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Spasmogenic activity of butanolic leaf extract of *Spondias mombin* in isolated uterine muscle of the rat: Role of calcium

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Abstract

Objective: To investigate the role of calcium [Ca^{2+}] on uterine muscle contraction induced by butanolic leaf extract of *Spondias mombin* (Anacardiaceae). Materials and methods: Dried leaves of *S.mombin* were exhausively extracted with n-hexane and 80% ethanol. The 80% ethanol extract was partitioned in dichloromethane, ethyl acetate, butanol, and water. The butanolic extract was tested for spasmogenicity on isolated uterine muscle strips of the rat using conventional methods. Results: The extract contracted the uterine muscle concentration-dependently (EC_{50}, 0.08 mg/ml). The responses to the extract (0.14 mg/ml) were abolished in Ca^{2+}-free media with or without EGTA and by verapamil (0.18 µg/ml). The contractions were similarly abolished by $LaCl_3$ but significantly ($P<0.05$) attenuated by amiloride (72 µmol). The extract altered the pattern of contraction induced by high K^+ depolarization (20 mmol) from fast phasic to a tonic contracture. Both isoprenaline (0.1 µmol) and salbutamol (0.1 µmol) inhibited the extract induced contractions which were reversed by propranolol (0.03 µmol). Conclusion: Butanolic leaf extract of *S. mombin* contracts uterine muscle of the rat primarily through voltage-dependent extracellular Ca^{2+} entry channel.

Keywords: Calcium, contraction, extract, *Spondias mombin*, uterus.

1. Introduction

The mammalian uterine smooth muscle is known to perform two primary functions namely, to transport the male sex cells (spermatozoa) to the oviduct, and to expel the foetus at term during labour. This dual role is accomplished through peristaltic movement of the myometrium and powerful muscular contractions that are associated with parturition in the species respectively. Data from several animal species suggest that parturition is initiated by hormonal changes at term, especially of oxytocin and the prostaglandins which exert powerful stimulatory effects on the myometrium during this period [1,2]. Uterine sensitivity to oxytocin, in turn, is governed by concentration of oxytocin receptors on the

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myometrial cell membrane which are known to increase near term [3,4] rather than increase in the concentration of the hormone per se. This may explain why larger doses of this hormone are needed to elicit uterine muscle contraction in non-pregnant human subjects [5].

Spondias mombin Linn Holl. is an ubiquitous, medium sized decidous tree widely cultivated in farm lands and around towns and villages as live fence in southern Nigeria. In southeastern Nigeria, it is known as *Ichikara* and fresh infusion from the leaves is used by the natives as 'oxytocic' to facilitate delivery in sheep and goats.

As observed in other visceral smooth muscle preparations, Ca^{2+} ion is known to play a crucial role in the regulation of the contraction-relaxation cycle in the myometrium [6,7,8]. Contraction is initiated by a transient rise in cytosolic free Ca^{2+} ion concentration, which stimulates the calmodulin-dependent enzyme, myosin-light chain kinase. This Ca^{2+} increase may originate from influx across the sarcolemma and/or released from the sarcoplasmic reticulum [9]. The mitochondria have also been reported to transport and bind Ca^{2+} in the myometrium [10].

However, the degree to which uterine contractants are able to mobilize Ca^{2+} from these sources varies greatly [11,12]. Earlier, we had shown that butanolic leaf extract of *S. mombin* contracts the myometrium *in vitro* predominantly through muscarinic receptor activation [13], thus justifying the ethnomedical use of the leaf infusion as an 'oxytocic'. In this communication, we report on the role of Ca^{2+} on *S.mombin* mediated increase in uterine muscle contraction.

2. Materials and methods

2.1 Collection of plant material

Fresh leaves of *S. mombin* were harvested in June, 2002 from the medicinal garden of the Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Nigeria. They were certified authentic by Mr. J.M.C. Ekekwe of the Department of Botany of the same institution, where voucher specimen were deposited. The leaves were air dried and pulverised into fine powder, using a conventional hammer mill.

2.2 Extraction studies

About 4.0 kg of the milled sample was subjected to cold extraction initially with n-hexane (20 l) for eight days and subsequently with 80% ethanol (15 l) for twelve days, stirred at intervals. The n-hexane extract was concentrated in vacuo (40°C) in a rotary evaporator and subsequently air dried at room temperature. The 80% ethanol extract was also concentrated under reduced pressure (40°C) and lyophilized to give a final yield of 992.2 g (24.81% of starting material). The 80% ethanol extract was redissolved in distilled water and partitioned in dichloromethane, ethyl acetate, and butanol.

The solvents were removed off the extracts under reduced pressure (40°C) and each tested for biological activity. The potency of the various extracts (v/v) on uterine muscle motility was of the order, BuOH > Ethyl acetate > Aqueous > Dichloromethane. No contraction was observed with the n-hexane extract even when dissolved in 7% DMSO. The BuOH extract was therefore used in the present investigation.

2.3 In-vitro biological assay

Non-pregnant Wistar rats of breeding age, weighing between 180g and 240g were used for the in vitro bioassay studies. The rats were kept at the Laboratory Animal Unit of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka and were fed on standard ration and watered *ad libitum*. No artificial lighting was provided. Each of the rats received 0.1mg/kg stilboestrol in paraffin oil subcutaneously 24 h prior to the experiments.

The animals were sacrificed by stunning and decapitation. The uterine horns were unravelled and trimmed free of extraneous tissues. For isotonic contraction studies, about 12 mm segment was removed and attached by ligatures at one end to a metalic specimen holder and at the other to a writting lever connected to the smoked drum of a kymograph.

The preparation was suspended vertically in an organ bath containing physiological solution of the following composition (mmol): KCl (4.7); NaCl (118); $CaCl_2$ (2.5); KH_2PO_4 (1.2); $NaHCO_3$ (2.5); $MgSO_4$ (1.2); and glucose (11); and perfused continously with a gas mixture of 95% O_2 and 5% CO_2, maintained at 37°C. The tissue was allowed to be fully relaxed, with a resting tension of 1.0 g before the addition of standard drugs and/or the extract.

In experiments in which Ca^{2+}-free physiological solution was used, $CaCl_2$ in the perfusate was replaced with 2.0 mmol EGTA. EGTA was omitted in experiments involving the trivalent cation, $LaCl_3$, hence such solutions were considered norminally Ca^{2+} free. The tissues were also exposed to the voltage-dependent Ca^{2+} channel blocker, verapamil and to high K^+ (20 mmol) depolarization. To study the effect of other ion fluxes, amiloride HCl was used as an inhibitor of the Na^+/H^+ exchanger system. Salbutamol and isoprenaline were used as selective and non-selective β-adrenergic receptor agonists respectively.

The concentration of the extract and standard drugs given in the text are final nutrient bath concentrations.

2.4 Statistics

The results are expressed as the mean ± standard error of the means (SEM). Statistical analysis of differences between means was carried out using Students *t* - test, with the number of replicates (n) in each experiment indicated in parentheses.

3. Results

3.1 Effects of S. mombin and carbachol on uterine motility

The results of the study showed that both carbachol and the extract contracted the uterus in a concentration-related fashion, with EC_{50} values of 0.05µg/ml and 0.08mg/ml respectively (Fig 1). While the contractile responses to the lowest concentration of *S. mombin* (0.06 mg/ml) were single and transient, higher concentrations produced phasic contractions of short duration.

Responses to carbachol were tonic and sustained, even with the lowest concentration of the agonist. The extract (0.06 mg/ml) also potentiated the amplitude (force) of contraction induced by carbachol (0.43 µg/ml) (carbachol, 9.60 ± 0.49 mm; carbachol + *S. mombin*, 13.0 ±1.41 mm). However, the increase was not significant ($P > 0.05$).

3.2 Uterine contractility in Ca^{2+} free media and effect of verapamil hydrochloride

In Ca^{2+}-free physiological solutions containing the Ca^{2+} ion chelator, EGTA the uterine muscle was unable to respond to stimulations by *S. mombin* (0.14 mg/ml) and carbachol (0.29 µg/ml) (Table 1). However, the contractile responses to the excitants were restored when the tissues were again bathed in media containing Ca^{2+} (Ca^{2+} reloading).

The voltage-dependent Ca^{2+} channel blocker, verapamil produced a graded inhibition of uterine responses to carbachol whereas the maximum contractile signal produced by the extract (9.25 ± 0.83 mm by 0.57 mg/ml) was abolished by the lowest concentration of verapamil (0.18 µg/ml) employed in the study. This inhibitory effects of verapamil on the excitants were reversible as the contractile responses were restored after washout.

Table 1
Effects of (a) Ca^{2+}-free media and (b) verapamil on uterine force stimulated by carbachol (0.29 μg/ml) and leaf extract of *S. mombin* (0.57 mg/ml). Values are the mean ± SEM.

(a)

Extract/Drug	**Uterine muscle responses (mm)**	
	Ca^{2+}-free media	**Ca^{2+}-containing media**
Carbachol (n=4)	0.00	11.75 ± 2.28
S. mombin (n=4)	0.00	9.25 ± 0.83

(b)

	Uterine muscle responses (mm)	
Verapamil (μg/ml)	**Carbachol**	***Spondias mombin***
0.18 (n=5)	6.40 ± 0.62	0.00
0.36 (n=5)	3.40 ± 0.74	
0.72 (n=5)	3.00 ± 0.08	
1.44 (n=4)	2.75 ± 0.64	

3.3 Responses to $LaCl_3$ in Ca^{2+}-free and Ca^{2+}-containing media

When $LaCl_3$ was used to prevent transmembrane efflux of Ca^{2+} ions, the extract (0.14 mg/ml) failed to contract the myometrium both in norminally Ca^{2+}-free media and in normal physiological solution containing Ca^{2+} (Table 2). In contrast, carbachol (0.29 μg/ml) contracted the tissue in both solutions.

However, the response in Ca^{2+}-free media was single and significantly ($P<0.05$) of lower amplitude than that recorded in Ca^{2+}-containing medium. A second application of carbachol, after the initial response in Ca^{2+}-free medium failed to produce a response in the tissue. Responses to carbachol with or without this trivalent cation in Ca^{2+} containing solution were similar.

3.4 Effects of Amiloride and high K^+ depolarization

The use of amiloride (72 μmol) as an inhibitor of Na^+/H^+ exchange resulted in significant inhibition ($P<0.05$) of uterine responses to *S. mombin* (0.14 mg/ml; Table 3). Only the amplitude (force) of contraction was altered by this concentration of amiloride.

High K^+ depolarization (20 mmol) resulted in repetitive phasic contractions that were replaced by a sustained tonic contracture of long duration when *S. mombin* (0.06 mg/ml) was added to the perfusate (Table 3).

3.5 Effects of β-adrenergic receptor agonists and propranolol

Preincubation of the uterine tissue in solution containing the selective and non-selective β-adrenergic receptor agonists [salbutamol (0.01 μg/ml) and isoprenaline (0.01 μg/ml) respectively] caused abolition of contractions induced by submaximal concentration of *S. mombin* (0.06 mg/ml). However, this inhibition was reversed by propranolol (0.03 μg/ml), a β-adrenergic receptor antagonist (Table 4).

4. Discussion

The results of the present study showed a concentration-dependent increase in uterine motility induced by butanolic leaf extract of *S. mombin* (Fig. 1). The maximum contractile response to the extract (in terms of amplitude or force) was observed with 0.57 mg/ml which was equivalent to uterine motility exerted by 0.43 mg/ml of carbachol.

Like in other smooth muscle preparations [14] uterine muscle contraction is directly related to membrane electrical activity, the influx of Ca^{2+} from the extracellular space , as well as the release of Ca^{2+} from intracellular Ca^{2+} storing organelle, the sarcoplasmic reticulum [15,16]. It is known that while Ca^{2+} release from the internal store results in an initial transient increase in cytosolic

Table 2: Responses to lanthanum chloride (La^{3+}, 0.14 µg/ml) in Ca^{2+}-free (CFM) and Ca^{2+}- containing (CCM) media. Results are the mean ± SEM.

Drug/Extract	Uterine muscle responses (mm)	
	CFM	CCM
Carbachol (0.29 µg/ml) Control (n=4)	*3.25 ± 0.43	8.11 ± 0.99
Carbachol + La^{3+} (n=5)	3.80 ± 0.40	8.75 ± 0.83
S. mombin (0.14 mg/ml) Control (n=4)	0.00	6.20 ± 0.98
S. mombin + La^{3+} (n=4)	0.00	0.00

*Value significantly different from CCM at P<0.05

Table 3
Effects of amiloride (72 µmol) and high K^+ depolarization (20 mmol) on uterine force stimulated by leaf extract of *S. mombin.*

Drug/Extract	Uterine muscle responses (mm)
S. mombin (0.14 mg/ml), n=5	7.60 ± 0.90
S. mombin (0.14 mg/ml) + amiloride, n=4	*3.75 ± 0.43
KCl, n=6	13.50 ± 1.26
S. mombin (0.06 mg/ml), n=4	3.25 ± 0.43
S. mombin (0.06 mg/ml) + KCl, n=6	14.17 ± 0.69

Value significantly different at P<0.05

Ca^{2+} concentration, Ca^{2+} entry from the extracellular compartment produces a sustained increase [17].

That the contractile activity of *S. mombin*, in the present study, is dependent solely on extracellular Ca^{2+} is evident from the failure of the extract to elicit contractions in Ca^{2+}-free media either with or without the Ca^{2+} chelator EGTA. Supportive evidence for this Ca^{2+} release mechanism has been provided by the experiments in which the voltage-sensitive calcium channel blocker, verapamil was used to prevent Ca^{2+} entry into the cell.

In this context, contractile response in the presence of extracellular Ca^{2+} and verapamil would be assumed to be a result of Ca^{2+} release from intracellular store. The results showed that the lowest concentration of verapamil employed in the study (0.18 µg/ml) was sufficiennt to abolish the maximum contractile response induced by the extract (Table 1), suggesting that Ca^{2+} for *S. mombin* induced contractions derived from the extracellular compartment and further, that the Ca^{2+} entry was predominantly through voltage-gated channel.

In the rat uterus, Ca^{2+} entry to the cell cytosol occurs almost entirely via L-type Ca^{2+} channels [8] which is specifically blocked by verapamil. It is not unlikely that there may have been contributions from other sources other than a change in membrane potential considering the change in the pattern of contraction from phasic to tonic during high K^+ depolarization; and the substantial decrease in uterine motility on exposure to amiloride (Table 3), a potent inhibitor of Na^+/H^+ exchanger in agonist induced mobilization of Ca^{2+} in cells [18,19].

Indeed, the Na^+/Ca^{2+} exchanger have been demonstrated in the uterus of different species [20] including their relative contribution to the overall Ca^{2+} homeostasis in uterine muscle cell [21,22]. On the other hand the limited contraction exerted by carbachol in norminally Ca^{2+} free media shows the capacity of this

Table 4
Effects of salbutamol (0.01 μg/ml), isoprenaline (0.01 μg/ml), and propranolol (0.03 μg/ml) on uterine motility induced by leaf extract of *S. mombin* (0.06 mg/ml).

Drug/Extract	**Uterine muscle responses (mm)**
S. mombin (n=4)	3.25 ± 0.43
S. mombin + salbutamol (n=5)	0.00
S. mombin + isoprenaline (n=4)	0.00
Propranolol + S. mombin (n=4)	3.75 ± 0.83
Propranolol + salbutamol + *S. mombin* (n=5)	3.00 ± 0.89
Propranolol + isoprenaline + *S. mombin* (n=4)	4.50 ± 0.50

excitant to release Ca^{2+} from the internal store, presumably through inositol 1,4,5-trisphosphate release mechanism [8]. This store Ca^{2+} is small and easily depleted since a second application of carbachol to the Ca^{2+} free solution failed to initiate a response from the tissue.

In the present study, we found that La^{3+} abolished uterine muscle contractions stimulated by the extract in Ca^{2+}-free and Ca^{2+}-containing media but failed to alter carbachol stimulated increase in contractility in Ca^{2+}-containing media (Table 2).

We propose that unlike carbachol, a significant amount of the extracellular Ca^{2+} mobilised for uterine motility by the extract may be located superficially on the sarcolemma and prolonged exposure to this polyvalent cation substantially reduced or abolished this membrane bound Ca^{2+} conductance. A high membrane-binding affinity for La^{3+} should expectedly limit or block the normal voltage-dependent conductance increases.

The ability of La^{3+} to block excitation has been similarly demonstrated in barnacle muscle fibres [23] and in lobster axon [24] and was found to be the same in either sodium or calcium spike systems.

However, our results contrast with those reported in guinea pig and rat ureteric muscles [25] and rat mesenteric artery muscle [26], presumably due to tissue variability with respect to this trivalent cation activity.

Both salbutamol and isoprenaline, as selective and non-selective β-adrenergic receptor agonists respectively, caused abolition of *S. mombin* induced uterine force which was reversed by propranolol (0.03 μmol; Table 4).

This is considered consistent with the general action of β-adrenergic agonists in smooth muscle preparations [27,28]. β-adrenergic agonists inhibition of myometrial contractions is reported to be associated with activation of receptor-mediated adenylate cyclase, resulting in increased cytosolic cAMP content [29] and calcium extrusion from the cell [30].

These biochemical changes are concurrent with increased K^+ ion conductance and reduction in cytosolic-free Ca^{2+} content, via inhibition of voltage-gated Ca^{2+} channel [31] leading, consequently, to relaxation. Propranolol, and other β-adrenergic blocking agents can blunt the rise in cAMP [32] and possibly enhance Ca^{2+} release from membrane sites [33] and therefore promote the stimulatory activity of agonists

In conclusion, we have shown that butanolic leaf extract of *S. mombin* concentration-dependently contracts uterine muscle strips of the rat. This contraction is dependent solely on extracellular fluid Ca^{2+} via a voltage-dependent Ca^{2+} entry channel. The β-adrenergic receptor agonists caused an inhibition of this extract

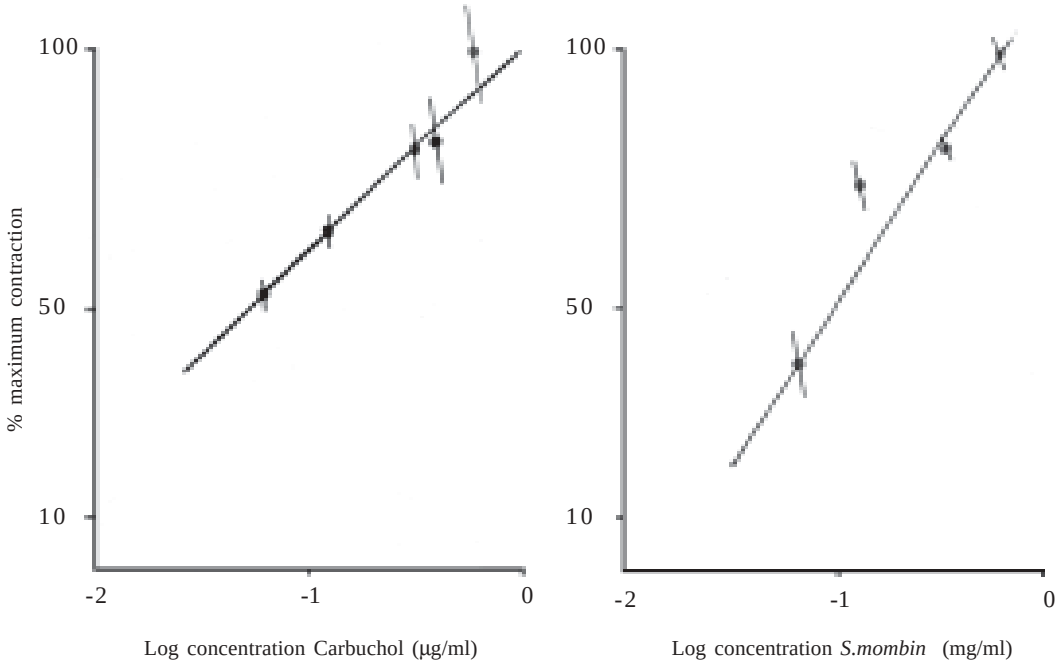

Fig 1. Log concentration-response curve of carbachol (µg/ml) and *S. mombin* extract (mg/ml) on isolated uterine muscle strip of the rat. Results are the mean ± standard error of the mean (SEM).

mediated increases in uterine motility. The use of leaf extract of *S. mombin* in the management of dystocia of small ruminants by the natives in southeastern Nigeria therefore has some physiological basis.

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