

**Pepper fruit (*Dennettia tripetala* [annonaceae]) seed inhibits uterine contractions via blockade of intracellular calcium release and extracellular calcium influx in *ex vivo* studies**Uchendu Adaeze Phina\*<sup>1</sup>, Bafor Enitome Evi<sup>1</sup><sup>1</sup>Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Nigeria.Submitted: 25<sup>th</sup> April, 2023; Accepted: 4<sup>th</sup> June, 2023; Published: 30<sup>th</sup> June, 2023DOI: <https://doi.org/10.54117/jcbr.v3i3.6>\*Corresponding author: Uchendu Adaeze Phina; [adaeze.uchendu@uniben.edu](mailto:adaeze.uchendu@uniben.edu); 08032065268**Abstract**

*Dennettia tripetala* (DT) is a tree found in rain forest belt of Nigeria, Cameroon and Ivory Coast. The seed is commonly used in preparing food for mothers after delivery because it is believed to play a role in postpartum uterine contraction. There is however no scientific evidence validating this claim. Hence, the study aimed to investigate the effects of DT seed on non-pregnant and pregnant uterus using mouse model.

The dried seeds of DT were macerated in methanol and the effects of DT extract were investigated on uterus tissues isolated from non-pregnant and pregnant mice. DT extract was examined on spontaneous uterine contraction, contractions induced by oxytocin, and high potassium chloride (KCl)-induced depolarization. Its effect was also evaluated on calcium ion (Ca<sup>2+</sup>) release from internal stores.

The DT extract suppressed spontaneous uterine contractility in both pregnant and non-pregnant uterus in a concentration-dependent manner. It significantly inhibited uterine contractions elicited by high KCl and oxytocin-induced contractions in both pregnant and non-pregnant uterus. The extract also inhibited uterine contractility induced by oxytocin in the Ca<sup>2+</sup>-free medium in pregnant and non-pregnant uterus.

The study has provided scientific evidence that DT seed extract has uterine relaxing effect in both pregnant and non-pregnant mouse uterus possibly by blocking

intracellular Ca<sup>2+</sup> released and influx of extracellular Ca<sup>2+</sup>. The result has shown that DT has no benefit in reducing the risk of postpartum hemorrhage, however, it may have potential benefits in management of dysmenorrhea and preterm labour in non-gravid and gravid uteri respectively.

**Keywords:** *Dennettia tripetala*, uterine contraction, pepper fruit, pregnant and non-pregnant uterus, oxytocin, high KCl

**Introduction**

*Dennettia tripetala* (DT) is a tree of West African origin with limited distribution in Ivory Coast, South Nigeria and West Cameroon. It is universally identified as pepper fruit, and also is known with different local names among the Igbo (mmimi), Yoruba (aka Igbere), Binis (Ako) and Niger Deltan (Imako) of Nigeria (Ikpi and Nku, 2008; Iseghohi, 2015).

Studies on its fruit ethanolic extracts has demonstrated the presence of tannins, alkaloids, steroids, flavonoids, cardiac glycosides, saponins and terpenoid (Ihemeja *et al.*, 2013). About 25 compounds including linoleic acid ethyl ester, caryophyllene, 3-caryophyllene, 3-carene, phenyl ethyl alcohol and cubebene have been isolated from the fruit (Iseghohi, 2015).

Nutritionally, the fruit is rich in carbohydrates, protein, fiber, lipids, vitamins and minerals. It is consumed widely generally because of its

spicy and peppery taste. In Southern Nigeria, it is used for the entertainment of guests and chewed occasionally (Ikpi and Nku, 2008; Iseghohi, 2015). It is also used in preparing food given to postpartum mothers, on the hypothesis that it aids in contraction of the postpartum uterus (Achinewhu *et al*, 1995; Okwu and Morah, 2004;).

It is also used in folklore medicine in treatment of various diseases including nausea, diabetes, fever, cough, toothache, and diarrhea. Experimental studies have demonstrated the antimicrobial, analgesic, anti-inflammatory, anticonvulsant, antioxidant, hypnotic, and anti-hyperglycemic effects of its fruit extracts (Iseghohi, 2015).

Despite the widespread use of the fruit, sufficient work has not been done to scientifically justify most of its uses in folklore medicine. Motivated by this knowledge gap, this study hopes to investigate the hypothesis that DT seed aids in contraction of postpartum uterus (Okwu and Morah, 2004).

## Materials and Methods

### 2.1 Plant materials and extraction

Fresh unripe fruits of DT were purchased from New Benin market, Ovia North-East Local government area in July, 2019 and identified by Dr. H.A Akinibosun, Department of Plant Biology Biotechnology, University of Benin, (Edo State, Nigeria). After, the skin of the fruits were peeled off, the seeds were air-dried for 3 weeks and pulverized. One thousand, two hundred grams (1200 g) of the seed powder was extracted by cold maceration with 7.5 L of methanol (99.8%) for 72 h while being constantly stirred. This was subsequently filtered and concentrated in a rotary evaporator (BUCHI Labortechnik AG, Flawil Switzerland) set at 60 °C to obtain 13.8% yield. The dried extract

was kept in a well labeled air-tight bottle and refrigerate until needed.

### 2.2 Animals

Pregnant albino mice (gestation day  $18 \pm 1$ , body weight range of 28 – 32 g) and non-pregnant (22.0 – 25.0 g body weight) were used in the research. The albino mice were procured through the Animal unit, Faculty of Pharmacy, University of Benin (Edo State, Nigeria). They were maintained under a natural light/dark cycle and at room temperature. Standard rodent pellet feeds (Top feeds limited, Ibadan, Nigeria) and clean water were made available to the mice *ad libitum*. They were allowed to adaptation for two weeks before the commencement of the study. All the procedures were carried out following the standard protocols for the Care and Use of Laboratory Animals (National Institutes of Health, 2015). All the protocols were approved by the Faculty of Pharmacy Ethics Committee, University of Benin (EC/FP/019/20).

### 2.3 Drugs and chemicals

Methanol (Pharmatrends, Nigeria) and Tween 80 (Kernel-KN, China) were utilized in this study. The physiological saline solution (PSS), Ringer's Locke solution was prepared with the following composition (mM/L): NaCl 154.00, NaHCO<sub>3</sub> 5.95, D-glucose 2.78, KCl 5.63, and CaCl<sub>2</sub>•2H<sub>2</sub>O 2.05. Physiological salts were obtained from Guangdong GuanghuaSci-Tech Co. Ltd China, Loba cheme PVT Ltd, India, and Sigma Aldrich, UK). Other drug used in this study include oxytocin (Roche pharmaceutical Ltd, UK).

### 2.3 Ex vivo assay

#### 2.3.1 Uterine tissue preparation

Non-pregnant (non-gravid) uterus was obtained from animals in estrus phase. The estrus phase was established by microscopic assessment of vaginal cells (cytology) by the

method described by Bafor *et al.*, (2019) and McLean *et al.*, (2012). Briefly, the squamous epithelia of vagina was flushed with about 0.1 mL of normal saline using a Pasteur pipette of diameter 0.1 mm. The content of the pipette was carefully placed on clean glass slide, air-dried and fixed with cold methanol. The vagina smear was stained with methylene blue (0.1%) and viewed under a microscope using X10 objective lens (Visiscope® VWR, UK). The validation of the estrus stage was confirmed by the prevailing presence of cornified epithelial cells (McLean *et al.*, 2012; Bafor *et al.*, 2019).

For pregnant (gravid) uterus, female mice were paired overnight with a male mouse of the same strain in ratio of 2:1. Gestation day 0 was ascertained by presence of vaginal plug and animals at gestation period of  $18 \pm 1$  days were used.

On the day of the experiment, animals (pregnant and non-pregnant mice) were euthanized by cervical dislocation. The abdomen was cut open and the uterine horns were immediately excised and placed in a dissecting dish containing previously warmed ( $37^{\circ}\text{C}$ ) and aerated physiological salt solution. The isolated uterine horns were freed of the connective and adhering fats and were cut into segments (1 – 2 mm length). The uterine segment was tied with surgical thread at both ends (a loop on one end and a long thread) using a sterile needle and was immersed vertically in a 10 mL organ bath containing Ringer's Locke solution, continuously aerated and maintained at  $37^{\circ}\text{C}$ . The loop was attached to the tissue holder and the long thread was attached to an isometric force transducer (Panlab ADInstruments, Spain) connected to bridge amplifiers which in turn was connected to a PowerLab data acquisition system (Powerlab 2/26 Model ML826 ADInstruments, Australia) for recording and displaying changes in force and frequency of contractions. LabChart 7 Reader software (v.

8.0, ADInstruments, North America, USA) was used for the measurements. The uterine strip was placed under a resting tension of 0.5 g and was allowed to equilibrate in the PSS until regular uterine contractions were obtained (Bafor *et al.*, 2019; Sukwan *et al.*, 2014).

### **Experimental protocols**

#### **DT extract on spontaneous uterine contractions**

After obtaining regular spontaneous uterine contractions which also served as control (100%), the effects of cumulative concentrations of DT extract (0.01 -12.21 mg/mL) were determined and a concentration-response curve was plotted. A contact time of 5 min was allotted to each concentration (Bafor *et al.*, 2019).

#### **DT extract on uterine contraction stimulated by oxytocin**

The uterine strip was pre-stimulated with oxytocin (11.62 nM) for 10 min, then washed with fresh PSS, and allowed recovery of regular contractions. Thereafter, the uterine strip was pre-contracted again with oxytocin (11.62 nM) for 10 min and a single concentration (half-maximal inhibitory concentration [ $\text{IC}_{50}$ ]) of DT extract (3.5 mg/mL and 4.12 mg/mL for non-pregnant and pregnant uterus respectively) was added in the continued presence of oxytocin (Bafor *et al.*, 2019).

#### **DT extract on high KCl-induced depolarization**

The effect of DT extract on high KCl-induced myometrial membrane depolarization was determined using the method described by Bafor *et al.* (2019). Briefly, the uterine tissue was pre-contracted with 80mM of high KCl for 10 min, then washed and allowed recovery of uterine contractions. Thereafter, tissue was pre-contracted again with 80mM of high KCl

for 10 min and DT extract (3.5 mg/mL and 4.12 mg/mL for non-pregnant and pregnant uterus respectively) was added in the continued presence of high KCl (80 mM).

#### **DT extract on uterine contraction induced with oxytocin in Ca<sup>2+</sup>-free medium**

The effect of DT extract on intracellular Ca<sup>2+</sup> release from Ca<sup>2+</sup> stores was assessed in a Ca<sup>2+</sup>-free PSS containing 0.1mM of ethylenediamine tetraacetic acid (EDTA). After obtaining regular spontaneous uterine contractions in the normal PSS (Ringer's Locke), the PSS was then replaced with zero Ca<sup>2+</sup> PSS containing EDTA (0.1mM). Without draining the Ca<sup>2+</sup>-free PSS, oxytocin (11.62 nM) was added and thereafter DT (3.5 mg/mL and 4.12 mg/mL for non-pregnant and pregnant uterus respectively) was added in the continued presence of oxytocin (Bafor *et al.*, 2019).

#### **Data analysis**

Data are expressed as mean ± standard error (SEM) where “n” represents the number of animals. Statistical differences between the drugs and the DT extract were analyzed using unpaired Student's t-tests, P values less than 0.05 was considered statistically significant. The GraphPad Prism v 8.0 (GraphPad software, San Diego, CA, USA) was used.

The bitmaps were analyzed using Labchart Reader Software 8.0 and the uterine contractility parameters analyzed include frequency and force (amplitude) of contractions. The mean log concentration-response curves were analyzed by fitting data to a four-parameter logistic equation, using non-linear regression and following equation values ( $Y = \text{Bottom} / (1 + 10^{-(\text{LogE}/\text{IC}_{50} - X) * \text{HillSlope}})$ ). Y represent the response which starts at the bottom and goes to the top in sigmoid shape, X represent logarithm of concentration.

## **Results**

### **Effect of DT extract on spontaneous uterine contractions**

Cumulative applications of the DT seed extract significantly decreased spontaneous contractions of the pregnant uterus (0.03 - 22.2 mg/mL) and non-pregnant uterus (0.01 - 12.21 mg/mL) in a concentration-dependent manner (Figure 1a). The result analysis showed that the amplitude and frequency of spontaneous contractions in both pregnant and non-pregnant uterus were reduced (Figure 1b). The half-maximal inhibitory concentration (IC<sub>50</sub>) of DT seed extract for amplitude and frequency of the non-pregnant ( $0.83 \pm 0.40$  mg/mL and  $0.99 \pm 0.06$  mg/mL respectively) and pregnant ( $1.29 \pm 0.04$  mg/mL and  $1.85 \pm 0.08$  mg/mL respectively) were estimated. There was immediate recovery of spontaneous uterine contractions after the washing out of DT seed extract with fresh PSS.

### **Effects of DT extract on oxytocin-induced uterine contraction**

The contractile response (amplitude and frequency) of the uterus tissues were increased by oxytocin (Fig 2a). However, application of DT extract in the continued presence of oxytocin significantly decreased frequency ( $p < 0.001$ ) and slightly decreased the amplitude of the non-pregnant uterus. In pregnant uterus, the amplitude of contraction was significantly decreased ( $p < 0.05$ ), but no significant change in frequency was observed. This is shown in Figs 2b and 2c.

### **Effects of DT extract on high KCl-induced depolarization**

The effect of the extract on KCl-induced contractions are shown in Figure 3a. The application of KCl produced a rapid and

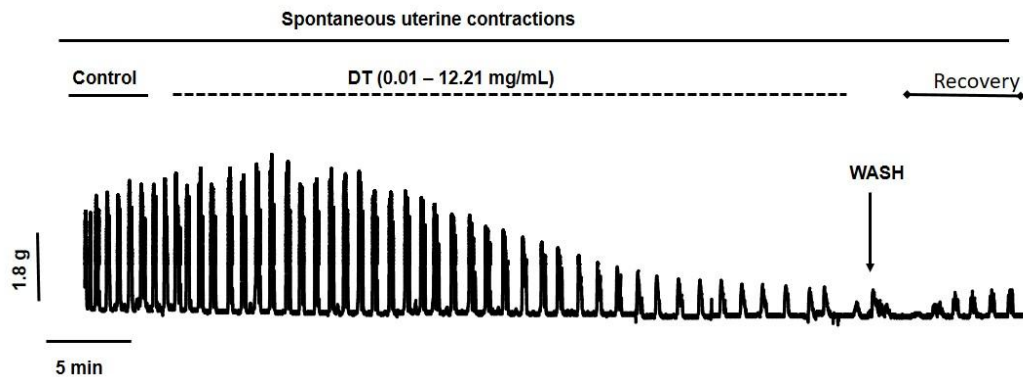
sustained increase in force of contraction. The introduction of DT in the presence of KCl (80 mM) significantly diminished the high KCl-induced force of contraction in both non-pregnant ( $p < 0.01$ ) and pregnant ( $p < 0.05$ ) uterus compared to high KCL alone (Figure 3b).

#### Effects of DT extract on oxytocin-induced contraction in calcium-deficient medium

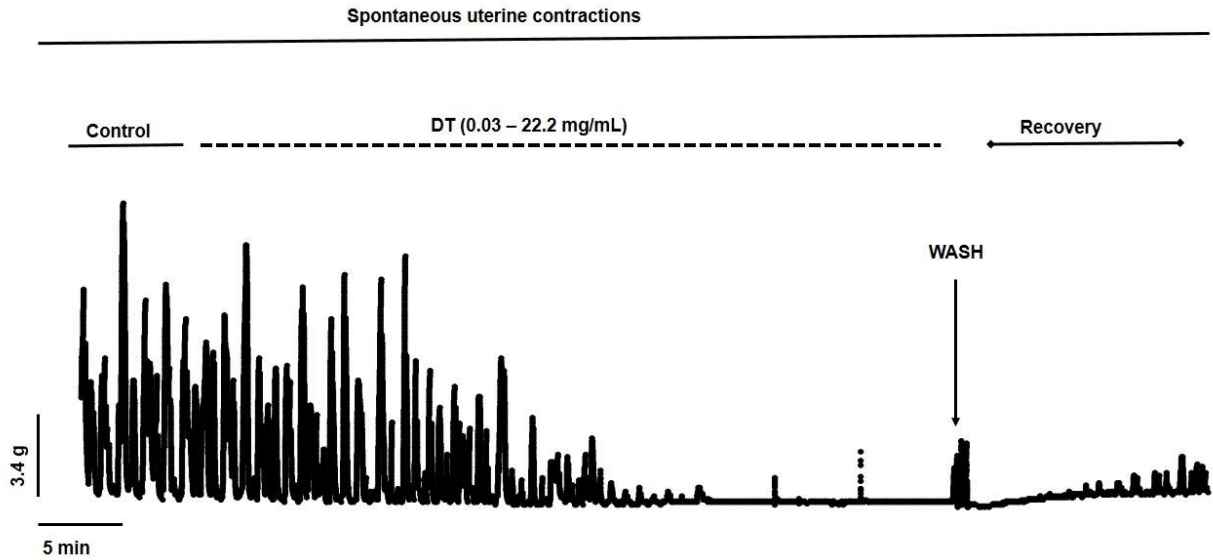
The effects of the DT extract on the release of  $Ca^{2+}$  from intracellular stores were shown in

Figure 4a. The application of oxytocin to the uterus tissue mounted in  $Ca^{2+}$ -free (containing EDTA, a calcium chelating agent) PSS slightly increased the spontaneous contractions. However, addition of the DT seed extract completely inhibited uterine contractions in both non-pregnant and pregnant uterus. The amplitude and frequency were significantly decreased in non-pregnant ( $p < 0.05$ ,  $p < 0.01$  respectively) and pregnant uterus ( $p < 0.05$ ,  $p < 0.001$  respectively) (Figs 4b and 4c).

A

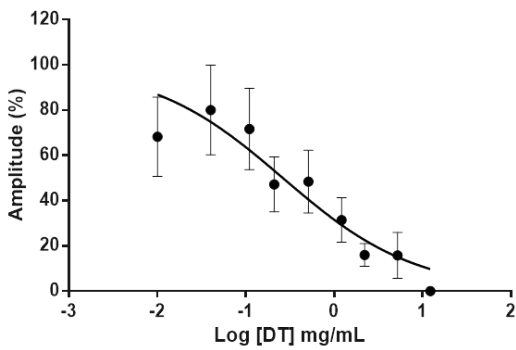


B

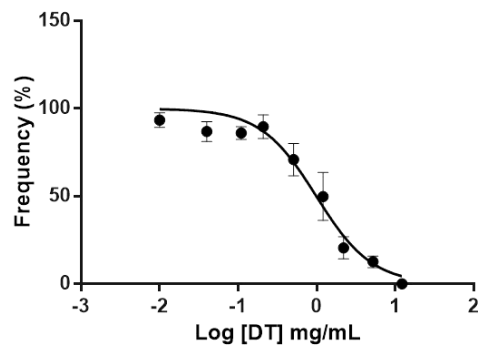


**Figure 1a.** Representative traces showing the response of isolated non-pregnant (A) and pregnant (B) mouse uterus to DT extract. DT, *Dennettia tripetala*.

A

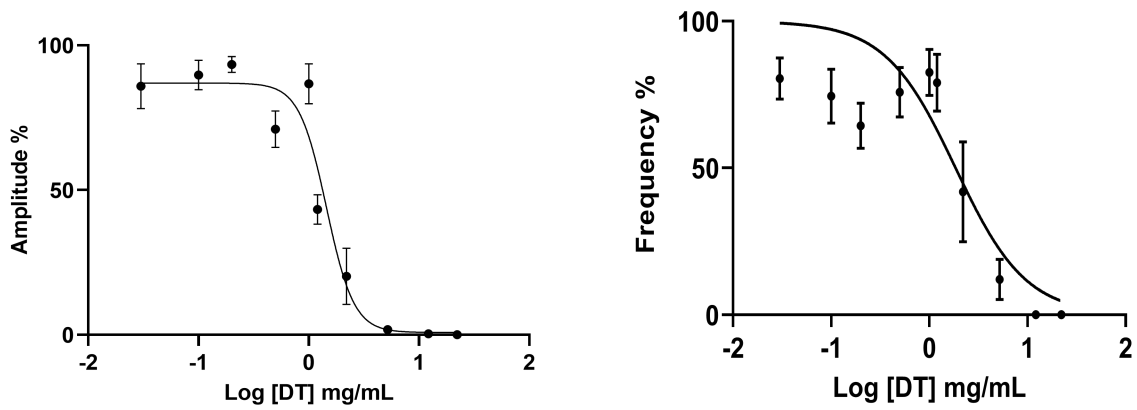


B



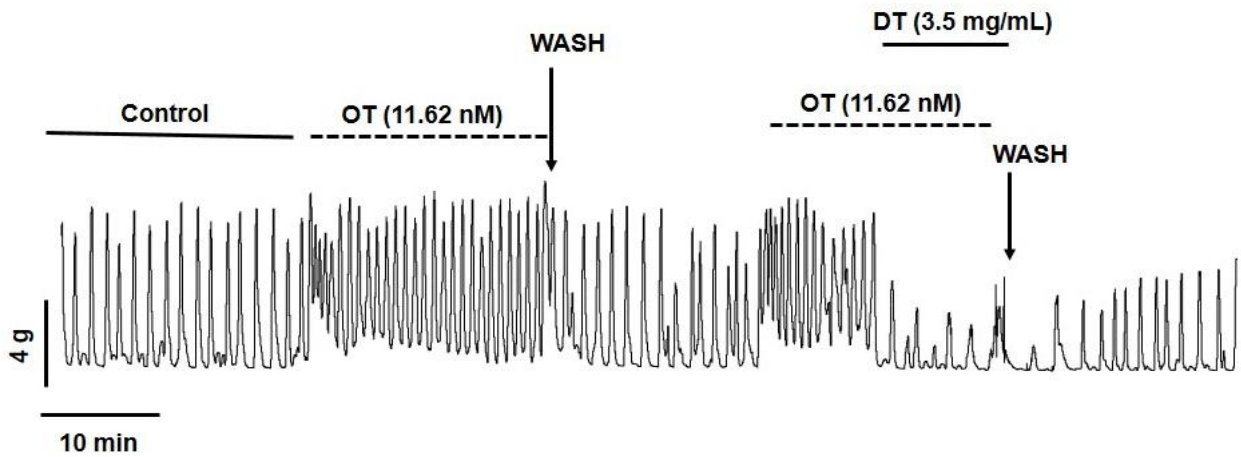
C

D

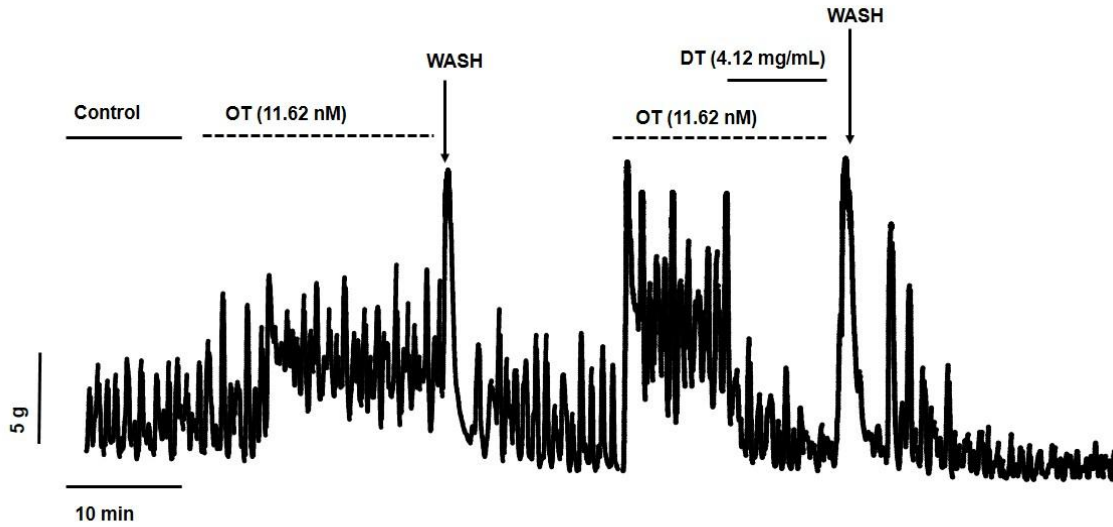


**Figure 1b.** Cumulative concentration-response effects of DT extract on the amplitude and frequency of spontaneous uterine contraction of non-pregnant mouse uterus (A, B) and pregnant mouse uterus (C, D). DT, *Dennettia tripetala*; n = 5 animals.

A

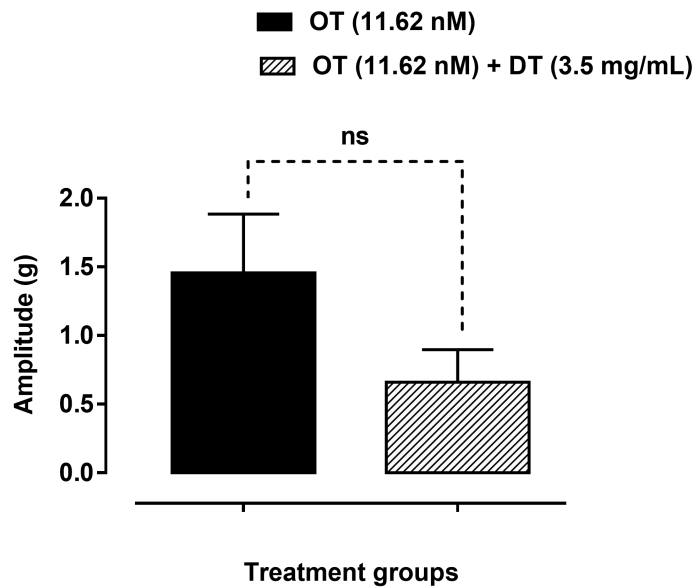


B



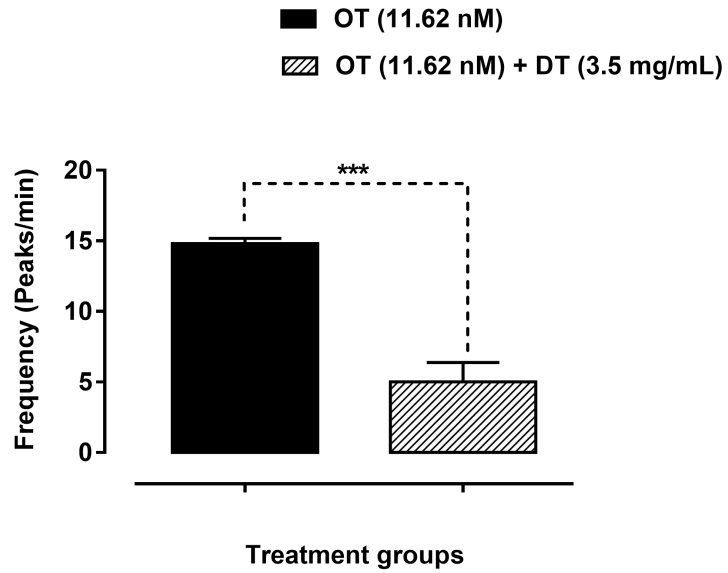
**Figure 2a.** Representative traces showing the effects of DT (3.5 mg/mL) and (4.12 mg/mL) on oxytocin-induced uterine contraction of the isolated mouse non-pregnant uterus (A) and pregnant uterus (B) respectively. DT, *Dennettia tripetala*; OT, oxytocin.

A



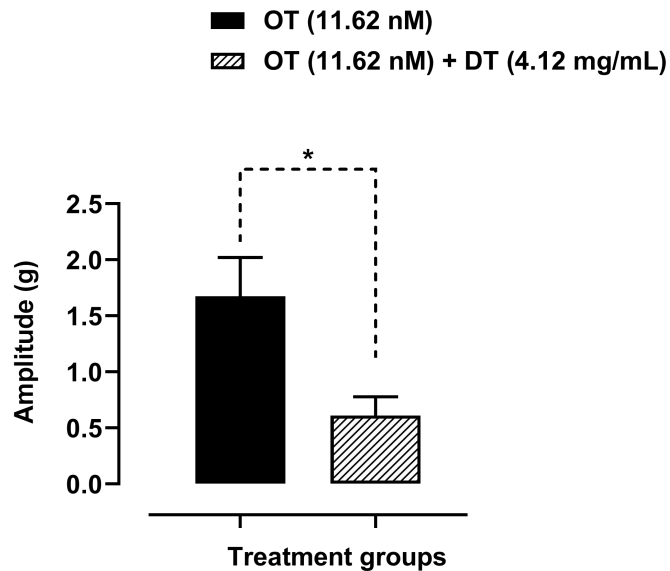


B

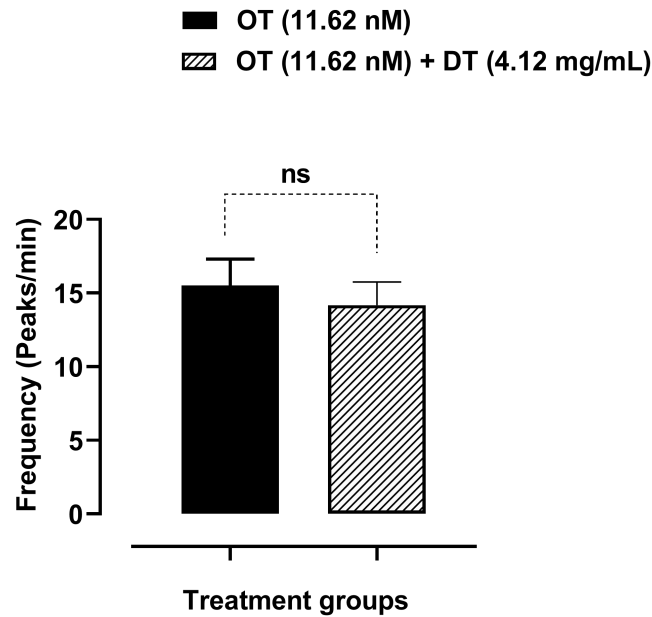


**Figure 2b.** Bar charts displaying the effects of DT extract on the amplitude (A) and frequency (B) of oxytocin-induced uterine contractions of isolated non-pregnant mouse uterus. DT, *Dennettia tripetala*; OT, oxytocin;  $^{***}p < 0.001$ ; ns, not significant; n= 5 animals.

A

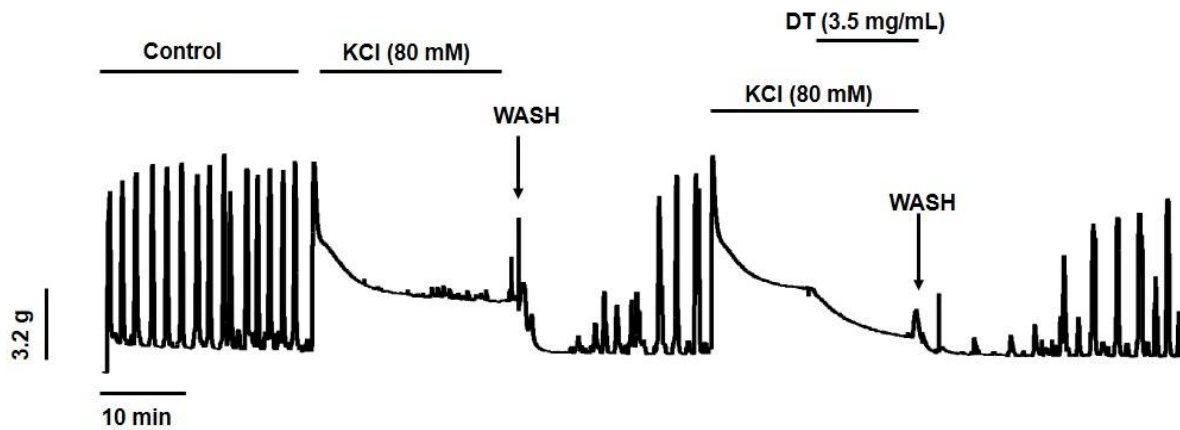


B

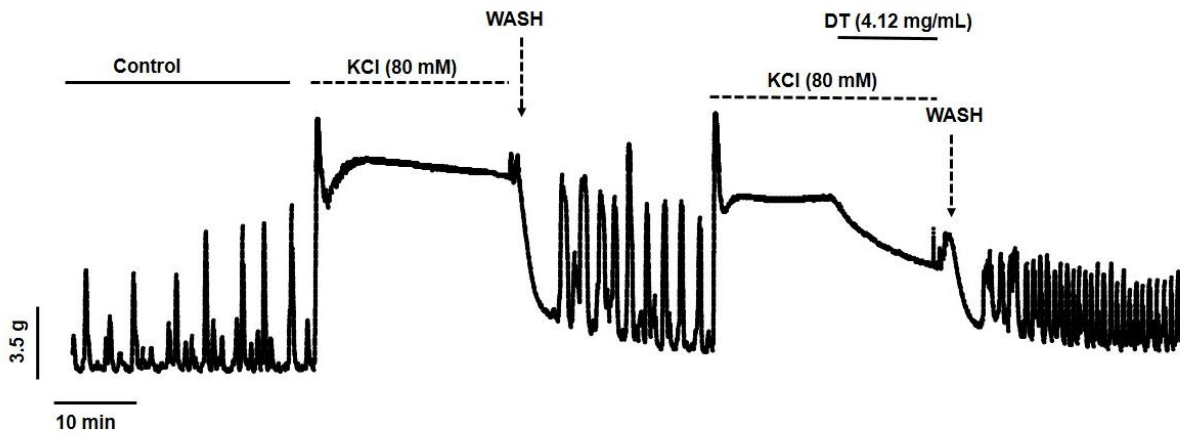


**Figure 2c.** Bar charts displaying the effects of DT extract on the amplitude (A) and frequency (B) of oxytocin-induced uterine contractions of isolated pregnant mouse uterus. DT, *Dennettia tripetala*; OT, oxytocin; \* $p < 0.05$ ; ns, not significant;  $n = 5$  animals.

A

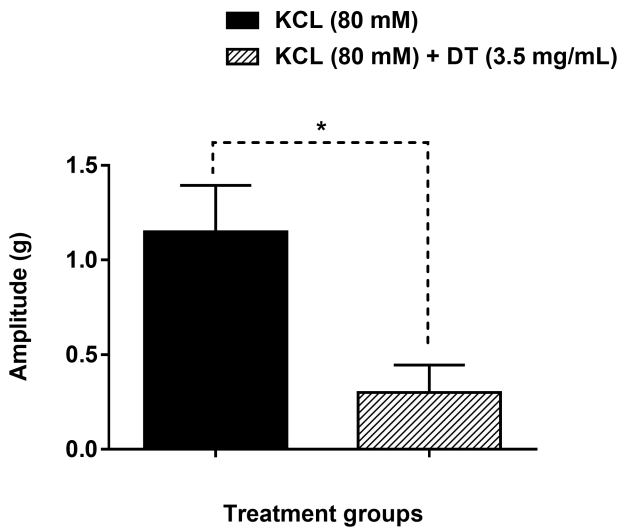


B

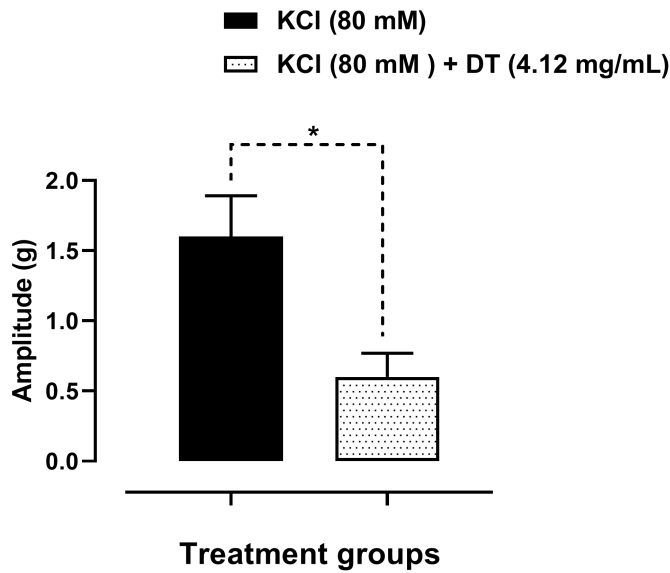


**Figure 3a.** Representative traces showing the effect of DT (3.5 mg/mL) and (4.12 mg/mL) on high KCl (80 mM)-induced uterine contraction of the isolated mouse non-pregnant uterus (A) and pregnant uterus (B) respectively. DT, *Dennettia tripetala*; KCl, potassium chloride.

A

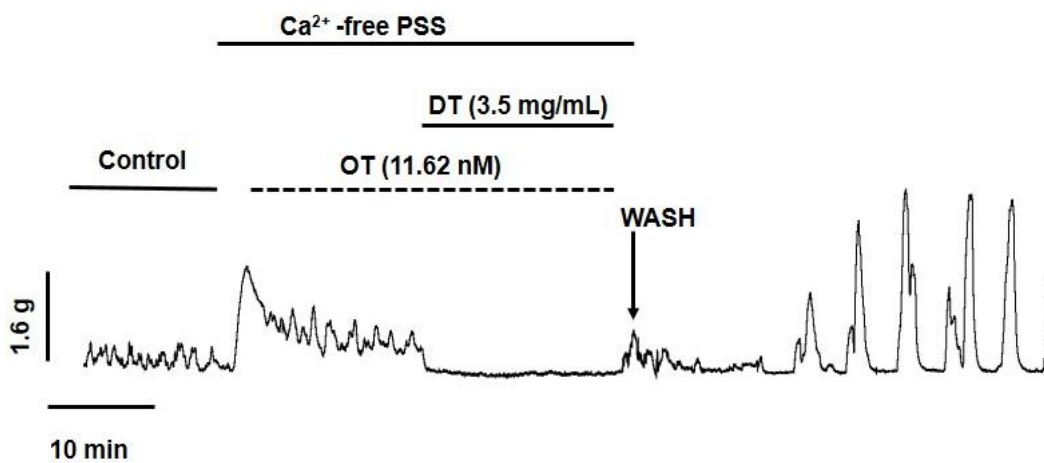


B

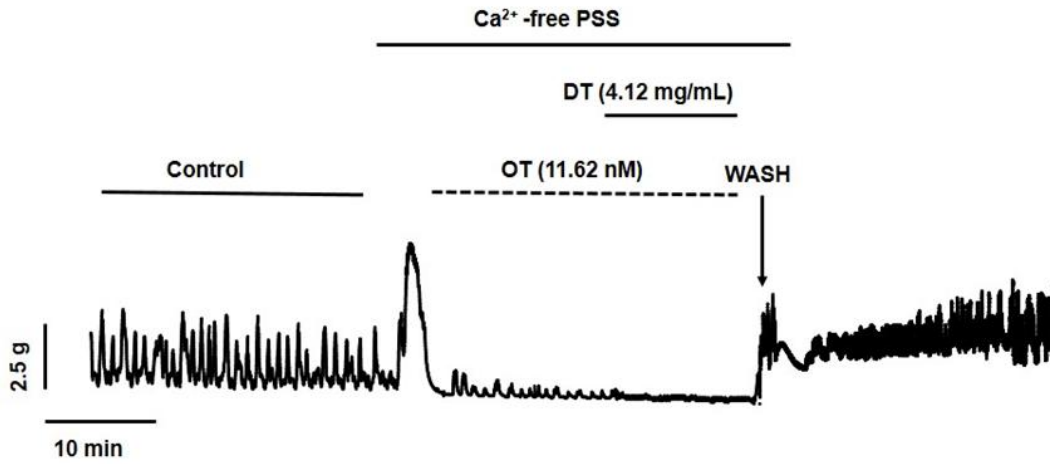


**Figure 3b.** Bar charts displaying the effects of DT extract on the amplitude (A) of high KCl (80 mM)-induced uterine contractions of isolated non-pregnant (A), and pregnant (B) mouse uterus. DT, *Dennettia tripetala*; KCl, potassium chloride; \* $p < 0.05$ ;  $n = 5$  animals.

A

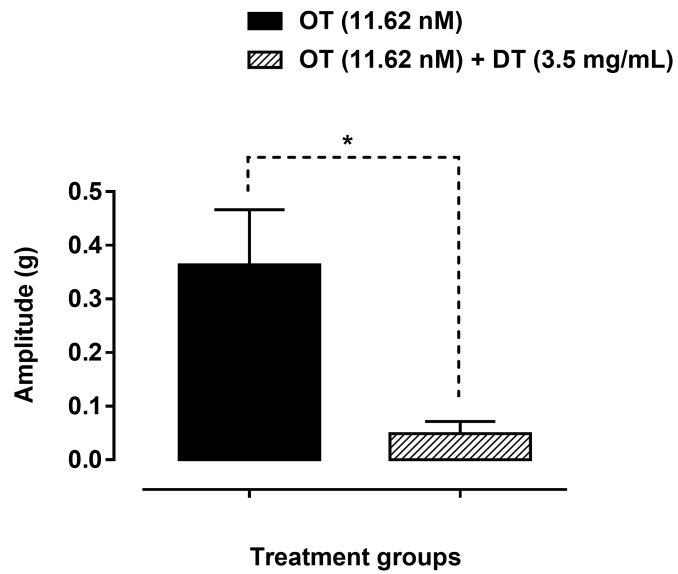


B

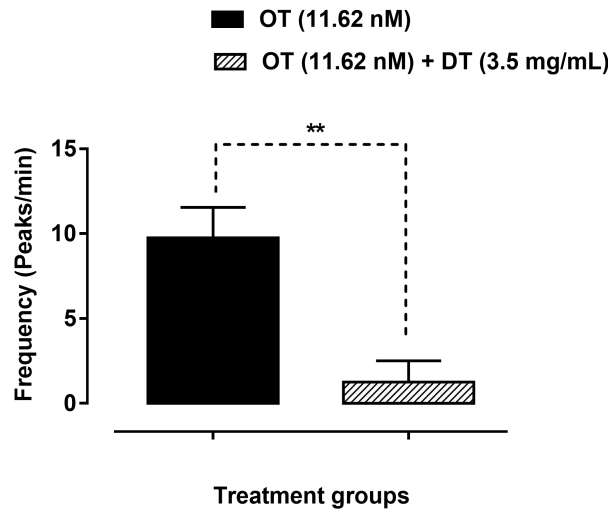


**Figure 4a.** Representative traces showing the effect of DT (3.5 mg/mL) and (4.12 mg/mL) on oxytocin-induced uterine contraction of the isolated mouse non-pregnant uterus (A) and pregnant uterus respectively in the absence of extracellular  $Ca^{2+}$  (free  $Ca^{2+}$  medium). DT, *Dennettia tripetala*; OT, oxytocin.

A

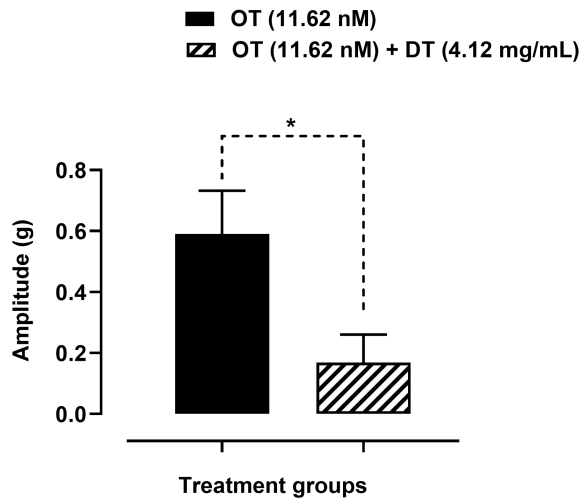


B

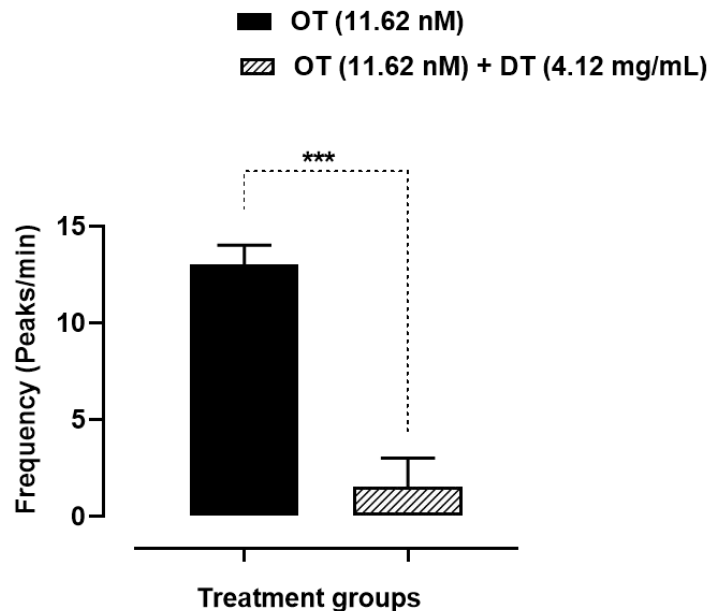


**Figure 4b.** Bar charts displaying the effects of DT extract on the amplitude (A) and frequency (B) of oxytocin-induced uterine contractions of isolated non-pregnant mouse uterus in the absence extracellular  $Ca^{2+}$ . DT, *Dennettia tripetala*; OT, oxytocin; \* $p < 0.05$ , \*\* $p < 0.01$ ; n= 5 animals.

A



B



**Figure 4c.** Bar charts showing the effects of DT extract on the amplitude (A) and frequency (B) of oxytocin-induced uterine contractions of isolated pregnant mouse uterus in the absence extracellular  $\text{Ca}^{2+}$ . DT, *Dennettia tripetala*; OT, oxytocin; \* $p < 0.05$ , \*\*\* $p < 0.001$ ;  $n = 5$  animals.

### Discussion

Uterine contraction is usually present in viable uterus, although there are variations in its frequency, amplitude, duration and direction of propagation of the contractions. These contractions are intended to achieve different functions such as expulsion of menstrual flow and transportation of sperm to the ovary in non-pregnant state; preterm labour and parturition in gravid uterus; and postpartum uterine contraction after delivery (Akerlung, 1998; Aguilar and Mitchell, 2010). Uterine contraction may therefore lead to undesirable complications if inappropriate for the given physiological states. Understanding these changing role will help the pharmacist in developing and improving drugs with different pharmacological roles in the uterus. The uterus is a hollow organ with inner layer of endometrium, middle layer of myometrium and outer layer of serosa. The myometrium is pivotal to uterine contractility which depends

on the concentration of the cytosolic ionized calcium  $\text{Ca}^{2+}$ . Calcium ion is mobilized from intracellular stores (the sarcoplasmic reticulum) and extracellular store through the L-type voltage operated  $\text{Ca}^{2+}$  channels (VOCC). The electrophysiology of uterine contraction is a complex mechanisms which is controlled by action potential, followed by entry of  $\text{Ca}^{2+}$  into the cytosol of myocytes. The cytosolic  $\text{Ca}^{2+}$  binds to a protein calmodulin (Cal) to form a complex, calcium-calmodulin, which leads to activation of myosin light chain kinase (MLCK). The activated MLCK enzyme increase the phosphorylation of the myosin light chain, which then initiate myosin cross-bridge to interact with actin filament, leading to contraction (McEnvoy and Sabir, 2022). To the best of our knowledge, this is the first scientific study to elucidate the effect of DT seed extract on pregnant and non-pregnant mouse uterus. In the present study, the results

indicate that DT seed extract inhibited spontaneous uterine contractions in a concentration-dependent manner. The amplitude and frequency of the spontaneous uterine contractility of pregnant and non-pregnant uterus were significantly decreased. This pharmacological effect may have potential benefit in its use as a tocolytic agent and this calls for further investigations. Its role in post-partum uterine contraction has been challenged by this study since it causes relaxation, rather than contraction, and therefore calls for further studies.

Spontaneous uterine contractions are primarily driven by the extracellular  $\text{Ca}^{2+}$  entry into the cytosol of myocytes through the L-type VOCC. In this study, the presence of high concentration of KCl (80 mM) in the medium caused depolarization of the myocyte membrane, activation of VOCC, and influx of  $\text{Ca}^{2+}$  into the myometrial cell, culminating into contraction of the uterus (Alotaibi, 2020). It therefore suggests that DT seed extract-induced uterine relaxant effect could be related to inhibition of influx of extracellular  $\text{Ca}^{2+}$ .

Furthermore, the study also demonstrated that DT inhibited oxytocin-induced uterine contractions in both pregnant and non-pregnant mouse uterus. This findings obviously indicate that the DT inhibitory effect on uterine contraction is associated with the extracellular and/or intracellular  $\text{Ca}^{2+}$  influx. The mechanistic pathway by which oxytocin induces uterine contractions include the depolarization of the myometrial membrane and the opening of VOCC, which allows the extracellular  $\text{Ca}^{2+}$  entry; and the activation of oxytocin receptors (belonging to a G-protein couple receptor) and subsequent activation of phospholipase C (PLC), leading to increased generation of inositol 1,4,5-triphosphate ( $\text{IP}_3$ ), and subsequently, increased  $\text{Ca}^{2+}$  released from the  $\text{Ca}^{2+}$  stores ((Vrachnis *et al.*, 2011; Alotaibi *et al.*, 2020). Therefore, the inhibitory effect of DT seed

extract on oxytocin-induced contraction in the  $\text{Ca}^{2+}$ -deficient medium in both non-pregnant and pregnant mouse uterus suggested the possible interaction of DT seed extract and mobilization of intracellular  $\text{Ca}^{2+}$  pathway.

### Conclusion

This study has provided scientific evidence that *Dennettia tripetala* (pepper fruit) seed extract inhibits uterine contractions in the pregnant and non-pregnant mouse uterus probably through the blockade of extracellular  $\text{Ca}^{2+}$  entry and intracellular  $\text{Ca}^{2+}$  released. Therefore, the inhibitory effect of *Dennettia tripetala* (pepper fruits) seed extract on uterine contractility in pregnant mouse uterus refute the earlier claim that it aids postpartum contraction. The study however underscores its potential benefits in the management of dysmenorrhea in non-pregnant uterus, and preterm labour in pregnant uterus.

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### Authors' contributions

U.A.P.: conceptualization, performed the experiment, analysis, and writing. E.E.B.: supervision and interpretation of the data.

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### Conflicts of interest

The authors declare no conflict of interest

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