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Effect of Methanol Extract of *Mangifera indica* on Mitochondrial Membrane Permeability Transition Pore in Normal Rat Liver and Monosodium Glutamate-induced Liver and Uterine Damage

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Authors' contributions

This work was carried out in collaboration between all authors. Author AOO designed the study, wrote the protocol, managed the analysis of the study, performed the statistical analysis and wrote the first draft of the manuscript. Authors OAA and OTA fed, treated and took care of the animals and also did the literature searches. Author OOO designed approved the study and read through the manuscript. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Introduction: The mitochondrion has been known to play a crucial role in the induction of apoptosis as a result of the opening of the mitochondrial permeability transition (mPT) pore which results to the release of cytochrome C and consequently, lead to cell death (apoptosis).
Aim: The aim of this study was to investigate the influence of crude methanol extract of *Mangifera indica* (MEMI) on mitochondrial-mediated apoptosis via induction of MMPT pore opening *in vivo* and *in vivo.*

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Methods: Mitochondria, isolated from female albino rat liver (between 90-100 g), were exposed to varying concentrations (10, 30, 50, 70, and 90 µg/ml) of MEMI. Opening of the pore, cytochrome c release, mitochondrial ATPase activity and extent of mitochondrial lipid peroxidation were assessed spectrophotometrically. Histological examinations were also carried out on the liver and uteruses of normal and monosodium glutamate (MSG)-treated rats.

Results: The *in vitro* results showed a significant concentration-dependent induction of pore opening by 1.5, 10.3, 11.5, 13.1 and 17.4 folds, respectively. Oral administration of MEMI at varying doses of 100, 200 and 400 mg/kgbw also showed an induction folds of 0.4, 1.9 and 2.3, respectively, after 14 days, and more significantly, induction folds of 3.4, 6.3 and 15.4, respectively, after 28 days of treatment. Also, MEMI caused a significant release of cytochrome C and enhancement of ATPase activity both *in vitro* and *in vivo* in a concentration and dose-dependent manner. The histological findings also showed that MEMI ameliorated the damage induced in the liver and uterus of MSG-treated rats. It also reduced the MSG-induced uterine hyperplasia in the co-administered group.

Conclusion: These results suggest that MEMI contains bioactive agents that can induce mitochondrial-mediated apoptosis and ameliorate MSG-induced liver damage and uterine hyperplasia. This might be relevant in disease conditions where apoptosis needs to be upregulated.

Keywords: Mangifera indica; mitochondrial membrane permeability transition pore; monosodium glutamate.

1. INTRODUCTION

Cells are programmed for death in order to maintain cellular homeostasis. Apoptosis, a form of programmed cell death, is one of the most potent defense mechanism by which potentially deleterious and mutated cells are eliminated from an organism while the integrity and architecture of the surrounding tissue is preserved [1]. Mitochondria have been shown to play a complex role in apoptosis via the induction of mitochondrial permeability transition (mPT) pore opening leading to the release of mitochondrial proteins into the cytosol which normally reside in the intermembrane space [2,3].

Studies have shown that the permeabilization of the inner mitochondrial membrane is a major event in the induction of mitochondrial pathway of apoptosis [4,5] and is a point of no return for apoptosis to take place [6,7]. Hence, deregulated apoptosis results in pathological conditions such as cancer, autoimmune diseases, neurodegenerative disorders, ischemic diseases, etc [8,9].

Experimental evidence has revealed that some medicinal plants elicit their chemoprotective effect by targeting mitochondrial apoptotic machineries through the induction of mPT pore opening towards efficient and selective treatment of diseases with too little apoptosis such as cancer [10]. *Mangifera indica* is a species of flowering plants belonging to the family of Anacardiaceae and it is popularly known as mango. It is used locally in the treatment of fibroid, asthma, cough, etc. One of the chemical constituents includes Mangiferin which is a polyphenolic antioxidant and a glucosyl xanthone [11]. It has strong antioxidant, wound healing, immunomodulation, cardiotonic, hypotensive, antidegenerative and antidiabetic properties [12,13]. It has also been shown to have anticarcinogenic effects [14].

Monosodium glutamate is the sodium salt of glutamate and it is generally used as a flavor enhancer. Its toxic and deleterious effects on various organs in rat model such as the uterus, ovaries and in tissues have been reported [15,16,17,18]. Due to paucity of information on the effect of the plant on mitochondrial permeability transition pore, this led to a pivotal study to investigate the influence of MEMI on rat liver mPT pore in normal and monosodium glutamate-treated rats.

2. MATERIALS AND METHODS

2.1 Experimental Animals

Two sets of virgin female rats each weighing between 100-120 g were obtained from the Preclinical Animal House, Physiology Department, University of Ibadan, Nigeria, and were kept at the Biochemistry Department Animal house, University of Ibadan, Nigeria, under light-controlled conditions (12 h-light/12 h-dark cycle) in well-ventilated plastic cages. The rats were grouped into four with eight animals

each, kept in ventilated cages with 12 hours light/dark cycling and fed with food and water *ad libitum*. The rats were acclimatized for two weeks. All experiments have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

2.2 First Set

The rats were grouped into: Control, 100 mg/kg, 200 mg/kg and 400 mg/kg (bw). Assays were carried out after 14 and 28 days of treatment. Histological study was also carried out on the liver.

2.3 Second Set

The rats were grouped into: Control, MSG only, MSG+MEMI and MEMI only. 200 mg/kg of MSG was administered as the toxicant while 100 mg/kg of MEMI was given as the potential candidate drug. The rats were sacrificed after 28 days and histology was carried out on their liver and uteruses.

2.4 Monosodium Glutamate

Ajinomoto (a brand of monosodium glutamate) was purchased from Bodija market, Ibadan, Nigeria, at a wholesale distributor. A stock solution was prepared by dissolving 10 g in 20 ml of distilled water.

2.5 Plant Material

The leaves of *Mangifera indica* were bought from Bode market in Ibadan, Oyo State and authenticated at Botany department, University of Ibadan, Ibadan, Nigeria, with voucher number UIH 22555.

2.6 Preparation of Extract

The leaves of *Mangifera indica* were cut into smaller pieces, washed, shade-dried under laboratory conditions for 4 weeks and pulverized to powder using a grinder. It was then soaked in methanol for 72 hours. The filtrate obtained was concentrated using a vacuum rotary evaporator (N-100, Eyla, Tokyo, Japan) and was later concentrated to dryness using a water bath at 37ºC. This was later transferred into a bottle and stored in a refrigerator until use.

2.7 Reagents

Mannitol, sucrose, N-2-hydroxy-ethyl-pipe-arizine-N-2-ethanesulfonic acid (HEPES), rotenone, spermine, Folin-Ciocalteu reagent, bovine serum albumin (BSA), and all other reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and were of the highest purity grade.

2.8 Isolation of Rat Liver Mitochondria

Rat liver mitochondria were isolated essentially according to the method of Johnson and Lardy [19] and as modified by Olorunsogo et al. [20].

2.9 Mitochondrial Swelling Assay

Mitochondrial membrane permeability transition was monitored by measuring changes in absorbance of mitochondrial suspension in the presence or absence of calcium (the triggering agent) in a T70 UV visible spectrophotometer essentially according to the method of Lapidus and Sokolove [21].

2.10 Determination of Mitochondrial Protein

Mitochondrial protein concentration was determined according to the method of Lowry et al. [22] using bovine serum albumin as standard.

2.11 Assesment of Mitochondrial F_OF_1 Atpase Activity

F_0F_1 Adenosine triphosphatase was determined by the method of Lardy and Wellman and as modified by Olorunsogo and Malomo [23]. Each reaction mixture contained 65 mM Tris-HCl buffer pH 7.4, 0.5 Mm KCl 1 Mm ATP and 25 Mm sucrose using 2,4 Dinitrophenol (2,4 DNP) as a standard uncoupling agent.

2.12 Estimation of Inorganic Phosphate Released

The concentration of inorganic phosphate released following the hydrolysis of ATP was determined according to the method described by Bassir [24] and as modified by Olorunsogo and Malomo [23]. The absorbance was read at 680 nm.

2.13 Inhibition of Lipid Peroxidation

2.13.1 *In vitro*

A modified thiobarbituric acid reactive species (TBARS) assay was used to measure the lipid peroxide formed using mitochondrial membrane

as lipid rich media [25]. The absorbance of the organic upper layer was measured at 532 nm. Percentage inhibition of lipid peroxidation by the extract was calculated as [AC-AE/AC] x 100. Where AC is the absorbance value of the fully oxidized control and AE is the absorbance in the presence of extract.

2.13.2 *In vivo*

Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) present in the test sample according to the method of Varshney and Kale [26]. Under acidic conditions, malondialdehyde (MDA) produced from the peroxidation of fatty acids reacts with the chromogenic reagent 2-thiobarbituric acid to yield a pink coloured complex with maximum absorbance at 532 nm.

2.14 Assay of Cytochrome C Release

The quantitative determination of cytochrome C released from isolated mitochondria was performed by measuring the Soret (γ) peak for cytochrome C at 414 nm (ε= 100 mM^{-1} cm^{-1}), according to method of Appaix et al. [27]. The optical density of the supernatant was measured at 414 nm which is the soret (γ) peak for cytochrome C.

2.15 Histological Study

The liver and the uterus were harvested, cleaned of blood and thereafter used for histopathology study.

2.16 Statistical Analysis of Data

The data were statistically evaluated using one way analysis of variance (ANOVA). All the results were expressed as mean ± standard deviation (SD). The $p < 0.05$ were considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1 Calcium-induced Mitochondrial Membrane Permeability Transition Pore Opening in Normal Rat Liver Mitochondria and its Reversal by Spermine (*in vitro*)

The data presented in Fig. 1 shows that there were no significant changes in the volumes of intact mitochondria respiring on succinate in the presence of rotenone over a period of twelve minutes. Upon the addition of calcium, there was a highly significant increase in MMPT pore opening which was almost completely reversed by spermine. This shows that the mitochondria used in this study were not uncoupled and suitable for use.

3.2 Effects of Varying Concentrations of Methanol Extract of *Mangifera indica* (MEMI) on the MMPT Pore in the Absence and Presence of Ca^{2+}

Fig. 2 shows the effect of various concentrations of MEMI on MMPT pore in the absence of calcium. In the absence of Ca^{2+}, the varying concentrations (20, 60, 100, 140 and 180 µg/ml) of MEMI significantly induced pore opening by 1.5, 10.3, 11.5, 13.1 and 17.4 fold respectively, and as shown in Fig. 3, calcium further potentiated opening of the pore by 10.1, 14.2, 17.4, 22.7 folds respectively.

3.3 Calcium-induced Mitochondrial Membrane Permeability Transition Pore Opening in Control Rat Liver Mitochondria and its Reversal by Spermine (*in vivo*)

Fig. 4 shows a representative profile of intact mitochondria of control animal respiring on succinate in the presence of rotenone over a period of twelve minutes at the end of 14 and 28 days of administration. When calcium was added, there was a highly significant increase in MMPT pore opening and were reversed by spermine. This implies that the mitochondria of the control animal used in this study were intact and suitable for the experiment.

3.4 Effects of Varying Doses of Methanol Extract of *Mangifera indica* (MEMI) on the MMPT Pore after 14 and 28 days of Treatment

Fig. 5 shows that the varying doses of MEMI (100, 200 and 400 mg/kgbw) caused an induction of pore opening by 0.4, 1.9 and 2.3 folds respectively, at the end of fourteen days of treatment. There was a further induction of pore opening by 3.4, 6.3 and 15.4 folds respectively, after 28 days of treatment as shown in Fig. 6.

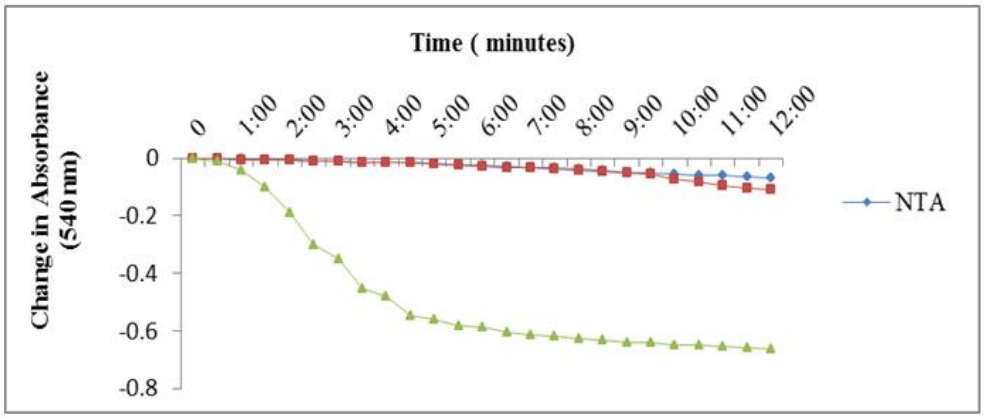

Fig. 1. Calcium induced mitochondrial membrane permeability transition pore opening and its reversal by spermine. (*In vitro*)

Abbreviations: NTA-No triggering agent; TA-Triggering agent

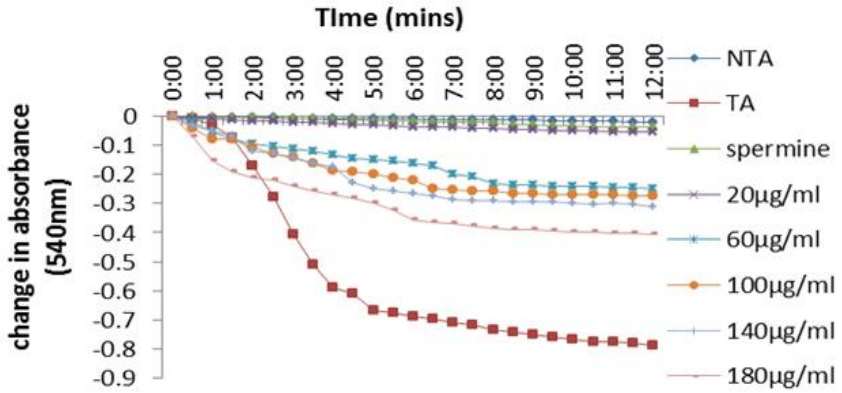

Fig. 2. Effect of varying concentrations of MEMI on rat liver mitochondrial membrane permeability transition pore in the absence of calcium

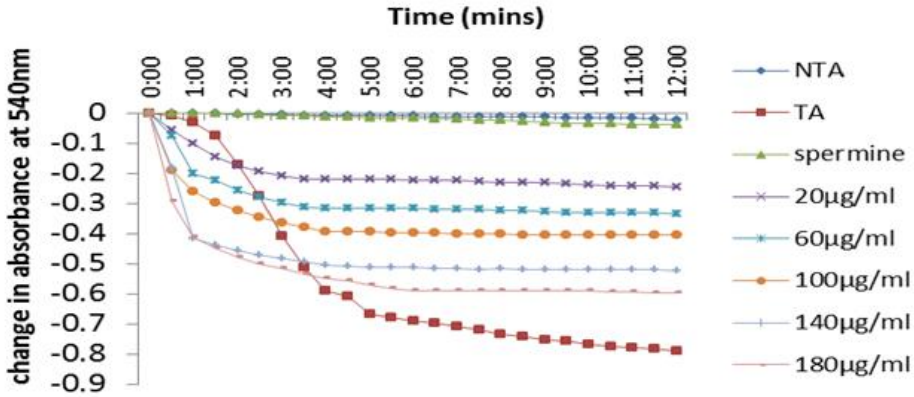

Fig. 3. Effect of varying concentrations of MEMI on rat liver mitochondrial membrane permeability transition pore in the presence of calcium

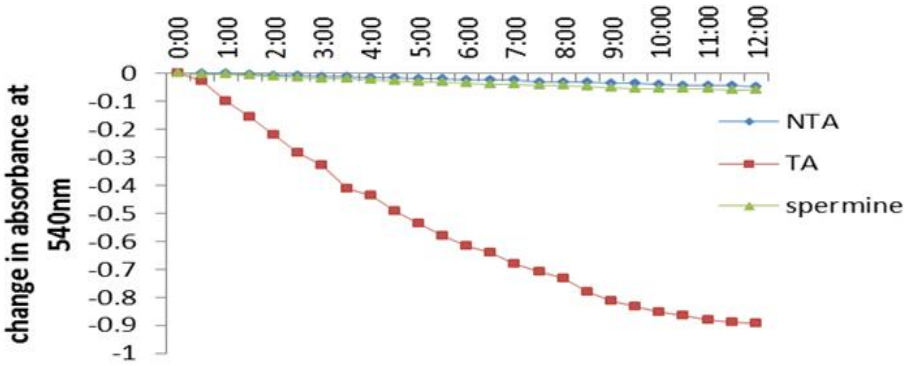

Fig. 4. Representative profile of calcium-induced mitochondrial membrane permeability transition pore opening and its reversal by spermine (*in vivo*)

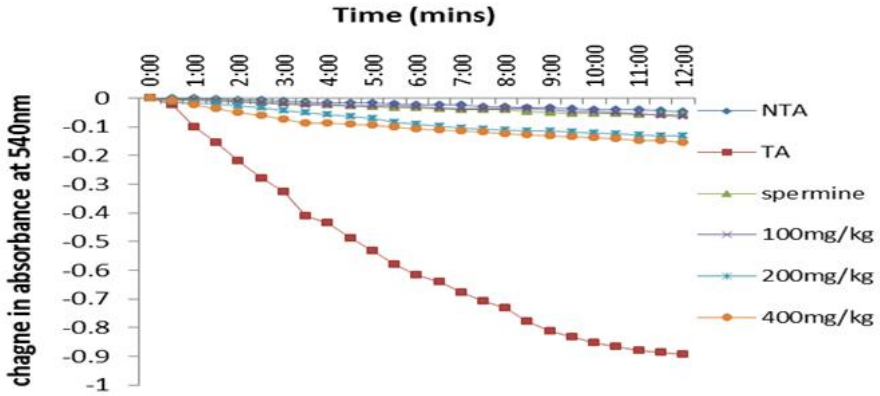

Fig. 5. Effect of MEMI on MMPT after 14 days of treatment

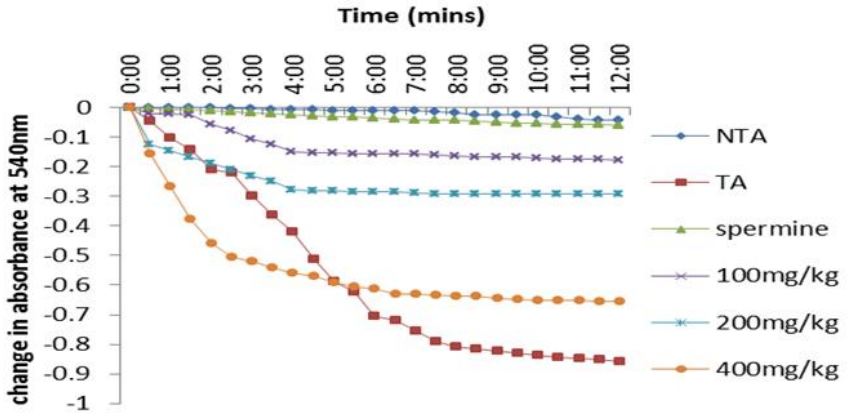

Fig. 6. Effect of MEMI on MMPT pore after 28 days of treatment

3.5 Effects of Methanol Extract of *Mangifera indica* on Mitochondrial FoF1 ATPase Activity (*in vitro* and *in vivo*)

Mitochondrial ATPase activity was enhanced by MEMI in a concentration-dependent manner (25 µg/ml, 75 µg/ml, 125 µg/ml, 175 µg/ml and 225 µg/ml) with 225 µg/ml having the highest ATPase activity when compared with the control as shown in Fig. 7. Also, oral administration of MEMI, at varying doses, as shown in Fig. 8 caused a slight enhancement of mitochondrial ATPase activity at the end of 14 days and a more

significant enhancement ($p<0.05$) after 28 days of treatment. As depicted in Fig. 9, dosage 400 mg/kgbw gave the highest enhancement of ATPase activity.

3.6 Effects of Varying Concentrations of Methanol Extract of *Mangifera indica* on Cytochrome c Release in Rat Liver Mitochondria

The effect of MEMI on cytochrome C release was depicted in Fig. 10. There was a significant release of cytochrome C in a concentration-dependent manner. The result showed that on addition of varying concentrations of MEMI to MSH-pre-incubated mitochondria, there was concentration-dependent release of cytochrome c.

3.7 Effects of Methanol Extract of *Mangifera indica* on Lipid Peroxidation in Normal Rat Liver Mitochondria (*in vitro* and *in vivo*)

Fig. 11 shows the effect of varying concentrations (50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml and 800 µg/ml) of MEMI on lipid peroxidation. The extract inhibited Fe^{2+}-induced lipid peroxidation in a concentration-dependent manner by 11.9%, 14.1%, 29.3%, 41.4% and 67.2%, respectively, with the highest concentration having the highest inhibitory effect.

Furthermore, as shown in Fig. 12, varying doses of MEMI inhibited lipid peroxidation as measured by the amount of malondialdehyde produced with increase in dosage. The highest dosage group (400 mg/kg) produced the least malondialdehyde.

3.8 Histological Assessment of the Effect of MEMI on the Liver and Uterus of Normal and MSG-treated Rats

Figs. 13a, b, c and d show the photomicrograph of the liver section of rats treated with varying doses (100, 200 and 400 mg/kgbw) of MEMI. The histological results show that there was no lesion at a lower dose while a toxic effect may be encountered at a higher dose. Figs. 14a, b and c show the effect of MEMI on the liver of normal and MSG-treated rats. The results show that there was a severe disseminated periportal infiltration by inflammatory cells in MSG-treated group. The group that received MSG co-administered with MEMI showed a moderate disseminated periportal infiltration by inflammatory cells when compared with the MSG-treated group. Results from the uterus in Figs. 15a, b, c and d show that the MSG-treated rats had an increase in collagen fibre and also, increase in the number of masson trichome-stained nuclei cells per unit area. The group that received MSG co-administered with MEMI showed a reduction in collagen fibre and also, reduction in the number of masson trichome-stained nuclei cells per unit area while MEMI-treated group showed a normal histology when compared with the MSG-treated group. These results suggest that MEMI was able to protect against MSG-induced liver damage and also alleviate uterine hyperplasia induced in the MSG-treated rats.

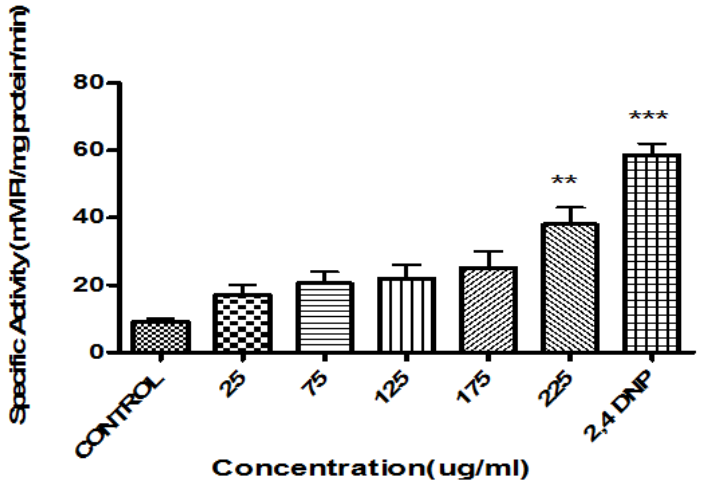

Fig. 7. Effect of varying concentrations of MEMI on the mitochondrial ATPase activity (*in vitro*)

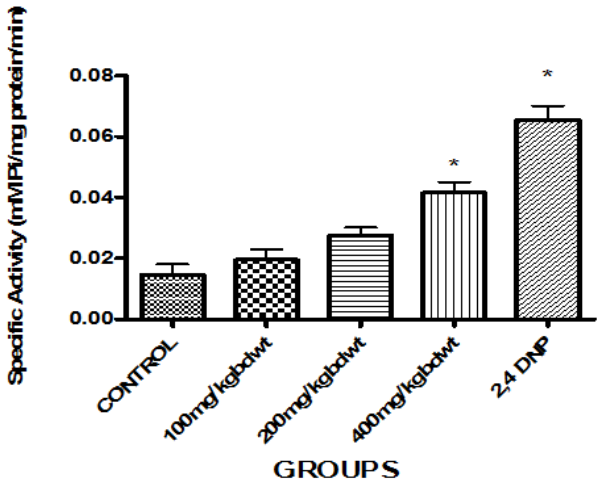

Fig. 8. Effects of MEMI on mitochondrial ATPase activity after 14 days of treatment (*in vivo*)

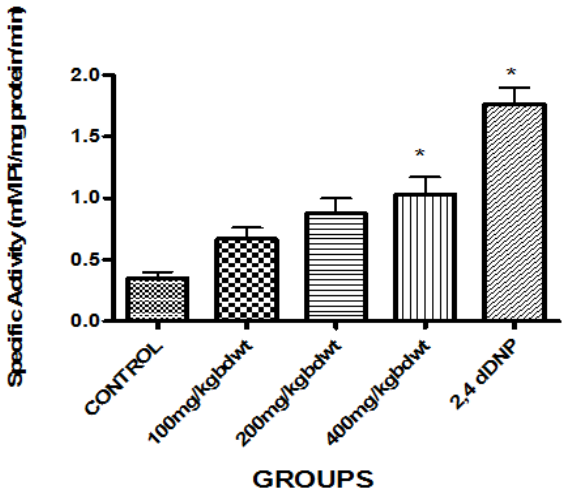

Fig. 9. Effects of MEMI on mitochondrial ATPase activity after 28 days of treatment (*in vivo*)

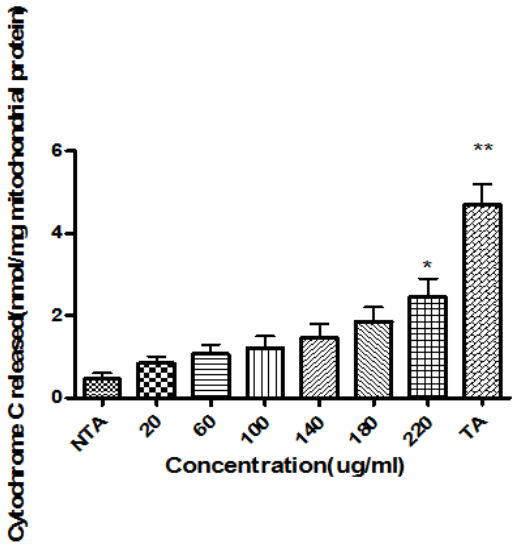

Fig. 10. Cytochrome C release by MEMI (*in vitro*)

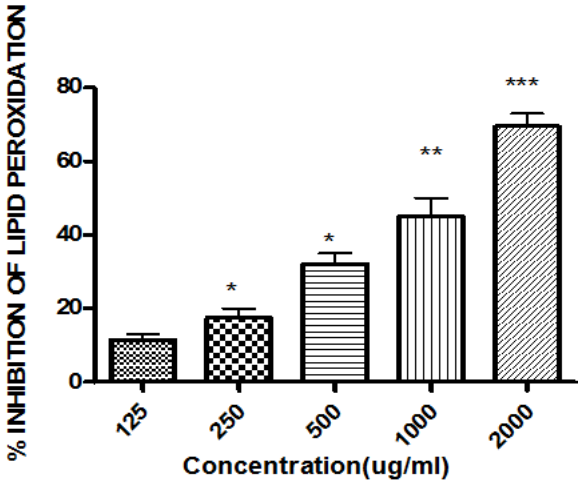

Fig. 11. Effects of MEMI on Fe^{2+} -induced lipid peroxidation in normal rat liver mitochondria (*In virto)*

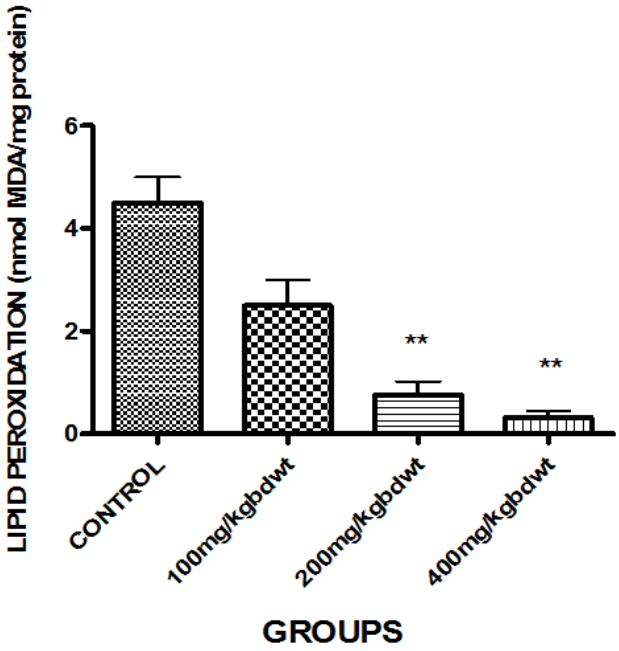

Fig. 12. Effects of varying doses of methanol extract of *Mangifera indica* on lipid peroxidation after 28 days of treatment. (*In vivo*)

4. DISCUSSION

The mitochondrion is an important organelle and plays a vital role in apoptosis. Apoptosis is a programmed cell death and it is one of mechanism for cellular defense against cancer, because it destroys potentially deleterious and mutated cells [28]. Intrinsically, the mitochondrion has been known to play a crucial role in the induction of apoptosis because, the opening of the mitochondrial permeability transition (mPT) pore results in the release of cytochrome C and other proapoptotic proteins which consequently leads to cell death. The mPT pore serves as a useful chemotherapeutic strategy for drug development in diseased conditions where the upregulation or downregulation of apoptosis is needed. In this study, the first experiment showed that exogenous calcium which is a potent inducer, caused an amplitude opening in the mitochondria membrane permeability transition (mPT) pore and in the presence of

spermine, there was a reversal of the calcium-induced pore opening. This shows that the mitochondria were intact and suitable for use. The varying concentrations (20, 60, 100, 140 and 180µg/ml) of MEMI in the absence of calcium induced pore opening by 1.5, 10.3, 11.5, 13.1 and 17.4 folds respectively. In the presence of calcium, varying concentrations of MEMI further potentiated the calcium-induced pore opening by 10.1, 14.2, 17.4, 22.7 and 26.1 folds respectively. The i*n vivo* results were also in accordance with the findings from the *in vitro* experiment. Varying doses (100, 200 and 400mg/kg) of MEMI also showed induction of pore opening by 0.4, 1.9 and 2.3 folds, respectively, after 14 days of treatment and 3.4, 6.3 and 15.4 folds respectively, after 28 days of treatment. These findings show that methanol extract of *Mangifera indica* contains bioactive agents that can induce mitochondrial-mediated apoptosis via induction of mPT pore opening. The release of inorganic phosphate (Pi) is an indication of uncoupling of phosphorylation in the mitochondrion and this happens during pathological conditions. The inorganic phosphate released is used as an index to measure the ATPase activity. MEMI was able to interact with the MMPT pore and ATPase activity was enhanced in a concentration-dependent manner. This is also in accordance with the *in vivo* results after 14 and 28 days of treatment which also showed significant enhancement of ATPase activity. The effect of MEMI on mitochondrial lipid peroxidation was examined and the extract was found to elicit an inhibitory effect on Fe^{2+}-induced lipid peroxidation. The results show that at varying concentrations, there was a significant inhibition of lipid peroxidation by 11.9%, 14.1%, 29.3%, 41.4% and 67.3% at 50, 100, 200, 400 and 800 µg/ml of MEMI respectively. The *in vivo* study on lipid peroxidation was also in consonants with the *in vitro* results. MEMI was found to elicit a dose-dependent reduction in the level of malondialdehyde produced by MEMI. This study suggests that MEMI possesses free radical scavenging activity that could protect the physicochemical properties of membrane bilayers from free radical-induced damage.

The relesase of cytochrome C from the intermembrane space is a sine qua non for apoptosis to take place. The MEMI caused the release of cytochrome C from the mitochondrial intermembrane space into the cytosol in a concentration-dependent manner. The histological results on the effect of varying doses of MEMI on rat liver showed that at a lower dose, MEMI is safe and tolerable while at a higher dose, might be toxic, as it causes marked disseminated microvesicular steatosis, thrombosis, periportal infiltration and disseminated congestion. Histological findings on the liver of MSG-treated rats showed a severe disseminated periportal infiltration by inflammatory cells. The group that received MSG co-administered with MEMI showed a moderate disseminated periportal infiltration by inflammatory cells. This suggests that MEMI contains phytochemicals that can alleviate MSG-induced damage in rat liver. Histological findings from the myometrium of the uterus of MSG-treated rats showed an increase in collagen fibre and increase in the number of stained nuclei cells, while the group that received co-administration with MEMI showed a reduction in collagen fibre and number of stained nuclei cells when compared with the MSG-treated group. These results suggest that MEMI was able to ameliorate the effect of MSG-induced damage in the treated rats and also cause a reduction in hyperplasia noticed in the uterus of MSG-treated rats.

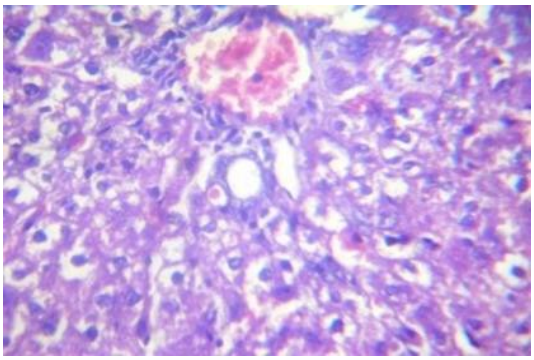

Fig. 13a. Control animal showing a Normal rat liver morphology (X400)

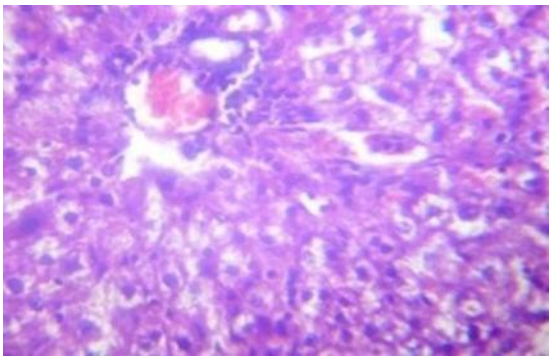

Fig. 13b. (100 mg/kg) Plate show moderate disseminated microvesicular steatosis (X400)

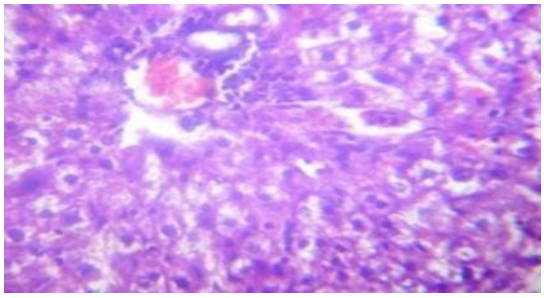

Fig. 13b. (100 mg/kg) Plate show moderate disseminated microvesicular steatosis (X400)

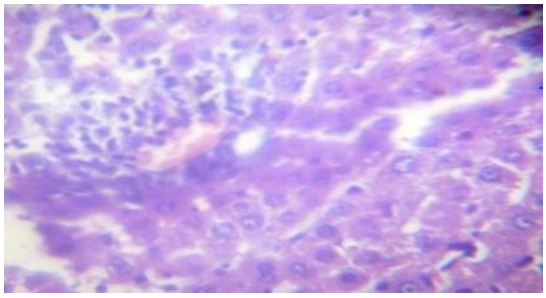

Fig. 13c. (200 mg/kg): Plate show moderate disseminated microvesicular steatosis and infiltration of zone 2 by inflammatory cells (X400)

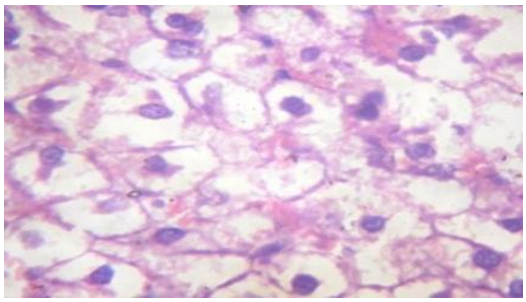

Fig. 13d. (400 mg/kg): Plates show marked disseminated microvesicular steatosis, thrombosis, marked periportal infiltration by inflammatory cells and disseminated congestion

Fig. 13. Photomicrograph of the liver section showing the effect of different doses of MEMI on the hepatocytes (H&E staining)

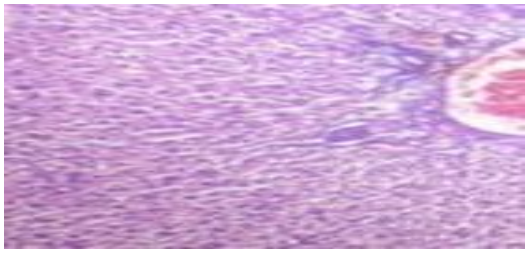

Fig. 14a. Section of the control liver showing normal morphology

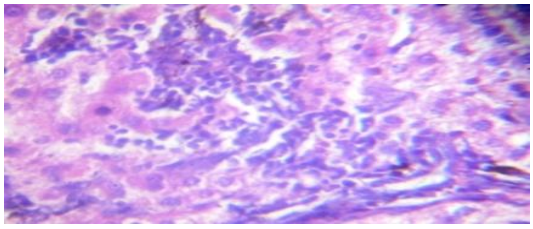

Fig. 14b. (MSG): There is severe disseminated periportal infiltration by inflammatory cells, disseminated congestion, multifocal area of thrombosis and focal area of ductal carcinoma

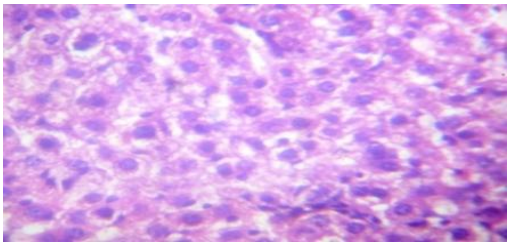

Fig. 14c. (MSG+MEMI): Plates show mild disseminated microvesicular steatosis, mild disseminated infiltration of zone 2 by inflammatory cells

Fig. 14. Photomicrograph of the liver section showing the effect of MEMI on normal and MSG-treated rats ((H&E staining)

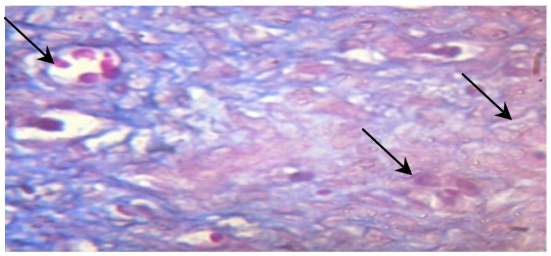

Fig. 15a. Control section showing the connective tissue and precursor cells within the endometrial submucosa (Mag. x400)

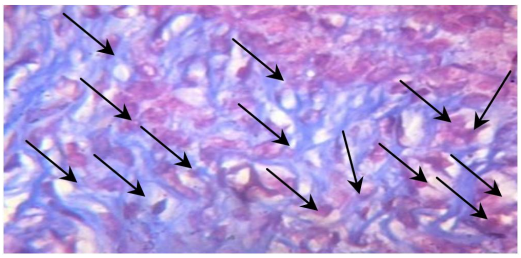

Fig. 15b. Treatment section of the myometrium (MSG) showing the connective tissue and precursor cells within the endometrial submucosa (Mag. x400). There is a severe hyperplasia of spindle shaped precursor cells

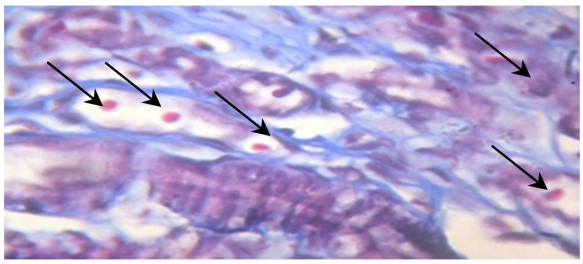

Fig. 15c. Treatment section of the myometrium (MSG & MEMI) showing a reduction of hyperplasia compared with MSG-treated group (Mag x400)

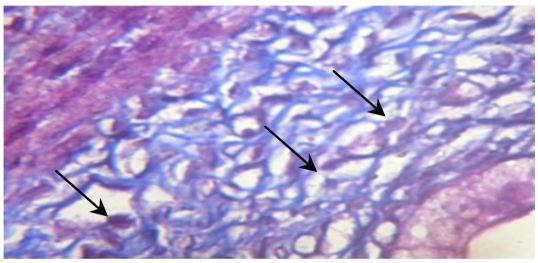

Fig. 15d. Treatment section of the myometrium (MEMI) showing the connective tissue and precursor cells within the endometrial submucosa (Mag. x400)

Fig. 15. Photomicrograph of the rat uterus (Masson trichrome staining)

5. CONCLUSION

In conclusion, this study suggests that MEMI contains phytochemicals that can induce mitochondrial-mediated apoptosis via induction of MMPT pore opening which may be relevant in the management and treatment of diseases where there is need for upregulation of apoptosis. Also, its ameliorative effect on MSG-induced rat liver damage and especially, uterine hyperplasia, justifies its folkloric use in the treatment of fibroid. It is also possible that the mechanism by which MEMI ameliorated MSG-induced uterine hyperplasia might be via upregulation of mitochondrial-mediated apoptosis. Though, the chemical nature of substances responsible for the effect shown by MEMI are still unknown, further work is necessary to elucidate and characterize the structure of putative agent(s) present in MEMI and their effect on induction of mitochondrial-mediated apoptosis.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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