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New Findings: What is the central question for the study? New potent myometrial inhibitory compounds are needed to combat uterine contractility disorders such as preterm labour. What is the main finding and its importance? This study sought to answer if Justicia flava leaf extract (JF) could inhibit human myometrial contractility as

was previously shown in mouse myometrium. The study has clearly shown that JF abolished human myometrial contractions and therefore presents as a lead plant in drug discovery studies involving drugs for preterm birth.

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Justicia flava leaves potently relaxes pregnant human myometrial contractility: a lead plant for drug discovery of new tocolytic drugs

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New Findings

This study sought to answer if *Justicia flava* leaf extract (JF) could inhibit human myometrial contractility as was previously shown in mouse myometrium. The study has clearly shown that JF abolished human myometrial contractions and therefore presents as a lead plant in drug discovery studies involving drugs for preterm birth.

Abstract

In the search for new potent therapies for preterm labour, *Justicia flava* leaves (JF) were previously shown to potently inhibit uterine contractility in both pregnant and non-pregnant mouse uterus. This study took the investigation a step further and investigated the activity of JF on pregnant human myometrial contractility. JF potently inhibited human myometrial contractility in a concentration-dependent manner. This pilot study provides evidence that JF should be further investigated as a lead plant in the drug discovery of new uterine relaxants.

Keywords: Tocolytic; uterus; preterm labour; plant; extract; spontaneous contraction; human uterus; *Justicia flava*; Acanthaceae; myometrium; preterm birth.

1.1 Introduction

Preterm birth (PTB) is a major cause of neonatal and maternal mortality and morbidity (Liu et al., 2016; van Vliet, et al., 2014) and is defined as birth or delivery that occurs before 37 weeks gestation (Frey & Klebanoff, 2016). Spontaneous preterm contractions constitute a major cause of PTB. Inhibition of uterine contractility (tocolysis) is therefore considered as part of therapeutic efforts to combat PTB (Simões-Wüst et al., 2018). Currently available drugs (such as, oxytocin receptor antagonists, β-adrenergic agonists, magnesium sulfate, calcium-channel blockers) have demonstrated varying efficacy in randomised controlled trials and some have side effects that limit their use (Berkman et al., 2003). This has led to the search for new effective tocolytics to combat preterm labour (PTL) and reduce the incidence of PTB. Plants are a source of new tocolytic therapies, and the plant *Bryophyllum pinnatum* has successfully made it to clinical trials as a tocolytic agent (Simões-Wüst et al., 2018). One other such plant of growing interest is *Justicia flava*. The leaves of *J. flava* are regularly used in the South of Nigeria to prevent miscarriages (Bafor et al., 2019) possibly

through myometrial contractility inhibition. For this reason, the plant was selected and explored extensively on mouse models for its activity on uterine contractility and the female reproductive system (Bafor et al., 2020; Bafor et al., 2019). Based on the success of mouse experimentation and the inhibitory activity of *J. flava* on mouse uterine contractility, it was, therefore, necessary to determine the efficacy of the plant on human myometrial contractility.

This pilot study, therefore, provides proof of concept that an extract from the leaves of *J. flava* potently inhibits pregnant human myometrial contractility and may be a good candidate for further clinical evaluation as a novel tocolytic.

2.1 Materials and Methods

2.1.1 Ethical Approval

Non-labouring human myometrial biopsies were obtained from women with full term, uncomplicated singleton pregnancies undergoing elective Caesarean section (CS) (median gestation 39 weeks, n = 9 at Liverpool Women's Hospital, UK. All women provided written informed consent and ethical approval was sought and granted by the North West (Liverpool East) Research Ethics Committee (REC Ref: 10/H1002/49) and by the Research and Development Director of Liverpool Women's NHS Foundation Trust, Liverpool, United Kingdom. Indications for CS were previous CS (4, one of them presented breech), hypermobility (1), maternal hip operation (1), idiopathic intracranial hypertension (1), planned elective CS (1), and breech presentation (1). Biopsies were taken from the upper edge of the lower uterine segment incision immediately after delivery of the baby (Luckas & Wray, 2000). All biopsies were placed in Hanks balanced salt solution at 4°C and experiments were performed within 24 h of biopsy excision. The study conformed to the standards set by the Declaration of Helsinki, except for registration in a database.

2.1.2 Plant Collection and Processing

J. flava leaves were collected in Edo State, Nigeria, and identified at the Department of Plant Biology and Biotechnology, University of Benin, Nigeria, by Dr. H. A. Akinnibosun. A herbarium number of UBHj386 was provided for the plant. The leaves were cleaned, air-dried for two weeks, and ground to powder. In order to prepare the methanol leaf extract of *J.*

flava (JF), the powdered leaves were macerated in methanol (500 g: 2 L) for 72 h. The mixture was continuously stirred during the period. After 72 h, the mixture was filtered, and the macerate was concentrated to a constant weight using a rotary evaporator set at 60°C (BUCHI Labortechnik AG, Flawil Switzerland). A yield of 20.14 w/w was obtained, and the constituted extract aliquot was stored at -80°C.

2.1.3 Experimental Protocol

Myometrial strips approximately 5 mm x 1 mm were isolated, such that the longitudinal axis of each strip was aligned with the direction of the muscle fibres (Arrowsmith et al., 2012). The uterine strips were mounted in a 1 mL chamber bath and secured with aluminium clips. The bath was continuously perfused with physiological saline of the following composition (in mM): 154 NaCl, 5.6 KCl, 1.2 $MgSO_4$, 7.8 glucose, 10.9 HEPES, and 2.0 $CaCl_2$, pH 7.4. The uterine strips were then placed under a resting tension of 2 mN. The rate of perfusion was set at 1.5 mL/min, and the temperature was maintained at 36°C. A tension transducer (FT03, Grass Technologies, Slough, UK) attached to one end of the strip and connected to a data acquisition system (Axon) was used for recording contractility. Regular spontaneous contractions were achieved within 2 h. Strips that failed to develop spontaneous contractions within 2 h, even after challenging with high K^+ (40 mM), were excluded.

Once regular spontaneous contractions were achieved, cumulative concentrations of JF (0.0014 – 0.514 mg/mL) were added to the uterine tissue with each subsequent concentration added as soon as responses to preceding concentrations plateaued. At the end of the experiments, the tissues were washed, and recovery monitored for 1-2 h.

All chemicals utilized were obtained from Sigma (Poole, Dorset, UK).

2.2 Data Analysis

The parameters of contraction (amplitude, frequency, duration, and area under the curve (AUC) were measured during a 20 min control period and in the last 20 min of each JF concentration using Origin Pro Software (Origin Lab, v8.1). Contractions in the last 20 min before cumulative additions of JF was taken as control and subsequent data expressed as a percentage of this control response. Measurement of duration included half maximal

amplitude, AUC calculations included increases in baseline where it occurred and was based on the mean of several contractions. The nonlinear regression sigmoidal dose response equation model was used to fit data from the amplitude, duration and AUC (GraphPad Prism Software, v. 5.01, California, USA). The sigmoidal dose response equation is represented by:

$$Y = Bottom + \frac{(Top-Bottom)}{(1+10\,\hat{}\,(\mathrm{Log}_{10}\,IC50-x)*Hillslope))}$$

Where X is log of concentration and Y is response.

Data are presented as mean ± SD with one way ANOVA and Dunnett's post hoc test for multiple comparisons done with GraphPad Prism Software (v. 5.01, California, USA). $P<0.05$ was considered as minimum statistical significance and n = number of women.

3.1 Results and Discussion

Once contractions were established, the tissues contracted rhythmically for several hours (data not shown). JF inhibited spontaneous contractions of the human myometrium (Fig. 1). On analysis of contraction parameters, JF inhibited the amplitude of contraction (Fig. 2a) in a concentration-dependent manner, with significant inhibition obtained at 0.044, 0.114, 0.214 and 0.514 mg/ml (P <0.01; 0.001; 0.001 respectively, n=9). As amplitude decreased, the frequency simultaneously increased until contractions were abolished (Fig. 1). JF did not significantly alter duration except at 0.514 mg/ml (by which time most tissues had ceased contracting; data not shown). JF inhibited total contractility (AUC) in a concentration-dependent manner with significant inhibitions ($P < 0.05$) obtained at 0.114, 0.214 and 0.514 mg/mL (n=9) (Fig. 2b). Some of the uterine tissues were monitored for 1-2 h after JF was removed and these showed up to ≈ 30-40% recovery (Supplementary Figure 1). To support this observation, our previous study in mice showed similar recovery (less than 50%) after cumulative additions of JF, and when single concentrations of JF were utilized (which will be the likely scenario when utilized clinically), almost total recovery was achieved (Bafor et al., 2019). The IC_{50} of JF on the amplitude and area under the curve were calculated as 0.01 ± 1.10 mg/ml and 0.13 ± 1.18 mg/ml respectively. The data utilized for plots and analysis in this study are presented as Supplementary Data.

JF has been shown in this study to inhibit the isolated human myometrium as potently as it inhibited the mouse myometrium within very similar concentrations (Bafor et al., 2019). The inhibition pattern observed with the mouse uterus was also observed on the human

myometrium. JF inhibits the amplitude of contraction in a concentration-dependent manner with a corresponding increase in frequency, which was only reduced when contractions were completely abolished (Bafor et al., 2019). JF was also shown to inhibit Ca^{2+}- entry through voltage-gated channels (VGCCs) and also inhibits intracellular Ca^{2+} release through inositol triphosphate (IP_3) and ryanodine receptors (RyRs) in the non-pregnant mouse uterus (Bafor et al., 2019). The mechanisms of JF in the mouse myometrium maybe similar to the human myometrium, although further studies will be required to confirm this. This is vital in the context of this study, as increases in Ca^{2+}- within the myometrium drives myometrial contractility via myosin light chain kinase-dependent calcium-calmodulin pathway (Sanborn, 2001). Nifedipine is an L-type Ca^{2+}-channel blocker (Moynihan, Smith, & Morrison, 2008) that additionally acts through inhibition of outward K^+ currents and inhibition of capacitative Ca^{2+} entry (Young et al., 2001), has shown clinical relevance in inhibiting uterine contractions in women with threatened PTL compared to atosiban (Al-Omari et al., 2008). However, use of nifedipine results in maternal complications (Al-Omari et al., 2006) such as severe hypotension and can also result in fetal death (van Veen et al., 2005). Thus JF which has shown similar calcium-blocking effects might prove safer and more efficacious in the management of uterine contractions arising from PTL.

The increased frequency associated with JF may have resulted from the phytosterols contained in JF (Bafor et al., 2019). It appears that progesterone has the unique property of producing a simultaneous inverse occurrence with the amplitude and frequency of uterine contractions (Fanchin et al., 2000; Fanchin et al., 2001) and that this unique activity of increased frequency in the presence of decreasing amplitude is common with substances or plants with a dominance of phytosterols (Gwehenberger et al., 2004; Sukwan et al., 2014). The inverse activity may also be a property modulated by chloride conductance in the uterus, which is known to regulate uterine contraction frequency (Dodds et al., 2015) specifically. Interestingly, progesterone has been shown to attenuate the inhibitory effect of nifedipine on uterine contractility *in vivo* (Hajagos-Tóth et al., 2009). This is despite induction of rapid relaxation of the amplitude of myometrial contractions by progesterone (Anderson et al., 2009). Though yet to be investigated, it can be speculated that the combined property of Ca^{2+}-channel blockade and presence of phytosterols, may contribute to better efficacy of JF over nifedipine.

This is a pilot study that clearly shows the tocolytic potential of JF for human uterine contractility disorders. Previous safety studies on mice showed JF to be relatively safe, even

at high doses of 5 g/kg (Bafor et al., 2019). Our previous studies have also shown JF to have mild estrogenic activities at low doses (Bafor et al., 2020), which is beneficial to its continued use on humans. That JF reproducibly and effectively inhibited human myometrial contractility in this study, places JF as a lead plant in the drug discovery process for the development of tocolytic drugs. There are however some limitations to the study. With JF being a crude plant extract with several interacting compounds, we were unable to determine molecular weights and therefore molarities of specific compounds which would then have been compared with existing tocolytic drugs. Nonetheless, future studies on the plant should involve bioassay-guided fractionation, isolation and purification of active secondary metabolites from the plant involved in myometrial inhibition. Off-target effects of JF should also be studied.

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Author Contributions

EE conceptualized the study and contributed in performing experiments, data analysis and article writing; CP contributed in performing experiments, data analysis and article writing; SW contributed in study conceptualization and article review.

Data Availability

The data that support the plots of this study are available in the Supplementary section. Other personal identifiable information data are not publicly available due to privacy and/or ethical restrictions.

References

Al-Omari, W. R., Al-Shammaa, H. B., Al-Tikriti, E. M., & Ahmed, K. W. (2006). Atosiban and nifedipine in acute tocolysis: A comparative study. *European Journal of Obstetrics and Gynecology and Reproductive Biology*, *128*(1–2), 129–134. https://doi.org/10.1016/j.ejogrb.2005.12.010

Anderson, L., Martin, W., Higgins, C., Nelson, S. M., & Norman, J. E. (2009). The effect of progesterone on myometrial contractility, potassium channels, and tocolytic efficacy. *Reproductive Sciences*, *16*, 1052–1061. https://doi.org/10.1177/1933719109340926

Arrowsmith, S., Quenby, S., Weeks, A., Burdyga, T., & Wray, S. (2012). Poor spontaneous and oxytocin-stimulated contractility in human myometrium from postdates pregnancies. *PLoS ONE*, *7*(5). https://doi.org/10.1371/journal.pone.0036787

Bafor, Enitome E, Ukpebor, F., Elvis-offiah, U., Uchendu, A., Omoruyi, O., & Omogiade, G. U. (2020). Justicia flava Leaves Exert Mild Estrogenic Activity in Mouse Models of Uterotrophic and Reproductive Cycle Investigations. *Journal of Medicinal Food*, *23*(4), 395-408.

Bafor, Enitome E, Ukpebor, F., Omoruyi, O., Ochoyama, E., & Odega, K. (2019). Acute Toxicological Evaluations of the Methanol Leaf Extract of Justicia flava (Vahl) Acanthaceae in Mouse Models. *Tropical Journal of Natural Product Research*, *3*(4), 138–144.

Bafor, Enitome Evi, Ukpebor, F., Omoruyi, O., Ochoyama, E., Ekufu, J., & Edrada-Ebel, R. (2019). Tocolytic activity assessment of the methanol leaf extract of Justicia flava Vahl (Acanthaceae) on mouse myometrial contractility and preliminary mass spectrometric determination of secondary metabolites. *Journal of Ethnopharmacology*, 243, 112087.

Berkman, N. D., Thorp, J. M., Lohr, K. N., Carey, T. S., Hartmann, K. E., Gavin, N. I., … Idicula, A. E. (2003). Tocolytic, treatment for the management of preterm labor: A review of the evidence. *American Journal of Obstetrics and Gynecology*, 188,1648–1659.

Dodds, K.N., Staikopoulos, V., & Beckett, E. A. (2015). Uterine contractility in the nonpregnant mouse: changes during the estrous cycle and effects of chloride channel blockade. *Biology of Reproduction*, 92(6), 1-15.

Fanchin, R, Ayoubi, J. M., Olivennes, F., Righini, C., de Ziegler, D., & Frydman, R. (2000). Hormonal influence on the uterine contractility during ovarian stimulation. *Hum Reprod.*, *15*(Suppl 1), 90-100. https://doi.org/10.1093/humrep/15.suppl_1.90

Fanchin, Renato, Righini, C., De Ziegler, D., Olivennes, F., Ledée, N., & Frydman, R. (2001). Effects of vaginal progesterone administration on uterine contractility at the time of embryo transfer. *Fertility and Sterility*, *75*(6), 1136–1140. https://doi.org/10.1016/S0015-0282(01)01787-3

Frey, A. H., & Klebanoff, A. M. (2016). The epidemiology, etiology, and costs of preterm birth. *Seminars in Fetal and Neonatal Medicine*, *21*(2), 68–73.

Gwehenberger, B., Rist, L., Huch, R., & Von Mandach, U. (2004). Effect of Bryophyllum pinnatum versus fenoterol on uterine contractility. *European Journal of Obstetrics and Gynecology and Reproductive Biology*, *113*, 164–171. https://doi.org/10.1016/S0301-2115(03)00370-1

Hajagos-Tóth, J., Falkay, G., & Gáspár, R. (2009). Modification of the effect of nifedipine in the pregnant rat myometrium: The influence of progesterone and terbutaline. *Life Sciences*, *85*(15–16), 568–572. https://doi.org/10.1016/j.lfs.2009.08.008

Liu, L., Oza, S., Hogan, D., Chu, Y., Perin, J., Zhu, J., … Black, R. E. (2016). Global, regional, and national causes of under-5 mortality in 2000–15: an updated systematic analysis with implications for the Sustainable Development Goals. *The Lancet*, *388*(10063), 3027–3035. https://doi.org/10.1016/S0140-6736(16)31593-8

Luckas, M. J. M., & Wray, S. (2000). A comparison of the contractile properties of human myometrium obtained from the upper and lower uterine segments. *British Journal of Obstetrics and Gynaecology*, *107*, 1309–1311. https://doi.org/10.1111/j.1471-0528.2000.tb11626.x

Moynihan, A. T., Smith, T. J., & Morrison, J. J. (2008). The relaxant effect of nifedipine in human uterine smooth muscle and the BKCa channel. *American Journal of Obstetrics and Gynecology*, *198*(2), 237-e1. https://doi.org/10.1016/j.ajog.2007.08.074

Sanborn, B. M. (2001). Hormones and calcium : mechanisms controlling uterine smooth muscle contractile activity. *Experimental Physiology*, *86*(2), 223–237.

Simões-Wüst, A. P., Lapaire, O., Hösli, I., Wächter, R., Fürer, K., Schnelle, M., … Von

Mandach, U. (2018). Two Randomised Clinical Trials on the Use of Bryophyllum pinnatum in Preterm Labour: Results after Early Discontinuation. *Complementary Medicine Research*, *25*(4), 269–273. https://doi.org/10.1159/000487431

Sukwan, C., Wray, S., & Kupittayanant, S. (2014). The effects of Ginseng Java root extract on uterine contractility in nonpregnant rats. *Physiological Reports*, *2*(12), e12230–e12230. https://doi.org/10.14814/phy2.12230

van Veen, A. J., Pelinck, M. J., van Pampus, M. G., & Erwich, J. J. (2005). Severe hypotension and fetal death due to tocolysis with nifedipine. *BJOG: An International Journal of Obstetrics and Gynaecology*, *112*, 509–510. https://doi.org/10.1111/j.1471-0528.2005.00771.x

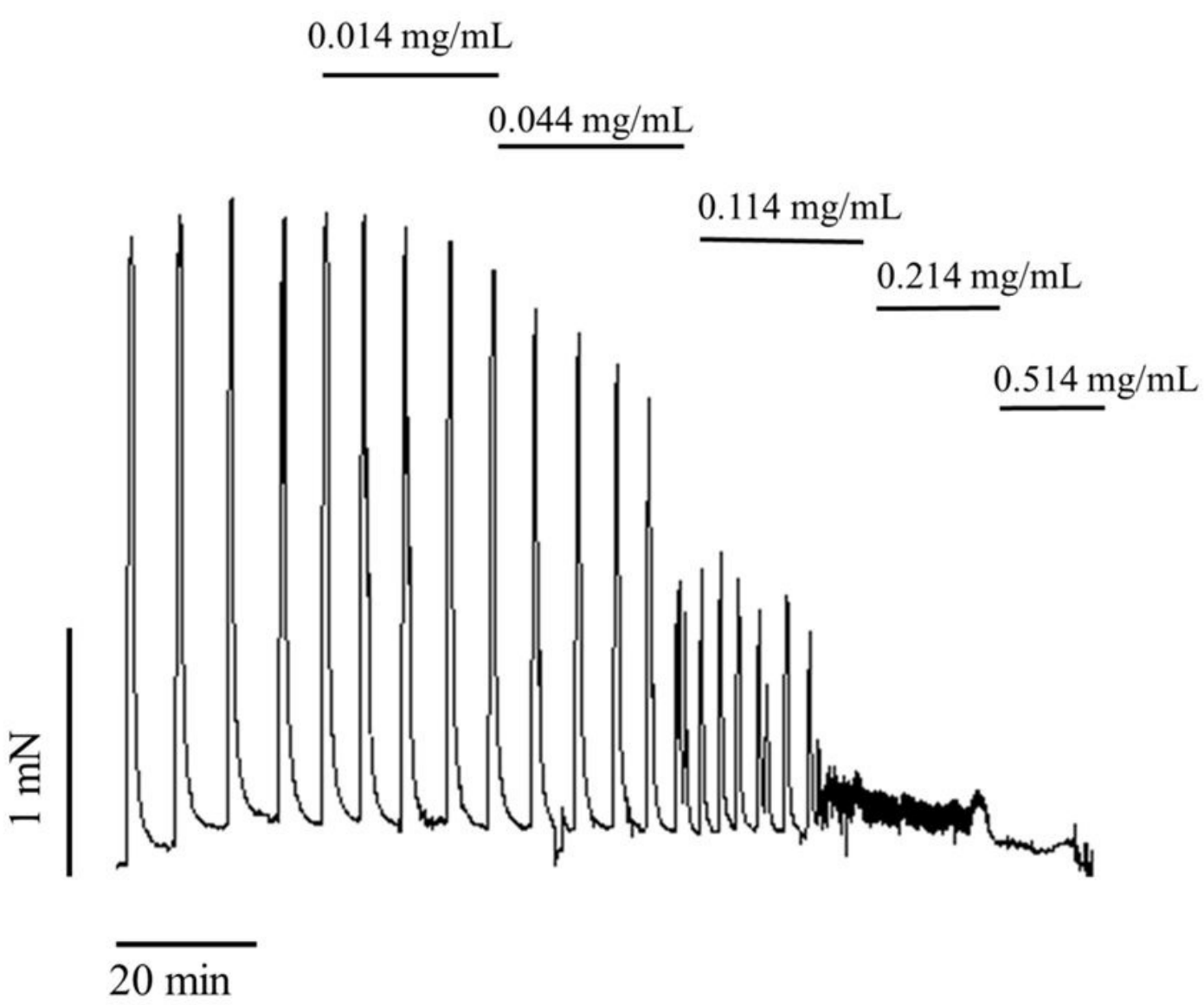
van Vliet, E. O. G., Boormans, E. M., De Lange, T. S., Mol, B. W., & Oudijk, M. A. (2014). Preterm labor: Current pharmacotherapy options for tocolysis. *Expert Opinion on Pharmacotherapy*, *15*, 787–797.

Young, R. C., Schumann, R., & Zhang, P. (2001). Nifedipine block of capacitative calcium entry in cultured human uterine smooth-muscle cells. *Journal of the Society for Gynecologic Investigation*, *8*, 210–215. https://doi.org/10.1016/S1071-5576(01)00109-5

Figure Legends

Figure 1. Original representative recording showing the *ex-vivo* effect of JF (0.0014 – 0.514 mg/mL) on spontaneous myometrial contractility.

Figure 2. Effect of JF on *ex-vivo* parameters of contractility in the pregnant human myometrium. (a) JF inhibited the amplitude of spontaneous myometrial contractility; (d) JF also inhibited total contractility in the pregnant myometrium n = 9 women; **P<0.05; ***P<0.0001; ****P<0.00001 compared to control.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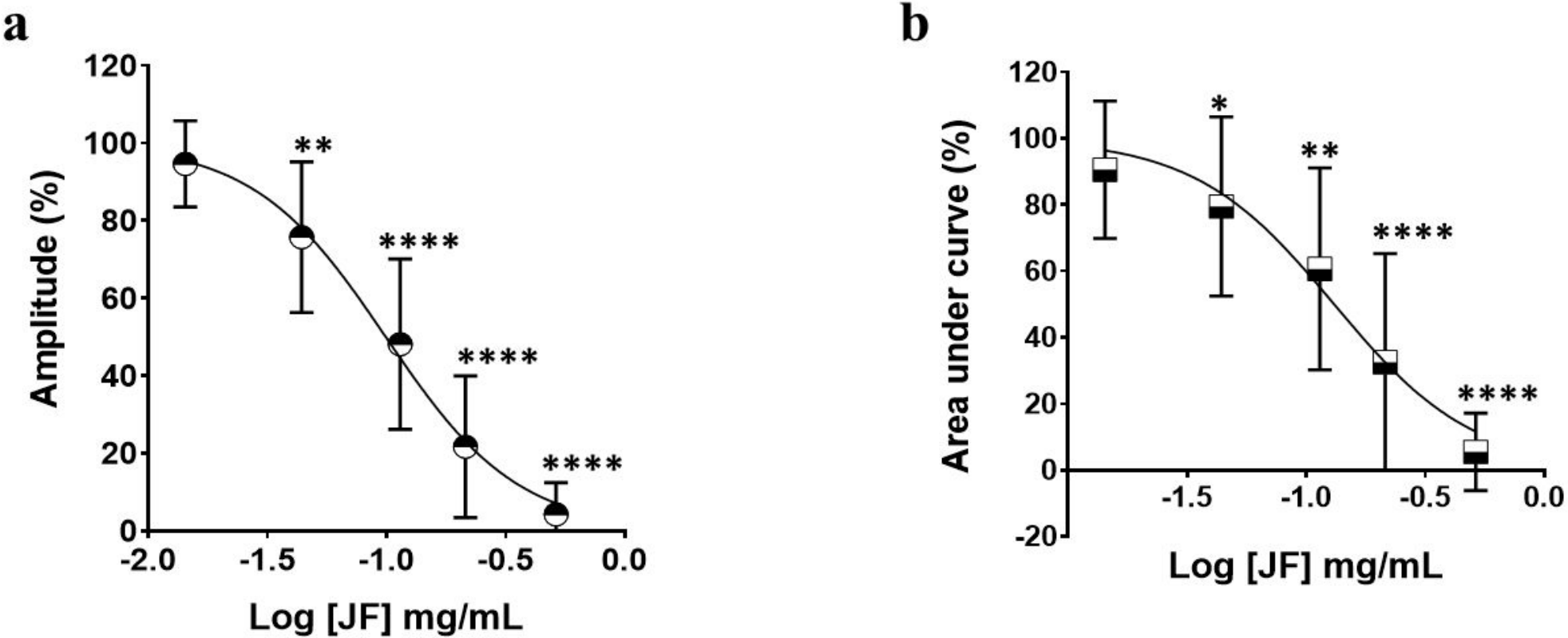
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Amplitude (%)
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-2.0
-1.5
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Log [JF] mg/mL
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Area under curve (%)
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100
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-20
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-1.5
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Log [JF] mg/mL