

## ***Annona muricata* Linn stem bark protects against uterine proliferative disorder induced by estradiol benzoate in female rat**

### **Abstract**

**Aim:** The opening of mitochondrial permeability transition (mPT) pore is an important event in the execution of mitochondrial-mediated apoptosis. Some bioactive compounds elicit their chemotherapeutic effects against tumor/cancer cells via the induction of mitochondrial-mediated apoptosis. *Annona muricata*, a medicinal plant, is folklorically used in the treatment of tumors and cancers. This study therefore aimed at investigating the effect of methanol stem bark extract of *Annona muricata* (MEAM) on apoptosis via mPT pore and estradiol benzoate (EB)-induced proliferative disorder using female Wistar rats.

**Methodology:** Mitochondria were isolated using differential centrifugation. The mPT pore opening, cytochrome c release and mitochondrial ATPase activity were determined spectrophotometrically. The levels of estrogen (E2), luteinizing hormone (LH), follicle stimulating hormone (FSH), malondialdehyde (MDA) and activities of superoxide dismutase (SOD), glutathione peroxidase (GSH), were determined using ELISA technique. Histological and histochemical assessments of the uterine sections were carried out using standard methods. Phytochemical constituents of MEAM were determined using Gas Chromatography-Mass Spectroscopy (GC-MS).

**Results:** The *in vitro* results showed a significant induction of mPT pore opening, release of cytochrome c and enhancement of mitochondrial ATPase (mATPase) activity in a concentration-dependent manner. However, oral administration of MEAM did not induce rat uterine mPT pore opening, neither any significant release of cytochrome c nor enhancement of mATPase activity

at the dosages used. Interestingly, MEAM reversed the EB-induced increase in E2, LH and FSH. The MEAM also improved the antioxidant milieu by reducing MDA level and increasing the SOD and GSH-Px activities in the treatment groups. Administration of EB induced endometrial hyperplasia in the model group which was mitigated by MEAM in the treatment group. The GC-MS analysis of MEAM revealed the presence of some important phytochemicals that are pharmacological relevant in cancer treatment.

**Conclusions:** This study suggests that the methanol stem bark extract of *Annona muricata* contains bioactive compounds that protect against EB-induced uterine proliferative disorder in female Wistar rats.

**Keywords:** *Annona muricata*, estradiol benzoate, endometrial hyperplasia, apoptosis

## 1 Introduction

Endometrial hyperplasia is a form of disordered proliferation which occurs within the uterus. It occurs as a result of unrestricted or uncontrolled supply of estrogen to the endometrial tissue [1]. Endometrial hyperplasia could develop into cancer if not given the necessary therapeutic attention on time [1,2,3]. Several mechanisms must be physiologically coherent in order to maintain the equilibrium between endometrial proliferation and apoptosis. Altered physiological processes in these factors could lead to endometrial pathological disorders including hyperplasia and carcinoma [4]. It has been proven that phytochemicals especially those that possess anti-oxidative and anti-inflammatory potentials can inhibit tumor growth [5,6]. Also, bioactive agents from medicinal plants that can induce mitochondrial permeability transition pore (mPT) opening could serve as a potential drug candidate to induce cell death [7,8,9]. *Annona muricata* Linn, a

medicinal plant is folklorically used in the treatment of tumors and cancers [10]. Its name has been checked with <http://www.theplantlist.org> and also with <http://www.worldfloraonline.org>. Its anti-viral [11], anti-microbial [12], wound healing, anti-carcinogenic and genotoxic [13] properties have been reported. The leaf extract of *A. muricata* has been suggested to repress tumor growth [14]. Several studies have also demonstrated anticancer activity of some bioactive isolates of *A. muricata* leaves. Its cytotoxic effects on EACC, MDA, and SKBR3 tumor cell lines have equally been demonstrated [15,16,17]. Apart from the leaves, other parts like fruits, stems and seeds of *A. muricata* plant have been reported to possess anticancer activities [18]. *Annona muricata* is employed in tropical Africa as insecticidal and pesticidal agents besides being used for the treatment of coughs, pain and skin diseases [19]. In Latin America, the leaves, seeds, unripe fruits and roots are being used traditionally as biopesticides, bioinsecticides and topical insect repellents.

In addition, previous study in our laboratory has shown the potency of methanol stem bark extract of *Annona muricata* with respect to its induction of rat liver mPT opening [20]. The study also demonstrated its anti-proliferative effect on monosodium glutamate-induced uterine hyperplasia in female rats; however, its biochemical basis of action was not unraveled. The present study intended to investigate its effect on uterine mPT pore opening as well as its possible anti-proliferative potential on estradiol benzoate (EB)-induced uterine dysfunction looking at the biochemical basis of action.

## **2 Materials and methods**

### **2.1 Chemicals and reagents**

All the reagents including estradiol benzoate (EB) were purchased from Sigma-Aldrich Chemical Co. (USA). The EB was administered intraperitoneally (2mg/kg) based on the weight of animals. The chosen dose was based on pilot study and literature search [21,22].

### **2.2 Collection of plant materials**

The stem bark of *A. muricata* was obtained from Botany Department, University of Ibadan, Nigeria, taxonomically identified and confirmed.

#### **2.2.1 Preparation of plant extract**

It was washed, dried, pulverized, soaked in methanol for 72 hours, filtered and concentrated at 40 °C to obtain methanol extract of *A. muricata* (MEAM).

### **2.3 Experimental animals**

Virgin female rats, acclimatized for two weeks in standard and well ventilated cages were used for this study. The study was conducted according to the guidelines of National Institute of Health (NIH publication 85–23, 1985) for laboratory animal care and use.

First set: Thirty virgin rats (180 to 200 g) were equally grouped into: Control, 50, 100, 200 and 400 mg/kg. The rats were orally treated with MEAM for 28 days, after which they were anesthetized with an intraperitoneal injection of pentobarbital (70 mg/kg) and euthanized by cervical dislocation

Second set: Forty-two virgin rats (180 to 200 g) were equally divided into six intervention

groups: A {control}, B {MEAM: 100 mg/kg}, C {MEAM: 200 mg/kg}, D {EB: 2 mg/kg}, E {EB + MEAM (100 mg/kg)} and F {EB + MEAM (200 mg/kg)}. The treatment was by oral gavage for 12 weeks except for EB which was administered intraperitoneally. A day after the experimental schedule, blood samples were collected and the animals were anaesthetized and euthanized by cervical decapitation. Blood samples were centrifuged at 3000g for 30 minutes and the separated serum stored at  $-20^{\circ}\text{C}$ . The uterus was rapidly excised and washed with ice cold normal saline. Assays were carried out and histological assessment of the uterus was performed following standard procedures.

#### **2.4 Isolation of rat uterine mitochondria**

The rats were sacrificed by cervical dislocation while mitochondrial isolation was carried out using differential centrifugation [24].

#### **2.5 Determination of mitochondrial protein**

This was determined following the method of Lowry et al. [25] using bovine serum albumin as standard.

#### **2.6 Assessments of mitochondrial $F_0F_1$ ATPase activity**

The  $F_0F_1$  ATPase activity was determined by the method of Lardy and Wellman [26 as modified by Olorunsogo and Malomo [27].

#### **2.7 Estimation of inorganic phosphate released**

This was determined following the method described by Olorunsogo et al. [28].

## **2.8 Assay of cytochrome c release**

The cytochrome c released from isolated mitochondria was quantified by measuring its sores peak at 414 nm ( $\epsilon = 100 \text{ mM}^{-1} \text{ cm}^{-1}$ ), according to method of Appaix et al. [29].

## **2.9 Histological preparation**

Buffered Formalin-fixed uterine tissues sections were used for the histology. Hematoxylin and eosin (H&E) and Masson's Trichrome (MT) stains were used for study. The histological pictures were taken using an Olympus microscope, Japan. The morphometrical analyses of the density of spindle shaped cells within the endometrial submucosa were done using TS View CX Image® Software, File version 6.2.4.3 Motic Image 2000 (China).

## **2.10 Determination of sex hormones concentrations**

The effect of MEAM on sex hormones was determined by using the estradiol (E2), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) detection kits.

## **2.11 Oxidative index detection**

Malondialdehyde (MDA) level, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities in the serum were detected using commercially available detection kits following the manufacturer's instructions.

## **2.12 Phytochemical screening**

Phytochemical screening of MEAM was performed according to the method of Treas and Evans [32].

### **2.13 GC-MS analysis of methanol stem bark extract of *Annona muricata* (MEAM)**

The phytochemical profile of MEAM was carried out using Gas Chromatography-Mass Spectrum technique.

#### **Statistical Analysis**

The reported data on mPT are representative of multiple ( $\geq 4$ ) experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Turkey's post test. p value  $< 0.05$  was considered statistically significant.

### **3.0 Results**

#### **3.1 Determination of mitochondrial intactness**

The data presented in Fig. 1a showed that there was no significant change in the absorbance of mitochondria over a period of 12 minutes. Nevertheless, addition of exogenous calcium caused marked increase in swelling of mitochondria which was almost completely reversed by spermine. This shows that the mitochondria used were intact and not compromised.

#### **3.2 Effects of varying concentrations of MEAM on the uterine mPT pore**

Figure 1b shows induction of mPT pore opening by MEAM. At concentrations 10, 30, 50 and 70  $\mu\text{g/ml}$ , induction fold of 7.5, 8.6, 10.5 and 13.4, respectively, were recorded when compared to the NTA (No Triggering Agent).

### **3.3 Effects of varying concentrations of MEAM on cytochrome c release in rat uterine mitochondria**

As revealed in Figure 1c, MEAM caused release of cytochrome c which was significant at 30, 50 and 70 µg/ml when compared to NTA.

### **3.4 Effects of varying concentrations of MEAM on mitochondrial F<sub>0</sub>F<sub>1</sub> ATPase activity**

The MEAM caused enhancement of mATPase activity which was significant at concentrations 30 µg/ml and above (Fig. 1d).

### **3.5 Effect of oral administration of MEAM on the mPT Pore after 28 days of treatment**

Figure 2a shows that there was no significant induction of pore opening at 50, 100, 200 and 400 mg/kg after 28 days of treatment compared with the control (NTA).

### **3.6 Effect of oral administration of MEAM on mitochondrial F<sub>0</sub>F<sub>1</sub> ATPase activity**

Also, as shown in figure 2b, there was no enhancement of mitochondrial ATPase activity at all the doses used compared to the control.

### **3.7 Effect of oral administration of MEAM on sex hormones in normal and EB-treated rats**

The effects of oral administration of MEAM on sex hormones after 12 weeks of treatment are illustrated in Figs. 3 a, b and c. The results showed significant increase in the levels of estradiol (p<0.001), LH (p<0.05) and FSH (p<0.001) in the model (EB-treated) group relative to control. Nevertheless, co-administration with MEAM at both 100 and 200 mg/kg significantly ameliorated the levels of the sex hormones relative to the model group.

### **3.8 Effects of oral administration of MEAM on oxidative indices**

Figures 4 a, b and c illustrate the effects of MEAM on some oxidative indices in normal and model rats. As shown in Fig. 4a, MDA level was significantly elevated in the EB-treated group relative to the control ( $p < 0.01$ ). Nevertheless, MEAM co-administration at 100 and 200 mg/kg remarkably decreased the MDA level in the treated group. Also, Fig. 4b showed significant reduction in the activity of SOD in the model group relative to control ( $p < 0.01$ ). However, co-administration with MEAM at the two doses significantly improved the SOD activity in the treatment group. Similarly, the activity of GSH-Px was evidently reduced in the EB-treated group relative to control ( $p < 0.01$ ). Nevertheless, co-administration with MEAM at 200 mg/kg evidently caused increase in the activity of GSH-Px (Fig. 4c).

### **3.9 Histological assessment of uterine sections following oral administration of MEAM**

Figure 5 depicts histological features of the uterine sections in normal and EB-treated rats after oral treatment with MEAM (using H&E stains). The cellular architecture of control, MEAM (100) and MEAM (200) groups were normal and no visible lesion was recorded. However, the EB-treated group showed poor cellular morphology, severely degenerated epithelial layers of the endometrium and hyperplasia. Co-treatment with MEAM at the two doses improved cellular architecture and no uterine hyperplasia was recorded. Similar results were recorded when MT stain was used (Fig. 6). There was moderate deposition of collagen fiber in the control, MEAM (100) and MEAM (200) groups. However, the EB-treated group showed high deposition of collagen, severe cellular aggregation and hyperplasia. Co-administration with MEAM at the two doses attenuated the anomaly and no hyperplasia was recorded.

### **3.10 Results on phytochemical screening of MEAM**

The results obtained from phytochemical analysis of MEAM showed the presence of some secondary metabolites as contained in Table 1

### **3.11 The Gas Chromatography-Mass Spectroscopy (GCMS) analysis of MEAM**

The results showed the presence of some pharmacologically relevant phytochemicals as reported in Figure 7. These include 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, n-Hexadecanoic acid, Hexadecanoic acid methyl ester, Octadecanoic acid, 9,12,15-Octadecatrienoic acid, Tetradecanoic acid, 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6, cis-10-Heptadecenoic acid and 2,6,10-Trimethyl,14-ethylene-14-pentadecne.

## **Discussion**

The mitochondria play prominent role in controlling cell death [32,33]. The mPT pore now serves as a pharmacological target for drug development; as it determines the life and death of a cell [34,35]. In this study, the intactness and suitability of the isolated mitochondria was first of all ascertained [36,37]. The opening of mPT pore by MEAM (in vitro) suggests its potential to interact with the pore. This suggests the presence of some phytochemicals in MEAM which can induce mPT pore opening. This may be developed, processed and targeted towards diseased conditions which require upregulation of mitochondrial-mediated cell death [38,39,40].

When mPT pore opens, cytochrome c is released and this could lead to cell death [37,41]. The release of cytochrome c by MEAM occurred as a result of its potential to induce mPT pore opening. The  $F_0F_1$  ATP synthase has been suggested to be the mPT pore [42,43]. The hydrolysis

of mitochondrial ATP caused by MEAM could possibly be linked to the induction of uterine mPT pore opening which resulted to cytochrome c release [44]. However, the pattern of results recorded in the *in vivo* experiment was different as oral administration of the methanol stem bark extract (MEAM) did not induce uterine mPT pore opening and no mATPase activity enhanced. This could be possible if the doses were not sufficient enough to induce uterine mPT pore opening and mATPase activity. Also, it might possibly be due to biotransformation. However, the fact that MEAM did not **affect** uterine mPT pore opening *in vivo* does not mean it has no anti-tumor/anti-proliferative property. Medicinal plants/drugs could elicit their anti-tumor/anti-proliferative potentials via several mechanisms [45]. This finding is similar to our previous work on MEAM where it caused significant rat liver mPT pore opening, mATPase activity and cytochrome c release *in vitro* and no significant effect *in vivo* [20]. Estrogen has been reported to be crucial in leiomyoma pathogenesis and growth [46,47,48]. In addition, its involvement in the development of endometrial hyperplasia and endometrial cancer has been proven [49,50]. In this study, the elevated estrogen level in the model group could be attributed to the prolonged EB-treatment. Also, the upraised FSH and LH levels in the EB-treated group could possibly be due to the increased estrogen level as a consequence of the prolonged EB-inducement. However, MEAM co-administration mitigated the EB-induced increase in the sex hormones. This finding suggests the presence of phytochemicals in MEAM which possess anti-estrogenic property. This may be relevant in the treatment of estrogen-dependent uterine pathophysiological disorder. Interestingly, this is comparable with the findings of Zhenqiang et al. [51], where fermented carica papaya was shown to significantly reduce FSH and LH levels in estrogen-induced mammary gland hyperplasia. Studies have shown that excessive reactive oxygen species (ROS) results in the generation of malondialdehyde (MDA) [14]. Large amount of ROS are generated

by long-term high estrogen inducement which could generate oxidative stress [52,53]. Elevated concentration of MDA observed in the model group could be attributed to the prolonged EB-inducement. Nonetheless, MEAM administration ameliorated the MDA level in the treatment group. A previous study on *Annona muricata* leaf extract also demonstrated its potential to ameliorate MDA formation in colon tissue [14]. The reduction in activity of antioxidant enzymes (SOD and GSH-Px) in the EB-treated group could be as a result of accumulation of ROS caused by prolonged EB treatment [54,55]. However, the administration of MEAM in the treatment group caused significant improvement in the activity of the enzymes. These findings suggest the presence of certain phytochemicals in MEAM that have protective effect against EB-induced oxidative stress in rats.

The poor cellular architecture, degenerated epithelial layers of endometrium and hyperplasia observed in the uterine sections of EB-treated group (using H&E stains) could be ascribed to the prolonged EB-inducement. Comparably, the histochemical analysis carried out using Masson's trichome stain revealed a high deposition of collagen fiber and severe cellular aggregations at the sub-epithelial region in the EB-treated category. Treatment with MEAM improved the histological changes of uterine tissue and mitigated the EB-induced endometrial hyperplasia. This is in accord with the findings of Evy Sulistyoningrum et al. [56], where *Annona muricata* leaves extract was shown to reduce proliferation and improve cellular architecture in rat's breast. This suggests that MEAM contains bioactive principles that possess anti-tumor/anti-proliferative property as it protected against EB-induced uterine hyperplasia. Previous studies have equally shown that *A. muricata* leaves extract protected against DMBA-induced melano carcinoma in mice [57].

The phytochemical screening of *Annona muricaca* stem bark extract (MEAM) revealed the

presence of various phytochemical components which have been associated with several beneficial effects [58,59]. Furthermore, the major identified compounds using GC-MS have been documented to have anti-oxidant and anti-cancer properties. Hexadecanoic acid possess antioxidant, hypocholesterolemic, nematicidal, pesticidal, antiandrogenic and hemolytic potentials [60], while hexadecanoic methyl ester, has also been reported to have antioxidant, anti-tumor, aromatase inhibitory and other pharmacological properties [61,62,63]. The 2,6,10-Trimethyl,14- Ethylene-14-Pentadecne identified from MEAM has been reported to exhibit antiproliferative property [64]. Furthermore, 3,7,11,15-Tetramethyl-2- hexadecen-1-ol has been documented to possess cancer-Preventive, antimicrobial, anti-inflammatory and anti-diuretic potentials [65]. Tetradecanoic acid has antioxidant, cancer preventive, antifungal, nematicidal and hypercholesterolemic properties [66]. Phytol has been documented to show anticancer, anti-inflammatory and antimicrobial properties [60].

**Conclusion:** The pharmacological property displayed by MEAM in this study could be attributed to the phytochemical compounds present in it. Therefore, it could be inferred that MEAM contains phytochemicals which have anti-tumor/proliferative potentials. Nevertheless, the active principle(s) present in the methanol stem bark extract (MEAM) are still unknown. Further work is therefore required to elucidate and characterize the structure of the bioactive agent(s) responsible for these pharmacological activities. This may prove useful in the management of estrogen-dependent gynecological dysfunction (such as uterine/endometrial hyperplasia) and perhaps, serve as an alternative therapy to many of our women with such gynecological disorder.

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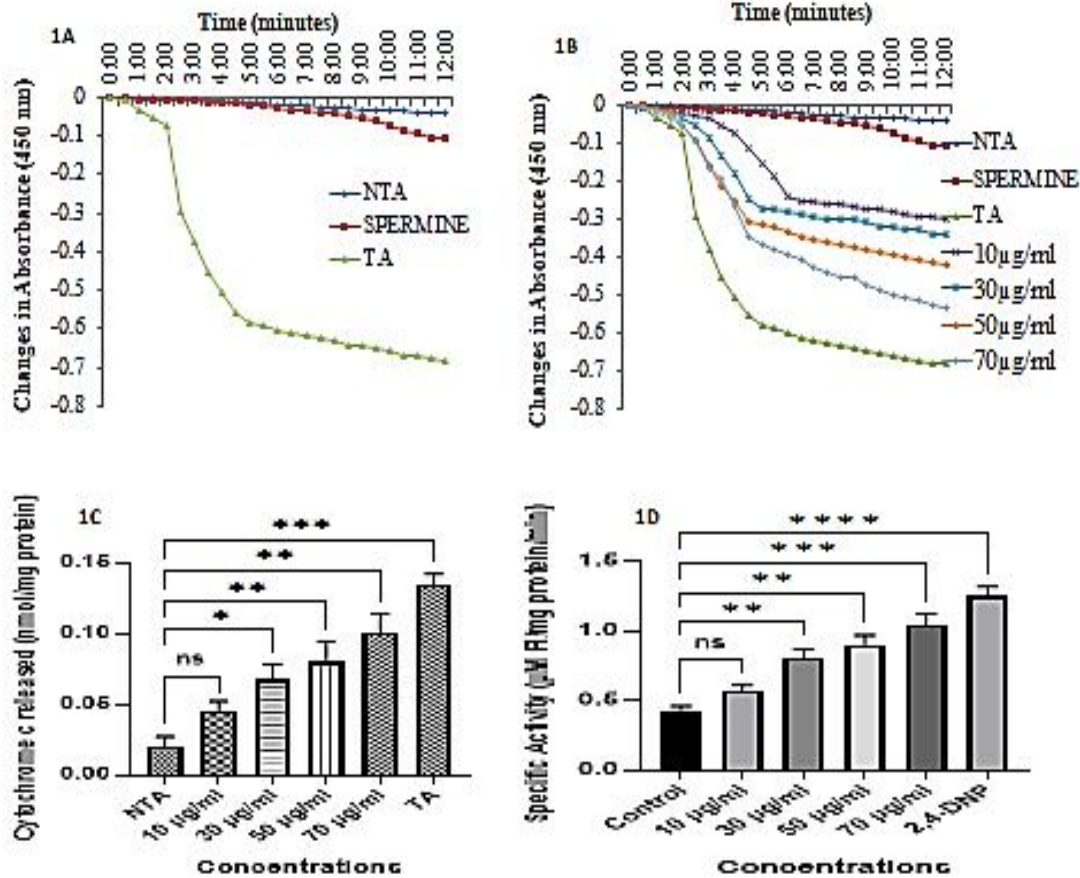
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**Figure 1:** (A) Calcium-induced mitochondrial permeability transition pore opening in normal rat uterus mitochondria and its reversal by spermine.

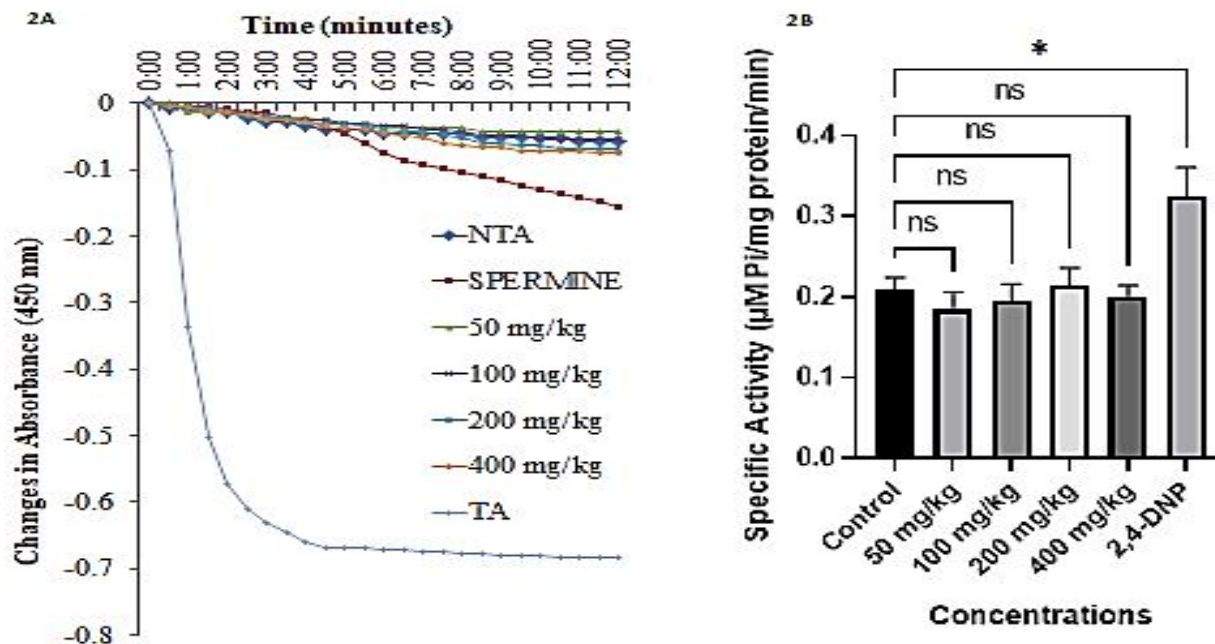
(B): Varying concentrations of MEAM induced rat uterine mitochondrial permeability transition pore opening

(C): Varying concentrations of MEAM caused the release of Cytochrome C form rat uterine mitochondrial inter membrane space

(D): Varying concentrations of MEAM enhanced rat uterine mitochondrial ATPase activity

**NTA:** no triggering agent (without calcium), **TA:** triggering agent (calcium), **Spermine:** standard inhibitor of mPT pore opening, **2,4-DNP:** (2,4,Dinitrophenol) standard uncoupler

The data reported on mPT are representative of multiple ( $\geq 4$ ) experiments while other values are expressed as mean  $\pm$  SD of four independent replicates, and analyzed using one-way ANOVA method followed by Turkey's post test. ns: not significant, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  compared to the control/NTA

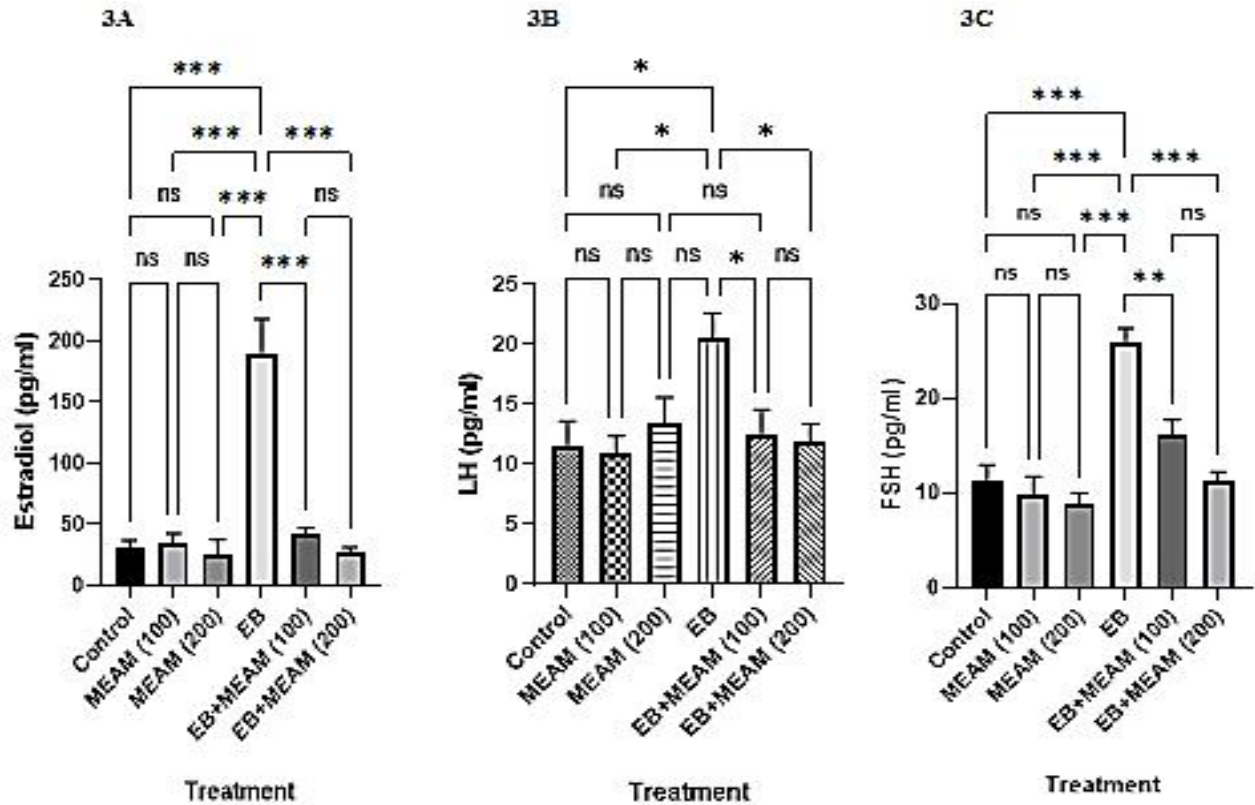


**Figure 2 (A):** Oral administration of MEAM did not induce uterine mitochondrial permeability transition pore opening at the tested doses after 28 days of treatment

(B): Oral administration of MEAM did not enhance uterine mitochondrial ATPase activity after 28 days of treatment

**NTA:** no triggering agent (without calcium), **TA:** triggering agent (calcium), **Spermine:** standard inhibitor of mPT pore opening, **2,4-DNP:** (2,4,Dinitrophenol) standard uncoupler

The data reported on mPT are representative of multiple ( $\geq 4$ ) experiments while other values are expressed as mean  $\pm$  SD of four independent replicates, and analyzed using one-way ANOVA method followed by Turkey's post test. ns: not significant, \* $p < 0.05$ , compared to the control/NTA



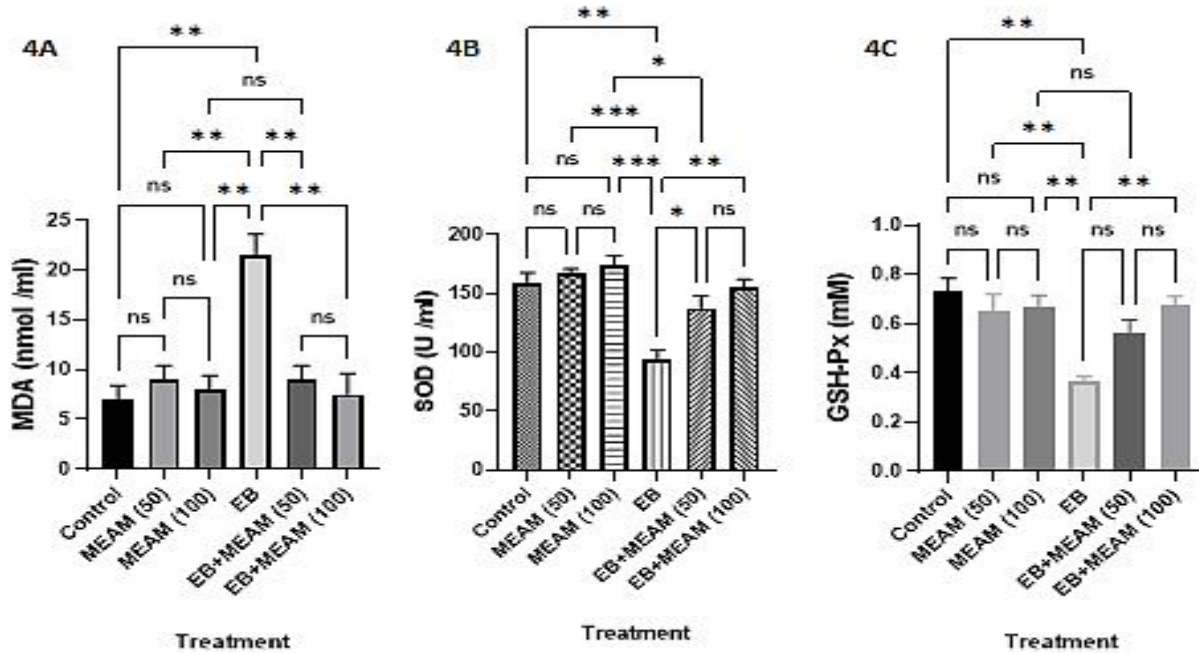
**Figure 3:** Effects of oral administration of MEAM on sex hormones;

(A): MEAM co-administration reversed EB-induced increase in estradiol (E2)

(B): MEAM co-administration reversed EB-induced increase in Luteinizing Hormone (LH)

(C): MEAM co-administration reversed EB-induced increase in Follicle Stimulating Hormone (FSH)

The values are expressed as mean  $\pm$  SD of four independent replicates, and analyzed using one-way ANOVA method followed by Turkey's post test. ns: not significant, \* $p < 0.05$ , \*\* $p < 0.01$  \*\*\* $p < 0.001$



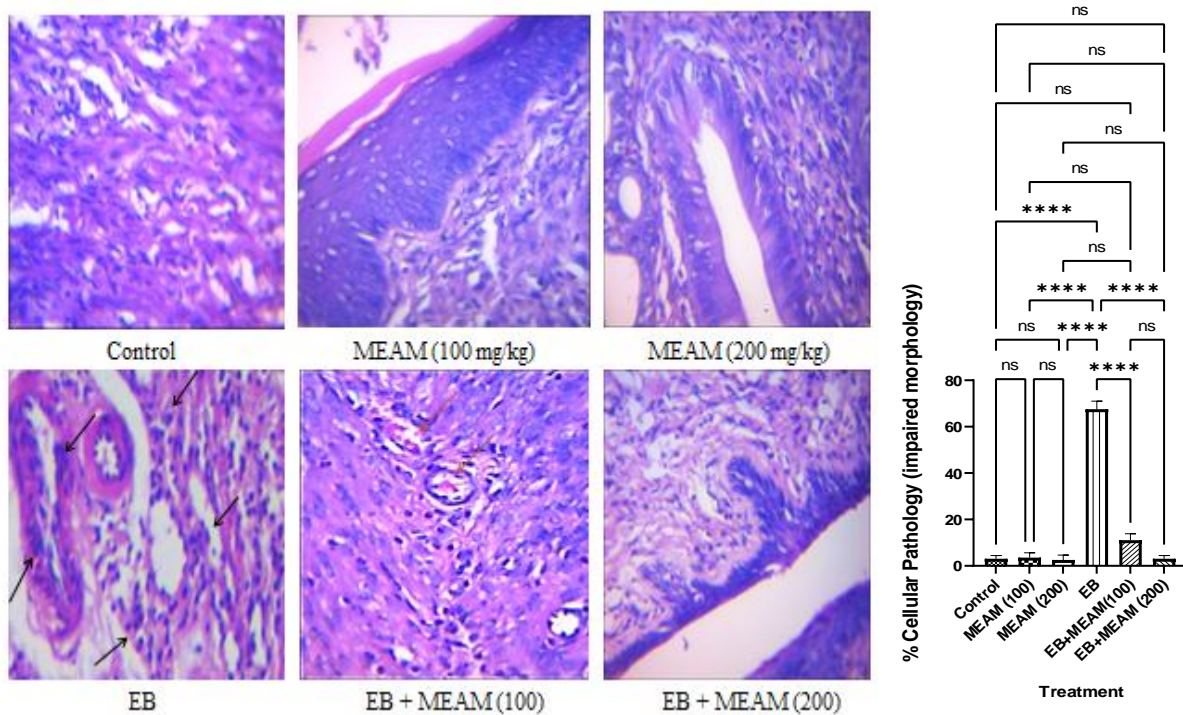
**Figure 4:** Effects of oral administration of MEAM on oxidative indices

(A) MEAM ameliorated the increase in MDA level caused by EB treatment

(B) MEAM co-administration caused upregulation of SOD activity

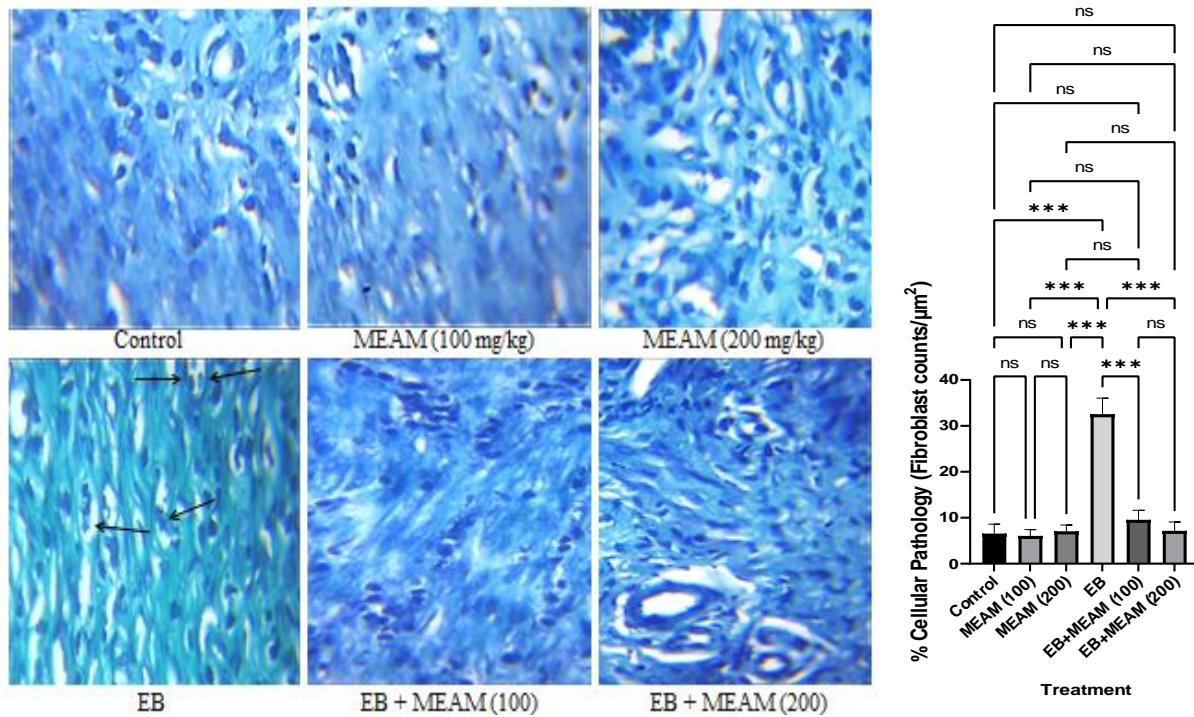
(C): MEAM upregulated the activity of GSH-Px

The values are expressed as mean  $\pm$  SD of four independent replicates, and analyzed using one-way ANOVA method followed by Turkey's post test. ns: not significant, \* $p < 0.05$ , \*\* $p < 0.01$  \*\*\* $p < 0.001$



**Figure 5:** Photomicrographs showing the effect of MEAM on the uterine section of normal and EB-treated female rats using haematoxylin and eosin stain (Mag. X 400).

**Control, MEAM (100) and MEAM (200):** Plates show normal cellular morphology, the endometrium epithelial layers and endometrial gland appear normal. **EB:** Plates show poor cellular architecture, the epithelial layers of the endometrium appear severely degenerated and show hyperplasia (black arrow) and the endometrial gland also show degenerated epithelial cells (black arrow), there is periglandular. **EB +MEAM (100) and EB +MEAM (200):** Plates show improvement on the cellular architecture, the pathology of the endometrial gland and epithelial layer improved on treatment, no hyperplasia was recorded, although glandular congestion was recorded at EB+MEAM (100).



**Figure 6:** Photomicrographs showing the effect of MEAM on the uterine section of normal and EB-treated female rats using Masson's Trichome stain (Mag. X 400).

**Control, MEAM (100) and MEAM (200):** Plates show normal cellular morphology and moderate deposition of collagen fiber. **EB:** There is a severe cellular aggregation (lymphocytes and macrophages) at the subepithelial region. Plates show high deposition of collagen fibers within the myometrium and severe hyperplasia. **EB+CFDC (100) and EB+CFDC (200):** There was reduction in collagen fiber within the myometrium and no uterine hyperplasia recorded

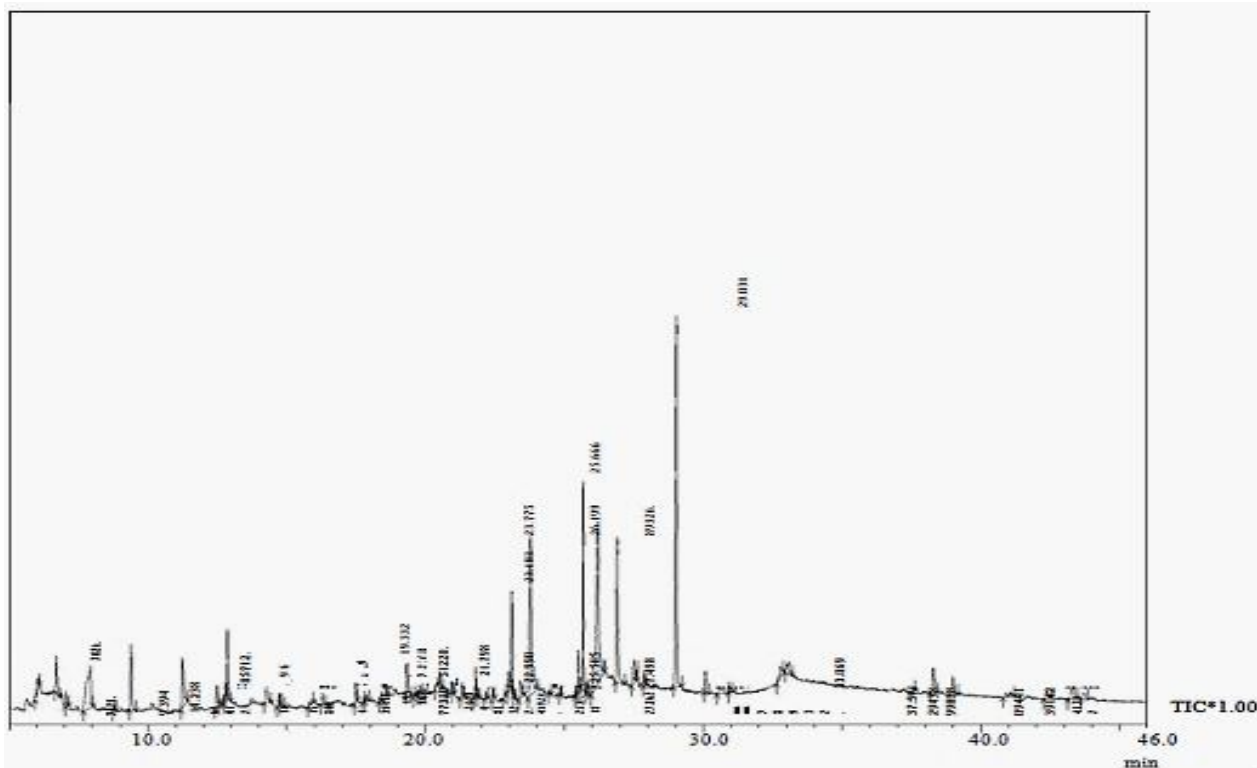
**Table 1:** Phytochemical screening of methanol stem bark extract of *Annona muricata* (MEAM)

Phytochemical Screening	Results
Flavonoids	++
Tannins	++
steroids	++
Saponins	-
Cardiac glycosides	+
Anthraquinones	+
Reducing sugars	-
Oil	++
Phlobatannins	-
Terpenoids	+

+ Mild presence

++ Strong presence

- Absence



**Figure 7:** The GC-MS analysis of methanol stem bark extract of *Annona muricata* showing its major constituents.

