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Tocolytic action of essential oil from *Annona leptopetala* R. E. Fries is mediated by oxytocin receptors and potassium channels

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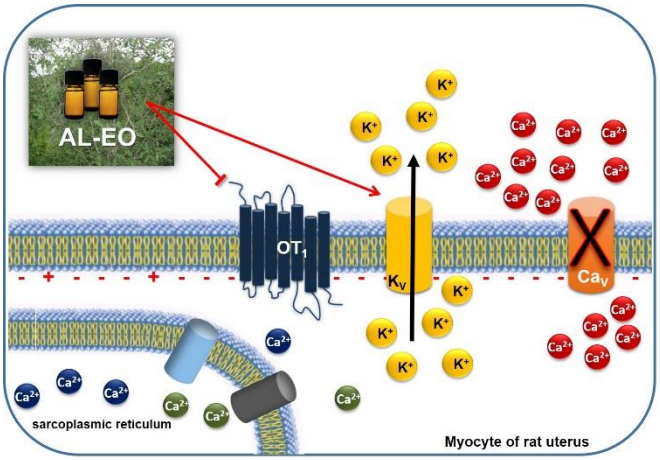
Graphical Abstract



Abstract

Annona leptopetala R. E. Fries (basynonymy *Rollinia leptopetala* R. E. Fries) is an endemic tree or shrub from Brazil. Some Annona species such as *Annona muricata* and *Annona squamosa* showed uterus activities. Thus, in the search for new agents to combat the uterine disorders, it was aimed to investigate a possible AL-EO tocolytic effect on isolated rat uterus and the underlying mechanisms. Uterine horns were removed, cleaned of adhering fat and connective tissue, and suspended by in organ baths containing Locke-Ringer solution, bubbled with carbogen mixture and the contractions were registered using a force transducer. All experimental procedures were previously approved by Ethic Comission on Animal Use of UFPB (protocol n° 0104/2014). AL-EO was more potent to relax pre-contracted uterus with oxytocin than with KCl. Moreover, AL-EO shifted to the right, in a non-parallel manner, oxytocin induced cumulative contraction curves, with Emax reduction, indicating a pseudo-irreversible noncompetitive antagonism of oxytocin receptors. In the presence of propranolol, β-adrenergic antagonist, an AL-EO action increase was

observed. Nitric oxide and cyclooxygenase pathways participation were assessed and discarded. The tocolytic potency of AL-EO was attenuated by CsCl, a non-selective K^+ channel blocker, suggesting a positive modulation of these channels. Additionally, in the presence of 4-aminopyridine (4-AP) but not in apamin, glibenclamide or tetraethylammonium (1 mM), the relaxant potency of AL-EO was reduced, indicating the voltage-dependent potassium channels participation, but not small- or big- conductance Ca^{2+}-activated and ATP sensitive potassium channels. Tocolytic mechanism of AL-EO on rat uterus involves pseudo-irreversible noncompetitive antagonism of oxytocin receptor and β adrenergic receptors and positive modulation of voltage-dependent potassium channels. Thus, AL-EO is presented as a promising drug with activity on uterine conditions, as dysmenorrhea, and after further evaluation in clinical studies, it would be used as an alternative drug on current pharmacotherapy.

Keywords: uterine smooth muscle, potassium channels, natural product, *Rollinia leptopetala*, dysmenorrhea.

Introduction

Annona leptopetala R. E. Fries (basynonymy Rollinia leptopetala R. E. Fries) is cited as a digestive, antitumor and anti-inflammatory agent [1], diuretic and against prostate inflammation [2]. Additionally, some *Annona* species, such as *Annona muricata* [3] and *Annona squamosa* [4] showed uterus activities. Essential oil from *A. leptopetala* leaves (AL-EO) was chemically analyzed by partners that identified 22 constituents in a complex mixture of monoterpenes (54.5%) and sesquiterpenes (45.5%). The main components found in the leaves were bicyclogermacrene (22.47%), cis-4-thujanol (17.37%) and germacrene (7.72%) [5].

The development of new drugs is a long process associated with a powerful advance in experimental pharmacology. The use of isolated organs, various animal models and contemporary in vitro methods [6] are applicable tools to be used as an initial step to discover potential medicinal drugs. Experimental animal models are known to be useful and effective in this process. [6, 7, 8]. In addition, there are several articles that use in vitro models to investigate the medicinal properties of essential oils [9, 10, 11].

Thus, in the search for new agents to combat the uterine disorders, we decided to investigate a possible tocolytic effect of AL-EO on isolated rat uterus and thus assign this natural product as a tool to improve the pharmacotherapy associated to these conditions.

Materials and Methods

Virgin female Wistar rats (150–250 g) (n = 5) were pretreated with diethylstilbestrol 1.0 mg/kg (s.c.) 24 h prior to the estrus induction experiment. They were euthanized using a guillotine and uterus was immediately extracted, drenched in Locke-Ringer solution [12] and bubbled with a carbogenic mixture (95% O_2 and 5% CO_2). The organ was cut longitudinally and corns were suspended by a cotton thread in organ baths at 32 °C and the contractions were registered using a force transducer.

After the stabilization period, two similar concentration-response curves were obtained with carbachol (CCh) (10^{-6} M) or oxytocin (10^{-2} IU/mL). Then, AL-EO was pre-incubated with the uterus corns for 15 min with a single concentration of the essential oil in independent experiments before adding CCh or oxytocin [13]. Additionally, similar concentration-response curves or two similar cumulative concentration-response curves for OXY (10^{-6}-10^{-1} IU/mL) were obtained.

A contraction was induced by OXY (10^{-2} M) and during the tonic phase, AL-EO was cumulatively added. After, the AL-EO relaxant activity was assessed in the presence of propranolol

(300 nM), a β-adrenergic antagonist; or L-NAME (100 µM), a NOS inhibitor; or indomethacin (10 µM), a COX inhibitor; or CsCl (5 mM), a non-selective potassium channels blocker; or 4-AP (3 mM), voltage-dependent K^+ channel (K_V) blocker; or glibenclamide ($3x10^{-5}$ M), ATP-sensitive K^+ channel (K_{ATP}) blocker; or apamin (100 nM), small-conductance Ca^{2+}-activated K^+ channel (SK_{Ca}) blocker; or TEA^+ (1 mM), big-conductance Ca^{2+}-activated K^+ channel (BK_{Ca}) blocker [13-19].

All trial procedures were done going along with the guidelines for the ethical use of animals in applied etiology studies and released by the Animal Use Ethics Committee of UFPB (protocol nº 0104/2014). The values were expressed as the mean and standard error of the mean (S.E.M.) and statistically analyzed by the Student's *t*-test or one-way variance analysis (ANOVA) followed by Tukey's test. The null hypothesis was rejected when $p < 0.05$. IC_{50} or EC_{50} were calculated by nonlinear regression [20] and E_{max} values were used as a measure of effectiveness. The data were analyzed by GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

Results

AL-EO (3-729 µg/mL, n = 5) antagonized the phasic contractions induced by oxytocin (IC_{50} = 20.6 ± 3.3 µg/mL) or CCh (IC_{50} = 87.1 ± 1.0 µg/mL) on rat uterus in a concentration-dependent manner. E_{max} values for all were 100%. AL-EO (0.01-729 µg/mL, n = 5) relaxed pre-contracted uterus with KCl (EC_{50} = 22.4 ± 2.4 µg/mL) or OXY (EC_{50} = 4.1 ± 0.4 µg/mL) in a concentration-dependent manner. An analysis of EC_{50} values indicated that AL-EO was more potent in inhibiting the contractions induced by OXY, around 6 folds.

AL-EO (9, 27 and 81 µg/mL, n = 5) inhibited the cumulative concentration-response curves to OXY. These curves were shifted to the right in a non-parallel manner, with decreasing E_{max} from 100% to 96.9 ± 2.4; 72.7 ± 3.3; 55.4 ± 1.6 and 0%. EC_{50} values of OXY were attenuated from 6.7 ± 0.1 x 10^{-5} IU/mL (control) to 9.2 ± 0.4 x 10^{-5}, 3.8 ± 0.3 x 10^{-5} and 3.8 ± 0.6 x 10^{-4} IU/mL in the presence of 3, 9 and 27 µg/mL of AL-EO, respectively

In the presence of propranolol (300 nM), AL-EO tocolytic effect was potentialized around 3 folds (EC_{50} = 4.1 ± 0.4 µg/mL and 1.4 ± 0.05 µg/mL, respectively). AL-EO (0.01 27 µg/mL, n = 5) tocolytic effect on OXY-induced tonic contraction was equipotent in the absence or presence of L-NAME (100 µM) (EC_{50} = 4.1 ± 0.4 µg/mL and 2.8 ± 0.4 µg/mL, respectively). Similarly, indomethacin (10 µM) did not alter AL-EO tocolytic effect on OXY-induced tonic contraction (EC_{50} = 4.1 ± 0.4 µg/mL and 2.9 ± 0.4 µg/mL, respectively).

AL-EO (0.01-81 µg/mL, n = 5) tocolytic effect (EC_{50} = 4.1 ± 0.4 µg/mL) was attenuated in the presence of CsCl, a non-selective potassium channels blocker, around 2 folds (EC_{50} = 8.9 ± 1.1 µg/mL, n = 5). On the other hand, AL-EO tocolytic effect did not modify statistically in the presence of glibenclamide, a K_{ATP} blocker, (EC_{50} = 5.1 ± 0.7 µg/mL, n = 5); apamin, a SK_{Ca} blocker, (EC_{50} = 4.8 ± 0.2 µg/mL, n = 5) or TEA^+ 1 mM, a BK_{Ca} blocker, (EC_{50} = 3.4 ± 0.3 µg/mL, n = 5); but in the presence of 4-AP, a K_V blocker, AL -EO tocolytic potency was attenuated around 2 folds (EC_{50} = 10.0 ± 0.6 µg/mL, n = 5).

Conclusions

Thus, this study shows that the essential oil from *Annona leptopetala* R. E. Fries exerts its tocolytic activity on rat uterus through a non-competitive pseudo-irreversible antagonism of OT receptors and a positive activation of K^+ channels, primarily the K_V channels, which indirectly blockade the Ca_V, leading to a reduction in Ca^{2+} influx and uterine smooth muscle relaxation.

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