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Original Research Article

In vitro inhibitory effect of methanol leaf extract of Stachytarpheta jamaicensis (Verbenaceae) on non-pregnant rat uterus

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Abstract

Purpose: *To investigate the effect of the methanol extract of the plant, Stachytarpheta jamaicensis (Verbenaceae) (SJ) on uterine smooth muscles of non-pregnant female rats, with the aim of examining the oxytocic or otherwise effect of the extract.*
Methods: *In the first phase of experiments, the effects of SJ (0.41 and 4.01 mg/ml) on oxytocin (OT) induced uterine contractions were determined and repeated after addition of salbutamol (SBL) (41.7 nM). In the second phase, verapamil (VER) (2.03 µM), SBL (41.7 nM), and SJ (0.41 and 4.01 mg/ml) were applied to the tissues after pre-contraction with K+ (80 mM). In the third phase, the effects of VER, SBL, and SJ on $CaCl_2$-induced contractions (0.03 – 10.83 mM) were examined. In the fourth and final phases, the second phase experiments were repeated in a calcium-free medium and in the absence and presence of propranolol (1.54 mM) respectively.*
Results: *SJ exhibited significant inhibitory effects on OT and $CaCl_2$ induced uterine contractions ($p < 0.05$). The EC_{50} for OT increased from 1.92 ± 0.12 to 7.16 ± 0.16 nM and that for $CaCl_2$ increased from 0.19 ± 0.09 to 0.76 ± 0.11 mM in the presence of the extract. SJ also significantly inhibited KCl- induced contraction by 39.15 ± 2.13 and 53.23 ± 1.58 % for 0.41 and 4.01 mg/ml of the extract, respectively ($p < 0.01$); this inhibition was unaffected by propranolol. On the other hand, SBL and VER showed inhibition of 77.25 ± 1.85 and 79.44 ± 2.27 %, respectively.*
Conclusion: *SJ exerts uterine inhibitory effects in rats which appear unrelated to β_2-adrenergic receptor stimulation but possibly through inhibition of calcium entry into the cytoplasm.*

Keywords: *Oxytocin, Calcium-free, Uterine contraction, β_2-Adrenergic receptor stimulation, verapamil, salbutamol, Propranolol, S. jamaicensis*

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INTRODUCTION

Starchytarpeta jamaicensis (L.) Vahl (Verbanaceae) popularly called Gervao, Brazillian tea and Bastard vervain is used extensively by indigenous people throughout the Amazons and tropical countries for the treatment of various ailments with a wide range of uses in ethnomedicinal practice. The plant has been reported to have reasonable antimicrobial and insecticidal properties [4]. Previous laboratory studies have verified its longstanding use in the treatment of gastrointestinal tract, and cardiovascular disorders. The aqueous extract of the leaves has been shown to cause a dose dependent decrease in the mean systolic, diastolic; mean arterial pressure and heart rate in normotensive male albino rabbits [5]. *S. jamaicensis* is also claimed to have abortifacient properties. Anecdotal evidence indicate that and

some Nigerian communities use some parts of the plant to induce abortion. Despite the claim of its abortifacient activity in traditional medicine practice, no record of scientific validation has been reported to our knowledge. This work was thus set out to investigate the effect of the methanol leaf extract of *S. jamaicensis* (SJ) on the uterine smooth muscles of non-pregnant adult female Sprague-Dawley rats, and possibly establish the mechanism(s) of its uterine effects.

EXPERIMENTAL

Drugs and chemicals

Diethylstilboesterol, salbutamol and propranolol were obtained from Sigma (UK), oxytocin (Laborate Pharmaceuticals, India), verapamil (May and Baker, UK). These were all prepared fresh on the day of the experiment.

Plant preparation and extraction

The leaves of SJ were collected from the premises of the University of Benin, Benin City, Nigeria, identified and authenticated by Professor Macdonald Idu of the Department of Plant Biology and Biotechnology of the University of Benin, Nigeria. The plant was provided with a herbarium number of UBHv0259a and a herbarium specimen was prepared and stored in the Herbarium Section of the Department of Plant Biology and Biotechnology, University of Benin. The leaves of the plant were washed free of earthy materials, air dried for seven days and further dried in an oven at 40 ºC for 2 h. A portion of the powdered plant material (500 g) was extracted with methanol, using cold maceration for 72 h and the extract was then concentrated to dryness in an oven maintained at 40 ºC. The resulting yield was calculated as 20.75 %.

Animal experiments

Adult female Sprague-Dawley rats (170-200 g) bred in the animal unit of the Department of Pharmacology and Toxicology, University of Benin were used. Animal care and experimental procedures complied with standard guidelines for use of laboratory animals (National Institute of Health USA: Public Health Service Policy on Humane Care and Use of Laboratory animals, 2002) [6]. This involved maintenance of animals under standard conditions with free access to standard diet (Bendel Feeds and Flour Mill, Ewu, Nigeria) and water. Ethical clearance was obtained from the Ethical Committee for Animal Studies, Faculty of Pharmacy, University of Benin, Benin City prior to commencement of the study.

Preparation of animals for uterine studies

Uterine segments of the rats were obtained and prepared for the *in vitro* studies as previously described [7]. Briefly, the animals were treated with diethylstilboesterol (0.2 mg/kg i.p.) 24 h prior to the commencement of the experiment. The oestrus stage was confirmed by microscopic observation of vaginal smears and macroscopic observation of the vulva. Rats were sacrificed under diethylether anaesthesia and the uterine horns rapidly dissected out and placed in previously warmed and aerated physiological salt solution (PSS) composed of Cl (154 mM), KCl (5.63 mM), $CaCl_2.2H_20$ (0.648 mM), $NaHCO_3$ (5.95 mM) and D-glucose (2.77 mM). Uterine segments, 2 cm in length, were cut and freed of adhering connective tissues and fat. The segments were mounted longitudinally in 40 ml organ baths containing PSS of the following. The lower ends of the tissue were attached to tissue holders by means of silk suture and the upper ends to an isometric force-displacement transducer (Model 82145, Ugo Basile, Monvalle VA, Italy) connected to a unirecorder (Model 7050, Ugo Basile, Monvalle VA, Italy). The PSS was maintained at 37 ^oC and continuously aerated. Each uterine segment was placed under optimum resting tension of 0.75 g and equilibrated for 45 min before the start of the experiment. During the equilibration period, the preparations were washed with the PSS every 10 min [8].

Study on the inhibition of concentration – response curves to oxytocin

Concentration – response curves for oxytocin (OT) (0.79 – 159.88 M) were obtained for the uterine tissues in the absence and presence of salbutamol (SBL) (41.7 nM) or SJ (0.41 and 4.01 mg/ml).

Study on the inhibition of K^+-induced contraction

To determine the possibility of calcium channel blockade by SJ, the uterine preparations were depolarized with K^+ using a modified method (9). Addition of K^+ (80 mM) produced a sustained contraction to which verapamil (VER) (2.03 µM), SBL (41.7 nM) or SJ (0.41 and 4.01 mg/ml) were added cumulatively.

Study on the inhibition of $CaCl_2$-induced contractions

The effect of SJ on $CaCl_2$-induced uterine contractions was examined as previously described [10]. The uterine tissue was

equilibrated in normal PSS for 30 min, which was then replaced with Ca^{2+}-free PSS containing ethylenediamine tetra-acetic acid (EDTA, 0.1 mM) for 30 min. This solution was further replaced with K^+-rich and Ca^{2+}-free PSS, composed of KCl (50 mM), NaCl (154 mM), $NaHCO_3$ (5.95 mM), D-Glucose (2.77 mM), $CaCl_2.2H_2O$ (0.648 mM) and EDTA (0.1 mM). The tissues were re-equilibrated for a period of 30 min. Concentration – response curves (CRCs) for Ca^{2+} (0.03 – 10.83 mM) alone were constructed and regarded as control. The tissues were subsequently incubated with SJ (0.41 and 4.01 mg/ml) for 15 min and without washing, the CRC to Ca^{2+} was repeated. This was necessary in order to examine the possible calcium channel blocking effect of the extract. The CRCs of Ca^{2+} were also repeated in the presence of VER (2.03 µM) and SBL (41.7 nM) in separate experiments.

Study on the inhibition of OT-induced contraction in Ca^{2+}- free PSS

A modified method [11] was employed for this phase of the study. Briefly, the uterine tissues were equilibrated for 60 min under a resting tension of 0.7 g and washed every 15 min. Subsequently, the medium was changed to Ca^{2+}-free PSS containing 0.1 mM EDTA and the tissues were further equilibrated for 50 min with intermittent washings every 10 min. Cumulative CRC to OT (0.04 – 3.19 mM) was repeated in the presence of SJ, VER, and SBL in concentrations previously mentioned.

Study on the effect of propranolol on inhibition of oxytocin – induced contraction

Propranolol (1.54 mM) was added to the organ bath for 15 min, thereafter CRCs to oxytocin was determined in the presence of SJ, SBL or VER in concentrations previously mentioned.

Curve fitting and statistical analyses of contraction assays

Values were presented as mean ± standard error of mean (SEM) and n represented the number of animals for each set of experiments. The responses were expressed as a percentage of control amplitude (g) of uterine contractions. In datasets 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 4.0 (GraphPad Software, San Diego, CA, USA) to determine the pEC50 values (Y = Bottom + (Top-Bottom)/(1+10^((LogEC50-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} is the concentration that produces half the maximal responses. Average potencies for frequency and amplitude of contractions were calculated and compared after curve fitting where possible. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparison tests. $P \leq 0.05$ indicated statistical significance in all cases.

RESULTS

Inhibition of concentration–response curves to oxytocin

SJ significantly ($p < 0.05$) inhibited OT-induced uterine contractions. This was observed in the rightward shift of the CRC to OT, significant increase ($p < 0.05$) of the EC_{50} (Table 1) and reduction of the E_{max} (Fig. 1) of OT in a concentration-dependent manner. Similarly, SBL and VER significantly increased ($p < 0.01$) the EC_{50} (Table 1) and reduced the E_{max} (Figure 1A) of OT respectively.

Inhibition of $CaCl_2$- induced contractions

SJ shifted the CRC of $CaCl_2$-induced uterine contractions to the right (Figure 1B) and significantly increased ($p < 0.05$) the EC_{50} (Table 1) and reduced the Emax (Figure 1B).VER, and SBL also significantly inhibited $CaCl_2$ ($p < 0.01$) (Figure 1B and Table 2).

Inhibition of K^+ - induced contraction

SJ significantly inhibited ($p < 0.01$) the sustained uterine contraction produced by, K^+ (80 mM) in a concentration-dependent manner (Table 2). VER, and SBL also produced similar but more potent inhibition of K^+ ($p < 0.01$) (Table 2).

Inhibition of OT-induced contraction in Ca^{2+}-free PSS

SJ and VER did not significantly inhibit OT-induced contractions in Ca^{2+} free medium (Figure 3). It was also observed that there was no significant difference in the EC_{50} (Table 3). However, SBL was observed to produce significant reduction ($p < 0.01$) in the EC_{50} of OT (Table 3) and a rightward shift in the CRC of OT (Figure 2).

Effect of propranolol on inhibition of oxytocin–induced contraction

After addition of propranolol (1.54 mM) for 15 min, it was observed that propranolol failed to attenuate the effect of SJ and VER on OT-

induced contractions; however the effect of SBL was antagonized in the presence of propranolol (Table 3).

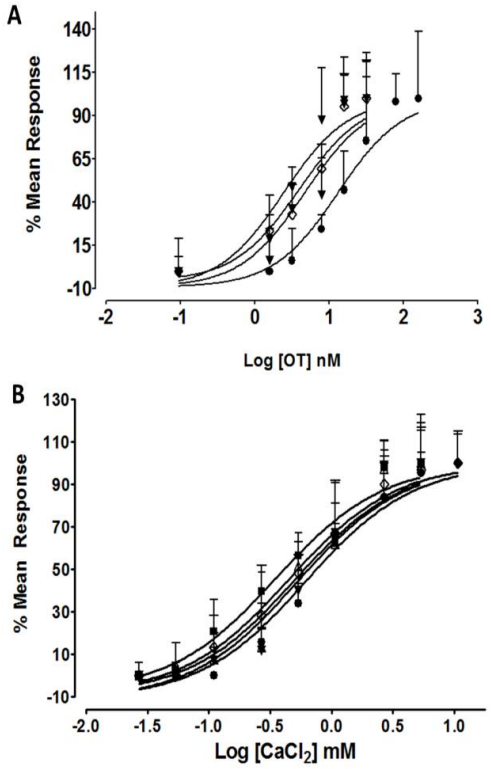

Figure 1: Concentration–response curves (CRC) for OT in the presence and absence of SJ and SBL (A) and CRC for $CaCl_2$ in the presence and absence of SJ, VER and SBL (B). n= 6 rats. SJ shifted the CRC of OT and $CaCl_2$-induced uterine contractions to the right and significantly reduced ($p<0.05$) the EC_{50} (Table. 1) and E_{max}. VER and SBL also significantly inhibited $CaCl_2$ and OT ($p<0.01$). For Figure 1A: ■ OT alone; ▲OT + SJ (0.41 mg/ml); ▼OT + SJ (4.01 mg/ml); ♦ OT + SBL (41.7 nM). For Figure 1B: ■ $CaCl_2$ alone; Δ $CaCl_2$ + SJ (0.41 mg/ml); ▼ $CaCl_2$ + SJ (4.01 mg/ml); ◊ $CaCl_2$ + VER (2.03 µM); ● $CaCl_2$ + SBL (41.7 nM)

Table 1: EC_{50} values obtained from the concentration–response curves to OT and $CaCl_2$ in the rat uterus

Drugs	EC_{50}
OT	
OT alone (Control)	1.92 ± 0.12 (nM)
OT + SJ (0.41 mg/ml)	6.59 ± 0.23 (nM)*
OT + SJ (4.01 mg/ml)	7.16 ± 0.16 (nM)*
OT + SBL (41.71 nM)	24.54 ± 0.16 (nM)**
$CaCl_2$	
$CaCl_2$ alone (Control)	0.19 ± 0.09 (mM)
$CaCl_2$ +SJ (0.41 mg/ml)	0.47 ± 0.11 (mM)^
$CaCl_2$ + SJ (4.01 mg/ml)	0.51 ± 0.25 (mM)^
$CaCl_2$ + SBL (41.71 nM)	0.76 ± 0.11 (mM)^^
$CaCl_2$ + VER (2.03 µM)	0.92 ± 0.18 (mM)^^

*$P < 0.05$; **$p < 0.01$ all compared to OT alone; ^$p < 0.05$; ^^$p < 0.01$ all compared to $CaCl_2$ alone; n = 6 rats

Table 2: Effect of *S. jamaicensis* (SJ), verapamil (VER) and salbutamol (SBL) on K^+ - induced sustained contraction

Concentration	Inhibition of KCl (%)
SJ (0.41 mg/ml)	39.15 ± 2.12**
SJ (4.01 mg/ml)	53. 23 ± 1.58**
SBL (41.71 nM)	77.25 ± 1.85**
VER (2.03 µM)	79.44 ± 2.27**

**$P < 0.01$ compared to K^+ alone (80 mM) = 100%; n= 6 rats

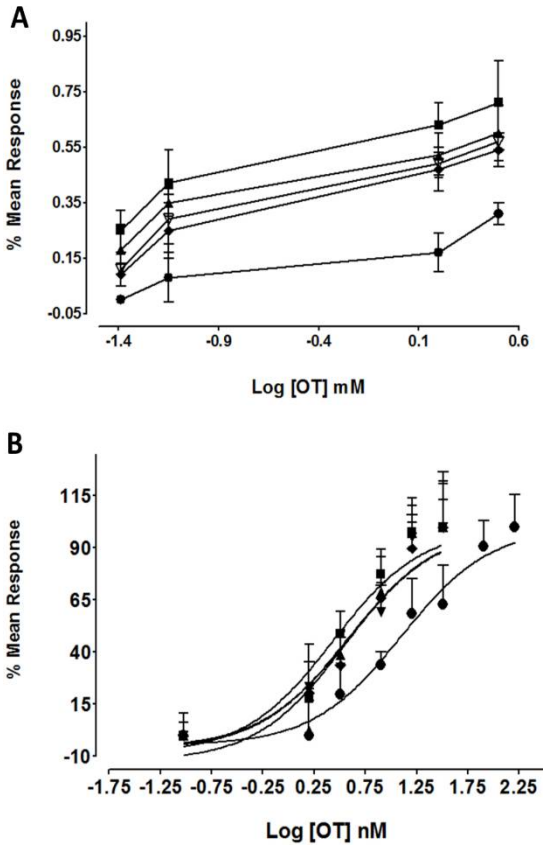

Figure 2: Concentration – response curves for OT in Ca^{2+} free medium (A) and in the presence of propranolol (B). SJ and VER did not significantly inhibit OT-induced contractions (A). However, SBL produced a rightward shift in the CRC of OT (B). In the presence of propranolol (1.54 mM), the effect of SJ on OT was not attenuated however the effect of SBL on OT was significantly ($p < 0.05$) overcome (B); n = 6 rats. ■ OT alone; ▲OT + SJ (0.41 mg/ml); ▼OT + SJ (4.01 mg/ml); ♦ OT + SBL (41.7 nM); ● OT + VER (2.03 µM)

DISCUSSION

The methanol leaf extract of *S. jamaicensis* (SJ) has been shown in this study to inhibit oxytocin-induced uterine contractions. On comparison, a similar effect was observed with salbutamol and verapamil both of which possesses inhibitory actions on uterine contraction.

Salbutamol is a ß-adrenoceptor agonist that acts

Table 3: EC_{50} values obtained from the CRC to OT in Ca^{2+} free medium and in the presence of propranolol (1.54 mM)

Drug	EC_{50} (in Ca^{2+} free medium)	EC_{50} (in presence of propranolol)
OT alone	0.51 (mM)	1.96 ± 0.17 (nM)
OT + SJ (0.41 mg/ml)	0.66 (mM)	6.63 ± 0.14 (nM) **
OT + SJ (4.01 mg/ml)	0.69 (mM)	7.18 ± 0.31 (nM) **
OT + SBL (41.71 nM)	1.47 (mM)*	2.02 ± 1.63 (nM)
OT + VER (2.03 µM)	0.58 (mM)	15.87 ± 0.76 (nM) **

** $p < 0.01$ compared to OT alone; n = 6 rats; ** $p < 0.01$

physiologically to antagonize contractions of the uterus this effect makes it a clinically useful tocolytic agent [11], while verapamil, a phenylalkylamine calcium channel blocker also antagonizes contractions of the uterus by inhibition of voltage-dependent calcium channels [12]. Oxytocin contracts the uterine smooth muscle by stimulating specific receptors, which amongst other actions activates second messenger systems to release intracellular stored calcium from the sarcoplasmic reticulum [13].

Thus SJ may have inhibited the calcium influx into the cell cytoplasm or interfered with one of the biochemical processes associated with the influx of calcium into the myometrial smooth muscle cells. This observation was further supported by the concentration-dependent relaxant effect of SJ on high K^+-induced contractions. Relaxation of K^+-induced sustained contractions by SJ just like the Ca^{2+} channel blocker, verapamil suggests possible calcium channel blockade by SJ [14]. Contraction of the isolated uterine smooth muscles by high K^+ in extracellular fluid is known to depolarize smooth muscle membranes, and to open the voltage - operated calcium channels (VOCs), in particular, the L-type calcium channel [13,14]. The resultant effect is the influx of calcium into the smooth muscle cells which culminates in contraction. The contraction is usually a biphasic response that consists of an initial, rapid, transitory contraction, the phasic response, followed by a slower more sustained contraction, the tonic response (14). This mechanical response to high K^+ is completely inhibited by several calcium channel blockers through blockade of L-type channel. Inhibition of both OT and $CaCl^2$-induced contractions may indicate that the spasmolytic principle contained in the extract is not a specific receptor antagonist.

Possible intracellular activity of SJ was examined by observing the influence of SJ on contractions induced by OT in Ca^{2+} free PSS. The contractions obtained in these conditions, are described as related only to release of calcium from intracellular stores [14]. Interestingly, SJ and VER were found in this study to show insignificant inhibition and it has been reported that nifedipine (also a Ca^{2+} channel antagonist) failed to inhibit the sustained contractions induced by OT in Ca^{2+} free PSS [15]. SBL however was observed to inhibit OT under this condition. This observation is supported by reports that $ß_2$-stimulants inhibit OT-induced contractions in Ca^{2+} free medium [16].

The possibility of ß- adrenoceptor activation by the extract of SJ was also investigated and it was observed that SJ as well as VER inhibited OT in the presence of propranolol. However, SBL failed to produce similar response in the presence of propranolol, apparently due to blockade of ß-adrenoceptors. This result suggests that the inhibitory effect of SJ is completely unrelated to ß-adrenoceptor activity.

CONCLUSION

The findings of this study reveal that at concentrations used in this study, SJ inhibits OT-induced uterine contractions in diethylstilboesterol-pretreated rats. This inhibitory effect is unrelated to β-adrenoceptor stimulation but might be through the inhibition of extracellular calcium influx or by some other mechanism which affects calcium influx and utilization in the uterine smooth muscle. Further studies however, are needed to determine the precise mechanism of uterine relaxation and to examine other fractions of the plant for activity on the uterus.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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