

Uterotonic Effects of Aqueous and Methanolic Extracts of *Lannea Acida* in Wistar Rats: An in Vitro Study.

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Abstract

Background: *Lannea acida* (Anacardiaceae), commonly called Kikié in the Noun division (West-Cameroon), is a tree whose bark is used locally to solve difficult childbirth. This study aimed to evaluate the *in vitro* uterotonic effects of aqueous and methanolic extracts of *L. acida* in female Wistar rats. Uterine strips isolated from female rats pretreated (48h) with oestradiol (5µg) were mounted in a single-organ bath containing a well aerated and thermostated De Jalon solution (37°C). The effects of *L. acida* extracts were recorded in a non-cumulative manner after application. The effect of the methanolic extract (the most active extract) was monitored in the presence of atosiban (a competitive antagonist of oxytocin receptors), atropine (a specific type 3 muscarinic receptor antagonist), nifedipine (an L-type calcium channel antagonist) and 2-Aminoethoxydiphenyl borate (2-ADB, a specific antagonist of inositol 1,4,5-triphosphate receptors type 1), and in calcium-free medium containing EGTA.

Results: *L. acida* induced uterine contraction in a concentration-dependent manner with the methanolic extract (1.506 ± 0.032 gf) being the most effective. Administration of atosiban (2 µmol/l), atropine (1 µmol/l), nifedipine (5 µmol), 2-APB (100 µmol), and calcium free medium containing EGTA (2 mmol) reduced the contractile effect of *L. acida*. Complete inhibition was observed with nifedipine, 2-APB, and calcium free medium containing EGTA.

Conclusions: These results suggest that *L. acida* possesses an uterotonic effect mediated through oxytocin receptors with mobilization of extracellular calcium.

Background

Parturition is the process of delivery of a fully-grown fetus on the completion of the normal pregnancy period. This biological process is characterized by an increase in myometrial contractility and the dilatation of the uterine cervix [1]. In fact, as term approaches, the quiescent uterus becomes activated by estrogens. This leads to increased expression of various contraction-associated proteins (CAPs) such as prostaglandin and oxytocin receptors [2]. Also, an increase in gap junction formation between adjacent myometrial cells allows electrical synchrony within the myometrium and ensures effective coordination of contractions [3]. During labor process, the frequency and intensity of myometrial smooth muscle contractions are essential contributing factors to normal delivery [4]. In women with slow and/or weak uterine activity during labor, uterotonic molecules (oxytocin and prostaglandins) are regularly used [5, 6]. In extreme cases, caesarian sections can be required [7]. However, these modern techniques are limited due to postpartum bleeding commonly associated to uterotonic compounds and financial and esthetic consequences of the surgical solution [8]. Furthermore, in developing countries where the accessibility of health services is limited, people largely rely on medicinal plant for primary health problems [9].

Many medicinal plants with uterotonic properties have been reported. These include among other *Newbouldia laevis* [10], *Ananas comosus* [11] and *Foeniculum vulgare* [12]. *Lannea acida* (Anacardiaceae

family), commonly called *Kikié* in the Noun Division (West-Cameroon) is a small deciduous tree of about 8 to 12 meters height. The decoction of its stem barks is traditionally used as fertility enhancer and at late gestational stage to facilitate parturition. Scientific evidences showed that this plant possesses androgenic [13], estrogenic [14] and contractile properties in isolated rat vas deferens and seminal vesicles [15]. Phytochemical screening of this plant revealed the presence of alkaloids, glycosides and tannins compounds [16, 17]. However, less is known on the effects of this plant on uterine contractility in rats. This study was therefore undertaken to determine the effect of *L. acida* on uterine activity in healthy non-pregnant rat and its mechanism of action.

Results

Effects of the aqueous and methanolic extracts of *L. acida* on uterine contractility

A dose-dependent contractile effect on the rat myometrial strips was recorded after application of aqueous extract of *L. acida* (Fig. 1A). On the contrary, the effect of methanolic extract was not dose-dependent (Fig. 1B). Indeed, the maximum contractile effect was observed at moderate concentration (2.18 mg) while the highest concentration (4.36 mg) was less active. As shown in Fig. 1C, the contraction force increased gradually after the application of the aqueous extract of *L. acida*. In the uterus samples treated with the methanolic extract of *L. acida*, the contraction force increased at low (1.09 mg/ml) and moderate (2.18 mg/ml) concentrations, but decreased at high concentrations (3.27 and 4.36 mg/ml). The methanolic extract was more effective at low concentrations while the aqueous extract produced the highest effect at high concentration (Fig. 1A, B, C).

Relative potency of the aqueous and methanolic extracts of *L. acida* and other uterotonics

The effect of *L. acida* extracts and agonists on the relative potency of rat uterus is shown in Table 1. The tension recorded after application of oxytocin was increased by 24.17% and 15.41% compared to aqueous and methanolic extracts of *L. acida* respectively. Acetylcholine also increased the uterus tension by 28.29% and 20% compared to aqueous and methanolic extracts of *L. acida* respectively. The uterus tension increased by 50.49% and 44.77% in the samples treated with potassium chloride, compared to aqueous and methanolic extracts of *L. acida* respectively (Table 1). Potassium chloride was the most effective drug.

Table 1
Relative potency of *L. acida* extracts compared to other uterotonics

Treatments	Tension (gf)
Methanolic extract of <i>L. acida</i>	1.506 ± 0.032
Aqueous extract of <i>L. acida</i>	1.680 ± 0.230
Oxytocin	1.986 ± 0.063
Acetylcholine	2.100 ± 0.087
Potassium chloride	3.042 ± 0.041

Effects of some antagonists on uterine contraction induced by plant extracts and agonists

Effect of atosiban and atropine on uterine contraction induced by *L. acida*

Oxytocin, acetylcholine and *L. acida* induced uterine contraction after application. The contractile effects of oxytocin were totally inhibited by atosiban (Fig. 2A). Atosiban also inhibited the contractile effect of the methanolic extract of *L. acida*. However, the second administration of the plant extract (before washout period) caused moderated contractions of the uteri strips (Fig. 2B). The force of contraction increased significantly ($p < 0.05$) after oxytocin and *L. acida* applications compared to control (Fig. 2C).

The contractile effect of acetylcholine was totally inhibited after atropine (a specific type 3 muscarinic receptor antagonist) application (Fig. 2D). Additionally, atropine partially inhibited the contractile effect of the methanolic extract of *L. acida* (Fig. 2E). The force of contraction recorded after atropine injection was significantly ($p < 0.05$) elevated compared to control (Fig. 2F).

Effect of nifedipine and Ca²⁺ free medium with EGTA on uterine contraction induced by *L. acida*

The contractile effects of KCl and methanolic extract of *L. acida* were completely abolished in the presence of nifedipine (5 µmol, an L-type calcium receptor antagonist) (Fig. 3A and B). The maximum contraction forces of KCl and *L. acida* were reduced significantly ($p < 0.05$), compared to control (Fig. 3C). Compare to the effect in De Jalon solution, acetylcholine or *L. acida* had no contractile effect in calcium free medium with 2 mMol/l of EGTA (Fig. 4A-C).

Effect of 2-aminoethoxydiphenyl borate (2-APB) on uterine contractions induced by oxytocin and *L. acida*

The contractile effect of oxytocin and *L. acida* on uterine smooth muscle was significantly ($p < 0.05$) inhibited by 2-APB (a specific antagonist of inositol 1,4,5-triphosphate receptors type 1) with the E_{max} values $14.82 \pm 3.64\%$ and $9.53 \pm 1.66\%$ respectively (Fig. 5A-C).

Discussions

This study demonstrated the *in vitro* uterotonic effect of aqueous and methanolic extracts of *L. acida*, which may justified its traditional used to facilitate parturition. The uterine strips isolated from rats pretreated (subcutaneously) with 17β -estradiol were used in this work because of its high sensitivity to uterotonic agents [2]. *L. acida* extracts (1.09 to 4.36 mg/ml) stimulated uterine contraction with a maximal effect obtained at a concentration of 2.18 mg/ml with the methanolic extract and 4.36 mg/ml with the aqueous extract. Comparatively, the methanolic extract of *L. acida* was more effective at low concentrations (1.09 and 2.18 mg/ml) while the aqueous extract had its highest effect at high concentrations (3.27 and 4.36 mg/ml). Over 2.18 mg/ml of methanolic extract in the medium, the contraction force decreased with increasing concentration. This adverse effect carried out *in vitro* could be attributed to the high concentration of the active principle such as alkaloids, saponins and flavonoids [17] in methanolic extract than the aqueous extract that can trigger an internalization or desensitization of the uterine receptors. Recently, it has been demonstrated that alkaloids and saponins induce contractile activities on smooth muscle [22, 23]. The alkaloids (Imperialine- 3β -D-glucoside) are known for their ability to contract uterine smooth muscle in contrast to the flavonoids (spinosine) that relax it [24]. Apart from *L. acida* (Anacardiaceae) other Anacardiaceae species including *Spondias mombin* [24] were also reported to stimulate uterine contraction (0.75 mg/ml), suggesting that the uterotonic effect is common to the Anacardiaceae family. Similar studies using rat [12, 25] and mouse [26] uterine strip have led to conclude that bioactive compounds found in *L. acida* are responsible for the contractile effects. In the current study, the maximal concentration (2.18 mg/ml) which gives the maximal effect is highest than the maximal concentration (1.6 mg/ml and 2 mg/ml) used respectively by Watcho [19] and salleh and Ahmad [27].

Like oxytocin, acetylcholine and potassium chloride which are good standard uterotonic agents, *L. acida* induced a concentration-dependent contractile effect. Despite several pharmacological and molecular studies on the elucidation of signaling pathways including metabotropic receptors [28], the mechanism of action with the most active extract (methanol) was carried out using atosiban (a competitive antagonist of oxytocin receptors) and atropine (a non-competitive antagonist of muscarinic channels) which are the major influence of extracellular calcium in the uterus strips. The contractile effect of the methanolic extract of *L. acida* was inhibited after atosiban (2 μ mol) or atropine (1 μ mol) application. Our findings suggest that *L. acida*-induced uterine contraction was mediated mainly via the oxytocin receptor as evidenced by the highest degree of inhibition of the atosiban while the lowest inhibition by atropine

suggests that *L. acida* binding to the muscarinic receptors produced the least degree of contraction. These results are similar to the action observed by Watcho *et al.* [19] and Salleh and Ahmad [27], which showed that *F. asperifolia* and *F. deltoidea* respectively increase the contractile activity of rat uterine smooth muscle via multiple membrane receptors. The mechanism of action of stimulants depends mainly on pharmaco-mechanical coupling since membrane depolarization receptors appear to belong to the G-protein family. Activation of the receptor which is coupled to G protein alpha stimulates uterine contraction by activating the phospholipase C/ Ca^{2+} dependent pathway [29]. G protein-coupled membrane receptors mobilize extracellular calcium via L-type calcium channels activated by DAG/PKC and/or intracellular calcium via IP₃ receptors [30]. In addition, acetylcholine opens ion channels without depolarization of membrane while KCl induced contraction by depolarizing membrane which causes the influx of Ca^{2+} [31]. Ca^{2+} then binds to calmodulin, which activates the myosin light chain kinase leading to phosphorylation of myosin light chains, triggering contraction [32].

In order to verify the involvement of L-type calcium channels and in turn the involvement of extracellular calcium in the mechanism of action of *L. acida*, a test with nifedipine and in free- Ca^{2+} De Jalon with 2 mM EGTA were performed using potassium chloride and acetylcholine. Nifedipine (1 μMol), an L-type calcium channel antagonist, suppressed the stimulatory effects of *L. acida*. Though all contractions of *L. acida* or acetylcholine were abolished in free- Ca^{2+} De Jalon with 2 mM EGTA after 30 minutes. In myometrial smooth muscle, calcium is sequestered in caveolae and recycles through L-type Ca^{2+} channels. The system can recycle Ca^{2+} efficiently between the caveolae and the SR using L-type Ca^{2+} channels and IP₃ mediated Ca^{2+} release from the SR (by acetylcholine) [33]. These results suggest that *L. acida* could mobilize extracellular calcium by stimulating L-type calcium channels receptors.

Given the intracellular origin of calcium in the uterine smooth muscle contraction process, an additive experiment was performed using 2-APB, a non-specific type 1 IP₃ receptor [34]. Because 2-APB inhibited the contractile effects of *L. acida* and oxytocin, we can therefore suggest that these drugs act through IP₃ pathway. Moreover, the inhibitory effect of atosiban suggests that *L. acida* act through oxytocin receptors and mostly depend of extracellular calcium. Nevertheless, the contraction produced does not depend solely on extracellular Ca^{2+} as evident from the total inhibition on Emax by 2-APB.

Oxytocin binds to its G protein-coupled receptor and activates phospholipase C (PLC), which in turn increases inositol-trisphosphate (IP₃) and diacylglycerol (DAG) levels. DAG induced extracellular Ca^{2+} influx through voltage-operate channels such as L-type calcium channel. IP₃ activates the IP₃ receptor at the sarcoplasmic reticulum membrane which release Ca^{2+} into the cytosol and amplify contractions [35]. Since 2-APB inhibits the contractile effect of *L. acida*, 2-APB may interact with TRPC and IP₃ receptors [36] because Inositol 1,4,5-triphosphate activates TRPC3 channels and increases extracellular Ca^{2+} influx in smooth muscle cells [35]. These results suggest that bioactive compounds present in the methanolic extract of *L. acida* could induce (via the myometrial membrane receptors) the release of intracellular calcium. This moderate Ca^{2+} release induces the opening of the calcium channels thus causing an

increase in the calcium flow at the origin of the contractions. These results are similar to those of Sharma et al. [37] who showed that histamine acts by first mobilizing calcium reserves and extracellular calcium.

Conclusion

Based on these findings, it appears that *L. acida* triggered uterine smooth muscle contraction *in vitro*. These uterotonic effects of *L. acida* are mediated through oxytocin receptors with mobilization of extracellular calcium. This result justifies the use of *L. acida* in traditional medicine to facilitate childbirth.

Methods

Collection of plant material and preparation of extracts

Fresh stem barks of *L. acida* (Anacardiaceae) were collected in January 2018 in the Noun Division (West-Cameroun). A sample was authenticated at the Cameroon National Herbarium (HNC-IRA) by Mr. Victor Nana, by comparison to the specimen deposited under the voucher number 40942 HNC. The barks were shade-dried and grinded into powder prior to aqueous and methanolic extracts preparation.

Extracts preparation

To obtain the aqueous extract, 500 g of the plant powder were mix in 3 L of distilled water and boiled for 10 minutes. The solution was allowed to cool at room temperature and filtered using Whatman paper No 4. The filtrate was oven-dried to obtain 20.4 g of the aqueous extract (extraction yield: 4.08%). The methanol extract was prepared by maceration of 250 g of the powder of *L. acida* stem barks in 1 L of methanol for 72 h at room temperature. The filtrate was evaporated under reduced pressure and oven-dried to obtain 13.5g of the methanol extract, giving an extraction yield of 5.4%. For bioactivity investigations, the aqueous and methanol extracts were dissolved in distilled water.

Animals

Healthy non-pregnant adult female Wistar rats weighing 150-170 g were obtained from the animal house of the Department of Animal Biology, Faculty of Science of the University of Dschang-Cameroon. They were housed in plastic cages and had access to water and standard rat chow *ad libitum*. All procedures were validated by the scientific committee of the Department of Animal Biology, University of Dschang, which follows the internationally accepted standard ethical guidelines for laboratory animal use and care as described in the European Economic Community guidelines; EEC. 2010 Council Directive 2010/63/EU of 22 November 2010 [18].

Experimental design

Isolated rat uterus preparation

The preparation of estrogenized uterus was performed according to the procedure described by Watcho et al. [19]. Briefly, 24h before the experiment, virgin female rats were subcutaneously injected with 17- β -estradiol benzoate (13.28 nM per animal). To collect the uteri, animals were sacrificed by cervical dislocation under anesthesia and the uteri were promptly removed, cleaned of the connective tissue and cut into strips of about 1 cm of length. Each uterine strip was vertically mounted in an organ bath of 20 mL capacity containing fresh De Jalon solution of the following composition (mM): NaCl 153.85, KCl 5.64, CaCl₂ 0.55, MgSO₄ 0.08, NaOH 12.5 and glucose 2.78, and thermostated at 37°C. Strip tension was adjusted to 0.71 g and allowed to equilibrate for 45 min during which the physiological solution was changed every 15 min. Spontaneous and drug-induced myometrial contractions were recorded using an isometric force transducer (SS12LA, BSL: Variable Force Transducer) connected to an MP36 amplifier (Biopac student lab pro version 3.7.3) and displayed on a monitor.

Drugs challenges

After the equilibration period during which spontaneous contractions were registered, non-cumulative concentration-response curves to oxytocin (0.054 - 3×10^{-10} mol/l), acetylcholine ($2.13 - 17 \times 10^{-6}$ mol/l), potassium chloride (5.3 - 42.1 mmol/l) and *L. acida* extract (1.09 - 4.23 mg/ml) were recorded during 5 min. The tissue was then washed by changing the bathing solution and allowed to rest for 15 min before the next stimulation. The experiment was repeated 5 times for each drug/extract concentration. At the end of this phase, the most active extract (methanol extract at lowest concentration) was chosen to investigate the mechanism of action of the plant uterotonic activity. **Determination of the mechanism of**

action of *L. acida* To determine the mechanism of action of *L. acida*, the tissue was pre-incubated for 30 min with atosiban (2 μ mol) (an oxytocin receptor inhibitor), atropine (1 μ mol) (a specific type 3 muscarinic receptor antagonist) and nifedipine (5 μ mol) (an L-type calcium channel antagonist) before administration of oxytocin, acetylcholine and KCl. The experiment was repeated with the plant extract in the presence of each antagonist. Furthermore, the effects of the plant extract was tested in the presence of 2-amino-ethoxyphenylborate (100 μ mol) (2-ADB, a specific antagonist of inositol 1,4,5-triphosphate receptors type 1) and in free calcium medium containing EGTA (2 mmol) to investigate the involvement of the intracellular and extracellular calcium in the plant activity. The results were expressed as inhibition percentage and calculated as follows: $\text{Inhibition\%} = (\text{CF without antagonist} - \text{CF with in the presence of the antagonist}) / (\text{CF without antagonist}) \times 100$ CF = contraction force. The calcium-free De Jalon solution was prepared by substitution of CaCl₂ with EGTA as describe by Aziba[20]. **Drugs** Estradiol benzoate (17- β -diol 3-benzoate), acetylcholine hydrochloride [ethanaminium, 2-(acetyloxy)-N,N,N-trimethyl-, chloride], Potassium Chloride, EGTA(Ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid) and Atosiban were purchased from Sigma Chemical (St Louis, MO, USA). Atropine sulfate [α -(hydromethyl) benzene acetic 8-methyl-8-azabicyclo (3.2.1) oct-3yl-ester], oxytocin and nifedipine were purchased from local suppliers. All chemicals were dissolved in distilled water except 2-APB (DMSO) [21].

Statistical analysis

The data was expressed as means \pm SEM. One-way analysis of variance (ANOVA) followed by Tukey HSD post hoc were used to assess statistical difference among groups using Statistica Software (version 8.0). The results were significantly different when $p < 0.05$.

Abbreviations

2-ADB

2-Aminoethoxydiphenyl borate

EGTA

(ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid)

CAPs

contraction-associated proteins

DAG

diacylglycerol

TRPC3

Transient Receptor Potential Cation Channel Subfamily C Member 3

PKC

Protein kinase C

IP3

inositotrisphosphate

Declarations

Acknowledgement

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Authors' Contributions

Esther Ngadjui, Pierre Watcho, Jibril Yves Kouam and Georges Romeo Fozin Bonsou participated in the study design. Aimé Césaire Tetsatsi Momo, Jibril Yves Kouam and Patrick Brice Defo Deeh collected the data and carried out the statistical analysis. Pierre Watcho, Modeste Wankeu-Nya, Patrick Brice Defo Deeh and Telesphore Benoit Nguелеfack drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

Ethics approval and consent to participate

All procedures were validated by the scientific committee of the Department of Animal Biology, University of Dschang, which follows the internationally accepted standard ethical guidelines for laboratory animal use and care as described in the European Economic Community guidelines; EEC. 2010 Council Directive 2010/63/EU of 22 November 2010 [18].

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interest.

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Figures

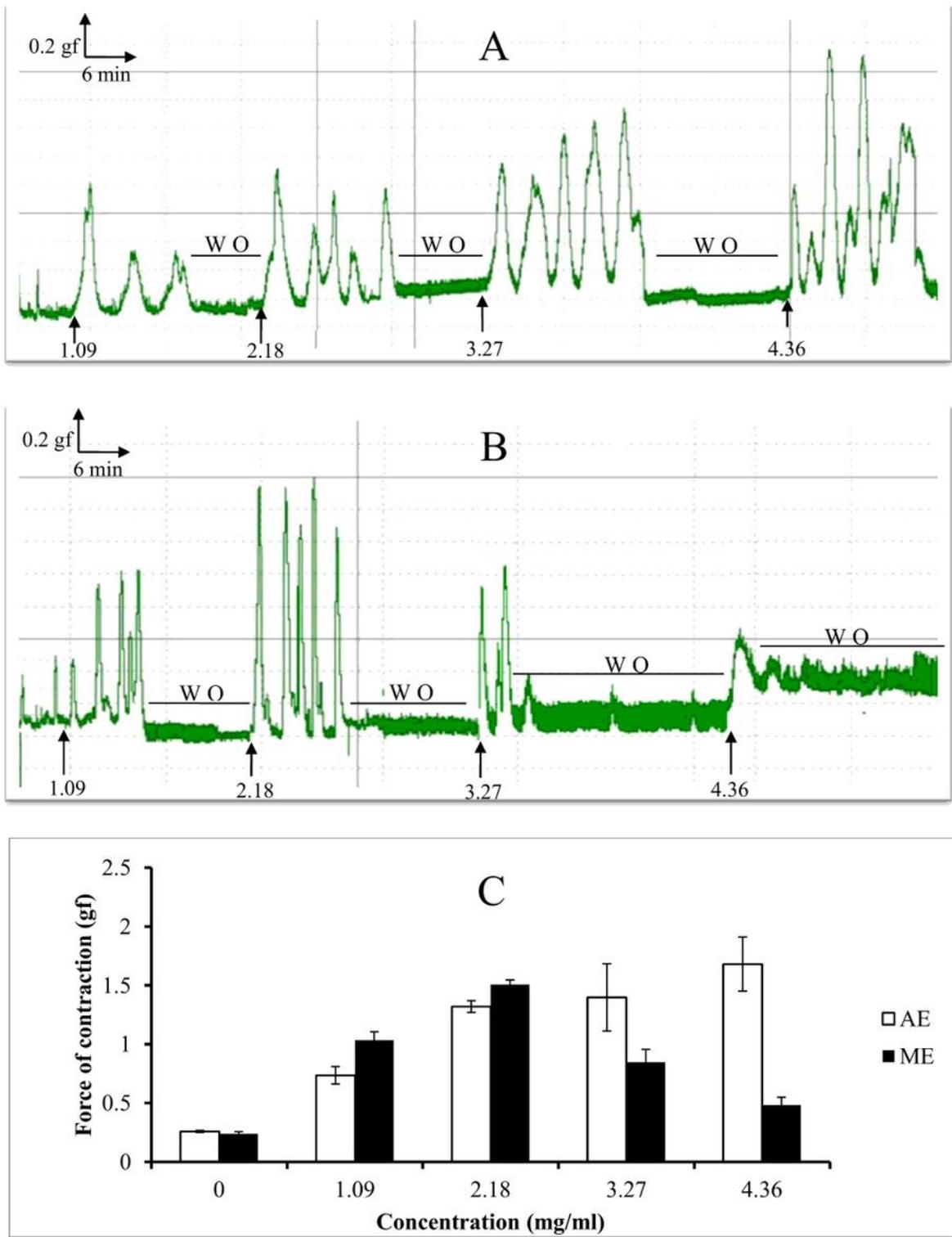


Figure 1

Effects of aqueous (A) and methanolic (B) extracts of *L. acida* on isometric uterine contractions. C: mean force contraction generated from five isolated uterine horns obtained from different oestrogenized rats, which were exposed to various concentrations of *L. acida* (0; 1.09; 2.18; 3.27 and 4.36 mg/ml) W O= washout period, AE= aqueous extract, ME= methanolic extract.

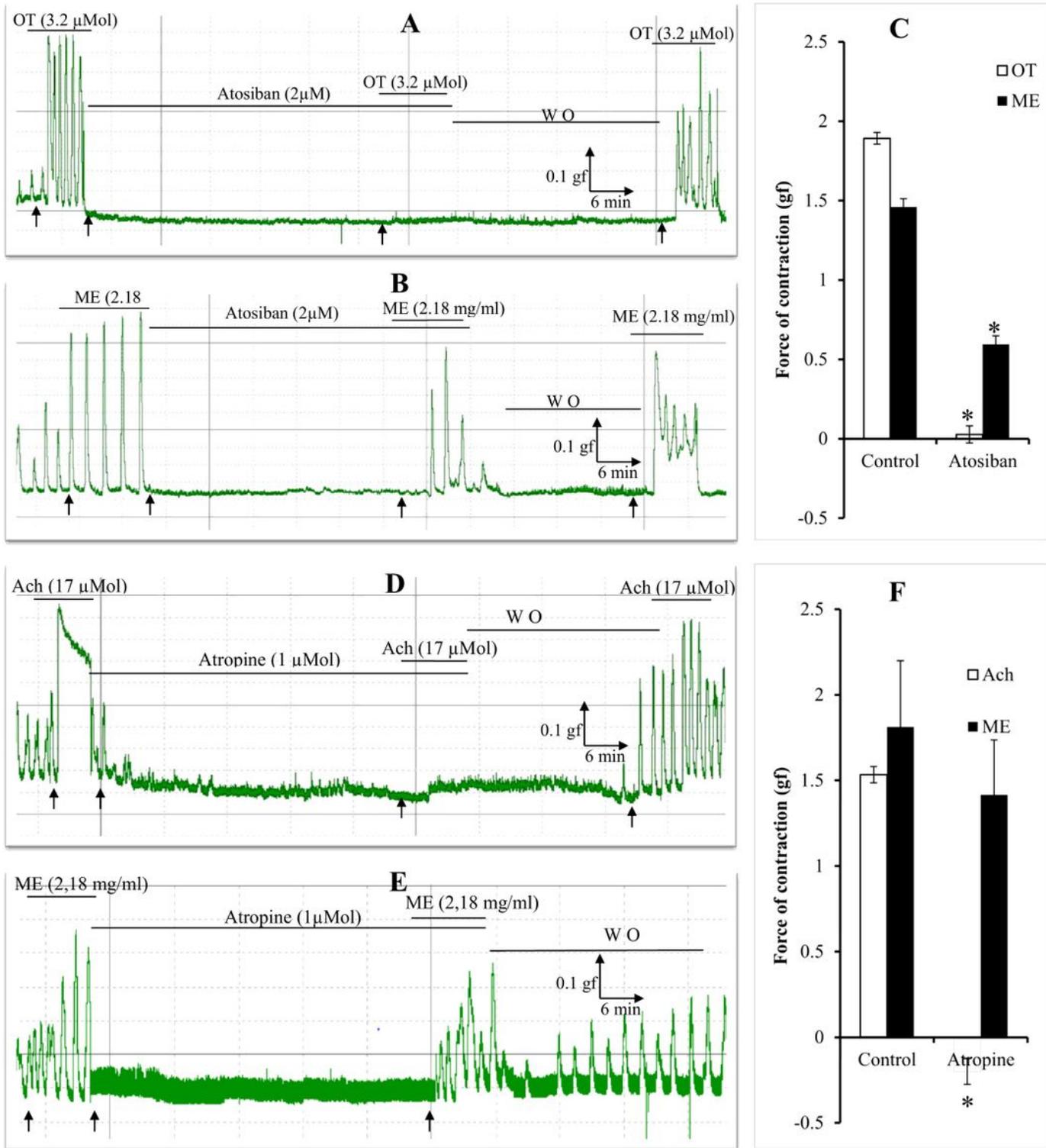


Figure 2

Effect of methanolic extract of *L. acida* and agonists in the presence of selected antagonists. (a,b) Representative tracings of isometric uterine contraction following agonists (a) and *L. acida* (b) administration in the presence of various antagonists and (c) mean Emax following administration of agonist and *L. acida*. n= 5 rats per group, ME= methanolic extract; OT= oxytocin; Ach= acetylcholine; W O= washout period.*: p<0.05 compared to control.

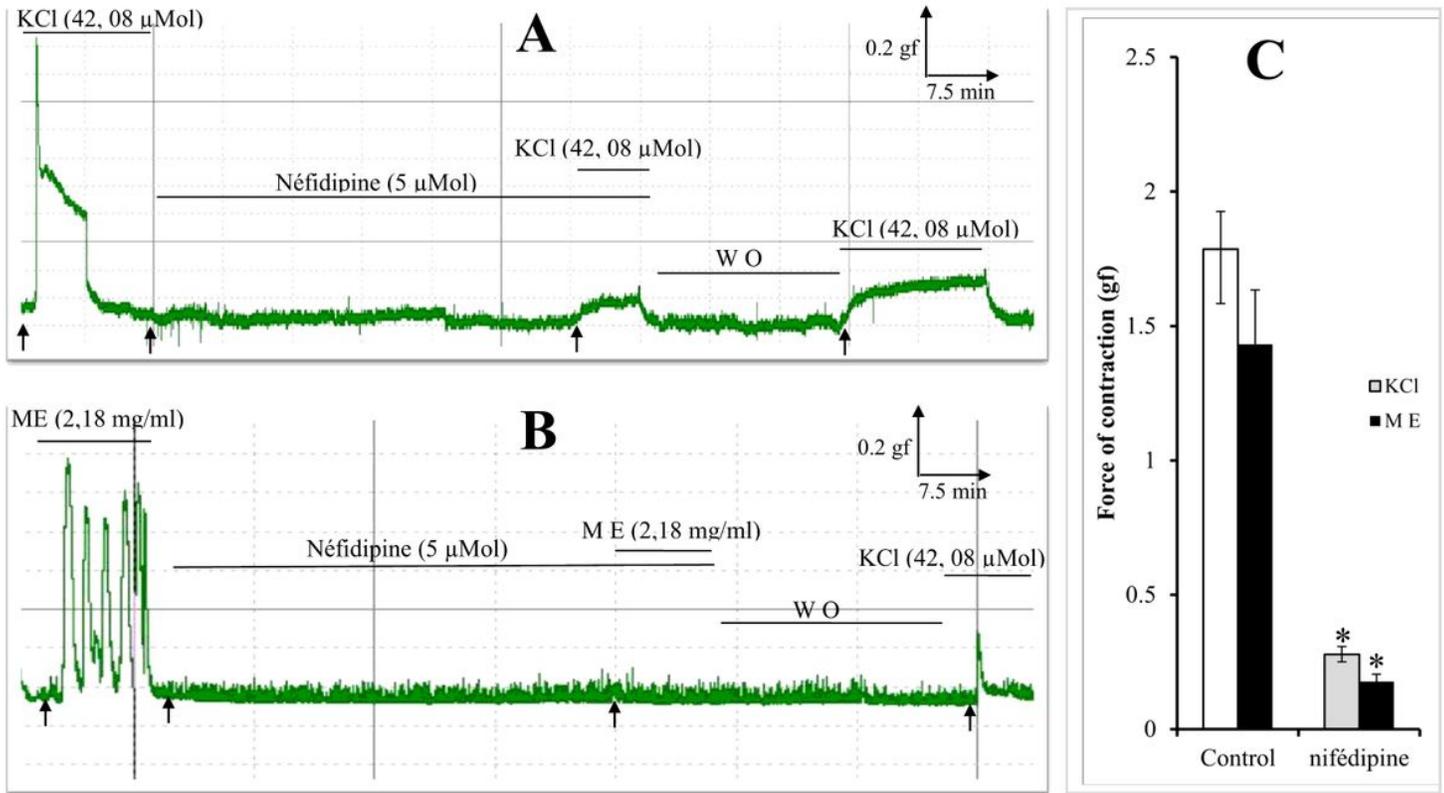


Figure 3

Effect of potassium chloride and methanolic extract of *L. acida* in the presence of nifedipine. (A,B) Representative tracings of isometric uterine contraction following potassium chloride and *L. acida* administration in the presence of antagonists and (C) mean E_{max} following administration of *L. acida* at 2.18 mg/ml and agonists alone and in the presence of nifedipine. $n=5$ rats per group, ME= methanolic extract; KCl= Potassium chloride. W O= washout period.

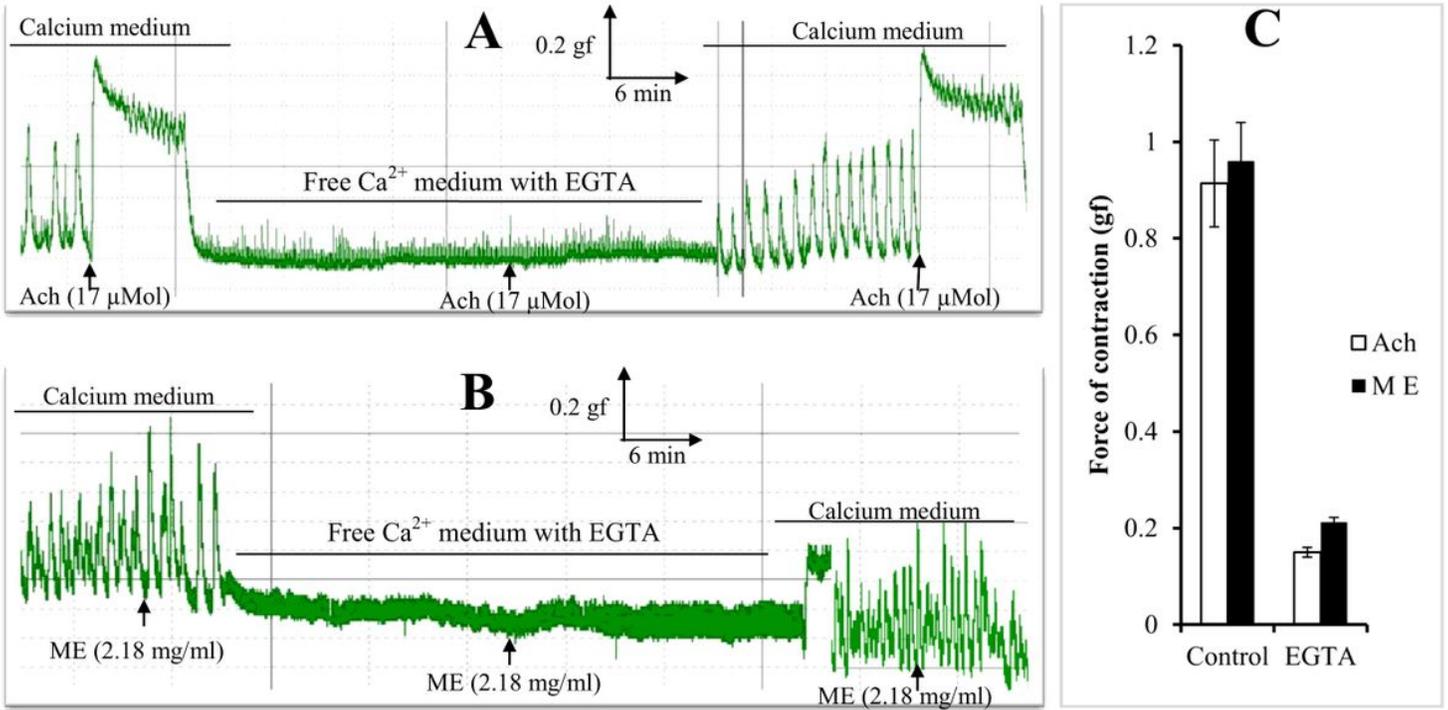


Figure 4

Representative physiographic recording of acetylcholine (A) and L. acida (B) De Jalon and in free-Ca²⁺ solution with EGTA on myometrial rat strips. Chart (C) shows the Emax of acetylcholine and L. acida drugs administrated in De Jalon and in free Ca²⁺ solution. L. acida have any effect without Ca²⁺.

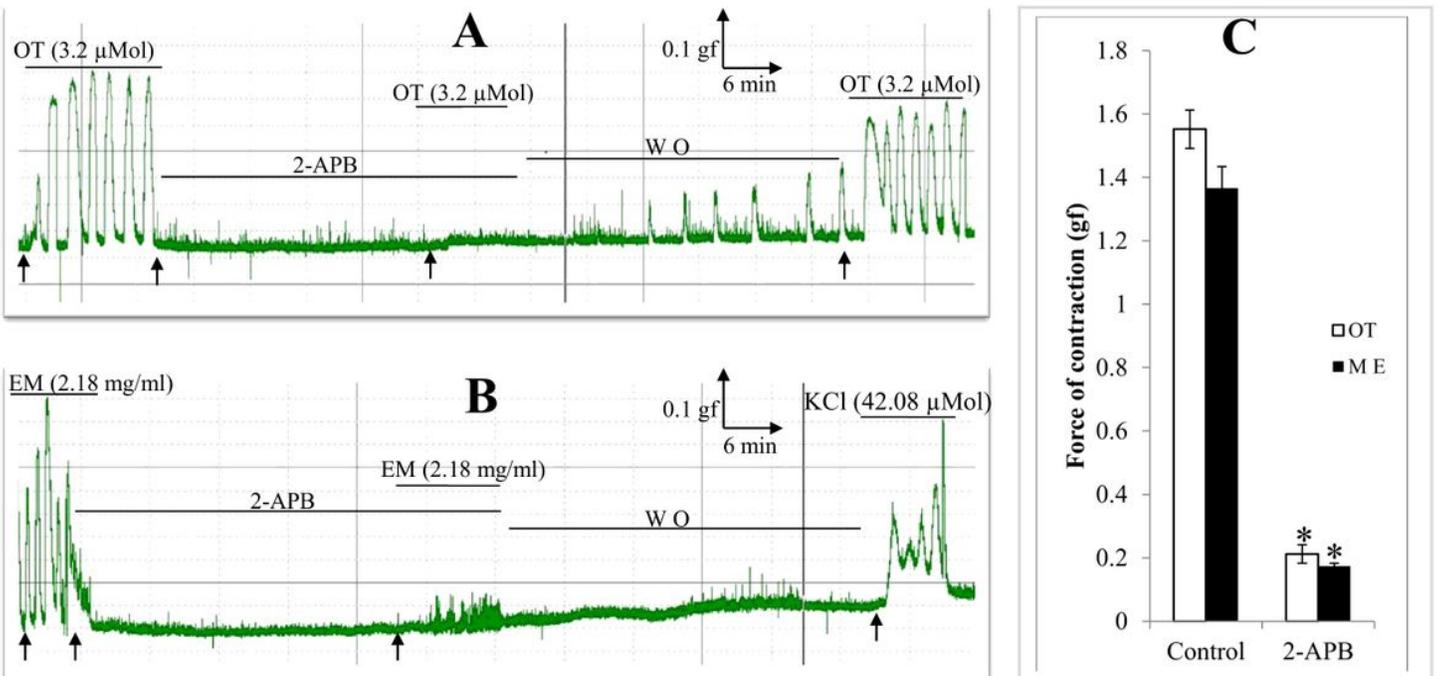


Figure 5

Effect of IP3 receptors blocker on uterine contraction induced by oxytocin (A) and methanolic extracts of *L. acida* (B). (C) Show the chart of oxytocin and *L. acida* drugs administrated. OT= ocytocine and M E = methanolic extract. W O= washout period.