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

Abstract

Ethnopharmacological relevance: 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.

Aim of the study: 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.

Materials and methods: 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 milleu 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 (Parkash et al., 2015). Endometrial hyperplasia could develop into cancer if not given the necessary therapeutic attention on time (Sherman, 2000; Parkash et al., 2015; Van der Meer and Hanna, 2017). 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 (Kaaks et al., 2002). It has been proven that phytochemicals especially those that possess anti-oxidative and anti-inflammatory potentials can inhibit tumor growth (Mishra et al.,

2013; Xiu et al. 2015). 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 (Green and Walczak, 2013; Wu et al., 2018; Olowofolahan et al., 2020; Olowofolahan et al., 2021). *Annona muricata* Linn, a medicinal plant is folklorically used in the treatment of tumors and cancers (Morton, 1980). Its name has been checked with http://www.theplantlist.org and also with http://www.worldfloraonline.org. Its anti-viral (Alvarez-Gonzalez, 2008), anti-microbial (Falodun, 2010), wound healing, anti-carcinogenic and genotoxic (Padmaa-Paarakh, 2009) properties have been reported. The leaf extract of A. muricata has been suggested to repress tumor growth (Liu et al., 2016). Several studies have also demonstrated anticancer activity of some bioactive isolates of A. muricata leaves (Moghadamtousi et al., 2014; Banerjee et al., 2017). Gavamukulya et al. (2014) equally demonstrated the cytotoxicity of the ethanol extract of A. muricata leaves on EACC, MDA, and SKBR3 tumor cell lines. Apart from the leaves, other parts like fruits, stems and seeds of A. muricata plant have been reported to possess anticancer activities (Oberlies et al., 1995; Villo, 2008). Annona muricata is employed in tropical Africa as insecticidal and pesticidal agents besides being used for the treatment of coughs, pain and skin diseases (Moghadamtousi et al., 2015). In Latin America, the leaves, seeds, unripe fruits and roots are being used traditionally as biopesticides, bioinsecticides and topical insect repellents in Latin America.

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 (Olowofolahan et al., 2021). 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 (El-Demardash, 2011; Jing et al., 2019).

2.2 Collection of plant materials

The stem bark of A. muricata was obtained from Botany Department, University of Ibadan, Nigeria. It was taxonomically identified and confirmed with voucher number UIH-23116 deposited.

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 approved by the University of Ibadan Animal Care and Use Research Ethics Committee (UI-ACUREC) and approval number (UI-ACUREC/17/0096) was assigned to the study.

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°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 (Costa et al., 2006).

2.5 Determination of mitochondrial protein

This was determined following the method of Lowry et al. (1951) using bovine serum albumin

as standard.

2.6 Assessments of mitochondrial F_oF_1 ATPase activity

The F_oF_1 ATPase activity was determined by the method of Lardy and Wellman (1953) as modified by Olorunsogo and Malomo (1985).

2.7 Estimation of inorganic phosphate released

This was determined following the method described by Olorunsogo et al. (1979).

2.8 Assay of cytochrome c release

The cytochrome c released from isolated mitochondria was quantified by measuring its soret peak at 414 nm (ε= 100 mM^{-1} cm^{-1}), according to method of Appaix et al. (2000).

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 (Masson, 1929; Luna, 1968). 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 (1989).

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 (≥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 μg/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_0F_1 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_0F_1 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 (Kroemer and Galluzzi, 2007; Sabharwal and Schumacker, 2014). The mPT pore now serves as a pharmacological target for

drug development; as it determines the life and death of a cell (Javadov and Karmazyn, 2007; Tait and Green, 2010). In this study, the intactness and suitability of the isolated mitochondria was first of all ascertained (Lapidus, 1993; Green and Kroemer, 2004). The opening of mPT pore by MEAM (in vitro) suggests it 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 (Deniaud et al., 2006; Constance and Lim, 2012; Gonzalez et al., 2018).

When mPT pore opens, cytochrome c is released and this could lead to cell death (Green and Kroemer, 2004; D'Souza et al., 2011). 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 (Angelin et al., 2008; Haq et al., 2013; Bernadi et al., 2015). 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 (Seidlmayer et al., 2012). 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 noteffectuterine 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 (Neagu et al., 2019). 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* (Olowofolahan et al., 2021). Estrogen has been reported to be crucial in leiomyoma pathogenesis

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and growth (Yue et al., 2003; Al-Hendy et al., 2004; Maruo et al., 2004). In addition, its involvement in the development of endometrial hyperplasia and endometrial cancer has been proven (Moloney and Cotter 2018; Pescatori et al. 2021). 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. (2017), 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) (Moghadamtousi et al., 2015). Large amount of ROS are generated by long-term high estrogen inducement which could generate oxidative stress (Gupta et al., 2015; Chen et al., 2015). 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 (Moghadamtousi et al., 2015). 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 (Peji et al. 2003; You et al. 2017). 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. 2017, 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 (Hamizah et al., 2012).

The phytochemical screening of *Annona muricaa* stem bark extract (MEAM) revealed the presence of various phytochemical components which have been associated with several beneficial effects (Pereira et al., 2007; Katalinic et al., 2004). 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 hemolyticpotentials (Rajalakshmi and Mohan, 2016), while hexadecanoic methyl ester, has also been reported to have antioxidant, anti-tumor, aromatase inhibitory and other pharmacological properties (Vijisaral and Subramanian, 2014; Dandekar et al., 2015; Olowofolahan et al., 2020). The 2,,6,10-Trimethyl,14- Ethylene-14-Pentadecne identified from MEAM has been reported to exhibit antiproliferative property (Parthipan et al., 2015).

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Furthermore, 3,7,11,15-Tetramethyl-2- hexadecen-1-ol has been documented to possess cancer-Preventive, antimicrobial, anti-inflammatory and anti-diuretic potentials (Shibula and Velavan, 2015). Tetradecanoic acid has antioxidant, cancer preventive,antifungal, nematicidal and hypercholesterolemic properties (Farina et al., 2015). Phytol has been documented to show anticancer, anti-inflammatory and antimicrobial properties (Rajalakshmi and Mohan, 2016). 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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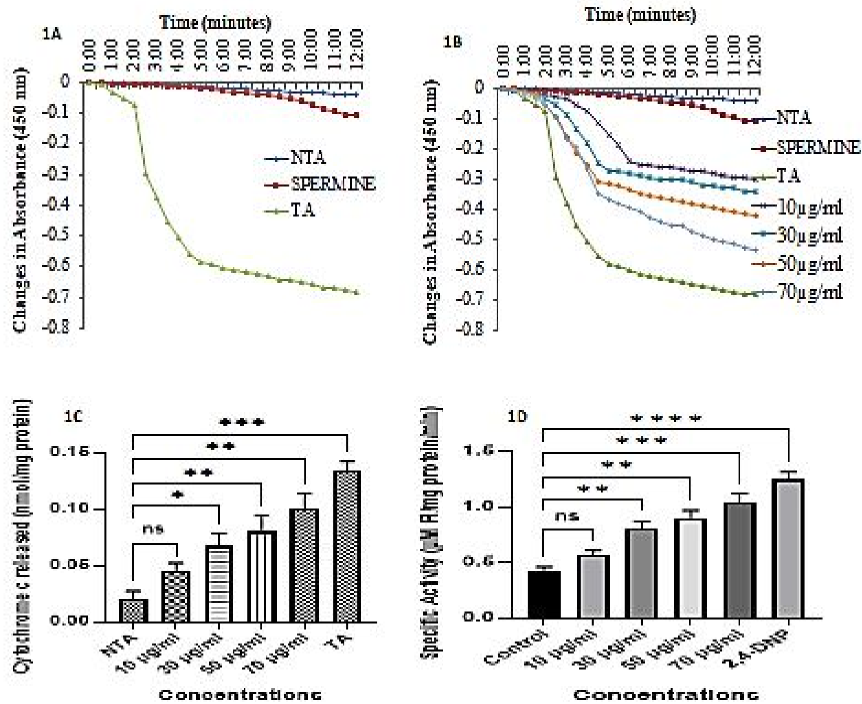

Figure 1: (A) Calcium-induced mitochondrial permeability transition pore opening in normal rat uterus mitochondria and its reversal by spermine.

(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 (≥4) experiments while other values are expressed as mean ± 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

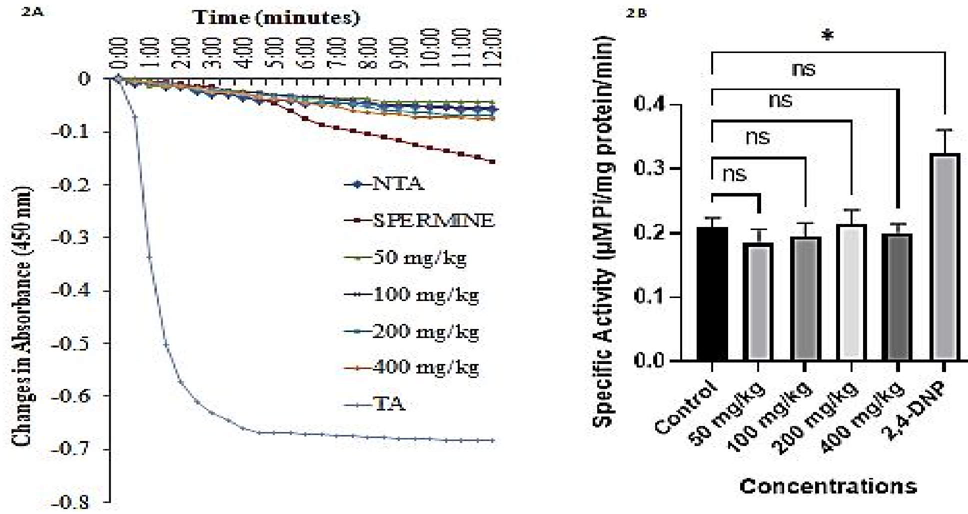

Figure 2 (A): Oral administration of MEAM did not induce uterine mitochondrial permeability transition pore opening at the tested doses after 28 days of treatment

(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 (≥4) experiments while other values are expressed as mean ± 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

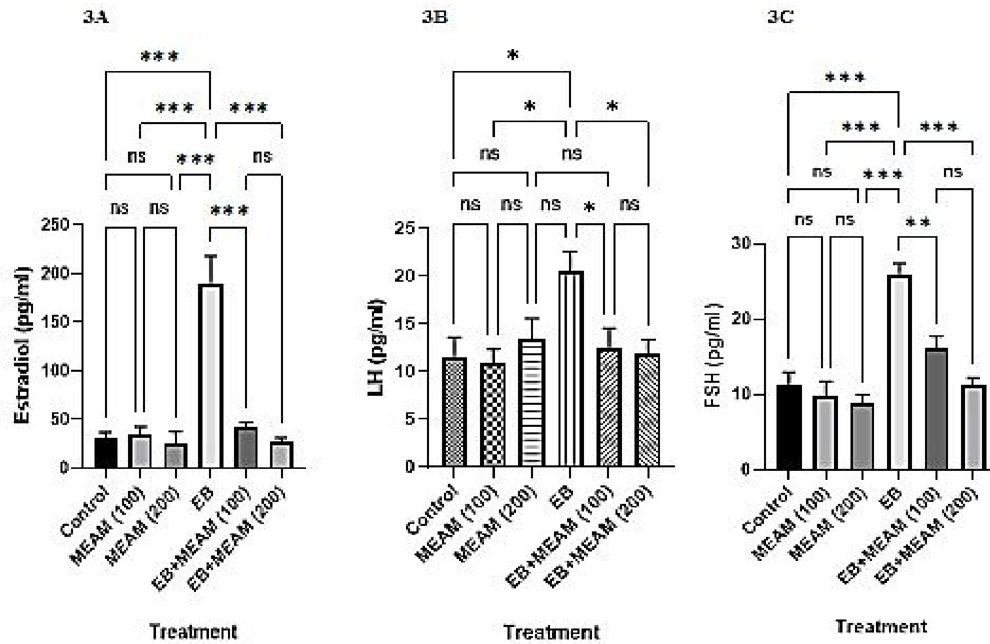

Figure 3: Effects of oral administration of MEAM on sex hormones;

(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 ± SD of four independent replicates, and analyzed using one-way ANOVA method followed by Turkey's post test. ns: not significant, *$p < 0.05$, **$p < 0.01$ ***$p < 0.001$

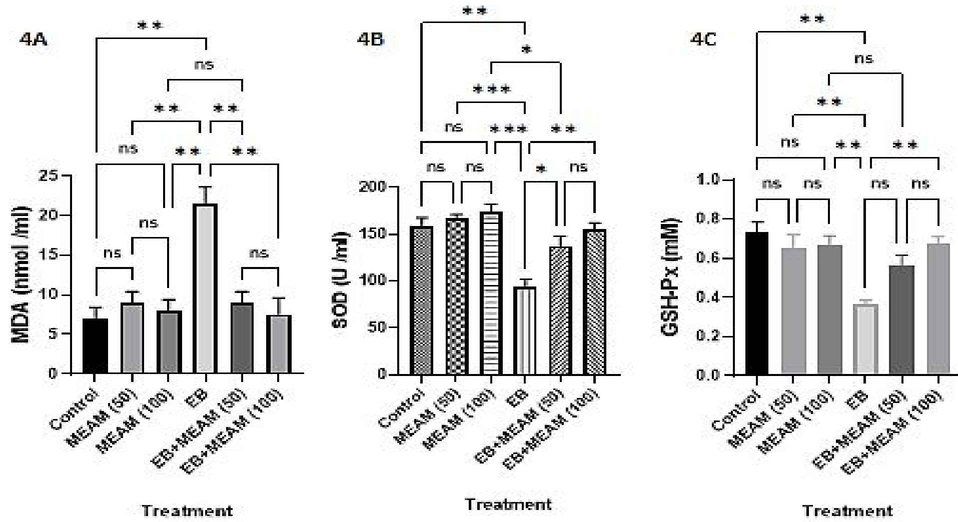

Figure 4: Effects of oral administration of MEAM on oxidative indices

(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 ± 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

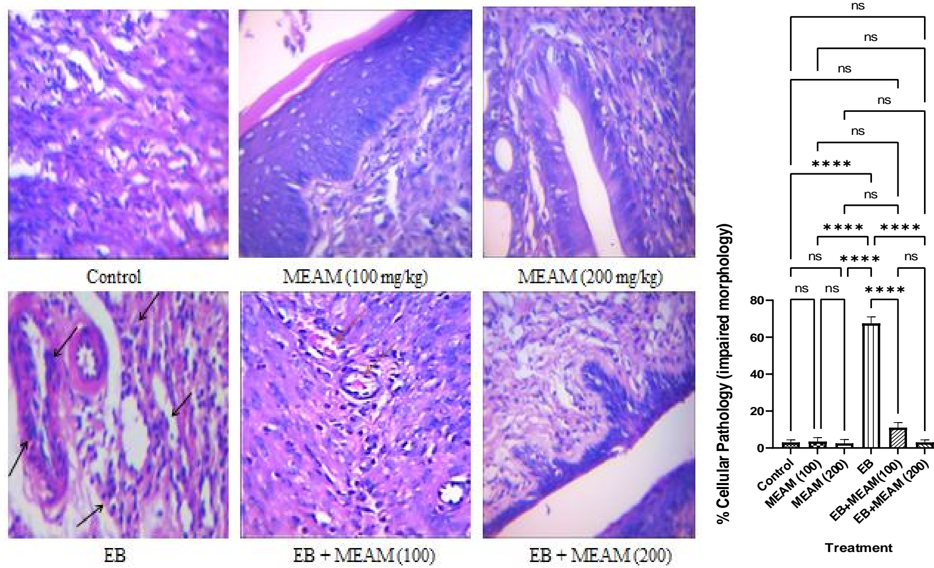

Figure 5: Photomicrographs showing the effect of MEAM on the uterine section of normal and EB-treated female rats using haematoxylin and eosin stain (Mag. X 400).

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).

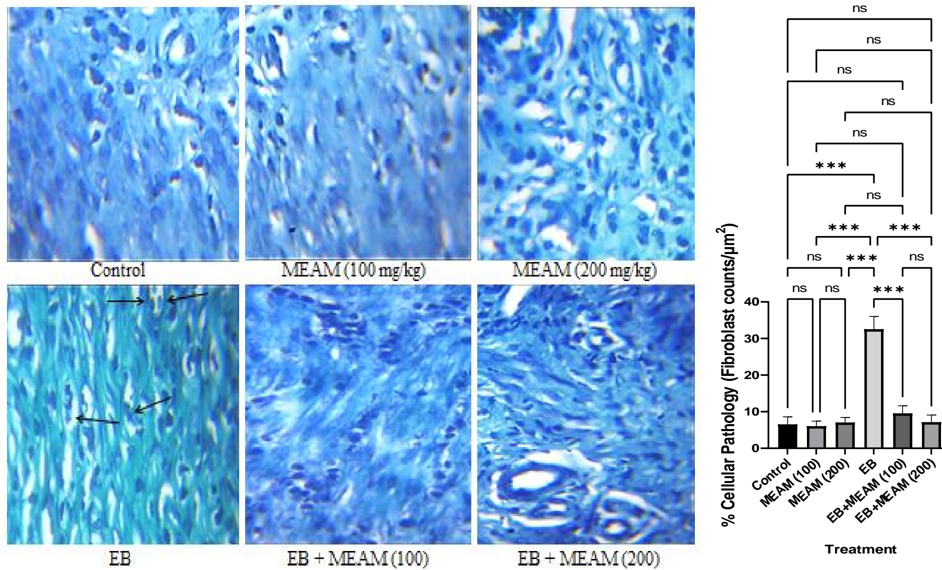

Figure 6: Photomicrographs showing the effect of MEAM on the uterine section of normal and EB-treated female rats using Masson's Trichome stain (Mag. X 400).

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

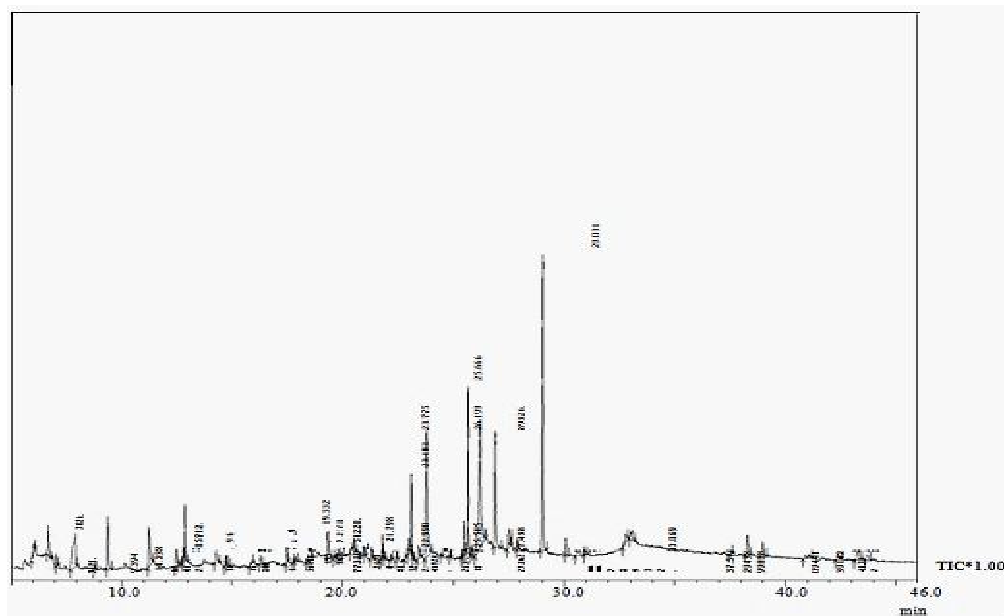

Figure 7: The GC-MS analysis of methanol stem bark extract of *Annona muricata* showing its major constituents.