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STIMULATION OF AUTONOMIC CHOLINOCEPTORS IN THE RAT UTERUS BY A CRUDE EXTRACT FROM *ELEOPHORBIA DRUPIFERA* LEAVES

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ABSTRACT

The crude extract from *E. drupifera* was tested for its effects on isolated rat uterine preparations. The extract (2.0–300 µg/ml) produced graded increases in uterine contractions. The contractions were potentiated by neostigmine ($1.64 \times 10^{-6}M$) and abolished by atropine ($4.4 \times 10^{-4}M$). The extract-induced contractions were not prevented by previous addition to the bath of hexamethonium or 5-hydroxytryptamine and bradykinin antagonist. However, extract-induced increase in uterine contraction was abolished by either lidocaine ($4.2 \times 10^{-5}M$) or tetrodotoxin ($5 \times 10^{-8}M$). These data seem to indicate that the crude extract-induced increase in myometrial contractility is due to actions on post-ganglionic autonomic nerve endings, with acetylcholine release and stimulation of muscarinic receptors.

INTRODUCTION

Plants of the Euphorbiaceae are frequently used in the indigenous system of medicine. Little is known about the pharmacological actions of the species *Elaeophorbium drupifera* (Thonn.) Stapf, although it is listed among the plants that “heal” (Ampofo, 1977). The leave is used as a filaricide and for guinea worm infestation (Comley, 1990). It is also reported to contain hypoglycemic agent(s) (Eno & Itam, 1995). The fruit is succulent (Kinghorn & Evans, 1974) but the latex has skin irritant effects (Kinghorn & Evans, 1975).

Apart from the use of *E. drupifera* leaves for the treatment of various ailments, traditional herbalists claim it is also effective in aiding or inducing labor or abortion. Ground leaves (paste) are dissolved in either water or soft drink (cola), and administered orally in doses determined by age.

Also, although the innervation of the mammalian uterus has been studied extensively, precise information regarding the intrinsic autonomic neural pathways and the relationship between the cholinergic and adrenergic components is lacking (Adham & Schenk, 1969; Garfield, 1986). Much work has been devoted to uterine sympathetic nerves with no corresponding interest concerning the cholinergic nerves, despite evidence for cholinergic innervation in the uterus of different mammalian species, particularly rat (Stjernquist & Owman, 1985; Hollingsworth, 1974; Adham & Schenk, 1969).

The main objective of this paper was to study the effects of *E. drupifera* leaf extract on isolated rat uterus in an attempt to elucidate the scientific basis for justifying the action of the herb as claimed by the herbalists, and possibly, speculate on its probable mode of action in the uterus. Some mediators of smooth muscle action outside the cholinergic and adrenergic systems, together with their specific antagonists, have also been considered.

MATERIALS AND METHODS

Preparation of the Crude Extract

Fresh leaves of *E. drupifera* were collected. The leaves were first washed free of soil and debris. Wash water was blotted off and the leaves ground to a paste. A quantity of the ground sample (50 g) was weighed and Soxhlet-extracted with 150 ml distilled water at 100°C for 8–10 h. Where larger ground samples (500–1000 g)

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were used, extraction was done under reflux with an appropriate volume of water. The extract was slowly evaporated to dryness *in vacuo* at 40°C using a rotary evaporator. A starting sample of 50 g of fresh material gave a mean yield of 1.58 ± 0.47 g of extract (mean \pm SEM, $n = 10$) which was stored at -4°C until use. Weighed samples of the extract were then used to make up test solutions of the desired concentrations (mg/ml) (expressed in terms of the weight of extract).

Animal Preparation

Eighty non-pregnant female white Wistar rats weighing 180–250 g were treated with stilboestrol (1 mg/kg i.p.) 24 h prior to the experiments. The animals were killed by a single blow to the head. The abdomen was opened and the uterine horns were removed, freed of fat, and immersed in a modified Tyrode solution with the following composition in mmole/liter: NaCl 136.8; KCl 2.7; MgCl₂ 2.1; CaCl₂ 0.72; NaH₂PO₄ 0.4; NaHCO₃ 11.9; glucose 5.5 (pH 7.4).

One uterine horn segment measuring 3 cm was suspended in a 20 ml organ bath containing Tyrode solution, bubbled with atmospheric air and maintained at $37 \pm 1^\circ\text{C}$. The isometric recording of uterine musculature activity was done with a FT 03 transducer connected to a Washington recorder (model 400 D). The transducer was previously calibrated to establish a relationship between the force applied to the transducer and the gauge deflection (1 g = 25 mm). The preparations were allowed to equilibrate in the bath for 30 min before the experiments.

E. drupifera leaves extract, agonists, and antagonists drugs were directly added to the reservoir containing the uterus. The tissue preparation was washed once with the bathing solution (Tyrode solution) after addition of the extract or drugs, and allowed to rest for 2 min before the next addition. The effects of the extract were compared with those of the other substances utilized and the results were analyzed based on the amplitude of contractions (expressed as tension in grams) in the absence or presence of antagonists.

Statistical Analysis

Regression lines with confidence limits were calculated for the linear portions of log-concentration response curves. The log-concentration limits at 50% of the maximum response were used in the analysis of the significance of concentration differences as described by Birmingham et al. (1970). Maximum responses were compared by unpaired Student's *t*-test.

Drugs

Nicotine alkaloid (95–98%), atropine sulphate, hexamethonium bromide, tetrodotoxin, 5-hydroxytryptamine (5-HT), and acetylcholine chloride, were from Sigma (U.S.A.). Lidocaine hydrochloride (Cristalia, Brazil); neostigmine bromide (Roche, Brazil); bradykinin, methysergide and oxytocin were from Sandoz, Brazil. HOE 140:D-Arg [Hyp-3-di-5-D-Tic-7-Oc-8] bradykinin was from Hoechst, Japan.

RESULTS

Effects of Extract on Uterine Contractions

The rat uteri showed spontaneous and rhythmic contractions in Tyrode solution. Although the amplitudes of these contractions varied from one preparation to another, the mean amplitude was about 15.4 ± 3.8 mm (about 0.61 g tension), and this was regarded as the control tension.

Investigations have shown that the crude extract from *E. drupifera* leaves (2–300 µg/ml) dose-dependently increased the amplitude of uterine contractions with an ED₅₀ value of 23.6 µg/ml (Fig. 3, control). High doses of the extract (above the ED₅₀ value) produced sustained contractions which were characterized by a rise in the basal tone of the tissue preparation. Based on this ED₅₀ value, test doses of 5 and 30 µg/ml were selected.

The latency of response was less than 2 s in all tissue preparations studied. When the tissue was exposed to the extract for about 30 min without wash, the effect of the extract (15 µg/ml) on the tissue was maintained to about 58.5% at the end of 30 min exposure. However, a longer period (about 2 h) was required before the tissue could return to its resting tension. However, it was possible to terminate the action of the extract as desired, by washing the preparation with Tyrode solution.

Effect of Drugs on Extract-induced Uterine Contractions

Results are summarized in Table 1.

Atropine Sulphate and Neostigmine

Addition of neostigmine (1.64×10^{-6} M) to the bath did not evoke any response. However, neostigmine (1.64×10^{-6} M) in Tyrode solution, potentiated the effects of the extract (5 µg/ml) (Fig. 1b) and acetylcholine (ACh) (1.1×10^{-7} M) (Fig. 1c). When the extract or ACh was added to neostigmine (1.64×10^{-2} M) - treated uterus, sustained contractions were observed (Figs. 1b and c). However, atropine sulphate (4.4×10^{-4} M) abolished

Table 1. Influences of drugs on the contractions induced by the crude extract, acetylcholine, oxytocin, 5-HT and bradykinin on isolated rat uterus.

Treatment	N	Extract (30 µg/ml)	Acetylcholin (1.1×10^{-8} M)	Oxytocin (1×10^{-2} UI)	5-HT (2×10^{-5} M)	Bradykinin (3.8×10^{-10} M)
None	10	2.58 ± 1.85	2.44 ± 2.09	2.62 ± 2.31	2.67 ± 2.57	2.55 ± 3.06
TTX (5×10^{-8} M)	10	0	1.42 ± 1.76	—	—	1.35 ± 1.78
Lidocaine (4.2×10^{-5} M)	10	0	1.55 ± 2.45	1.69 ± 1.48	—	1.71 ± 2.59
Atropine (4.4×10^{-4} M)	12	0	0	2.41 ± 2.63	—	1.67 ± 1.75
Hexamethonium (2×10^{-6} M)	8	2.64 ± 2.58	2.73 ± 1.93	1.72 ± 3.11	—	—
Methysergide (1.1×10^{-5} M)	5	2.23 ± 1.97	2.35 ± 1.55	—	0	1.78 ± 2.45
Hoe140 (7.6×10^{-8} M)	8	2.41 ± 3.32	1.75 ± 2.66	—	—	0

The isolated rat uterus was immersed in a 25 ml Tyrode solution at $37 \pm 1^\circ\text{C}$ and constant oxygenation.

N = Number of experiments. The blockade is expressed as 0, — indicates experiments were not performed.

The values for each group are the means \pm S.E.M. of the tension (grams).

the sustained contractions produced by the extract or ACh (Figs. 2a and b), but failed to abolish oxytocin-induced contractions (Fig. 2f).

Tetrodotoxin (TTX)

The addition of the crude extract (30 µg/ml) to the bath solution caused sustained contractions of the uterus. This effect was abolished by TTX (5×10^{-8} M) (Fig. 2d). After repeated washing, the uterus started to con-

tract again with the same pattern as observed in TTX-free preparations. TTX also abolished oxytocin-induced contractions (Fig. 2e).

Lidocaine

When lidocaine hydrochloride (4.2×10^{-5} M) was added to the bath, the contractile effect of the extract was abolished (Fig. 2c). However, the tissue recovered from this effect after repeated washing.

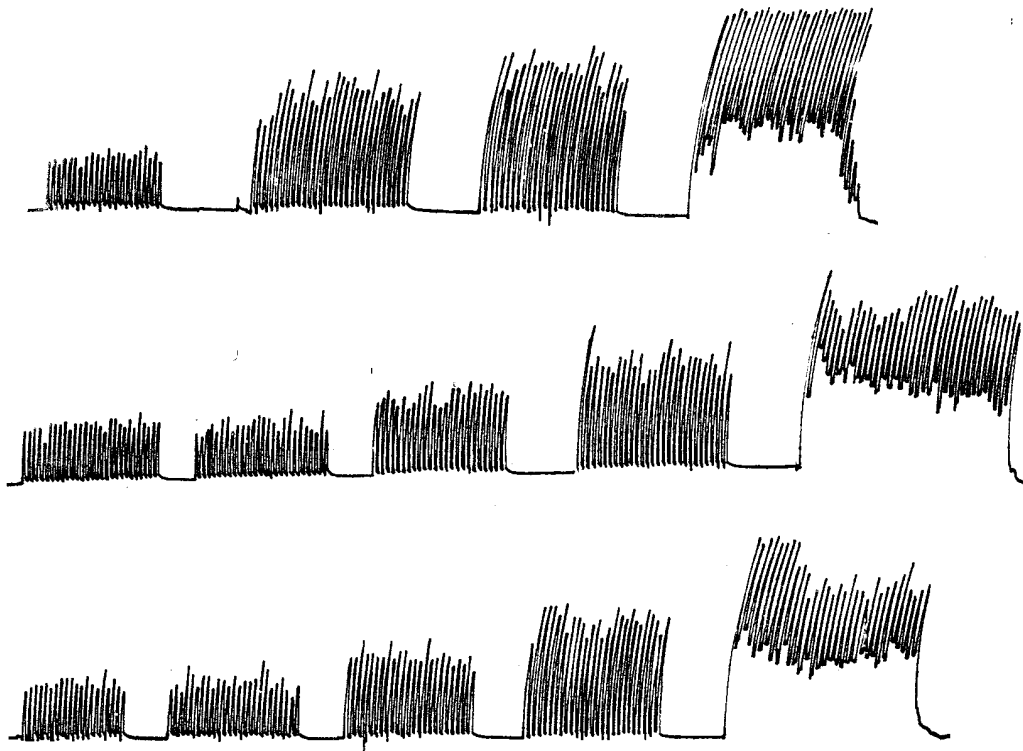


Fig. 1. Typical recordings showing the effects of the extract (5, 15 and 30 µg/ml) on the isolated uterine muscle (a); the potentiation of extract's effect on the tissue by neostigmine (1.64×10^{-6} M and 1.64×10^{-2} M) in Tyrode solution (neo-Tyr) (b); and of ACh-induced contractions (c). The tissue preparation was washed after each drug treatment.

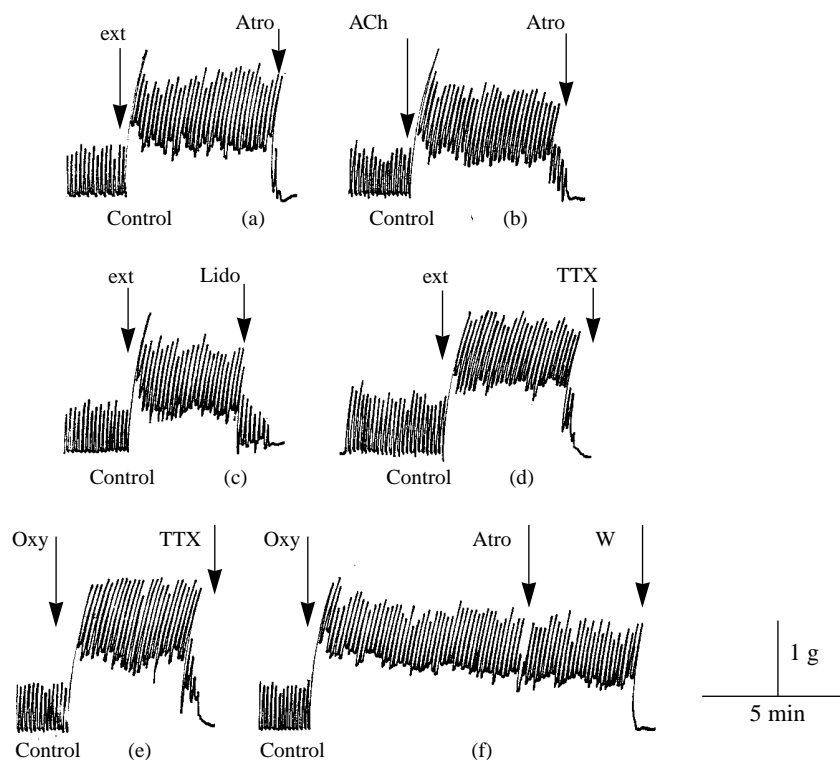


Fig. 2. Mechanical recordings showing the effects of atropine (4.4×10^{-4} M) (a); lidocaine (4.2×10^{-5} M) (c); TTX (5×10^{-8} M) (d) on the contractions induced by the extract (30 μ g/ml). Atropine also blocked ACh-induced contractions (b). Contractions caused by oxytocin (1×10^{-2} M) were blocked by TTX (5×10^{-8} M) (e); but not by atropine (4.4×10^{-4} M) (f). The tissue was washed (W) with Tyrode solution after each drug treatment.

Methysergide (5-HT Blocker)

5-Hydroxytryptamine (2×10^{-5} M) produced increased uterine contractions which were prevented by methysergide (1.13×10^{-5} M). This dose of methysergide failed to prevent the increase in uterine contractions produced by the extract (30 μ g/ml) (not shown).

Hexamethonium (Hex.)

The ganglion blocker, Hex. (1.9×10^{-6} M) failed to abolish the contractions produced by the extract (30 μ g/ml) or acetylcholine (1.1×10^{-7} M) (not shown).

Nicotine

Nicotine (from 1×10^{-7} to 1×10^{-3} M) did not increase uterine contractions (not shown).

Bradykinin Blocker (HOE 140)

The addition of 7.6×10^{-8} M HOE 140 to the bath was able to prevent the contraction induced by 3.8×10^{-10} M bradykinin, but had no effect on the contraction induced by 30 μ g/ml extract (not shown).

Agonist Action of the Extract on the Uterus

Concentration-response curves were established by graded increases in the concentration of the crude extract (2.0–300 μ g/ml). The bathing solution of the tissue was then changed to atropinized-Tyrode solutions. Atropine concentrations in Tyrode were: 1×10^{-9} M, 1×10^{-6} M or 1×10^{-4} M. From the results (Fig. 3) the presence of atropine (1×10^{-9} – 1×10^{-4} M) in the bathing fluid dose-dependently inhibited extract-induced increases of uterine contractions, and shifted the dose-response curve of the extract (control) to the right. Also, the atropine inhibition of contractions was surmounted by raising the concentration of the extract in the bathing fluid. The atropine antagonism is probably of a competitive type.

DISCUSSION

Clinically, drugs used to induce labor or abortion contract the uterine smooth muscle. Such drugs include

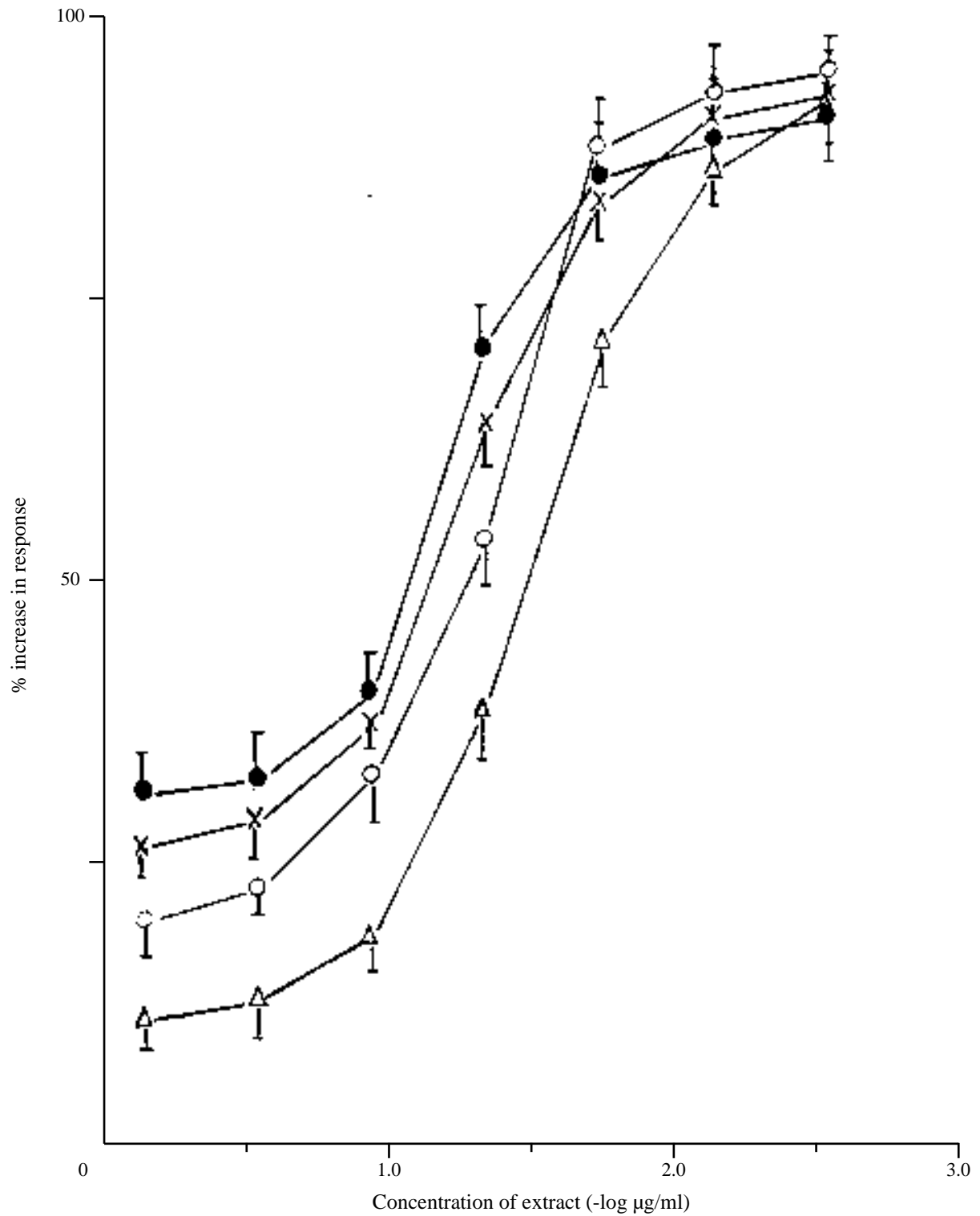


Fig. 3. Mean contraction of the isolated uterus to graded increases in the concentration of extract (2–300 µg/ml). Responses (% controls) in the absence (●) or presence of 1×10^{-9} M (X), 1×10^{-6} M (○), and 1×10^{-4} M (Δ) atropine sulphate in Tyrode solution. Each point represents the mean value \pm S.E.M., $n = 5$, $P < 0.05$ compared with controls by unpaired Student's t -test.

oxytocin, ergometrine and quinine (Bowman & Rand, 1980). From the results of the present study, there is sufficient evidence to believe that the crude extract from *E. drupifera* leaves contains a uterus-contracting agent(s). Small doses of the extract increased the amplitude of spontaneous uterine contractions, and large doses produced sustained spasms of the uterus, which was evidenced by the rise in basal tension of the tissue preparations. These tissue responses to the extract are similar to that reported for oxytocin and other standard uterus-contracting agents (Bowman & Rand, 1980). It was observed that the uterus-contracting action of the extract was fast in onset and could be totally terminated by washing with extract-free Tyrode solution. This may indicate the low molecular weight compound present in the extract, which may penetrate rapidly to its site of action. However, the fast onset of action exhibited by the extract could merely reflect the high concentration of the active compound present.

Additions of specific antagonists of 5-HT and bradykinin to the bath prevented uterine contractions induced by these mediators, but did not prevent the effect of the extract. Therefore, the data indicate that the uterus contraction elicited by the extract is not related to the activity of these mediators. As the effects of the extract are potentiated by neostigmine and prevented or abolished by atropine, it seems likely that the action of the extract depends on stimulation of cholinergic muscarinic receptors. The atropine antagonism could be competitive in nature, since it was surmounted by raising the concentration of the extract. The data (Table 1) showed that TTX did not prevent the contractions induced by ACh or bradykinin, but totally abolished the contractions elicited by the extract. These probably suggests the interaction of the extract with the sodium channels. Therefore, it is likely that the extract acts on the sodium channels, releasing ACh from post-ganglionic nerve fibers with subsequent stimulation of muscarinic cholinceptors. These findings are consistent with reports on the autonomic innervation of the rat uterus (Hollingsworth, 1974; Garfield, 1986; Mendonca et al., 1995). Hexamethonium (Hex) abolishes nicotinic actions just as it abolishes the actions of nicotine itself (Bowman & Rand, 1980). This probably explains why this ganglion blocking drug (Hex) failed to abolish the contractions produced by the extract, suggesting that the uterine smooth muscle cells could be devoid of nicotinic receptors, or that the extract was acting post-synaptically.

However, it is premature to speculate on the actions of this extract on smooth muscle since it may contain more than one active compound in its crude stage. Further progress must await refinements in its separation techniques.

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