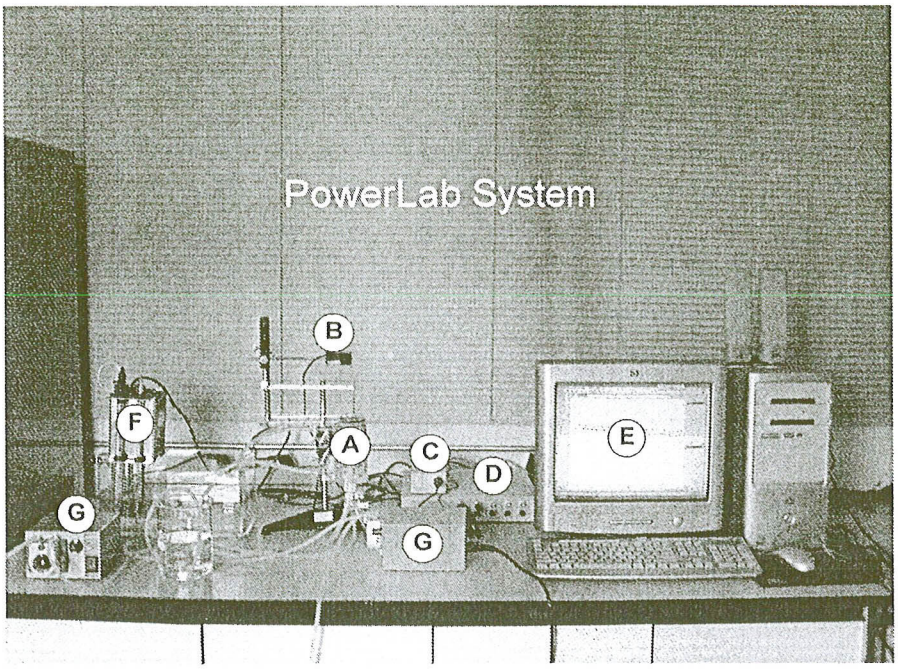
# ภาคผนวก ก




เครื่องมือที่ใช้วัดการหดตัวของกล้ามเนื้อเรียบมดลูก

A, Organ bath chamber

B, Transducer

C, Bridge Amp

D, PowerLab

E, Computer Set

F, Water bath chamber with temperature controller

G, Peristaltic pumps

# ภาคผนวก ข

สารเคมีที่ใช้ในการเตรียมสารละลาย Physiological Saline Solution

| | ความเข้มข้น (mM) | หน่วย g/L |
|---|---|---|
| NaCl | 154 | 9 |
| KCl | 5.6 | 0.42 |
| $Mg.SO_4.7H_2O$ | 0.12 | 0.29 |
| HEPES | 10.9 | 2.6 |
| Glucose | 8 | 1.44 |
| $CaCl_2$ | 2 | 2 |

# ประวัติผู้วิจัย

นางศจีรา คุปพิทยานันท์ ตำแหน่งอาจารย์ เกิดวันเสาร์ที่ 7 มีนาคม พุทธศักราช 2513 ที่อำเภอบัวใหญ่ จังหวัดนครราชสีมา สำเร็จการศึกษาระดับปริญญาตรีสัตวแพทยศาสตร์บัณฑิตเกียรตินิยม จากมหาวิทยาลัยขอนแก่นในปีพุทธศักราช 2537 จากนั้นได้รับทุนจากบริติสเคาน์ซิลและรัฐบาลไทยให้ไปศึกษาต่อระดับมหาบัณฑิตและดุษฎีบัณฑิตในสาขาสรีรวิทยา ที่มหาวิทยาลัยลิเวอร์พูล ประเทศอังกฤษ สำเร็จการศึกษาในปีพุทธศักราช 2546 ขณะกำลังศึกษา ณ สถานศึกษาดังกล่าวได้รับทุนนักสรีรวิทยารุ่นเยาว์ (Young Physiologist) จากมหาวิทยาลัยฯ เพื่อนำเสนอผลงานวิจัย ปีละ 1,000 ปอนด์ตลอดระยะเวลาการศึกษา ปัจจุบันปฏิบัติงานที่ สาขาวิชาชีววิทยา สำนักวิชาวิทยาศาสตร์ มหาวิทยาลัยเทคโนโลยีสุรนารี 111 ถนนมหาวิทยาลัย ตำบลสุรนารี อำเภอเมือง จังหวัดนครราชสีมา รหัสไปรษณีย์ 30000 มีประสบการณ์ในการวิจัยและผลงานทางวิชาการทางด้านสรีรวิทยาระบบสืบพันธุ์ที่ได้รับการตีพิมพ์ในช่วงปี 2543-2554 ผลงานฉบับเต็มในวารสารนานาชาติจำนวน 16 เรื่อง วารสารไทยจำนวน 3 เรื่อง และบทคัดย่อในวารสารระดับชาติ 5 เรื่องและวารสารระดับนานาชาติจำนวน 14 เรื่อง

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# The Effects of Wild Ginger (*Costus speciosus* (Koen) Smith) Rhizome Extract and Diosgenin on Rat Uterine Contractions

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## Abstract

The aim of this study was to investigate the effects of wild ginger (*Costus speciosus* (Koen) Smith, Costaceae) rhizome extract on uterine contractility. We particularly examined the effects on spontaneous phasic contractions and the mechanisms whereby it exerts its effects. Wild ginger rhizomes were ethanolic extracted and their constituents analyzed. Isometric force was measured in strips of longitudinal myometrium and the effects of the extract studied. The extract (10 mg/100 mL) increased spontaneous contractions. The amplitude and frequency of the phasic contraction were significantly increased along with basal tension. Force produced in the presence of the extract was abolished by inhibition of L-type calcium channels or myosin light chain kinase (MLCK). The actions of the extract were not blocked by the estrogen receptor blocker, fulvestrant. Although significant amounts of diosgenin were present in the extract, we found that, depending upon its concentration, diosgenin had either no effect or was inhibitory on force. Interestingly, the extract induced significant amounts of force in the absence of extracellular calcium, which could be blocked by inhibition of the sarcoplasmic reticulum calcium-ATPase (SERCA), but not fulvestrant. We conclude that wild ginger rhizome extract stimulates phasic activity in rat uterus. Our data suggest that the uterotonic effect is due to nonestrogenic effects and not those of diosgenin. Wild ginger was able to increase contraction via calcium entry on L-type calcium channels and sarcoplasmic reticulum (SR) calcium release. We suggest that wild ginger rhizome extract may be a useful uterine stimulant.

## Keywords

*Costus speciosus* (Koen) Sm, Costaceae, wild ginger, sarcoplasmic reticulum calcium-ATPase, potassium channel, calcium, smooth muscle, SR

Wild ginger (*Costus speciosus* (Koen) Sm) is a medicinal plant wildly distributed in Asia. The plant is traditionally used in the treatment of fevers, cough, worm infections, skin diseases and snake bites.[1] Wild ginger plants also contain diosgenin, a steroidal saponins, in its rhizome.[1] It is, therefore, widely used as starting material for commercial production of steroidal hormones including sex hormones.[2] Other compounds have also been isolated from this plant, including β-sitosterol, which is a uterine stimulant.[3]

It has been shown that saponins isolated from the plant rhizome have estogenic activities as they increased the uterine weight of spayed albino rats[4] as well as terminated pregnancy in rats.[5] It has also been reported that the fresh juice of wild ginger rhizome increased the tone, amplitude and frequency of spontaneous contractions of the uterus isolated from rats, guinea-pig, rabbit, dog, and human.[6] Increases in contraction were not blocked by atropine and pentolinium barbiturate,[6] suggesting that the juice did not act through muscarinic and GABA receptors. However, the underlying mechanisms of action of wild ginger are unclear.

It has been reported that diosgenin glycosides can alter myocardial activities in both isolated frog heart[7] and cultured

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embryonic myocardial cells[8] via the modulation of extracellular Ca flux across the plasma membrane. In vascular smooth muscle cells, diosgenin is reported to be a potent vasodilator, as it induced an acute, endothelium-independent coronary artery relaxation via protein kinase G signaling cascade and activation of large conductance Ca-activated K (BK) channels.[9] Furthermore, it has been reported to induce relaxation in isolated rat superior mesenteric artery via the same pathway.[10] The effect of diosgenin on the uterus is not known, but as extracellular Ca influx is important and BK channels are expressed, it may be anticipated to affect uterine contractility.

To the best of our knowledge, the mechanisms underlying the uterotonic effect of wild ginger rhizome have not been studied. As there is a clinical need to find better drugs to help control uterine activity,[11,12] and novel compounds are sought, the aim of the study was therefore to investigate the effects of wild ginger rhizome extract on uterine contractions. We particularly examined the effects on spontaneous phasic contractions and the mechanisms whereby wild ginger exerts its effects. Previous work has identified both the internal Ca store, the sarcoplasmic reticulum (SR)[13,14] and excitation-Ca entry[15,16] as being important for uterine force production, and thus both these components were examined. The effects of the extract were also compared to 2 of the major components in it, diosgenin and β-sitosterol, both of which are phytoestrogens.[4,17] Finally, we used fulvestrant, a blocker of estrogen receptors, to examine whether the effects occurred through these receptors.

## Materials and Methods

### Plant Material

Fresh wild ginger rhizomes were collected from fields in the area of Nakhon Ratchasima, Thailand, in August. The plant and its rhizome was identified and confirmed by the Royal Forest Department of Thailand and a voucher specimen (BKF161284) deposited in the laboratory for future reference.

### Extraction and Isolation

The wild ginger rhizomes were manually isolated. They were cleaned, air-dried, powdered, and subjected to Soxhlet extraction with ethanol. The extract was filtered through a filter paper, evaporated in a rotary evaporator, and dried by a lyophilizer. The yield was 7.21%.

Crude extract was analyzed for the constituent by GC-MS (A Agilent Technologies 6850 gas chromatograph, coupled with an Agilent Technologies 5973 (EI) mass spectrometer). The separation was performed on an HP-5MS column, 30 m × 0.25 mm ID × 0.25 μm film thickness. The temperature of the column was programmed from 50 to 200°C at 10°C/min, 200 to 260°C at 12°C/min respectively. The injector temperature and the detector temperature were 270°C. Helium was used as the carrier gas with a constant flow rate of 1.0 mL/min. All separated compounds were identified from the recorded mass spectra by comparison with the mass spectra from the NIST and Wiley libraries. The rest of the extract obtained was stored at 4°C until use in the physiological experiments. A working solution was obtained by dissolving the extract in physiological solution. We also purchased chemical-grade diosgenin and β-sitosterol, which were 95% and 75% pure, respectively.

### Animals

Nonpregnant Wistar rats (200-250 g) were used in this study and maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council, Thailand. The experiments performed on rats were conducted in accordance with the advice of the Institutional Animal Care and Use Committee, Suranaree University of Technology, Thailand.

The rats were humanely killed by cervical dislocation under $CO_2$ anesthesia. The uterus was removed and immediately immersed in buffered physiological solution (pH 7.40) containing (mmol/L) 154.0 NaCl; 5.4 KCl; 1.2 $MgSO_4$; 8.0 glucose; 2.0 $CaCl_2$; and 10.0 *N*-[2-hydroxyethyl]piperazine-*N*-[2-ethanesulfonic acid] (HEPES). The uterus was placed in a shallow dissecting dish containing physiological solution at 37°C, and under a microscope, the longitudinal muscle layer was separated from the endometrium and circular muscle layer. Five or six strips (1-2 mm × 0.5 mm × 10 mm) were dissected and either used immediately or stored for a maximum of 12 hours at 4°C.

### Tension Measurement

The uterine strips were mounted vertically under a resting tension of 1 g in a tissue bath (25 mL Panlab s. l. for AD Instruments Pty Ltd, Spain) connected to a force transducer (AD Instruments Pty Ltd, Spain) using silk threads. The electrical signal from the transducer was amplified and converted to a digital signal and recorded on a computer using Chart software (AD Instruments Pty Ltd, Australia). The tissue-bathing medium used was physiological saline solution maintained at pH of 7.4, temperature of 37°C, and gassed with 100% $O_2$. The strips were allowed to contract spontaneously and an equilibrium period of at least 30 minutes was given before the application of any chemical. The measurements were made whilst the tissue was continually perfused with physiological solution (control), high K solution (40 mmol/L), or solution containing wild ginger rhizome extract between 10 and 70 mg/100 mL. In some experiments, known components of the extract, diosgenin or β-sitosterol (dissolved in physiological solution), were used. In some experiments, 0-Ca solutions were used; physiological solution in which $CaCl_2$ had been omitted and 1 mmol/L Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-t etraacetic acid (EGTA) added. Wortmannin, an inhibitor of myosin light chain kinase (MLCK);[18] nifedipine an inhibitor of L-type Ca entry;[19] cyclopiazonic acid (CPA) an inhibitor of the sarcoplasmic reticulum calcium-ATPase (SERCA) pump[20]; and fulvestrant, an estrogen receptor antagonist,[21,22] were also used, as indicated in the text.

### Chemicals

All chemicals were purchased from Sigma, Singapore.

### Statistical Analysis

The data were analyzed using Microcal Origin Software. The following parameters of contraction were measured: force integral, frequency, amplitude, and duration. The phasic contractions in wild ginger were measured over 30 minutes from the start of their application. Results were expressed as percentages of control contractions (ie, the control is 100%). To test the effects of applications of diosgenin, β-sitosterol, or fulvestrant following wild ginger extract, contractions were compared for the 30 minutes in wild ginger rhizome extract (i.e. 30-60 minutes after start of wild ginger extract exposure), to the 60 to 90 minutes in wild ginger extract with the addition of diosgenin, β-sitosterol, or fulvestrant. Integrated force (area under the contraction) was measured over a 30-minute period. In some experiments, changes in force amplitude are expressed with respect to basal (resting) force level (0%) and the peak force (100%) in control condition. Throughout, data are presented as mean ± SEM and "n" represents the number of samples, each one from a different animal. Significance was tested using appropriate *t* tests and *P* values <.05 taken to be significant.

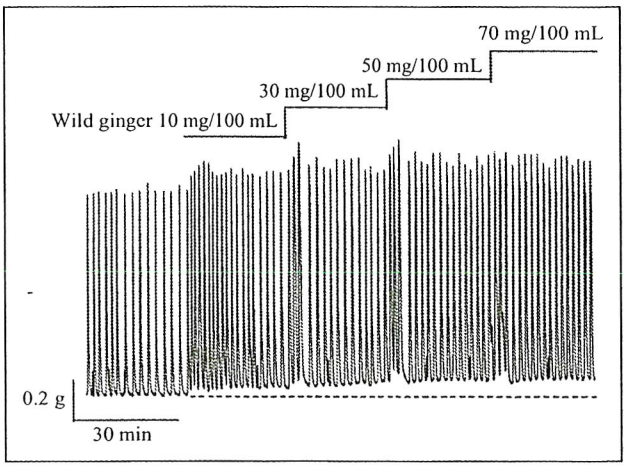


**Figure 1.** Dose dependency of wild ginger rhizome extract. The effects of increasing cumulative concentrations of wild ginger rhizome extract (10-70 mg/100 mL) on spontaneous contractions of nonpregnant rat uterus are shown (typical of 4 other traces from different animals). In this and subsequent figures, the uterine strips were perfused with oxygenated physiological saline, pH7.4, and temperature 37°C. The wild ginger extract significantly ($P < .05$) increased contractions amplitude, frequency, and area under the curve, measured over a 30-minute period (n = 11 animals). In subsequent figures, the effects of 10 mg/100 mL extract are examined. All n numbers are from different rats.

## Results

### GC/MS Analysis

The GC/MS analysis showed 6 main compounds that had retention times (minutes) of 48.81 (56.31%), 54.69 (28%), 22.94 (5.91%), 20.54 (5.87%), 22.83 (4.28%), and 50.52 (3.87%). These corresponded to diosgenin, 9,19-cycloergost-24(28)-en-3-ol, 9,12,15-octadecatrien-1-ol, hexadecanoic acid, 9,12-octadeca - dienoic acid, and β-sitosterol respectively. Traces of 14 other known compounds, mainly essential oil (0.02%-3%), and 8 unknown compounds were detected (data not shown).

### Spontaneous Uterine Activity—Dose Dependency of Wild Ginger Rhizome Extract

Under control conditions, spontaneous contractions of consistent amplitude and frequency could be recorded for several hours allowing the effects of the different concentrations of the extract to be examined (see figures throughout). The effects of increasing cumulative concentrations of wild ginger rhizome extract (10-70 mg/100 mL) were examined; each concentration was applied for 30 minutes. Wild ginger rhizome extract increased uterine contractility arising spontaneously (n = 5). An example of this is shown in Figure 1A. At 10 mg/100 mL, the extract increased the amplitude and the frequency of the contractions, and increased basal tension. With higher doses (30, 50, 70 mg/mL), further increases in force were usually seen, but this was not consistently observed. The stimulatory effects of the extract could be seen within 5 minutes of application and were maintained as long as it was present in the bath. These effects were irreversible over the timescale of the experiments. As the stimulatory concentration on myometrium contractility occurred between 10 and 70 mg/100 mL, the concentration of 10 mg/100 mL was used throughout the remainder of the study.

### Effects on Parameters of Contraction

Application of wild ginger rhizome extract (10 mg/100 mL) to the rat myometrial preparations produced significant potentiating effects on spontaneous force (n = 11). The frequency of the contractions increased significantly to 126 ± 12% (from 0.67 ± 0.07 per minute to 0.82 ± 0.07 per minute). The amplitude of force was also significantly increased; 109.11% ± 2%, (from 1.19 ± 0.17 g to 1.29 ± 0.17 g) and these changes resulted in a significant increase in the area under the contractions; 141.31 ± 6% (compared with control, 100%). In addition, wild ginger rhizome extract consistently increased basal force by 26.17 ± 5% ($P < .05$).

### Effects of Ca-Dependent Force Pathway Modulation

Uterine force can be produced by several pathways, but the main mechanism involves Ca-calmodulin-MLCK.[23] To investigate whether the increases in uterine force seen with the extract were dependent on the calcium-calmodulin MLCK pathway, we studied its effects in the presence of inhibitors of the L-type calcium channels and MLCK inhibitors (Figure 2). As can be seen in Figure 2A, the application of 10 μmol/L nifedipine in the continued presence of the extract, rapidly inhibited and then

abolished force transients ($n = 5$). However, basal force did not return to control levels but remained somewhat elevated and oscillatory, for example, compared with Figures 2D, E, and 6A.

The effects of wild ginger rhizome extract in the presence of a potent inhibitor of MLCK, wortmannin, are shown in Figure 2B. Wortmannin (4 μmol/L) in the continued presence of the extract gradually reduced active and basal force in all preparations studied (n = 5). In the presence of wortmannin, the area under the curve was reduced to 25 ± 2% ($P < .05$) compared with 100% control. When the extract was added after, but in the continued presence of wortmannin, a small but consistent tonic force was produced (n = 5). An example of this is shown in Figure 2C.

In the uterus, some uterotonic agents can elicit a contraction in 0-Ca solution or when L-type Ca channels are blocked,[20,24] and it has been suggested that this contraction occurs independently of the calcium-calmodulin-MLCK pathway.[20,24] To investigate this, the extract was applied in 0-Ca solutions or after application of nifedipine. As can be seen in Figure 2D, spontaneous force was abolished by applying of 0-Ca (EGTA) solutions. The extract, however, could elicit transient force with oscillatory in nature. The area under the contraction (measured for initial 5 minutes) in the presence of wild ginger rhizome was 17% ± 5% significantly increased compared with the (also measured over 5 minutes) in 0-Ca solution, which was 2.4% ± 0.2% (n = 5). We next investigated the effects of the rhizome extract in the presence of nifedipine. As can be seen in Figure 2E, force transients are observed during the application of the extract. The area under the contraction in the presence of wild ginger rhizome was 46% ± 5%, compared with the 6.6% ± 2.1% contraction in the presence of nifedipine alone (n = 6). We also investigated the effect of the extract when intracellular Ca is maintained at a high level, by exposing the uterus to high K solution. When the extract was added in the continued presence of 40 mmol/L K solution (n = 3), as can be seen in Figure 2F, a tonic force was produced upon addition of the extract.

## Effects of Diosgenin

As shown above, wild ginger rhizomes potentiate uterine contraction. As diosgenin, a steroidal saponin and phytoestrogen is one of the major components found in the extract, it was of interest to determine whether the effects of the extract were due to this compound. The effects of increasing cumulative concentrations of diosgenin (0.02, 0.2, 2 mg/100 mL, 30 minutes for each concentration) were examined. Diosgenin up to 2 mg/100 mL produced no significant effects on the amplitude of spontaneous uterine contractions (n = 5). At the highest dose (2 mg/100 mL), diosgenin, however, significantly decreased the frequency of the contraction (66.5% ± 7%, n = 6). An example of this is shown in Figure 3A. At a very high dose, 40 mg/100 mL, diosgenin significantly decreased both the frequency and the amplitude of spontaneous contraction to 64.75% ± 8% and 81.25% ± 4% (n = 4), respectively, compared to control, 100% (Figure 3B). We next investigated whether wild ginger rhizome could elicit force in the presence of diosgenin. To investigate this, the extract was applied in the presence of the highest diosgenin dose (40 mg/100 mL). As can be seen in Figure 3B, application of wild ginger rhizome significantly increased the amplitude and frequency of force (n = 4). The amplitude was increased from 0.97 ± 0.21 g in diosgenin alone to 1.13 ± 0.22 g after the extract was added and the frequency was increased from 4.24 ± 1.11 per 10 minutes in diosgenin alone to 6.5 ± 1.26 per 10 minutes after the extract was added.

## Effects of β-Sitosterol

β-sitosterol, another component of the extract, has previously been found to stimulate the uterus.[3] These effects are demonstrated in Figure 4A; β-sitosterol (1 mg/100 mL) significantly increased spontaneous force in all preparations studied ($n = 5$). The frequency and the amplitudes of the contractions increased significantly to 138.8% ± 9% and 116.8% ± 5%, respectively (all compared with spontaneous control, 100% frequency means values 0.55 ± 0.06 per min compared 0.75 ± 0.05 per min and amplitude means values 1 ± 0.13 g compared 1.15 ± 0.13 g, respectively). An application of the wild ginger rhizome in the continued presence of β-sitosterol produced further increases in force, although its effect could be transient (Figure 4A). The area under the contraction was 133.23% ± 7%, compared with β-sitosterol alone (n = 6). The frequency and the amplitudes of the contractions increased significantly to 116.8% ± 6% and 114.8% ± 7%, respectively (all compared with β-sitosterol alone, 100% frequency means values 0.75 ± 0.05 per minute compared 0.87 ± 0.05 per minute and amplitude means values 1.15 ± 0.13 g compared 1.30 ± 0.08 g, respectively). However, when β-sitosterol was added after wild ginger rhizome; no further increases in force were observed (Figure 4B, n = 8).

## Effects of Fulvestrant

As discussed earlier, the effects of wild ginger rhizome extract on uterine activity could be occurring through estrogen receptors (ERs). We therefore blocked these, with fulvestrant for 30 minutes (1 μmol/L),[21,22] and studied the effects of the extract (n = 5). Application of fulvestrant to spontaneously active uterus produced no significant changes in uterine contractions (Figure 5A) but reduced amplitude and frequency of contractions in estradiol-stimulated preparations (SK unpublished observations). Application of wild ginger rhizome extract in the continued presence of fulvestrant produced significant increases in contraction (Figure 5A). The increase in the area under the contraction was 188 ± 16% (all compared with fulvestrant control, 100%). When fulvestrant was added after wild ginger rhizome extract, no significant change in force occurred, Figure 5B, n = 5.

## Effects of Ca-Independent Force Pathway Modulation

As shown above, it is clear that wild ginger rhizomes potentiate uterine contraction in the presence and absence of extracellular calcium. We next further investigated the underlying mechanisms of the force elicited in the absence of external

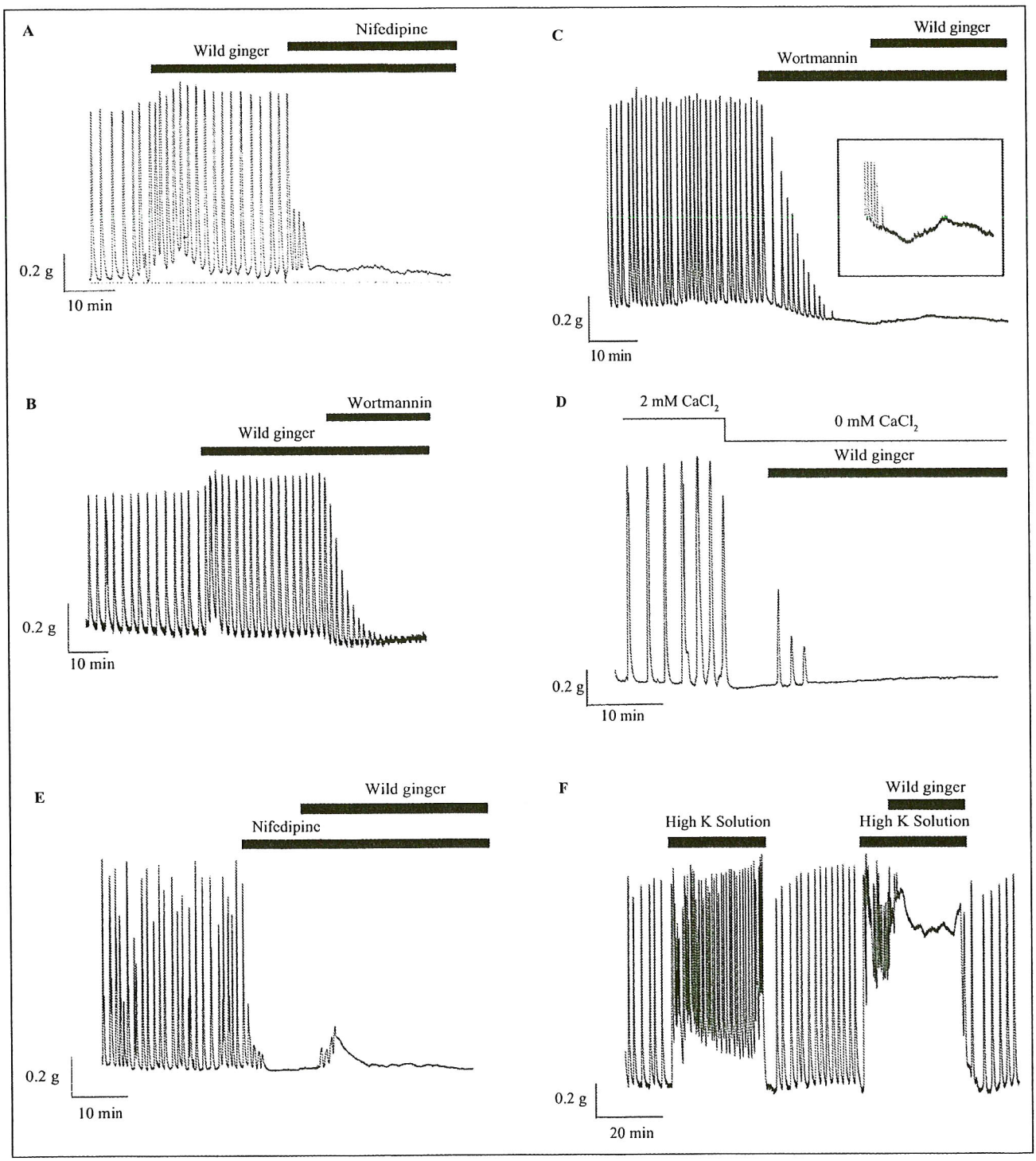


**Figure 2.** Effects of Ca-dependent force pathway modulation. A, The effects of nifedipine (10 μmol/L), a blocker of Ca entry channels, on force induced by wild ginger rhizome extract (10 mg/100 mL; typical of 4 other preparations). B, The effects of wortmannin (4 μmol/L) on force induced by wild ginger rhizome extract. Wortmannin inhibits myosin light chain kinase (MLCK) and this trace is typical of 4 others. C, The effects of wild ginger rhizome extract in the continued presence of wortmannin (4 μmol/L). Note small but consistent stimulation of force produced by the extract. D, The effects of wild ginger rhizome extract in the continued presence of 0-Ca (EGTA containing) solution. Note how following abolition of contractions by 0-Ca, significant force can still be produced by the extract, n = 5. E, The effects of wild ginger rhizome extract in the continued presence of nifedipine (10 μmol/L) typical of 5 other preparations. Note again stimulation of force by extract. F, The effects of wild ginger rhizome extract in the continued presence of high K solution (40 mmol/L), right-hand side, compared to high-K alone, left-hand side (n = 3).

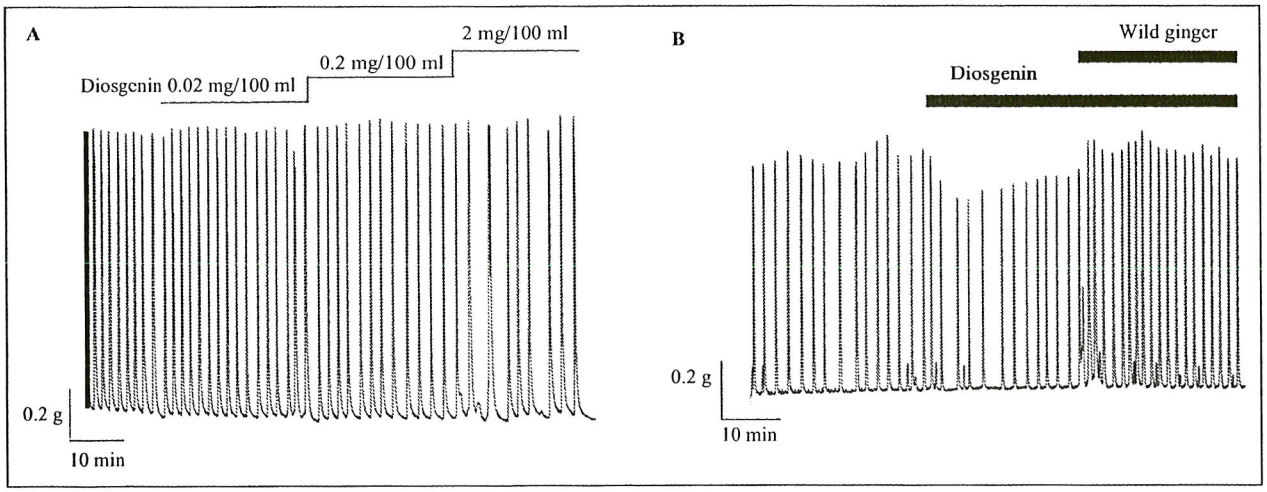


**Figure 3.** The effects of diosgenin on uterine contractions. A, The effects of increasing cumulative concentrations of diosgenin (0.02-2.0 mg/100 mL, n = 5) on spontaneous contractions of rat uterus. No significant effects at these doses were seen as shown. B, The effects of a high dose of diosgenin (40 mg/100 mL) and then in the presence of wild ginger extract, trace typical of 3 others.

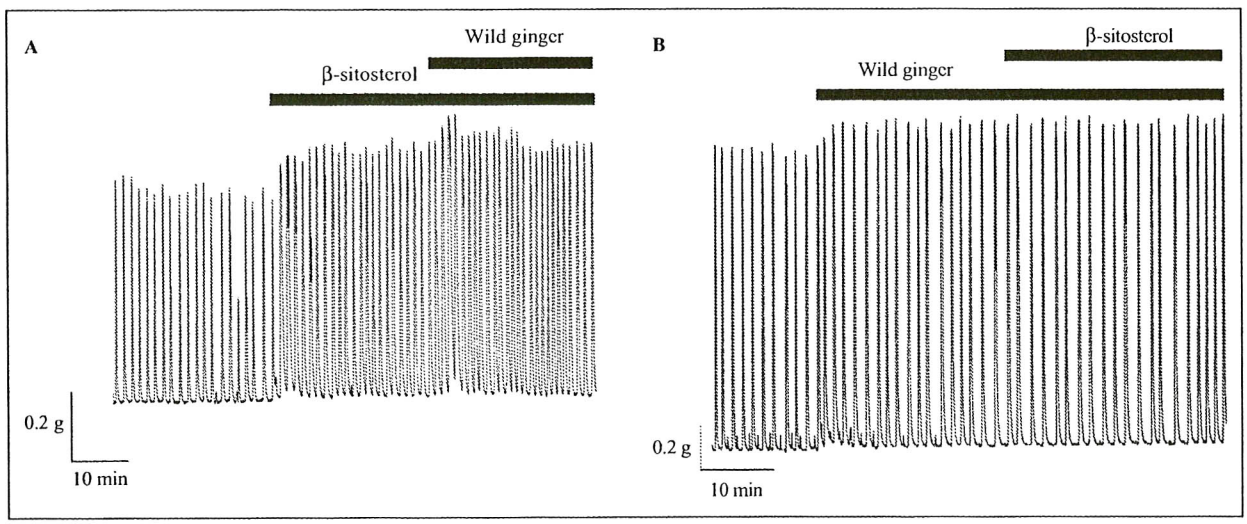


**Figure 4.** The effects of β-sitosterol and wild ginger rhizome extract. A, β-sitosterol (1 mg/100 mL) produced significant stimulation of the rat uterus (n = 5). Addition of wild ginger rhizome further stimulated the uterus, as shown. B, Applications of β-sitosterol after wild ginger rhizome extract were without further effect on the uterus (n = 8).

calcium. As can be seen in Figure 6A, in the continued presence of fulvestrant, force was abolished by nifedipine but the extract applied 15 minutes later was able to elicit significant amount of force, often oscillatory in nature. The area under the contraction induced by the extract in the continued presence of nifedipine plus fulvestrant was 37% ± 12%, compared with fulvestrant control, 100% (n = 3). Under these conditions, it is expected that the extract will be stimulating SR Ca release.

To test this, the SR was inhibited using CPA, a blocker of the SR Ca-ATPase.[20] As can be seen in Figure 6B, in agreement with previous findings,[20] CPA greatly stimulates spontaneous uterine activity. The area under the contraction induced by CPA was 252% ± 39%, compared with spontaneous control, 100% ($P < .05$; n = 5). After 20 minutes, nifedipine was applied and force was abolished. Fifteen minutes later, the extract was applied, in the continued presence of CPA and nifedipine. However, under these conditions, the extract was not able to elicit any force, $n = 5$. The area under the contraction in the absence of the rhizome was 5.6% ± 1.8%, compared with those in the presence of the rhizome, 6.9 ± 2% ($P > .05$; $n = 5$).

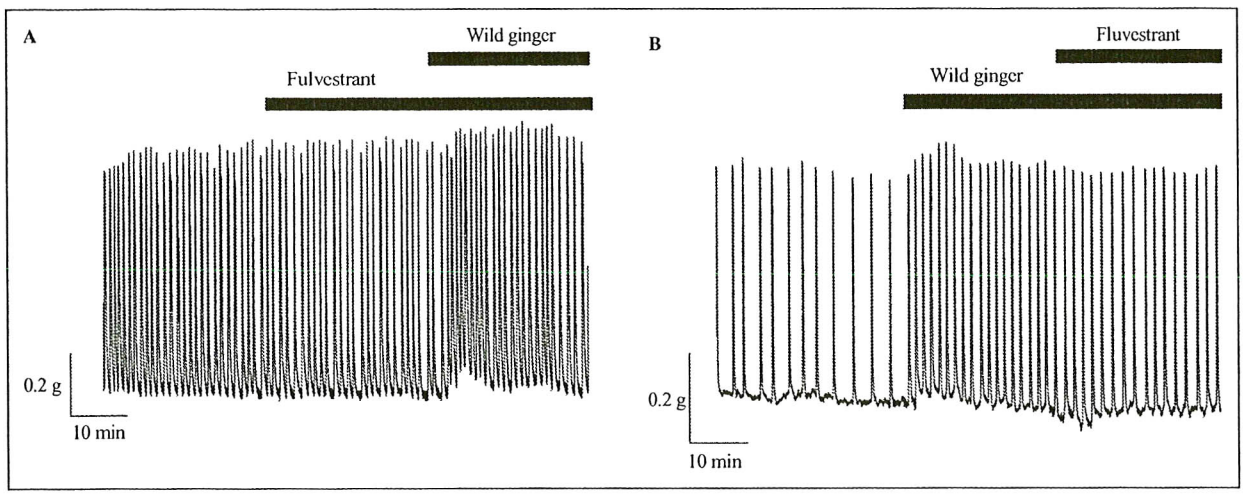


**Figure 5.** The effects of fulvestrant. A, The estrogen receptor blocker fulvestrant (1 μmol/L) applied to the rat uterine preparation produced no significant changes in contractile activity (n = 5). The effects of wild ginger rhizome extract added after fulvestrant can be also seen; force was stimulated. B, Following stimulation with wild ginger rhizome extract, fulvestrant had no effect (n = 5).

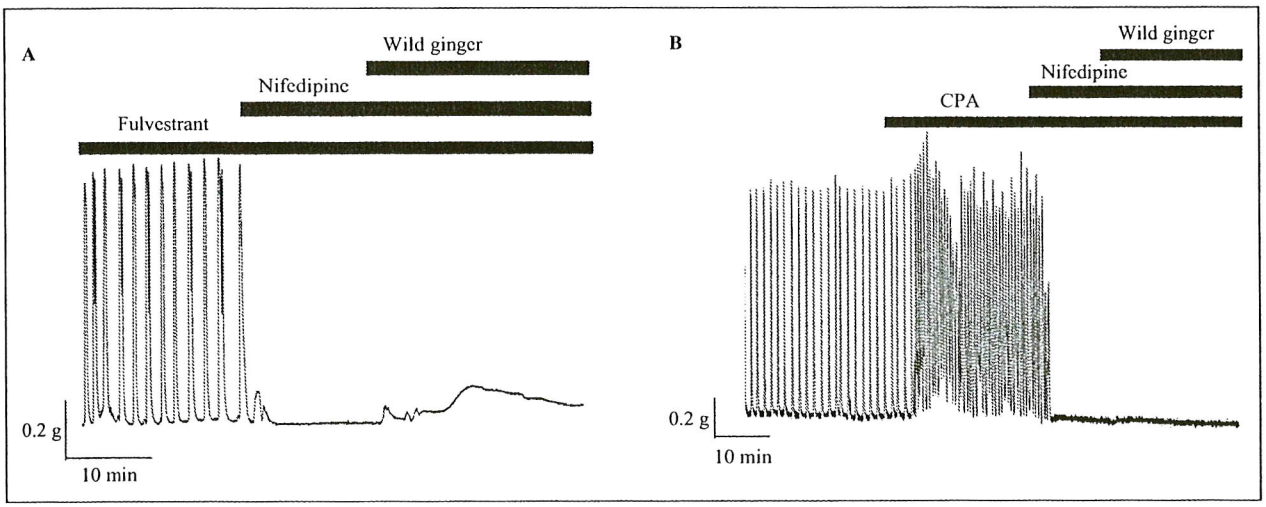


**Figure 6.** Effects of Ca-independent force pathway modulation. A, The effects of wild ginger rhizome extract induced contraction in the presence of nifedipine (10 μmol/L) and fulvestrant (1 μmol/L) typical of 2 other traces. Note the stimulation of force. B, The effects of rhizome extract induced contraction in the presence of nifedipine (10 μmol/L) and cyclopiazonic acid (CPA; 20 μmol/L), an inhibitor of the SR Ca-ATPase, n = 5.

## Discussion

This study is the first to show the underlying mechanisms of the effects of wild ginger rhizome extract on uterine contraction and demonstrates that it significantly potentiates uterine contractions. Both the amplitude and frequency of the phasic contraction were significantly increased as well as the basal tension. The effects of wild ginger rhizome extract on the uterus were shown not to be due to diosgenin. The effects of the extract were however reduced following β-sitosterol administration, suggesting that its actions are in part mediated by this component. The potentiation of spontaneous force induced by the extract was dependent upon the Ca-calmodulin-MLCK pathway, but the extract was also able to stimulate force via SR Ca release. When the effect of wild ginger rhizome extract was studied after fulvestrant treatment, it was still effective. Thus, our conclusion is that wild ginger rhizome extract is uterine stimulant acting via a nonestrogen mechanism on both Ca entry and SR Ca release.

In agreement with an early study,[1] we showed that constituents isolated in wild ginger rhizome extract are diosgenin and β-sitosterol. Diosgenin is found in a variety of other

plants, particularly yams and fenugreek as well as wild ginger.[25] It has a variety of medicinal uses, for example in treatment of diabetes[26] and hypercholesterolemia.[27] It is structurally similar to endogenous $E_2$ hence it is also a phystoestrogen (steroidal saponin). It has not previously been studied in the uterus, but in vascular smooth muscle it has recently been found to reduce tone, partly through activating BK channels and reducing Ca entry.[9,10] At high concentrations, we also found diosgenin to relax the rat uterus, suggesting a common action on contractility in the smooth muscle. The relaxant effect of diosgenin was overcome by the wild ginger extract (Figure 3B), suggesting a more potent effect of other components, such as β-sitosterol. The stimulatory effect we show of β-sitosterol is consistent with our earlier data with respect to pomegranate seeds.[3] β-sitosterol is a common plant estrogen and appears to be the major uterotonic in pomegranate seeds.[3] In other cell types, an inhibitory action of β-sitosterol on the SERCA has been reported.[28] We have previously shown that such an inhibition will increase uterine intracellular [Ca] and contractions, consistent with this, see also Figure 6B.[20] Thus, β-sitosterol while probably not the only agent in the extract responsible for the potentiation of uterine force is a key mediator of its actions. It remains to be established which additional component of the extract contributes to potentiating spontaneous contraction. Significant amounts of the ergostane steroid, 9,19-cycloergost-24(28)-en-ol, were also found in the rhizome extract, and this may be contributing, although we can find nothing in the literature concerning such activity.[29]

We found that the pathway to increase uterine contraction by wild ginger rhizome extract occurred via a calcium-dependent pathway. Support for this conclusion comes from the experiments with MLCK inhibition; force transients were no longer produced by the extract. In addition, although force transients are abolished in nifedipine or wortmannin, some force can still be produced via SR Ca release (Figure 2A and B, discussed below). When high K was used to depolarize the uterus and maintain intracellular Ca at high levels, the extract was still able to alter force, with activity shifting from phasic to tonic. This could be due to Ca-independent effects on the uterus, although there is little evidence for Ca sensitization in this tissue[30] or due to SR Ca release. We and others have shown that agonists can affect Ca signaling pathways even in depolarized preparations.[31] Thus, together our data support a mechanism of action involving the Ca-calmodulin-MLCK pathway-elevating tone. The increase in contraction frequency could also suggest a stimulation of the intrinsic pace-making mechanism in the uterus, but it is currently unclear how such mechanisms work.[32]

In addition, we suggest there is involvement of the internal Ca store, that is, the SR.[20] The evidence for this comes from the effects of the wild ginger extract after nifedipine, which had clearly blocked L-type Ca channels (Figure 2E) and after removal of external Ca (in presence of EGTA, Figure 2D). Clear increases of force were seen when the extract was added. This is one of our most interesting findings as few agents including oxytocin have previously been found to be so active. That this oscillatory release is coming from the SR was shown by using CPA—inhibition of SR function abolished the releases of Ca (Figure 6B). Many factors including Ca itself and pH can influence SR Ca release.[33,34] Future studies investigating the mechanisms underlying the effects of wild ginger extract on SR Ca releases would be of interest.

The experiments with fulvestrant, a blocker of ERs, clearly demonstrated that despite being a source of phytoestrogens, the action of the wild ginger rhizome extract is via a nongenomic ER pathway. This is also consistent with our data showing that diosgenin, another phytoestrogen, does not stimulate contractions, but the extract does. Previous work with β-sitosterol has shown it to stimulate, through a nonestrogen receptor mechanism,[3] and our results are also consistent with this.

## Conclusion

In conclusion, we have presented novel data demonstrating a significant stimulation of uterine activity by wild ginger rhizome extract, which can largely but not entirely be accounted for by its constituent, β-sitosterol, acting via non-estrogen receptor mediated mechanism. The stimulation of uterine activity of wild ginger rhizome may be a useful source of uterine stimulant for slowly progressing labours,[35] although further studies in human myometrium are required to develop these suggestions. In particular, the ability of wild ginger to elicit repetitive SR Ca releases is of interest and worthy of further study.

### Declaration of Conflicting Interests

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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