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Aqueous extracts of clary sage (*Salvia sclarea*) contract isolated strips of mouse uterine tissue

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Abstract

Salvia sclarea, commonly known as clary sage, is a plant native to the Mediterranean basin. Essential oils from clary sage are highly aromatic and have been used in aromatherapy during labor, with the goal of reducing stress and anxiety, and increasing in uterine contractility. While there is growing evidence to suggest that some of the essential oils (linalyl acetate, linalool, α -terpineol) may be effective in the reduction of anxiety, other studies have shown that these same oils do not contract smooth muscle *in vitro*. It remains to be empirically determined whether other clary sage constituents can contract the uterus. The specific objective of this project was to determine whether an aqueous extract of clary sage had the ability to contract smooth muscle from isolated uterine tissues. Results showed that clary sage produced contractile responses at almost all concentrations applied and these were greater than the tissue's own spontaneous motility ($P < 0.0001$). It is proposed that the amphipathic triterpenoid saponins, contained within the applied extract, are involved with the muscle contractions, and that the hydrophobic essential oils involved are in the anxiolytic effects. Together, they provide some evidence that clary sage aromatherapy during labor can potentially allow the birthing process itself to be less impeded.

Keywords: *Salvia sclarea*, saponins, uterus, smooth muscle contractions, labor, clary sage

Introduction

The practice of herbal medicine is deeply embedded in history. Herbal remedies are estimated to be the primary means of medicine for at least 80% of the world's population [1, 2]. Obstetrics and midwifery are closely allied to herbal medicines, and serve as an important accessory in traditional and complementary medicine [3, 4, 5].

The use of aromatherapy with essential oils has been supposed to reduce stress and anxiety during labor [6]. Observations from midwifery surveys [6, 7, 8] report that clary sage (*Salvia sclarea*) aromatherapy is considered helpful for women with dysfunctional or slow labor. This was indicated by the fact that fewer women opted for oxytocin infusion, and thus proceeded to a spontaneous vaginal delivery.

These claims were based on interviews with attending midwives and/or the mothers themselves. Even though they are subjective, the noted outcomes were consistently reported, and have led to the opinion that the use of clary sage aromatherapy during labor can act as a stress reducer and coincidentally, enhance uterine contractility [9]. It remains to be empirically determined whether the chemical constituents in the herbal clary sage itself actually contract the uterus, or if the reduction in the anxiety associated with labor allows the birthing process itself to be less impeded.

Clary sage, *Salvia sclarea* family Lamiaceae, is native to southern Europe and Syria [10] and is cultivated in central Europe, Russia, England, Morocco, and the United States [11]. Some of the chemical constituents isolated from clary sage are commonly used in food flavorings [12, 13] and perfumes [14, 15]. The medicinal applications of clary sage are based on the bioactive constituents found in the essential oils, and have been demonstrated *in vitro* to be anti-inflammatory [16], antibacterial [17], antiviral [18], and antifungal [19].

The project reported herein investigated the capabilities of clary sage to contract uterine tissues *in vitro*, and apply those results to assess the validity of the *in vivo* claims as put forth by midwifery and oral traditions of clary sage behaving as an oxytocic agent. Thus, the specific objectives were to 1) determine whether clary sage has the ability of contracting smooth muscle from isolated uterine tissues; 2) determine whether the resulting changes in contractile forces were directly proportional to the concentrations of clary sage administered, 3) reflect on which constituents may be biologically active in the aqueous extract, and 4) provide some empirical support for the use of clary sage aromatherapy to enhance or augment labor.

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Methods and Materials

The experimental design employed herein was adapted from Bristol and DeGolier^[20].

Specimens

Eighteen virgin female mice, *Mus musculus* (outbred ICR-CD-1) each weighing 25-30 grams were obtained from Envigo, Inc. (Indianapolis, Indiana, USA).

The mice were kept in Bethel University's animal room and were given sufficient water and food *ad libitum*. All procedures were completed in accordance with the Institutional Animal Care and Use Committee of Bethel University (protocol #1712001).

Tissue preparation

Twenty-four hours prior to specimen sacrifice, an injection of 0.2 mg diethylstilbestrol (DES) was given to the mice. DES is a synthetic non-steroidal estrogen agonist used to promote mice into the estrus stage of their estrous cycle^[21]. It increases the number of gap junctions in the uterine tissue and allows the uterus to contract more effectively as a single unit^[22, 23].

The morning of an experiment, a DeJalons Ringer's solution (g/4 L: 36 g NaCl, 1.68 g KCl, 2 g NaHCO₃, 2 g D-glucose, 0.32 g CaCl₂) was made to mimic uterine extracellular fluid. The mice were then euthanized via CO₂ asphyxiation, pinned down to a dissection board, and an incision on the ventral abdomen was made. The uterine horns were extracted. Each uterine horn received two sutures, each on opposing ends, to stabilize the horn in an organ bath. One end was tied to a stationary rod, which was anchored into the organ bath, and the other end was tied to a force transducer which was connected to an amplifier and a PowerLab data acquisition system (AD Instruments, Colorado Springs, Colorado, USA). Each uterine horn was left in the organ bath for an hour under 0.8 g of tension^[24] and continually aerated (~2 psi) with 95% O₂/5% CO₂. The uterine horn tissues were flushed every fifteen minutes with fresh DeJalons solution and waveform activities were collected via the PowerLab system.

Testing protocol

After this equilibration period, oxytocin (OXY 10⁻⁵ M), an endogenous hormone that contracts uterine tissue in the body^[25] was applied to the tissues and served as a positive control. The resulting contractions were observed and recorded for 10 min. The tissues were then flushed (subjected to a washout) and allowed to return to their normal spontaneous rhythm before any clary sage extract treatments were administered. All treatments were pipetted directly onto the tissues, and tissue responses were recorded for 10 min.

Clary sage preparation

Clary sage seeds were purchased from Richters (Goodwood, Ontario, Canada). Seeds were finely ground in a coffee grinder and 1.8 g of ground clary seed was mixed with 100 mL of boiling deionized water, and allowed to cool. This resulted in a highly mucilaginous mixture, which contained a network of cellulose fibers with a matrix of other polysaccharides (i.e. pectins and hemicelluloses) important for water binding and storage^[26].

This mixture was then vacuum filtered through Whatman filter papers via a Buchner funnel in an effort to obtain an

aqueous extract. It was found that 1 ml of this filtrate contained 1.0 mg of clary sage residue following evaporation of the aqueous component. The clary sage concentrations tested herein were 0.06 mg/ml (n=1), 0.125 mg/ml (n=2), 0.25 mg/ml (n=8), 0.5 mg/ml (n=7), 1.0 mg/ml (n=8), and 2.0 mg/ml (n=4). Thus, the varying concentrations of clary sage extract given to the muscle tissues were based on the volume of the extract applied to a 20 ml organ bath. It was beyond the scope of this project to determine which chemical constituents remained in the aqueous extract, as opposed to those that were bound within the mucilage. It was observed that the applied extract remained water soluble as there was no evidence of a hydrophobic partition settling to the top of the water bath.

Measurements

The resulting waveform data was used to measure changes in contractile force from the baseline tension of the waveform to the maximal force produced within the treatment exposure. To control for the possible force contribution that the tissue's own spontaneous motility might have on the treatments, these forces were also measured in a similar manner before the treatment applications, and were considered as the control, or the "0" treatment. To normalize for slight variation in the uterine tissue masses, each tissue's maximal contractile response to any given treatment was expressed as a percent of its initial contractile response to 10⁻⁵ M OXY.

Statistical analysis

The data were summarized as means ± SE for contractile force for each treatment. Data with a sample size of three or greater were analyzed using ANOVA for multiple comparisons among the treatment means.

Resulting *P* values < 0.05 were subjected to the Tukey-Kramer post hoc test (JMP 4.0, SAS Institute, Cary, NC) which indicated which treatment (or concentration means) were considered to be statistically different from each other.

Results

Uterine smooth muscle response to oxytocin

Tissue viability was affirmed by the presence of spontaneous motility at the beginning of an experiment, which produced an average force of 5.60 ± 5.56 mN (n=30). Tissues that did not exhibit spontaneous motility during the pretreatment 60 min equilibration period, did indeed present these endogenous rhythms after the application of OXY.

A representative uterine tissue waveform illustrating spontaneous motility as well as responses to both OXY and a clary sage extract is shown in Figure 1. OXY generated an immediate increase in contractile force (29.8 mN) followed by a sustained plateau response that gradually declined over time. These tissues usually needed to be flushed twice over 20 min to ensure that the tension returned close to baseline prior to adding the experimental treatment, as partially contracted tissue would have been a confounding variable.

The general contractile response from all the clary sage applications was to produce a rapid increase in contractile force, but in contrast to OXY, this response typically returned back to baseline tension within 5 min (Figure 1).

In all cases, spontaneous motility returned to pre-clary sage rhythms. Changes in their frequency were nonremarkable and were not subject to further analysis and thus, are not reported herein.

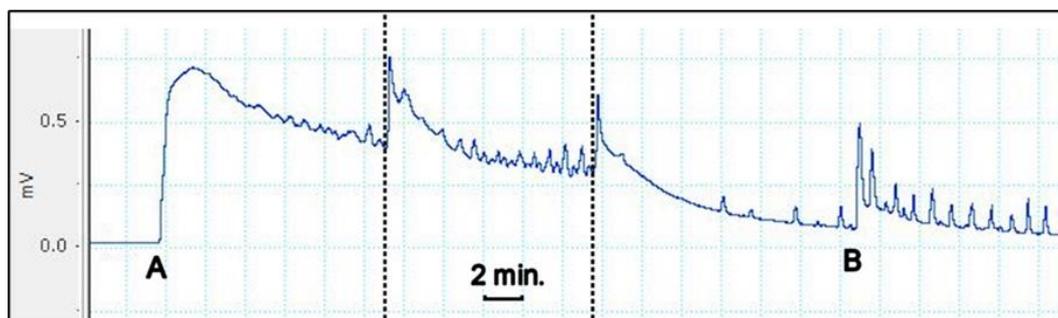


Fig 1: Typical uterine smooth muscle waveform before and after an application of 10^{-5} M oxytocin followed by a clary sage treatment. In this sample oxytocin (A) generated an increase in contractile force (29.8 mN). Two tissue bath washouts occurred before a 0.5 mg/ml clary sage application was given at letter (B). This resulted in an immediate increase in contractile force (18.2 mN), though it was not sustained as long as the contraction produced by oxytocin. The vertical axis represents contractile force in mV, later converted to mN.

Uterine smooth muscle response to clary sage

The average contractile forces (mN) produced from the clary sage treatments (mg/ml) were as follows: 0.06 mg/ml: 8.78 mN, 0.125 mg/ml: 27.55 mN, 0.25 mg/ml: 17.09 ± 15.56 mN, 0.5 mg/ml: 18.89 ± 9.11 mN, 1.0 mg/ml: 27.80 ± 12.79 mN, and 2.0 mg/ml: 11.04 ± 3.10 .

The overall effect of the clary sage treatment (presented as % OXY) did show an increase in contractile force when compared to the average "0" treatment ($P < 0.0001$; Figure 2). Significant increases were found between "0" treatment ($17.26 \pm 3.02\%$ OXY) and the forces produced by clary sage at 0.5 mg/mL ($56.83 \pm 10.9\%$ OXY), 1.0 mg/mL ($86.89 \pm 14.35\%$ OXY), and 2.0 mg/mL ($67.32 \pm 17.34\%$ OXY).

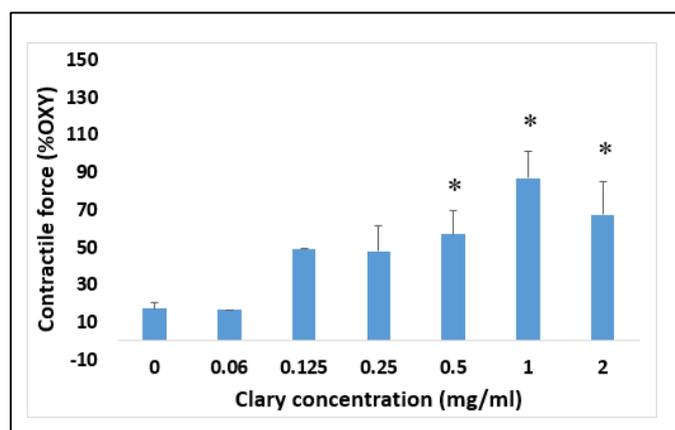


Fig 2: Means \pm SE of uterine contractile force (% OXY) in response to increasing extract concentrations of clary sage. Extract concentrations are expressed as mg of plant tissue mass per 1 ml organ bath fluid. The uterine contractile responses produced by the 0.5 mg/ml, 1.0 mg/ml, and 2.0 mg/ml treatments (*) were statistically greater than the "0" treatment, the tissue's own spontaneous motility ($P < 0.0001$)

Discussion

Clary sage produced large contractile forces from the isolated uterine tissues at almost all concentrations applied. These results support the hypothesis that this aqueous seed extract would result in an oxytocic effect when applied directly onto uterine tissues.

Clary sage seeds have an average oil content of 25-30% and are rich in unsaturated fatty acids such as linolenic, linoleic, and stearic acid [27, 28]. Since these fatty acids are principally insoluble in water at 25 °C (linolenic 0.000124 mg/L [29], linoleic 1.59 mg/L [30], stearic acid 0.597 mg/L [31]), they may have been trapped by the high mucilage polysaccharide content of the ground seed mixture and not available as part of the filtered extract. Thus, their direct contribution to uterine

contractility is not likely. Furthermore, a visual inspection of the organ baths following the administration of the clary sage extract did not show any partition of lipids in the bath. It seems reasonable, then that the biologically active constituents in the seed extract were water soluble.

Saponins, a secondary metabolite of plants composed of triterpene glycerol, are both water and lipid soluble [32]. Saponins have been reported in several different plant species, and are often found to be plant specific with variable distributions within the plant, depending upon seasonal changes and stages of development [33]. Triterpenoids, specifically oleanolic, betulinic, and ursolic acids, have been isolated from clary sage *Salvia* family Lamiaceae [33, 34, 35].

The reported biological activities of saponins *in vitro* and *in vivo* are numerous and are considered to be anticarcinogenic, anti-inflammatory, antioxidant, antimicrobial, antiviral, and immunomodulating behaviors [32, 33, 34]. It is the hydrophilic/hydrophobic behavior of saponins that contributes to its ability to interact with cell membranes [36], and act as permeating agents due to their detergent-like properties [37-41]. It has been proposed that the hydrophobic aglycone moieties of the saponin molecules form insoluble complexes with membrane cholesterol leading to saponin-cholesterol micelles which disrupt the lipid bilayer [42, 43]. This can result in invaginations and subsequent pore formation in the membrane [39, 44].

Research done by Bristol and DeGolier [20], using a protocol similar to that presented herein, found that aqueous extracts from the soap tree *Quillaja saponin*, contracted isolated uterine smooth muscle. It was proposed that the calcium ions from the DeJalons organ bath solution was able to diffuse into the uterine smooth muscle cells through the saponin-induced pores, and thus initiate contraction. This was supported by the observation that when calcium channels were blocked with nifedipine, the contractions still occurred, indicating that calcium entry for contraction may have been through the saponin created pores. Furthermore, there was no smooth muscle contraction when a calcium free DeJalons solution used, supporting the idea that the saponins themselves do not mediate the contraction by entry through the pores.

Since saponins are considered both water and lipid soluble [45], it can be proposed that saponins can be absorbed by carrier oils administered topically and rectally [46]. However, the bioavailability of saponins once orally ingested, cannot be taken for granted. In fact, dietary saponins are poorly absorbed [39]. In both *in vivo* and *in vitro* experiments in ruminants, it was demonstrated the carbohydrate (glycone) portion of the saponin is attacked by rumen microbes [47], leaving the sapogenin (aglycone) portion intact and absorbed in the duodenum [48]. It has also been shown *in vitro*, that

saponins may increase permeability of intestinal mucosa cells and thus facilitate the uptake of substance not normally absorbed [49].

Additional research which supports the proposed role that saponins may have in contracting smooth muscle has also been reported by Mendel *et al.* [50]. It was shown that triterpenoid saponins enhanced acetylcholine-evoked contractions of isolated smooth muscle from bovine ruminant abomasum and duodenum, likely through an increase in ion flow.

Aromatherapy employs the sense of smell as well as topical applications of essential oils for promoting its stress reducing, and proposed labor inducing effects. Tadokoro [51] examined the effects of clary sage inhalation on oxytocin levels in pregnant women. The results indicated that clary sage acted as an effective inducer of increased oxytocin levels, which could then proceed to produce uterine contractions.

Even though the direct detection of saponins using crude sample powders from medicinal plants has been demonstrated using fourier transform infrared spectroscopy [52], it is possible that whole animal uterine contractile activity, such as those resulting from clary sage aromatherapy, may be the result of constituents other than or in addition to the saponins. At the fruit set stage of clary sage development, the monoterpenoids linalool, linalyl acetate, and α -terpineol are the major essential oil components [53, 54]. Their solubilities in water at 25 °C are listed as follows: linalyl acetate 8.2 mg/L [55], linalool 1590 mg/L [56], and α -terpineol 7100 mg/L [57].

Linalyl acetate has been shown to relax rabbit vascular smooth muscle that was precontracted with phenylephrine [58]. This was thought to be partially mediated through both the activation of nitric oxide/cyclic guanosine monophosphate pathway and MLC dephosphorylation activating MLC phosphatase. In another study, linalyl acetate in conjunction with nicotine on phenylephrine pre-contracted tissues also reduced contractility in mouse aortic rings in [59]. It was proposed that the relaxation effect was related to its inhibition of Ca²⁺ channels.

Linalool has been shown to inhibit both 5-HT and ACh precontracted smooth muscle in rat ileal tissues as well as oxytocin precontracted smooth muscle in isolated rat uterine tissues. It was put forward that these responses might be mediated by an inhibition of G_q subfamily pathways [60]. Linalool was also shown to reduce arteriolar smooth muscle tone in isolated rat tissues, which could lead to hypotension *in vivo* [61]. This relaxation pathway was considered to be independent of the presence vascular endothelium and K⁺ channels.

Sabino [62] found α -terpineol to reduce arterial pressure in rat mesenteric arterial rings, and likewise proposing that the mechanism was independent of vascular endothelium. Ribeiro *et al.* [63] however, when employing both *in vitro* and *in vivo* cardiovascular studies in rats, supported the participation of a NO-cGMP pathway in the hypotension and relaxation as induced by α -terpineol. Using isolated rat ileal tissue, Sadraei *et al.* [64] demonstrated that ACh and KCl induced contractions were mildly inhibitory following applications of α -terpineol. Ponce-Monter *et al.* [65] was also able to show that α -terpineol relaxed KCl induced contraction of rat uterine rings, possibly by blocking calcium influx into uterine smooth muscle cells.

All three of these essential oils (linalyl acetate, linalool, α -terpineol) reduced contractile activities in variable isolated tissues which contained smooth muscle. Therefore, they are not candidates for inducing or augmenting labor. This

logically reinforces that saponins contained within the carrier oil applied either topically or suspended in aromatherapy, may be a contributor to smooth muscle contraction.

Linalyl acetate, linalool, and α -terpineol are also found in lavender oil, from *Lavandula augustifolia* [66, 67, 68]. There is growing evidence to suggest that the use lavender oil during labor, may be an effective medical treatment in the reduction of a number of anxiety disorders and related conditions [6, 7, 8, 69, 70]. These same essential oil anti-anxiety behaviors could reasonably be expected as sourced from clary sage.

Conclusion

The specific outcomes of this project did determine that an aqueous extract of clary sage contracted smooth muscle from isolated uterine tissues. These results support the hypothesis that the herbal clary sage would result in an oxytocic effect when applied directly onto uterine tissues. It is proposed that the triterpenoid saponin constituents are involved with the smooth muscle contractions, and that the hydrophobic essentials oil constituents are involved with the anxiolytic effects. Together, these constituent behaviors provide some empirical support that clary sage aromatherapy during labor can potentially allow the birthing process to be less impeded.

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