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Potent tocolytic activity of ethyl acetate fraction of *Ananas comosus* on rat and human uteri

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ABSTRACT

The aim of this study was to investigate the tocolytic properties of Ananas comosus extract in rat and human uterine tissue in vitro and in the rat in vivo. Organ bath technique was employed to perform functional studies in vitro. The PhysioTel transmitter was implanted in SD rats to measure the changes in intrauterine pressure (IUP) in vivo. Analyses of F2 was performed using LC-HRMS.

F2 produced a non-selective inhibitory response on oxytocin, prostaglandin F2α, acetylcholine and KCl. The inhibitory activity of F2 on oxytocin-induced contraction was not attenuated by propranolol, TEA, glibenclamide and indomethacin. Nω-Nitro-L-arginine, a nitric oxide synthase inhibitor, suppressed the maximal tocolytic activity of F2 by 25%. DIDS, an inhibitor of chloride channels, appeared to suppress the relaxant effect of F2. F2 suppressed the oxytocin-induced contraction in Ca^{2+} free solution. The in vivo tocolytic activity of F2 and ritodrine were observed in non-pregnant rats during the estrous stage by suppressing the frequency and amplitude of IUP peaks following intrauterine administration. Chemical analysis confirmed the involvement of citric acid in the tocolytic activity of F2. However, another less polar fraction is essential to accompany citric acid to produce such potent inhibitory response of F2.

It is likely that F2 exerted tocolytic activity by multiple mechanisms, including antagonizing L-type Ca^{2+} channels, interfering with the intracellular Ca^{2+} release mechanism and releasing nitric oxide. F2 would be a promising candidate to develop as a tocolytic agent.

1. Introduction

Uterine activity regulation, in terms of quiescence and contractions, is a prerequisite to maintain the fetus until term and the process of labor, respectively. Abnormality in this physiological process can result in preterm birth, post term pregnancy and postpartum hemorrhage. These clinical complications can eventually lead to a serious problem regarding disability, mortality and cost to society. Preventing preterm delivery remains as one of the major challenges in obstetrics because despite significant advances in neonatal care, preterm delivery is still the largest cause of neonatal morbidity and mortality. Preterm birth contributes to pulmonary and neuro-cognitive morbidity with increasing prevalence and globally causes 28% of neonatal deaths [1]. Due to the side effects and lack of satisfactory potency, several tocolytic agents, like cyclooxygenase inhibitors, calcium channel blockers, nitric oxide donors and oxytocin receptor antagonists were not approved by the US Food and Drug Administration [2].

In folkloric medicine, *Ananas comosus* (common name: pineapple) has been reported to act as an abortifacient [3,4]. However, no scientific evidence supported the efficacy of *A. comosus* in inducing abortion. While some research has been carried out on the uterotonic activity of *A. comosus* [5] there is no study discussing the inhibitory effect of *A. comosus* on uterine activity. Interestingly, the pilot study has identified tocolytic activity of ethyl acetate fraction of *A. comosus* extract.

Several organic acids like malic acid [6], sinapic acid, p-coumaric acid [7] and bioactive amines such as spermidine, putrescine, agmatine and serotonin have been identified in the edible part of *A. comosus*. Citric acid was identified as a major organic acid in all the pineapple species [8]. These compounds are formed during metabolic processes and have been implicated not only in diverse physiological functions in plants but also may play a crucial role in mammals by triggering several responses following oral absorption [9].

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Previous studies have shown the contribution of p-coumaric acid in the regulation of estrous cycle in mice [10] and increasing the serum estradiol level [11]. Sinapic acid was demonstrated as an efficient free radical scavenger [12]. Malic acid displayed a significant increase in the muscle regeneration and wound healing [13].

In the light of diverse biological activities of the aforementioned compounds, this study aimed to investigate the tocolytic properties of *Ananas comosus* extract with a range of maturities in rat and human uterine tissue in vitro and in rat in vivo.

2. Materials and methods

2.1. Extract preparation

Crude extracts and fractions were prepared according to the procedure described previously. Briefly, the freeze dried powder of edible part was extracted with 70% ethanol using sonication method. The concentrated crude extract was fractionated through a series of liquid-liquid partitions using hexane (F1), ethyl acetate (F2) and 1-butanol (F3). The aqueous fraction was labeled F4. Phytochemicals, including alkaloids, flavonoids, anthraquinones, saponins, carbohydrates, steroids, triterpenes and tannins had been screened in the crude extract with a range of maturities [5]. The F2 extract was dried down using rotary evaporator and further dried fully by a centrifugal vacuum concentrator (temperature = 42 °C). The yield of F2 extract was 0.5%. The extract was kept at −20 °C until use.

2.2. Human tissue

The study was approved by the National Healthcare Group Domain Specific Review Board (DSRB-ID-2011-00211) and all patients gave their written informed consent to participate. Myometrium specimens were obtained from the lower segment of pregnant women undergoing routine elective caesarean.

2.3. Animals

All the experimental procedures were performed in accordance with the international guideline for animal research under due approval from institutional animal care and committee, National University of Singapore (R12-0216). Pregnant SD rats (Day 21) were acquired for the in vitro study. Non pregnant rats were used for the in vivo telemetry study. The stage of estrous cycle was examined by vaginal smear and those in the estrus stage were employed for in vivo experiment. Animals were allowed free access to standard laboratory diet and tap water.

2.4. Surgical procedure

The PhysioTel PA series transmitter model PA-C40 (Data Sciences International, St. Paul, MN) was implanted to measure intrauterine pressure. Briefly, under general isoflurane anesthesia, using aseptic techniques, small vertical midline incision (2–3 cm) was made on the skin and underlying abdominal muscle. The uterus was taken gently out of the body cavity. The catheter was inserted and secured in the uterus through small hole made on the uterus. Transmitter body was sutured carefully to the abdominal muscle and then skin closed using staple. Post-operative care was performed routinely.

2.5. Experimental protocol

Rat and human uterine strips were prepared according to the experimental protocol detailed by Monji et al. (2016). In different models, including spontaneous phasic contraction and agonists induced contraction, uterine strips were pretreated with established inhibitors or blockers of various pathways or receptors to characterize the mechanism involved in tocolytic activity. In stimulated model, myometrial strips were contracted with standard agonists for at least 1 h to check the stability of induced contraction before testing the tocolytic activity of the F2 and reference drugs. In the next cycle after stabilization of the induced contraction, cumulative concentrations of F2 and standard tocolytic drugs were added to the bath.

To evaluate the tocolytic activity of the fraction in calcium free solution, uterine tissue was first equilibrated in the Krebs bicarbonate solution for 1 h, then in calcium free solution containing 1 mM EDTA for 1 h. Subsequently, uterine tissue was incubated for 0.5 h in calcium free solution containing 0.01 mM EDTA. Oxytocin produced a sustained tonic contraction under described condition. Following stabilization of the response, cumulative concentration of F2 was added to the bath.

The possible contributions of the known compounds of *A. comosus* in tocolytic activity of F2 were also evaluated either on KCl or oxytocin induced contraction

For in vivo study, F2 and controls were administered by intrauterine route in postpartum and non-pregnant rats. To evaluate the tocolytic activity, F2 was administered after uterus had a stable regular phasic contraction at least for 2 h.

To prepare the animals for intrauterine administration, rats were anesthetized using isoflurane for 5 min. A customized sterile speculum was placed gently into the rats' vagina. A sterile oral dosing catheter was inserted into the speculum and through the cervix. Fractions and standard drugs were sterilized using sterile filtrate syringe (pore size = 0.2 μm). The volume of the fractions and controls were adjusted to 200 μl for intrauterine administration.

2.6. LC-HRMS analysis of F2

F2 (ethyl acetate fraction) from ripe pineapple extract was dissolved in ultrapure water at 2 $mg.mL^{-1}$. The sample was sonicated for 15 min and centrifuged at 14,000 for 5 min, prior to injection. Analytical HPLC was performed on an Agilent UHPLC 1290 Infinity coupled to an Agilent 6540 accurate-mass quadrupole time-of-flight (QTOF) mass spectrometer equipped with a splitter and an ESI source. The analysis was performed with a C18 4.6 x 75 mm, 2.7 μm column at flowrate of 2 $mL.min^{-1}$. Mobile phase solvent A was 0.1% (v/v) formic acid in water and mobile phase solvent B was 0.1% (v/v) formic acid in acetonitrile. The gradient was started at an initial composition of 0% B for 0.5 min, to 50% B over 9.5 min, then 2 min linear gradient to 100% B, held for 4 min, and returned to the initial conditions. HRMS and MS/MS data was acquired in positive mode. The HRMS data acquired from F2 was searched against an in-house library, and subsequently compared with reference standards for unambiguous identification.

2.7. Bioassay guided fractionation of F2

F2 (ethyl acetate fraction, 300 mg) from ripe pineapple extract was dissolved in 1.5 mL of water, sonicated for 15 min. and centrifuged at 14,000 for 5 min. The supernatant was separated by preparative reversed-phase high performance liquid chromatography (RP-HPLC) on a C18 column (19 × 100 mm, Waters Atlantis T3). Mobile phase solvent A was 0.1% (v/v) formic acid in water and mobile phase solvent B was 0.1% (v/v) formic acid in methanol. The initial mobile phase was isocratic elution at 100% A for 6 min. Solvent B was increased linearly to 15% B between 6 and 12 min; then to 50% B in 8 min; finally to 100% B in 15 min and held for 10 min. Sub-fractions collected were submitted for biological testing.

2.8. Drugs and chemicals used

The following compounds were used in this study: oxytocin (1 mIU), prostaglandin F2α (PGF2α, 10 μM), acetylcholine (15 μM), propranolol (10 μM), ritodrine (0.03–1 μM), tetraethylammonium (TEA, 10 mM), glibenclamide (10 μM), diazoxide (10–200 μM), Nω-Nitro-L-arginine (100 μM), indomethacin (10 μM), bromelain (1–1000 $\mu g.ml^{-1}$);