ภาคผนวก

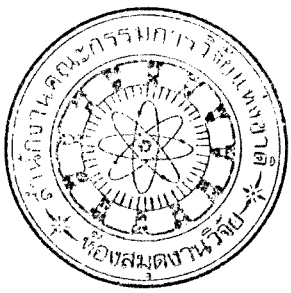
สารเคมีที่ใช้เตรียมสารละลาย Physiological Saline Solution

ส่วนประกอบ

	mM		1 L
NaCl	154	9	g/L
KCl	5.6	0.42	g/L
$Mg.SO_4.7H_2O$	0.12	0.29	g/L
HEPES	10.9	2.6	g/L
Glucose	8	2.44	g/L
$CaCl_2$	2	2	g/L

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



PH25 Estrogenic effects of pomegranate extracts in ovariectomized rats

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Pomegranates (*Punica granatum* L.) have been wildly used for health benefits. They have been found effective in reducing heart disease risk factors [1] and may be effective against prostate cancer [2] and osteoarthritis [3]. Recently, their uterotonic effects have been demonstrated [4]. However, their estrogenic effects are not well understood. The aims of the study were to investigate the estrogenic effects of pomegranate seed and peel extracts on ovariectomized rats. We particularly examined the effects of the extracts on vaginal cornification. Pomegranate seeds and peel were collected from local gardens and extracted using methanol. Rats were ovariectomized and treated with distilled water (DW), estradiol valerate (1 mg/kg BW), pomegranate seed extract (400, 500 mg/kg BW), and pomegranate peel extract (400, 500 mg/kg BW) for 14 days. The vaginal cytology was checked daily. The treatments of DW did not influence the vaginal epithelium, but the injection of estradiol valerate induced a vaginal cornification from day 5 to day 14 of treatment period. Interestingly, vaginal cornification was inducible when the rats were fed with either pomegranate seed or peel extract. The occurrence of vaginal cornification during treatment was dependent on types and dosages of the extracts. Thus, the cornification was fist found on day 9 and day 7 with 400 and 500 mg/kg BW pomegranate seed extract, respectively. With 400 and 500 mg/kg BW pomegranate peel extract, the cornification was fist found on day 10. The effects of both seed and peel extracts lasted until day 14. These data suggest that pomegranates have estrogenic effects in ovariectomized rats and that they could be useful for health benefits in menopause. References: 1. Aviram, M. & Dornfeld, L. (2001) Atherosclerosis 158: 195 – 8. 2. Malik, A. et al. (2005) Proc Natl Acad Sci USA 102: 14813 – 8. 3. Adhami, V.M., Mukhtar, H.B. (2006) Free Radic Res 40:1095 – 104. 4. Promprom, W. et al. (2007) Planta Medica 9: P1007.

PH26 Effects of noni fruit extract on intestinal contractility in rats

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Noni (*Morinda citrifolia*, Rubiaceae) fruits have been wildly used for health benefits. They have been found effective in reducing blood cholesterol levels [1] and may be effective against breast cancer [2] and have antioxidant properties [3]. However, the effects of noni fruit extract on intestinal contractility have never been investigated. The aims of the study were to investigate the effects of noni fruit extract on intestinal contractility in male rats. We particularly examined the effects of the extract on contractions arising spontaneously and by acetylcholine stimulation and investigated the underlying mechanisms. Noni fruits were collected from local gardens and extracted using methanol. The extract was then analyzed by GC/MS. Rats were killed by asphyxiation with CO_2 and longitudinal duodenal smooth muscles isolated. Isometric force was measured and the effects of the extract studied. Several agents, previously reported to decrease contraction in other smooth muscles, were found in noni fruit extract. When the extract was applied to spontaneously duodenal contractions, it significantly decreased the contractions. Its effect was dose dependent. The half maximal effect of the extract was observed at the dose of 50 mg/100 ml (20 – 100 mg/100 ml). An elevation of extracellular Ca^{2+} concentration could not reverse the inhibitory effects of the extract. The extract (100 mg/100 ml) also inhibited the phasic, but not tonic components of acetylcholine-induced contractions. In conclusion, noni fruit extract has inhibitory effects on intestinal contractility. These may involve contractile machinery that regulates phasic components of contraction. References: 1. Henley, E. et al. (2006) 46th AHA Annual Conference. 2. Hornick, C.A. et al. (2003) Angiogenesis 6:143 – 9. 3. Wang, M.Y. & Su, C. (2001) Annals of the New York Academy of Sciences 952:161 – 8.

PH27 Dietary supplementation with bilberry extract prevents macular degeneration and cataracts in senescent accelerated OXYS rats

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Cataracts and macular degeneration remain the major cause of blindness and acuity of vision deterioration in the elderly. Both pathology have been attributed to damage by free radicals, there has been a great deal of interest in antioxidants. Bilberry's (*Vaccinium myrtillus* L.) flavonoids are known as potent antioxidants, scavenging free radicals and used for multiple age-releted ocular disorders. There are no experimental studies, devoted to estimation of bilberry effect. To explore this one the senescence-accelerated OXYS rats were used. Developed at the Institute of Cytology and Genetics of Russian Academy of Sciences the OXYS rat strain is an animal model of accelerated senescence and age-related disease including early cataract and chorioretinal degeneration with clinical presentations correspond to age-related macular degeneration (AMD). From 1,5 to 3 month OXYS rats were given control diets or those supplemented with 25 % bilberry extract (BE, 20 mg on kg of body weight including 4,5 mg of antocianidin) or vitamin E (20 mg/kg) as drag for comparison. The ophthalmoscopy testing at 3 month have showed that more than 80% of control OXYS rats had cataract and macular degeneration while the supplementation of BE completely prevented impairments in the lenses and retina. The VE had no significant effects but both antioxidants decreased lipid peroxides in the retina, lens and serum of OXYS rats. The results suggest that long-term supplementation with BE is effective in prevention age-related macular degeneration and senile cataract and the OXYS rat strain is the useful model of these disorders and for screening new drugs for treatment of them.

PH28 Antiradical efficiency and aldose reductase inhibitory capacity of Cornelian cherry *(Cornus mas L.)* fruits' extracts

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Cornelian cherry (*Cornus mas, Cornaceae*), is a shelf shown tree in northern Greece with edible fruits, used for the preparation of marmalades, beverages, liqueurs and distillates. Fruits were found rich in anthocyanins and ursolic acid, substances preventive against diabetes and heart disease [1 – 4]. Aqueous extracts showed significant antioxidant activity in vitro, in various antioxidant systems [5, 6]. The aim of the study was the estimation of the antiradical efficiency (DPPH· assay and Co(II)/EDTA -induced luminol chemiluminescence test) [7] as well as the inhibitory capacity of aldose reductase enzyme (AR, ALR2, E.C. 1.1.1.21) [8] of several extracts and fractions of increasing polarity. For these tests, well matured fruits were exhaustively extracted with methanol and the dry remaining was partitioned with solvents of increasing polarity (diethyl ether, ethyl acetate, *n*-butanol, water). According to the DPPH test the initial methanolic extract, the diethyl ether and the ethyl acetate fractions had significant antioxidant capacity in comparison to the standards used (quercetin and trolox), while only the diethyl ether fraction was effective according to the CL test. The diethyl-ether and ethyl acetate fractions were efficient ALR2 inhibitors in comparison to sorbinil at a concentration of 25 µg/ml, indicating a strong potential of *Cornus mas* fruits against the long term diabetic complications [9]. References: 1. Jayaprakasam, B. et al. (2006) J.Agric.Food Chem. 54: 243 – 248. 2. Vareed, S. et al. (2006) Life Sci. 78: 777 – 784. 3. Char-Thanh, D. et al. (1973) Phytochem. 12: 2487 – 2489. 4. Seeram, N. et al. (2002) J. Agric.Food Chem. 50: 2519 – 2523. 5. Gulcin, I. et al (2005) Act. Alimentaria 34: 193 – 202. 6. Pantelidis G.E. et al. (2007) Food Chem. 102: 777 – 783. 7. Termentzi, A. et al. (2006) Food Chem. 98: 599 – 608. 8. Nicolaou, I. et al. (2004) J. Med. Chem. 47: 2706 – 2709. 9. Matthew, J. et al. (2002) J Amer Med Assoc 288: 2579 – 2582

P3AM-12-11
OLFACTORY PREFERENCE OF MALE RATS AFTER DESTRUCTION OF THE MEDIAL AMYGDALA OR THE MEDIAL PREOPTIC AREA

Sunil Dhungel, Susumu Urakawa, Yasuhiko Kondo, Yasuo Sakuma
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P3AM-12-12
ANALYSIS OF NURSING ABILITY AND LACTATIONAL FUNCTION OF CIN85 DEFICIENT MICE

Jun Ikezawa[1], Nobutaka Aotsuka[1], Kazutoshi Sayama[2], Noriaki Shimokawa[1], Noriyuki Koibuchi[1]
[1]Department of Integrative Physiology, Gunma University, Japan, [2]Laboratory of Animal Physiology and Biochemistry, Shizuoka University Graduate School of Agriculture, Shizuoka, Japan

P3AM-12-13
EFEECTS OF CENTRAL INFUSION AND IMMUNONEUTRALIZATION OF GROWTH HORMONE ON TIMING OF PUBERTY AND PLASMA LEPTIN LEVELS IN THE FEMALE RAT

Saeed Zeinoaldini[1], Bert J.M. Van de Heijning[2], Hans J.M. Swarts,[2]
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P3AM-12-14
POSSIBLE ROLE OF CYP450 2E1-INDUCTION IN ADVERSE REACTION ON REPRODUCTIVE SYSTEM OF MALE AND FEMALE RATS

Ganna Shayakhmetova, Taisiya Byshovetz, Alla Voronina, Valentina Kovalenko
Department of General Toxicology, Institute of Pharmacology and Toxicology of AMS of Ukraine, Ukraine

P3AM-12-15
CHEMICAL IMPACTS ON NEUROBEHAVIORAL DEVELOPMENTS: TIME- SEX- AND SPECIES DEPENDENCY

Shuji Aou[1], Makoto Monda[1], Ai Kanemaru[1], Tetsuya Fujimoto[2], Kazuhiko Kubo[3]
[1]Department of Brain Science and Engineering, Kyushu Institute of Technology, Japan, [2]Department of Physioloty, Osaka Dental University, Japan, [3]Department of Otorhinolaryngology, Chidoribashi Hospital, Japan

P3AM-12-16
NONYLPHENOL INHIBITS THE L-TYPE Ca^{2+} CURRENT IN GUINEA-PIG VENTRICULAR MYOCYTES

Tong Zhu[1], Qing-hua Gao[2], Lei Yang[1], Mei-mi Zhao[2], Gang Li[2], Hui-yuan Hu[2], Rui Feng[2], Jin-sheng Zhao[2], Li-fei Ren[2], Feng guo[2], Li-ying Hao[2]
[1]Lab of Environmental Biology and Engineering, Northeastern Univ., China, [2]Dept. of Pharmaceutical Toxicology, Dept. of Physiology, China Medical Univ., China

P3AM-12-17
SOY-ISOFLAVONE GENISTEIN AS A POSSIBLE THERAPEUTIC OPTION FOR REDUCING CARDIOVASCULAR RISK IN POSTMENOPAUSAL WOMEN WITH METABOLIC SYNDROME

Elena Bianca Adamo[1], Rolando Marini[1], Rosario D' Anna[2], Francesco Squadrito[3], Herbert Marini[1]
[1]Department of Biochemical, Physiological and Nutritional Sciences, Sect.of Physiol.and Hum.Nutrition, University of Messina, Italy, [2]Department of Obstetrical and Gynecological Sciences, University of Messina, Italy, [3]Department of Clinical and Experimental Medicine and Pharmacology, University of Messina, Italy

P3AM-12-18
EFFECTS OF THAI POMEGRANATE TREATMENT IN MAMMARY GLAND, UTERUS, AND VAGINA

SAJEERA KUPITTAYANANT[1], WILAWAN PROMPROM[2], KORAKOD INDRAPICHATE[2], PAKANIT KUPITTAYANANT[3]
[1]DEPARTMENT OF PHYSIOLOGY, SURANAREE UNIVERSITY OF TECHNOLOGY, Thailand, [2]SCHOOL OF BIOLOGY, INSTITUTE OF SCIENCE, SURANAREE UNIVERSITY OF TECHNOLOGY, Thailand, [3]SCHOOL OF ANIMAL PRODUCTION TECHNOLOGY, INSTITUTE OF AGRICULTURAL TECHNOLOGY, SURANAREE UNIVERSITY OF TECHNOLOGY, Thailand

P3AM-12-19
EFFECT OF DIET CONTAINING PLUKENETIA CONOPHORA ON THE REPRODUCTIVE SYSTEM OF SPRAGUE DAWLEY RATS

Mistura Oyebisi Azeez, Muritala Adefemi Adesola, Sarah Funke Ige, Waid Adeoye Saka, Mujeeb Salau
Department of Physiology, Ladoke Akintola University of Technology Ogbomoso, Nigeria

IV-1 Neural development and repair: Axon outgrowth, circuit formation, regeneration and degeneration

P3AM-13-1
THE EFFECTS OF PROXIMAL BRANCHES ON THE DEGENERATION OF THE NEURITES OF CULTURED DORSAL ROOT GANGLION NEURONS

Aydin Him[1], Gurkan Ozturk[1], Nurettin Cengiz[2], Ender Erdogan[2], Elif Oguz[3]
[1]Department of Physiology, Yuzuncuyil University, Turkey, [2]Department of Histology and Embryology, Yuzuncuyil University, Turkey, [3]Neuroscience Research Unit, Yuzuncuyil University, Turkey

P3AM-13-2
ELECTROACUPUNCTURE ON PERIPHERAL NERVE REGENERATION IN RAT

Motohiro Inoue[1], Miwa Nakajima[2], Tatsuya Hojo[3], Megumi Itoi[2], Hiroshi Kitakoji[1]
[1]Department of Clinical Acupuncture and Moxibustion, Meiji University of Integrative Medicine, Japan, [2]Department of Orthopedic Surgery, Meiji University of Oriental Medicine, Japan, [3]Faculty of Health and Sports Science, Doshisha University, Japan

P3AM-13-3
REGENERATION OF THE OLFACTORY NERVES FOLLOWING MILD AND SEVERE INJURY AND EFFICACY OF STEROID TREATMENT

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[1]Department of Otorhinolaryngology-Head and Neck Surgery, Mie University Graduate School of Medicine, Japan, [2]Department of Physiology and Biophysics, Virginia Commonwealth University School of Medicine, USA

P3AM-13-4
AXONAL DEGENERATION IN *HuC*-DEFICIENT CEREBELLUM

Kyoko Kakumoto[1], Hirotaka James Okano[1], Robert B Darnell[2], Hideyuki Okano[1]
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The Effects of Pomegranate Seed Extract and β-Sitosterol on Rat Uterine Contractions

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*The aim of this study was to investigate the effects of pomegranate (*Punica granatum *L., Punicaceae) seed extract on uterine contractility. Pomegranate seeds were methanolic extracted and their constituents analyzed using gas chromatography and mass spectrometry. Isometric force was measured in strips of longitudinal rat myometrium and the effects of pomegranate seed extract studied. We found β-sitosterol to be the main constituent of the extract (16%) and its effects were also investigated. Pomegranate seed extract and β-sitosterol increased spontaneous contractions* in a concentration-*dependent* manner *with a maximum effect at 250 mg/100 mL and 1 mg/100 mL, respectively. The amplitude and frequency of the phasic contraction were significantly increased along with basal tension. The effects of pomegranate seed extract were very similar to those of β-sitosterol. Force produced in the presence of pomegranate seed extract was abolished by the inhibition of L-type calcium channels or myosin light chain kinase (MLCK). Contractions were not potentiated by pomegranate extract following the inhibition of K channels or inhibition of the sarcoplasmic reticulum calcium ATPase (SERCA). The actions of β-sitosterol and the extract were not blocked by the estrogen receptor blocker, fulvestrant. We conclude that pomegranate seed extract is a potent stimulator of phasic activity in rat uterus. Our data suggest that the uterotonic effect is due to nonestrogenic effects of β-sitosterol acting to inhibit K channels and SERCA and thereby increasing contraction via calcium entry on L-type calcium channels and MLCK. We suggest that pomegranate extract and β-sitosterol may be a useful uterine stimulant.*

KEY WORDS: *Punica granatum* L, Punicaceae, pomegranate, smooth muscle, potassium channel, calcium, sarcoplasmic reticulum calcium ATPase, SR.

The pomegranate (*Punica granatum* L., Punicaceae) is an ancient, mystical, and highly distinctive fruit that has been used in folk medicine in many cultures.[1] Pomegranates are a rich source of crude fibers, pectin, sugars, and several tannins.[2] They also contain species of flavonoids and anthocyanidins in their seed oil and juice.[3] The seeds have been shown to contain a variety of estrogenic compounds,[4,5] as well as other steroids such as testosterone, β-sitosterol, and coumesterol.[5,6]

It has been shown that the extracts of all parts of the fruit appear to have therapeutic properties.[7] Most research has focused on its antioxidant, anticarcinogenic, and anti-inflammatory properties.[7] In addition, pomegranate seed extract has a uterotropic effect as it increased uterine weight and induced vaginal cornification in ovariectomized animals.[8]

To the best of our knowledge, the uterotonic effect of pomegranate seed has not been studied. As there is a clinical need to find better drugs to help control uterine activity,[9] and novel compounds are sought, the aim of the

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study was therefore to investigate the effects of pomegranate seed extract on uterine contractions. We particularly examined the effects on spontaneous phasic contractions and the mechanisms whereby pomegranate exerts its effects. The effects of the extract were then compared to β-sitosterol, one of the common plant estrogens isolated from pomegranate seeds.[5,6] Recently, it has been reported that plant sterols such as β-sitosterol may act to inhibit the sarcoplasmic reticulum calcium ATPase or SERCA[10] and that sterols can affect calcium-activated K channels[11]; we therefore investigated these possibilities. We find that pomegranate extract significantly stimulates uterine activity. Some of these data have been reported in abstract form.[12]

MATERIALS AND METHODS

Plant Material

Fresh pomegranate fruits were collected from fields in the area of Nakhon Ratchasima, Thailand, during April to May. The plant and its fruit was identified and confirmed by the Royal Forest Department of Thailand and a voucher specimen (Herbarium No 080252) deposited in the laboratory for future reference.

Extraction and Isolation

The pomegranate seeds were manually isolated. They were cleaned, air-dried, powdered, and subjected to Soxhlet extraction with methanol. The extract was filtered through a filter paper, evaporated in a rotary evaporator, and dried by a lyophilizer. The yield was 25.06%.

Gas chromatography/mass spectrometry (GC/MS) analyses were performed on a Hewlett-Packard 5973(IE) MS selective detector coupled with a Hewlett Packard 6890 gas chromatograph equipped with a cross-linked 5% PHME siloxane HP-5MS capillary column (30 m × 0.25 mm; film thickness, 0.25 μm). The gas chromatographic conditions were as follows: carrier gas, helium with a flow rate of 1.0 mL/min; column temperature, 50°C at 6°C/min; injector temperature, 250°C; volume injected, 0.1 μL of the oil; split ratio, 250:1. Compound identification was based on comparisons with mass spectra and retention indices of authentic reference compounds where possible. 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 β-sitosterol, a major component of pomegranate seeds.

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 carbon dioxide (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 $MgSO_4$; 12.0 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 7.40, 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 min was given before the application of any chemical. The measurements were made while the tissue was continually perfused with physiological solution (control) or solution containing pomegranate seed extract between 200 and 260 mg/100 mL. In some experiments, the known component of the extract, β-sitosterol (0.5-1.5 mg/100 mL, dissolved in physiological solution) was used. Wortmannin, an inhibitor of myosin light chain kinase (MLCK)[13]; nifedipine an inhibitor of L-type Ca

entry[14]; tetraethylammonium (TEA), an inhibitor of calcium-activated potassium channels; and cyclopiazonic acid (CPA), an inhibitor of the SERCA pump[15]; and fulvestrant, an estrogen receptor antagonist,[16,17] were also used, as indicated in the text.

Chemicals

All chemicals were purchased from Sigma unless stated otherwise. The purity of β-sitosterol, which was used as a positive control, was 75%.

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 pomegranate extract or β-sitosterol were measured over 20 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 stimulation with CPA, TEA, or fulvestrant following pomegranate extract, contractions were compared for 10 minutes in CPA and pomegranate extract (ie, 11-20 minutes after start of pomegranate extract exposure), to the 11 to 20 minutes in pomegranate extract without the addition of CPA, TEA, or fulvestrant. Integrated force (area under the curve) was measured over a 10- or 20-minute period as appropriate. 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.

RESULTS

Gas Chromatography/Mass Spectrometry Analysis

The GC/MS analysis showed 4 main compounds that had retention times (minutes) of 40.55 (18.30%), 18.23 (15.72%), 30.16 (14.93%), and 17.01 (11.04%). These corresponded to tocopherol, 6-butyl-1,4-cycloheptadiene, β-sitosterol, and octadecadienoic acid, respectively. Traces of 22 other known compounds, mainly essential oil (0.1%-5%), and 4 unknown compounds were detected (data not shown).

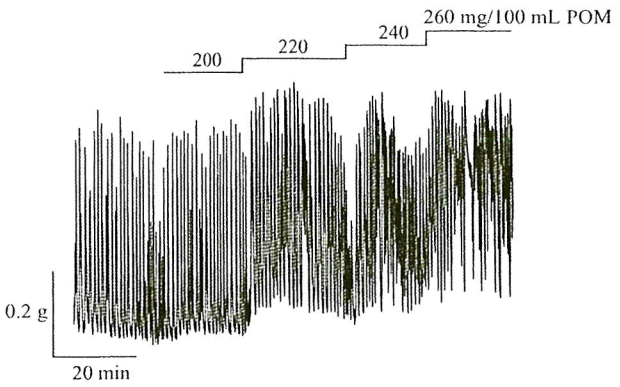

Figure 1. The effects of pomegranate seed extract (POM) on spontaneous contraction. The presence of increasing cumulative concentrations of POM (200-260 mg/100 mL) is shown.

Spontaneous Uterine Activity—Dose Dependency of Pomegranate 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 Figure 1). The effects of increasing cumulative concentrations of pomegranate seed extract (200-260 mg/100 mL) were examined; each concentration was applied for 30 minutes. Pomegranate seed extract, in a concentration-dependent manner, increased uterine contractility arising spontaneously (n = 5). An example of this is shown in Figure 1. At each concentration, the extract increased the amplitude and the frequency of the contractions and increased basal tension. The stimulatory effects of pomegranate seed 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. The threshold concentration at which an effect was consistently observed with 220 mg/100 mL of pomegranate seed extract, and the maximal stimulatory concentration on myometrium contractility occurred between 240 and 260 mg/100 mL (n = 5). Thus, the concentration of 250 mg/100 mL was used throughout the remainder of the study.

Effects on Parameters of Contraction

The application of pomegranate seed extract (250 mg/100 mL) to the rat myometrial preparations produced significant potentiating effects on spontaneous force (n = 5). The frequency of the contractions increased significantly

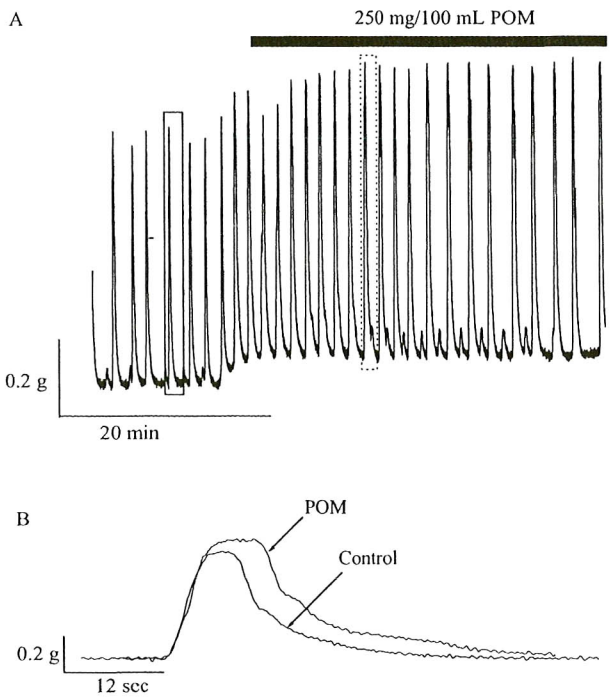

Figure 2. The effects of pomegranate seed extract (POM) on spontaneous contraction. Pomegranate seed extract (250 mg/100 mL) is added to spontaneously contracting uterus (A). Superimposed force records taken from (A), under control conditions and in the presence of POM (dotted trace; B).

to 134% ± 11%. The amplitude of force was also significantly increased; 130% ± 12%, as was its duration; 163% ± 3% (all compared with control, 100%). The mean increase in integrated force over the last 20 minutes in extract was 146% ± 12%. A typical example is shown in Figure 2A. It can be seen in Figure 2B, where control and pomegranate seed extract records have been expanded and overlapped, that there is a clear effect of pomegranate seed extract to prolong the force transient, due to an effect of prolonging the plateau phase and also significantly slowing the relaxation rate (172% ± 23%). In addition, pomegranate seed extract consistently increased basal force by 6% ± 1% (see Figure 2A).

Effects of β-sitosterol

As shown above, it is clear that pomegranate seeds potentiate uterine contraction. As β-sitosterol is one of the major components found in the extract and has previously been found to be a phytoestrogen, it was of

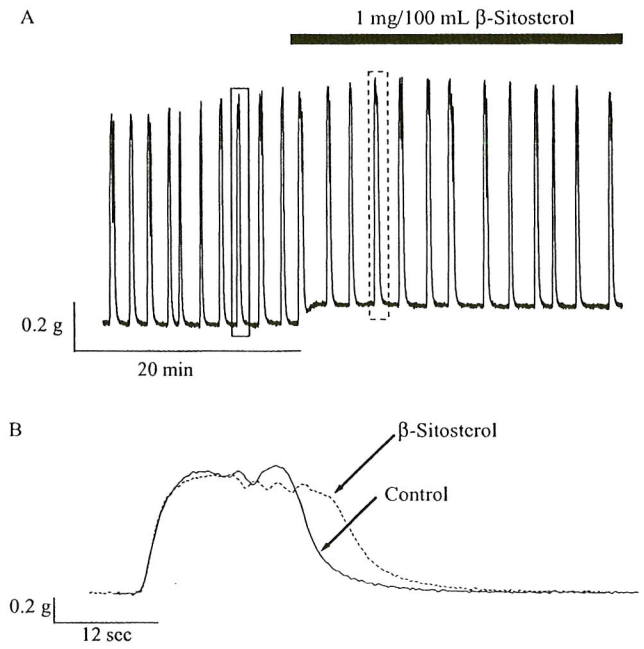

Figure 3. The effects of β-sitosterol on spontaneous contraction. β-sitosterol (1 mg/100 mL) is added to spontaneously contracting uterus (A). Superimposed force records taken from (A), under control conditions and in the presence of β-sitosterol (dotted trace; B).

interest to determine whether the effects of pomegranate seed extract were due to this compound. The estrogenic activity of phytoestrogens range from 1/500 to 1/1000 of the activity of estrogen.[18] Initial dose response curves over the range 0.5 to 1.5 mg/100 mL showed that maximal effects were achieved at around 1 mg/100 mL, and thus, a dose of 1 mg/100 mL of β-sitosterol was used (n = 5). As shown in Figure 3A, application of β-sitosterol produced significant increases in force (125% ± 3% compared with control integrated force). The amplitude of force was significantly increased; 111% ± 3%, as was its duration; 138% ± 8% (all compared with control, 100%). However, the frequency of the contractions was not significantly increased (107% ± 3%; compared with control, 100%). As with the pomegranate seed extract, β-sitosterol increased the basal force to 7% ± 1% (see Figure 3A). It is interesting to note that β-sitosterol also prolonged the force transient (Figure 3B).

Effects of Ca-dependent force pathway modulation

Uterine force can be produced by several pathways, but the main mechanism involves Ca-calmodulin-MLCK.[19] To investigate whether the increases in uterine force were

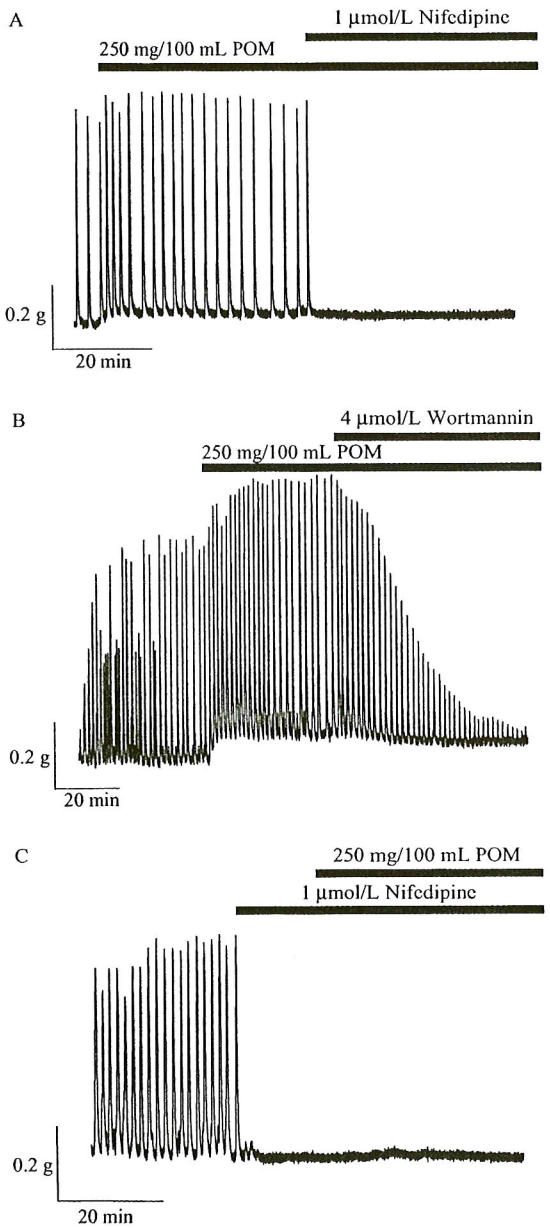

Figure 4. The effects of nifedipine and wortmannin on rat uterine contractions induced by pomegranate seed extract (POM). Control POM application followed by POM and nifedipine (1 μmol/L; A). Control POM application followed by POM and wortmannin (4 μmol/L; B). Nifedipine (1 μmol/L) application followed by POM (250 mg/100 mL; C).

dependent on the calcium-calmodulin MLCK pathway, we studied the effects of the extract in the presence of the L-type calcium channels and MLCK inhibitors (Figure 4). As can be seen in Figure 4A, the application of 1 μmol/L nifedipine in the continued presence of the extract rapidly inhibited and then abolished force ($n = 5$). Basal force, however, did not return to control levels but remained elevated.

We next investigated the effects of pomegranate seed extract in the presence of a potent inhibitor of MLCK, wortmannin (Figure 4B). As shown in Figure 4B, wortmannin (4 μmol/L) in the continued presence of the extract gradually reduced force in all preparations studied ($n = 5$); a significant reduction occurred after 10 minutes (mean amplitude of contractions 86% ± 1%) and by 45 ± 9 minute contractions were abolished.

In the uterus, some uterotonic agents can elicit a contraction in a 0-Ca solution or when L-type Ca channels are blocked,[15,20] and it has been suggested that this contraction occurs independently of the calcium-calmodulin-MLCK pathway.[15,20] To investigate this, the extract was applied after application of nifedipine. As can be seen in Figure 4C, no force is observed during the application of the extract ($n = 5$).

Role of K Channels

The effects of pomegranate extract on force resembled those of potassium channel blockers, which prolong the action potential and thereby potentiate force.[15,21] Thus, the question arose whether the extract effects were mediated by effects on potassium channels. We therefore blocked potassium channels, with TEA (5 mmol/L) and studied the effects of pomegranate seed extract ($n = 6$). Application of TEA produced increases in force (137% ± 4% compared with control integrated force), but no further significant increase occurred upon addition of pomegranate seed extract in the continued presence of K channel inhibition (110% ± 12%; Figure 5A). Similarly, if TEA was added after pomegranate seed extract, it produced no further increases in force (Figure 5B). These data suggest that the potentiating effect of TEA was prevented by pomegranate seed extract.

Role of Sarcoplasmic Reticulum

Release of Ca from the sarcoplasmic reticulum (SR) can potentiate force in smooth muscles,[22] and thus the SR may be a target for pomegranate extract. In addition, the increase in basal tone found with the extract could be due to the release of Ca from the SR or inhibition of Ca reuptake by SERCA. We therefore elucidated the effect of pomegranate extract after the inhibition of SERCA by cyclopiazonic acid (20 μmol/L).[15,23] As expected, inhibiting SERCA increased uterine force. As can be seen in Figure 6A, the extract was unable to potentiate force after

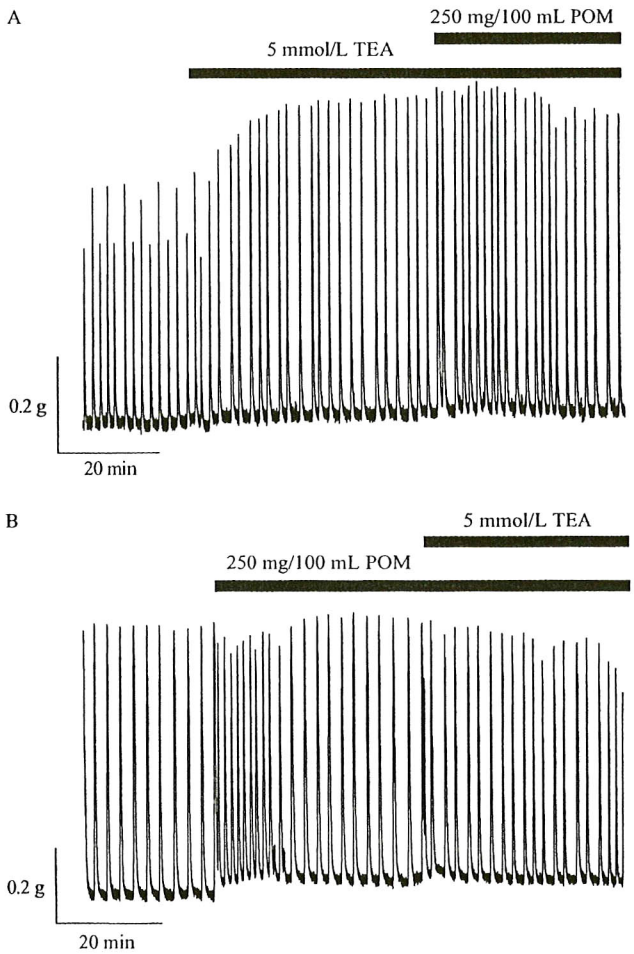

Figure 5. The effects of pomegranate seed extract (POM) on contraction in the presence of the calcium-activated potassium channel inhibitor. Tetraethylammonium (TEA, 5 mmol/L) is added before (A) and after (B) POM (250 mg/100 mL).

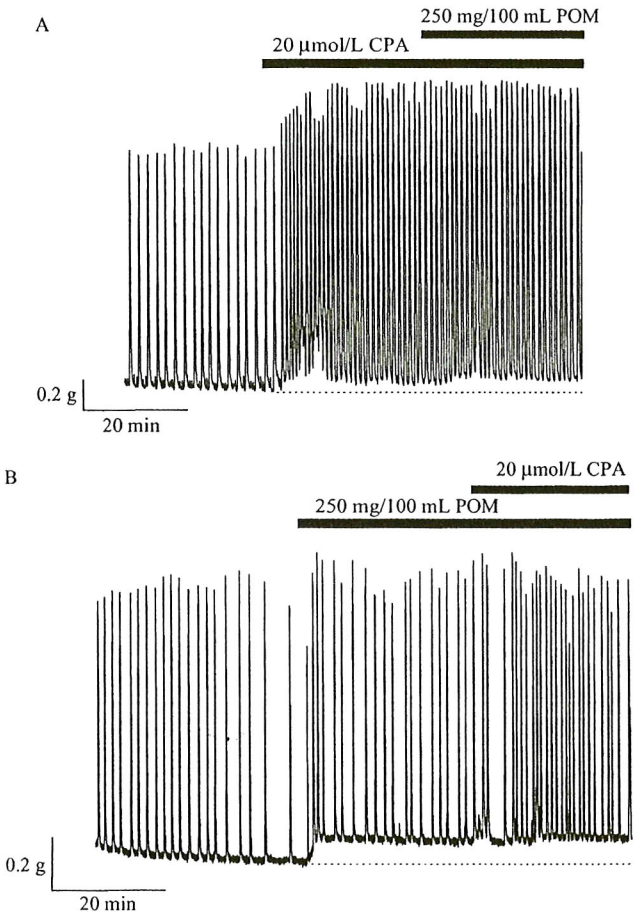

Figure 6. The effects of pomegranate seed extract (POM) on contraction in the presence of the sarcoplasmic reticulum calcium-ATPase inhibitor. Cyclopiazonic acid (CPA, 20 μmol/L) application is added before (A) and after (B) POM (250 mg/100 mL).

CPA treatment (n = 5). The application of CPA produced increases in force (227% ± 18% compared with control integrated force). No further increase occurred upon addition of pomegranate seed extract in the continued presence of CPA (103% ± 4%; Figure 6A). As can be seen in Figure 5A, CPA caused an increase in basal tone (≅5%); no further increase occurred in the presence of pomegranate. If CPA was added after pomegranate seed extract (n = 5), it produced no further increases in force amplitude (Figure 6B), or basal tone, but the contraction frequency was increased.

Effects of Fulvestrant

The above results clearly show the effects of pomegranate seed extract, which could be occurring through either the nonreceptor estrogen-like actions of β-sitosterol or via its estrogen receptor-mediated actions. We therefore blocked the estrogen receptors, with fulvestrant (1 μmol/L),[16,17] and studied the effects of pomegranate seed extract (n = 5) and β-sitosterol (n = 6). Application of fulvestrant to spontaneous active uterus produced no significant changes in uterine contractions. The amplitude, frequency, and area under the curve of spontaneous contraction after the application of fulvestrant were 105% ± 2%, 104% ± 10%, and 97% ± 10% (all compared with control, 100%). Application of pomegranate seed extract in the continued presence of fulvestrant produced significant increases in the amplitude of the contraction, frequency, and area under the curve (Figure 7). The increases in the frequency and area under the curve were 105 ± 3, 142% ± 11% and 131% ± 4% (all compared

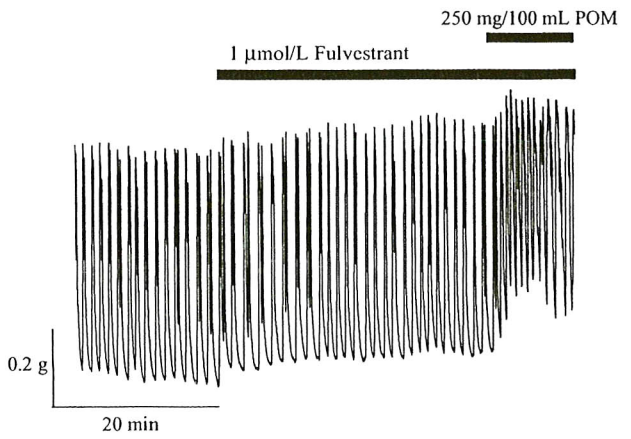

Figure 7. The effects of pomegranate seed extract (POM) on contraction in the presence of the estrogen receptor antagonist. Fulvestrant (1 μmol/L) application is added before POM (250 mg/100 mL).

with fulvestrant control, 100%). β-sitosterol was also able to significantly increase force in the presence of fulvestrant (data not shown).

DISCUSSION

This study is the first to show the effects of pomegranate seed extract on uterine contraction and demonstrates that it potently potentiates spontaneous contractions. Both the amplitude and the frequency of the phasic contraction were significantly increased as well as the basal tension. The effects of pomegranate seed extract were very closely matched to those of β-sitosterol. The potentiation of force induced by the extract was dependent upon the Ca-calmodulin-MLCK pathway. Pre-incubation with TEA or CPA prevented the extract exerting its effects, suggesting that K channels or SERCA are the targets for its actions. When the effects of pomegranate seed extract and β-sitosterol were compared after fulvestrant treatment, they were still effective. Thus, our conclusion is that pomegranate seed extract is a powerful uterine stimulant acting via a nonestrogen mechanism.

In agreement with previous studies,[5,6] we showed that one of the major constituents isolated in pomegranate seed extract is β-sitosterol. Other constituents, mainly essential oils, have been demonstrated to relax rather than contract uterine smooth muscle.[24] Thus, the predominant effect on uterine contractions of an extract of pomegranate is potentiating, and we show that β-sitosterol is the active agent responsible for this effect. It also increased basal tone as did the extract.

We found that the pathway to increase uterine contraction by pomegranate seed extract occurred via calcium-dependent pathway as: (a) addition of the extract could not produce force in the absence of external calcium entry and, (b) force produced in the presence of the extract was abolished when Ca entry through L-type Ca channels was inhibited. Further support for this conclusion comes from the experiments with MLCK inhibition; force was no longer produced by the extract. Thus, our data support a mechanism of action involving the Ca-calmodulin-MLCK pathway rather than that of Ca-sensitization. Nifedipine application, however, did not reverse the increase in basal tone caused by the extract, although it did abolish the spontaneous contractions. This suggests that the mechanism causing the elevation of basal tone is not dependent upon Ca entry and may therefore involve the internal Ca store, that is, the SR. This is discussed further later.

Our data suggest that the extract is potentiating force by an inhibition of K channels and an effect on the SR. The effects of pomegranate seed extract on the uterus resembled those of the K channel inhibitor 5 mmol/L TEA,[15,23] and after exposure to TEA, the extract was without effect. There are data from other sterols, especially cholesterol, that these compounds can modulate K channel activity. Specifically in the uterus, cholesterol manipulation can have marked effects on Ca signaling and contractility[25] via effects on Ca-activated K channels.[11] Cholecalciferol, a vitamin D_3 precursor, has also been shown to affect K channel activity in vascular smooth muscle.[26] Further studies are suggested to determine which type of K channels are the main targets of the extract, along with measurements of electrical activity.

The experiments in which SERCA was inhibited using CPA point to an involvement of the SR in mediating the effects of pomegranate extract. Pre-incubation with CPA prevented any additional effects of the extract occurring. However, CPA was still able to produce some stimulation of the uterus following pomegranate application. The role of the SR in spontaneous activity of the uterus remains enigmatic, as its inhibition promotes contractility, suggesting it is not acting as a source of Ca for spontaneous contractions.[27] Nor is it likely that it is acting via the release of Ca sparks from the SR that activate Ca-activated K channels, as such a mechanism is absent in the uterus.[28] Thus, it is difficult to interpret our findings in further detail. We can find no evidence in smooth muscle that β-sitosterol potentiates force by inhibiting SERCA. There is evidence however that phytoestrogen can affect SERCA in cardiac muscle.[29] In addition, there

is clear evidence in macrophages that plant sterols such as sitosterol can inhibit SERCA.[9]

Inhibition of SERCA may explain the increase in basal tone found with extract application. Our previous studies have shown that when SERCA is inhibited; there is an increase in intracellular [Ca],[15] which may lead to change of basal tone. Our data with nifedipine support the source of Ca being intracellular, as nifedipine abolished phasic contractions but did not restore basal force to resting condition.

CONCLUSION

In conclusion, we have presented novel data demonstrating a significant stimulation of uterine activity by pomegranate seed extract, which can largely be accounted for by its constituent, β-sitosterol, acting as a nonestrogen receptor-mediated mechanism. The stimulation of uterine activity may contribute to the antifertility effects of pomegranates, and they may be a useful source of uterine stimulant for slowly progressing labours,[30] although further studies in human myometrium are required to develop these suggestions. Although not studied, it is speculated that, as the pathways for calcium signaling and contractility are similar in the pregnant to nonpregnant myometrium, and in human and rat myometrium, that similar effects of pomegranate seed extract will be seen in the pregnant human uterus.

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