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# Investigations on the source and role of calcium ions in the *in vitro* uterine muscle contraction stimulated by ethanol leaf extract of *Luffa cylindrica*

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**Abstract**

Traditionally, leaves of *Luffa cylindrica* are known and used for their uterotonic activity, and our earlier studies on isolated rat uterine muscle strips corroborated this. The present study investigated the source and influence of calcium ions ($Ca^{2+}$) on the isolated uterine smooth muscle contractions induced by ethanol leaf extract of *L. cylindrica*. Dried leaves of *L. cylindrica* were subjected to Soxhlet extraction using 80% ethanol. The extract produced a concentration-dependent increase in uterine muscle contraction, with 0.14 mg/ml as the lowest active concentration. Oxytocin (0.5 ng/mg) similarly produced a dose-dependent contraction of the uterine muscle preparation that was fast and phasic in nature. Verapamil hydrochloride significantly ($p < 0.05$) caused inhibition of the extract-mediated contractions with complete abolition of contractions at 0.67 mg/ml. Nifedipine (0.02 mg/ml) also abolished the spontaneous contractions of the myometrium and contractions induced by the extract, even when a higher concentration (2.4 mg/ml) of the extract *was* added to the perfusate. Both the extract and oxytocin were unable to contract the uterine muscle preparation in physiological salt solution (PSS) that was devoid of $CaCl_2$. However, the contractions were restored when the tissue was again bathed in normal PSS containing $CaCl_2$. Caffeine exerted no influence on uterine muscle contractions stimulated by the extract. The results of the study suggest that the ethanol leaf extract of *L. cylindrica* led to contractions of isolated rat uterine muscle strips solely through mobilization of $Ca^{2+}$ from the extracellular compartment to boost the intracellular $Ca^{2+}$ concentration, which is a prerequisite for myometrial contraction.

**Keywords**: *Luffa cylindrica*, Ethanol leaf extract; Uterotonic activity; Calcium ions; Myometrium.

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## Introduction

Contractions of the myometrium are usually phasic in nature, and their wavelength, frequency, duration and intensity varies (Wray *et al*., 2003). The mechanisms that control contractions and relaxation of the uterus are determined by individual cell signaling systems that are uniquely adapted to control their particular functions (Berridge, 2008; Jackson and Boerman, 2018). Myometrial contraction occurs from the action potentials across the plasma membranes, resulting from an increase in the cytosolic free calcium ion ($Ca^{2+}$) concentration (Young, 2007), which depends on the activating stimulus. The stimulus can either be due to $Ca^{2+}$ influx through voltage-gated or ligand-gated plasma membrane channels, efflux from intracellular stores through the ryanodine receptors (RyR), and/or through inositol triphosphate receptor (IP3R) $Ca^{2+}$ channels, or a combination of these channels (Barrett *et al*., 2010), as well as the presence of contractile elements and the conducting system between uterine muscle cells (Wray *et al*., 2003). Agonist binding of the *α*1-adrenergic receptor stimulates inhibitory G proteins (Gi), which inactivate the adenylate cyclase-mediated production of cAMP from ATP. cAMP results in cell relaxation in many ways, including inhibition of MLCK and the efflux of $Ca^{2}$+ through sodium/calcium (Na+/$Ca^{2}$+) exchanger channels (Halls and Cooper 2011).

*Luffa cylindrica* is an edible and medicinal plant which belongs to the Cucurbitaceae family. It has many common names, including smooth luffa, sponge luffa and vegetable sponge gourd (Partap *et al*., 2012). Locally in Nigeria, it is commonly referred to as Ogbo or Asisa in Igbo, kankan or kankan oyibo in Yoruba, while the Hausas call it soo soo (Iwu, 1993). The flowers, leaves, stem and roots of the plant have been reported to exhibit a wide spectrum of pharmacological activities: It has been shown to possess anti-inflammatory and antiemetic (Khan *et al*., 2013), anti-diabetic, anti-viral, wound healing, and anti-cancer properties (Panthong *et al*., 2003). The plant (leaves, bark, root and seeds) also possesses antifungal, anti-bacteria, anthelmintic, hypoglycemic, antioxidant activities. It has also been reported to have a hepato-protective effect in animals (Partap *et al*., 2012). The traditional use of the leaves of this plant for its uterotonic activities have been reported in Uganda (Kamatenesi-Mugisha *et al.,* 2013) and corroborated by our studies on isolated uterine muscle strips of the rat (unpublished data). Information on the source and role of the $Ca^{2+}$ involved in the contractions has not been reported in available literature. The present study investigated the source and role of $Ca^{2+}$ on the *in vitro* spasmogenic activity of the ethanol leaf extract of *Luffa cylindrica*.

## Materials and Methods

**Plant Material and Extraction:** The leaves of *Luffa cylindrica* were collected from a rural community in Lassa, Askira/Uba Local Government Area, Borno State, Nigeria. It was identified by a botanist in the Department of Biological Sciences at the University of Maiduguri, Borno State, Nigeria. The leaves were air-dried under shade and ground into fine powder using a milling machine. Three hundred and eighty grammes of the dried leaves were extracted with three litres of 80% ethanol, following the Soxhlet extraction method (Jensen, 2007). The extraction product was filtered using Whatman filter paper (18 cm) using air dried hot air oven at 40 – 50$^{o}$C. After evaporation of the solvent, the dried extract was kept in a clean container and stored at 4 °C for further use.

**Ethical Approval:** Ethical approval for the use of rats for the study was obtained from the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka (Approval Reference N**umber:** FVM-UNN-IACUC-2023-08/112).

**Experimental animals and Tissue preparation:** Adult female Wistar rats weighing between 180 and 205 g were used for the isolated uterine tissue studies. They were kept in plastic cages for two weeks for acclimatization. They were fed (Vital feeds® Nig. Ltd., Jos, Nigeria) and provided clean water *ad libitum*. Each rat was administered estrogen 0.1 mg/kg body weight subcutaneously 24 hours before humane sacrifice. The rats were humanely sacrificed after diethyl anesthesia, the abdomen was opened to expose the internal organs. The uterine horns were identified, dissected out, and transferred to a dish containing De Jalon's physiological salt solution. De Jalon's physiological salt solution is composed of sodium chloride, potassium chloride, calcium chloride, sodium bicarbonate, glucose and distilled water (Kurle *et al*., 2016).

The isolation and mounting of the non-gravid rat uterus were done by the procedure described by Uchendu (2000). A strip of the horn, about 1 – 2 cm, was cut out. A thread was then attached to one end of the isolated strip and tied to the aerator tube in the organ bath containing 37 mL of De Jalon's solution. Another thread was attached to the other end of the uterine tissue and fixed to a lever system fitted on the stylus. The load on the tissue was 0.5 g. The tissue was aerated with ordinary air using an aquarium air pump in a thermostatically regulated organ bath (37 ± 0.50 °C). The set up was connected to the transducer of the physiograph to record responses of the tissue to perturbations.

In another set of experiments, $CaCl_2$ was omitted from the De Jalon's solution in order to verify the role of calcium in the extract-mediated contractions

**Data Analysis and Presentation:** Analysis of Variance (ANOVA) was used to analyze the extent of variation between the groups using the computer statistical software GraphPad InStat$^R$ 3.0, and P – value equal to or less than 0.05 were considered significant. From the data collected the response curves were plotted, using Microsoft Excel.

## Results

The extraction process produced a dark brown, sticky semi-solid substance.

The ethanol leaf extract of *L. cylindrica* produced a concentration-dependent increase in uterine smooth muscle contraction, with 0.14 mg/ml as the lowest active concentration and 2.4 mg/ml as the highest concentration (Figure 1). The contraction was progressive and phasic in nature ($EC_{50}$ 1.96 mg/ml).

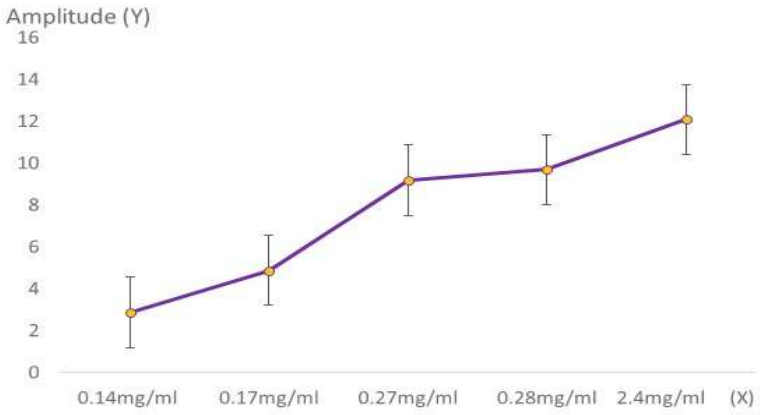


**Figure 1**. Effect of *L. cylindrica* ethanol leaf extract on isolated uterine smooth muscle of the rat.

When the effects of ethanol leaf extract of *L. cylindrica* was compared with oxytocin, both the extract and oxytocin produced concentration dependent contractions of the rat uterine muscle preparation (Figure 2). The contractions evoked by oxytocin was fast-phasic and affected both the frequency and amplitude (force) of contraction (Figure 2).

Addition of Verapamil hydrochloride (0.14 µg/ml), an L-type calcium channel blocker to the perfusate caused a significant ($p < 0.05$) inhibition in the frequency but not the amplitude of contraction induced by aqueous ethanol extract of *L. cylindrica* (Figure 3a). However, there was a complete abolition of the contractions with a higher concentration (0.67 µg/ml) of verapamil. The spontaneous, as well as the extract (2.4 mg/ml) and oxytocin (0.5 ng/ml) mediated contractions were restored after wash (Figure 3b).

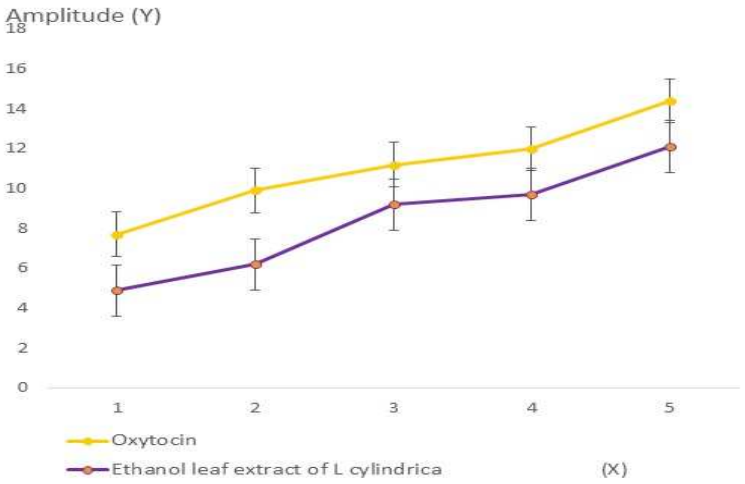


**Figure: 2**. Comparative effects of oxytocin and ethanol leaf extract of *L cylindrica* on isolated rat uterine smooth muscle.

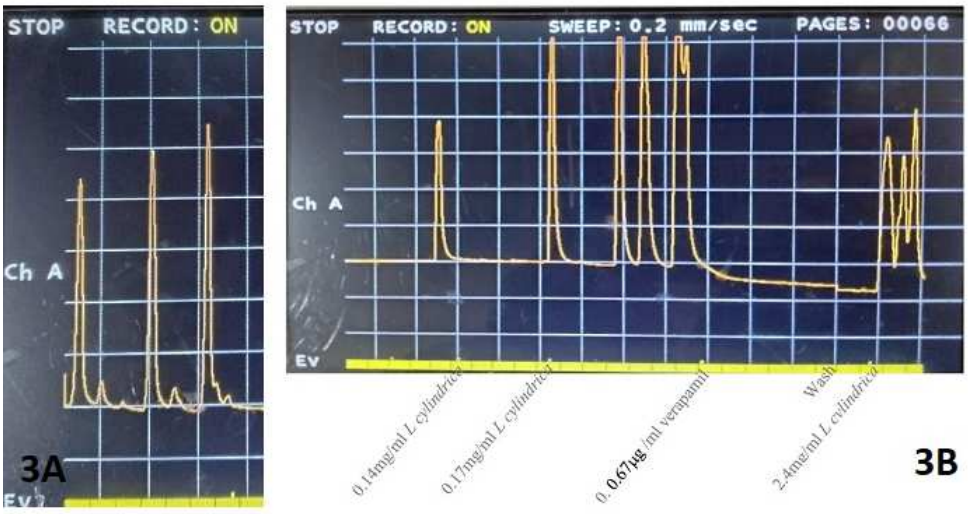


**Figure 3**. Effect of Verapamil hydrochloride on spontaneous uterine muscle contractions and on contractions induced by ethanol extract of *L. cylindrica.*

Nifedipine (0.2mg/ml), a dihydropyridine subclass calcium channel blocker abolished the extract (0.14 – 0.17 mg/ml) mediated contractions, even when a higher concentration of the extract (2.4 mg/ml) was added to the bath medium (Figure 4). The spontaneous contractions of the uterine muscle preparation were not restored after wash, until oxytocin (0.5 ng/ml) was added to the perfusate (Figure 4).

The effect of $Ca^{2+}$ on uterine contraction stimulated by aqueous ethanol extract of *L cylindrica* is shown in Figure 5. The normal, spontaneous uterine muscle contractions were abolished in the absence of $CaCl_2$ in the physiological salt solution (PSS). Application of aqueous ethanol extract of *L*. *cylindrica* (0.14 mg/ml) similarly, did not evoke any contraction in the $Ca^{2+}$-free PSS. Furthermore, the uterine tissue failed to respond to caffeine (2 mmol), known to release $Ca^{2+}$ via the calcium-induced, calcium-release (CICR) mechanism in this $Ca^{2+}$-free PSS (see A in Figure 5). However, the spontaneous contractions were restored in $Ca^{2+}$-containing medium, likewise the tissue responses to the contractants (see B in Figure 5).

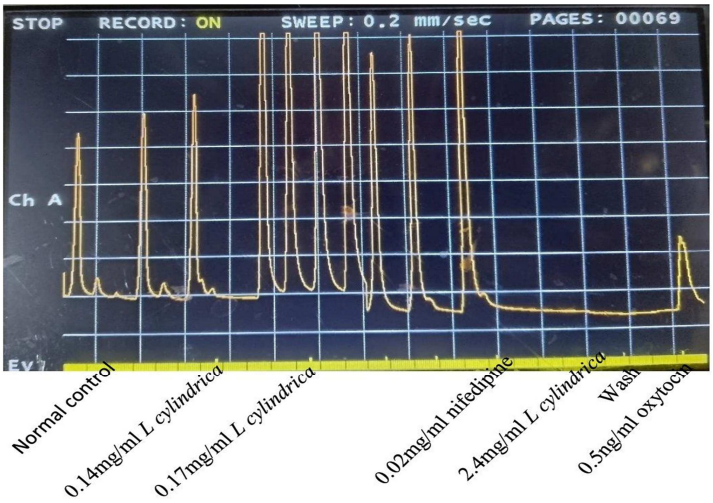


**Figure 4**. Effect of nifedipine on spontaneous uterine muscle contractions and on contractions induced by ethanol extract of *L. cylindrica.*

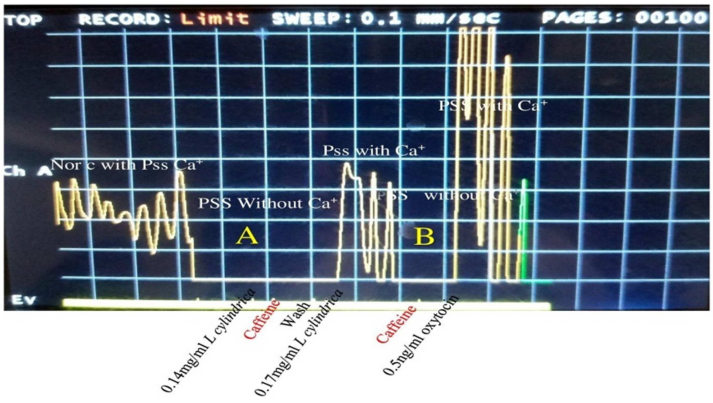


**Figure 5**. Effect of extracellular calcium ($Ca^{2+}$) and caffeine (2mmol) on uterine contractions induced by 0.17mg/ml ethanol extract of *L cylindrica* and 0.5 ng/ml oxytocin

## Discussion

Activation of alpha (α) 1 receptor on the myometrium stimulates uterine contraction, firing of action potentials, depolarization, and the opening of voltage-gated L-type $Ca^{2+}$ channels (Wray *et al*., 2003). The opening leads to a flood of $Ca^{2+}$ into the cell, which leads to contraction (Wray *et al*., 2003). Thus, the major source of $Ca^{2+}$ for contraction is from the extracellular fluid, and the $Ca^{2+}$ channels are regulated by a wide variety of second messengers, e.g., protein kinases A, C, and G (PKA, PKC, and PKG), that are produced when agonists bind to receptors on the myometrial membrane (Keef *et al*., 2001).

The abolition of spontaneous contractions as well as contractions stimulated by the extract of *L. cylindrica* and oxytocin in $Ca^{2+}$-free media in the present study, are testaments to the requirement for extracellular $Ca^{2+}$ for uterine muscle contractions and agrees well with the report of other investigators that the uterine myocytes have a bulk of voltage-operated $Ca^{2+}$ channels, and it is by massive influx of extracellular $Ca^{2+}$ through these channels into the cell cytosol that myometrial contraction occurs (Luckas *et al*., 1999; Wray *et al*., 2003; Chiroma *et al*., 2021).

The inability of the uterine muscle tissue to respond to caffeine, a stimulator of the $Ca^{2+}$-induced $Ca^{2+}$-release (CICR) mechanism, is evidence that the extract of *Luffa cylindrica* was unable to trigger $Ca^{2+}$ release from the intracellular storage site to support the $Ca^{2+}$ pool that is required to initiate the contractile process. However, there are contractile agonists that are able to access $Ca^{2+}$ from the sarcoplasmic reticulum of myocytes through inositol trisphosphate (IP3)-induced $Ca^{2+}$ release (IICR) or through $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) mechanisms (Santo-Domingo and Demaurex, 2010; Pehlivanoğlu *et al*., 2013), but the overall contribution from this site to the cytosolic $Ca^{2+}$ pool has been reported to be minimal (Kupittayanant *et al*., 2002; Pehlivanoğlu *et al*., 2013).

In the present study, it was recorded that Verapamil, a voltage-dependent calcium channel blocker abolished spontaneous uterine contractions as well as contractions stimulated by varied concentrations of the extract. This abolition of extract-mediated myometrial contractions was also evident with nifedipine, a dihydropyridine calcium channel blocker, which further re-emphasized the fact that the $Ca^{2+}$ required for the extract-mediated contractions was derived solely from the extracellular compartment.

In conclusion, the findings in the present study have demonstrated that the calcium for the *L. cylindrica* extract-mediated uterine muscle contraction is mobilized predominantly from outside of the myocytes (that is, through influx into the cell cytosol), with no contribution from intracellular storage sites such as the sarcoplasmic reticulum and the mitochondria.

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## Conflicts of Interest

The authors declare no conflict of interest.

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