lowry, et al., [30] using bovine serum albumin as standard.

Assessment of mitochondrial swelling

Mitochondrial swelling was assessed according to the method described by Lapidus and Sokolove [31]. Intact mitochondria (0.4mg/ml) were pre-incubated in the presence of 0.8μM rotenone and MSH buffer for 3.5 minutes prior to the addition of 5mM sodium succinate. In order to assess Ca^{2+}-induced swelling, mitochondria were pre-incubated in 0.8μM rotenone and MSH buffer for 3 minutes. $Ca^{2+}$was then added to the reaction mixture while sodium succinate was added 30 seconds later in a total reaction volume of 2.5 ml. Spermine was used as the standard inhibitor of mitochondrial swelling, and was added prior to mitochondrial pre-incubation with rotenone. Change in absorbance was estimated at 540nm at 30 seconds interval for 12 minutes in a T70 UV-visible spectrophotometer, PG Instrument Ltd. To determine the effect of the extracts of *C ferruginea* on Ca^{2+} - induced MMPT pore opening, graded concentrations of each of the extract fraction–MECF, CF, EF, BF and WF were separately pre-incubated with mitochondria for 3 minutes following addition of Ca^{2+} to the reaction mixture. But calcium was excluded in the reaction mixture when the direct modulatory effect of the extracts on intact mitochondria was desired. Mitochondrial swelling was measured as decrease in absorbance at 540nm. Swelling rate was quantified as 540/min/mg.

Assessment of Mitochondrial FoF_1 ATPase Activity

Adenosine triphosphatase was determined by a modification of the method of Lardy and Wellman [32] and as described by Olorunsogo and Malomo [33]. Each reaction mixture contained 65mM Tris-HCl buffer pH 7.4, 0.5mM KCl, 1mM ATP and 25mM sucrose. The reaction mixture was made up to a total volume of 2ml with distilled water. The reaction was started by the addition of mitochondrial suspension (of known protein content) and was allowed to proceed for 30 minutes with constant shaking at 27°C. The reaction was stopped by the addition of 8ml of a 10 percent solution of trichloroacetic acid and each reaction mixture was then centrifuged at 3000g. The supernatant was kept for phosphate determination. The zero time tube was prepared by adding the solution of ATP to the reaction vessel after the addition of trichloroacetic acid.

Estimation of Inorganic phosphate released

The inorganic phosphate released was measured according to the procedure described by Olorunsogo and Malomo [33]. To 5ml of the de-proteinized supernatant in a test tube was added 0.4ml of a solution of perchloric acid. This was followed by the addition of 0.4ml of 5 percent ammonium molybdate and 0.2ml of a 0.2 percent freshly prepared solution of ascorbic acid. The tube was thoroughly mixed by gentle shaking and allowed to stand for 20 minutes. A standard solution of potassium dihydrogen phosphate (0.2mg Pi per 5ml) was similarly treated and the intensity of the blue colour developed was read at 680nm. A water blank was used to set the instrument at zero.

Results

Effects of increasing concentrations of MECF, CF, EF, BF and WF on calcium –induced opening of MMPT pore.

Figure 1 shows the pattern of inhibition of calcium-induced MMPT pore opening by varying concentrations (0.2 – 1.4 mg/ml) of MECF in normal rat liver mitochondria energized by succinate. The basal change in absorbance obtained in the absence of calcium was ($0.074 \pm 2.83 \times 10^{-3}$) indicating the intactness of the mitochondria. As seen from the figure, calcium induced mitochondrial swelling by 85%. Swelling was significantly lowered in a concentration-dependent manner up to 1mg/ml of MECF above which the degree of inhibition began to dwindle.

In this regard, the opening of the pore was inhibited by -5, 22, 49, 60, 75 and 46% at 0.2, 0.4, 0.6, 0.8, 1, and 1.4 mg/ml, respectively. Inhibition of Ca^{2+}-induced swelling by 1 mg/ml of MECF was greater by 1/3 than that due to 0.1mM spermine, the standard inhibitor. Figures 2, 3, 4 and 5 show the pattern of inhibition of varying concentrations of different fractions of methanol extract of *Cnestis ferruginea* (EF, CF, BF, and WF) on Ca^{2+}-induced swelling in normal rat liver mitochondria. In general, all the fractions significantly ($p<0.05$) inhibited Ca^{2+}-induced swelling in a concentration-dependent manner compared to mitochondria incubated with calcium alone. Specifically, EF at 0.2, 0.4, 0.6, 0.8, 1, and 1.4 mg/ml reduced Ca^{2+}-induced swelling by 8, 17, 59, 68, 71, and 49%, respectively (Figure 2) whereas similar concentrations of CF inhibited pore opening by 63, 75, 80, 80, 83, and 94%, respectively (Figure 3). Furthermore, the same concentrations of BF also inhibited calcium-induced pore opening by 32, 39, 42, 65, 88, and 88%, respectively (Figure 4) while these concentrations of WF reduced the pore opening by 9, 39, 42, 46, 68, and 66%, respectively (Figure 5). As seen from the results, all the fractions inhibited calcium-induced opening of the MPT pore in a concentration-dependent manner only up to 1mg/ml after which the inhibition started to diminish except in the case of CF which inhibited pore opening by 94% at 1.4mg/ml.

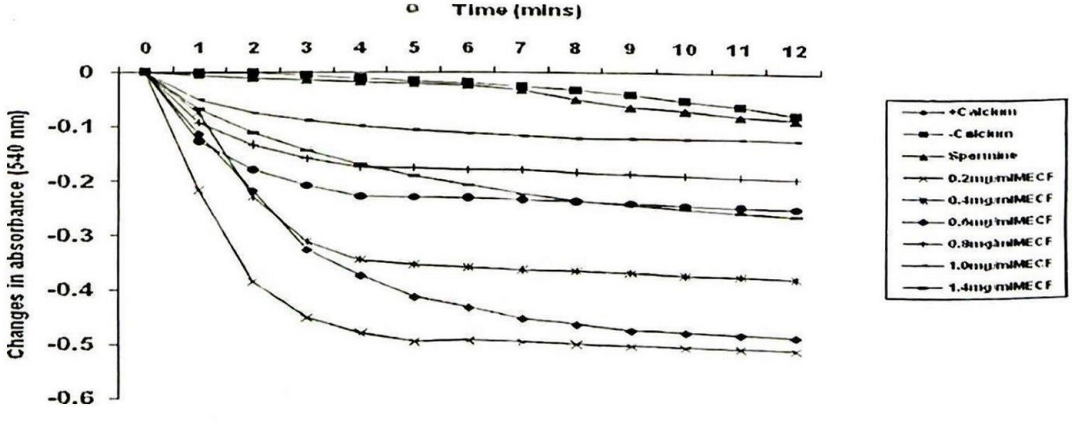

Fig. 1: Inhibition of Ca^{2+}-induced MPTP opening by MECF in normal rat liver mitochondria energized by succinate

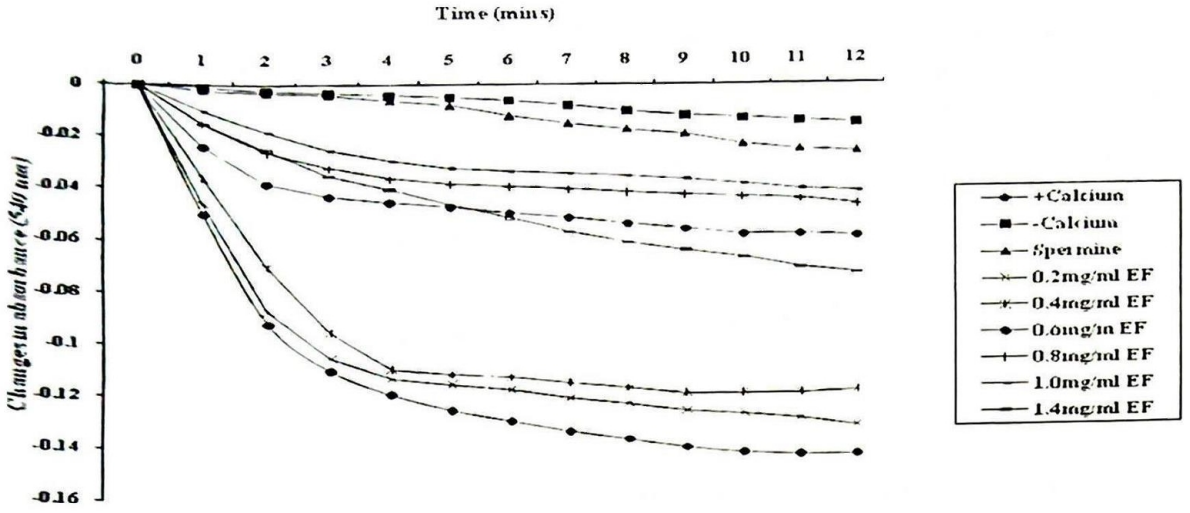

Fig. 2: Inhibition of Ca^{2+}-induced MPT pore opening by EF in normal rat liver mitochondria

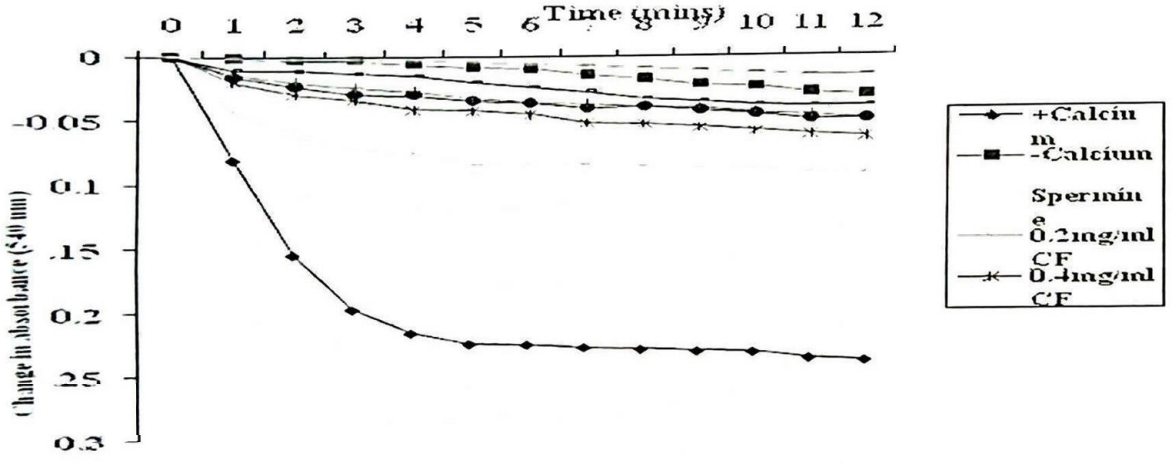

Fig. 3: Inhibition of calcium-induced MPT pore opening by varying concentrations of CF in normal rat liver mitochondria energized by succinate

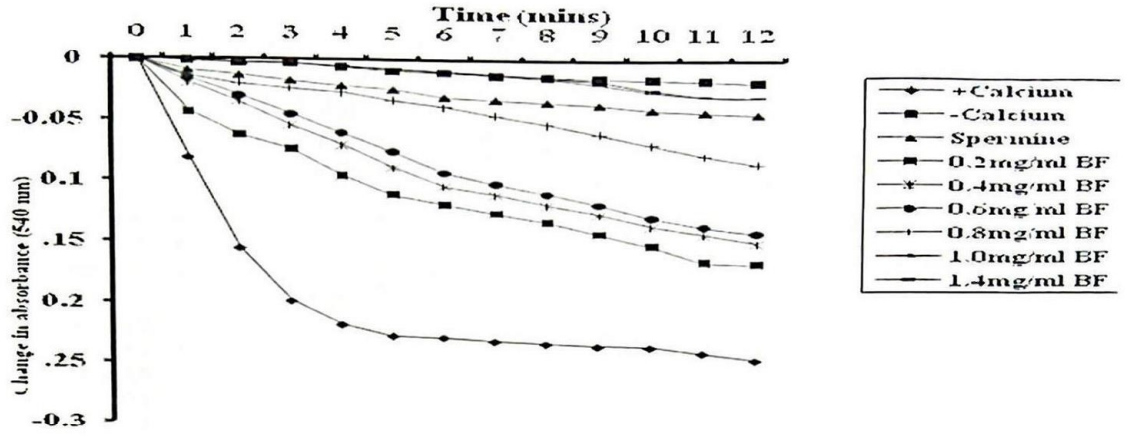

Fig. 4: Inhibition of calcium-induced membrane permeability transition pore opening by BF in sodium succinate energized mitochondrial rat liver

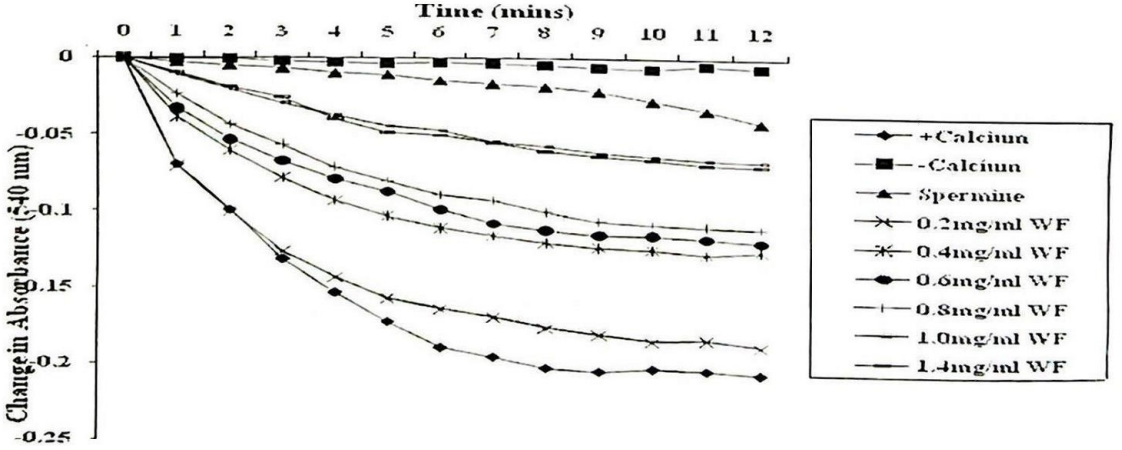

Fig. 5: Inhibition of calcium-induced MPT pore opening by WF in normal rat liver mitochondria energized by succinate

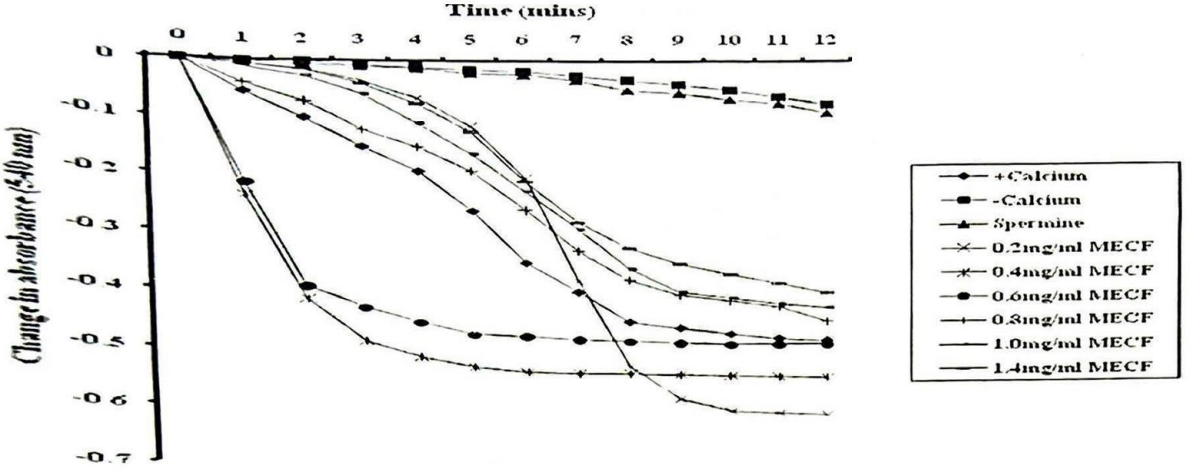

Fig. 6: Induction of MPT pore opening by MECF in the absence of calcium in rat liver mitochondria

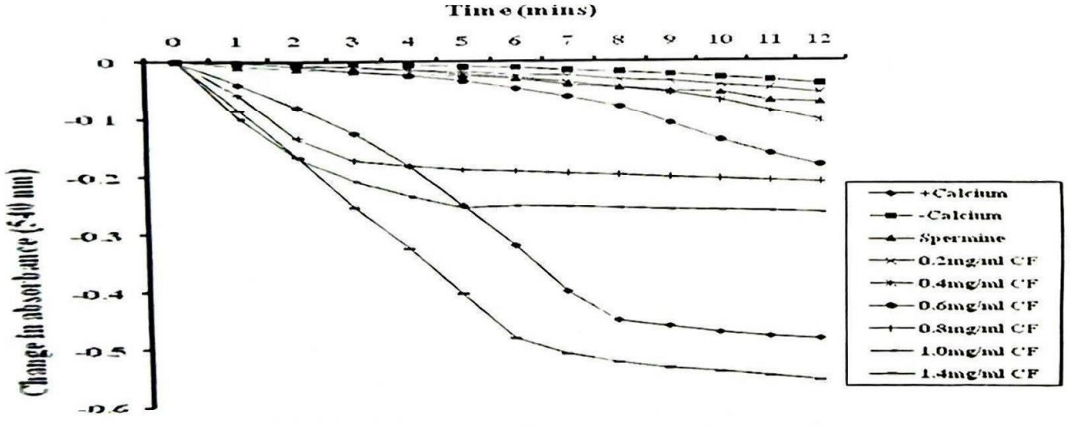

Fig. 7: Induction of MPT pore opening by CF in normal rat liver mitochondria energized by succinate in the absence of calcium

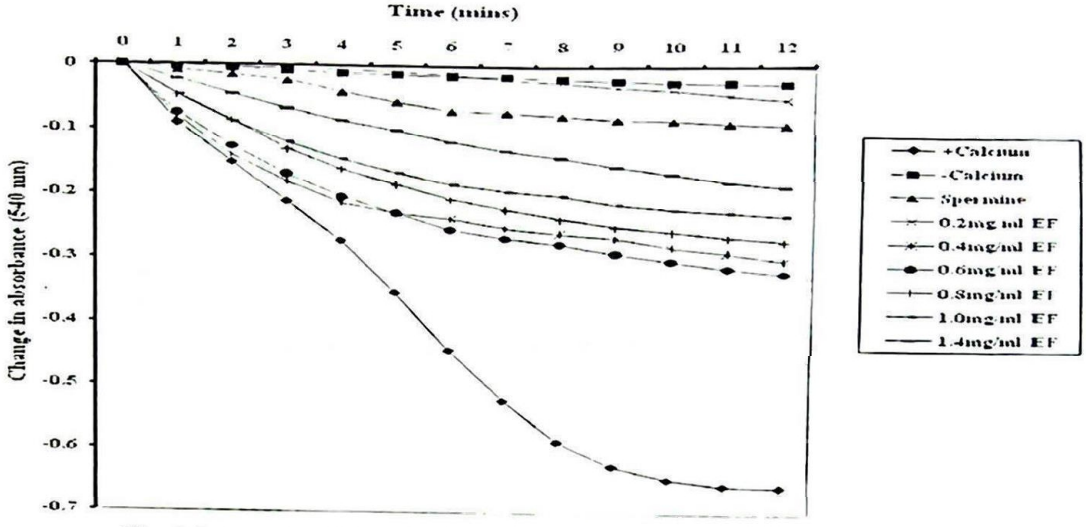

Fig. 8: The effect of EF on MPT pore opening in normal rat liver mitochondria energized by succinate in the absence of calcium

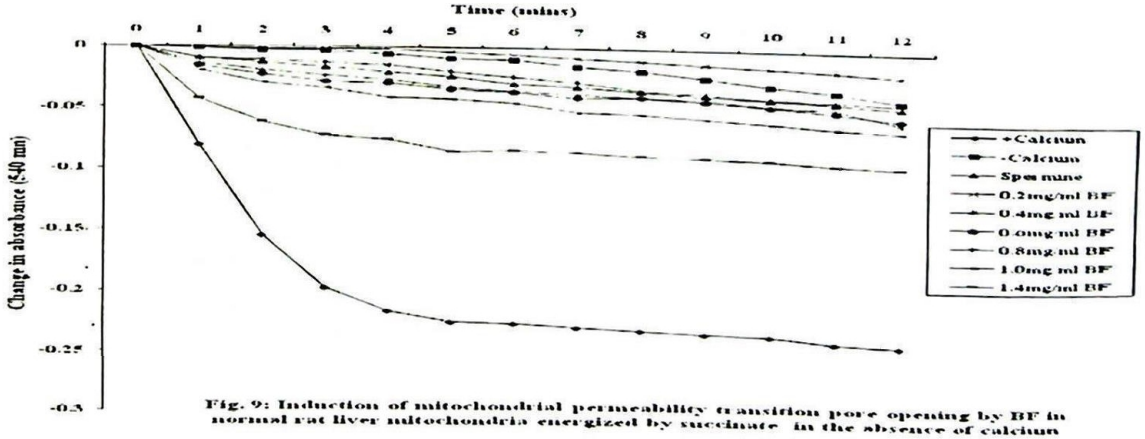

Fig. 9: Induction of mitochondrial permeability transition pore opening by BF in normal rat liver mitochondria energized by succinate in the absence of calcium

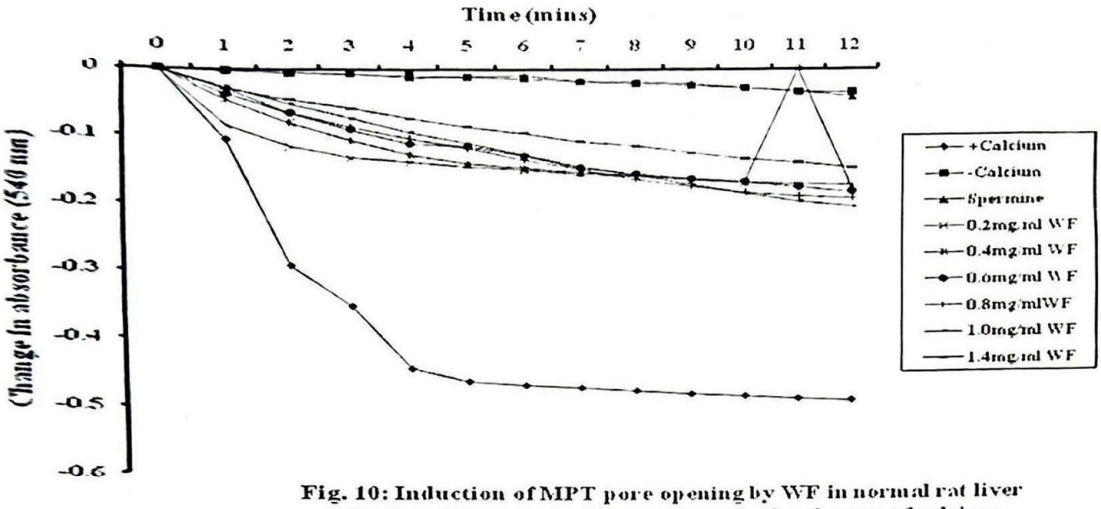

Fig. 10: Induction of MPT pore opening by WF in normal rat liver mitochondria energized by succinate in the absence of calcium

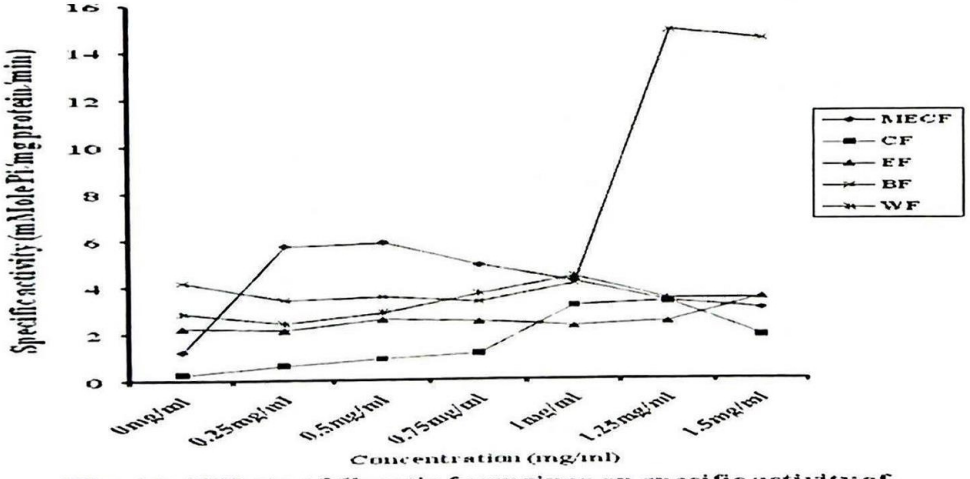

Fig. 11: Effects of Cnestis ferruginea on specific activity of rat liver mitochondrial F0F1 ATPase

Effects of increasing concentrations of MECF, CF, EF, BF and WF on MMPT pore in the absence of calcium.

On the contrary, pre-incubation of normal healthy mitochondria with MECF and different fractions of *C ferruginea* in the absence of calcium resulted in induction of opening of MMPT pore to varying extents by the varying concentrations of the fractions. For example, MECF induced pore opening by 7.0 and 6.2 folds at 0.2 and 0.4 mg/ml, respectively. Higher concentrations of MECF up to 1.4 mg/ml actually caused insignificant ($p>0.05$) decreases in

the extent of swelling (Figure 6). Furthermore, varying concentrations (0.2, 0.4, 0.6, 0.8, 1.0, and 1.4 mg/ml) of CF and EF induced opening of MMPT pore by 0.4, 1.4, 3.3, 4.0, 5.2, 11.4 folds (Figure 7) respectively, and 0.85, 10.5, 11.3, 9.4, 7.6, 6.1 folds (Figure 8), respectively. Moreover, similar concentrations of BF also induced opening of the pore by 0.07, 0.07, 0.26, 0.33, 0.47, 1.14 folds (Figure 9), respectively whereas, WF induced swelling by 3.94, 4.03, 4.23, 4.49, 4.83, and 3.2 folds (Figure 10) at 0.2, 0.4, 0.6, 0.8, 1.0, and 1.4 mg/ml, respectively. It was also observed that 1.4 mg/ml of CF and 0.6 mg/ml of EF induced mitochondrial swelling to the same extent.

Effects of increasing concentrations of MECF, CF, EF, BF and WF on mitochondrial F_oF_1-ATPAse

Figure 11 shows the modulation of the specific activity of the mitochondrial F_0F_1 -ATPase by methanol extracts and its fractions. The data obtained show that MECF and its purified fractions (CF, BF, and WF) significantly ($p<0.05$) stimulated the specific activity of the mitochondrial F_0F_1 ATPase although to varying extents. Specifically, varying concentrations of CF and BF (0.25 – 1.5 mg/ml) stimulated this enzyme activity linearly by 12.7 and 3.5 folds respectively. Also, MECF at 0.5 mg/ml stimulated the enzyme activity by 4.7 folds. However, this stimulatory effect is not significantly ($p<0.05$) different from the effects produced by other concentrations used. Surprisingly, all the concentrations of EF tested did not have any significant stimulatory effect on the specific activity of the mitochondrial ATPase when compared to the basal activity of the enzyme (control), whereas WF at 1 mg/ml stimulated the specific activity of the enzyme by 1.53 fold.

Discussion

Mitochondria have been demonstrated to play a pivotal role in apoptosis of mammalian cells by releasing apoptogenic proteins into the cytoplasm [34]. It is established that mitochondria are the chief sources of endogenous oxidant, including hydrogen peroxide, superoxide and hydroxyl radicals [35]. The presence of these oxidants damage the cell and the mitochondria in which they are produced causing oxidative damage and also adversely affecting the inner mitochondrial membrane which is a specialized structure serving mainly as the site of oxidative phosphorylation [35]. Thus, apoptosis could be triggered by the mitochondria in order for these cells to be eliminated [36]. This suicide pathway is one of the important functions of apoptosis especially with regard to elimination of preneoplastic and neoplastic cells. However, in pathological disease states such as ischemic injury, anorexia, diabetes etc, irreversible insults to the cells will cause excessive apoptosis which will lead to necrosis and disease.

The list of agents that promote onset of the mitochondrial swelling or opening of mitochondrial permeability transition (MPT) pore is long. The notable ones include Ca^{2+} which must be transported into mitochondria, inorganic phosphate, reactive oxygen species (ROS), and some other oxidant chemicals. In addition, membrane depolarization, atractylate and cross- linking of thiols in the pore complex promote pore conductance [37, 38]. Other factors block onset of the MPT and these include Mg^{2+}, (pH below 7), a variety of phospholipase inhibitors including bibucaine, mepacrine and trifluoperazine, the immunosuppressive cyclic endecapeptide cyclosposin A, ADP, bongkreckate and spermine [39, 40].

In the present study, exogenous Ca^{2+} was used to induce opening of mitochondrial membrane permeability transition pore as described by Lapidus and Sokolove, [31]. All the concentrations of MECF (0.2 – 1.4 mg/ml) used inhibited Ca^{2+}-induced opening of MMPT pore in a concentration-dependent manner up to 1mg/ml where it fully attained saturation with 75% pore closure, compared to 0.1mM spermine that inhibited the pore by about 60%. The different fractions of methanol extract of *C ferruginea*, CF, EF, WF and BF also inhibited calcium-induced opening of MMPT pore in a concentration- dependent manner. In this regard, EF and WF inhibited the pore by 71 and 68% respectively, at 1 mg/ml, whereas 1.4 mg/ml each of CF and BF inhibited the pore opening by 94 and 88%, respectively. It seems that the observed inhibition by the fractions especially CF is due to the presence of a high amount of the potent bioactive agents compared to the EF and WF. Consequently, calcium-induced MMPT pore opening was prevented (94%) in the presence of CF or minimized in the case of EF, BF and others. These findings correlate very well with our earlier reports that constituent phytochemicals in plants used as herbs such as *Buccholzia coriacea* and *Alstonia boonei* elicit chemoprotective activity on Ca^{2+}-induced MMPT pore opening in rat liver [41,42]. Thus, once opening of the pore is precluded, other systematic events such as release of cytochrome c and other apoptogenic proteins into the cytosol, dissipation of membrane potential and activation of the executioner caspases [43] would not occur and hence apoptosis would not happen.

Essentially, these results suggest that certain constituent phytochemicals in the crude extracts and the different partially purified fractions of *C ferruginea* may be responsible for the inhibition of calcium-induced MMPT pore opening observed in the present study. The purification of these substances in pure form could give rise to drug candidates that may find therapeutic use in situations that require reduction of excessive calcium-induced cell death or apoptosis possibly as in acquired immuno deficiency syndrome (AIDS), sarcopenia, ischemia, neurodegenerative disorders such as Alzheimer's disease, parkinson's disease, cerebellar degeneration, and pathological conditions including ischemic injury which may induce myocardial infarction, stroke and reperfusion injury [44].

Surprisingly, when intact mitochondria were pre-incubated with the extracts of *C ferruginea* in the absence of calcium, mitochondrial swelling was induced to a large extent by all the extracts. For instance, not less than 4 folds induction of swelling was observed when intact mitochondria were pre-incubated with MECF and the least concentration used induced pore opening by 7.2 folds whereas CF induced pore opening in a concentration- dependent manner with the least and highest concentrations of 0.2mg/ml and 1.4mg/ml inducing pore opening by 0.35 and 11.4 folds, respectively. Furthermore, the saturation concentration for EF was 0.6 mg/ml which opened the pore by 11.3 folds while WF (1 mg/ml) opened the pore by about 4.8 fold. It is surprising that this same leaf extract that inhibited calcium-induced swelling could also be inducing the pore opening in the absence of calcium- the triggering agent. It is possible that the extracts apart from facilitating pore formation may interact with the mitochondrial membrane components such that the membranes become leaky, admits in water and swells as observed in this study. Obviously a leaky mitochondrial membrane negates the criterion of the chemiosmotic theory and may result in mitochondrial swelling, collapse of the mitochondrial membrane potential, reduced oxidative phosphorylation, rupture of outer mitochondrial membrane, cytochrome c release, ATP hydrolysis and apoptosis [43]. This proposed mechanism of alteration of mitochondrial membrane structure by the extracts of *C ferruginea* for an inductive effect on MMPT is in agreement with the reports of Omotuyi *et al.*, 2011 [45] that alterations in membrane phospholipids distribution, key enzyme distribution and phospholipid to protein ratio occur in liver mitochondria and peroxisomes during tumorigenesis. This was suggested to be a mechanism of evading apoptosis in tumor cells; therefore the modification of mitochondrial membrane structure by inducers of apoptosis as suggested in this study would no doubt be among the therapeutic strategies for inducing apoptosis.

The modulatory activity of MECF, CF, EF, BF and WF on mitochondrial F_0F_1 ATPase was also evaluated. The highest stimulation of the ATPase activity was observed at 1.25mg/ml of CF (12.7 folds), followed by 0.5 mg/ml of MECF (4.7 folds) and mg/ml of BF (3.6 folds). Indeed, these data confirm the presence of phytochemicals that uncouples electron transport from oxidative phosphorylation either directly or indirectly as a result of opening of the MMPT pore because the assay was carried out in the absence of calcium. It seems probable that there is the existence of a structure-function relationship being responsible for the extent of the observed stimulation of the enzyme activity. For example, hydrophobic bioactive agents fractions such as CF would easily diffuse across the hydrophobic mitochondrial membrane lipid bilayer, therefore, possibly encouraging a formation of the MMPT pore or this may be a plausible explanation for the high level of induction of MMPT pore opening and stimulation of F_0F_1 ATPase activity by CF. Although it is not clear how EF induced MMPT pore opening but if the type of phytochemicals in CF that were responsible for induction of MMPT were present in EF, then a higher concentration of EF may be required to elicit the same level of induction as observed in this study. Therefore further work is necessary to isolate and characterize the bioactive components in the extracts to serve as beneficial drug candidates for cancer chemotherapy.

References

1. Reed J. Mechanism of apoptosis avoidance in cancer. Curr. Opin. Oncol. 1999; 11: 68 - 75
2. Parone P, Priault M, James D, Nothweler SF, and Martinou JC. Apoptosis: bombarding the mitochondria. In essays in Biochemistry, programmed cell death. Cotter TG; ed Portland press, London U.K.2003, 41-51,
3. Nazareth W, Yafei N, and Crompton M. Inhibition of anoxia-induced injury in heart myocytes by cyclosporine A. J Mol Cell Cardiol 1991; 23: 1351-1354
4. Griffiths EJ, and Halestrap AP. Protection by Cyclosporin-A of ischemia/reperfusion –induced damage in isolated rat hearts. J Mol Cell Cardiol 1993; 25: 1461-1469

5. Vanderluit JL, McPhail LT, Fernandes KJ, Kobayashi NR and Tetzlaff W. In vivo application of mitochondrial pore inhibitors blocks the induction of apoptosis in axotomized neonatal facial motoneurons. Cell Death Differ 2003; 10: 969-976
6. Bernardi P, Krauskopf A, Basso E, Petronilli V, Bladley-Dyson E, Di Lisa F, and Foite MA. The mitochondrial permeability transition from *in vitro* artefact to disease target. FEBS Journal 2006; 273: 2077-2099
7. Petronilli V, Nicole A, Costantini P, Colonna R and Bernardi P. Regulation of the permeability transition pore, a voltage-dependent mitochondrial channel inhibited by cyclosporine A. Biochim Biophys Acta 1995; 1187: 225-259
8. Silva PMS, Tanabe E, Munhos-Hermoso AP, Bersani-Amado CA, Bracht A, Ishii-Iwamoto EL and Salgueiro-Paradigorria CL. Changes in calcium-dependent membrane permeability properties in mitochondria of livers from arthritic rats. Cell Biochem Funct 2008; 26: 443-450
9. Inoue I, Suzuki R, Koide T, Sakaguchi N, Ogihara Y, and Yabu Y. Antioxidant, gallic, induces apoptosis in HL-60 cells. Biochem Biophys Res Commun 1994; 204: 898-904
10. Davis JN, Singh B, Bhuiyan M and Sarkar FH. Genistein-induced upregulation of p21WAF1, downregulation of cyclin B, and induction of apoptosis in prostate cancer cells. Nutr Cancer.1998; 32: 123-131
11. Hsu SH, Tsai TR, Lin CN, Yen MH. and Kuo KW. Solamargine purified from *Solanum incanum* Chinese herb triggers gene expression of human TNFR I which may lead to cell apoptosis. Biochem Biophys Res Commun 1996; 229: 1-5
12. Buttke TM, and Sandstrom PA. Oxidative Stress as a mediator of apoptosis. Immunol Today 1994; 15: 7-10
13. Martin KR. Targeting Apoptosis with Dietary Bioactive Agents. Soc Exp Biol Med 2006; 231: 117 – 129
14. Berridge MV, Herst PM and Lawen A. Targeting mitochondrial permeability in cancer drug development. Mol Nutr Food Res 2009; 53: 76-86
15. Hatefi Y. ATP synthesis in mitochondria. Eur J Biochem 1993; 218: 759-767
16. Dietze EC, Caldwell LE, Grupin SL, Mancini M and Seewaldt VL. Tamoxifen but not 4-hydroxytamoxifen initiates apoptosis in p53 (-) normal human mammary epithelial cells by inducing mitochondrial depolarization. J Biol Chem 2001; 276: 5384-5394
17. Morin D, Barthelemy S, Zini R, Labidalle S and Tillement J. Curcumin induces the mitochondrial permeability transition pore mediated by membrane protein thiol oxidation. FEBS Lett 2001; 495: 131-136
18. Hail N Jr and Lotan R. Examining the role of mitochondrial respiration in vanilloid-induced apoptosis. J Natl Cancer Inst 2002; 94: 1281-1292
19. Parvez M and Rahman A. A novel antimicrobial Isoflavone Galactoside from *Cnestis ferruginea* (Connaraceae) J Chem Soc Pak 1992; 14 (3): 221 -223
20. Declume C, Assamoi A and Akre TB. Anticonvulsant activity of *Cnestis ferruginea,* D.C: Connaraceae Annales pharmaceutiques francaise 1984; 42(1): 35 41
21. Ogbechie AK, Olugbade TA and Oluwadiya JO. Chemical constituents of *Cnestis ferruginea* DC II: Petroleum etter extract of the fruit. Niger J Pharm Sci 1987; 3 (2): 36 -38.
22. Parvez M and Rahman A. A novel antimicrobial Isoflavone Galactoside from *Cnestis ferruginea* (Connaraceae) J Chem Soc Pak (1992; 14 (3): 221 -223
23. Adisa RA, Oke JM, Olomu SA and Olorunsogo OO. Inhibition of human haemoglobin glycosylation by flavonoid-containing leaf extracts of *Cnestis ferruginea.* J Cameroon Acad Sci 2004; 4: 351 – 359
24. Adisa RA, Choudhary MI, Adewoye EO and Olorunsogo OO. Hypoglycaemic and Biochemical Properties of *Cnestis ferruginea.* Afr J Trad CAM 2010; 7 (3): 185-194
25. Adisa RA. Modulation of rat liver mitochondrial membrane permeability transition and hapatoprotection by phenolic compounds purified from leaves of *Cnestis ferruginea* (de Candolle). A PhD thesis submitted to University of Ibadan 2009; 147-161
26. Finley J. Proposed criteria for assessing the efficacy of cancer reduction by plant foods enriched in carotenoids, glucosinolates, polyphenols, and selenocompounds. Ann Bot 2005; 95: 1075 – 1096
27. Kelloff GJ, Crowell JA, Steele VE, Lubet RA, Boone CW, Malone WA, *et al.* Progress in cancer chemoprevention. Ann NY Acad Sci 1999; 889: 1-13
28. Schneider WC. Intracellular distribution of enzymes in the oxidation of octanoic acid by rat liver fractions. J Biol Chem 1948; 176: 259-266

Johnson D, and Lardy H. Isolation of liver or kidney mitochondria. Methods Enzymol 1967; 10: 94 –96

Lowry OH, Rosenbrough NJ, Farr AL and Randall RJ. Protein measurement with Folin phenol reagent. J Biol Chem 1951; 193: 265-275

Lapidus RG, and Sokolove PM.. Spermine inhibition of the permeability transition of isolated rat liver mitochondria: an investigation of mechanism. Arch Biochem Biophys 1993; 306: 246-253.

Lardy HA, and Wellman H. The catalytic effect of 2,4-dinitrophenol on adenosine and soluble enzymes. J Biol Chem 1953; 201: 357 – 370

Olorunsogo OO and Malomo SO Mitochondrial bioenergetics during exposure of rats to Perfluidone, a florinated aryl alkylsulphonamide. Chem Biol Inter 1984; 52: 67 -78

Martinou JC, and Green RD. Breaking the mitochondrial barrier. Nature Rev 2001; 2: 63-67

Hagen T, Ingersoll RT, Liu J, Lykkesfeldt J, Wehr CM, Vinarsky V, Bartholomew JC, and Ames BN. (R)-á-Lipoic acid-supplemented old rats have improved mitochondrial function, decreased oxidative damage, and increased metabolic rate. FASEB J 1999; 13: 411-418

Lowe SW, Ruley HE, Jacks T, and Housman DE.. P53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 1993; 34: 957 – 967

Bernardi P, Vassanell S, Veronese P, Colonna R, Szabo I, and Zoratti M. Modulation of the mitochondrial permeability transition pore. Effect of protons and divalent cations. J Biol Chem 1992; 267:2934-2939.

Costantini P, Chernyak BV, Peronilli V, and Bernardi P. Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites. J Biol Chem 1996; 271: 6746-6751.

39. Lapidus RG and Sokolove PM. The mitochondrial permeability transition, Interactions of Spermine, ADP and inorganic phosphate. J Biol Chem 1994; 269(29): pp. 18931 -18936.
40. Fontaine E, and Bernardi P. Progress on the mitochondrial permeability transition pore, regulation by complex I and ubiquinone analogs. J Bioenerg Biomembr 1999; 31 (4): 335 – 345
41. Adisa RA, Ajewole I, Omolohunnu I, and Olorunsogo OO. Induction of Membrane Permeability Transition (MPT) Pore Opening by *Buccholzia coriacea* (Engl.) seeds *in vitro* in rat liver mitochondria. Afr J Med Med Sc 2010; **39:** 129-138
42. Salako TA, Adisa RA, Alao OO, Adeniran OO and Olorunsogo OO. Effects of Methanolic and Chloroform Extracts of Leaves of *Alstonia boonei* on rat liver Mitochondrial Membrane Permeability Transition Pore. Afr J Med Med Sc 2010; 39: 109-116
43. Lemasters JJ, Nieminen AL, Qian T, Trost LC, Elmore SP, Nishimura Y, Crowe RA, *et al.* The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. Biochim Biophys Acta 1998; 1366: 177-196
44. Martin KR. Targeting Apoptosis with Dietary Bioactive Agents. Soc Exp Biol Med 2006; 231: 117 – 129
45. Omotuyi IO, Ologundudu A, Okugbo TO, Oluyemi KA and Omitade OE. Cytoplasmic, peroxisomal and mitochondrial membrane phospholipid alteration in 3'-diamino-azo-benzene (3'-DAB)- induce he[patocellular carcinoma. Afr J Biochem Res 2011; 5(1): 7-13