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Crescentia cujete (calabash tree) seed extract and fruit pulp juice contract isolated uterine smooth muscle tissues from *Mus musculus*

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Abstract
Traditional Mayan healers have recommend the fruits of the calabash tree (*Crescentia cujete)* to force menses, birth, after birth, or trigger abortions. The purpose of this research was to directly apply either an aqueous seed extract or raw juice from the fruit pulp directly to isolated uterine tissues from *Mus musculus,* and evaluate the resulting smooth muscle contractile responses. The seed extracts (0.1 - 10%) increased the force and frequency of contractions when compared to the tissue's spontaneous motility (P = 0.0575; P = 0.0048, respectively). The fruit pulp juice (50 - 500 µL) also produced increases in contractile forces (P = 0.0049) when compared to the tissue's spontaneous motility. Changes in frequency were less remarkable (P = 0.4855). These observations collected at a reduced model of investigation support traditional claims from Mayan healers that the prescriptive consumption of *Crescentia cujete* fruit evokes a contractile response from the uterus.

Key words: *Crescentia cujete*, calabash seeds, pulp, uterine contractions, mice, *in vitro*

1. Introduction

The Mayan civilization became vastly prominent in southern Mexico and other South American counties around 2000 BC. The tropical regions inhabited were an ideal environment for parasites and other infectious bacteria [1]. When diseases occurred, local Mayan healers often took advantage of the variety of medicinal plant resources provided in the local rain forest [2]. Mayan healers used herbal remedies to cure many different diseases, often relating the disease with the plant to be used. For example, red plants were used for rashes, blood disorders, and burns; blue plants were used for neural disorders; and yellow plants were associated with the liver and spleen. Often white plants were avoided because white was associated with death [3].

Calabash *Crescentia cujete* L. (family Bignoniceae) is a tree found in the West Indies, tropical America, and tropical areas of the Old World [4]. Calabash blooms during the month of June and its fruits grow and ripen slowly as they remain on the tree for six to seven months. During this time, the fruit changes from green to yellow and is harvested during the dry season from December to May [5].

The hard outer shell of the calabash fruit has been used for food containers, bowls, tobacco pipes, and as musical tools [6]. The white spongy pulp inside the shell contains numerous flat seeds [6]. The fruit itself can be mixed with milk, heated, and consumed for treating colds and asthma [7]. Ripe fruit has also been regarded as a laxative [8]. Traditional Mayan healers have recommend that the fruit may be ingested to force menses, birth, after birth [9, 10], or trigger abortions [11]. The latter has been observed in cattle [12, 13].

The purpose of this study was to determine if the constituents of the calabash fruit as it is available to the consumer, would directly influence the contractility of uterine smooth muscle tissue *in vitro* following the application of either an aqueous seed extract or raw fruit pulp juice. Our specific objectives were to characterize the resulting contractile waveform force and frequency responses and compare them to standard uterine positive contractile agents, namely oxytocin and acetylcholine. The results from this investigation might then advance our knowledge base about the claims within traditional medicine and provide some credibility to the Mayan medicine man's prescriptive outcomes.

2. Material and Methods

2.1 Preparation of the seed extract

Two *Crescentia cujete* gourds were obtained from Guatemala in 2014, provided by the IX Chel Tropical Research Field Station in Santa Athena Cay, Belize. The gourds were cut in half and the seeds were extracted and hand separated from the intertwined pulp. The seeds were rinsed in deionized water and then dried under a hooded vent for 10 days. Once the seeds were fully dried, they were ground in a coffee grinder and kept refrigerated at 3°C for the duration of the investigation. Prior to each experiment the seeds were made into different aqueous solutions at 0.1, 1, and 10%. Once dissolved in deionized water the aqueous solution was filtered through Whatman's paper using vacuum filtration. The seed extract solution was made fresh at the beginning of each experiment as it was found to grow mold if left in the refrigerator.

2.2 Preparation of the fruit pulp juice

The remaining pulp was removed from the shell, blended, and excess fibrous tissues were removed by vacuum filtration. The extract was kept refrigerated at 3°C for the duration of the investigation and brought to room temperature and gently stirred prior to use.

2.3 Animal specimens

Twenty-seven virgin female mice, *Mus musculus* (outbred ICR CD-1), each weighing 25-30 g, were obtained from Envigo (Indianapolis, IN). They were housed in cages in the Department of Biological Sciences at Bethel University (St. Paul, MN) and had access to water and standard mice chow *ad libitum*. All procedures were completed in accordance with the Institutional Animal Care and Use Committee of Bethel University.

2.4 Preparation of uterine tissues

Twenty-four hours prior to uterine horn extraction, mice were given an injection of diethylstilbestrol (DES). DES is a synthetic non-steroidal estrogen agonist used to promote the mouse into the estrus stage of their estrous cycle [14], thereby increasing the responsiveness of the smooth muscle [15]. The epigenetic change induced by DES stimulates the formation of gap junctions and allows the uterus to function as a single-unit of smooth muscle through endometrial thickening within the uterine wall [16].

On the day of the experiment, fresh DeJalons Ringer's solution (g/4 L: 36g NaCl, 1.68g KCl, 2g $NaHCO_3$, 2g D-glucose, and .32g $CaCl_2$) was made to simulate extracellular fluid conditions. Mice were then euthanized via CO_2 asphyxiation, placed on a dissection board, and the uterine horns were removed by means of a 4 cm abdominal incision made cranially from the vaginal orifice. The two uterine horns were individually isolated from each mouse and a suture was tied on each end of a horn; one suture was attached to a stationary rod for eventually placement into a 20 mL organ bath, and the other for eventual attachment to a force transducer.

At the start of each experiment the organ baths were flushed multiple times with DeJalons warmed to 32° C, and continually aerated (~2 psi) with 95% O_2/5% CO_2. A prepared uterine horn was lowered into the organ bath; the stationary rod was anchored into the bath and the other sutured uterine horn was attached to an isometric force transducer (MLT500, ADInstruments, Colorado Springs, CO), and placed under 0.8 g of tension [17]. The force transducer was connected to an amplifier and a PowerLab data acquisition system (ADInstruments, Colorado Springs, CO) that collected data from the tissue and translated the tissue's contractile responses into visual waveforms.

The tissue samples were equilibrated in the individual baths for one hour with flushes every 15 minutes, replenishing the system with fresh DeJalons. During this time, tissues demonstrated spontaneous motility representative of healthy uterine smooth muscle under tension.

2.5 Experimental protocol

For the calabash seed extract, 10^{-5} M oxytocin (Oxy) was added to elicit a control contractile response. Oxytocin is an endogenous hormone known to evoke contractions of uterine smooth muscle via oxytocin receptors [18], or the calabash pulp juice, 10^{-5} M acetylcholine (ACh) at was added to elicit a control contractile response. Acetylcholine is an endogenous neurotransmitter known to evoke contractions of uterine smooth muscle via cholinergic receptors [19]. Following a ten minute exposure to oxytocin or acetylcholine, the tissues were flushed and allowed to return to their normal spontaneous rhythm.

Each tissue was then given only one of the desired aqueous seed extracts (0.1, 1, 10%) or volumes of pulp juice (50, 100, 200, 300, 500 µL) and left in the organ bath for 15-20 minutes. Changes in uterine contractile force and frequency were observed, recorded, and measured. Calabash treatments were not repeated on the same tissues as they were observed to show fatigue following a second application.

2.6 Measurements

All treatment applications were made after the completion of a full spontaneous motility cycle and under baseline tension. Changes in contractile force were measured from the baseline tension to the maximal force produced within the first five minutes of treatment exposure. To control for the possible force contribution that the tissue's spontaneous motility might have on the treatments, the amplitude of these forces were also measured in a similar manner five minutes before the application of either calabash seed extract or fruit pulp juice. They were considered as the control, or the "0" treatment.

To normalize for the slight variation in the uterine tissue masses, each tissue's maximal contractile response to any given 1) seed extract was expressed as a percent of its initial contractile response to 10^{-5} M Oxy, and 2) fruit pulp juice as a percent of its initial contractile response to 10^{-5} M ACh.

To determine changes in contractile frequency, the waveforms produced were counted five minutes prior to the application of the treatment and five minutes after.

2.7 Statistical analysis

The data was summarized as means ± SE for each treatment [calabash seed extract, calabash fruit pulp juice] for both contractile force and frequency. Each set of means included data 1) with a sample size greater than three, 2) which had experienced spontaneous motility prior its respective positive contractile control, and 3) responded to its respective positive contractile control. Individual data were further analyzed using ANOVA for multiple comparisons among the means. Resulting P values $\leq$ 0.05 were subjected to the Tukey-Kramer post-hoc test (JMP 4.0, SAS Institute, Cary, NC) which indicated which means were considered to be significantly different from each other.

3. Results

3.1 Smooth muscle waveform responses: spontaneous motility, oxytocin, acetylcholine, and calabash

Tissue viability was confirmed by the presence of spontaneous motility at the beginning of the experiment as well as a positive response to 10^{-5} M oxytocin or 10^{-5} M acetylcholine. Oxytocin produced an immediate contraction that slowly decreased from its plateau response (Fig 1A). The average contractile force in response to oxytocin was 42.81± 2.59 mN (n = 24). Tissues that received 10^{-5} M acetylcholine also produced an immediate contraction, but no plateau was established as acetylcholine was rapidly hydrolyzed. The average contractile force in response to acetylcholine was 28.80 ± 2.96 mN (n = 19), approximately 67% of the contractile response produced by 10^{-5} M oxytocin.

Following a tissue washout, the flush response was followed by the return of spontaneous motility (Fig 1B). A typical waveform response to *Crescentia cujete* seed extract is shown in Figure 1C. There is no long sustained contraction as previously seen in oxytocin (Fig. 1A). In contrast, the responses showed increases in both contractile force and frequency when compared to the tissue's spontaneous motility. A typical waveform response to *Crescentia cujete* fruit pulp juice typically produced a single strong contraction that was sustained for ~ 30 seconds before returning to baseline tension.

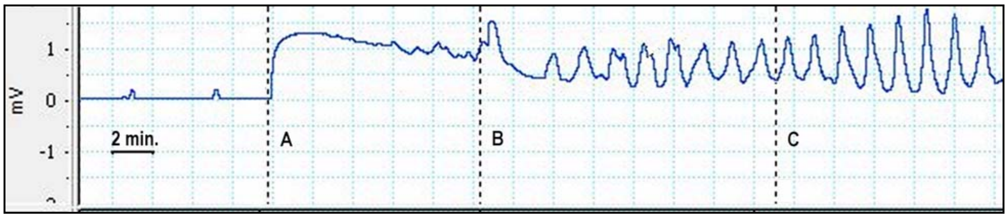

Fig 1: Typical uterine smooth muscle waveform response to oxytocin and calabash. Letter **A** shows a waveform response following the application of 10^{-5} M oxytocin which was typically a long contraction that eventually plateaued. Letter **B** shows the flush response as oxytocin was replaced with fresh DeJalons solution. Letter **C** shows typical waveform response to 1% *Crescentia cujete* aqueous seed extract. The y-axis scale is in units of mV and was later converted to mN of force.

3.2 Change in uterine contractile activity in response to calabash seed extract

Crescentia cujete seed extract increased contractile forces following the applications of 0.1% (39.44 ±10.76 mN; n=5), 1% (55.64 ±10.54 mN; n=8), and 10% (60.21 ±10.62 mN; n=6). When normalized to their respective oxytocin responses, all contractile responses were greater than that of the control treatment, but not significantly greater (P = 0.0575. Fig.2A).

There was a significant change in the contractile frequency induced by increasing concentrations of calabash seed extract (P = 0.0048; Fig. 2B). The 0.1 and 1% solutions produced a gradual increase in frequency, but at 10% the contractile frequency was decreased and similar to that of spontaneous motility. The significant difference was found between the "0" treatment and the 1% solution.

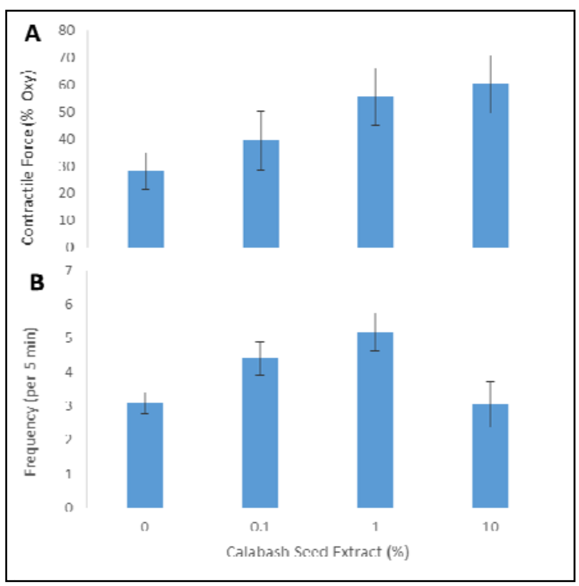

Fig 2: (A). Means ± SE uterine contractile force (% Oxy) in response to increasing concentrations of calabash seed extract. *Crescentia cujete* seeds did contract the uterine smooth muscle tissue, however, the responses observed were not statistically different from that observed from treatment "0", the tissues endogenous spontaneous motility (P = .0575). **(B)** Means ± SE increases in uterine contractile frequency in response to increasing volumes of applied calabash seed extract. There is an increase in frequency produced from the 0.1% and 1% solutions, with the 1% yielding a statistical increase (P = 0.0048) relative to the "0"treatment.

3.3 Change in uterine contractile activity in response to calabash fruit pulp

Crescentia cujete fruit pulp juice increased contractile forces from those of baseline spontaneous motility (9.56 ± 2.47 mN; n = 15) following the administration of 50 µL (18.30 ± 6.40 mN; n = 5), 100 µL (30.10 ± 10.68 mN; n = 4), 200 µL (27.94 ± 6.40 mN; n = 4), 300 µL (17.94 ± 5.86 mN; n = 3), and 500 µL (26.11 ± 7.91 mN; n = 3). When normalized to that of their respective acetylcholine responses, all contractile responses from the calabash treatments were greater from that of the control treatment (Fig. 3A), with the 100 µL volume producing a significant increase ($P = 0.0049$).

The mean ± SE contractile frequency responses to each volume of raw calabash pulp juice applied are shown in Fig. 3B. After the application of 50, 200, and 500 µL, there was some increase in contractile frequency, but overall, the changes were not significant within the 5 minute assigned measurement parameters ($P = 0.4855$).

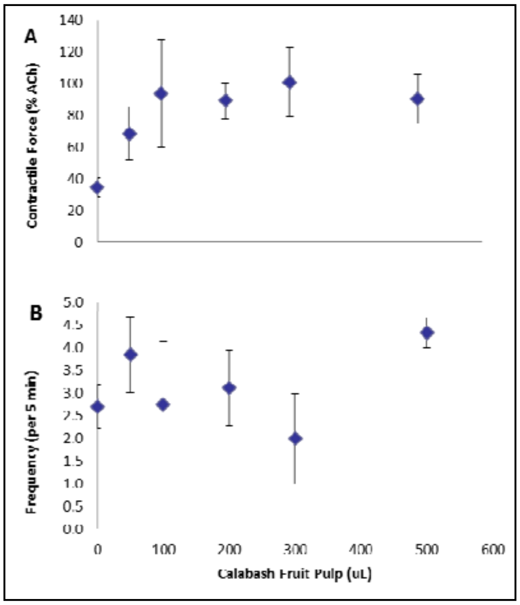

Fig 3: (A) Means ± SE uterine contractile force (% ACh) in response to increasing volumes of calabash pulp juice. Each increase in volume resulted in a forceful contraction, nearing 100% of their ACh response. The 100 µL volume evoked a significant increase ($P = 0.0049$) when compared to the control treatment. **(B)** Means ± SE changes in uterine contractile frequency in response to increasing volumes of applied calabash pulp juice. Although there is a slight increase in frequency at the 50, 200, and 500 µL applications, the differences are not statistically greater than the control treatment ($P = 0.4855$, n = 16).

4. Discussion

4.1 A positive uterine contractile response from *Crescentia cujete* seeds and pulp

The purpose of this study was to determine if the constituents of calabash fruit as it is available to the consumer would directly influence the contractility of uterine smooth muscle *in vitro*. After each application of calabash seed extract or pulp juice, increases in contractile force occurred within five minutes. Increases in contractile frequency were less remarkable. These observations collected at a reduced model of investigation support traditional claims from Mayan healers that the prescriptive consumption of *Crescentia cujete* fruit evokes a contractile response from the uterus.

4.2 Biological constituents

Several of the phytochemicals that have been identified in *Crescentia* fruit may contribute to human health when based on an understanding of their biological activities garnered from other investigations [20]. For example, phenolic compounds and tannins are reported to have disinfecting and bacteriocidal properties [6, 9] and flavonoids may act as antioxidants [21]. Some of the alkaloids may serve as analgesics [22] and anti-spasmotics [23], further supporting calabash as a treatment for asthma, bronchitis, coughs and lung congestion [7, 10, 24]. Saponins have been shown to stimulate the immune system [25], demonstrate antifungal and antiviral properties [26, 27], and in calabash, are further proposed to act as anti-inflammatory and antibiotic agents [20]. Of interest to our investigation is whether or not any of the isolated calabash phytochemicals show any biological activity on smooth muscle. Folklore reports the use of calabash fruit as a purgative [6], a laxative [8], an oxytocic [9, 10], and an abortive [11]. While none of these uses have been clinically tested [28], each could potentially involve smooth muscle. Furthermore, no previous studies of its contractile effects on uterine smooth muscle have been reported.

4.3 Contractile responses from the seed extract constituents

The seeds of calabash contain oils are similar to a peanut or olive oil [6] and linoleic acid [29]. We propose that the role of linoleic acid in calabash seed extract may contribute to the uterine contractile activity. Linoleic acid is a precursor for prostaglandins, so it is reasonable to believe this could be triggering the smooth muscle contraction of the uterus and inducing labor [30, 31]. Linoleic acid is considered an active constituent in evening primrose [32, 33] has been shown to contract isolated uterine horns using a protocol similar to that reported herein [34].

4.4 Contractile responses from the fruit pulp constituents

We also propose that the saponin constituent in calabash may contribute to the uterine contractile activity observed in this study. Saponins are cell membrane permeating agents, which work because of their detergent-like properties [21, 35-38]. The hydrophobic aglycone moieties of the saponin molecules likely form insoluble complexes with membrane cholesterol leading to saponin-cholesterol micelles disrupting the lipid bilayer [39]. These disruptions in the lipid bilayer result in invaginations and subsequent pore formation [21]. This would allow for the influx of Ca^{2+} ions as found in the DeJalons solution bathing the isolated tissues. This is supported by recent work showing positive uterine contractile responses from saponins directly isolated from *Quillaja saponin* [40].

4.5 Seed and pulp potency

A small separate study (n=8) was conducted to determine if the potency of calabash fruit pulp juice and seed extract were comparable to each other when both of the tissue's maximal contractile responses were compared to 10^{-5} M oxytocin. Isolated tissues given 200 µL fruit pulp juice produced a response of 59.47 ± 7.75 (% Oxy; n =4)) and tissues given 10% aqueous calabash seed extract produced an average response of 60.21 ± 10.62 (% Oxy; n =4). These contractile responses were not statistically different from each other. Interestingly, the same data for the 10% fruit pulp juice standardized to its 10^{-5} M acetylcholine response was 89.33 ± 11.43, indicating that at equal molar concentrations, oxytocin produced a contractile response almost 50% greater than that produced by acetylcholine. If the mean contractile values plotted in Fig. 3A (expressed as % ACh) were reduced by 50%, the values would look more similar to those plotted in Fig. 2A (expressed as % Oxy).

However, quantification of the biologically active constituents unique to the seed extract and the fruit pulp has not yet been determined. Without knowing these comparative values, it cannot be claimed whether the seeds or fruit are more potent. Reports from Arvigo and Balick [10] indicate that calabash seeds can be used as an abortive [11] and the fruit pulp to force menses, birth, and afterbirth [9]. Such a specific prescription for oral consumption does imply unique expected outcomes, which likely reflects the distinct chemical makeup of the seeds and fruit.

4.6 Conclusions and recommendations
Our research results collected at an *in vitro* level of investigation do support traditional claims from Mayan healers that the prescriptive consumption of *Crescentia cujete* fruit produces a contractile response from the uterus. Attempts to characterize the resulting uterine contractile responses with what may happen following the oral consumption of calabash were done with the understanding that the contributions of the active constituents may be synergistic, additive, or antagonistic with reproductive processes at an organismal level.

Nutrient values indicate that *Crescentia cujete* fruit is of nutritional value and is notably high in sodium and phosphorus [20]. However, high levels of the anti-nutrient hydrogen cyanide [6, 12, 13] would interfere with oxygen consumption in mitochondrial cytochrome oxidase [41] and would deter one from continual consumption [20].

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