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In vitro biochemical evaluation of methanol extract of *Moringa oleifera* pods on rat liver mitochondrial membrane permeability transition pore and lipid peroxidation

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Abstract

Background: *Moringa oleifera* is a well-known world herbal plant for its amazing medicinal and nutritional properties. The effect of methanol extract of *M. oleifera* can be useful in managing diseases associated with mitochondrial membrane permeability transition pore and lipid peroxidation.

Methods: Evaluation was done at varying concentrations of the methanol pods extract on mitochondrial membrane permeability transition pore opening (swelling) and Fe^{2+}- H_2O_2-EDTA (Fenton reaction)-induced lipid peroxidation *in vitro*. Five male albino rats (weighed 120-250 g) were anaesthetized and sacrificed; the liver was excised and homogenized to obtain mitochondria pellets. This study analyzed the effect of varying concentrations of methanol pods extract of *M. oleifera* at 50, 150, and, 300 µg/ml respectively. The effects of *M. oleifera* varying concentration *in vitro* was determined using malondialdehyde reaction quantified at 532 nm in a UV- spectrophotometer as index for lipid peroxidation and spectrophotometric absorptions at 520 nm was observed as an index of mitochondrial membrane permeability pore respectively.

Results: Varying concentrations of methanol pods extract of *M. oleifera* at 50, 150, and 300 µg/ml in the presence and absence of triggering agent (Ca^{2+}) inhibited opening of mitochondria membrane permeability transition pore while 0.25, 0.50 and 1.00 mg/ml inhibited lipid peroxidation induced mitochondria of the rat liver respectively in a concentration dependent mode.

Conclusion: The results suggest that methanol extract of *M. oleifera* pods at high concentrations (such as 300 µg/ml and 1.00 mg/ml respectively) may inhibit mitochondrial membrane permeability transition pore opening and lipid peroxidation.

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Introduction

Plants are essential for human kind, animals, and environment in all viable ecosystems. They produce primary and secondary bioactive compounds which encompass a wide array of functions [1]. According to World Health Organization, medicinal plants are the most important source to derive a variety of novel herbal medicine. Around 80 % of persons from advanced and developing nations use conventional medicine, which has composites derived from medicinal plants. The *Moringa oleifera* tree is one of the world's most useful herbs [2] and it is a well-known plant herb for its unique medicinal and nutritional properties [3]. The plant gives a rich exceptional mixture of zeatin, kaempferom, quercetin, β-sitosterol and caffeoylquinic acid [4,5] with substantial medicinal effects. Various parts of the plant serves as cardiac and circulatory stimulants, anti-tumour, anti-inflammatory, antioxidant, antipyretic, antiepileptic, antiulcer [6] and many other important medicinal properties.

The mitochondria are the powerhouse of the cells, which is useful for creating energy as adenosine triphosphate (ATP) and heat. They are also involved in the apoptosis-signaling pathway. One main functional attribute of mitochondria is the production of a huge transmembrane potential through the inner mitochondrial membrane. This process generates proton gradients which drives the production of ATP. The mitochondrial potential also powers the buildup of calcium into mitochondria. Calcium "overload" in the mitochondrial has been associated repeatedly in diverse disease mechanisms. The main process understood to facilitate calcium-induced mitochondrial damage is the mitochondrial membrane permeability transition (MMPT) pore [7], a huge conductance pathway in the inner mitochondria membrane that leads to ATP depletion, and necrotic cell death [8].

The MMPT pore may signify a channel for calcium to elude from excess mitochondria under biological states [9], it may play an essential function in form of some apoptotic cell death, as the opening of pores causes swelling of the mitochondrial and subsequent opening of the outer membrane with the release of cytochrome c [10]. Mitochondria are a main spring of free radicals, which an elevated concentration poses the threat of damage to cell membranes and DNA.

In these findings we explored the medicinal properties of *M. oleifera* effects on liver mitochondrial membrane permeability and lipid peroxidation to proffer additional information and knowledge to drug production for the treatment of lipid peroxidation and membrane permeability associated disorders.

Methods

Plant Collection, Processing and Extraction

Fresh pods of *M. Oleifera* were taken from a farmland at Irolu, Ikenne local government, Ogun state, Nigeria and were ascertained by Professor E.B. Esan of the Department of Basic sciences, Babcock University, Ilishan Remo, Ogun State. The pulverized test sample (100g) was dissolved in 70% methanol and shaken intermittently for 48 hours. Filtration of the test samples was done using the No.1 filter paper and the filtrate was concentrated in the water bath and preserved in the refrigerator pending further use.

Experimental Animals

Five male albino Wistar rats (weighing 120-250 g) were acquired from the department of physiology animal house, University of Ibadan, Ibadan, Nigeria. The rats were adapted for 15 days while been given water and rat chow *ad libitum*, and kept under accepted conditions of temperature and 12-hour dark/light cycle.

Experimental Design

A total of 5 male albino rats were used for the experimental study. The animals were anaesthetized with chloroform and subsequently sacrificed. The liver was excised and excess tissues trimmed, it was homogenized and centrifuged to obtain mitochondria pellets. This was further subjected to the swelling buffer assay test for the assessment of mitochondria membrane permeability transition (MMPT) pore using varying concentrations (50, 150 and 300µg/ml) of *M. oleifera* pods extract and the effect was investigated on the induction of lipid peroxidation in the rat liver mitochondria.

Preparation of Low Ionic Strength Rat Liver Mitochondria

Buffer C: 0.12 g of Hepes (Sigma Aldrich, USA) was dissipated in 70 ml of distilled water. 3.83 g mannitol (Sigma Aldrich, USA) was added and standardized to pH 7.4 with KOH (Sigma Aldrich, USA Products) and

the whole solution made up to 100 ml. The buffer was then stored in the refrigerator at 4°C.

Buffer D: 0.12 g of Hepes (Sigma Aldrich, USA) was dissolved in 70 ml of distilled water. 3.83 g mannitol (Sigma Aldrich, USA) and Bovine Serum Albumin (Sigma Aldrich, USA) of 0.5 % were also added to the mixture and standardized to pH 7.4 with KOH (Sigma Aldrich, USA). The whole solution made up to 100 ml. The buffer solution was stored in the refrigerator at 4 °C.

Isolation of Liver Mitochondria from Rats

The liver mitochondria were isolated using the method by Johnson and Lardy (1967) combined with that of Schneider [11]. The excised and weighed rat liver was washed several times in buffer C until a clear wash was obtained and minced with a pair of scissors. The suspended liver homogenate in buffer C was implored into a refrigerated MSE centrifuge. The nuclear fraction and cellular debris were pelleted down by low speed centrifugation at 2300 x g for 5 minutes. The supernatant was re-centrifuged at the same speed and time to remove unbroken cells. The supernatant obtained was centrifuged at 13,000 x g for 10 minute to sediment the mitochondria. The brown mitochondrial pellet was washed by re-suspending in buffer D and centrifuged twice at 12,000 x g for 10 minutes. The obtained mitochondria were instantly suspended in an appropriate volume of ice cold Mannitol, Sucrose, Hepes pH 7.4-KOH (MSH) buffer then immediately dispensed in 1.5 ml eppendorf tubes in aliquot and kept on ice for immediate se. All assays were carried out in an ice-cold medium in order to preserve the integrity of the mitochondria.

Assessment of effects of methanol pods extracts *M. oleifera* on MMPT in Rat Liver Mitochondria

Swelling of the mitochondria was determined using the Lapidus and Sokolove method [12]. Intact mitochondria (0.4 mg/ml) were pre-incubated in 1.5 ml eppendorf tubes in the presence of varying concentrations of coartemether (50, 150 and 300 µl) (which were all in iced conditions for 5 minutes), and in 0.8 µM rotenone for 3.5 minutes before adding 5 mM sodium succinate. Mitochondria were pre-incubated in 0.8 µM rotenone for 3 minutes, and Ca^{2+} was added, and Na succinate was added 30 seconds later. Swelling rate was quantified as a decrease in absorbance at 540 nm wavelength at a time interval of 30 seconds for 12 minutes with the use of JENWAY 6305 spectrophotometer. The sample blank was also taken into consideration every other condition including the mitochondrial buffer volume but excluding the mitochondrial therein.

Effects of Methanol Pods Extract *M. oleifera* on Lipid Peroxidation

The induction of lipid peroxidation was done using Fe^{2+}-EDTA system in rat liver mitochondria in the presence and absence of methanol pods extracts of *M. oleifera* of varying concentrations to produce thiobarbituric acid reacting constituents (TBARS) which contained a mixture of 0.1 ml of mitochondria in MSH buffer pH 7.4, 0.1 ml of $FeSO_4$, 0.1 ml of EDTA, 0.1 ml of H_2O_2, and 0.5 ml of various concentrations (0.25, 0.5 and 1.0 mg/ml) of methanol pods extract of *M. oleifera* to a concluding volume of 1 ml. The reaction mixture was incubated for 1 hour at 37 °C. After incubating 0.5 ml of the reaction mixture was treated with 0.2 ml of SDS, 1.5 ml of TBA and 1.5 ml of acetic acid respectively. The TBARS thus formed was determined by the method of Ohkawae *et al.* [13]. The total volume was measured up to 4 ml by distilled water and kept in a water bath at 95-100°C for 1 hour. Two milliliters of the reaction mixture was then mixed with 3 ml of n-butanol (BDH Chemicals Ltd, England), vortexed and centrifuges at 3000 x g for 10 min, the organic layer was removed and its absorbance was measured at 532 nm in a UV-spectrophotometer. Tubes without Fenton's reaction mixture or *M. oleifera* pod extract but with rat mitochondria were included as blank, while those without *M. oleifera* pods extract alone were taken as control. The mean absorbance of the *M. oleifera* pods extract treated tubes was compared with that of Fenton's reaction alone to determine the lipid peroxidation. The experiments were done in triplicates.

Data analysis was carried out using Microsoft Office Excel, Standard version 2007 to determine percentage inhibition.

Results

Data analysis showed an intact mitochondrial membrane in figure 1 when the absorbance of the mitochondria suspension was read with no calcium chloride (triggering agent). In the presence of calcium

chloride there was decrease in absorbance of the mitochondrial suspension at 540 nm, an indication mitochondrial swelling, while the spermine inhibited mitochondrial swelling (figure 1). The rate of mitochondria swelling decreases with increasing concentrations (50, 150 and 300 µg/ml) of methanol pods extract of *M. oleifera* (figure 2). Minimal (71.29%) and maximal (86.11%) inhibitions of mitochondria swelling were at 50 and 300 µg/ml of methanol pods extract of *M. oleifera* respectively (table 1). Furthermore, spermine inhibited MMPT at 50, 150 and 300 µg/ml concentrations of methanol pods extract of *M. oleifera* (figure 3).

The varying concentrations (0.25, 0.50 and 1.00 mg/ml) of methanol pods extract of *M. oleifera* stimulated an increase in the percentage inhibition of lipid peroxidation in a concentration dependent manner with minimal (21.31%) and maximal (37.22%) inhibitions at 0.25 and 1.0 mg/ml respectively (table 2).

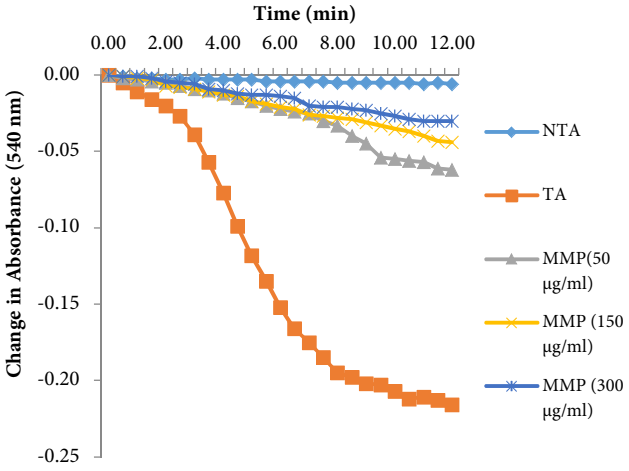

Figure 2: The effect of varying concentrations of methanol pods extract of *M. oleifera* on mitochondrial membrane permeability transition pore in the presence of a triggering agent (Ca^{2+}) energized by sodium succinate
TA- Triggering agent (Presence of Ca^{2+})
NTA- Non triggering agent (Absence of Ca^{2+})
MMP- Methanolic extract of *M. oleifera* pods

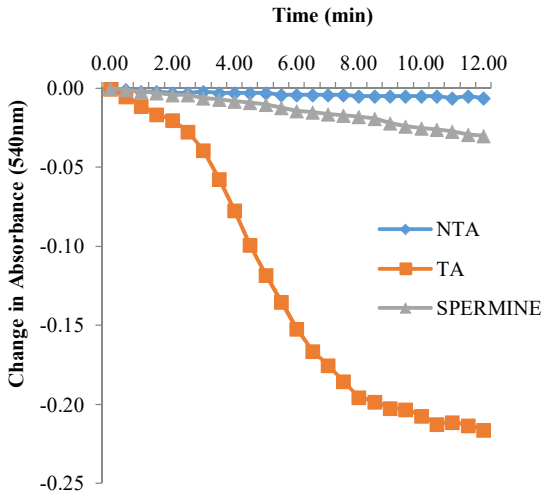

Figure 1: The effect of the presence and absence of triggering agent Ca^{2+} on mitochondrial membrane permeability transition pore in the presence of an inhibitor (spermine) energized by sodium succinate monitored as swelling at 540 nm for 12 minutes.
TA- Triggering agent (Presence of Ca^{2+})
NTA- Non Triggering agent (Absence of Ca^{2+})

Concentrations (µg/ml)	Extent of Inhibition (%)
50	71.29
150	79.62
300	86.11

Table 1: Percentage inhibition of varying concentrations of methanol pods extract of *M. oleifera* on mitochondrial membrane permeability transition pore opening of rat liver mitochondria at the 12th min.

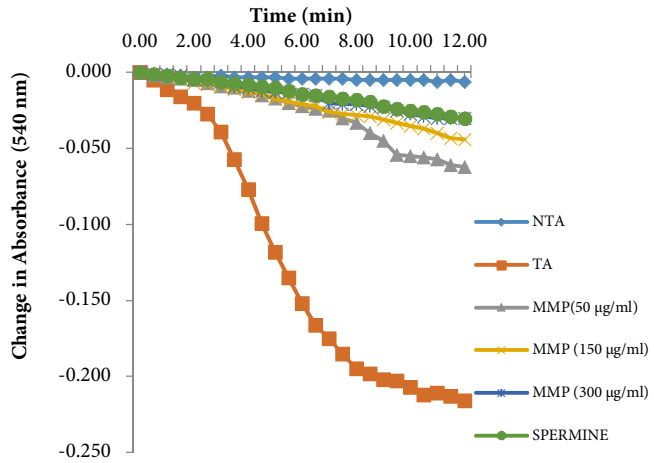

Figure 3: A graph showing the effect of varying concentrations of methanol pods extract of *M. oleifera* on mitochondrial membrane permeability transition pore in the presence of an inhibitor (spermine) energized by sodium succinate.
TA- Triggering agent (Presence of Ca^{2+})
NTA- Non triggering agent (Absence of Ca^{2+})
MMP- Methanol pods extract of *M. oleifera*

Concentrations (mg/ml)	Extent of Inhibition (%)
0.25	21.31
0.50	24.15
1.00	37.22

Table 2: Percentage inhibition of varying concentrations of methanol pods extract of *M. oleifera* on lipid peroxidation induced in rat liver mitochondria.

Discussion

The varying concentrations of methanol pods extracts of *M. oleifera* inhibited the opening of the MMPT pore in a concentration dependent manner. Thus, *M. oleifera* pods might be able to improve mitochondrial function by reducing the rate of occurrence of mitochondrial related diseases [14]. Oxidative damage occurs and pileup in the mitochondria when reactive oxygen species (ROS) such as the superoxide radical are not converted to water (H_2O) fast enough by superoxide dismutases and peroxidases. Radical species, such as hydroxyl ions (OH^-) and hydrogen peroxide (H_2O_2) may be present in high concentrations, posing a risk of lipid peroxidation. A major significance of uncontrolled oxidative stress (imbalance between the prooxidant and antioxidant levels in favor of prooxidants) is cells, tissues, and organs injury caused by oxidative damage. It has long been acknowledged that high levels of free radicals or ROS can perpetrate direct damage to lipids. An equilibrum between the production of oxidants and the scavenging of those oxidants by antioxidants establishes the extent of lipid peroxidation.

In this study, the various concentrations (0.25, 0.50 and 1.00 mg/ml) of *M. oleifera* pods used protected against Fe^{2+}- H_2O_2-EDTA (Fenton reaction)-induced lipid peroxidation in rat liver mitochondria homogenate in a concentration dependent manner at 21.31, 24.15 and 37.22 percentage inhibition respectively. The methanol pods extract of *M. oleifera* may have performed protection either by neutralizing the H_2O_2 or by scavenging the superoxide (.OH) generated from the Fenton's reaction. This coincide with similar report by Kumar and Pari (2003) [15], that *M. oleifera* pods possess anti-oxidant properties which may help in maintaining health when continuously taken as part of dietary foods, spices or drugs.

The protective property of methanol extract of *M. oleifera* pods could be due to the fact that *M. oleifera* pods contain essential bioactive compounds] isothiocyanates, like glucosinolates, flavonoids, and thiocarbamates [16]. These compounds regenerate membrane-bound antioxidants, satiates ROS, and chelate metal ions. This finding is consistent with earlier studies, which demonstrated the antioxidant activity of *M. oleifera* pods extracts [14,15].

The methanol extract of *M. oleifera* pods could be an inhibitor of MMPT pore opening and an inhibitor of lipid peroxidation by reducing the risk of diseases associated with mitochondria dysfunction and oxidative stress when regularly taken as constituent of herbs, dugs or dietary foods. The alkaloids, flavonoid and phenol components of the *M. oleifera* can be credited for these observed effects. Thus, the isolation and characterization of specific compounds involved in the observed effect by methanol extract of *M. oleifera* pods will give more credence to these findings.

Conflict of Interest Statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

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