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The Possible Biochemical Mechanism of Action of Petroleum-Ether Extract of *Calotropis procera* Leaves (Asclepiadaceae) - A Potent Abortifacient in Gravid Dawley Rats

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Abstract: *Calotropis procera* (Asclepiadaceae) is one of the traditionally used antifertility plants in Nigeria. Previous studies have shown that this antifertility plant has abortifacient property but none of them has reported its possible biochemical mechanism of action. Organ bath experiments using cumulative doses of the extract on rat uterine rings in Dejalon's solution aerated with 95% O_2 + 5% CO_2 produced an increase in tension (% response) with an EC_{50} value of 0.1064 mg/mL. Blocking muscarinic receptors with 35 μg/mL atropine caused the dose-response curve to slightly shift to the right (EC_{50} shift = 0.1064-0.1242), with a significant ($p<0.05$) decrease in maximal tension (%response). However, the β_2-adrenergic blocker (1 μM propanolol) caused a two-fold shift in the dose-response curve (EC_{50} shift = 0.1064-0.2591). The extract also exhibited its effect on cAMP modulation of β_2-adrenergic receptors by causing a significant ($p<0.05$) decrease in cAMP level after treatment of cultured uterine cells with the extract. In conclusion, this study suggests that pet-ether extract of *Calotropis procera* (Asclepiadaceae) may exerts its abortifacient effect by inducing myometrial contractions of the uterus by binding to β_2-adrenergic receptors thereby causing a decrease in the level of cAMP. cAMP reduction reduces the activation of Protein Kinase A (PKA) thereby preventing PKA from inactivating Myosin Light Chain Kinase (MLCK).

Key words: Abortifacient, β-adrenergic receptor, *Calotropis procera*, cAMP, muscarinic receptor, Myosin Light Chain Kinase (MLCK), Protein Kinase A (PKA), uterine contraction

INTRODUCTION

Medical abortion has emerged as a valuable alternative to surgical abortion and will contribute to safe reproductive control worldwide (Reynolds, 1996; Gan *et al*., 2008). Although synthetic abortifacients of known biochemical mechanisms are effective and popular, but the risks associated with these drugs have triggered the need to develop new molecules from medicinal plants. Hence, there is a need for elucidating the physiologic and biochemical mechanisms of suitable natural products with known abortifacient property from indigenous medicinal plants that could be used as therapeutic alternatives for women in developing world.

Calotropis procera (Asclepiadaceae) is a perennial shrubby treelet with thick cottony tomentose leaves when young and frequently glabrescent when fully developed (Huber, 1985). It is distributed widely in the tropics, especially in the waste places (Hussein *et al*., 1994). It is a common plant in Nigeria called "bom-bom" but it is more abundant in the northern part of the country (Sofowora, 1984; Mbako *et al*., 2009). Investigators (Saha *et al*., 1961; Malhi and Trivedi, 1972; Jain *et al*., 1996; El-Badwi and Bakhiet, 2010) have described the plant to be an abortifacient by stimulating a spontaneous contraction on the myometrium but none of any previous studies have documented the possible biochemical mechanism of this effect.

Phytochemically, the plant has been investigated for cardenolide from latex and leaves of plants, triterpenoids, anthrocyanins from flowers and hydrocarbons. The leaves and latex of *Calotropis procera* were found to have cardiac glycosides which include calotrogenin, calotropin, uscharin, calotoxin, calactin (Al-Robal *et al*., 1993; Mueen *et al*., 2005). Alkaloids, flavonoids sterols have also been found to be present in the entire plant (Edman, 1983; Hussein *et al*., 1994). A new norditerpenyl ester named, calotropterpenyl ester and two unknown

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pentacyclic triterpenoids namely calotropursenyl acetate and calotropfriedelenyl acetate have been isolated from the root bark of *Calotropis procera.*

However, most elucidated abortifacient mechanisms involve contractility of the uterine smooth muscle. In smooth muscle, β-adrenoreceptors decreases contractility: PKA phosphorylates MLCK, which thereby becomes inactivated. In contrast, α_1-adrenoceptors increase smooth muscle contractility. They activate phospholipase C, which in turn releases inositoltriphosphate (IP_3) from the endoplasmic reticulum by binding to a cognate receptor channel. Ca^{++} then binds to calmodulin, which in turn activates myosin light chain kinase (Michael, 2007). In this study, we provided evidence that the possible biochemical mechanism of action of the abortifacient property of *Calotropis procera* (Asclepiadaceae) modulates the activity of β_2-adrenergic receptor on the endometrium.

MATERIALS AND METHODS

This research was done in the departments of physiology and biochemistry, College of Medicne-University of Lagos, Nigeria in 2008.

Experimental animals: White Sprague-Dawley female rats were purchased from the animal house of the College of Medicine, University of Lagos, Idi-araba, Lagos-Nigeria. Animals were maintained under controlled standard animal house conditions. They had access to standard rat feed and water *ad libitum*.

Plant: 500 g of fresh leaves of *Calotropis procera* (Asclepiadaceae) were collected at a garden in Okokomaiko town, Ojo Local government, Lagos. The plant was identified by the Botany department Lagos State University and authenticated at the Forestry Research Institute, Jericho, Ibadan with voucher no 107093.

Preparation of extract (Pet-ether): 750 g of *Calotropis procera* leaves was weighed and oven dried at 40ºC in an oven. On the third day, the leaves were pulverized using a laboratory blender.

Approximately 300 g of the powdered *Calotropis procera* was placed in a soxhlet extractor's thimble and 2500 mL of 45% pet-ether solution was used to percolate the thimble in a continuous extraction process. The extract was concentrated using a rotary vacuum evaporator and the crude extract (concentrate) was oven dried at 40ºC. The dried solid was weighed and kept in an air-tight container and was stored in a refrigerator.

Cell isolation and culture: Animal uteri were removed following the laboratory protocol of the College of Medicine, University of Lagos Animal Laboratory Centre. Uteri were washed twice in warm (37-39ºC) sterile Phosphate Buffered Saline (PBS) and trimmed of excess connective tissue. 1% penicillin-streptomycin solution was added [the antibiotic stock contained 10,000 U/ml penicillin and 10,000 μg/mL streptomycin].

Primary cell culture was established by suspending collagenase washed cells in Dulbecco's Modified Eagle's Medium (DMEM) that had been supplemented with 5% Fetal Calf Serum (FCS) and 1% pen-strep.

cAMP assay: cAMP production in cultured uterine myometrial cells in response to graded (2.44×10^{-3}-2.5 μg/μL) concentrations of *Calotropis procera* extract (dissolved with Tween 20) on protein-coupled receptors (GPCRs) was monitored using cAMP-Glo™ Assay kit (Promega, U.S.A.).

Organ bath experiments (Uterotonic Activity): Mounting of uterine strips in the organ bath (Calixto *et al*., 1991): Unprimed gravid horns of the uterus were dissected out and freed from surrounding tissues. Each horn was then mounted in an organ bath containing DeJalon's solution. This solution was constantly aerated (5% CO_2+95% O_2) with an aerator. The bath temperature was adjusted between 32-34ºC to reduce spontaneous uterine contractions. The whole preparation was allowed to equilibrate for 45 min according to the method described y Calixto *et al*. (1991). The contractile activity was then measured using an isometric force transducer (Grass Model 7E Polygraph, USA).

Isotonic contractions of the uterine muscle with different extract concentrations were recorded, and concentration-response curves were constructed. The extract additions were cumulative (FBC = 0.0312, 0.625, 0.125, 0.25, 0.5, 1.0, 2.0 mg/mL). The effect of the extract after thorough washing of the preparation was observed and recorded.

Elucidation of the probable mechanism of action: The effect of the extract was also investigated in the presence of two uterine muscle contractions inhibitors (35 μg/mL atropine and 1 μM Propanolol), which were equilibrated with the tissue for 45 min. Extract concentration- response curves were constructed after the tissues were incubated with above mentioned uterine muscle contraction inhibitors. The (EC_{50}) value for the extract, i.e. the concentration causing half maximal contraction was determined.

Calculations and statistical analyses: All calculations and statistical analyses were done using the computer software GraphPad Prism® 5. Organ bath experiments were expressed as percent maximum contractions. A

Table 1: Table showing percent responses of cumulative extract administration to the organ-bath

Final bath conc. (FBC) (mg/mL)	Conc. of stock	Log FBC	Percent response [without inhibitor] ±SEM	Percent response [with 35 μg/mL Atropine] ±SEM	Percent response [with 1 μM Propanolol] ±SEM
0.0312	3.12	- 1.5058	65.000±7.21	52.500±14.43	81.250±2.17
0.0625	1.58	- 1.2041	66.250±7.94	42.500±5.77	73.750±12.27
0.1250	8.93	- 0.9030	92.500±5.77	60.000±4.33	58.750±10.83
0.2500	6.25	- 0.6021	101.250±10.83	69.000±2.02	76.250±12.27
0.5000	35.71	- 0.3010	103.625±7.87	72.500±17.32	91.250±2.17
1.0000	25.01	0.0000	103.625±5.25	70.000±12.99**	88.750±6.50

Values are Mean±S.E., No of samples = 3, **: p<0.05 versus no inhibitor group

p-value of less than 0.05 was considered significant. EC_{50} values were calculated using the software GraphPad Prism 5.0.

RESULTS

Organ bath experiments: Experiments with the Pet-ether extract of *Calotropis procera* produced a non-significant (p>0.05) increase in tension (% Response) of the uterine tissue as shown in Table 1 and Fig. 1 indicating an EC_{50} value of 0.1064 mg/mL.

Blocking the tissues' muscarinic receptors with an antagonist (35 μg/mL Atropine) caused the concentration-response curve to shift (Fig. 1) dextrally with a significant (p<0.05) decrease in maximal tension (% Response) as shown in Table 1 and Fig. 1. Subsequent incubation of uterine rings with another blocking agent, a β_2-adrenergic blocker (1 μM Propanolol) showed a two-fold shift of the curve to the right (Fig. 2).

cAMP assay: SYNOPIS PAGE: *Calotropis procera* (Asclepiadaceae) is one of the traditionally used anti-fertility plants in Nigeria. Studies have shown that this plant has abortifacient property but none is yet to present its possible biochemical mechanism of action. This research article provides an experimental physiological/biochemical explanation of the abortifacient mechanism of action of Petroleum Ether extract of *Calotropis procera*. Figure 4 shows the effect of Pet-ether extract of *Calotropis procera* on cAMP modulation in epithelial uterine cultured cells. The extract caused a significant (p<0.05) increase in oxy-luciferin absorption which indicates a reduction in cAMP modulation in cultured cells.

DISCUSSION

Termination of pregnancy has been practiced since antiquity. Although synthetic abortifacients of known mechanisms are effective and popular, but the risks associated with these drugs have triggered the need to develop new molecules from medicinal plants. The antifertility effect of the Pet-ether extract of *Calotropis procera* might be attributed to one or more mechanisms of

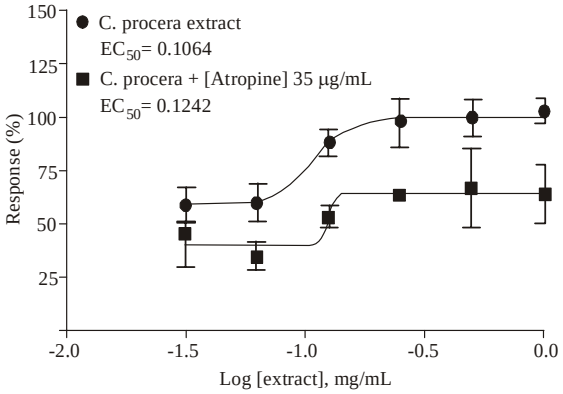

Fig. 1: Cumulative dose response curve of pet-ether extract of *Calotropis procera* without and with 35 μg/μL atropine

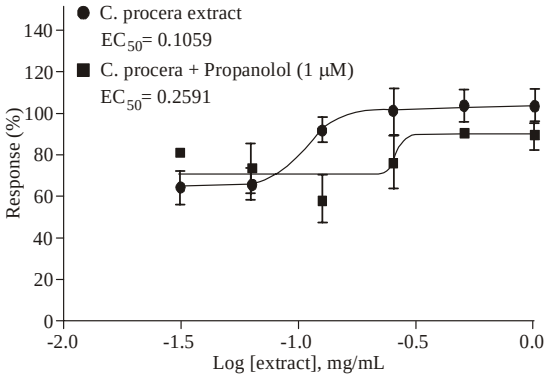

Fig. 2: Cumulative dose response curve of pet-ether extract of *Calotropis procera* without and with 1 μM propanolol

approved drugs. General mechanisms for approved drugs used to terminate pregnancy may include any or the following-- inhibition of synthesis of progesterone, induction of myometrial contractions, antagonizing action of progesterone, or inhibition of development of the trophoblast (Stewart *et al*., 2001).

The observed uterotonic response by the extract in this study as shown in Fig. 1 was characterized by an increase in the magnitude and frequency of uterine contractions indicating the abortifacient effect of the extract. The finding from the isolated uterine muscle studies also showed that the extract produced an equal

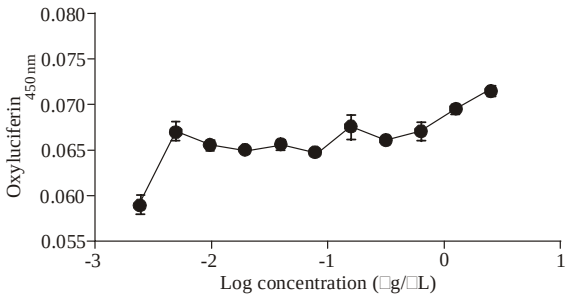

Fig. 3: cAMP modulation by pet-ether extract of *Calotropis procera*

maximal effect (an intrinsic activity greater than 1) on uterine muscle contraction to that of the standard drug, oxytocin, inferring that the extract may be more potent. This is inline with prostaglandins and oxytocin, which stimulate uterine contractions by binding to specific receptors (α_1 and β_2 adrenergic receptors) on the myometrial-cell surface (Izumi *et al*., 1994). This action results in increased calcium production by the endoplasmic reticulum and, consequently in uterine contraction (Izumi *et al*., 1994).

The significant ($p<0.05$) decrease in the magnitude of the maximal effect upon incubating the tissue with muscarinic antagonist (35 µg/mL atropine) indicates that the effect of the extract on the tissue may be mediated through activation of the muscarinic receptor (Fig. 1). The extract caused the EC_{50} of the concentration response curve to greatly shift to the right when β_2 receptor was blocked with a non-significant ($p>0.05$) decrease in maximal upon incubating the tissue with β_2 adrenergic receptor antagonist (1 µM Propanolol) suggests that β_2 receptors may also be involved in the extract induced contraction (Fig. 2). All of these decreases in maximal contractility caused by these antagonists did not produce a total abolishment of contraction as expected.

Contraction and relaxation of myometrium (and other smooth muscles) are regulated by phosphorylation and dephosphorylation of the 20-kD light chain of myosin (Haeberle *et al*., 1985; Csabina *et al*., 1986; Hai and Murphy, 1989; Kamm and Stull, 1985). In smooth muscle, contraction is slower and longer lasting than in striated muscle. Regulation of actin and myosin does not work by way of troponin/tropomyosin but by phosphorylation of the regulatory myosin light chain.

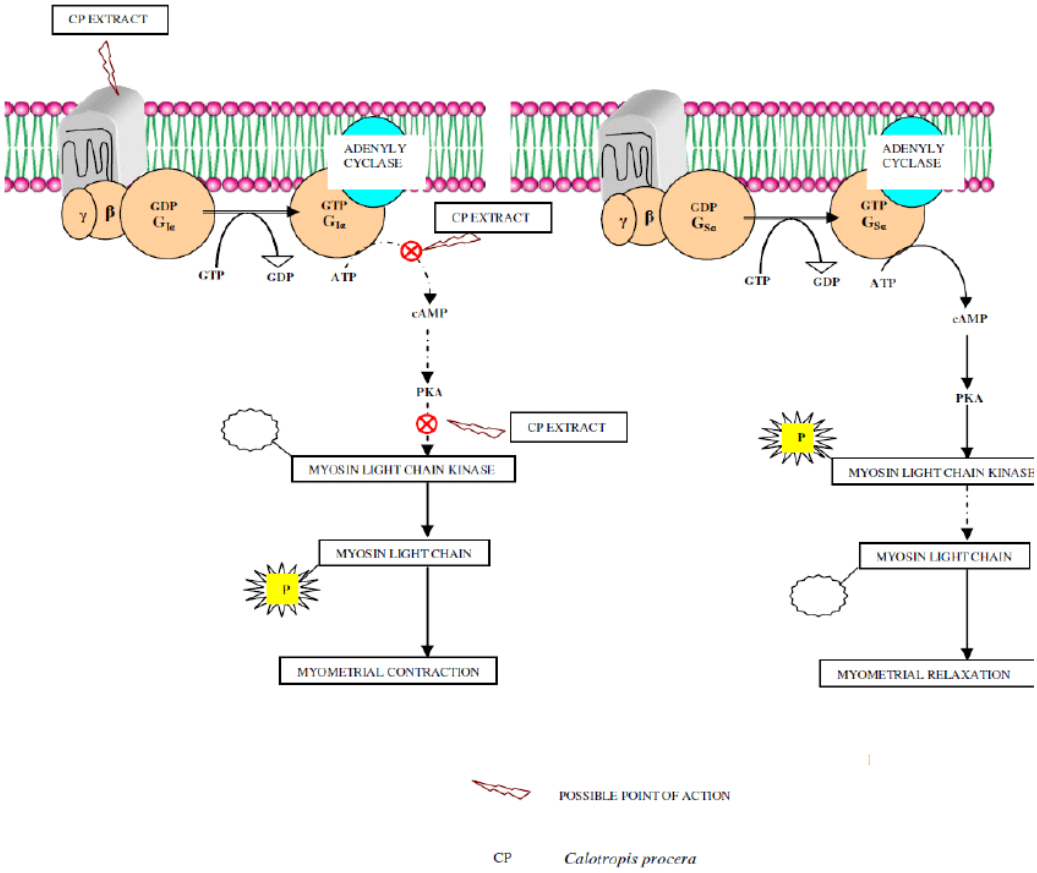

Fig. 4: Possible points of action of *Calotropis procera* extract

This is catalyzed by Myosin Light Chain Kinase (MLCK), which is calmodulin-dependent and, hence, again under the control of calcium. However, less calcium is necessary in this regulatory mechanism, because MLCK provides an extra amplification stage not present in the direct binding of calcium to troponin (Squire and Morris, 1998; Michael, 2007).

In smooth muscle, b-adrenoceptors *decrease* contractility: PKA phosphorylates MLCK, which thereby becomes inactivated. In contrast, a_1-adrenoceptors *increase* smooth muscle contractility. For PKA to be activated, cAMP must be produced by the action of adenylylcyclase. In this study, the administration of the extract to cultured uterine epithelial cells caused a significant ($p<0.05$) increase (Fig. 3) in the absorption of oxyluciferin, a bioluminescent compound used to measure the level of cAMP produced in cultured cells. The higher the absorption of oxyluciferin, the lower the level of cAMP produced. The extract therefore showed a cAMP lowering effect. This infers that the extract binds to b-adrenoceptors and inhibits the action of adenylyl cyclase, preventing the production of cAMP, which then reduces the amount of cAMP necessary to activate protein kinase A (PKA) that phosphorylates myosin light chain kinase (MCLK) (Fig. 4). When myosin light chain kinase (MCLK) is not phosphorylated (deactivated) by protein kinase A, it (unphosphorylated-MCLK) phosphorylates Myosin Light Chain (MLC). Phosphorylation of myosin light chain results in actin activation of myosin ATPase activity, the development of force and shortening of the muscle (myometrium) (Kamm and Stull, 1985).

CONCLUSION

The present study suggests induction of myometrial contraction of the uterus by binding of a phytochemical of *Calotropis procera* extract to β_2-adrenergic receptors of the uterus thereby inhibiting the production of cAMP as a possible mechanism. Inhibition of cAMP through β_2-adrenergic receptors of the uterus reduces the level of PKA required to phosphorylate (inactivate) Myosin Light Chain Kinase (MLCK) in which unphosphorylated (active) MLCK initiates the contraction process in the myometrium by phosphorylating Myosin Light Chain (MLC).

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