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Induction of rat hepatic mitochondrial membrane permeability transition pore opening by leaf extract of *Olax subscorpioidea*

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ABSTRACT

Background: The induction of the mitochondrial membrane permeability transition (MMPT) pore has been implicated in the cascade of events involved in apoptosis (programmed cell death). *Olax subscorpioidea* is traditionally used for the treatment of several diseases and infection. However, its role on MMPT is not yet established. This study was aimed at evaluating the effects of varying concentrations of the methanol leaf extract of *O. subscorpioidea* (MEOS) on MMPT pore opening, mitochondrial adenosine triphosphatase (ATPase), and mitochondrial lipid peroxidation. **Materials and Methods:** Opening of the pore was spectrophotometrically assayed under succinate-energized conditions. **Results:** In the absence of triggering agent (calcium), MEOS induced MMPT pore opening by 350, 612, 827, 845% at 36, 60, 86 and 112 μg/ml, respectively. MEOS further induced MMPT pore opening in the presence of a triggering agent by 866, 905, 831, 840, 949% at 12, 36, 60, 86 and 112 μg/ml, respectively. The extract significantly induced mitochondrial membrane lipid peroxidation in all the concentration used. MEOS also significantly increased mitochondrial ATP hydrolysis by mitochondrial ATPase in all concentration of the extract used. **Conclusion:** It may be deduced from this results, that MEOS contains certain bioactive components that may find use in pathological conditions that require an enhanced rate of apoptosis.

Key words: Apoptosis, mitochondrial membrane permeability transition pore, *Olax subscorpioidea*

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INTRODUCTION

The intrinsic or mitochondrial pathway of apoptosis is initiated by DNA damage and endoplasmic reticulum stress, which leads to permeabilizations of the mitochondria outer membrane.[1] However, permeabilizations can either be permeability transition (PT) pore dependent or independent.[2] The PT pore is a calcium-sensitive mitochondrial channel, which allows the passage of molecules <1.5 kda in size.[2,3] It comprises of the matrix protein cyclophilin D, the inner mitochondrial membrane protein adenine nucleotide translocator, and the outer mitochondria membrane protein, voltage-dependent anion channel.[4] This definition dates back to four decades ago.

Mitochondrial membrane PT (MMPT) pore opening disrupts the proton gradient of the inter-membrane space configured by the electron transport chain of the inner mitochondria membrane, thus, uncoupling oxidative phosphorylation, rupture of the outer mitochondrial membrane, release of apoptogenic proteins such as cytochrome C, apoptosis-inducing factor, endonuclease G, small mitochondrial dependent activator of caspase (SMAC).[5,6] The apoptosome, a complex of cytochrome C and apoptosis protease activating factor-1 activates caspase 9 by cleavage of its zymogen (pro-caspase 9). Caspase 9 further activates downstream effector or execution caspases (caspase 3 and 7).[7] MMPT has therefore become a surrogate marker of cell death in pathologies such as AIDS, septic shock, and leukemias under chemotherapy.[2]

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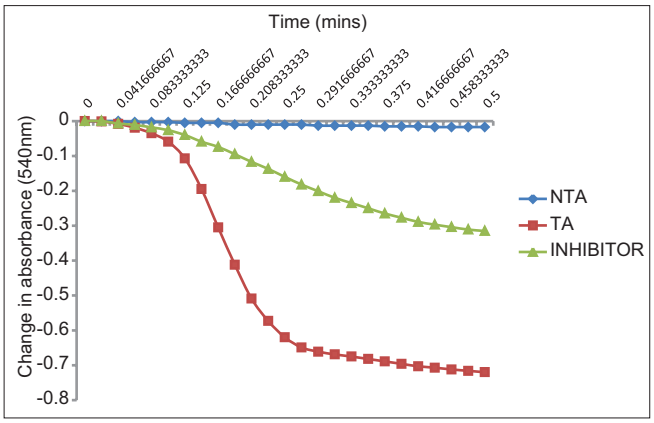

Figure 1: *In vitro* $Ca2^{+}$ - induced opening of rat liver mitochondrial membrane permeability transition pore and inhibition of the opening by spermine. NTA: No triggering agent, TA: Triggering agent, INHIBITOR: Spermine

The use of the medicinal plant in Africa for the treatment of diseases has risen over the years.[8] *Olax subscorpioidea,* which belong to the family of *Olaceae* is widely distributed in Nigeria and neighboring countries such as Zaire and Senegal.[9] It is recognized as "Ifon" in South-Western Nigeria and "Aziza" in the Eastern part of Nigeria. Bioactive components reported to be present in this plant material are flavonoids, saponins, alkaloids, glycosides, tannins, and steroids.[8] Certain antimicrobial activities elicited by the plant have also been reported,[9] saline and alkaline extracts have also shown membrane stabilizing and anti-protease activities respectively. Although, advancement in the field of medicine has led to the unpopularity of medicinal plants globally, however, the World Health Organization places medicinal plants as the best source of a variety of drugs.[10] Many compounds from plant origin have been tested for their apoptosis-inducing capability.[11] We established the possible apoptotic potential of methanol extract of *O. subscorpioidea* by elucidating the role it plays in the induction of MMPT pore opening which is an important hallmark in mitochondrial apoptosis. Induction of apoptosis in cancer cells is one useful strategy for anticancer drug development.[12]

MATERIALS AND METHODS

Materials

Mannitol, sucrose, 4-(2-Hydroxyethyl) piperazine -1-ethanesulfonic acid (HEPES), ethylene glycol tetraacetic acid (EGTA), spermine, rotenone, sodium succinate hexahydrate, bovine serum albumin (BSA), methanol, folin C, sucrose were products of Sigma-Aldrich Co, USA. All chemicals were of analytical grade.

Extraction of plant material

The authenticated leaves were washed and air-dried at room temperature (28°C–30°C) for 60 days. The air-dried leaves were pulverized into particulate matter.

Preparation of methanol extract of Olax subscorpioidea leaves

Thousand gram of *O. subscorpioidea* leaves was macerated (soaked) in 5 l of absolute methanol in an air-tight glass container and left on standing at room temperature for 72 h, filtered and then soaked for another 24 h and filtered by muslin cloth and cotton buds. The combined filtrates were concentrated in vacuo to remove solvents by evaporating in a rotary evaporator at a temperature below 40°C, producing 200 g of methanol-fraction of the leaves. The yield was then refrigerated prior to use at 4°C.

Methods

Mitochondria isolation

Albino Wistar rats obtained from the National Institute of Medical Research, Lagos, Nigeria were sacrificed and their mitochondria isolated essentially according to the modified method of Johnson and Lardy by Olorunsogo *et al.* which was reported by Lapidus and Sokolove.[13-15] Liver sample was rapidly excised, trimmed to remove excess tissues in a buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 M KOH, and 1 mM EGTA, pH 7.4.

The liver samples were weighed, chopped and suspended in the same buffer to make a 10% homogenate. The suspension was immediately homogenized on ice using a porter glass homogenizer. The homogenate was centrifuged in a high speed refrigerated centrifuge (SM-18B, Surgifield medical, England) and the mitochondrial fractions obtained were washed with a washing buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES-KOH and 0.5% BSA, pH 7.4.

The mitochondrial pellets were suspended in swelling buffer (210 mM mannitol, 70 mM sucrose, and 5 mM HEPES-KOH, pH 7.4) and immediately dispensed in 2 ml Eppendorf tubes. Isolated mitochondria were used within 3 h of isolation.

Mitochondrial protein determination

Mitochondrial protein was determined according to the method described by Lowry *et al.*[16] Using BSA as standard.

Mitochondria swelling assay

Mitochondria (0.4 mg of protein/ml) were preincubated in a 1 cm light path glass cuvette in the presence of 0.8 μM rotenone in swelling buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES-KOH, (pH 7.4) prior to the addition of 120 μM $CaCl_2$ (triggering agent). Mitochondrial permeability was quantified as changes in absorbance at 540 nm in the presence of 5 mM sodium succinate. 120 mM $CaCl_2$ was omitted in assays without triggering agent.[17]

Mitochondria lipid peroxidation

Mitochondrial lipid peroxidation was determined by the method described by Ruberto *et al.*[18]

Mitochondrial adenosine triphosphatase activity

Adenosine triphosphatase activity was determined at an absorbance of 680 nm according to the method described by Lardy and Wellman.[19]

Statistical analysis

Statistical package for the social sciences (SPSS) version 15.0 was used for the statistical analysis (SPSS Inc. Chicago Illinois, USA). Group comparison was done using one-way analysis of variance (ANOVA) test. Test for statistical significance was carried out at 95% confidence interval.

RESULTS

All increase in MMPT pore opening of the intact mitochondria were reversed by 4 mM spermine, a reference inhibitor of MMPT pore opening [Figure 2]. In the absence of triggering agent, hydrated calcium chloride [Figure 3], methanol leaf extract of *O. subscorpioidea* (MEOS) did not induce MMPT pore opening at 12 μg/ml but induced MMPT pore opening by 350, 612, 827, 845% at 36, 60, 86 and 112 μg/ml MEOS, respectively. MEOS induced MMPT pore opening in the presence of a triggering agent by 866, 905, 831, 840, 949% at 12, 36, 60, 86 and 112 μg/ml MEOS, respectively. However, varying concentration of MEOS significantly ($P < 0.05$) induced mitochondrial membrane lipid peroxidation when compared with the control, observed inductive fold are 0.9, 0.7, 1.2, 0.8 and 0.9 at 60, 120, 240, 480 and 960 μg/ml respectively [Figure 1]. In addition, we observed that MEOS also significantly ($P < 0.05$) increased mitochondrial phosphate release by mitochondrial adenosine triphosphatase (ATPase) in all concentration of the extract when compared with the control. Observed percentage induction fold are 105, 74, 49 and 79% at 12, 36, 60, 86 μg/ml MEOS, respectively [Figure 4].

DISCUSSION

This study shows the results of the leaf methanol extracts of *O. subscorpioidea* on MMPT pore opening. *O. subscorpoidea* induced MMPT pore opening of intact mitochondria suggesting the presence of certain phytochemicals in the methanol extract that are capable of altering the integrity of the mitochondrial membrane, albeit, degree and the pattern of induction were varied for the different concentration of the extract that was assessed. Since MMPT pore is a nonselective, high-conductance channel with multiple macromolecular components,[20] such as adenine nucleotide translocase and cyclophilin D, which form at sites where the inner and outer membranes of the mitochondrion meet,[21,22] opening of the pore is considered

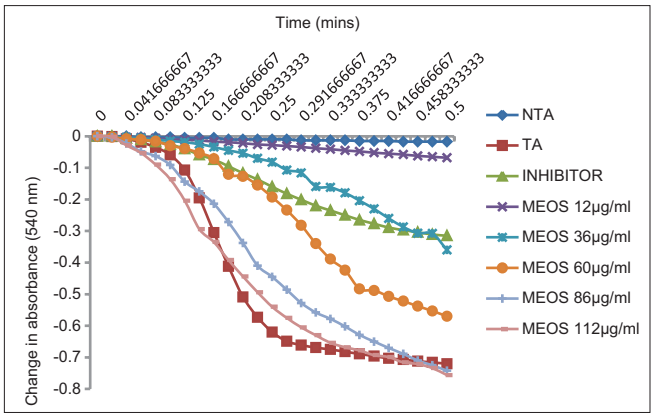

Figure 2: *In vitro* induction of rat liver mitochondrial membrane permeability transition pore opening by varying concentrations of methanol leaf extract of *Olax subscorpioidea* in the absence of calcium. NTA: No triggering agent, TA: Triggering agent, INHIBITOR: Spermine

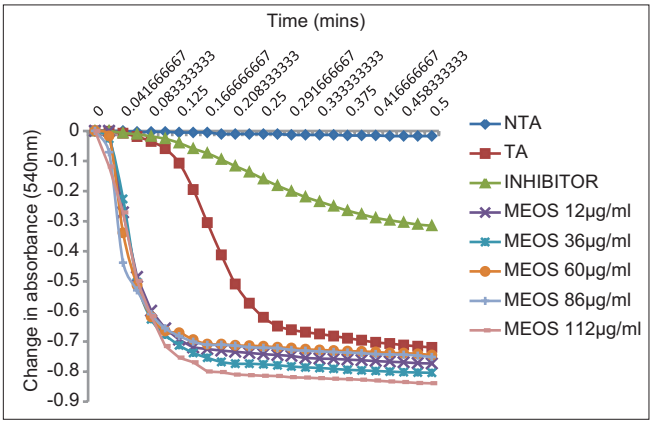

Figure 3: *In vitro* induction of rat liver mitochondrial membrane permeability transition pore opening by varying concentrations of methanol leaf extract of Olax subscorpioidea in the presence of calcium. NTA: No triggering agent, TA: Triggering agent, INHIBITOR: Spermine

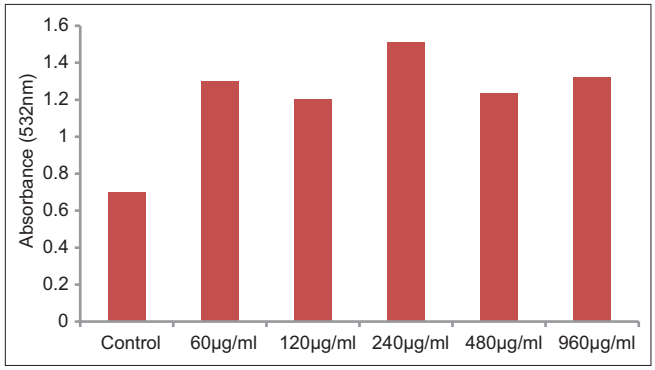

Figure 4: *In vitro* induction of rat hepatic mitochondrial lipid peroxidation by varying concentration of methanol leaf extract of *Olax subscorpioidea*. Values represent the mean ± standard error of mean

a major marker of the intrinsic pathway of apoptosis, which results in the destruction of mitochondrial function, being the power house of the cell.[23] In the absence of calcium [Figure 3], induction of MMPT pore opening was

observed for 36, 60, 86 and 112 μg/ml of the extract however, the extract concentration of 12 μg/ml did not induce opening of the MMPT pore. Otori and Mann reported that *O. subscorpioidea* is high in mineral content such as calcium, iron, magnesium and phosphorus to mention a few,[24] these elements are most likely to be responsible for the activities of this plant extract in our research. Hunter and Harworth reported that calcium, phosphate, arsenate and oleic acid were inducers of MMPT pore opening while inhibitors of MMPT pore opening include cyclosporin A, bongkrekic acid, EGTA, adenine nucleotides and magnesium.[25-27] Cyclosporine A, a fungal peptide interacts with cyclophilin D a peptidyl prolyl cis-trans isomerase to effect inhibition of pore opening. Prolonged opening of MMPT pore makes the mitochondrial inner membrane permeable to solutes whose molecular size are above 1500 Da consequently compromising the mitochondrial integrity thereby releasing certain pro apoptotic proteins such as SMAC/DIABLO, cytochrome C, endonuclease G and some other apoptogenic molecules. The release of cytochrome C-mediated by MEOS is currently ongoing in our laboratory. MEOS executed an increased significant increase in MMPT pore opening in the presence of the used triggering agent [Figure 5]. All the concentration used induced MMPT pore opening beyond the standard triggering agent. The result exhibited no form of inhibition, this result is similar to the observation of Adisa *et al.* on methanol fraction of *Cnestis ferruginea*.[28] This suggests that MEOS is abundant in phytochemicals and inorganic minerals that aid mitochondrial apoptosis since MMPT pore opening prompts the demise of the cell.[3] Induction of apoptosis by certain phytochemicals in human prostate adenocarcinoma cells has also been reported.[29] Since, *O. subscorpioidea* is reported to contain alkaloids, anthocyanins, flavanoids, and tannins,[8] therefore, MMPT pore modulating activities of MEOS can also be hinged on steroid alkaloid in this fraction in addition to elements such as calcium and phosphate present in the plant extract.

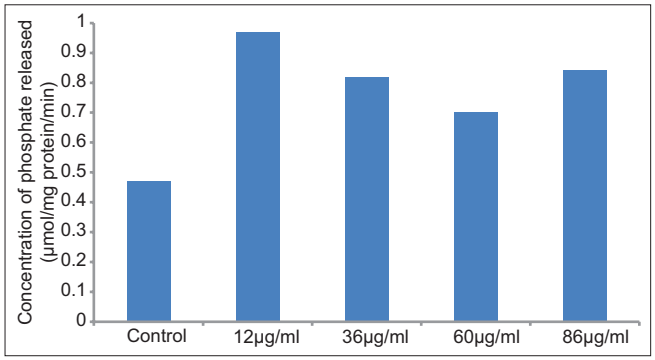

Figure 5: *In vitro* induction of rat hepatic ATP hydrolysis by mitochondrial ATPase activity induced by varying concentration of methanol leaf extract of *Olax subscorpioidea*. Value represents the mean ± standard error of mean

A similar medicinal plant that has shown MMPT pore opening, *Calliandra portoricensis* root bark was reported by Oyebode *et al.*[3] In addition to MMPT pore opening, we have observed that MEOS also induced the hydrolysis of ATP, which was characterized by the release of inorganic phosphate during mitochondrial stress [Figure 4]. The mitochondrial ATP synthase contributes to most of the ATP synthesized by the cell except for substrate level phosphorylation that occur during glycolysis.[14] Giorgio *et al.* have reported that mitochondrial ATPase is an integral part of the MMPT pore;[30] therefore external induction of MMPT pore opening is most likely to compromise the activities of the mitochondrial ATPase thus, changing the flux of the reaction to the hydrolysis of ATP catalyzed by the same enzyme, that is, the mitochondrial ATPase. Cyclophilin D an important component of the MMPT pore reversibly binds the mitochondrial ATPase thereby preventing it from ATP synthesis. It can also be suggested that MEOS induces MMPT pore opening by providing the phosphate ion needed by cyclophilin D, this reversibly bind mitochondrial ATPase thereby hydrolyzing ATP releasing more phosphate ions consequently. Another appropriate vantage point of the possible induction of MMPT pore opening by MEOS is the C subunit of the mitochondrial ATPase that forms a voltage sensitive channel. The entry of calcium down its concentration gradient causes membrane depolarization.[31] Since MEOS is abundant in inorganic minerals as calcium, it probably explains the effectiveness of MEOS in the induction of MMPT pore opening and its role in the hydrolysis of ATP, which certainly is caused by inner mitochondrial membrane depolarization. Despite the mitochondrial membrane depolarization, increased phosphate release aided by mitochondrial ATPase and MMPT pore opening, we also observed that MEOS significantly ($P < 0.05$) induced mitochondrial membrane lipid peroxidation. Although the extent of lipid peroxidation was not concentration dependent [Figure 1], however, all concentration of MEOS significantly induced lipid peroxidation of the mitochondria. The mitochondria are a significant source of free radicals in the cell due to the intense oxidative process that ultimately result in the synthesis of ATP, thus, the "power house" of the cell.[32] Oxidants that originated from the matrix are capable of inducing lipid peroxidation of the inner mitochondrial membrane making the membrane more permeable to hydrophilic ions and high molecular structures thereby depolarizing this supramolecular structure.[33] ATP becomes hydrolyzed instead of being synthesized by the mitochondrial ATPase,[14] this in turn compromises the integrity of the mitochondria by opening the membrane PT pore. Scavenging excessive free radicals generated by a dying cell is vital against oxidative damage. The role *O. subscorpioidea* play in the induction of antioxidant

enzymes has not been reported. Since antioxidant enzymes are upregulated by reactive oxygen and nitrogen species capable of becoming free radicals, these activate redox-sensitive transcription factors such as Nrf2 and NF-kB, which increases the expression of antioxidant enzymes such as catalase, superoxide dismutase, glutathione reductase and peroxidases. MEOS can, therefore, induce mitochondrial apoptosis without necessarily causing a free radical out-burst. Persistent free radical can lead to a more dangerous type of cell death, necrosis.[34] We have observed that MEOS possesses phytochemicals such as phenols, flavonoids, and terpenoid, these are capable of restricting or salvaging free radical damage [Table 1]. *O. subscorpioidea* has been reported as a nontoxic plant if ingested,[8] from our result, induced MMPT pore opening by MEOS may result in the release of cytochrome C leading to the activation of initiator and executioner caspases such as caspases 9 and caspase 3 respectively, these are mediators of programmed cell death also known as apoptosis.[35,36] Identifying drugs and phytochemicals from natural products that can induce apoptosis is a growing research area around the world. Recently, Nikhil *et al.* were able to show that *Acacia catechu* can induce apoptosis by altering Bax/Bcl-2 ratio.[37] This apoptosis induction is also achieved via the mitochondrial outer membrane permeabilization. Similarly, *Crocus sativus* has been reported to possess anticarcinogenic properties,[38] however, its mode of preferential apoptosis has not been elucidated. Apoptosis induction is an integral part of cancer research,[39,40] apoptotic agents are potential tumor suppressors or inducers of the expression of tumor suppressors, however, other area of cancer research are the study of oncogenes and epigenetics which determines the expression of either these oncogenes or tumor suppressor proteins. Therefore, we propose that *O. subscorpoidea* is a potential pro-apoptotic plant material, which promises a future in the treatment of cancer via preferentially inducing apoptosis on target cells by inducing MMPT pore opening. It is however, noteworthy that the extraction of biologically active compounds from plant material depends on the types of solvent used in the extraction procedure. Certain pro-mitochondrial apoptosis compounds will dissolve in polar solvents because of the aqueous environment in which the mitochondria are suspended in the cell, that is, the cytosol. Added to the long list of the medicinal properties of *O. subscorpioidea*, we have proposed that it is potentially pro-apoptotic, and this property is mediated via the induction of MMPT pore opening. Our experiment, therefore, could serve as a pivotal to prospective findings. In addition, extract potency could be affected by certain factors as the type of solvent used for extraction as well as the method employed in extraction.

Table 1: Phytochemical analysis of methanolic extract *olax subscorpioidea*

Phytochemical	Status
Tanins	Positive
Saponin	Positive
Flavanoids	Positve
Alkaloids	Positive
Glycosides	Negative
Terpenoids	Positive
Phenols	Positive
Steroids	Positive

CONCLUSION

The *in vitro* data here suggests that the presence of pro-apoptotic properties in methanol leaf extract of *O. subscorpioidea* makes it a plant open for novel discoveries. Basically, extraction of bioactive components from medicinal plant is known to permit the demonstration of their physiological activity as well as facilitate pharmacological studies on the plant leading to synthesis and discovery of pure and potent compounds that have low toxicity when used as drugs. This will require further isolation and characterization.

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