EVALUATION OF *In Vitro* TOCOLYTIC EFFECTS OF FRUIT EXTRACT OF *Dennettia tripetala* (pepperfruit) IN MOUSE

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A PROJECT WORK SUBMITTED TO THE DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF THE DOCTOR OF PHARMACY (PHARM.D) DEGREE

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CERTIFICATION

This is to certify that Ogbebor John Timothy with matriculation number PHA1305058, a student of Faculty of Pharmacy, Department of Pharmacy, University of Benin has successfully completed his project work under close supervision.

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_____ _____

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(Head of Department)

DEDICATION

I wish to dedicate this work to God almighty who has been my source of strength and wisdom, my parent, Mr and Mrs. Ogbebor who are my pioneers, my supervisor, Pharm. Mrs. Uchendu, who is my source of inspiration and motivation.

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I express profound gratitude to God for granting me the grace and to conduct this research.

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ABSTRACT

It has been reported (Umoh 1998), that the peppery fruits of Dennettia tripetala usually find application in food meant for pregnant women. Moreover, Dennettiatripetala seeds are very important in the diets of women after childbirth, during which time it is claimed that spices and herbs aid the contraction of the uterus (Achinewhel et al., 1995).

The uterine activities of this important fruit have not been fully documented. Little is known of the uterine activity of Dennettiatripetalafruit despite its widespread multipurpose uses as food and drugs. The present study was designed to determine the uterine activity of Dennettiatripetala fruit and, consequently.The cumulative concentrations (0.01-12.21 mg/ml) of the methanolic extract of DT was tested on rhythmic spontaneous uterine contraction, while the most potent concentration 3.5 mg/kg of DT extract was tested on oxytocin (OT), high potassium chloride (KCl)-induced uterine contractions and OT-induced uterine contractions in a calcium-deprived state. DT extract produced significant ($P<0.05$) decrease in the frequency and amplitude of spontaneous contractions with IC_{50} of 0.99 ± 0.06 and 0.83 ± 0.40 mg/ml respectively. As well as OT (11.62 nM)-induced frequency of myometrial contractions and OT (11.62 nM)-induced contractions in calcium deprived state containing ethylenediaminetetraacetic acid (EDTA), and significant changes on high KCl (80 mM)-induced myometrial contractions. These observations may explain the effect of DT and its folkloric use in the food of pregnancy women, however further studies are advised to know the direct mechanism of action and to characterize and isolate specific bio-constituents responsible for the observed effects.

CHAPTER ONE

1.1 INTRODUCTION

Plants are integral part of nature and have been used for health and medical purposes for several thousands of years. Plants have an almost endless variety of metabolites, which is very useful to human being (Surash*et al,* 2011). Plant kingdom is a treasure house of potential drugs and in recent years, there have been an increased awareness of the importance of medicinal plants. Drugs from these plants are usually less expensive, available, safe, and efficient and rarely have side effects (Thite*et al* 2013). The number of higher plant species on earth is about 250,000 and it is estimated that over 35,000 to 70,000 species have medicinal purposes (FAO, 2015).

A majority of the world population in developing countries still relies on herbal medicine to meet its health needs. Herbal medicine are usually used to provide first- line and basic health services both to people living in remote areas where it is their only available health services, and to people living in poor areas where it offers only the affordable remedy. Even in areas where modern medicines are available, the interest in herbal medicine and their utilization have been increasing rapidly in recent years.

Medicinal plants are important sources for pharmaceutical manufacturing, and they account for a significant percentage of the pharmaceutical market. Hence, finding new secondary metabolite is a prerequisite for the development of novel pharmaceuticals. This thematic series on the biosynthesis and function of secondary metabolites deals with the discovery of new biologically active compounds from all kinds of sources, including plants (Dickshat*et al,* 2011). According to

(Owuanibe, 1979), it was concluded that the various bioactive chemicals present in divers plants make them treat or alleviate one disease or illness or the other.

1. cardiovascular disease: e.g Hypertension, stroke, etc., for which the antihypertensive herbs, the African Rauwolfia and Negro coffee have been used,
2. Disease of the nervous system:e.g Convulsions, insomnia etc. for which the parrot's beak and the African Rauwolfia also offer a good remedy.
3. Disease of the alimentary system:e.g Diarrhoea, dysentery etc. for which basil is useful.
4. Disease of the endocrine system:e.g Diabetes etc. for which the leaves of the common Roused periwinkle or mormodica are valuable.
5. Disease of the respiratory system :e.g asthma, cough etc. for which the lemon grass is of value.
6. Disease of the genito urinary system: e.g gonorrhoea, haematuria, etc. for which the bush banana is useful.
7. Disease caused by microbes, viruses, insects etc: e.g infections, malaria etc. for which garlic, clove, the African mahogany etc. was found useful (Owanibe, 1979).

The WHO notes however, that "in appropriate use of traditional medicines or practice can have negative or dangerous effects" and that "further research is needed to ascertain the efficacy and safety of several of the practices and medicinal plants used by traditional systems".

The use of plants to facilitate birth or to protect the young embryo appears to be a common practice among traditional healers. Over the years, traditional cultures have relied on the beneficial effects of herbal remedies during pregnancy, birth and postpartum care. The

knowledge and correct use of these natural medicines, have been acquired and improved over many generations. It is estimated that 85% of the population in developing countries depend mainly on traditional healthcare system (centre for gender and social policy studies, 2012) and factors such as accessibility, availability, affordability and inherent trust in this method have necessitated their frequent use (Ogbe *et al*, 2009). It has been known that during pregnancy and child birth, traditional medicine relies on the use of certain herbs for their beneficial effects to tone the uterus muscle, induce labour, in the removal of retained placenta and management of postpartum bleeding (Gruber and O'Brien, 2011).

Dennettiatripetala, often referred to as DT, is also known as pepperfruit. It is widely grown in the rain forest zones of Nigeria and some parts of West Africa. DT is commonly consumed for its spicy taste. It is known in Nigeria by the following names: Ako (Edo), Mmimi (Ibo), AtaIgbere (Yoruba). It is also used in traditional medicine as a remedy for cough, fever, toothache, diarrhea, diabetes, and nausea in pregnant women. It grows as a small woody shrub. The tree can grow to a height of 12–15 m and have a girth of 0.6 m. The wood is white in color and The fruits are green when developing but start to turn red with ripening. The moisture content also increases with ripening. The fruits possess a very strong characteristic smell. The leaves are 3–6 inches long and 1.5–2.5 inches broad. They are elliptic in shape. The fruits are mainly made up of the seeds and a bit of hard, spicy flesh. The fruit and seeds are edible and are consumed because of the spicy nature (Ejechi and Akpomedaye 2005; Enwere1998 ;Okwu and Morah 2004).

It has been reported (Umoh 1998), that the peppery fruits of *Dennettiatripetala* usually find application in food meant for pregnant women. Moreover, *Dennettiatripetala* seeds are very

important in the diets of women after childbirth, during which time it is claimed that spices and herbs aid the contraction of the uterus (Achinewhel *et al*., 1995).

The uterine activities of this important fruit have not been fully documented. Little is known of the uterine activity of *Dennettiatripetala*fruit despite its widespread multipurpose uses as food and drugs. The present study was designed to determine the uterine activity of *Dennettiatripetala* fruit and, consequently, assess its potential usefulness as a pharmaceutical raw material in the formulation of drugs.

Aim of Research

To investigate the activity of pepperfruit (*Dennettiatripetala*), on uterine contractility of isolated non-pregnant mice uteri.

Specific objectives of proposed project include

1. To determine the direct effect of pepperfruit (*Dennettiatripetala*) on spontaneous and oxytocin-induced uterine contraction of non-pregnant mice uteri.
2. To evaluate the effect of pepperfruit (*Dennettiatripetala*) on high potassium chloride (KCl)-induced uterine contractility in non-pregnant uteri.
3. To evaluate the effect of pepperfruit (*Dennettiatripetala*) on uterine contractility in Ca^{2+} free medium of non-pregnant uteri.

LITERATURE REVIEW

Dennettiatripetala(commonly known as Pepperfruit) is widely consumed by the inhabitants of West Africa due to its distinctive spicy taste. It is also used traditionally as a remedy for cough, fever, toothache, diabetes, and nausea. The highly nutritious fruit is rich in protein, carbohydrates, as well as the antioxidant vitamins A, C, and E. The plant possesses phytochemicals that have been shown to elicit antimicrobial, insecticidal, analgesic, and anti-inflammatory properties. The plant has also been shown to possess chemotherapeutic, antihyperglycemic, and antioxidant properties. In addition, *D. tripetala*finds application in food preservation and seasoning (Sylvia, 2015).

1.1 Description

*Dennettiatripetala*G. Baker (Annonaceae) also known as pepperfruit tree is a woody plant of at least 3meters height with simple leaves and abundant fruits widely consumed in Southern Nigeria. It is found in the tropical rainforest region of Nigeria and sometimes in Savanaareas (Okwu *et al*., 2005). It is locally called "Nkarika" by the Efiks of Calabar,"Nmimi" by the Igbos and "Igbere" by the Yorubas. The young leaves and fruits have instinctive spicy taste (Achinewhu *et al*., 1995). The mature fruits constitute the main edible portions. Some communities in parts of Southern Nigeria also utilized the leaves and roots, in addition to the fruits for medicinal purpose (Iwu, 1989).

They are elliptic in shape. The fruits are mainly made up of the seeds and a bit of hard, spicy flesh. The fruit and seeds are edible and are consumed because of the spicy nature. The wood is used as fuel. The plant usually produces fruit between the months of March and May. For this reason, local traders preserve the seeds of pepperfruit by drying it under the sun in order to

ensure continuous availability until the next harvest (Ejechi and Akpomedaye 2005; Enwere1998 ;Okwu and Morah 2004).

Figure 1: showing Ripe (red) and unripe (green) *Dennettiatripetala*fruits (Sylvia, 2015).

*Dennettiatripetala*is used as masticators, which when chewed produces unique peppery effect (Keay, 1989). The peppery spicy taste of mature *D. tripetala* fruits usually serves as a mild stimulant to the consumer. The fruits are sometimes taken with kolanut, garden egg and palm wine in parts of Nigeria, especially in Southern part of Nigeria where it serves also for cultural entertainment of guests, particularly during coronation, new yam festivals, weddings and marriage festivals (Enwere, 1998; Keay, 1989). *D. tripetala*fruit has also been reported to be used as spice in flavouring food, and as seasoning which are added to prepared food such as meat, sausages, soups and vegetable (Lebouef and Caver, 1972). The peppery fruits of *D. tripetala*are applied to the food meant for pregnant women and are important in the diets of postpartum

women, during which time it is claimed that spices and herbs aid uterine contraction (Okwu and Morah, 2004; Achinewhu *et al*., 1995). Okwu *et al*. (2005) also reported that *D. tripetala* fruits contain important nutritive substances such as vitamins, minerals and fibre. However, there is limited information on the antioxidant activity of *D. tripetala*fruits as a dietary source of antioxidant.

1.2 Classification of *Dennettiatripetala* (pepperfruit)

*Dennettiatripetala*is classified as follows:

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnolidae

Order: Magnoliales

Family: Annonaceae

Genus: Denettia

Species: *Dennettiatripetala* (Sylvia, 2015).

1.3 Nutritive and chemical composition of pepper fruit

Dennettia tripetala fruit is a good source of dietary nutrients. It has high protein content (15.31%). Plant protein may be consumed as the raw plant or cooked (Enwere 1998). The fruit is not only rich in protein but also in calories. *Dennettia tripetala*has food energy of 480.24 g calories. This high-energy value might be due to high oil content. The fruit contains both

essential oils and volatile oils (Osisiogu 1975). The essential oil contains fragrant, aromatic and pungent principles. The volatile oils are responsible for the aroma and taste of *D. tripetala*fruit (Enwere 1998). This prompted its inclusion in food as a spice and flavouring agent for meal preparations, soups, sauces and canned foods (Enwere 1998). The total carbohydrates available in the fruit are high (62%). They comprise sugars such as glucose, sucrose and fructose, hemicellulose and pectin, which act as dietary fibre, add bulk to the diet and may, sometimes, act as a mild natural laxative for human beings (Enwere 1998). *D. tripetala*fruit contains crude fibre (9.8%). Most ripe fruits contain no starch while some unripe fruits may contain a reasonable amount of starch. The fruits therefore are regarded as healthy foods and their consumption is beneficial in many ways.

The hydrogen cyanide level in the fruit was low (0.02 mg·kg–1). The level of the cyanogenicglucosides is so low that man can consume this fruit raw without it being deleterious to health. The moisture content of *D. tripetala*fruits is high (8.00%). This characteristic is a function of quality and this definitely determines how fresh fruit was at harvest or how long it has been stored before analysis (Umoh 1998). Potassium and calcium of *D. tripetala*fruits are very high [(2.48 and 1.80)%, respectively]. The sodium content is fair (0.72%). Zinc, copper, manganese, cobalt, nickel and cadmium are available in trace quantities. The zinc content could mean that it can play a valuable role in the management of diabetes, which results from insulin malfunctioning. Zinc is essential for the production of insulin, a hormone and carbonic anhydrase, an enzyme in the body (Okaka and aokaka 2001). The iron level is high in the fruit (17.75 mg·kg–1). This element is known to be important in the human body because it is a component of haemoglobin. It helps oxygen transport and, together with haemoglobin and ferrodixin, it plays a vital role in man's metabolism. Chromium was not detected. *D. tripetala*

fruit contains several nutrients and biologically active components that prolong and enhance life. It is a good source of ascorbic acid, riboflavin, thiamine andniacin. Therefore, this fruit is nutritionally necessary for a well-balanced diet because it contributes important vitamins such as vitamin C (ascorbic acid) which can be used for the treatment of the common cold and the control of other diseases such as prostrate cancer (Okogun 2002).

Twenty-five compounds were identified in the *n*-hexane seed extract, including linoleic acid ethyl ester, caryophyllene, 3-carene, phenyl ethyl alcohol, and cubebene. Phytochemical screening of the ethanolic extract revealed the presence of tannins, alkaloids, steroids, flavonoids, cardiac glycosides, saponins, and terpenoids (Elekwa*et al*., 2011).These constituents provide a scientific basis for the use of DT in traditional medicine. Saponins, tannins, and flavonoids, for instance, are effective against diabetes. They also possess antimicrobial and anti-inflammatory properties (Sparg*et al*., 2004; Reihemann*et al*., 1999). Cardiac glycosides can be used in the treatment of asthma (Trease and Evans 1989). Alpha-linoleic acid has been shown to reduce the risk of cardiovascular

1.4 Medical uses of *D. tripetala*fruit

Antimicrobial Properties of Dennettiatripetala

Researchers at Delta state University, Abraka, have found that the essential oil and phenolic acid extract of DT can inhibit the growth of food-borne microorganisms such as *Staphyloccocusaureus*, *Salmonella* sp., *Escherichia coli*, and a host of others (Ejechi and Akpomedaye 2005). This points to a role for pepperfruit in the preservation of food substances such as meat which is prone to rapid decomposition in places without constant electricity. More recently, the leaves of DT were found to be effective in inhibiting the growth of the rot-causing

fungus *Sclerotiumrolfsii*in cocoyam both *in vitro* and *in vivo* (Nwachukwu and Osuji 2008). Several other reports show the antimicrobial activity of DT (Anyaede and Amusan 2003).

1.4.1 Analgesic and Anti-Inflammatory Effects of Dennettiatripetala

The essential oil of DT fruits has been found to possess analgesic effects as great as that induced by the powerful opioid morphine as well as aspirin and indomethacin. This oil also relieved inflammation in rodents with edema to levels comparable with that of dexamethasone (Oyemitan*et al*., 2008). The mechanism by which DT exhibits its analgesic effects was inferred by the fact that Naloxone, which inhibits the analgesic effect of morphine, was also able to inhibit that of DT. This result backs up the use of DT in pain and fever in folk medicine.

Other Effects of Dennettiatripetala on the Nervous System

More recently, researchers have discovered a component of the essential oil of the fruits, leaves, and seeds of DT which is largely responsible for the observed neuropharmacological effects of the oil. This compound, 1-nitro-2-phenyl ethane, exhibits hypnotic, anticonvulsant, and anxiolytic effects in mice (Oyemitan*et al*., 2008).

1.4.2 Antihyperglycemic Effect of Dennettiatripetala

Recent research has provided evidence and a preliminary mechanism for the antihyperglycemic effect of the ethyl acetate extract of DT. Anaga and Asuzu in 2010 (Anaga and Asuzu 2010) showed that DT can reduce the plasma glucose level in drug-induced hyperglycemic rats to levels comparable with that of normal rats. This effect was found to be more pronounced than that of Tolbutamide. Using 3T3-L1 adipocytes and brefeldin, these same researchers investigated the possible mechanism for this observed phenomenonin DT. It was found that DT exerts this

effect partly by recruiting glucose uptake proteins from the interior of the cell to the plasma membrane (Anaga and Asuzu 2011).

1.4.3 Antioxidant Effect of Dennettiatripetala

In living organisms, reactive oxygen species (ROS) are generated as a part of metabolism. These ROS are usually hindered from causing oxidative damage to cellular constituents by antioxidants present in the organism. Some of these antioxidants are produced in the body in the form of antioxidant enzymes, while others have to be consumed from plants in the form of antioxidant nutrients (Oboh and Akindahunsi 2004).

Preliminary phytochemical analysis has revealed the presence of antioxidants such as flavonoids and ascorbic acid in DT (Ihemeje*et al*., 2006). Recently, a group of researchers at the Federal University of Technology, Akure, evaluated the changes in antioxidant content and potentials of fresh DT fruits with ripening. Using the aqueous extract, they found that the phenol content increased with ripening, while the ascorbic acid and flavonoid content did not change (Adedayo and Oboh 2010). Intriguingly, their results showed that the aqueous extract of unripe DT possesses greater antioxidant ability compared to ripe DT as typified by higher reducing power, greater ability to scavenge ABTS, DPPH, and OH, as well as higher Fe reducing and chelating potential (Adedayo and Oboh 2010). They therefore concluded that, as DT undergoes ripening, its total phenol content increases, but its antioxidant potentials decreases. In 2011, a different group of researchers isolated two flavonoid glycosides from the ethyl acetate fraction of a 20% aqueous methanol extract of DT leaves which were found to instantly bleach the purple color of DPPH, indicating free radical scavenging potential (Aderogba*et al*., 2011). In 2014, researchers at the University of Benin evaluated the antioxidant activity of the roots of DT. They discovered

that the ethanolic extract of DT roots exhibits the ability to reduce ferric ion in a concentration-dependent manner (Okolie*et al*., 2014). The extract also possesses a H2O2-scavenging ability similar to that of ascorbic acid. Furthermore, the extract inhibits lipid peroxidation in frozen animal samples to an extent comparable with that of vitamins C and E [20]. They therefore concluded that DT may be useful in the preservation of frozen meat.

Other *in vitro* antioxidant studies have been done using the methanol extract of DT leaves and results show that DT possesses strong antioxidant potentials *in vitro* (Odoh*et al*., 2014).

From the preceding paragraphs, it is obvious that a lot of investigations have been carried out on various parts of DT using a wide range of solvents, but to the best of my knowledge, there has been no attempt to confirm the antioxidative potentials of this plant *in vivo*.

Toxicity of Dennettiatripetala

A number of studies have been carried out to ascertain the toxicity of DT. Although DT has been reported to contain uvariopsin, an alkaloid which improves bile secretion and attenuates hepatic disorders (Ofem*et al*., 2014; Lopez-martin *et al*., 2002), a study by Ofem and colleagues (Ofem*et al*., 2014) showed that the ethanolic extract of DT fruits administered at a certain dose reduces bile production in normal healthy rats. The extract also caused an increase in sodium, potassium, and bicarbonate ions in bile and reduced the chloride and unconjugated bilirubin content of bile (Ofem*et al*., 2014).

The effect of the ethanolic extract of DT on hematological parameters in normal healthy rats has also been investigated by Ikpi and Nku(Ikpi and Nku 2008). Firstly, they carried out an acute toxicity test to determine theLD50 of DT and a moderately high value of 251.19 g/kg·bw was gotten when the ethanolic extract of DT was administered intraperitoneally to normal healthy mice. Subsequently, they administered DT in normal saline orally to normal healthy rats and

observed that, at low to moderate dose, DT may be hematotoxic to rats. Interestingly, the observed toxicity seemed to be relieved when the dose of DT administered was increased (Ikpi and Nku 2008).

The toxic effects of the ethyl acetate root extract of DT have also been studied. An LD50 value of1120mg/kg was gotten from the intraperitoneal administration of the extract (Anaga*et al*., 2006). Although, the extract exhibited mild toxicity on the liver, kidney, spleen, and blood cells, it was seemingly beneficial to the hearts of mice following prolonged exposure (Anaga*et al*., 2006).

The hexanolic extract of DT fruits has been found to be toxic to the larvae of the *Aedesaegypti*mosquito and this points to the potential for generating insecticides from *Dennettia*essential oil (Anyaede and Amusan 2003).

1.4.4 Effect of Dennettiatripetala on Healthy Humans

The seeds of DT have been found to be effective in reducing the intraocular pressure of normotensive emmetropic humans (Timothy and Okere 2008). This suggests that DT could be put to use in the possible prevention and management of glaucoma.

1.4.5 Effect of Dennettiatripetala on Cancer

A recent report from the University of Illinois at Chicago showed that DT extract inhibits the growth of prostate cancer cells (Jagla 2013). In the study, the ethanolic extract of DT seeds was tested for its efficacy on prostate cancer cell lines PC3 and LNCaP. The extract of DT was found to possess growth-inhibitory and cytotoxic effects on the prostate cancer cell lines *in vitro* (Jagla 2013).

1.5 THE UTERUS

The uterus (from the Latin "uterus", plural) is the main female reproductive hormone organ of most mammals, including humans. One end of the cervix opens into the vagina, while the other end is connected to one or both fallopian tubes, depending on the type. The uterus, also known as womb, is a hollow muscular organ that is responsible for the development of the embryo and fetus during pregnancy. This is an extraordinary distillation that can grow to the size of a closed fist during pregnancy to become large enough to hold a full baby. It is also a very strong organ that can contract to force the baby out during labor (Innerbody 2015).

The uterus is an important part of the female reproductive tract. Here the embryo is implanted. The fetuscontinues to grow, develop and differentiate in a beneficial uterine environment and use an abundant supply of maternal blood until it is able to survive in the external environment. At this stage of pregnancy, the uterus goes through labor, i.e Delivery the product of conception. Therefore, contractility of the uterus plays a key role, so that it can fulfill multiple functions. The uterus is a relatively relaxed organ during pregnancy when it performs the developing fetal harvest function. Instead, it develops into a very active and reactive state during birth, characterized by strong, rhythmic, and synchronous contractility.

It is believed that this change in uterine contractility is caused by complex interactions between a number of systems and events.

1.6 ANATOMICAL STRUCTURE OF THE UTERUS

The uterus can be anatomically divided into three segments: cervix, body and fundus. The narrow lower region, called the cervix, connects the uterus to the vagina underneath and acts as a sphincter muscle to control the flow of material inside and outside the uterus. The body (or body)

of the uterus is a wider area of the uterus that is superior to the cervix. The body is much thicker than the cervix because it protects and supports the developing fetus and contains the muscles that push the fetus out of the uterus. The fallopian tubes extend laterally from the angle of the fundus (Romer *et al*., 1977).

1.6.1 Layer

Three separate layers of tissue form the uterine wall

1.6.2 **ENDOMETRIUM**

The lining of the uterine cavity is called "endometrium". It consists of the functional endometrium and the main endometrium from which the first originated. Damage to the basal endometrium causes adhesion and / or fibrosis (Asherman's syndrome). In all placental mammals, including humans, the endometrium periodically forms a mucous membrane that peels or is absorbed when pregnancy does not occur. The functional function of the endometrial mucosa is responsible for menstrual bleeding (colloquially referred to as "period" in humans with a cycle of about 28 days ± 7 days of flow and ± 21 days in the process) during the woman's fertile years and sometimes outside. (Blackburn *et al*., 2011).

Depending on the nature and characteristics of physical and physiological health, body weight, environmental factors, daily rhythm, photoperiodism (physiological response of the body to day and night), the effect of the menstrual cycle on the reproductive function of uterine hormone production, cell regeneration, and other biological activities. Menstrual cycles can vary from a few days to six months, but can vary in the same person and are often interrupted for several cycles before recovery.

1.6.3 **MYOMERIUM**

The uterus is composed mostly of smooth muscle known as "myometrium". The deepest layer of the myometrium is known as the thickened junction area with adenomyosis. This middle layer accounts for the majority of uterine volume and is a muscle layer consisting mostly of smooth muscle cells.

1.6.4 **PERIMETRIM**

The outer lining of the uterus, serous or perimeter, i. Thin tissue layer of epithelial cells that surround the uterus.

1.7 FUNCTION OF THE UTERUS

The uterus consists of the body and cervix. The cervix sticks out into the vagina. The uterus is held in place by condensation of endopelvic fascia, referred to as ligation, in the pelvis (Blackburn *et al*., 2011). These compounds include the cervical, transverse, or macenrodhalous ligaments, or cardinal ligaments and uterine sacral ligaments. Covered with peritoneal, broadband and uterine folds, which are important for sexual reactions by directing blood flow to the pelvis and external genitalia, including the ovaries, vagina, labia, and clitoris.

The reproductive function of the uterus is to take a fertilized egg that passes through the fallopian tubes from the fallopian tubes. It is implanted in the lining of the uterus and receives food from blood vessels that develop exclusively for this purpose. The fertilized egg becomes an embryo, binds to the uterine wall, forms the placenta, and develops into a fetus (pregnancy) until birth.

1.8 THE PHYSIOLOGY AND PHARMACOLOGICAL RESPONDS OF THE UTERUS

The physiological and pharmacological responses of the uterus vary at various stages of the menstrual cycle, and during pregnancy (Rang and Deles, 2007) physiological processes at birth (ie, childbirth, childbirth, and childbirth) require complex interactions of hormonal actions, nerve activity and contraction of the uterine smooth muscle. During the first two trimesters of pregnancy, the uterus remains silent and shows little or no myometrial contraction. This inactivity is largely due to the inhibitory effect of high levels of circulating progesterone on the uterine muscles. In the first trimester, smooth uterine muscles become restless, so that mild muscle contractions are seen (Braxton-Hicks contractions); From time to time, they increase in both strength and frequency, and can even be seen as a sign of the onset of labor, a phenomenon known as false labor. In part, labor requires the integration of processes that involve dilation of the cervical canal and contraction of uterine smooth muscle that is strong enough to expel the fetus (Charles and Robert 2007). The muscles of the uterus rhythmically contract both in vivo and in vitro, with contractions originating from the muscles themselves. Myometrial cells in the fundus act as pacemakers and produce action potentials. The electrophysiological activity of the pacemaker is regulated by sex hormones (Rang and Deles, 2017).

The non-pregnant uterus contracts spontaneously, but every week, during the first part of the cycle, and strengthens during the luteal phase and during menstruation. Uterine movement is suppressed during pregnancy, because estrogen which is potentiated by progesterone increases myometrial cells. It suppresses spontaneous contractions. However, at the end of pregnancy, contractions begin; They become stronger and more frequent and fully coordinated during labor. Nerve delivery to the uterus including the sympathetic component and inhibitory excitation; Adrenaline acts on β2 adrenergic receptors inhibiting uterine contractions, while norepinephrine

acts on β-adrenoceptors and stimulates contractions (Rang and Deles, 2007). Pregnancy and childbirth are natural events, and both, as with most bodily functions, are the main mechanisms for controlling the pregnant uterus. They are used to stimulate the uterus (uterotonic or oceanic) in three main clinical scenarios:

1. Perform uterine activity to induce labor or abortion.
2. Increase progressive work that is slow and
3. To stimulate placental birth and prevent postpartum hemorrhage.

They are used to release the uterus (tocolytics) in threatening or opening premature births, to prevent or delay preterm birth. Because of its occurrence and severe side effects besides death and neonatal morbidity, premature birth is the most widely studied in both tocolytics and oxytocytics, and is therefore very important for managing various pregnancies and preventing related diseases. Both of these classes of drugs target the pathway that triggers and triggers uterine contractions.

1.9 ANATOMICAL BASIS OF UTERINE CONTRACTION

The uterine wall consists of three layers. The innermost layer, the endometrium, breaks down the organ lumen and consists of columnar epithelium and underlying stromal tissue. The middle layer, myometrium, which consists mainly of smooth muscle cells, also contains blood and lymphatic vessels, nerves, immune cells, and connective tissue. The outer layer, serosa, is a thin layer that covers most of the uterus and consists of mesothelioma cells (Venu*et al.*, 2015).

1.91 Uterine smooth muscle

Individual smooth muscle cells are physiological units of uterine contractions. They are small cells in the form of spindles with a length of 50 to 800 mm and a width of 2 to 10 mm. An increase in pregnancy is associated with uterine smooth muscle hypertrophy and hyperplasia. The increase in size can reach three to five times until the end of pregnancy. Structurally, the smooth muscle of the uterus is very similar to other types of smooth visceral muscles. Each cell is bound to the plasma membrane and contains an elongated central nucleus and various cytoplasmic organelles (Figure I). The smooth structure of smooth muscle cells is similar to other cell types except for two important modifications: contractile apparatus and sarcoplasmic reticulum (Venu*et al.*, 2015).

1.9.2 Contractile apparatus

Contractions in smooth muscle cells are achieved by interaction of myofilaments: thick filaments and thin filaments. Thick filaments consist of myosin, while thin filaments consist mainly of actin but also contain other proteins such as tropomyosin, caldesmon and filamine. The arrangement of myofilaments is random; As a result, smooth muscle development becomes slow, unlike jagged muscle cells (skeletal or heart), where strength is quickly generated in a collection of myofilaments that are spaced regularly. Thick filaments form bridges with thin filaments, which in turn are attached to solid objects and form longitudinal or skewed fibers in smooth muscle cells. The solid body contains α-actinin, a protein that binds to vinculin, which in turn binds to actin in thin filaments (Venu *et al.*, 2015). At the ends and sides of muscle cells, thin filaments are inserted into the dense cytoplasmic spots formed by the adherence of the dense body to the plasma membrane. Cytoplasm also contains intermediate filaments consisting of desmin. They should form a cytoskeleton that supports contractile devices.

1.9.3 Sarcoplasmic reticulum

Sarcoplasmic reticulum is a network of tubules and sacs in the cytoplasm of smooth muscle cells. It functions as a calcium reserve which plays an important role in muscle contraction. However, unlike striped muscles, the sarcoplasmic smooth muscle reticulum does not develop properly.

1.9.4 Cell to cell contact

Neighbouring cells in muscle bonds are tightly localized in certain specific regions of their plasma membrane and form cells from cells. With the exception of this specific area, smooth muscle cells in muscle bonds are separated by a distance of = 50-100 nm. These contacts, which are functionally important for the control of smooth muscle contractility, are called gap junctions.

Cross-gap is a modification of the opposite plasma membrane from adjacent cells that functions to bind it electrically and metabolically. They are present in all types of networks. Under the electron microscope they appear in the adjacent area between cells as a paired area with a parallel membrane with extraordinarily fine lines separated by a short distance of about 2 or 3 nm (gap). Each connection node consists of several thousand channels and each channel consists of two half-symmetric channels, i. Connections, each in two opposite cell membranes. Six connexin proteins form compounds, with the main connexin in the uterus being connexin 43. The channels formed by connexin allow molecular diffusion of less than = 1kDa. In this way, inorganic ions and small molecules can easily move from one cell to another (Venu *et al.,* 2015).

1.10 MECHANISM OF UTERINE CONTRACTION

The smooth uterine muscle is spontaneously active like other types of smooth visceral muscle. The uterus is composed of billions of smooth muscle cells. During pregnancy, the contractile activity of these cells is poorly coordinated, resulting in ineffective uterine contractions and, thus,

relative weakening of the uterus (Venu *et al*., 2015). However, during birth, increased bond between cells and cells leads to the formation of functional syncytium. As a result, uterine contractions are well synchronized and can effectively move the product from conception. The sequence of events that causes uterine contractions is explained in detail.

1.10.1 Spontaneous Electrical Activity ofUterine Smooth Muscle

Contractile activity in the uterus is a direct result of electrical activity in uterine smooth muscle cells. During reduction, the smooth muscle cell plasma membrane maintains a difference of -40 to -70 mV potential, which is internally negative (resting membrane potential). Decreased potential across this membrane is the result of different permeability from plasma membranes to ions, especially potassium (Kf), sodium (Na ') and calcium (calf). Kf is present in higher intracellular concentrations, while Naf and Calf are present in higher extracellular concentrations. Usually, membranes are more permeable to K^+, which moves down the electrochemical gradient, i. from intracellular to extracellular space, creating negative potential in cells.

The smooth uterine muscle shows spontaneous electrical activity characterized by cyclic depolarization and repolarization of the plasma membrane and the possible effects indicated. The electrical activity in the longitudinal muscle is in the form of potential action potential for spikes. Circular muscles show during pregnancy the potential for individual action in the highlands, which turns into an action potential similar to longitudinal muscles during birth. The action potential is caused by changes in voltage and time on membrane permeability for different ions (Figure 2). Membrane depolarization is mainly causedby increased permeability of Ca^{2+} and to a lesser extent Na^+. Both ions have higher concentrations in the extracellular space and therefore move intracellularly, which makes the membrane potential more positive. The membrane is repolarized by increasing the permeability to K^+. The resulting external K^+ motion consists of

components that are voltage dependent (fast) and components activated by Ca^{2+} (slow). It can be observed that the movement of only a few ions through the plasma membrane is sufficient to change the membrane potential without significant changes in intracellular / extracellular ion concentration (Venu *et al*., 2015).

2.5.1 Excitation-Contraction Coupling

Increased intracellular Ca^{2+} forms the basis for binding contraction-excitation in all types of contractile cells, including smooth muscle cells. At rest, intracellular Ca^{2+} in smooth muscle cells is below 10^{-7}M. Contraction is associated with an increase in intracellular Ca^{2+} more than 10^{-7}M. The main source of this increase in intracellular Ca^{2+} is extracellular space, which has a concentration of Ca^{2+} 10^{-3}M. This enormous chemical gradient is maintained by the low permeability of the plasma membrane to Ca^{2+} and by an efficient mechanism for sequencing Ca^{2+} in cells. Smooth muscle contraction is preceded by an increase in free intracellular Ca^{2+} levels. Ca^{2+} combined with cytoplasmic protein that binds to cytoplasm. Ca^{2+}calmodulin forms an active complex with myosin light chain kinase (MLCK), which phosphorylates the myosin light chain. Myosin Mg^{2+} -ATPase can now hydrolyze ATP. As a result, a cross bridge between myosin and actin moves, causing thin filaments to slide along the narrow filament and contract smooth muscle cells. Calmodulin is perhaps the most important CaL^{+} receptor for contractile proteins, but there is evidence that Ca^{2+} can also directly activate MLCK. In addition, MLCK can be phosphorylated by various protein kinases such as cyclic adenosine monophosphate (CAMP) and cyclic guanosin monophosphate (Venu *et al*., 2015).

1.11 Significance of uterine contraction

The smooth uterine muscle has a phase pattern that alternates between contractile models and maintenance of the test tone with discrete periodic contractions of various frequencies, amplitudes, and durations (Aguilarb *et al*., 2010).

The uterus, which is not pregnant, contracts spontaneously but weekly in the first part of the cycle, and strengthens during the luteal phase and during menstruation (Rang and Dele 2007). However, in early pregnancy, spontaneous contractions are reduced, and hence uterine movement is disrupted due to the fact thatestrogen-potentiated progesterone hyperpolarizes myometrial cells.

Oxytocin causes regularly coordinated contractions that move from the fundus to the cervix. Both the amplitude and frequency of contraction depend on the dose. During low doses, the uterus is completely relaxed between contractions. Higher doses increase the frequency of contractions and there is incomplete relaxation between them. However, higher doses produce permanent contractions that inhibit blood flow through the placenta and cause fetal distress or death (Rang and Dele 2007).

Uterine relaxants that inhibit spontaneous or oxytocin-induced contractions in selective patients, to prevent preterm birth that occur in other pregnancies without complications between 22 and 33 weeks' gestation.

Myometrial activity in pregnant women is indispensable for expulsion of endometrium and spilled blood during menstruation, as well as for the transport of sperm to the fallopian tubes or oocytes of newborns for implantation (Akerlund 1998).

Proper uterine contractions can ensure the transportation of gametes / embryos through the uterine tube and the success of embryo implantation. In spontaneous or assisted reproduction,

inadequate uterine contractility can cause ectopic pregnancy, dysmenorrhea, and endometriosis (Bulletti and Deziegher 2005).

AIM AND OBJECTIVES

The aim of this study was to investigate the effects of *Dennettiatripetala* fruit/seed extract on uterine contractility in non-pregnant mouse model.

The following were the objectives of the study;

i. To investigate the effect of the DT fruit/seed extract on spontaneous uterine contractility.
ii. To investigate the effect of the DT fruit/seed extract on oxytocin-induced uterine contractility.
iii. To examine the effect of the DT fruit/seed extract on high KCl-induced uterine contractility.
iv. To determine the effect of the DT fruit/seed extract on oxytocin-induced contraction in calcium-free medium.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Plant materials and extraction

Fresh unripe fruits of *D. tripetala* were purchased in July, 2019 from a market in Benin City, Ovia North-East Local government area, Benin City Edo State Nigeria. Botanical identification was done by Dr H.A Akinibosun from the Department of Plant Biology Biotechnology, Faculty of Life Science, University of Benin, Edo State Nigeria.

The fruits were sliced and air-dried under shade for 3 weeks. Dried fruits were ground to fine powder. The powdered material (1200 g) was subjected to cold maceration extraction by immersing it in 7.5 L of 99.8% methanol for 72 hours with frequent shaking. The extract was filtered using a funnel and a filter cloth, the filtrate was further filtered using cotton wool to remove any particle in the filtrate. The filtrate was then concentrated to dryness by freezer drying. The yield was 13.8%w/w. The dried extract was preserved in an air-tight container and stored in a refrigerator at 4^{o}C until required.

2.2 Animals

Mature non-pregnant female Swiss albino mice weighing between 22.0 – 28.0 g were used to carry out all the experiments. Animals were procured from and maintained in the Animal house in the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Edo state, Nigeria. They were housed in plastic cages with bedding materials made of wood shavings. Animals were allowed to acclimatization for two weeks before the commencement of the study. They were maintained on dry rodent pellet feeds (Top feeds limited, Ibadan, Nigeria) and water (borehole). All studies were carried out following the standard protocols for the use of laboratory animals (National Institute of Health,2002).

2.3 Drugs and chemicals

Methanol of high analytical grade (Pharmatrends, Nigeria) and tween 80 (Kernel-KN, China) were utilized in this study. Physiologicalsolution salts were obtained from Guangdong GuanghuaSci-Tech Co. Ltd China,Lobacheme PVT Ltd, India, and Sigma Aldrich, UK). Other drug used in this study oxytocin (Roche pharmaceutical Ltd, UK

2.4 Experimental protocols on the mouse uterus

2.4.1 Uterine tissue preparation

On the day of the experiment estrus was confirmed by visual assessment of the vulva and by microscopic assessment of vaginal cells. Prior to the experiment proper, vaginal lavage was performed by flushing with normal saline using a Pasteur pipette (0.1 mm in diameter) (McLean *et al*., 2012). The collected cells were placed on clear glass slides. The slides were dried, fixed with cold methanol and subsequently stained with methylene blue (0.01%). The cells were then viewed under a microscope using X10 objective lens (Visiscope® VWR, UK). Estrus stage was confirmed by the dominant presence of cornified epithelial cells (McLean *et al*., 2012). On validation of the estrus stage, the mice were then utilized for *ex vivo* uterine contractility assay.

Non-pregnant mice were humanely killed by cervical dislocation and the uterine horns were immediately removed and placed into a petri dish containing previously warmed and aerated physiological salt solution. Connective and adhering tissues were removed from the isolated uterus and one horn was dissected in half to obtain a uterine horn segment of approximately 1-2 mm in length. The uterine segment obtained was mounted in a warmed organ bath (10 mL) maintained at 37^{o}C and containing aerated physiological solution. The

physiological salt solution used was of the following composition in mM/L: NaCl 154.00, $NaHCO_3$ 5.95, D-glucose 2.78, KCl 5.63, and $CaCl_2 \cdot 2H_2O$ 2.05 (Bafor *et al*., 2015).

Uterine tissue strips were mounted vertically in tissue organ baths (10 mL) and activity recorded using isometric force transducers (PanlabADInstruments, Spain) connected to bridge amplifiers which in turn was connected to a PowerLab data acquisition system consisting of a recording unit (Powerlab 2/26 Model ML826 ADInstruments, Australia) coupled to a LabChart software (ADInstruments). The tissue was equilibrated under resting tensions of 4.90 mN for 30-45 min or until regular contractions were obtained (Bafor *et al*., 2019; Sukwan *et al*., 2014). The amplitude and frequency of uterine contractions were recorded and measured.

2.4.2. Experiment on spontaneous uterine contractility

Spontaneous uterine contractions were allowed to reach equilibrium in the physiological salt solution for at least 30 min. The rhythmic contractions were observed for 10 min and was used as the control value (100%).The concentration-response effects of the DT on spontaneous contractions were tested by increasing concentrations of the extract in a cumulative manner (0.01–12.21 mg/mL). Each concentration was allowed acontact time of 5min (Bafor*et al*., 2017a).

2.4.3. Experiment on oxytocin-induced uterine contraction

To determine the effects of extract on oxytocin (OT) induced uterine contraction, the uterine strips were stimulated by oxytocin (11.62 nM) for10 min. Then, DT (3.5 mg/mL) was added to the strips in the continued presence of oxytocin. A contact time of 5 min was allowed for the extract concentration. The bathing solutionwas replaced with the physiological saline solution andthe recovery monitored.

2.4.4. Experiment on high KCl-induced uterine contractility

To determine the effect of DT on high KCl-induced uterine contraction, the strips were stimulated by high KCl (80 mM) for 10 min. DT (3.5 mg/ml) was added to the strips in the continued presence of high KCl. At the end of the experiment, the bathing solution was replaced with the physiological saline solution and recovery was monitored.

2.4.5. Experiment on the effect of the extract in Ca2+ - free medium

To determine the effects of the extract on intracellular Ca release, Ca-free physiological saline solution was used and 0.1 mMEthylenediaminetetraacetic acid (EGTA) was added.After the equilibrium period of spontaneous contractions for 30 min, the physiological saline solution was replaced with Ca-free solution containing (0.1 mM EDTA). In the continued presence of Ca-free solution,oxytocin (11.62 nM) was added to the organ bath and 5 min contact time was allowed. Without washing the uterine strips, DT (3.5 mg/mL) was added and a contact time of 5 min was allowed for the extract.

2.5 Data analysis

All data were analyzed using the GraphPad Prism, (version 6.01; GraphPad software Inc, San Diego, CA, USA). All data are expressed as mean ± standard error (SEM) where “n” represents the number of samples from different animals or experiments as indicated. Significance was evaluated using appropriate t-tests, P values ≤ 0.05 was considered statistically significant in all cases.

Contractions occurring at the last 5 or 10 min of the phasic contractions were used to calculate the mean frequency and amplitude. The results were expressed as percentages of control applications (control = 100%).

In data sets with sufficient data points, mean log concentration-response curves were analyzed by fitting data to a four-parameter logistic equation, using non-linear regression with GraphPad Prism 6.0 (GraphPad software, San Diego, CA, USA) using the following equation values (Y = Bottom) (1 + 10^(LogE/IC_{50}-X)*HillSlope). Where Y = response which starts at the bottom and goes to the top in sigmoid shape, X = logarithm of concentration and EC_{50} or IC_{50} is the concentration that produces half the maximal responses.

CHAPTER THREE

3.0 RESULTS

3.1 Concentration-response effects of the DT on the parameters of spontaneous contractions

Cumulative applications of the DT extract significantly decreased spontaneous contractions in a concentration-dependent manner (Fig 3.1A). At each concentration (0.01 – 12.21 mg/mL), the amplitude (Fig 3.1B) and frequency (Fig 3.1C) of spontaneous contractions were gradually reduced until contractions were completely inhibited. The IC50 of DT on amplitude was 0.83 ± 0.40 mg/mL, while frequency was 0.99 ± 0.06 mg/mL.

3.2 Effects of DT extract on oxytocin-induced uterine contraction

The effects of DT extract on contraction induced by oxytocin (11.62 nM) were investigated. As expected the contractile response of the uterine stripswere augmented by oxytocin (Fig 2A). In the presence of the DT extract, a significant reduction ($p<0.001$) in the frequency occurred, and insignificant decrease in the amplitude. This is shown in Fig 2C and Fig 2B respectively.

3.3 Effects of DT extract on high KCl-depolarization

The effect of the extract on KCl-induced contractions are shown in Fig 3A and 3B. The application of KCl produced the expected rapid increase in force of contraction. The introduction of DT in the presence of KCl (80 mM) significantly diminished $p<0.01$ the force of contraction produced by high KCl.

3.4 Effects of DT extract on oxytocin-induced contraction in calcium-free medium

The effects of the extract on the release of Ca^{2+} fromintracellular stores were also examined. In Ca-free(EGTA) solution spontaneous contractions were abolished,as L-type Ca2+ channel entry of Ca2+ is absent (Fig 4A). DT produced a significant reduction in the amplitude ($p<0.05$) and

frequency ($p<0.01$) of OT-inducedcontractions in the presence of zero calcium as shown in Fig 4B and 4C.

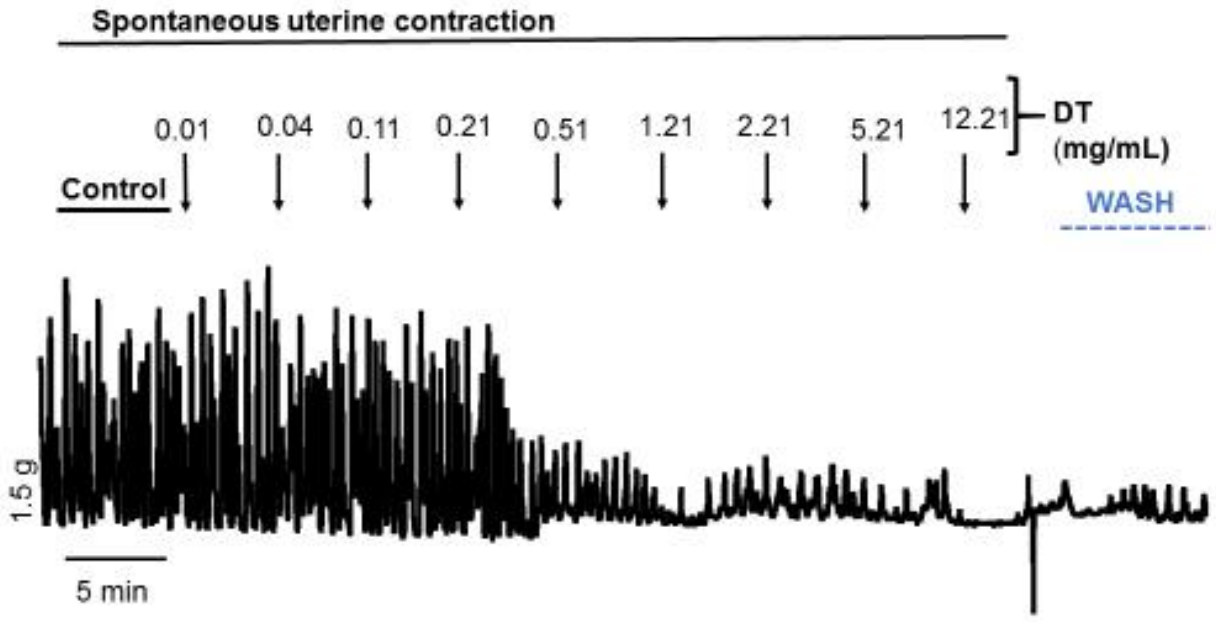

Figure 3.1A. Representative recording showing cumulative effect of DTon the isolated non-pregnant mouse uterus. DT= *DennettiaTripetala.*

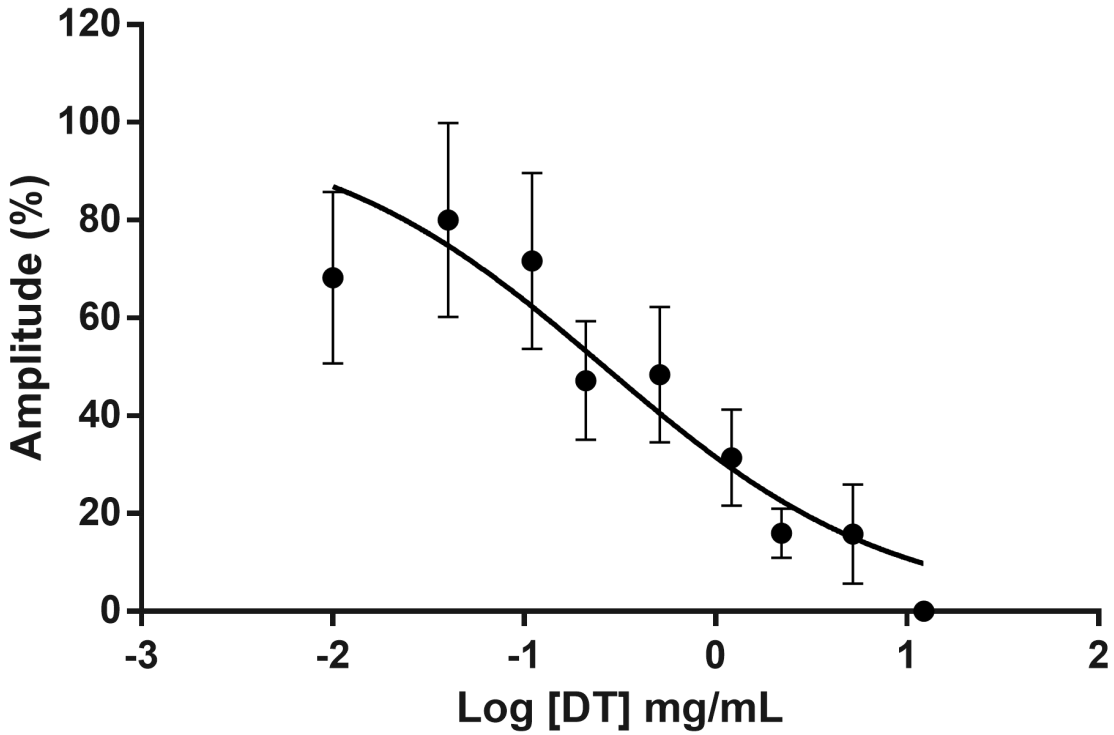

Fig. 3.1B. Effect of DT on spontaneous contractions in the non-pregnant uterus, Concentration-response curve showing the effect of DT on frequency. n=5 animals. A gradual decrease in the amplitude of spontaneous uterine contraction with complete inhibition at higher concentration was observed.

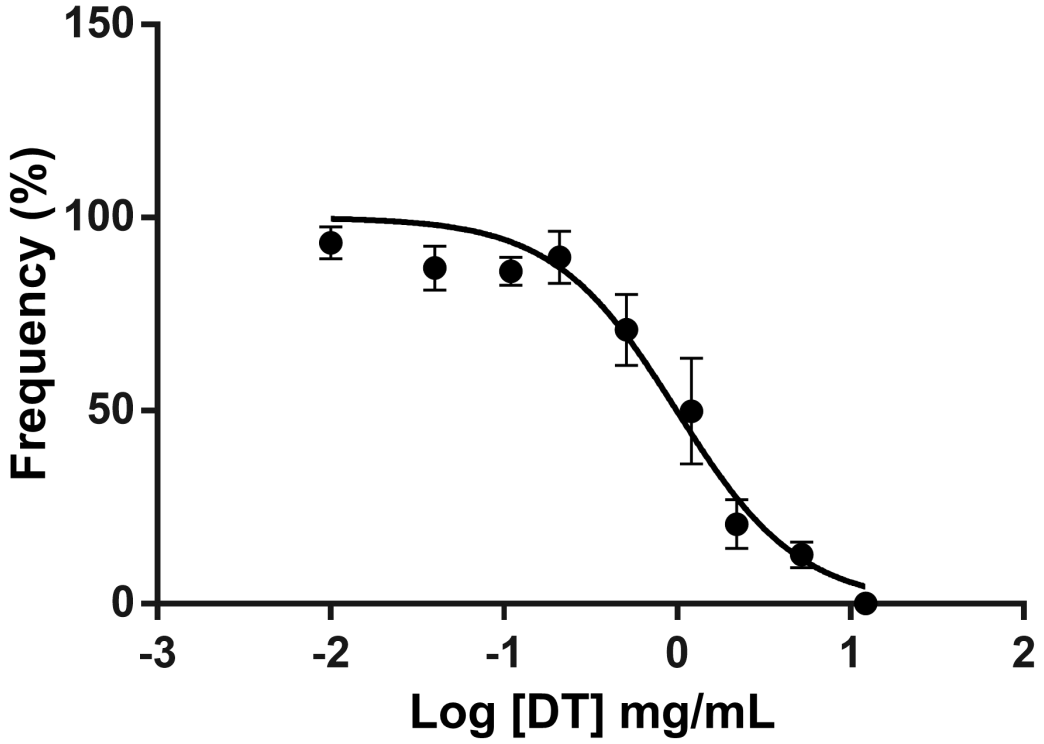

Fig. 3.1C. Effect of DT on spontaneous contractions in the non-pregnant uterus, Concentration-response curve showing the effect of DT on frequency. A progressive reduction of the frequency of spontaneous contractions was observed. n=5 animals.

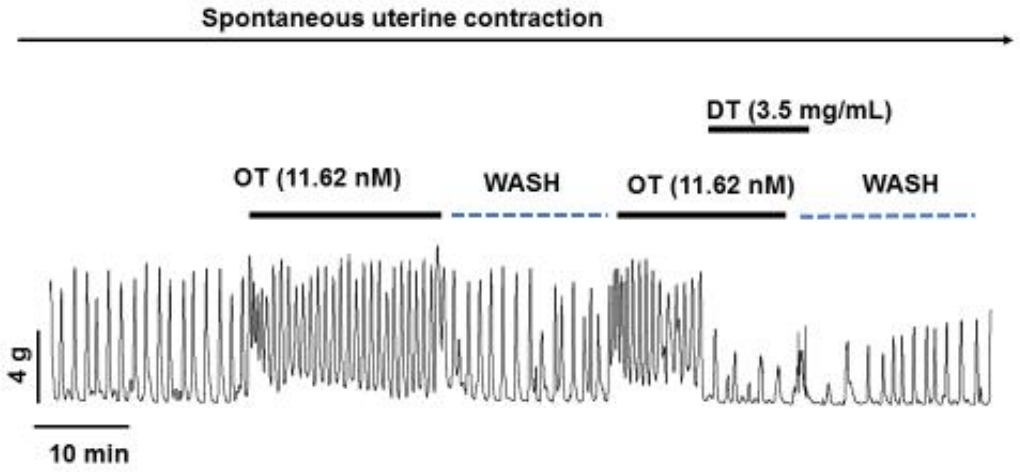

Fig 3.2A Representative recording showing the effect of DT (3.5 mg/mL) on spontaneous uterine contraction in the isolated non-pregnant uterus, DT = *Dennettiatripetala.*

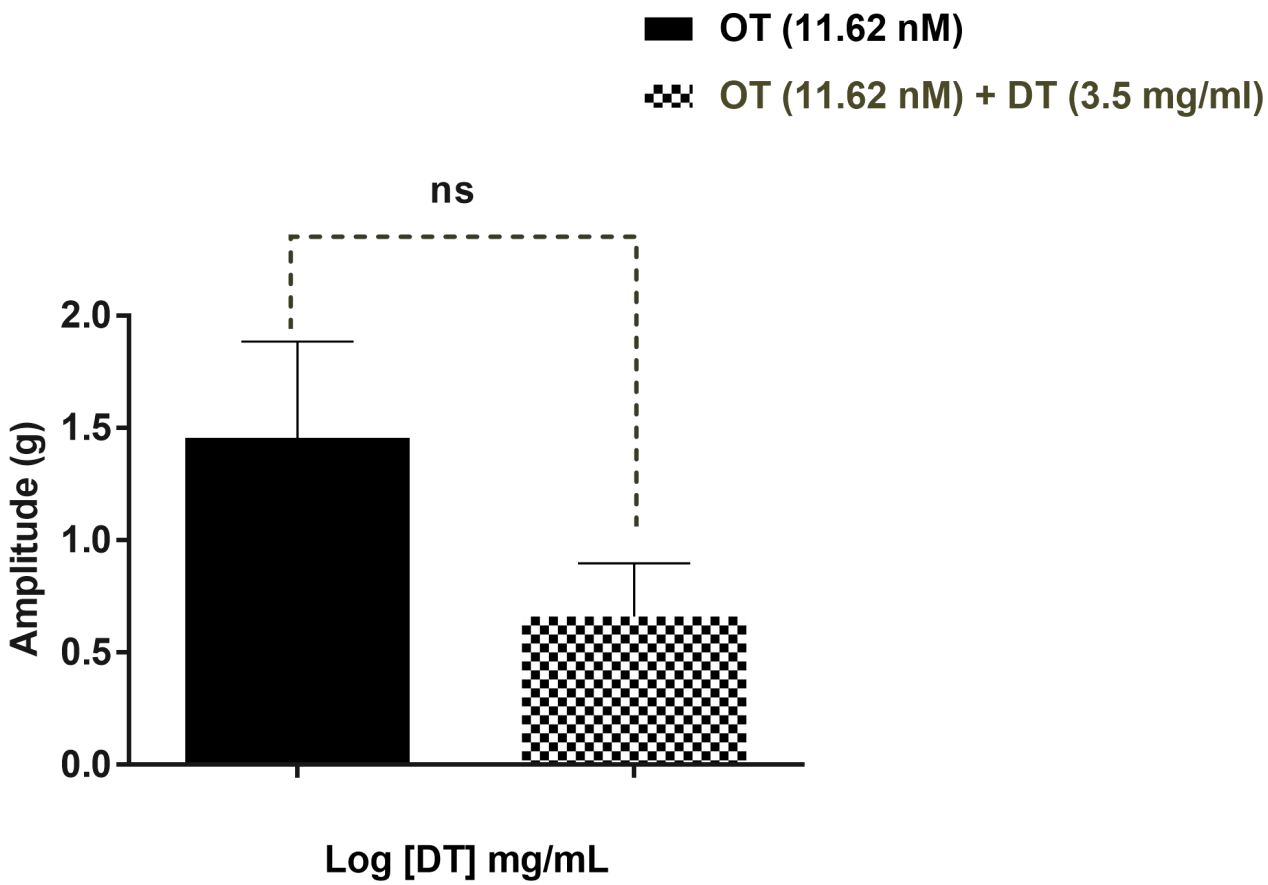

Fig. 3.2B. Effect of DT on oxytocin-induced contractions in the non- pregnant uterus.

Bar charts showing the effects DT on amplitude of oxytocin-induced contractions. The extract was observed to produce an insignificant change in the amplitude of oxytocin-induced contractions. n = 5 animals; OT = oxytocin.

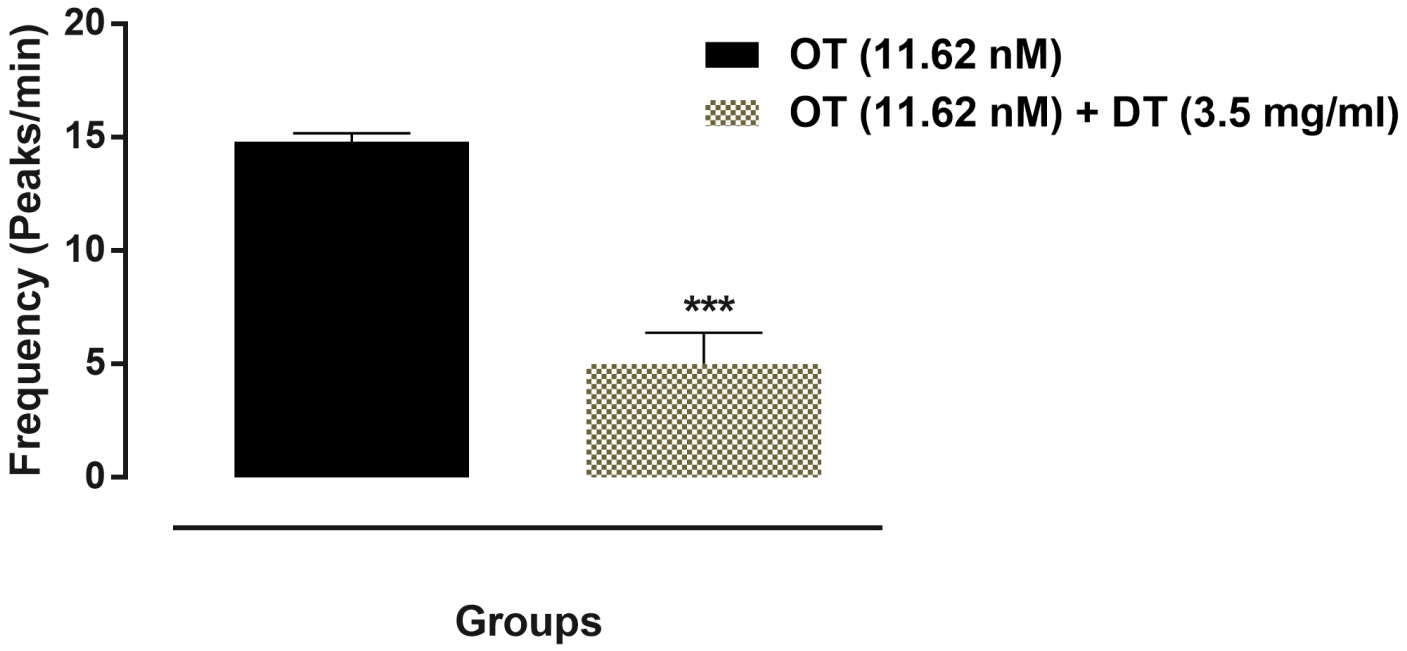

Fig. 3.2C Effect of DT on oxytocin-induced contractions in the non- pregnant uterus.

Bar charts showing the effects DT on amplitude of oxytocin-induced contractions. n = 5 animals; ***$p < 0.001$ compared to oxytocin alone; OT = oxytocin.

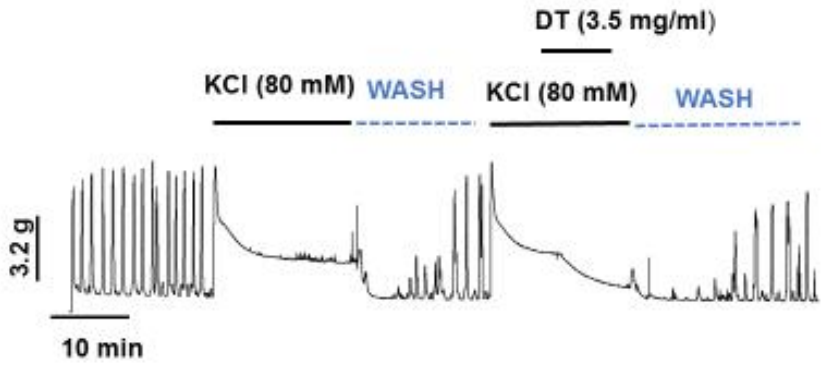

Fig 3.3A Representative recording showing the effect of DT on KCl-induced contractions of the non-pregnant uterus. DT= *Dennettiatripetala*

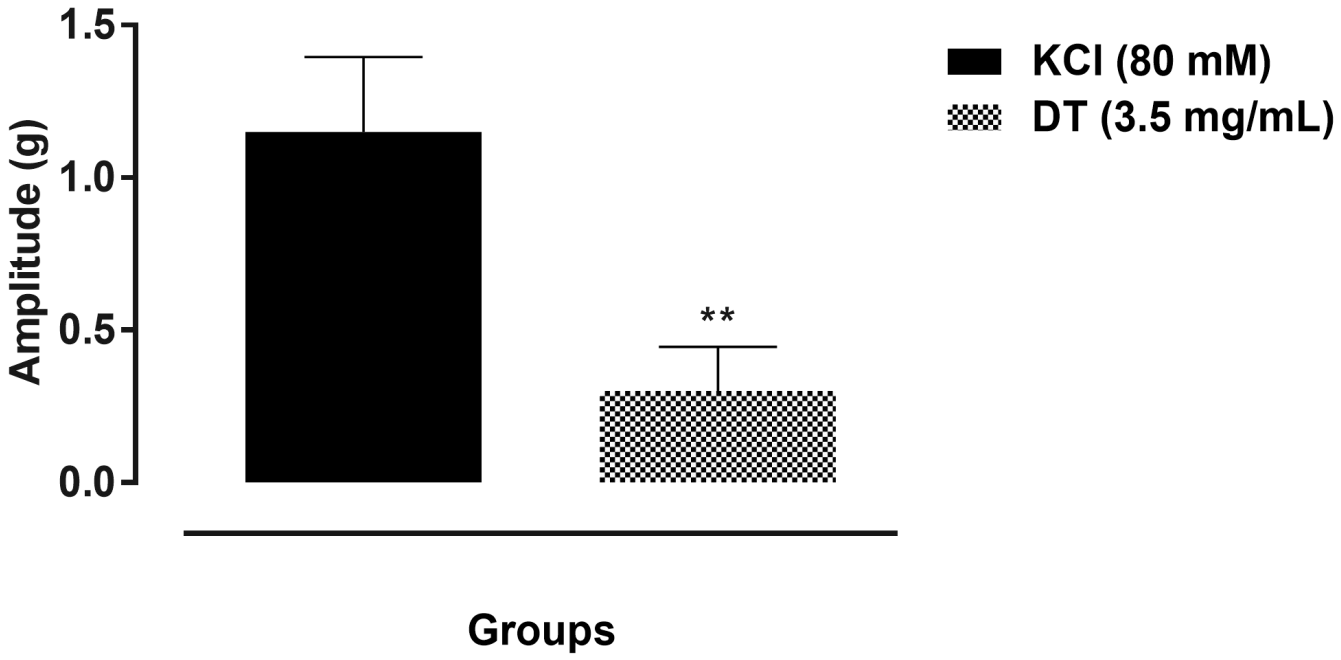

Fig.3B Effect of DT on high KCl-induced contractions (80mM) in the non-pregnant uterus. Bar charts showing the inhibitory effect of DT on amplitude KCl-induced contraction. n = 5 animals; **p < 0.01 compared to KCl alone; KCl = potassium chloride.

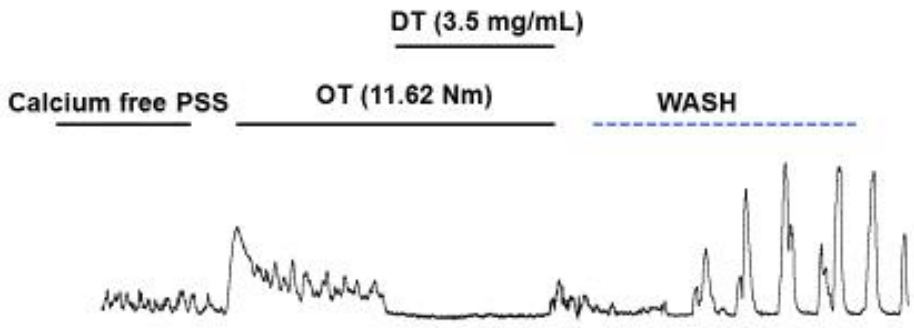

Fig 3.4A Representative recording showing the effect of DT on OT-inducedcontractions in Ca^{2+}-free medium. DT= *Dennettiatripetala*

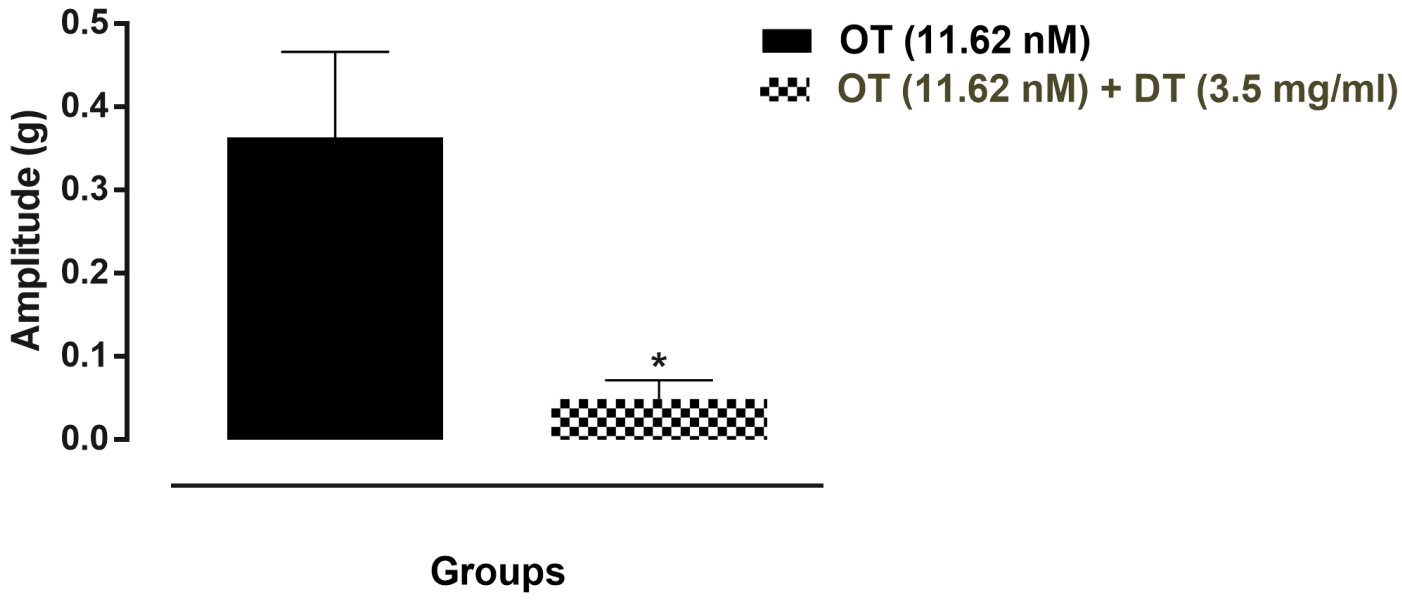

Fig. 3.4B Effect of DT on oxytocin-induced contractions in Ca^{2+} free medium in the non-pregnant uterus. Bar charts showing significant decrease in the amplitude of the uterine contractions in the presence of zero Ca^{2+} medium. n = 5 animals; *p < 0.05 compared to OT alone; OT = oxytocin.

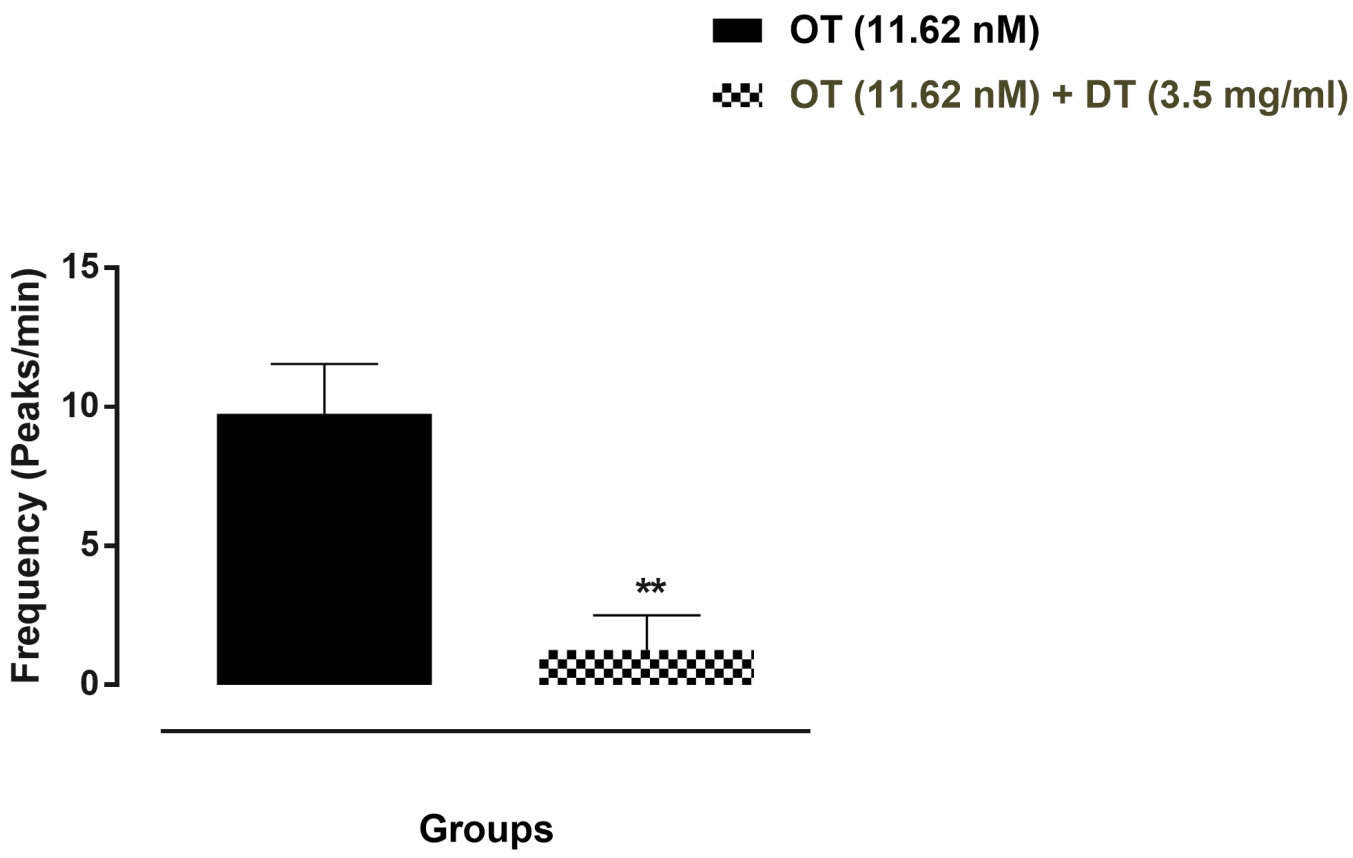

Fig. 3.4C Effect of DT on oxytocin-induced contractions in Ca^{2+} free medium in the non-pregnant uterus. Bar charts showing significant decrease in the frequency of the uterine contractions in the presence of zero Ca^{2+} medium. n = 5 animals; **p < 0.01 compared to OT alone; OT = oxytocin.

DISCUSSION OF RESULT

4.1 Discussion

The myometrium of the uterus contracts constantly in both the pregnant and non-pregnant states in all mammals (Aguilar and Mitchelle, 2010). The non-pregnant spontaneous uterus contractions help to allow sperm pass through the cervix (Pehlivanoğlu *et al*., 2013) and also plays an important role in sloughing of endometrium (Wray and Noble, 2008).

The cumulative concentration of DT extract from 0.01 to 12.21 mg/ml successfully decrease ($p < 0.05$) the amplitude (Force) of uterine contraction in a concentration-dependent manner with maximal effect observed at the highest concentration. However, a high significant decrease was observed on frequency of spontaneous uterine contraction compared to baseline (control) value, The extract, at cumulative concentrations from 0.01 to 12.21 mg/ml, also caused a significant decrease ($p< 0.05$) on the frequency of spontaneous uterine contraction in a concentration-dependent manner with maximal effect observed at the highest concentration. The reduction of amplitude and frequency of spontaneous contractions of the pregnant uterus by the extract, suggests the possible interaction with myosin light chain phosphorylation by possible inhibition of MLCK as well as inhibition of extracellular voltage-gated calcium channels or decrease in intracellular calcium stores, resulting in the depletion of calcium levels in the cell. High calcium levels are required for spontaneous uterine contractions (Pehlivanoglu*et al.,* 2013).

DT significantly inhibits the amplitude of spontaneous uterine contraction at low concentration more than its frequency. The greater effect of EM on one parameter of spontaneous contractions than the other is suggestive that there might also be involvement of myometrial gap junctions, which regulate the frequency, and amplitude of the contraction (Mackler *et al.*, 1999; Garfield *et al*., 1980).

It was observe that the extract produce a highly significant inhibitory effect on the amplitude and a relatively low inhibitory effect on frequency at low concentration on the spontaneous contraction as shown in figure 3.1B and C. A probable reason for the difference in effect on the amplitude and frequency at low dose, is due to the effect of the extract on endogenous pacemakers' cells which has been reported to reside in uterine tissue (mackler *et al.*, 1999). The pacemaker cells in the uterus regulates the gap junction assembly and will either increase or decrease cellular communication and consequently uterine contraction. These endogenous oscillators in the uterus regulate the amplitude of contraction (mackler *et al.*, 1999). These pacemaker cells possess an oscillator present in the cytosol and this oscillator is able to generate repetitive Ca^{2+} transient that are able to activate the inward current and cause them to spread through structures known as gap junction and this provide the signals that result in contraction.

The extract, at effective concentration (3.3 mg/ml) used caused a significant decrease in the frequency of oxytocin-induced uterine contractions, but its decrease in the amplitude was insignificant. The action of Oxytocin (OT) is mediated via Oxytocin receptors (OT-Rs), which are located on the plasma membrane of smooth muscle cells, the number of binding sites are more in the myometrium than in the endometrial layer. Occupied OT- receptors, then mediate release of Prostaglandin$F_{2\alpha}$ ($PGF_{2\alpha}$). $PGF_{2\alpha}$ is known to induce uterine contractions. (Samper *et al.,* 2007). OT also exerts its action by impairing Ca^{2+} efflux from the cell, altering Ca^{2+} entry as well as releasing Ca^{2+} from the sarcoplasmic reticulum via Inositol 1,4,5-triphosphate (IP_3) induced calcium release. (Wray, *et al.,* 2001). The extract thus reduces the frequency of OT-induced contractions, by possibly inhibiting the response of the uterus to oxytocin either by blocking the OT receptors or inhibition of one or more of the mechanisms through which oxytocin exerts its contractile effects on the uterus.

In Calcium deprived state with EDTA, DT extract at effective concentration (3.3 mg/ml) used caused a significant decrease in the frequency of OT-induced contractions, and a similar significant decrease in amplitude. Since there was absence of calcium in the physiological salt solution, the effect exhibited by DT on the frequency of OT-induced contractions suggests that it interacts with intracellular calcium stores rather than extracellular calcium channels.

At effective concentration used in this study, the extract of DT was observed to significantly reduce the amplitude of KCl-induced uterine contraction when compared to the control. KCl acts as a calcium-sensitizing stimulus, and has been used to activate smooth muscle contraction by activation of voltage-operated Ca^{2+} channels which leads to increases in free cytosolic Ca^{2+}. (Ratz*et al.,* 2005). Since the amplitude of KCl-induced contractions was significantly reduced, it suggests that the effect of the extract might involve extracellular calcium channels as well as intracellular calcium-stores as corroborated by the calcium-free state results.

Uterine contractions reoccur throughout the menstrual cycle in human females. The contractions are of low frequency and amplitude during the early follicular phase of the cycle. The frequency of contractions however increase during ovulation with the amplitude and frequency decreasing again during the luteal phase to promote implantation. In the absence of implantation, the frequency remains low but the amplitude of contractions increases radically during the period of menstruation (Aguilar *et al.,* 2010).

Several agents have been used to control uterine contractility and are broadly classified into Uterotonic and Tocolytic agents. Uterotonic agents are agents that are used to induce contraction or increase tonicity of the uterus. Tocolytic agents are agents used to suppress contraction. Uterotonics have found their use in the stimulation of labor and reduction of postpartum hemorrhage while tocolytics are used to suppress premature labor (Payton *et al.,* 1999). The

effects of EC on the pregnant uterus resulting in the reduction of contractions suggest its possible use as a tocolytic agent. Tocolytic therapy is used when delivery if continued, would result in preterm birth, thus helping to delay delivery until the administration of glucocorticoids, which speed up maturation of fetal lung, but may take one or two days before manifestations are noticed.

Although the uterus of humans and rodents have structural differences, their physiological regulatory mechanisms responsible for controlling uterine contractility are quite similar since they are both mammals. Therefore, the results from this study may prove very useful to humans, shedding more light on the usefulness of the plant in female reproductive disorders.

CHAPTER FIVE

5.2 CONCLUSION

In conclusion, this research has displayed that the methanol extract of fresh unripe fruits of *D. tripetala* produces uterine relaxant effects showing possible inhibitory interaction with intracellular calcium stores, myometrial gap junctions and possibly extracellular calcium channels which is in line with literature. This study therefore is contrary to the traditional use of the plant as a uterotonic given to women after child birth. Further studies are however necessary to identify specific bio-constituents responsible for the observed effects and the various mechanisms of action through which the plant extract elicit its effect.

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Appendix

APPENDIX B

Calculations and Derivations

Calculation of final bath concentration of extract

Formula

$C_1V_1 = C_2V_2$

Where;

C_1= Final bath concentration

C_2= Stock solution concentration

V_1= bath volume

V_2= Volume given

For 10ul of 0.4mg/ml stock solution of extract;

$C_1 = C_2V_2 \div V_1$

Given that;

C_2= 10 mg/ml

xV_2= 10ul

V_1= 10ml ×1000= 10,000ul

C_1= 10×10÷10000= 0.01 mg/ml

For 30ul of 10 mg/ml stock solution of extract;

$C_1= C_2V_2÷ V_1$

Where V_2= 30ul;

C_1= 10×30÷10000= 0.03 mg/ml. C_1= 0.03+0.01= 0.04 mg/ml

For 70ul of 10mg/ml

$C_1= C_2V_2÷ V_1$

Where V_2= 70ul;

C_1= 10×70÷10000=0.07 mg/ml. C_1= 0.07+0.04=0.11 mg/ml.

The following equations were used to derive the final bath concentration for the other stock solution preparations of the extract; 100 mg/ml and 1000 mg/ml. The calculated final bath concentrations were added to the previous concentrations.

Calculations of final bath concentration of Oxytocin

$C_1V_1 = C_2V_2$

Where;

C_1= Final bath concentration

C_2= Stock solution concentration

V_1= bath volume

V_2= Volume given

For concentration of 0.1 IU Oxytocin;

To covert iu to microgram =1iu×670 therefore 0.1iu×670=67µg

$C_1 = C_2V_2 \div V_1$

Given that C_2= 67µg

V_2=70ul

V_1=10,000ul

C_1= 67×70÷10000= 0.45µg/ml

Calculation of final bath concentration of KCl

$C_1V_1 = C_2V_2$

Where;

C_1= Final bath concentration

C_2= Stock solution concentration

V_1= bath volume

V_2= Volume given

For a concentration of 3.33M KCl,

$C_1 = C_2V_2 \div V_1$

Given that

C_2=3.33M

V_2=240.24ul

V_1=10,000ul

C_1 3.33×240.24÷10000=0.080M

0.080M÷1000=80nM.

APPENDIX C

Preparation of Stock solutions, Reagents and Chemicals

Preparation of stock solution

4g of the extract was weighed and dissolved in 2ml of tween 80 and made up to10ml by adding 8ml of distilled water to get 40mg/ml

Serial dilution was used to get the subsequent concentrations of 40mg/ml, 4mg/ml and 0.4mg/ml by taking 1ml from the previous concentration and adding 9ml of distilled water.

Composition of Ringer's physiological salt solution

NaCl- 4.5g

KCl- 0.21g

D-glucose-0.5g

$NaHCO_3$- 0.1g

$CaCl_2.2H_2O$-0.16g

Calcium free physiological salt solution was prepared by replacing 0.16g of $CaCl_2.2H_2O$ with 0.146 0f EDTA.

Preparation of Oxytocin

To prepare 1iu of oxytocin, the purchased oxytocin which was 10iu was dissolved in 9ml of distilled water. To prepare 0.1iu of oxytocin, the 1iu oxytocin was dissolved in 9ml of distilled water.

Preparation of high 3.33M KCl.

1.86g of KCl was dissolved in 7.5ml of distilled water.