Journal of Medicinal Plants for Economic Development
ISSN: (Print) 2519-559X

Dryopteris filix-mas (Dryopteridaceae) leaves inhibit mouse uterine activity

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Dates:
Received: 20 May 2017
Accepted: 03 Sept. 2017
Published: 25 Oct. 2017

How to cite this article:
Bafor, E.E., Omokaro, W.O., Uwumarongie, O.H., Elvis-Offiah, U.B., Omoruyi, O., Viegelmann, C.V. et al., 2017, '*Dryopteris filix-mas* (Dryopteridaceae) leaves inhibit mouse uterine activity', *Journal of Medicinal Plants for Economic Development* 1(1), a25. https://doi.org/10.4102/jomped.v1i1.25

Background: The plant *Dryopteris filix-mas* has been used traditionally for its uterine-stimulant effects.

Aim: The current study is therefore aimed at investigating and determining the effect of the leaves of *D. filix-mas* on uterine contractility *in vitro*.

Setting: Fresh leaves of *D. filix-mas* were collected from a river bank in the south-western part of Nigeria.

Methods: The leaves of *D. filix-mas* were cleaned, dried and extracted in methanol. The extract (0.07 µg/mL–21.0 µg/mL) was tested on the isolated mouse uteri in order to determine activity on spontaneous-induced uterine contractions. Subsequently the extract (0.005 mg/mL and 0.05 mg/mL) was tested on oxytocin-induced contraction (0.00017 ng/mL–4.98 ng/mL) in calcium-containing media, submaximal oxytocin-induced contraction (0.116 ng/mL) in calcium-free media and in the presence of high KCl-induced uterine contractions (80 mM). The extract was also subjected to mass spectrometric determination of secondary metabolites.

Results: The plant extract inhibited spontaneous-induced contractions with IC_{50} amplitude = 658.41 ng/mL ± 0.11 ng/mL and IC_{50} frequency = 175.32 ng/mL ± 0.53 ng/mL. The plant extract inhibited oxytocin-induced and high KCl-induced uterine contractions ($p < 0.01$ at 0.5 mg/mL). The plant extract had no effect on oxytocin-induced contractions under calcium-free conditions. Secondary metabolites belonging to classes of fatty acids, alkaloids, saponin glycosides, amino acids, limonoids, terpenes and porphyrins were identified.

Conclusion: The current study reports an inhibitory effect of the plant on uterine contractility in this study, suggesting possible application as a tocolytic or as a contraceptive, as most contraceptive plants have shown uterine-relaxing effect.

Introduction

The myometrium is a myogenic organ which generates regular spontaneous contractions by its own mechanisms without any input from the hormonal or nervous systems (Wray 1993). The contraction of the myometrium also exhibits phasic properties accompanied with variations in the frequency, amplitude and duration of contraction. All of these properties enable the myometrium to perform its physiological function adequately and efficiently. These properties are also very often examined individually so as to provide a more holistic insight into the action and activity of the myometrium. Uterine contractions (or myometrium contractions) are activated on increases in intracellular calcium concentrations ($[Ca^{2+}]_i$), which are initiated and regulated by action potentials in the myometrium (Burdyga, Wray & Noble 2007). The myometrium (uterine smooth muscles) acts to prepare the uterus for the processes of maintaining and expelling the foetus (Wray 2007). For instance, the myometrium undergoes significant changes in the non-pregnant uterus, which allows for the successful implantation of the fertilised embryo (Wray 2007). In the non-pregnant uterus, the myometrium is also responsible for the contractions that occur during menstruation (primates) or oestrous (mammals), which potentiates the cramping observed often referred to as dysmenorrhoea (Togashi 2007). In this state, the myometrium is involved in a uterine peristaltic action that supports and contributes to the endometrial sloughing that occurs during menstruation (Bulletti et al. 2000). Changes in female steroid hormones released during this time also act to regulate the sequence of myometrial activity (Wray & Noble 2008). For purposes of this study, it is important to emphasise that the pattern of contractile activity in the non-pregnant uterus is similar to uterine contraction in the pregnant uterus as well (Lyons et al. 1991). Contractions occurring in antegrade manner and propagating from the fundus towards the cervical end of the uterus are necessary for emptying or discharge of uterine content,

which may be the menstrual blood (non-pregnant uterus) or foetus (pregnant uterus) (Lyons et al. 1991). The cervico-fundal contractions also assist in electrolyte retention as well as sperm transport (Kunz & Leyendecker 2002). Retrograde contractions in the myometrium during pregnancy may contribute to the maintenance of early pregnancies within the uterine cavity (de Vries et al. 1990).

From the foregoing, it is clear that uterine contractility constitutes an important parameter of female reproductive health.

Maternal morbidity and mortality arising from female reproductive health disorders are a serious concern. Women in Africa bear a disproportionately large share of the global burden of disease and death, particularly in maternal morbidity and mortality. Africa as a whole accounts for more than half of all cases of maternal deaths worldwide, and African women have a 1 in 42 lifetime risk of dying during childbirth compared with 1 in 2900 in Europe. Understanding the roles of drugs or natural products in uterine contractility therefore will assist in providing useful information going forward in the improvement of female reproductive health in general. For many women, current therapies offer inadequate treatment, and this includes management of a range of health issues from dysmenorrhoea to complications of pregnancy (Marsden, Strickland & Clements 2004) and the search for new treatment options continues. Interests in medicines from natural products (plants inclusive) have soared through the years. Plant preparations in the forms of decoctions, concoctions, macerations or infusions are used to treat a wide range of diseases. Use of plant medicines has also been utilised to tackle human reproductive health issues, which as stated earlier remains a public health concern worldwide. Use of medicinal plants has therefore become a mainstay in several cultures, including Africa (Dugoua 2010; Fakeye, Adisa & Musa 2009). This therefore indicates that research into plants hold a strong potential in the elaboration of new therapies to tackle reproductive health issues. One such plant that has been reported to have effect on some reproductive concerns is the plant, *Dryopteris filix-mas*.

Dryopteris filix-mas is a hardy ornamental fern (Duke 2001). It is considered a traditional wild vegetable (Mohammed Abdus Satter 2016). It is commonly referred to as 'wild fern', or 'bear's paw' and botanically described as *Dryopteris filix-mas* (Linn.) Schott, and the family name is Dryopteridaceae (Nwosu 2002). The local Nigerian names are '*Ihi*' and '*Erinji*' (Nwosu 2002). It grows in a wide range of habitats such as open ground, but is commonly found in moist environs and deciduous forests. The plant grows in all parts of Europe, temperate Asia, North India, North and South America, the temperate parts of the USA and also grows in Africa (Uwumarongie, Enike & Bafor 2016). The plant is highly adaptable and can grow well in both arid and fertile soils (Duke 2001). It favours damp shady areas in the understory of woodlands but also shady places in hedge-banks and rocks. The plant is sometimes referred to in ancient literature as Worm Fern because it has been used in traditional medicine for the treatment of worm infections (Mohammed Abdus Satter et al. 2016). There is however some documented information on the biological or medicinal activities of *D. filix-mas* with most reports focused on ferns as a class. Some of the available reports are described here. The early physician, Theophrastus, recognised the value of the fern for treating tinea (ringworm) infections (Duke 2001). Male Fern has also sometimes been used as a tonic and vulnerary in China; it is used for wounds and haemorrhages, such as epistaxis, menorrhagia and postpartum haemorrhage (a reproductive complication). The plant has the potential to arrest embryonic development in insects (suggestive of a potential reproductive function) (Snehlata & Tiwari 2011). The plant has been reported to have uterine stimulant effects traditionally and has been advised to be avoided during breastfeeding as the plant contains anthraquinones, which may induce diarrhoea and colic in infants (Cock 2015). The plant has also been reported to increase the size of the male reproductive organ through mechanisms yet unknown (Kantemir, Akder & Tulunay 1976); however, this action may be suggestive of an oestrogenic or androgenic effect. The young coiled fronds of the plant are used in different parts of Nigeria as an antihelminthic, and infusion of the leaves is additionally used as an aphrodisiac (Nwosu 2002). The plant has also been reported to have potent antioxidant and cytotoxic activities (Sekendar Ali et al. 2012), insecticidal activity (Shukla & Tiwari 2011) and antimicrobial activities (22). The plant has been reported to contain a high concentration of K^+ (1065.45 ± 1.13 mg/100 g) and reasonable concentrations of Ca^{2+} (279.16 ± 1.33 mg/100 g), Mg^{2+} (148.50 ± 0.65 mg/100 g) and Na^+ (94.44 ± 0.66 mg/100 g) (Mohammed Abdus Satter et al. 2016).

Based on its reported use in postpartum haemorrhage and uterine stimulation, this study sets out to verify this use and examines some parameters on possible mechanisms of activity. The study is therefore aimed at investigating the activity of the plant extract on uterine contractility, investigating preliminary mechanisms of activity and determining secondary metabolites contained in the leaf, which can serve as a basis for further drug discovery studies.

Materials and method

Plant material

Fresh leaves of *D. filix-mas* (hereafter DF) were collected between July and August, 2015, from the river bank of Osun River at Osogbo, Osun State, Nigeria. It was authenticated by Dr H.A. Akinnibosun from the Department of Botany, Faculty of Life Sciences, University of Benin, Edo State, Nigeria. A voucher specimen has been prepared with voucher number of UBN/PCG/1658 and deposited at the Department of Pharmacognosy, University of Benin, Nigeria.

Animals

Mature non-pregnant female albino mice weighing an average of 24 ± 0.66 g were obtained from the Animal House Department of Pharmacology and Toxicology, Faculty of

Pharmacy, University of Benin, Edo State, Nigeria. They were housed in plastic cages at an environmentally controlled room temperature of approximately 27 ± 5°C and environmentally controlled lighting conditions of approximately 11 h/13 h light and dark cycles. Relative humidity ranged from 85% to 88%. The animals were acclimatised to these conditions. Handling was done as much as possible according to standards of the Public Health Service policy on humane care and use of Laboratory Animals (Office of Laboratory Animal Welfare & NIH 2015; National Research Council 2010). The animals were maintained on standard diet of animal pellets and clean tap water.

Drugs and chemicals

Methanol of high analytical grade (Pharmatrends, Nigeria) and tween 80 (Sigma Aldrich, UK) were solvents used in this study. Salts for the physiological solution were obtained from BDH chemicals, England. Other drugs used in this study include oxytocin (Laborate Pharmaceuticals, India) and diethylstilboesterol (Sigma Aldrich, UK). For the mass spectrometric experiments, methanol (MeOH), dichloromethane (DCM), acetonitrile (MeCN) and formic acid were purchased (Fisher Scientific, Hemel Hempstead, UK). All reagents were of analytical grade.

Preparation of extract

The plant material was air-dried for 5 days followed by controlled oven-drying at 40 °C after which it was reduced to powder form by using an electric milling machine (Christy Norris, England). The powdered samples were kept in airtight containers and stored in the refrigerator at 4 °C until needed. Five hundred grams (500 g) of the powdered plant material was macerated in 2.5 L methanol, and the extract obtained was concentrated under pressure by using a rotary evaporator maintained at 40 °C. The extract weight was 98.65 g giving a yield of 5.07 %w/w.

Contractility studies

Uterine tissue preparation

Twenty-four hours prior to the day of experiments, each mouse was administered 1.0 mg/kg diethylstilbestrol orally by using a feeding syringe (Bafor, Omogbai & Ozolua 2010). This dose and route of administration had been previously determined in our laboratory to effectively induce oestrous. Diethylstilbestrol was constituted in Tween 80 and distilled water (1:1). On the day of the experiment prior to the experiment proper, vaginal smears were obtained and prepared (Caligioni 2009; Cora, Kooistra & Travlos 2015). Briefly the smears were collected by flushing with distilled water by using a Pasteur pipette (0.1 mm). A smear was made on a clean glass slide, fixed with ethanol and stained with a drop of gentian violet. The smear was then viewed under a microscope. Once the stage of oestrous was ascertained, the mouse was then humanely sacrificed. Animals in pro-oestrous and oestrous states (Figure 1) were used for the experiments (Bafor et al. 2014). The selected mouse was humanely killed by cervical dislocation and the uterine horns were immediately excised and immediately placed into a petri dish containing previously warmed and aerated physiological salt solution. The uterine tissues were cleaned of connective tissues and one horn was transected medially in half. Tissue lengths of approximately 1–2 mm each were obtained. The uterine segment was then mounted in a warmed 10-mL organ bath maintained at 37 °C and containing aerated physiological solution. The physiological salt solution used was of the following composition in M: NaCl 154.00, $NaHCO_3$ 5.95, D-glucose 2.78, KCl 5.63 and $CaCl_2 \cdot 2H_2O$ 2.05 (Bafor et al. 2014).

Experimental protocol

The mounted tissues were equilibrated under resting tension of 4.90 mN for 30–45 min (time duration used was dependent on when regular contractions were obtained) (Bafor et al. 2014; Sukwan, Wray & Kupittayanant 2014). The force and frequency of uterine contractions in the longitudinal muscle layers were measured by using a 7003E-isometric force

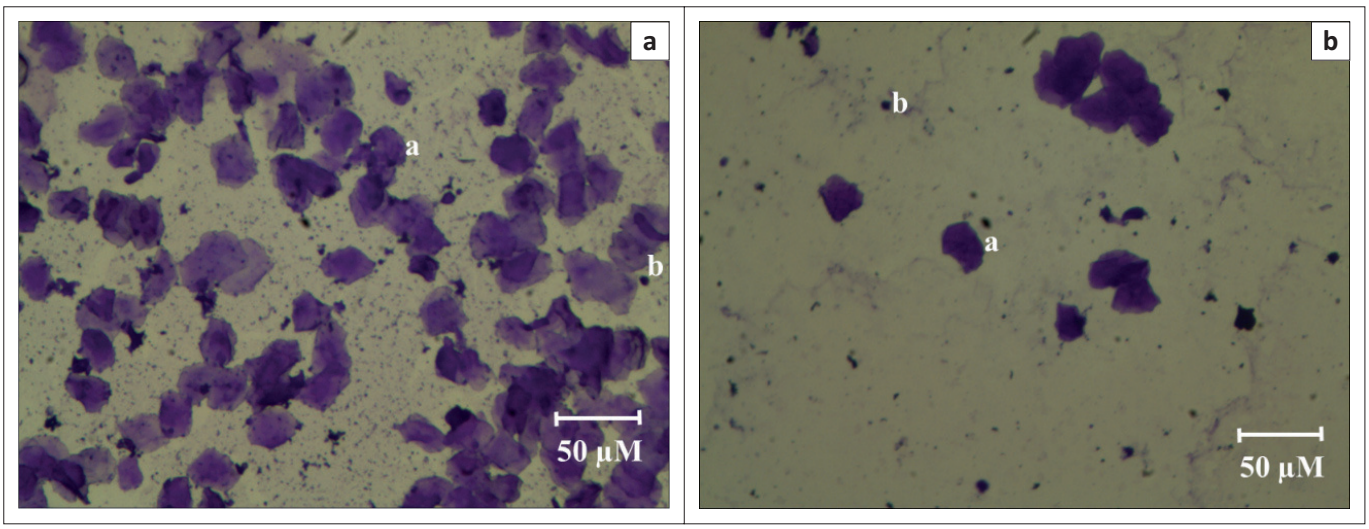

FIGURE 1: Representative ×100 images of gentian violet-stained exfoliative vaginal cytology from animals used in this study corresponding to: (a) oestrus with mainly anucleated cornified epithelial cells (a), (b) late pro-oestrus with nucleated cells undergoing cornification (a) and few lymphocytes (b).

transducer (Ugo Basile, Varise, Italy) connected to a 17 400 data capsule digital recorder with an inbuilt bridge amplifier (Ugo Basile, Varese, Italy).

Experiment on the effect of extract on spontaneous uterine contraction: The direct effect of cumulative concentrations of the extract on uterine smooth muscle contractility was investigated (Bafor et al. 2014, 2017; Elvis-offiah, Iyawe & Bafor 2016; Sukwan, Wray & Kupittayanant 2014). Concentration–response relationships were obtained by using concentrations between 0.07 µg/mL and 21.0 µg/mL. The concentration used had been predetermined in our laboratory as concentrations covering the range of effects of the extract. A contact time of 3 min was allowed following each concentration of extract administered. After each set of administration, the tissues were washed three times and a wash-out over a minimum period of 10 min was allowed for the tissue to recover before the next administration.

Experiment on the effect of extract on oxytocin-induced uterine contraction: Conversion of oxytocin from international units (IU) to milligram (mg) was in accordance with the World Health Organization recommendation where 10 IU = 16.6 µg/mL (World Health Organization 2010). After tissue equilibration, the effect of the extract on oxytocin-induced uterine contraction was investigated. This was carried out by first performing a concentration–response to oxytocin (0.00017 ng/mL–4.98 ng/mL) in the absence of the extract. This was then repeated in the presence of cumulative sub-maximum concentrations of the extract at 0.005 and 0.05 mg/mL at 5 min each.

Experiment on the effect on high KCl-induced uterine contractility: The effect of the extract was determined in the presence of high KCl (80 mM). KCl (80 mM) was added to the bath containing the uterine tissues and left in contact for 5 min and without washing cumulative concentrations of the extract (0.0005 mg/mL–0.5 mg/mL) were determined.

Experiment on the effect of the extract in Ca^{2+}-free medium: After tissue equilibration for 30 min, the physiological salt solution was changed to one without calcium and containing 0.1 mM of ethylenediaminetetraacetic acid. The tissue was then equilibrated in the Ca^{2+}-free solution for a further 15 min. After equilibration, oxytocin (0.116 ng/mL) was added and a contact time of 5 min was allowed. Without flushing, cumulative concentrations of the extract (0.07 µg/mL–210.00 µg/mL) were added. A contact time of 3 min was allowed for each extract concentration.

Liquid chromatography-high resolution Fourier Transform mass spectrometry identification of constituents in extract

Samples and medium control samples were prepared at a concentration of 1 mg/mL in 80:20 MeOH:DCM. A solvent blank was also included. Liquid chromatography-high resolution Fourier Transform mass spectrometry (LC-HRFTMS) analysis was performed on a Dionex UltiMate-3000 (DIONEX, Sunnyvale, CA, USA) coupled to a Thermo Scientific Exactive Orbitrap system (Thermo Fisher Scientific [Bremen], GmbH, Bremen, Germany). The mass accuracy was set to less than 3.0 ppm. The instrument was externally calibrated according to the manufacturer's instructions before the run. The column used was an ACE 5 C18 75 mm × 3.0 mm column from Hichrom Ltd., Reading, UK. Ten micro-litres of the sample was injected from the vial, and compounds were eluted with a flow rate of 300 µL/min by using water (A) and acetonitrile (B), both of which contained 0.1% formic acid, by a gradient starting with 10% B and increasing to 100% B in 30 min. The mobile phase was maintained at 100% B for 5 min after which the column was equilibrated with 10% B. The UV absorption wavelength was set at 254 nm, the sample tray temperature was maintained at 4 °C and the column was maintained at 20 °C. The files were sliced into positive and negative data sets by using ProteoWizard (Kessner et al. 2008) prior to data mining by using MZmine 2.10 (Pluskal et al. 2010). Peak detection was accomplished by using the centroid mass detector and a noise level of 1000. The chromatogram builder generated peak lists from the mass lists obtained from the previous step. The minimum time span was 0.2 min, minimum height was 10 000, and the *m/z* tolerance was set to 0.0001 *m/z* or 5 ppm. Chromatogram deconvolution was accomplished by using the local minimum search algorithm with the following parameters: threshold (90%), search minimum in retention time (RT) range (0.4 min), minimum relative height (5%), minimum absolute height (10 000), minimum ratio of peak top/edge (Burdyga et al. 2007) and peak duration range (0.2–5.0 min). The peak lists were de-isotoped by using the isotopic peaks grouper with an *m/z* tolerance of 0.001 *m/z* or 5 ppm, RT tolerance of 0.1 min (absolute) and maximum charge of 2. The representative isotope was the most intense. The peak lists were then merged by using the Alignment function. The weight for *m*/z and for RT was 20, and the RT tolerance was 5%. The aligned peak lists were gap-filled by using the Peak Finder, with an intensity tolerance of 1% and RT tolerance of 0.5 min (absolute). Adducts were identified, together with other complexes that may have formed during ionisation. The chemical formulae of each peak were predicted by using the formula prediction tool developed by MZmine. Hits from the database were accessed by using ChemBioFinder version 13 (PerkinElmer Informatics, Cambridge, UK).

Data analysis

The mean frequency and amplitude were calculated from contractions occurring at the last 3 min of the phasic contractions by using the GraphPad Prism (version 7.03; GraphPad software Inc., San Diego, CA, USA). Results were obtained as percentages of control applications (control = 100%). In some experiments, changes in force (amplitude) were expressed with respect to basal resting force level (100% amplitude). All data shown were expressed as mean ± standard error of mean and n represents the number of samples each from different animal, and in this study $n = 5$ animals. Significance was evaluated by using appropriate

t-tests, and where necessary, one-way analysis of variance with Dunnett's *post hoc* and p values ≤ 0.05 was taken to represent minimum significance in all cases.

In datasets with sufficient data points, mean log concentration–response curves were analysed by fitting data to a four-parameter logistic equation, by using the following equation values (Y = Bottom)/(1 + 10^[(LogIC50-X)×HillSlope)], where Y = response which starts at the bottom and goes to the top in sigmoid shape, X = logarithm of concentration and IC_{50} is the concentration that produces half the maximal responses.

Ethical considerations

All animal experiments were performed according to approved guidelines for Humane Care and Use of Laboratory Animals and after prior approval of the institutional animal ethics committee, which is also stated within the article.

Ethical approval was obtained from the Ethical Committee, Faculty of Pharmacy, University of Benin, and an approval number was provided, EC/FP/016/04.

Results

Vaginal cytology of the experimental animals

Representative exfoliative vaginal cytology for the stage of the oestrous cycle of animals utilised for this study is shown in Figure 1. The relative prevalence of the cell type was used to ascertain the oestrous state for each recipient mouse. Typically animals in the oestrous and pro-oestrous stages were used for this study.

Effect of extract on spontaneous uterine contractions

Rhythmic contractions occurred in about 90% of uterine tissue preparations containing both longitudinal and circular smooth muscle in this study, which were subsequently utilised for studies on spontaneous uterine contractility. Cumulative concentration–response experiments were performed to determine the effects of the extracts on the force and frequency of spontaneous uterine contractions. Response curves were determined for DF (n = 5). DF extract was observed to induce mild inhibitions in the amplitude and frequency of spontaneous contractions at concentrations used in this study (Figure 2). Inhibitions were more pronounced at higher concentrations particularly at 210.00 µg/mL (Figure 2). Concentration producing 50% inhibition of responses (IC_{50}) were extrapolated to give IC_{50} amplitude = 658.41 ng/mL ± 0.11 ng/mL and IC_{50} frequency = 175.32 ng/mL ± 0.53 ng/mL.

Effect of extract on oxytocin-induced uterine contractions

Experiments were performed to characterise the activities of DF on oxytocin-induced uterine contractions. The DF extract was observed to decrease the response of oxytocin-induced uterine contraction (Figure 3). The effect on the amplitude (force) of oxytocin was more pronounced than the effect seen on the frequency, which had more variability (Figure 3). DF was observed to decrease both the amplitude and frequency of oxytocin-induced contraction in a concentration-dependent manner (Figure 4). A rightward shift was observed in the response to oxytocin at the concentrations of DF used in this study (Figure 4). DF effectively depressed the oxytocin's action on uterine contraction at both concentrations utilised in this study.

Effect of *D. filix-mas* on high KCl-induced (80 mM) uterine contractions

Characterisation of the actions of DF to high K^+ solution was performed. The possibility of interaction of DF with extracellular calcium channels involved in high K^+ depolarisation of smooth muscles (Triggle 1987) was investigated. As shown in Figure 5, high KCl solution produced a rapid increase in force, which was immediately preceded by a decline and rapidly oscillating production of force (Crichton et al. 1993). Application of DF extract altered this activity at the highest concentration used for this protocol (Figure 5). The DF extract was observed to decrease significantly ($p < 0.01$) the contractile force (amplitude) of KCl-induced contractions at 0.5 mg/mL used (Figure 5).

Effect of *D. filix-mas* in Ca^{2+}-free medium

Further experiments were designed to investigate whether DF extract could inhibit internal Ca mobilisation from the sarcoplasmic reticulum (Arrowsmith & Wray 2014; Wray & Arrowsmith 2010). To do so, internal Ca mobilisation was blocked by utilisation of a Ca-free medium. In the absence of Ca^{2+}, DF (0.07 µg/mL–210.00 µg/mL) was observed to have no significant effect on oxytocin-induced contractions (Figure 6). However, mild inhibitions were observed at the highest concentration of DF utilised (Figure 6).

Identified secondary metabolites in *D. filix-mas*

LC-HRFTMS was utilised to identify putative metabolites involved in the activity of DF on uterine contractility, which can be investigated further. The LC-HRFTMS results and database search (by using Dictionary of Natural Products) enabled the detection of 18 significant compounds (Tables 1 and 2), 10 of which were identified (Table 1) and 8 were unknown (Table 2). The identified compounds included, 4-acetyl-2,4-octadienoic acid (1), aurantiamide (2), trillenogenin (3), asperphenamate (4), 10,15-cyclo-11,14-dihydroxy-1,2-dinor-6-phyten-3-one (5), detigloyl-6-deoxy-2-hydroxyswietenine (6), 24(23→22)-abeo-16,23-epoxy-3,23-dihydroxycholesta-5,24-dien-18-al (7), chlorocruoroporphyrin (8), 17-hydroxyingenol (9) and phylloerythrin (10) (Figure 7). The identified compounds were observed to belong to a diverse range of phytochemical classes including fatty acids, phenols, terpenoids and tetrapyrroles.

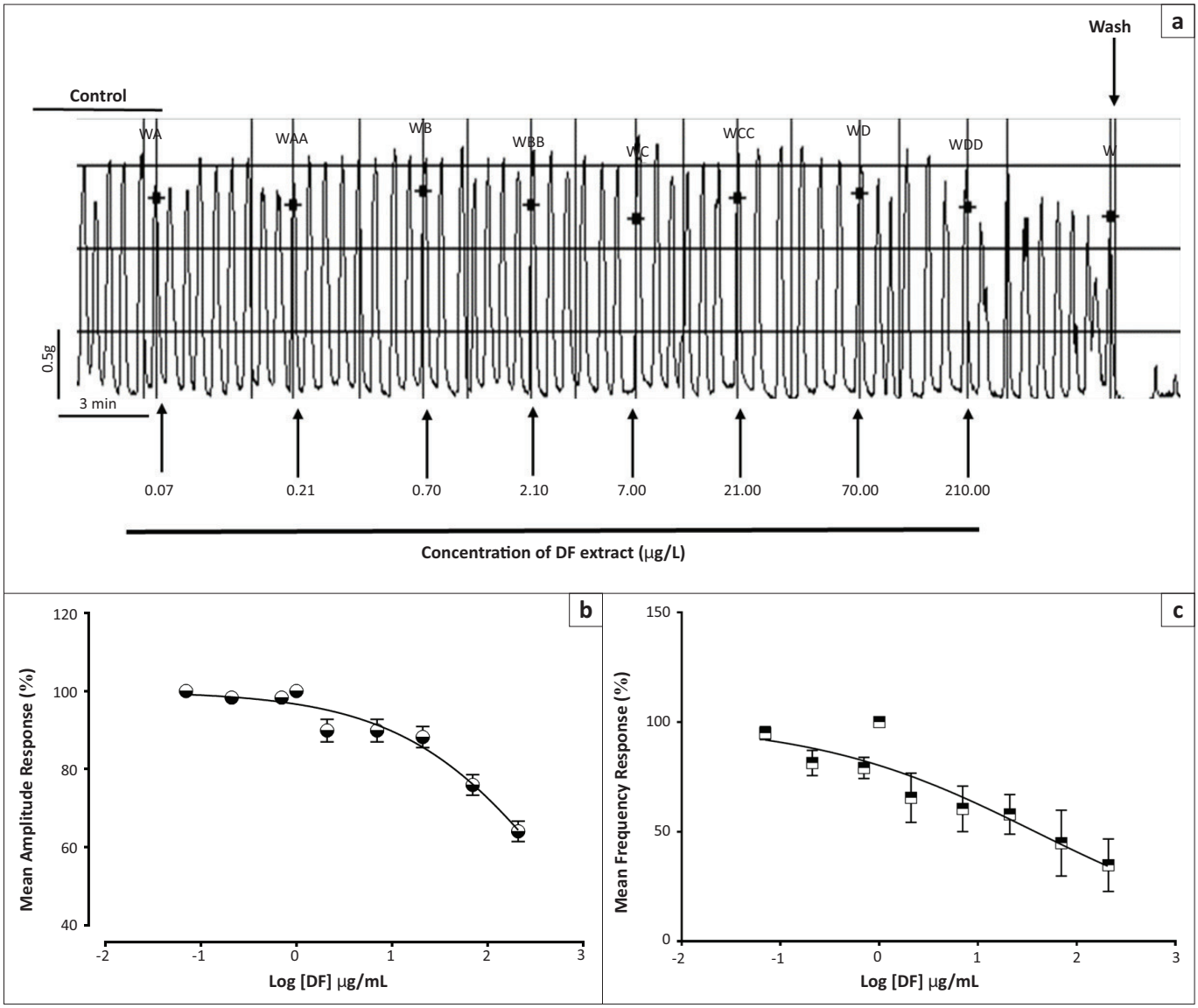

FIGURE 2: Effect of *D. filix-mas* (DF) on spontaneous uterine contraction. (a) Original recording showing the effect of DF on spontaneous contractility of the isolated mouse uterus. (b) Concentration–response curves showing the effect of DF on the amplitude of spontaneous uterine contractions. (c) Concentration–response curves showing the effect of DF on the frequency of spontaneous uterine contractions. A concentration-dependent inhibition for both the amplitude and frequency was observed. $n = 5$ animals.

Discussion

The myometrium (smooth muscle layer of the uterus) is active throughout life and not restricted to periods of labour and delivery. Uterine contraction therefore occurs throughout the menstrual cycle in non-pregnant states as well as the pregnant states, in a complex and dynamic physiological phenomenon (Aguilar & Mitchell 2010). Some of the unwanted but frequently observed results of myometrial dysfunction are contractions that are not timed leading to abortions or preterm delivery, or stronger than necessary contractions causing foetal distress, hypoxia and even death of the foetus (Aguilar & Mitchell 2010; Wray 2007). The non-pregnant myometrium exhibits contractions at different phases of the menstrual cycle; these include rhythmic, 'wave-like' contractions, oftentimes referred to as uterine peristalsis or spontaneous contractions, and the 'focal and sporadic bulging of the myometrium' (Togashi 2007; Togashi et al. 1993), leading to sustained contractions. These contractions are important in endometrial sloughing (Wray & Noble 2008) and assist in sperm passage (Pehlivanoğlu, Bayrak & Doğan 2013). In late-term pregnancy, the myometrium contracts via similar mechanisms as occurs in the non-pregnant uterus with differences in morphology and concentrations of circulating hormones to systematically ensure successful expulsion of the foetus in the absence of any abnormalities (Pehlivanoğlu et al. 2013). Therefore, regardless of the presence or absence of pregnancy, uterine contractions are dependent on the contractile activity of the cellular elements, the uterine myocytes. The uterine myocytes are cells of the myometrium, which often exhibits a phasic contractile pattern such that the resting tone of the uterus is maintained. This resting tone is often superimposed by separate intermittent set of contractions with varying frequency, amplitude and duration. It is predominantly regulated by intracellular calcium concentration ($[Ca^{2+}]_i$) (Aguilar & Mitchell 2010; Wray 2007). DF was found in this study to inhibit this spontaneous intrinsic uterine contraction suggesting possible inhibition of the force and frequency of

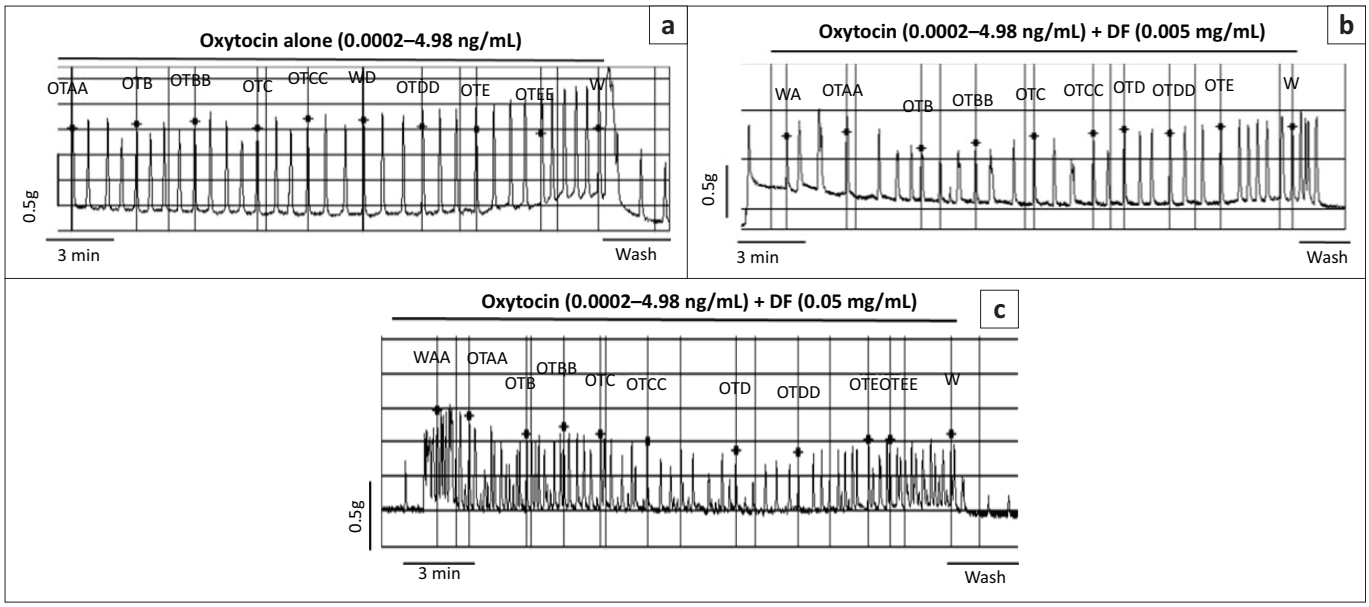

FIGURE 3: Original recordings showing the effect of *D. filix-mas* (DF) on oxytocin-induced uterine contraction. (a) Original recording oxytocin (0.0002 ng/mL–4.98 ng/mL) on uterine contractility of the isolated mouse uterus. (b) Original recording showing the effect of oxytocin (0.0002 ng/mL–4.98 ng/mL) in the presence of DF (0.005 mg/mL). (c) Original recording showing the effect of oxytocin (0.0002 ng/mL–4.98 ng/mL) in the presence of DF (0.05 mg/mL).

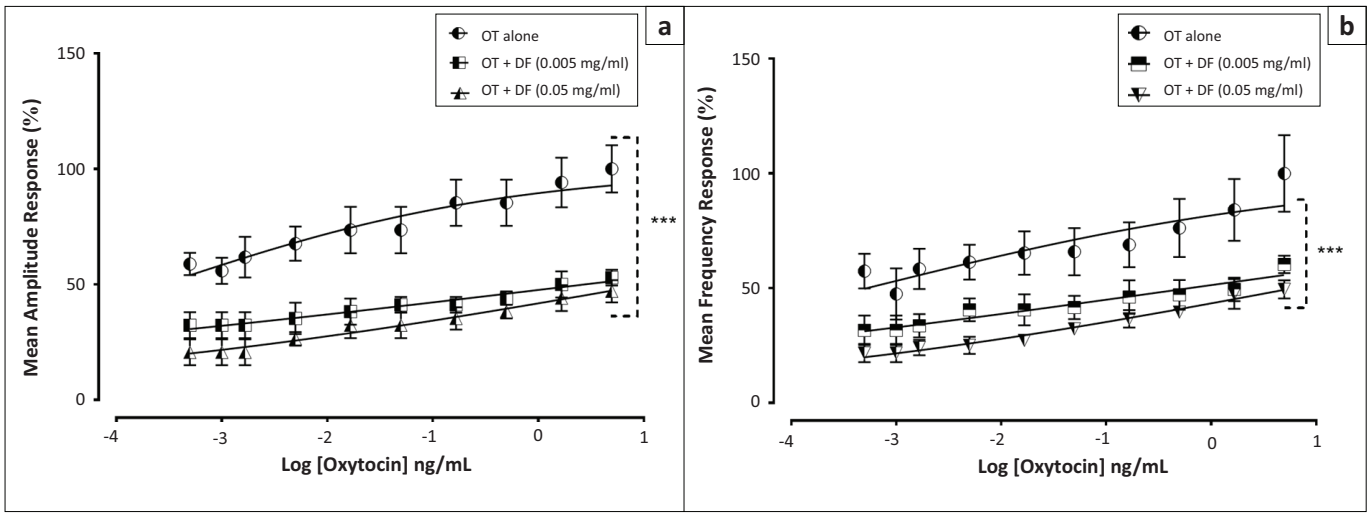

FIGURE 4: Concentration–response curves of oxytocin (0.0002 ng/mL–4.98 ng/mL) in the presence of *D. filix-mas* (DF). (a) Effect of DF (0.005 mg/mL and 0.05 mg/mL) on the amplitude of oxytocin-induced contractions. (b) Effect of DF (0.005 mg/mL and 0.05 mg/mL) on oxytocin-induced contractions. A concentration-dependent inhibition for both the amplitude and frequency was observed and a rightward shift in the concentration–response curves was observed. n = 5 animals.

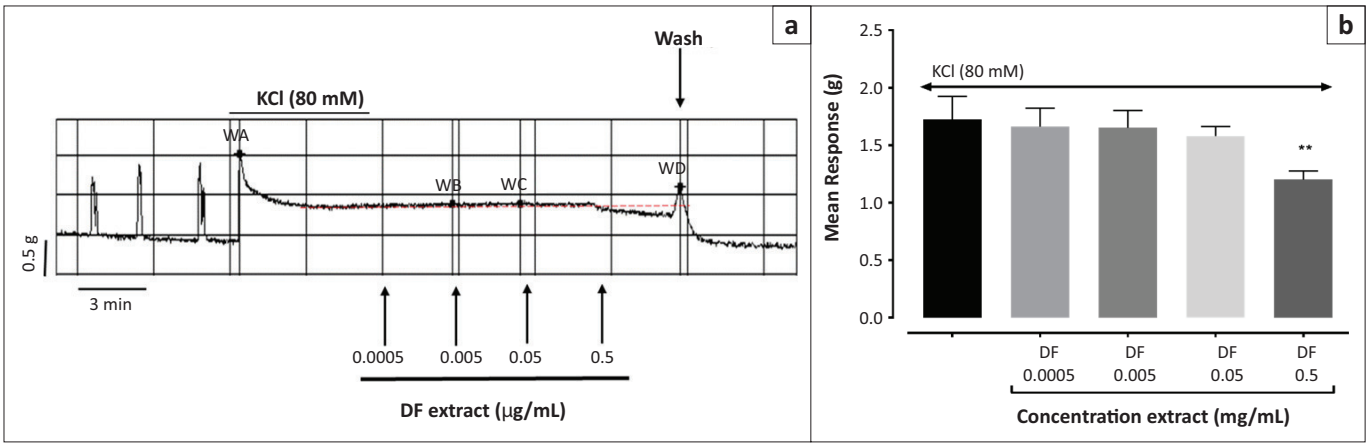

FIGURE 5: Effect of *D. filix-mas* (DF) on high KCl-induced (80 mM) uterine contraction. (a) Original recording of DF on high KCl-induced uterine contraction (80 mM). (b) Bar graphs showing the effect of DF (0.0005–0.5 mg/mL) on KCl-induced contraction. A significant depression (p < 0.01) was observed at the highest concentration of DF used in this study of $^{**}p$ < 0.01; n = 5 animals.

myocyte activity via inhibition of $[Ca^{2+}]_i$. An increase in $[Ca^{2+}]_i$ is reported to activate a calcium ion (Ca^{2+})-dependent cytosolic protein, calmodulin (CalM), which is capable of binding up to four Ca^{2+} ions (Johnson et al. 1996). Formation of the Ca^{2+}-CalM complex activates the enzyme myosin light chain kinase (MLCK) resulting in an increase in the phosphorylation of myosin regulatory light chain-20 (MLC20) and subsequent cross-bridge cycling (Shojo & Kaneko 2001). MLC20 phosphorylation by MLCK is the principal determinant of the amplitude and duration of contraction (Butler et al. 2013; McConnell & Wadzinski 2009). MLCK contains several phosphorylation target sites for protein kinase A, protein kinase C (PKC) and other kinases (Aguilar & Mitchell 2010; Wray et al. 2001) which may contribute to its activity. It may therefore seem that the inhibition of the amplitude of myometrial contraction markedly observed in this study may be attributed to the prevention of MLC20 phosphorylation by DF. Activation of MLCK by CalM translocation of activated MLCK towards the contractile apparatus may be the rate-limiting step of contraction (Wray et al. 2003) determining the contraction frequency of the myometrium. It would also seem that with the effect of DF being somewhat more pronounced on the amplitude than the frequency of spontaneous contractions, DF may exert less activity on MLCK activation and possibly a greater effect on prevention of MLC20 phosphorylation.

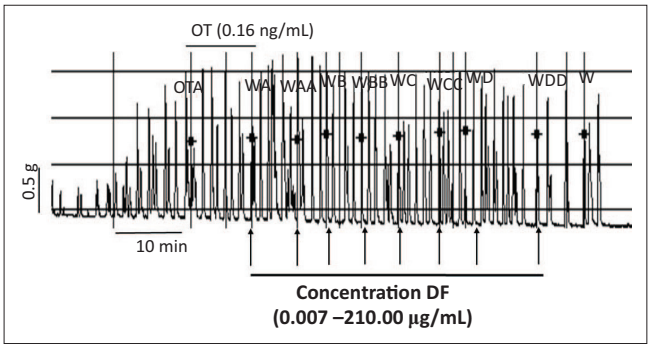

FIGURE 6: Original representative recording showing the effect of *D. filix-mas* (DF) on oxytocin-induced uterine contraction in calcium-deprived medium. DF appeared to have little effect on oxytocin when a calcium-free medium was used, although slight depressions were observed at the highest concentrations of DF used in this study. $n = 5$ animals.

This study additionally reports the inhibition of oxytocin-induced uterine contraction by DF. Oxytocin is known largely for its stimulatory actions on myometrial contraction (Pehlivanoğlu et al. 2013) where it is widely used to reinforce labour contractions. Oxytocin stimulates calcium entry and release from the sarcoplasmic reticulum. Coupling of oxytocin to its receptor activates phospholipase-Cβ, which hydrolyses phosphatidylinositol bisphosphate (PIP_2) releasing two second messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG) (Wray & Arrowsmith 2010). IP_3 activates $[Ca^{2+}]_i$ from the sarcoplasmic reticulum, which proceeds to open up more extracellular calcium channels, while DAG activates protein kinase C (Wray & Arrowsmith 2010). Oxytocin has also been reported to exert a stimulatory effect on $[Ca^{2+}]_i$ entry and release from the sarcoplasmic reticulum (Soloff & Sweet 1982) while also inhibiting $[Ca^{2+}]_i$ efflux, which may result in the inhibition of myosin light chain phosphate (Wray & Arrowsmith 2010). The net effect is a powerful enhancement of force and slowing relaxation which could be observed in this study. The effect of DF on oxytocin seen in this study therefore supports the

TABLE 1: Putatively identified compounds in *Dryopteris filix-mas*.

Serial Number	Compound name	Molecular formula	Molecular weight (g/mol)	*m/z*	RT (min)
1.	4-acetyl-2,4-octadienoic acid	$C_{11}H_{16}O_3$	196.1099	197.1172	6.64
2.	aurantiamide-O-acetyl	$C_{27}H_{28}N_2O_4$	444.2048	445.2121	16.79
3.	trillenogenin; 7β-hydroxy, 1-O-[β-D-apiofuranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside]	$C_{47}H_{70}O_{25}$	1034.4231	1035.4303	19.46
4.	asperphenamate	$C_{32}H_{30}N_2O_4$	506.2199	505.2126	19.47
5.	10,15-cyclo-11,14-dihydroxy-1,2-dinor-6-phyten-3-one	$C_{18}H_{32}O_3$	296.2345	295.2272	20.60
6.	detigloyl-6-deoxy-2-hydroxyswietenine	$C_{34}H_{42}O_{10}$	610.2792	611.2865	21.32
7.	24(23→22)-abeo-16,23-epoxy-3,23-dihydroxycholesta-5,24-dien-18-al	$C_{27}H_{40}O_4$	428.2923	429.2996	22.42
8.	chlorocruoroporphyrin; Di-Me ester	$C_{35}H_{36}N_4O_5$	592.2689	593.2762	29.33
9.	17-hydroxyingenol; 17-benzoyl, 3-angeloyl, 5-Ac	$C_{34}H_{40}O_9$	592.2689	593.2762	30.10
10.	phylloerythrin	$C_{33}H_{34}N_4O_3$	534.2635	535.2708	30.93

RT, retention time.

TABLE 2: Unidentified compounds in *Dryopteris filix-mas*. Double bond equivalence indicates number of rings and double bonds in the structure, where 1 ring = 1 double bond equivalence.

Serial Number	Predicted molecular formula	Double bond equivalence	Molecular weight (g/mol)	*m/z*	RT (min)
11.	$C_{16}H_{28}N_3O_2$		294.2189	$[M-H]^-$ 293.2116	19.09
12.	$C_{29}H_{28}N_4O_6$	18.0	528.2027	$[M+H]^+$ 529.2100	19.46
13.	$C_{35}H_{28}NO_5$	22.5	542.1969	$[M-H]^-$ 541.1896	19.47
14.	$C_{30}H_{28}N_5O_3$	19.5	506.2207	$[M+H]^+$ 507.2279	19.47
15.	$C_{33}H_{32}N_2O_6$	19.0	552.2256	$[M+H]^+$ 551.2183	19.47
16.	$C_{25}H_{34}N_3O_3$	10.5	424.2610	$[M+H]^+$ 425.2683	21.29
17.	$C_{23}H_{16}N_3O_4$	17.5	398.1147	$[M-H]^-$ 397.1074	23.51
18.	$C_{40}H_{54}N_6O_3$	17.0	666.4241	$[M-H]^-$ 665.4169	34.53
19.	$C_{55}H_{98}N_3O_6$	8.5	896.7443	$[M+H]^+$ 897.7515	34.61

RT, retention time.

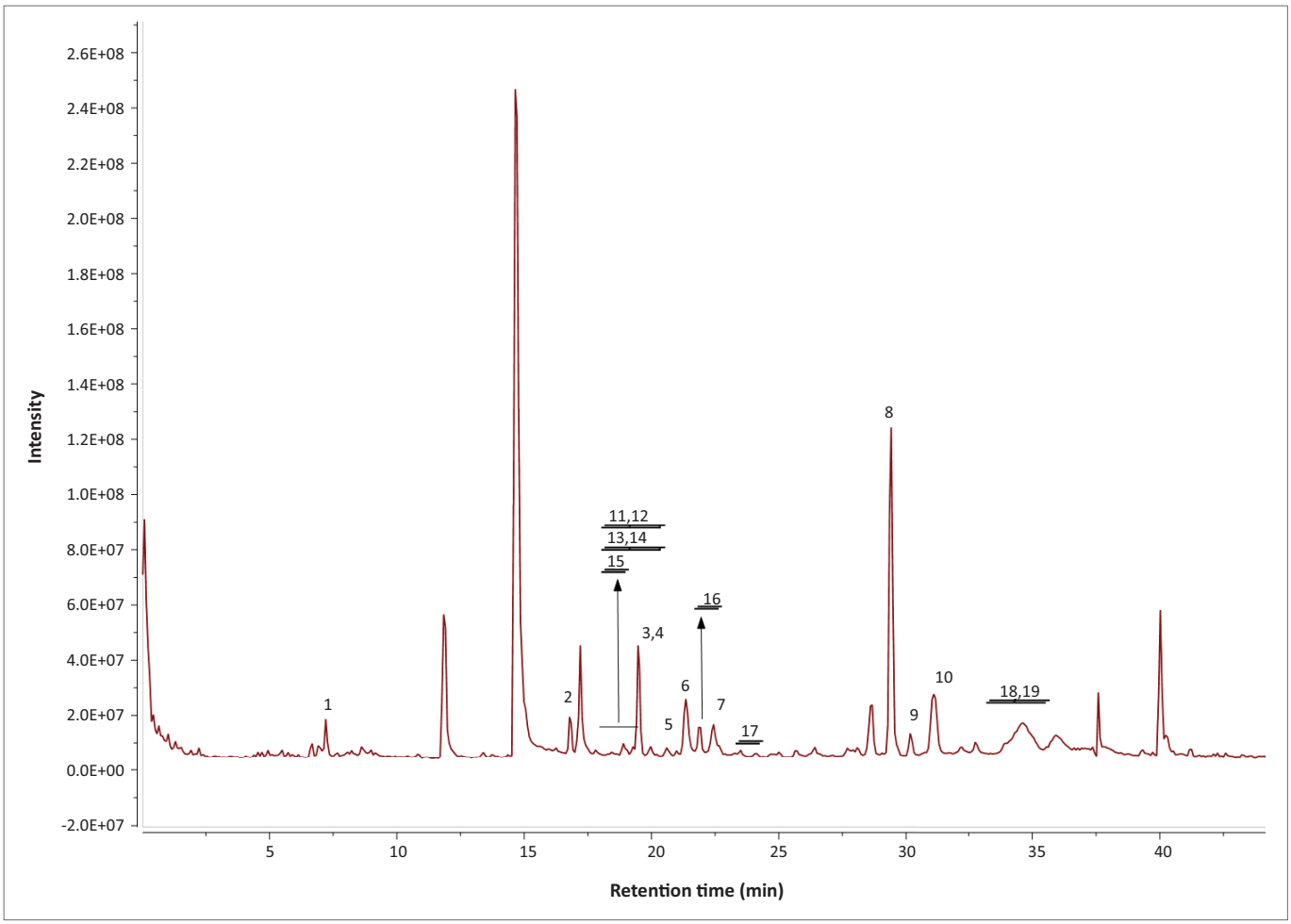

FIGURE 7: Total ion chromatogram for *D. filix-mas* (DF) showing identified metabolites (1–10) and unidentified but detected metabolites (11–19). The identification, molecular formula, molecular weight, mass to charge ratio (m/z) and retention time in min (RT) are indicated in Tables 1 and 2.

developing hypothesis that DF may be involved in the prevention of MLCK activation. However, the effect of DF on oxytocin also suggests possible interaction with the associated second messengers IP_3 and DAG, but this remains to be investigated.

KCI-rich solutions have been reported to depolarise the surface membrane of muscle cells resulting in contraction (Niedergerke 1956). The resting membrane potential of uterine smooth muscle cells has been recorded to be between -35 mV and -80 mV (Togashi 2007). Potassium-rich solutions cause changes in the ionic permeability of the myocyte membrane, which leads to the threshold for action potential generation being reached, resulting in the depolarisation of the cell membrane, markedly increased Ca^{2+} entry, and subsequent contraction (Arrowsmith et al. 2014). Together, these result in what is known as a 'complex action potential' which involves an initial spike-like depolarisation followed by a sustained plateau of depolarisation between -30 mV and -20 mV, and involves strong Ca^{2+} conductance (Arrowsmith et al. 2014) through the voltage-operated calcium channels (VOCs), in particular, the L-type calcium channel (Granger, Hollingsworth & Weston 1986). Gap junctions within the myocytes ensure propagation of the action potentials throughout the myometrium, prompting contraction synchronicity at the whole organ level (Arrowsmith et al. 2014; Little, Teaf & Hurwitz 1985). This mechanical response to high K^+ is completely inhibited by several calcium channel blockers through blockade of the L-type channel (Calixto & Sirley 1985). This therefore proposes potential inhibitory activity of DF on VOC which would have been responsible for the inhibition of KCl-induced contractions in this study.

In a calcium-deprived medium, the only available calcium originates from intracellular stores (Batra 1986). In this study, oxytocin was shown to produce contractions in the calcium-free solution suggesting the utilisation of intracellular calcium stores which has been previously documented (Arrowsmith & Wray 2014; Arrowsmith et al. 2014). The uterine sarcoplasmic reticulum actively takes up and stores Ca^{2+} by using the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), and luminal Ca^{2+} buffers. It is also well endowed with Ca-release channels with the major effect of IP_3 being to trigger Ca^{2+} release from the sarcoplasmic reticulum via the IP_3 receptors (Arrowsmith et al. 2014). DF was observed to have no significant effect on oxytocin-induced contraction under these conditions, suggesting a lack of interaction with $[Ca^{2+}]_i$ and rather a possible interaction with the extracellular voltage-gated calcium channels.

Taken together, it appears that DF may exhibit uterine inhibitory activity particularly through interaction with extracellular voltage-gated calcium channels, presumably the L-type calcium channels that are responsible for spontaneous contractions. Spontaneous contractions have been reported to have no involvement with intracellular calcium (Shmigol, Eisner & Wray 2001) suggesting that myogenic phasic contractions of the uterus are entirely dependent on Ca^{2+} influx through voltage-gated L-type Ca^{2+} channels, which are necessary and sufficient for normal spontaneous myometrial activity (Kupittayanant, Burdyga & Wray 2001; Shmigol, Eisner & Wray 1998). However, agonists (such as oxytocin) promote re-uptake of Ca^{2+} into the sarcoplasmic reticulum via the Ca^{2+}-ATPase of the sarcoplasmic reticulum (Shmygol & Wray 2004) suggesting a strong dependence of agonist on intracellular calcium (Arrowsmith et al. 2014). In this study, it was observed that under the influence of extracellular calcium channels on oxytocin activity, DF produced an inhibitory effect but in the absence of extracellular calcium, DF lacked activity on oxytocin. That DF could inhibit high K^+-induced contraction which is highly dependent on calcium from extracellular channels also supports the involvement of DF with VOCs. There are currently no scientific reports on the effect of DF on myometrial contractility; however, an earlier study reported the anthelminthic effect of DF (Urban et al. 2008), which supports possible calcium inhibitory effect. Studies performed with *Verbascum thapsus* showed a similar anthelminthic activity and also reported, in addition, the possible inhibition of VOCs by the same plant *V. thapsus* (Ali et al. 2012), giving credence to the interaction of DF with VOCs suggested in this study.

Biological effects of identified compounds in *D. filix-mas*

Several compounds were identified in DF belonging to general classes of fatty acids, alkaloids, saponin glycoside, amino acids, limonoids and terpenes, as well as porphyrins. Some reported biological effects of the identified compounds are briefly discussed here in order to extrapolate possible relationship with the activity of DF in this study. Aurantiamide identified in DF belongs to the alkaloid group of compounds (Liu et al. 2015), and while there is not much work performed on the effect of aurantiamide on the uterus, it was reported that aurantiamide acetate inhibits the release of pro-inflammatory cytokines, nitric oxide and prostaglandin E_2 (Liu et al. 2015), which may contribute to the inhibitory effect observed in this study. The trillenogenin also identified in DF is a steroid saponin polyglycoside (Wang et al. 2007), and similar to aurantiamide there has been no report on its actions on the uterus. However, it has been reported to inhibit cylclooxygenase 2 (COX-2) production (Wang et al. 2007) and therefore may contribute to alteration of uterine contractility. Labour is associated with increased synthesis of prostaglandin *in utero* (Keirse, Mitchell & Turnbull 1977). Prostaglandins which are formed from the precursor arachidonic acid, which is itself is a substrate for at least three enzyme groups (Slater et al. 1999), are known to facilitate cervical ripening, stimulate uterine contractions (Crankshaw & Dyal 1994) and indirectly increase fundally dominant myometrial contractility via up-regulation of oxytocin receptors (Garfield, Tabb & Thilander 1990). The cyclooxygenase (COX) pathway releases prostaglandins. It has been reported that in labour, arachidonic acid metabolism is increased and the ratio of COX to lipoxygenase metabolism is changed such that it favours prostaglandin E_2 synthesis (Bennett et al. 1993). The expression of COX-2 therefore increases drastically during labour (Hirst et al. 1995), which potentiates uterine contraction. Inhibition of COX-2 will therefore lead to an inhibition of uterine contractility. Asperphenamate is a phenylalanine derivative (Zheng et al. 2013) identified in DF. It is an unusual ester of *N*-benzoylphenylalanine and *N*-benzoylphenylalaninol and was first isolated from *Aspergillus flavipes* (Clark, Hufford & Robertson 1977). It is known majorly for its antitumor activity (Li et al. 2012; Wu et al. 2004; Yuan, Wang & Sun 2010), but there are currently no reports on the effect of asperphenamate on uterine contraction. Destigloylswietenine belongs to the limonoid class of plant secondary metabolites (Zhang et al. 2009) with swietenine being first isolated from the seeds of *Swetenia macrophylla* in 1964 (Conolly et al. 1964). While there are also no reports of the effect of destigloylswietenine on uterine contraction, limonoids from *Swietenia humilis* (Meliaceae) have been reported to cause a stimulatory effect on intestinal and uterine smooth muscle, and it was suggested to occur possibly through an interaction with oestrogens (Perusquía et al. 1997). The chlorocruorins are green blood-pigments which resemble the haemoglobins and erythrocruorins in having a ferrous iron-porphyrin complex as prosthetic group, but the porphyrin, chlorocruoroporphyrin, which was identified in DF is different from that of haem (Walsh 1961). Phylloerythrin identified in DF also belongs to the porphyrin group of secondary metabolites and finds clinical application currently in photodynamic therapy (Smith et al. 1996). The porphyrin group of compounds has been reported to have varying effects of inhibition and stimulation on uterine contractility (Bafor et al. 2014); chlorocruoroporphyrin and phylloerythrin may therefore contribute to the activity of DF on the uterine smooth muscle. Hydroxyingenol also identified in DF belongs to the diterpene class of plant secondary metabolites which are reported to exhibit analgesic and anti-writhing activity and to also inhibit phorbol dibutyrate receptors (Mbwambo et al. 1996; Wu et al. 1991). The phorbol esters are potent analogues of diacylglycerol and also bind actively to protein kinase C receptors (Ono et al. 1989), which promotes Ca^{2+} release and smooth muscle contraction. Therefore, inhibition of the phorbol dibutyrate receptors may lead to an inhibition of contractility and may contribute to the effect of DF in this study. Earlier studies reported significant antioxidant activity of DF possibly because of the presence of several phenolic compounds (Sekendar Ali et al. 2012; Soare et al. 2012). These support the compounds detected in these studies, which had varied components of phenol groups.

Conclusion

This study reports the inhibitory effect of the methanol leaf extract of DF on uterine contractility. DF was shown in this study to inhibit spontaneous, oxytocin-induced and high KCl-induced uterine contractions. DF was however shown to have no effect on oxytocin-induced contraction in calcium-free media. Taken together, this study reports the inhibitory effect of DF on uterine contractility and suggests possible interaction with calcium with preference to the extracellular voltage-gated calcium channels. Several secondary metabolites were also identified in DF which were found to include fatty acids, alkaloids, saponin glycoside, amino acids, limonoids, terpenes and porphyrins. Inhibition shown by DF in this study may therefore not support the reported traditional use in postpartum haemorrhage and uterine stimulation.

Acknowledgements

The authors wish to acknowledge the technical assistance of Miss Uyi Omogiade during this research.

Competing interests

The authors declare that they have no financial or personal relationships which may have inappropriately influenced them in writing this article.

Author's contributions

B.E.E. conceptualised and designed the study, participated in the experiments, analysed the data and wrote the article. O.O.W. participated in the experiments and data analysis. U.O.H. was primarily involved in the plant collection, preparation and extraction. E.U.B. and O.O. participated in the uterine contractility experiments. V.C. and E.R. were involved in the mass spectrometry experiments, interpretation of data and contributed in writing the article.

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