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Aqueous extracts from evening primrose seeds (*Oenethera biennis*) contract isolated uterine tissues but have no effect on isolated cervical tissues

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Abstract
The effect of aqueous extracts from evening primrose seeds, *Oenothera biennis*, on mouse uterine tissues and rat cervical tissues *in vitro* were investigated at various concentrations (10-40 mg) using standard 15 mL organs baths. All extract concentrations used increased the force of uterine contractions; the highest concentrations yielded significant increases (P=0.01972). These contractile forces were slightly greater than those evoked from acetylcholine (10^{-5} M). Cervical tissues that were pre-contracted with a high potassium solution did not demonstrate any relaxation following 15 min incubations with the evening primrose seed extracts. The β adrenergic receptor agonist isoproteronol did relax the cervical tissues by 97% (P=0.05). These results may indicate that some of the evening primrose constituents responsible for (1) uterine contractions are water-soluble and are present in the aqueous extract; and (2) cervical relaxation are primarily lipid-soluble and are present to a greater degree in the oil fraction.

Keywords: *Oenothera biennis*, uterine contractions, cervical ripening, rodents, *in vitro*

1. Introduction
Labor is generally induced as a standard part of obstetric practice when the benefits of delivery are greater than the risks of continuing the pregnancy [1, 2]. Some of these risk conditions include premature amniotic membrane rupture; postdate pregnancy, intra-amniotic infection, and intrauterine fetal death [2]. Typically, prostaglandins and oxytocin are used to induce labor by ripening the cervix and inducing uterine contractions [1-4]. A large number of Certified Nurse Midwives use alternative herbal preparations for the induction of labor in post-dates pregnancies. For example, the roots and rhizomes of blue cohosh (*Caulophyllum thalictroides)* and black cohosh (*Actaea racemosa)*, red raspberry leaves*(Rubus idaeus),* castor oil (*Ricinus communis)*, and evening primrose oil (*Oenethera biennis*) have all been cited as herbal preparations for stimulating labor [5, 6, 7]. Many have specifically been drawn to the use of evening primrose oil in concert with castor oil. Castor oil is thought to exhibit a larger uterine contractile force than evening primrose, and evening primrose is thought to have a greater effect in ripening the cervix [5, 8, 9]. Evening primrose is a flowering plant native to North America but has also been naturalized to certain parts of Asia, including the major modern producer China [10, 11]. Historically, evening primrose was prepared by mixing its crushed roots and leaves with water to form an aqueous solution. It was used medicinally for numerous conditions, including diabetic neuropathy, multiple sclerosis, rheumatoid arthritis, eczema, psychiatric disorders, and cancer [10, 12-14]. In clinical practice today, evening primrose is principally used in the form of oil [5, 8, 10, 14] and is obtained by pressing the oil from the plant's seeds [10, 14]. The seeds by weight are 15% protein, 43% cellulose and lignin, and 24% oil. About 9-13% of the fatty acids found in evening primrose oil isγ-linolenic, which is an essential fatty acid in human metabolism [13, 14]. Since γ-linolenic acids are precursors to prostaglandins, it is reasonable that the use of oils containing these acids would similarly induce labor and could be used as a medicinal alternative for labor induction [5, 9, 10, 11, 14]. Cervical ripening plays a large part in determining the success of labor induction [1]. Prostaglandin E_2 has been shown to be safe and effective for cervical ripening when applied topically [5]. It has been suggested that prostaglandins may dilate the cervix by physical relaxation of the smooth muscle [2, 9] and/or softening the tissue by rearrangement of the collagen fibers at a molecular level [2, 15, 16]. It is uncertain how evening primrose promotes cervical ripening. Vaginal evening primrose oil has been found to be useful as far as reducing total cervical dilatation time and providing greater cervical width [17]. Oral doses have been studied on low-risk nulliparous women, but the research has failed to demonstrate any significant effect on the length of pregnancy [8]. The use of muscle tissues *in-vitro* in an organ bath can maintain conditions that are representative of anintact organism [18].

This can allow for the investigation of topical effects of chemicals without contributions from the nervous or endocrine systems. *In vitro* studies of smooth muscle using rodent uterine and cervical tissues to simulate studies of the human have been extensively reported in the literature. The oil of evening primrose, however, is not compatible with the aqueous buffer employed in standard organ bath protocols. Recent investigations using simple aqueous extracts from pulverized root and rhizomes of blue cohosh [19], powdered red raspberry leaves [20], and crushed castor beans [21] have all effectively contracted isolated strips of mouse uterine tissue. Thus, the purpose of the investigation herein was to employ similar methods using an aqueous extract from the seeds of evening primrose and collect data that may support or refute claims that constituents of evening primrose would both contract uterine smooth muscle and relax the cervix. The specific objectives were to 1) observe and quantify the contractile effects of aqueous extracts of evening primrose seeds on isolated strips of mouse uterine horn sand rat cervical tissues; and 2) determine if the resulting responses produced a concentration-dependent relationship.

2. Materials and Methods

2.1. Evening primrose seed extract preparation

Seeds from *Oenethera biennis* were obtained from Mountain Rose Herbs Company (Eugene, Oregon, USA) and Richters Herbals (Good wood, Ontario, Canada). Herbarium samples preserved for future reference are housed at Bethel University. An aqueous extract of *Oenethera biennis* seed was prepared based on the methods published by Lis-Balchin and Hart [22] and Kaingu *et al*. [23]. The seeds were crushed using a mortar and pestle, massed, and an appropriate amount of deionized water was added to create the desired concentration of extract. The mixture was vortexed for several seconds, vacuum filtered through filter paper to remove seed particulates. The resulting filtrate was stored on ice in a closed container for the duration of each experiment, and warmed to approximately the temperature of the organ bath prior to application. The extract was made fresh for each experiment and concentrations of 10, 20, 30 and 40 mg per 15 mL organ bath were used. This aqueous extract was miscible with the polar DeJalons solution used in the organ baths.

2.2 Specimens

Twenty-two virgin, female, ICR CD-1 outbred mice (*Mus musculus*) weighing 25-30 g and five fully matured female Hsd: Sprague Dawley rats (*Rattus norvegicus*) weighing 200-250 g, were obtained from Envigo (Indianapolis, Indiana, USA). All specimens had *ad libitum* access to water and standard chow. All procedures were completed in accordance with the Institutional Animal Care and Use Committee of Bethel University. Since the estrous cycle of mice is four to five days, intra peritoneal (IP) injections of diethylstilbestrol (DES), a synthetic non-steroidal estrogen agonist, were given 24 hrs prior to each experiment to forward each mouse into the estrus stage of their cycle [24]. DES also facilitates the formation of gap junctions within the endometrial cells of the uterus, thus promoting the thickening of the endometrial layer of the uterus and allowing the organ to work as single-unit smooth muscle [25]. In order to simulate actual hormone levels present in gravid rats, IP injections of 0.2 mg DES were also given to each rat 24 hrs. prior to dissection.

2.3 Tissue preparations

On the day of the experiment, the animals were sacrificed via CO_2 asphyxiation. For mice, the uterine horns were extracted from the abdominal cavity and immediately placed in chilled De Jalons solution (g/5 L): 45 g NaCl, 2.1 g KCL, 2.5 g $NaHCO_3$, 2.5 g D-glucose, 0.4 g $CaCl_2$.Each uterine horn was dissected, cleansed of excess connective tissue, and ligated on either end. One end was attached to the distal portion of a fixed stationary hook to be placed inside a 15 mL organ bath, and the other attached to an isometric force transducer (MLT500, AD Instruments, Colorado Springs, Colorado, USA) coupled to Power Lab 4/SP data acquisition system with Chart 5.2 software (AD Instruments). The uterine tissue was then placed into the organ bath containing De Jalons solution, suspended at 0.8 g of tension, and allowed to equilibrate for 1 hr with the flushing of fresh De Jalons solution every 15 min. A mixture of 95%O_2 and 5%CO_2 was bubbled into the bath throughout the duration of the experiment. The tissues were maintained at 30°C so as to dampen their normal endogenous contractile patterns, herein called spontaneous motility, and enable contractions produced by the evening primrose extract treatment to be more easily distinguished [26]. Following rat euthanasia, the cervix was extracted from the abdominal cavity and segmented into four rings. Each circular ring was suspended at 2 g of tension in the organ bath [27]. Krebs-Henseleit solution was used for the cervical tissues (g/5L): 10.0 g D-glucose, 0.71 g $MgSO_4 \cdot H_2O$, 0.8 g KH_2PO_4, 1.75 g KCl, 34.5 g NaCl [27, 28]. A high potassium solution was also made for pre-contraction of the cervical tissues (g/5L): 21 g D-glucose, 2.9 g $MgSO_4 \cdot H_2O$,1.6 g KH_2PO_4,28 g KCl, 21 g $NaHCO_3$, 2.8 g $CaCl_2$ [26]. The remaining organ bath parameters were the same as that employed by the uterine tissues.

2.4.1 Mouse uterine tissue testing, response measurements, and analyses

All tissues were first given 10^{-5} M acetylcholine (ACh) to produce a positive contractile response and affirm tissue viability [26]. Tissues were then rinsed with fresh De Jalons solution, allowed to equilibrate for an additional 10 min, and return to baseline motility patterns. Each tissue was then given the desired treatment and the resulting contractile forces were recorded and measured. Each uterine horn was treated as a unique sample and the final extract concentrations used were 10, 20, 30, and 40mg/15 mL bath. A sample size of 2-6 uterine horns was used for each concentration. All treatment applications were made after the full completion of a spontaneous motility cycle under basal tension. Changes in contractile force were measured from baseline tension to the maximal force produced within the first 10 min of treatment exposure. The mean values for change in maximal contractile force relative to 10^{-5} M ACh at each evening primrose seed extract concentration were also determined.

Means ± S.E.M. were calculated for each concentration and differences among the contractile responses were analyzed using ANOVA for multiple comparisons among the means. Following the rejection of the null hypothesis that evening primrose seed extract would have no contractile effect on isolated uterine horn tissues, the Tukey-Kramer post hoc test was used to indicate which contractile responses produced from the extract concentrations were significantly different from each other.

2.4.2 Rat cervical tissue testing, response measurements, and analyses

The suspended cervical tissues were also equilibrated for approximately 1 hr. with flushing at 15 min intervals. Then the Krebs-Henseleit solution was flushed out, and the organ

bath was refilled with the high potassium solution so as to induce contraction in the tissues [26]. After 15 minutes of equilibration, the aqueous evening primrose extract treatment was added. Tissue motility was then observed for the next~ 30 minutes. If no change in contractile tension occurred, isoproterenol, a β adrenergic receptor agonist, was added as a positive control for rat cervical tissue relaxation [28] and motility was recorded for another 15 minutes. The cervical contractile forcesas induced by the high K^+ bufferwere measured before and after application of evening primrose, and the percent change was calculated for each experiment (n=5). Similar measurements were also taken for tissues subjected to isoproterenol (n=5). Paired t-test were performed to test for any statistical difference between the force before and after application of evening primrose, as well as before and after the isoproterenol treatment. A Mann-Whitney U test was performed to test for any statistical difference between the percent change in the tension of the tissues subjected to evening primrose and the percent change in the tension of tissues subjected to isoproterenol. For all analyses, data were considered to be statistically different at $P \leq 0.05$ (JMP 4.0, SAS Institute, Cary, NC).

2.5 Chemicals

All drugs were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Acetylcholine was dissolved in deionized water and isoproterenol was solubilized in diemthylsulfoxide.

3. Results

3.1. Uterine contractile responses to evening primroseaqueous extracts

The application of evening primrose aqueous extracts to isolated uterine strips of mouse tissue resulted in immediate contractions that exhibited a strong increase in force and a mild increase in basal tonus that gradually returned to baseline within 5-10 min (Figure 1). Contractile responses from the evening primrose extracts (10-40 mg/15 mL bath) ranged from 00.02-66.31.68 mN. Typically, rapid increases in the frequency of contractions occurred, but rates were difficult to consistently measure and thus were not further analyzed for this study. Since force was the most consistent and repeatable indicator of contraction, it is the focus of the quantified measurements presented herein.

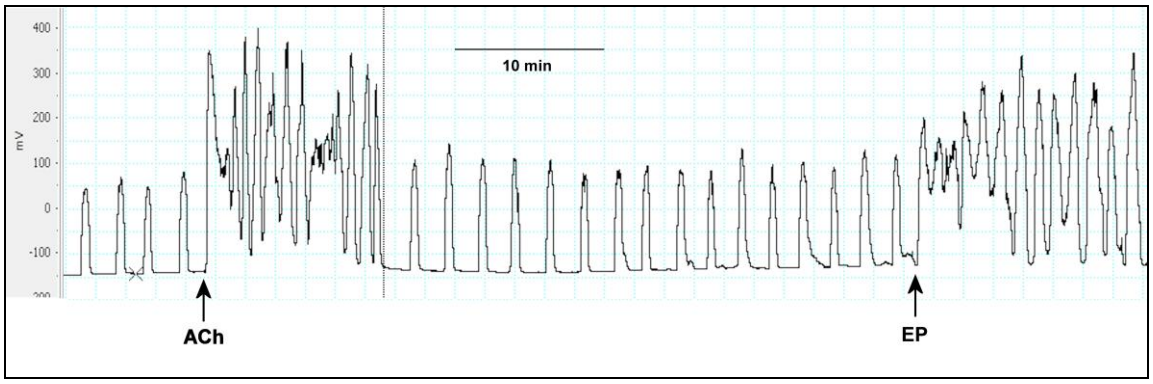

Fig 1: Typical contractile waveform responses following the application of 10^{-5}M acetylcholine (ACh) followed by 30 mg evening primrose (EP) aqueous extract as recorded from a single isolated mouse uterine horn. In this 72 min selection, the addition of ACh resulted in a contractile force 120% greater than that observed in the spontaneous motility just prior to application (from 20.49 mN to 45.43 mN). The addition of 30 mg EP resulted in a contractile force 86% greater than that observed in the spontaneous motility just prior to EP application (from 22.34 mN to 41.71 mN). The dotted vertical line indicates a tissue washout with fresh DeJalons. For analysis, the default y-axis was converted to mN based upon calibration of the force transducer. All uterine tissues exhibited spontaneous motility before the application of either ACh or evening primrose treatments. To control for the possible contributions these endogenous forces might have on the treatments, the amplitude of the spontaneous motility was subtracted from the peak contractile forces evoked from their respective ACh or evening primrose treatments. Furthermore, to normalize for the slight variation in the tissue masses, each tissue's maximal contractile response to an evening primrose extract was expressed as a percent of its initial contractile response to 10^{-5} M ACh. Figure 2 presents the mean ± S.E.M uterine contractile responses for each evening primrose extract concentration tested. All concentrations contracted the uterine tissues, and at the highest concentrations tested (30 and 40 mg) the extracts yielded significantly greater increases in contractile force (P=0.0057).

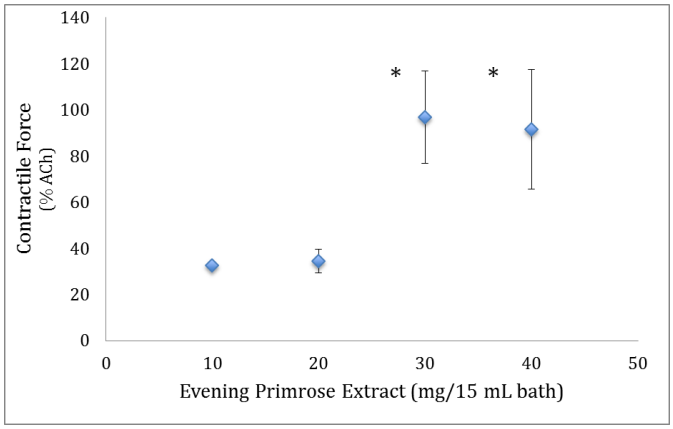

Fig 2: Mean ± S.E.M. uterine contractile forces (%ACh) in response to evening primrose aqueous extracts. Even though all concentration evoked a contractile response from the uterine tissues, only those marked with * produced forces that were significantly greater than "0" treatment (P=0.0057, n=2-6 tissues/concentration).

3.2. Cervix relaxation responses to evening primrose aqueous extracts

There was no significant relaxation of the pre-contracted cervical tissues in response to 30 mg evening primrose extract. As expected, isoproteronol did demonstrate a large decrease in pre-contracted cervical tissues tension (Figure 3).

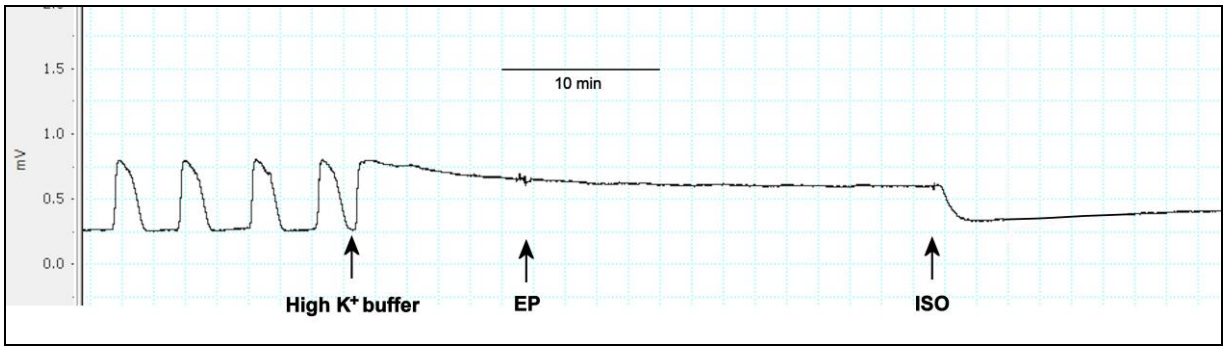

Fig 3: A typical waveform response of a pre-contracted uterine tissue followed by the application of 30 mg evening primrose (EP) aqueous extract, and then 10^{-5}M isoproterenol (ISO). In this 72 min selection, the addition of a high K^+ buffer resulted in a contractile force similar to a peak spontaneous motility force of 12.16 mN. After the addition of 30 mg EP extract 12 min later, the baseline tension was only reduced from 9.28 mN to 7.62 (an 18% reduction in tension). On this same tissue, the application of ISO reduced the tissue tension to 1.61 mN, a 79% reduction in contractile force. For analysis, the default y-axis was converted to mN based upon calibration of the force transducer. The mean pre-contracted cervical tissue force observed was 3.30± 0.14 mN (n=5). Following the addition of 30.0 mg evening primrose extract, the force observed was3.10 ± 1.5 mN, and no significant change in tissue force was observed (P=0.409). However, isoproterenol resulted in a significant decrease in muscle tension (P=0.05). The mean ± S.E.M contractile force for the tissues before and after isoproterenol addition was 3.35 ± 1.35 mN and 0.38 ± 0.37 mN, respectively. When considering the percent change in contractile tension in both pre- and post-treatments for evening primrose and isoproterenol, the overall percent tissue relaxation following isoproterenol was 97.08% and was significantly greater than that evoked from evening primrose, 13.70% (P=0.0018; Figure 4).

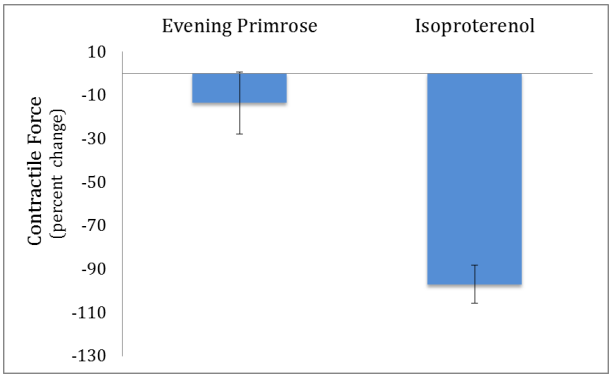

Fig 4: Changes in pre-contracted cervical tension (means ± S.E.M; n=5) in response to 30 mg evening primrose aqueous extracts or 10^{-5}M isoproterenol. The overall percent tissue relaxation following isoproterenol (97.08%) was significantly greater (P=0.0018) than that evoked from evening primrose (13.70%).

4. Discussion

4.1 Aqueous extracts of *Oenethera biennis* as an uterotonic

One of the important physiological roles of prostaglandins is the control of smooth muscle contraction in parturition [1]. Prostaglandins E_2 and F_2 are both powerful stimulators of uterine motility. Since γ-linolenic acids are precursors to prostaglandins [29, 30], and prostaglandin E_2 and F_2 augment uterine contractions [31-34], it makes sense that evening primrose oil can have an effect on the smooth muscle contractility of the uterine tissues.

In this preparation however, it was considered that the insolubility of γ-linolenic acid in aqueous environments [35], as found in both the evening primrose extract and the organ bath, did not contribute to uterine tissue contractility. Our results did demonstrate however, that the prepared aqueous extract of eveningprimrose seeds did act as an herbal uterotonicby producing a strong contractile response from the uterine tissues (see Figures 1, 2). Therefore, an assumption can be made that there may be other biological constituents in the evening primrose extract that can act as receptor-agonist to promote calcium entry and initiation of contraction in uterine smooth muscle [36].

Several of the secondary metabolites in evening primrose remain in the seed pomace following the removal of lipids and fatty acids [37]. Many are considered to be therapeutic. For example, triterpenoids and phenolics can serve as antioxidants [38] and may be used as anti-inflammatory treatments for irritable bowel disease [37]. Polyphenol extracts from defatted seeds have also been shown to reduce the viability of prostate and breast cancer cell lines [39].

One of the most biologically active phenolic compounds is gallic acid [40], which has been show to possess microbial activity against several human and plant pathogens [41]. Large quantities of gallic acid are found in evening primrose seeds [40] and may be found as both free and as a hydrolyzable tannin [39, 42].

Other herbal preparations used by Certified Nurse Midwives [5, 6, 9] have been investigated using the same methodology as reported herein. A comparison of three commonly used herbal uterotonics with the results from the evening primrose reported herein, is provided in Table 1. It can be seen the evening primrose is not as strong ascastor seed, red raspberry leaves, or blue cohosh when prepared as an aqueous extract and given to isolated uterine tissues at the same concentrations.

Table 1: Means ± S.E.M. uterine contractile forces evoked by four different herbals (30 mg) from isolated strips of mouse uterine tissues suspended in a 15 mL organ bath (n=3-4). All contractile data is presented as a percent of the positive contractile control, 10^{-5}M ACh. Endogenous forces from spontaneous motility havealso been factored out.

Herbal and reference	Plant organ	Contractile force (%ACh) (Means ± SEM; n=3-4)
Castor Bean [21] *Ricinus communis*	seeds	140 ± 35
Blue Cohosh [19] *Caulophyllum thalictroides*	roots, rhizomes	116.7 ± 8.5
Red Raspberry [20] *Rubus idaeus*	leaves	110.3 ± 25.6
Evening Primrose *Oenothera biennis*	seeds	96.77 ± 20.03

It would seem reasonable that all of the herbals listed in Table 1 contain some water-soluble phenolic or phenolic derivative that acts on uterine smooth muscle cell receptors and facilitates the entry of calcium and the concurrent contraction. An updated review presenting the different phytoconstituents present in *Oenethra biennis* seeds included steroids, phenolic acids, tannins, flavonoids, lipids, and terpenes [42]. Not included in this list are alkaloids, which are suggested by Udoh *et al*. [43] to be responsible for the uterotonic action in rats given *Piper guineense* leaves, an African relative of black pepper. The alkaloid compound groups as found in the roots and rhizomes of *Caulophyllum thalictroides* has also been proposed to be an important player in evoking uterine smooth muscle contractions [44, 45]. Aqueous extracts from castor seed also contracted isolated strips of uterine tissue, but that response is likely due to the presence of ricinoleic acid in the extract [46].It is more polar than other fatty acids and has been shown toactivateEP_3 prostanoid receptors on uterine smooth muscle cells [47]. Red raspberry leaves contain a large amount of the hydrolyzable tannin, ellagic acid [48]. Literature on the contractile effects of ellagic acidorgallic acid (as in evening primrose) directly on isolated uterine smooth muscle has not been reported and has warranted further investigation. Tannic acid, however, another specific type of tannin, has been shown to dose-dependently and non-competitively antagonize contractions to different agonists in isolated rat uterine tissues [49]. It is likely that in mostherbal products, there is not a single active ingredient that dominates over the others, but rather many active ingredients that work synergistically to createa response [5].

4.2 Aqueous extracts of *Oenethera biennis* as an agent to ripen the cervix

Several prostaglandins are produced in the cervix; it contains receptors for both prostaglandins E_2 and $F_{2\alpha}$which likely induce enzymes to remodel the tissue's collagen and proteoglycan content resulting in a softer cervix [30]. The aqueous extract of evening primrose prepared herein did not have any significant relaxing effect on the smooth muscle present in isolated rat cervical tissues (Figures 3, 4). Therefore, the lack of response in the cervical tissue may be due to the absence of essential hydrophobic components (such as the prostaglandins) when the aqueous solution was prepared for the smooth muscle bath.

5. Conclusions and Recommendations

5.1 Conclusions

Most documented research on the role of evening primrose in reproductive physiology reports on the oral consumption or vaginal placement of the oil as extracted from the seeds [1, 5, 7, 9, 17, 50, 51]. This project uniquely reported the effects of evening primrose on uterine contractions and cervical relaxation when prepared as an aqueous extract. The results presented in this study demonstrate that an aqueous extract prepared from the seeds of evening primrose increased the force of contractions from isolated uterine tissues of *Mus musculus*. Isolated rings of pre-contracted rat cervical tissue however, were unresponsive to evening primrose even though isoproterenol exhibited a strong relaxation effect. These results may indicate that the constituents in evening primrose responsible for (1) the uterine contractile behaviors are water-soluble and are present in the water extract; and (2) the cervical relaxation behaviors are primarily lipid-soluble and are present to a greater degree in evening primrose oil.

5.2 Efficacy and Safety

It is important to consider the applicability of results collected at the reduced level of isolated tissues, to the oral consumption and/or topical administration of evening primrose (oil or aqueous extract) at the level of an intact organism. While the mouse uterine horn serves as a good model for the effects of agents on human uteri, intact organisms have many other input systems that might interfere with the components of evening primrose acting on the receptors on the uterus. The bioavailability of the biological constituents of evening primrose to other parts of the body remains to be addressed.

Evening primrose is sold as a food supplement and is not subjected to the same evaluative standards seen in pharmaceuticals. Individuals experiencing anemic conditions may want to avoid consumption of aqueous extracts because the tannins contained in evening primrose [42] may bind to iron, possibly exacerbating the condition [52]. A recent study employing mice using leaf extracts from evening primrose provided preliminary data supporting its safety as evident by the lack of adverse behavioral, biochemical, or hematological parameters [53]. While literature reviews on herbal preparation for inducing or augmenting labor note that there is a lack of clinical trials with adequate sample size to demonstrate safety and effectiveness, most state that when protocols are followed as recommended by practitioners, no negative side effects to mother or baby have been observed [5, 7, 54, 55].

5.3 Future research

Additional questions can now be asked that can pave the way for future investigations. For example, the aqueous extract used in this investigation was not further refined. Since the seeds were crushed with a mortar and pestle, it is possible that some of the more polar lipid constituents may have remained in the aqueous extract. Thus, a chemical analysis of the prepared extract is appropriate. Secondly, since tannins appear to be found throughout a variety of plants [56, 57] and extracts from different organs of the plant species listed in Table 1all resulted in strong uterine contractions, it is of interest whether or not the hydrolizable tannins (for example, ellagic acid and gallic acid) can directly contract smooth muscle tissues in the uterus. If so, then receptor antagonism studies can indicate which receptors (e.g. catecholamine, acetylcholine, oxytocin) are being activated, resulting in an increase prostaglandin synthetase activity in the smooth muscle plasma membrane to form PGE_2 or $PGF_{2\alpha}$.

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