

# **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**

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## **Abbreviations**

JF = Methanol leaf extract of *Justicia flava*; preterm labour (PTL); Preterm birth (PTB); KCl = potassium chloride; Oxytocin = OT; EDTA = Ethylenediaminetetraacetic acid; NIF = Nifedipine; ATB = Atosiban; NM = Neomycin; TEA = Tetraethylammonium chloride; LC-HRFTMS = Liquid chromatography-high resolution Fourier Transform mass spectrometry; PR = propranolol; VGCCs = Voltage-gated calcium channels; PLC = phospholipase C; IP<sub>3</sub> = Inositol triphosphate; Ca<sup>2+</sup> = calcium ion; K<sup>+</sup> = potassium ion; RyR = Ryanodine receptor; OTR = oxytocin receptor.

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## **Abstract**

*Ethnopharmacological relevance:* The leaves of *Justicia flava* are traditionally used in the South of Nigeria to prevent preterm births.

*Aim of the study:* In this study, the activity of the methanol leaf extract of *J. flava* (JF) was investigated on uterine contractility in non-pregnant and pregnant isolated mouse tissues.

*Material and methods:* The effects on spontaneous, oxytocin, and KCl-induced contractions were determined. The effects in calcium-free media were also determined. Possible mechanisms of activity were investigated using receptor and channel modulators. Mass spectrometric analysis was additionally performed on the leaf extract to identify secondary metabolites.

*Results:* JF was observed to inhibit spontaneous, oxytocin and high KCl-induced uterine contractility. JF also inhibited contractions in  $\text{Ca}^{2+}$ -free media. JF was found to exert its inhibitory effect via interaction with inositol triphosphate and ryanodine receptors and also through modulation of  $\text{K}^+$ - channels. Lignans and alkaloids were identified with the lignans being the most abundant in JF.

*Conclusion:* JF has been shown to potently inhibit uterine contractions in non-pregnant and pregnant isolated mouse uterus. The inhibitory activity of JF has been shown to occur via blockade of extracellular and intracellular calcium entry and these effects may be due to the lignans identified in the JF. JF has therefore been shown in this study to be a lead plant in the discovery of new drugs with uterine inhibitory activity.

**Keywords:** *Justicia flava*; uterus; oxytocin; mass spectrometry; preterm labour; dysmenorrhoea

## **1. Introduction**

Contractility of the uterus is involved in dysmenorrhoea as well as in term and preterm labour (PTL). While dysmenorrhoea is considered a major reproductive problem for women of reproductive age leading to severe discomfort and poor quality of life (Bulletti et al., 2000), PTL which leads to preterm birth (PTB) has caused a significant number of neonatal/maternal mortalities and morbidities worldwide (Goldenberg, 2002; Goldenberg et al., 2008; Liu et al., 2015). Babies born preterm are prone to several health complications (Steer and Flint, 1999) creating a dire need to avert PTB as much as possible. There have been improvements in care to neonates through the years but PTB incidence still requires intervention. PTL is due to premature contraction of the pregnant uterus (Maltaris et al., 2006) and available uterine contractility inhibiting drugs (tocolytics) such as beta-adrenergic receptor agonists, are prone to several adverse effects and do not provide sufficient efficacy (Berkman et al., 2003). This therefore suggests that the ideal tocolytic has not been found (Keirse, 2003). There is also the issue of dysmenorrhea which occurs in non-pregnant women of reproductive age and manifests as uterine cramps which are particularly painful (Coco, 1999). Despite the popularity of dysmenorrhoea, it still remains under-diagnosed and treatment still remains far from successful (Campbell and McGrath, 1997; Coco, 1999; Proctor et al., 2010). Several limitations exist with conventional treatments for dysmenorrhoea and this has made herbal medicines a more viable source of therapy (Park et al., 2014) and there have been some positive reports from therapy with herbal medicines (Chen et al., 2006; Proctor and Murphy, 2001). In addition, herbal medicines can provide new leads and therapeutic alternatives for combating PTL and dysmenorrhoea.

Medicinal plants have proven useful through the years in the elaboration of several therapeutic agents including a number of current day drugs (Kinghorn et al., 2011; Newman and Cragg, 2012). Plants have also been utilized by several cultures for managing

dysmenorrhea, pregnancy management, birth facilitation and postnatal care supporting the recording and scientific validation of these cultural information on medicinal plants as it can provide scientists with useful leads and templates for targeted investigation of novel therapies for pregnancy and conditions related to uterine function (Gruber and O'Brien, 2011). In PTL and dysmenorrhea, increased uterine contractility occurs (Mirabi et al., 2014; Van Andel et al., 2014). Therefore for drug discovery processes, natural products or herbal medicines that are traditionally reputed to have uterine relaxing activities are of primary interest for such conditions.

*Justicia flava* (Forssk.) Vahl, of the Acanthaceae family is a medicinal plant whose leaves are used locally by traditional healers in the South of Nigeria to prevent miscarriages and PTB (personal communication with traditional healers in Edo State, Nigeria). It is called ‘Ighereje’ in Urhobo language of the South of Nigeria and “Afema” in local Asante-Twi language in Ghana. It is a perennial shrub often referred to as yellow Justicia of the Acanthaceae family due to its distinctive yellow flowers. It stands erect and grows to a height of about 1 m. It can be found growing widely in tropical and Southern Africa and eaten as a vegetable in parts of Guinea (Burkill, 1985). The plant is used to manage bleeding and for menorrhagia in Ivory Coast. It also used for painful menstruation as well as to induce menstruation in Ivory Coast (Burkill, 1985), suggesting the plant may have uterine contraction inhibiting effects as well as ability to modulate steroidal receptors. The leaves are used in Kenya to manage diarrhea (Burkill, 1985), this again suggests that the plant may have smooth muscle inhibiting effects and/or antimicrobial effects.

In the search for new targets and therapies for managing PTL as well as dysmenorrhea, this study was therefore aimed at investigating the activity of the plant leaves of *J. flava* on uterine contractility in non-pregnant and pregnant mouse models and to additionally investigate possible mechanisms of action. This study is also aimed at preliminary identification of

significant secondary metabolites present in the leaves of *J. flava* that may have contributed to the plant's biological activities.

## 2. Materials and Method

### 2.1. Drugs and Chemicals

Methanol of high analytical grade (Pharmatrends, Nigeria) and tween 80 (Kernel-KN, China) were utilized in this study. Physiological solution salts were obtained from Guangdong GuanghuaSci-Tech Co. Ltd China and Sigma Aldrich, UK). Other drug used in this study oxytocin (Roche pharmaceutical Ltd, UK), atosiban (Sigma-Aldrich, Inc), neomycin (Mancare Pharmaceuticals PVT. Ltd, India), nifedipine (Sigma-Aldrich, UK), propranolol, tetraethyl ammonium chloride (TEA) (Sigma-Aldrich, UK), heparin (Sigma-Aldrich, UK), gentian violet dye (Nomagbon pharmaceuticals Ltd, Nigeria), For the mass spectrometric experiments, methanol (MeOH), dichloromethane (DCM), acetonitrile (MeCN) and formic acid were purchased (Fisher Scientific, Hemel Hempstead, UK). All reagents used were of analytical grade.

### 2.2 Plant material

Fresh mature leaves of *J. flava* (**JF**) were collected from Ugonoba village, Edo State, Nigeria in the month of October 2016. The plant was identified by Dr. H.A. Akinnibosun of Plant Biology and Biotechnology Department, Faculty of Life Sciences, University of Benin, Benin City, Edo State Nigeria. The plant was provided an herbarium number UBHj 386 and a voucher specimen was deposited. The common and local names of the plant are listed in the introduction. The plant name has also been checked with <http://www.theplantlist.org>, accessed on the 6<sup>th</sup> of April, 2019.

### *2.2.1 Collection and extraction of the plant material*

The fresh leaves were freed of debris as much as possible and dried under shade for two weeks. The dried leaves were then ground into powder with the aid of a milling machine (Christy Norris, England). The powdered leaves (500 g) were subsequently macerated in methanol (2 L) for 72 h with constant stirring and the macerate was concentrated in a rotary evaporator (BUCHI Labortechnik AG, Flawil Switzerland) set at 60°C. The dried extract gave a yield of 20.14% (w/w) and was placed in an air-tight container and stored in a refrigerator at about 4 °C until needed.

### *2.3 Animals*

Healthy non-pregnant and pregnant (20-30 g) albino mice were utilized for this study and housed in the Animal Unit of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Edo State, Nigeria. The animals were maintained in accordance with the Public Health Service policy on humane care and use of Laboratory Animals (National Research Council, 2010; NIH, 2015). Ethical permission was obtained before the start of the experiments from the Faculty of Pharmacy Ethics Committee, University of Benin, Nigeria and the study was provided an approval reference number of: EC/FP/016/04. The animals were housed and maintained at an environmentally controlled room temperature of approximately  $27 \pm 5$  °C and natural light and dark cycles. The animals were maintained on standard diet of animal pellets and clean tap water provided *ad libitum*.

### *2.4 Experimental protocol*

The uterine tissue strips were mounted vertically in tissue organ baths (10 mL) connected to an isometric force transducer (7003E- Ugo Basile, Varise, Italy). The tissue was equilibrated under resting tensions of 4.90 mN for 30-45 min or till regular contractions were

obtained (Sukwan et al., 2014). The transducer was connected to a 17400 data capsule digital recorder with an inbuilt bridge amplifier (Ugo Basile, Varese, Italy).

#### *2.4.1 Uterine tissue preparation*

Animals were humanely killed by cervical dislocation and the uterine horns were immediately removed and placed into a petri dish containing previously warmed and aerated physiological salt solution. Connective and adhering tissues were removed from the isolated uterus and one horn was dissected in half to obtain a segment of the uterine horn of approximately 1-2 mm in length. The uterine segment obtained was mounted in a warmed organ bath (10 mL) maintained at 37 °C and containing aerated physiological solution. The physiological salt solution used was of the following composition in mM/L: NaCl 154.00, NaHCO<sub>3</sub> 5.95, D-glucose 2.78, KCl 5.63, and CaCl<sub>2</sub>·2H<sub>2</sub>O 2.05 (Bafor et al., 2015). For the pregnant animals, timed mated pregnant animals were used. On day 18, the animals were humanely killed by cervical dislocation and the pregnant uterus was carefully excised. The foetuses were carefully removed from the uterus and segments were obtained and mounted as described above.

#### *2.4.2 Experiment on spontaneous uterine contractility*

JF (0.001 – 0.647 mg/mL) was added cumulatively to the isolated uterine tissue to obtain concentration-response relationships. The concentrations used were previously determined in our laboratory constituting the total effect of the extract. Each concentration was allowed a contact time of 5 min. (Bafor et al., 2017a). This procedure was performed for both non-pregnant and pregnant mouse uteri.

#### *2.4.3 Experiment on oxytocin-induced uterine contraction*

Activity of JF on oxytocin (OT) induced uterine contraction was determined. Cumulative concentrations of JF (0.001 – 0.647 mg/mL) were added to the tissue pre-

contracted by oxytocin (11.62 nM). For the pregnant uterus, a single concentration of JF (0.1 mg/mL) was added to the OT-induced uterine contraction (11.62 nM). A time period of 5 min was allowed for each concentration of extract and OT.

#### *2.4.4 Experiment on high KCl-induced uterine contractility*

The effect of JF on high KCl-induced uterine contraction was additionally determined. JF (0.1 mg/mL) was added to KCl (80 mM) induced uterine tonic contraction. Determinations were done for 5 min per concentration of KCL or JF.

#### *2.4.5 Experiment on the effect of the extract in Ca<sup>2+</sup>-free medium*

In this experiment, zero calcium physiological solution was used in which CaCl<sub>2</sub> was omitted and Ethylenediaminetetraacetic acid (EDTA) was added. The uterine tissue was initially equilibrated for 30 min after which the physiological salt solution was changed to the zero calcium solution containing 0.1 mM EDTA. The tissue was then equilibrated in the Ca<sup>2+</sup>-free solution for 3-5 min (it was essential that contractions were not totally diminished during the experiment to allow for measurements). After equilibration, oxytocin (1.16 µM) was added and a contact time of 5 min was allowed. Without flushing, JF (0.1 mg/mL) was added. A contact time of 5 min was allowed for the extract concentration.

#### *2.4.6 Drug challenges to determine mechanism of activity*

In other experiments, some receptor antagonists were utilized in this study in order to determine possible mechanism(s) of activity associated with the effect of JF on uterine contractility. This included: propranolol, a beta-adrenoceptor antagonist (PR 0.02 mM), Heparin, an inositol triphosphate blocker (HEP 62.56 nM), Nifedipine, a calcium channel blocker (NIF 0.49 pM), Atosiban, an oxytocin receptor antagonist (ATB 2.01 µM), Neomycin, a phospholipase C inhibitor and ryanodine receptor blocker (NM 4.39 µM) and tetraethyl ammonium chloride, a blocker of calcium-dependent potassium channels (TEA 1.03 mM).

After tissue equilibration, the tissue was allowed to spontaneously contract for 5 min, then the receptor antagonist was added for 5 min before addition of JF (0.02 mg/mL) for an additional 10 min.

Time-matched controls were performed in all cases where appropriate.

## *2.5 LC-HRFTMS identification of constituents in extract*

Liquid chromatography-high resolution Fourier Transform mass spectrometry (LC-HRFTMS) analysis was performed on the crude extract of JF. The mass spectrometer, a Dionex UltiMate-3000 (DIONEX, Sunnyvale, CA, USA) was coupled to a ThermoScientific Exactive Orbitrap system (Thermo Fisher Scientific (Bremen) GmbH, Bremen, Germany). An ACE column C18 75 × 3.0 mm (Hichrom Ltd., Reading, UK) was used. Parameters used were as previously described (Bafor et al., 2016). The elution flow rate was set at 300 µL/min with water (A) and acetonitrile (B). Both mobile phases contained 0.1% formic acid. A gradient flow starting with 10% B and increasing to 100% B in 30 min was used in this analysis. The mobile phase was sustained for 5 min at 100% B thereafter equilibration of the column with 10% B was done. Data mining was performed using MZmine 2.10 (Pluskal et al., 2010). Prior to data mining, data acquired were sliced into positive and negative data sets with the aid of ProteoWizard (Kessner et al., 2008). Peaks were detected with the centroid mass detector with the noise level set at 1000. The chromatogram builder generated peak lists from the mass lists. The minimum time span was set at 0.2 min, with a minimum height of 10,000, and the *m/z* tolerance of 0.0001 *m/z* or 5 ppm. Chromatogram deconvolution was performed using the local minimum search algorithm with the following parameters: threshold (90%), search minimum in RT range (0.4 min), minimum absolute height (10,000), minimum relative height (5%), minimum ratio of peak top/edge (2), and peak duration range (0.2-5.0 min). The peak lists were de-isotoped with the isotopic peaks grouper and an *m/z* tolerance of 0.001 *m/z* or 5 ppm,

retention time tolerance of 0.1 minutes (absolute), and maximum charge of 2. The most intense isotope was selected as the representative isotope. The peak lists were subsequently merged with the aid of the Alignment function. The weight for *m/z* and for RT was set at 20, and the RT tolerance was set at 5%. The aligned peak lists were gap-filled using the Peak Finder, with an RT tolerance of 0.5 min (absolute) and an intensity tolerance of 1% (Macintyre et al., 2014). The adducts were identified with other complexes. Prediction of the chemical formulae for each peak was done using the formula prediction tool developed by MZmine. ChemBioFinder version 13 (PerkinElmer Informatics, Cambridge, UK) and was used to access hits from the database.

## *2.6 Data analysis*

The parameters of uterine contraction frequency and force were obtained and computed using the GraphPad Prism, (version 5.03; GraphPad software Inc, San Diego, CA, USA). Data were obtained as mean  $\pm$  standard error of mean (SEM) which in some cases were calculated and presented as percentages of control applications (control=100%). The number of animals used in this study were presented as ‘n’. Significance was evaluated using appropriate t-tests, and where necessary, one way analysis of variance with Dunnett’s post hoc and P values  $\leq 0.05$  was considered as minimum significance in all cases. Datasets having sufficient data points were computed as mean log concentration-response curves. For such datasets, analysis was done by fitting data to a variable slope logistic equation, using the following equation values  $Y = \text{Bottom} + (\text{Top}-\text{Bottom})/(1+10^{((\text{LogIC50}-X)*\text{HillSlope})})$ . Where Y represents response extending from the bottom to the Top in sigmoid shape, X represents the logarithm of concentration and IC<sub>50</sub> represents the concentration that gives a response half way between the top and bottom.

### **3. Result**

#### *3.1 JF on spontaneous uterine contraction*

Cumulative additions of JF significantly decreased spontaneous uterine contractions in a concentration-dependent manner (Fig. 1). In the presence of JF, force amplitude progressively decreased until contractions were abolished completely (Fig. 1B). An interesting pattern was however observed with frequency in the presence of JF. An increase in frequency was observed which decreased with increasing concentration of JF (Fig. 1C). The inhibitory activity of JF was similarly observed in the pregnant uterus (Fig. 2). The IC<sub>50</sub> of JF on amplitude for the non-pregnant uterus was  $0.014 \pm 0.077$  mg/mL while frequency was  $0.309 \pm 2.85$  mg/mL. The IC<sub>50</sub> of JF on amplitude for the pregnant uterus was  $0.002 \pm 0.076$  mg/mL while frequency was  $0.251 \pm 0.053$  mg/mL (Table 1).

#### *3.2 JF on oxytocin-induced uterine contraction*

OT stimulated an increase in amplitude and frequency of uterine contractions. However in the presence of JF, there was observed a concentration-dependent decrease in force and frequency of OT-induced contractions (Fig. 3A). This was clearly observed on analysis of the amplitude (Fig. 3B) and frequency (Fig. 3C). JF also exhibited significant inhibition ( $p < 0.001$ ) of the amplitude and frequency of the pregnant uterus (Fig. 4).

#### *3.3 JF on high KCl- depolarization*

JF was observed to inhibit the high KCl-induced uterine contractions (Fig. 5A). This was calculated as a significant decrease ( $p < 0.01$ ) in the force of contractions induced by KCl (80 mM) (Fig. 5B).

### *3.4 JF on OT-induced contraction in calcium-free medium*

In the presence of JF, OT-induced contraction in calcium-free medium was inhibited (Fig. 6A). JF induced a significant reduction ( $p < 0.01$ ) in the amplitude (Fig. 6B) and frequency (Fig. 6C) of OT-induced contractions in the presence of zero calcium.

### *3.5 JF on receptor antagonists and channel blockers*

In the presence of NIF, JF significantly ( $p < 0.001$ ) decreased the amplitude and frequency of spontaneous uterine contractions (Fig. S1 C and D respectively). In the presence of PR, JF significantly decreased ( $p < 0.01$ ) the amplitude of spontaneous uterine contractions (Fig. S1E) though a slight increase in frequency was observed (Fig. S1F). In the presence of ATB, JF significantly decreased ( $p < 0.001$ ) the amplitude of spontaneous uterine contractions (Fig. S1G) but with an accompanying slight increase in frequency (Fig. S1H). In the presence of TEA, JF significantly decreased ( $p < 0.05$ ) the amplitude of spontaneous uterine contractions (Fig. S1I) and a slight increase in frequency was also observed (Fig. S1J). In the presence of NM, JF non-significantly decreased the amplitude of spontaneous uterine contractions (Fig. S1K) while a significant increase ( $p < 0.01$ ) in frequency was observed (Fig. S1L). In the presence of HEP, JF significantly decreased ( $p < 0.05$ ) the amplitude of spontaneous uterine contractions (Fig. S1M) while a slight increase in frequency was observed (Fig. S1N). Original representative recordings are shown in Figures S2-S5.

In order to determine if these receptor and channel antagonists potentiated or attenuated the effect of JF on spontaneous contraction, a percentage computation was done for the activities of JF on spontaneous contractions in the presence of each antagonists. It was observed that NIF significantly potentiated ( $p < 0.01$ ) the inhibitory effect of JF on the amplitude of uterine contractions (Fig. 7A) while HEP significantly reduced ( $p < 0.05$ ) the inhibitory effect of JF on the amplitude (Fig. 7A). NM, ATB and TEA in the order of decreasing potency

reduced the inhibitory effect of JF on the amplitude of uterine contractions but these were statistically non-significant (Fig. 7A). PR had no significant effect on the inhibitory effect of JF on the amplitude of spontaneous uterine contractions (Fig. 7A).

On the frequency, JF at 0.02 mg/mL was shown to stimulate an increase in frequency of spontaneous uterine contractions. In the presence of NIF, JF the increase in frequency was significantly attenuated ( $p < 0.01$ ). This was similarly observed in the presence of PR and ATB, in order of decreasing potency, though the data was considered statistically non-significant. In the presence of TEA, NM and HEP (in order of decreasing potency), the stimulatory effect of JF on frequency of uterine contractions were potentiated though statistically non-significant (Fig. 7B).

### *3.6. Mass spectrometric identification of secondary metabolites in the extract*

Compounds of **JF** were analyzed and the known compounds were identified by LC-HRFTMS. The fingerprint chromatogram are shown (Figure 8). Seven (7) compounds were putatively identified in the leaves of JF (Table 1). These included, prostalidin D (1), justiciamide (2), vasicinol (3), vasicinone (4), 9,9'-demethylsecolintetralin (5), dimethylsecoisolariciresinol (6), and hypercratine (7) (Fig. 9).

## **4. Discussion**

The results of this study have shown JF to potently inhibit uterine contractions. This was evident in the inhibition of spontaneous uterine contractions in both non-pregnant and pregnant uterus. Slight contractions were observed on washout when cumulative concentrations of JF were used (Fig. S1). However, when single concentrations of JF were used (Fig. S3-S6). This suggests that the inhibition may not be due to irreversible damage (Loch-

Caruso et al., 2003). However, toxicity may not be ruled out and further studies are required to confirm this.

The uterus is composed of 3 layers of which one of them is the myometrium (smooth muscle cells of the uterus) which produces spontaneous contractility of the uterus (Kelly, 1962; Pehlivanoğlu et al., 2013). Contractions in the non-pregnant uterus, function to expel parts of the endometrial layer during the initial follicular phase (de Vries et al., 1990). However, excessive contractility of the uterus (dyskinesia) may lead to discomfort and pain (dysmenorrhea), and in the pregnant uterus this will lead to PTL and abortion (Bulletti et al., 1993; Kelly, 1962). The frequency of uterine contractions increases at the follicular phase of the menstrual cycle and this is decreased at the luteal phase (Bulletti et al., 1993; Lyons et al., 1991). The cycle phase of the non-pregnant animals used in this study was the late follicular phase as well as the ovulation phase where the frequency of uterine contractions are about at their highest (Bafor et al., 2017b; Bulletti et al., 1993). The influx of  $\text{Ca}^{2+}$  from both the intracellular and extracellular regions have been reported to play a primary role in spontaneous uterine contractions (Floyd and Wray, 2007; Shmigol et al., 1998; Wray et al., 2003). It may therefore be that JF inhibits  $\text{Ca}^{2+}$  influx in both the non-pregnant and pregnant uterus. JF was also shown to inhibit OT-induced uterine contractility. That JF inhibits OT-induced contraction in addition also suggests that JF inhibits both spontaneous and agonist-induced uterine contractions. OT induces uterine contraction through interaction with oxytocinergic receptors and this in turn activates the phospholipase C (PLC) system resulting in the eventual  $\text{Ca}^{2+}$  mobilization from intracellular stores (Arrowsmith and Wray, 2014; Wray, 2007). Contribution from extracellular  $\text{Ca}^{2+}$  also occurs with OT and occurs through activation of voltage-gated calcium channels (VGCCs) (Wray et al., 2001). Therefore the inhibition of OT by JF supports a likely interaction and possibly inhibition of  $\text{Ca}^{2+}$  related mechanisms in uterine contractility. There is also the possibility of JF interacting or modulating the secondary messengers involved

in OT-induced contractions that eventually lead to  $\text{Ca}^{2+}$ mobilization. This was further investigated with the receptor antagonists used in this study. At this stage it is unclear if JF interacts with both sources of  $\text{Ca}^{2+}$  influx into the cytosol (i.e either intracellular or extracellular). The VGCCs plays a role in both spontaneous and OT-induced uterine contractions and in order to investigate the likely interaction of JF with VGCCs, JF was tested on high KCl-induced tonic contractions.

High KCl activates L-type VGCCs (Granger et al., 1986; Niedergerke, 1956), leading to sustained tissue depolarization (Little et al., 1985). JF inhibited depolarization induced by high KCl lending support to the interaction of JF with extracellular  $\text{Ca}^{2+}$  channels. This was also evident as the inhibitory effect of JF was further potentiated in the presence of NIF an L-type calcium channel blocker which blocks VGCCS (Forman et al., 1979; Moynihan et al., 2008). As earlier mentioned, intracellular  $\text{Ca}^{2+}$  contributes to uterine contractility in addition to VGCCs. The activity of JF was therefore further investigated in  $\text{Ca}^{2+}$ -free medium. Once again, JF inhibited OT-induced contraction in  $\text{Ca}^{2+}$  free medium where the principal source of  $\text{Ca}^{2+}$ arises from intracellular stores (Kupittayanant et al., 2002; Luckas et al., 1999). It would therefore seem that JF inhibits  $\text{Ca}^{2+}$  from both the L-type VGCCs and intracellular stores. The inhibition of  $\text{Ca}^{2+}$  from intracellular stores also suggests possible interaction with inositol triphosphate ( $\text{IP}_3$ ).  $\text{IP}_3$  is a second messenger involved in downstream signaling of OT-induced uterine contraction and it also regulates intracellular  $\text{Ca}^{2+}$  release (Arrowsmith and Wray, 2014; Stepien and Ziecik, 2002; Zingg and Laporte, 2003). In order to investigate if the  $\text{Ca}^{2+}$ -blocking effects were mediated via specific receptors, the effect of JF was tested in the presence of some receptor blockers.  $\beta$ -adrenergic receptor agonists generally inhibit uterine contractions and are very often used to manage PTL (Conde-Agudelo et al., 2011). Failure of PR (a  $\beta$ -adrenergic receptor blocker (Sozzani et al., 1992)) to attenuate the inhibitory effect of JF suggests that JF does not interact with  $\beta$ -adrenergic receptors to produce its inhibitory effect. HEP blocks  $\text{IP}_3$

mediated  $\text{Ca}^{2+}$  - release from intracellular stores by blocking  $\text{IP}_3$  receptors on the sarcoplasmic reticulum (Guillemette et al., 1989). In this study, HEP attenuated the inhibitory effect of JF suggesting that JF activates  $\text{IP}_3$  receptors located in the sarcoplasmic reticulum. HEP was the most potent drug in this study to attenuate the inhibitory effect of JF. This was followed closely by NM. NM is a potent ryanodine receptor (RyR) blocker (Laver et al., 2007; Wang et al., 1996). RyR operates as a  $\text{Ca}^{2+}$  release channel in the myometrium similar to  $\text{IP}_3$  receptors and is also located on the sarcoplasmic reticulum (Mackrill et al., 2015). This therefore suggests that JF interacts with RyR in the myometrium. ATB was also found in this study to mildly attenuate the inhibitory effect of JF. ATB interacts with OT receptors (OTR) and competitively blocks OT or OTR agonists in the myometrium (Arrowsmith et al., 2010). Blockade of OTR would usually lead to inhibition of contractility, it was therefore surprising that ATB had a mild effect in attenuating the inhibitory effect of JF rather than potentiate it. OTR activation of  $\text{G}_q$  proteins have been widely studied and it results in the PLC activation of  $\text{IP}_3$  and diacylglycerol and consequent increase in  $\text{Ca}^{2+}$  (Sanborn, 2001). ATB is therefore popularly used clinically for its ability to block the  $\text{G}_q$  pathway in the myometrium (Phaneuf et al., 1994) by blocking OTR. However, a study have shown that ATB can selectively activate OTR coupling to  $\text{G}_i$  proteins. This is despite being an antagonist at OTR- $\text{G}_q$  coupling for which it is commonly known and utilized for. ATB has therefore been termed a ‘biased agonist’ (Reversi et al., 2005), a term which represent compounds which act as agonists on a particular G-protein receptor through a particular pathway but then act as antagonists at another (Kenakin, 2003). It may therefore seem that JF interacts with  $\text{G}_i$  signaling pathway, however further studies are required to confirm this. Potassium channels in the uterus have been reported and can serve to inhibit uterine contractility (Brainard et al., 2007; Piedras-Renteria et al., 1991).  $\text{K}^+$ - channels are also modulated through the G-protein signaling pathways particularly the  $\text{G}_s$  and  $\text{G}_i$  proteins (Zhou et al., 2006). The effect on JF on potassium channels was also investigated in this study using

TEA, a non-selective K<sup>+</sup>- channel blocker (Khodakhah et al., 2002). It was found that a mild attenuation of the inhibitory effect of JF was also observed. This suggests a possible activation of K<sup>+</sup>-channels by JF. However, that the attenuation was not large may suggest an indirect interaction.

An interesting feature of this research was the increasing frequency of contractions associated with decreasing amplitude (Fig. S2). The frequency was only abolished when contractions were completely abolished as well. This occurred in both the pregnant and non-pregnant uterus in this study. The reason for this seemingly opposing effect between amplitude and frequency uterine contractions are not well understood. However a few explanations maybe made at this stage. Dominic and Reinke had suggested that differences in the levels of estrogen may cause an inverse relationship between frequency and force of contraction (Dominic and Reinke, 1968), a response pronounced during estrus and after ovulation (Hamilton, 1951) when progesterone dominates (Dominic and Reinke, 1968). Progesterone has been reported to cause asynchronous electrical activity in the uterus (Csapo and Takeda, 1965), resulting in a decrease in amplitude and increase in frequency which is overcome as progesterone levels increases (Dominic and Reinke, 1968). Studies by Putnam et al. (1991) showed that at certain concentrations, progesterone inhibited the amplitude of uterine contractions with a corresponding increase in the frequency (Putnam et al., 1991). This effect of progesterone was abolished in the presence of a progesterone antagonist, RU486, suggesting a direct effect of progesterone possibly by interaction with progesterone receptors in the uterus (Putnam et al., 1991). Fanchin et al. (2000) while studying controlled ovarian hyperstimulation, observed a high uterine contraction frequency in the early luteal phase and suggested that this may have resulted from an insufficient response of the uterus to the relaxant effect of progesterone during the luteal phase (Fanchin et al., 2000). In other studies, Fanchin and colleagues consistently observed this dominance in increased uterine contraction frequency at the early stages of

progesterone rise which again is attenuated with a more intense progesterone exposure (Fanchin et al., 2001). They also maintained that at these times, the uterus previously stimulated by estrogen attempts to resist the relaxation induced by progesterone (Fanchin et al., 2001). A similar event of increase in frequency with an extract exhibiting inhibition of amplitude had also been observed on *Bryophyllum pinnatum* extract on spontaneous human uterine contraction (Gwehenberger et al., 2004). In a more recent study by Sukwan et al. (2014) it was observed that the herb Ginseng Java, potently inhibited uterine contractility but as the amplitude decreased, the frequency increased correspondingly until contractions were completely abolished (Sukwan et al., 2014). Ginseng Java like JF inhibited contractions due to extracellular and intracellular  $\text{Ca}^{2+}$  entry. *B. Pinnatum* (Fürer et al., 2013; Oufir et al., 2015), Ginseng Java and JF were found to contain a dominance of phytosterols, some of which has been attributed to be responsible for the inhibitory effect of Ginseng Java and *B. pinnatum* on the uterus (Gwehenberger et al., 2004; Sukwan et al., 2014). It may therefore seem that sterols possibly progesterone, has the unique property of inducing the inverse occurrence between amplitude and frequency of uterine contractions and that this effect is overcome as the concentration is increased. This also suggests that JF may contain a dominance of phytosterols and necessitated preliminary determinations of the constituents of JF which was achieved in this study using mass spectrometry.

Mass spectrometric analysis identified seven compounds at this stage which will be briefly discussed. Prostalidin D was identified and is an arylnaphthalide lignan containing a rare catechol unit (Rajasekhar et al., 2000). 9,9'-demethylsecolintetralin, and dimethyl secolintetralin were also identified in JF and are also lignans (Das et al., 1994; Ward, 1999). Lignans are phytoestrogens found in plants which can act as weak estrogen agonists and also potent estrogen antagonists (Morris, 2007). They are also known to decrease aromatase activity leading to a reduction in estrogen activity (Wang, 2002). Lignans have been reported to

increase progesterone concentrations and decrease estrogen levels (Fecteau et al., 2011) and also induces a down regulation of the estrogen receptor ER $\beta$  (Richter et al., 2010). Lignans have also been shown to inhibit smooth muscle contraction (Herrmann et al., 2014). These effects support the activity seen with JF in this study. Justiciamide also known as N - (2-hydroxy-4,5-dimethoxyphenyl) - (2 S,4 S) – g – hydroxyl glutamic acid (Lorenz et al., 1999) was also identified in JF. No clear activity have been reported on Justiciamide yet. Some alkaloids were additionally identified. The pyrroloquinazoline alkaloids, vasicinol and vascinone (Joshi et al., 1996) and the pyrrolidine alkaloid, hypercratine (Neukomm et al., 1983). Vascinone is known to produce bronchodilatation by relaxing the bronchial smooth muscle (Amin and Mehta, 1959) which suggests smooth muscle inhibiting effect. Not much is known on the pharmacological activities of the others. Therfore of the compounds identified in JF, lignans were found to be more abundant than the alkaloids with dimethylsecoisolariciresinol being the most abundant of all compounds present (known and unknown). This observation will also support the developing hypothesis of the phytosterols in JF being responsible for the inhibitory activity exerted on uterine contractions.

One limitation of this study is that the human uterus was not used. Studies using the human uterus will provide stronger basis for the progress of the use of this plant for clinical management of preterm labour. Further studies investigating the effect of JF on the isolated human myometrium are therefore recommended. Another limitation was that relatively high concentrations of the extract was used. The range of concentrations used for JF, were predetermined to be the concentrations in which activity was observed. This different types of metabolites contained in the crude extract of JF may have contributed to the concentrations used.

## **5. Conclusion**

This study shows the first evidence that the leaves of *Justicia flava* produces inhibitory effects on spontaneous and agonist induced contractions in the non-pregnant and pregnant uterus. The possible mechanisms of activity include blockade of Ca<sup>2+</sup>-influx through L-type Ca-channels, blockade of Ca<sup>2+</sup>- release from intracellular stores via interaction with IP<sub>3</sub> receptors and RyRs, and indirect modulation of K<sup>+</sup>- channels. The study also reports for the first time the presence of some lignans and alkaloids in JF and the high dominance of lignans may contribute to the uterine inhibitory effect of JF. This study therefore presents JF as a valid lead for further drug discovery process for the production of new drug treatments in conditions where uterine contractility inhibition is of necessity such as dysmenorrhea and PTL.

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### **Competing interests**

None declared.

### **Author's contribution**

EEB conceived and designed the research, supervised, wrote and revised the article. FU, OO, EO, GO and JE performed the experimental studies. RE performed, analyzed and interpreted the mass spectrometric studies and also revised the article. All authors have approved the final article.

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## TABLES

**Table 1. Estimates of JF on contractility parameters or pregnant and non-pregnant uterus**

	IC <sub>50</sub> AMP (mg/mL)	EC <sub>50</sub> Freq (mg/mL)
<b>Non-pregnant (Mouse)</b>	0.015 ± 0.074	0.072 ± 0.023
<b>Pregnant (Mouse)</b>	0.002 ± 0.076	0.251 ± 0.053
<b>Pregnant (Human)</b>	0.099 ± 0.101	0.095 ± 2.241

**Table 2. Putatively Identified compounds in JF**

P= positive; N= negative; RT = retention time; MW = molecular weight; ppm = parts per million

No.	Polarity	m/z	RT	MW	ppm	Formula	Name
<b>1</b>	P	337.0713	1.23	336.064	1.821	C <sub>19</sub> H <sub>12</sub> O <sub>6</sub>	prostalidin D
<b>2</b>	P	315.1178	2.13	314.111	-2.687	C <sub>13</sub> H <sub>18</sub> N <sub>2</sub> O <sub>7</sub>	justiciamide
<b>3</b>	P	205.0972	3.45	204.090	0.388	C <sub>11</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub>	vasicinol
<b>4</b>	P	203.0819	3.57	202.075	1.946	C <sub>11</sub> H <sub>10</sub> N <sub>2</sub> O <sub>2</sub>	vasicinone
<b>5</b>	P	373.1664	6.41	374.174	1.952	C <sub>21</sub> H <sub>26</sub> O <sub>6</sub>	9,9'-demethylsecolintetralin
<b>6</b>	N	389.1970	10.04	390.204	0.101	C <sub>22</sub> H <sub>30</sub> O <sub>6</sub>	dimethylsecoisolariciresinol
<b>7</b>	P	439.2236	12.15	438.216	1.903	C <sub>25</sub> H <sub>30</sub> N <sub>2</sub> O <sub>5</sub>	hypercratine

**Table 3****Summary of the pharmacological activities of JF**

OT – oxytocin, IP<sub>3</sub> – inositol triphosphate, DAG – diacylglycerol, KCl – potassium chloride, VOCC- voltage operated calcium channel, NIF – nifedipine, PR – propranolol, ATB – atosiban, TEA – tetraethylammonium chloride, NM – neomycin, HEP – heparin, RyR – ryanodine receptor

<b>Drug/Activity</b>	<b>Pharmacological mechanism of drug</b>	<b>Response observed in the presence of JF</b>	<b>Proposed mechanism of JF</b>
<b>OT</b>	Oxytocin receptor agonist, activates Ca <sup>2+</sup> mobilization:	Inhibition of OT-induced phasic contraction	Antagonizes OT receptors or inhibits Ca <sup>2+</sup> mobilization
<b>High KCl</b>	Opens extracellular VOCCs	Inhibition of high KCl-induced tonic contractions	Inhibits opening of VOCCs
<b>OT in Ca<sup>2+</sup>-free media</b>	Assessment of Ca <sup>2+</sup> -release from intracellular stores	Inhibition of OT in Ca <sup>2+</sup> -free media	Inhibits Ca <sup>2+</sup> from intracellular stores
<b>NIF</b>	Calcium channel blocker	Augmented inhibitory effect of JF	Inhibition of VOCCs
<b>PR</b>	β-adrenoceptor antagonist	No effect on inhibitory effect of JF	Does not act through β-adrenoceptors in the uterus
<b>ATB</b>	OT receptor antagonist	No effect on inhibitory effect of JF	Does not act through OT receptors
<b>TEA</b>	Non-selective potassium channel blocker	No effect on inhibitory effect of JF	Does not act through potassium channels
<b>NM</b>	ryanodine receptor (RyR) blocker	Attenuated the inhibitory effect of JF	JF antagonizes RyR located in the sarcoplasmic reticulum
<b>HEP</b>	IP <sub>3</sub> receptor blocker	Attenuated the inhibitory effect of JF	JF antagonizes IP <sub>3</sub> receptor located in the sarcoplasmic reticulum

## **Figure captions**

**Figure 1. Effect of JF on spontaneous contractions in the non-pregnant uterus.** (A) Original representative recording showing the effect of JF on spontaneous contractions of non-pregnant uterus; Concentration response curves showing the inhibitory effect of JF on amplitude (B) and frequency (C). n = 5 animals.

**Figure 2. Effect of JF on spontaneous contractions in the pregnant uterus.** (A) Original representative recording showing the effect of JF on spontaneous contractions of the pregnant uterus; Concentration response curves showing the inhibitory effect of JF on amplitude (B) and frequency (C). n = 5 animals.

**Figure 3. Effect of JF on oxytocin-induced contractions in the non-pregnant uterus.** (A) Original representative recording showing the effect of JF on oxytocin-induced contractions of the non-pregnant uterus; Concentration response curves showing the inhibitory effect of JF on amplitude (B) and frequency (C). n = 5 animals.

**Figure 4. Effect of JF on oxytocin-induced contractions in the pregnant uterus.** (A) Original representative recording showing the effect of JF on oxytocin-induced contractions of the pregnant uterus; Bar graphs showing the inhibitory effect of JF on amplitude (B) and frequency (C). n = 5 animals; \*\*\*p<0.001 compared to oxytocin alone; OT = oxytocin.

**Figure 5. Effect of JF on high KCl-induced contractions (80 mM) in the non-pregnant uterus.** (A) Original representative recording showing the effect of JF on KCl-induced contractions of the non-pregnant uterus; Bar graphs showing the inhibitory effect of JF on amplitude (B) of contraction. n = 5 animals; \*\*p<0.01 compared to KCl alone; KCl = potassium chloride.

**Figure 6. Effect of JF on oxytocin-induced contractions in Ca<sup>2+</sup>- free medium in the non-pregnant uterus.** (A) Original representative recording showing the effect of JF on OT-induced contractions in Ca<sup>2+</sup>-free medium; Bar graphs showing the inhibitory effect of JF on amplitude (B) and frequency (C) of contractions. n = 5 animals; \*\*p<0.01 compared to OT alone; OT = oxytocin.

**Figure 7. Effect of some antagonists and blockers on JF-mediated inhibition of uterine contractility in the non-pregnant uterus.** HEP produced the greatest attenuation of JF-induced inhibition of the amplitude of contraction (A) while NM, TEA and HEP in decreasing order of potency showed greater potentiation of the frequency increase induced in the presence of JF. NIF potentiated both the frequency and amplitude of JF-induced inhibition. n=6 animals; \*\*p<0.01 inhibition compared to JF alone; #p<0.05 attenuation compared to JF alone. NIF = nifedipine; PR= propranolol; ATB = atosiban; TEA = tetraethylammonium chloride; NM = neomycin; HEP = heparin.

**Figure 8. Total ion chromatogram for JF in positive ionisation modes showing identified metabolites (1-7).** The identification, molecular formula, molecular weight, mass to charge ratio (m/z) and retention time in min (RT) are indicated in table 1..

**Figure 9. Chemical structures of compounds identified in JF through LC-HRFTMS.** Prostalidin D (**1**), justiciamide (**2**), vasicinol (**3**), vasicinone (**4**), 9,9'-demethylsecolintetralin (**5**), dimethylsecoisolariciresinol (**6**), and hypercratine (**7**).

**Figure 10. Schematic diagram showing the proposed mechanism of JF on the myometrium.** JF specifically inhibits IP<sub>3</sub>R and RyR thereby inhibiting Ca<sup>2+</sup>-release from the sarcoplasmic reticulum. This process inhibits the downstream signalling involved when IP<sub>3</sub>R and RyR are activated to promote myometrium contraction. JF also inhibits release of Ca<sup>2+</sup>-from extracellular channels which are activated in the presence of high KCl and during spontaneous uterine contraction when PGRs are activated. This process leads to generalized inhibition of myometrial contraction.

-ve = inhibition, +ve = stimulation, OTR = oxytocin receptor, IP<sub>3</sub>R = inositol triphosphate receptor, RyR = ryanodine receptor, PGR = prostaglandin receptor, Blue X sign = inhibition of the pathway, PMCA = plasma membrane Ca<sup>2+</sup>-ATPase.