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Metabolomics-Coupled Functional Pharmacology of Chlorophyll Compounds isolated from the leaves of *Ficus exasperata* Vahl (Moraceae) Provides Novel Pathways on Myometrial Activity

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Short title: Investigation of chlorophyll compounds' activity on the myometrium

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Abstract

New chlorophyll derivatives (pheophytins along with pheophorbide-*derivatives*) were isolated from the leaves of *Ficus exasperata* and were found to have varying effects on uterine contractility. The current study was therefore aimed at the utilization of mass spectrometry and nuclear magnetic resonance spectroscopy coupled with isolated uterine tissue assay as a platform to assist in the determination of the mechanism of activity of the chlorophyll compounds isolated from the plant *F. exasperata*. The pheophytin and pheophorbide compounds were added to the isolated uterine tissues at 200 µg/ml. Mice uteri, treated with the pheophytin compounds, and the physiological buffer in which the uterine tissues were immersed, were rapidly collected then analyzed using HRFTMS and proton (^{1}H)-NMR for bioinformatics study. Resulting data were analyzed via pair-wise chemometric comparison models, with $P < 0.05$ considered statistically significant. Primary signaling pathways found to be correlated with the pheophytins in this study included cAMP, dopamine, extracellular signal-regulated kinases 1/2 and glutamate pathways.

Keywords: Pharmacology; Metabolomics; Uterus: Pheophytin; Chlorophyll

Introduction

New chlorophyll derivatives (pheophytins along with pheophorbide-*a* derivatives) were isolated from the leaves of *F. exasperata* and were found to have varying effects on uterine contractility. These new pheophytins included; compound **3**[1]: ethyl 3- docosa-4,6,8-trien-1-yl]-16-ethenyl-3-hydroperoxypentamethyl-4-oxo-7,23,24,25-tetraazahexacyclo-hexacosapropanoate (ficusphytin-a1); compound **4**[1]: ethyl 3-16-ethenyl-3-hydroperoxy-3,11,12,17,21,26-hexamethyl-4-oxo-7,23,24,25-tetraazahexacyclohexacosapropanoate (ficusphytin-a2); compounds **7**[1]: (1*E*)-but-1-en-1-yl 3-[(21S)-16-ethenyl-3-hydroperoxy-12-pentamethyl-4-oxo-7,23,24,25-tetraazahexacyclohexacosapropanoate (ficusphorbide–b1); compound **8**[1]: (1*E*,3*E*)-henicosa-1,3-dien-1-yl-3-[(21S)-16-ethenyl-3-hydroperoxy-12-pentamethyl-4-oxo-7,23,24,25-tetraazahexacyclohexacosapropanoate (ficusphorbide–b2); and compound **9**[1]: methyl 3-[(21S)-16-ethenyl-3-hydroperoxy-12-pentamethyl-4-oxo-7,23,24,25-tetraazahexacyclo hexacosapropanoate (ficusphorbide–b3). Apart from the new pheophytins, pheophorbide-*a* and pyropheophorbide-*a* were also isolated from the leaves of *F. exasperata*[1].

In our previous study, pheophorbide-*a* derivatives at concentrations of 70 – 200 µg/ml, were mainly observed to reduce the amplitude but increase the frequency of uterine contractions[1], this was similarly observed in **fraction 6-17** (**C3-5**). However, **fraction 4-31** (**C6**) inhibited uterine contractions[1]. The short chain pheophorbide-*b* derivatives exerted both a stimulatory and inhibitory effect on spontaneous uterine contractions while inhibiting oxytocin-induced uterine contractions[1]. Due to low available yields of the compounds at the stage of structure elucidation, it was impossible to continue with further characterization of the uterine activities of the compounds. Metabolomic investigation was therefore performed to enable characterization of activities due to the low quantities available. Metabolomics which involve the use of high resolution Fourier transform mass spectrometry (HRFTMS) and 1D/2D- nuclear magnetic resonance (NMR) enabled the study of changes in metabolomic

profile, which are produced by living tissues[2,3]. Metabolites have been described as functional units within cells, which are released in response to signals or stimuli. Measurement of these metabolites therefore can assist in the understanding of basic and underlying mechanisms related to metabolic actions[4]. Biofluids and/or tissues can be sampled for metabolomic analysis measurement and in such instances these samples can be used to investigate specific information associated with the tissue or organ while the study of biofluids can provide a projection of the metabolic changes that have occurred within organs or tissues[5].

This study was therefore aimed at the use of metabolomic techniques coupled with functional *ex vivo* uterine assay as a platform to determine fluxes in metabolite profiles during treatment with pheophytins isolated from *F. exasperata*. Knowledge of these changes in metabolomics profiles can provide more information into the mechanism(s) of activity of the chlorophyll derivatives on uterine contractility.

Materials and Methods

Animals

Female C57BL/6 strain laboratory mice weighing between 20-30 g, obtained from the Biological Procedures Unit of the University of Strathclyde, U.K. were used in this study. The animals were maintained in a controlled environment and in an air-conditioned room at 25°C, 60% relative humidity under a 12/12h cycle of light and dark with free access to food and water.

All experiments were carried out in accordance with the Animal Health and Welfare (Scotland) Act 2006 and the Public Health Service Policy on Humane Care and Use of Laboratory Animals 2002. Mice were euthanized under rising concentration of CO_2 and exsanguinated before excision of the uterine tissues.

Contractility Experiments

Methods described previously by Bafor and colleagues were employed[1]. Briefly, mice in pro-estrous and estrous stages were used. Contractility experiments were performed on the dissected uteri, which were cut into segments of approximately 0.5 - 0.8 cm in longitudinal length, cleaned of extra connective tissues to provide four preparations per mouse. The segments were then weighed and subsequently mounted in warmed (37 °C) 3 ml organ baths containing normal Krebs-Henseleit physiological solution which was composed of 118.4 NaCl, 25 $NaHCO_3$, 11.1 Glucose, 4.69 KCl, 2.41 $MgSO_4$, 1.18 KH_2PO_4, 2.5 $CaCl_2$ (mM) and continuously gassed with carbogen (95% O_2, 5% CO_2). Tissues were set up with an initial tension of 1 g and equilibrated for 30 min before the start of the experiments. The force and frequency of spontaneous contractions in the longitudinal muscle layer were recorded through Grass (FTO3) isometric force transducers connected via Quad Bridge modules to a PowerLab 4/20 data acquisition system using Chart 3.3 software (AD Instruments, UK) which was used to store and analyze acquired data.

Concentration-Response Studies

Single concentrations (200 µg/ml) of **C3-5** which were eluted as a mixture (ficusphytin-a1 and a2, and pheophytin-*a*) or **C6** (pyropheophorbide-a), were added to the bath for 10 min following stable rhythmic spontaneous contractions. This concentration was selected as the maximum concentration for observation of activity based on the previous study. ~~Single concentrations are preferred in metabolomic studies as it saves cost and ensures precise correlations of activity with the concentration involved.~~ Without washing and while the compounds were still in the organ bath, the uterine tissues were rapidly collected after 10 min, placed into pre-weighed cryovials and immersed in liquid nitrogen for 20 s. This was done in order to snap freeze the tissues and keep the internal environment of the metabolites in the

tissues intact. Snap freezing is critical for experiments where it is necessary to retain the activity within a biological sample at a specified period, as it prevents the cells responding to any handling perturbation[6]. Bath fluids where the tissues were immersed, were similarly collected (3 ml) and snap frozen. All samples were subsequently stored at −80 °C prior to further experiments. Four (4) animals each for individual experiments were employed. All graphs were prepared using Prism Software (version 4; GraphPad Software, CA).

Tissue Metabolite Extraction

All samples were placed in dry ice after retrieval from the −80 °C freezer. Each snap frozen tissue was homogenized in 400 µl cold methanol (MeOH) using a homogeniser (IKA®-WERKE GmbH & Co., Germany). The homogenate was vortexed for 60 s before being centrifuged at 13,000 rpm for 5 min per tissue sample extracted with MeOH (samples extracted with MeOH were subsequently referred to as the 'polar extracts'). The supernatant was collected using a micropipette syringe and dried in pre-weighed vials in preparation for further NMR and MS measurements. Using the same homogenized tissue residue, the process was repeated with 400 µl cold dichloromethane (DCM) and centrifuged at 13,000 rpm for 10 min. Tissues extracted with DCM were subsequently referred to as the 'non-polar extracts'. The time periods employed for centrifugation were predetermined from preliminary experiments. All extracts were freeze-dried and corresponding weights were recorded.

Bath Fluid Metabolite Extraction

Each fluid sample was freeze-dried in a freeze drier (Christ Alpha 2-4, SciQuip, UK) and weighed. The dried samples were then reconstituted with 500 µl of cold MeOH and vortexed for 1 min. The solution was pipetted out and centrifuged as previously described for polar extracts. The process was repeated with 600 µl cold DCM on the residue obtained after MeOH extraction. The entire extraction procedure was performed under cold conditions with samples

placed in dry ice at all times. All samples were freeze-dried as previously described and weighed.

^{1}H-NMR Data Acquisition

The dried polar extracts were weighed and reconstituted in 200 µl dimethylsulphoxide (DMSO) vortexed for 30 s and submitted for 1D ^{1}H-NMR measurements while the DCM extracts (non-polar extracts) were weighed and reconstituted in 200 µl $CDCl_3$ and similarly submitted for 1D ^{1}H-NMR measurements. Pre-saturated ^{1}H-NMR experiments were carried out on a JEOL-LA400 FT-NMR spectrometer system (JEOL Ltd, UK) with an AS400 magnet and measured at 400 MHz for ^{1}H using a Pulse Field Gradient "Autotune" 40TH5AT/FG broadband high sensitivity 5 mm probe. Samples were measured in a 3 mm NMR tube with a 5 mm tube adapeter. ^{1}H-proton NMR with presaturation of the residual water resonance were obtained using the following parameters; 32k data points, spectral width of 5998.4 Hz, acquisition time of 2.73 s, relaxation delay of 3.0 s., and pulse width of 5.47 requiring 3.85 min total acquisition time.

HPLC-HRFTMS Data Acquisition

Extracts of tissues and samples were also submitted for HRMS analysis. Polar extracts were reconstituted with 200 µl of 100 % methanol while the non-polar extracts were reconstituted in DCM : MeOH at a ratio of 30:70. The 30:70 ratio were predetermined from preliminary experiments as best suited for dissolving the non-polar extracts. Details of the HPLC analysis can be obtained from the supplementary data.

Data Processing and Analysis

HPLC-HRFTMS Profiles

The HPLC-HRFTMS metabolic profiles of polar and non-polar extract-treated tissues and surrounding bath fluids were analyzed for changes in metabolites before and after drug treatment as previously described[7]. Statistical determination was performed as previously described[10] in XCMS using the t-test with $p < 0.05$ considered significant for each pairwise comparison of control and treated groups. Identification of metabolites within XCMS online was done using METLIN (a web-based database). Details of the profiling can be obtained from the supplementary data.

Bioinformatic Analysis of HPLC-HRFTMS Data

The Ingenuity Pathway Analysis (IPA) bioinformatics tool (produced by Ingenuity Systems, Redwood City, CA) was used to perform bioinformatic analysis on resulting data. A search was additionally performed for regulated pathways with the aid of identified metabolites. Statistical significance was calculated using Fischer's exact test, $p<0.05$ was considered statistically significant. IPA automatically generates pathways related to specific metabolites for a given data set.

NMR Pre-processing and Analysis

All NMR data sets were pre-processed using Mnova v. 10.1. The imported spectra were manually phased and binned between δ 0.5 – 10 ppm at bin value of δ 0.04 ppm width. Residual water and solvent peaks were removed. The total spectra area was then normalized to a total value of 100 and a 2D matrix (n x d) was created which was then exported into SIMCA. Mean centering was performed before principal component analysis (PCA) and OPLS-DA was applied to the datasets. Potential outliers as well as similarities between the metabolic fingerprints was enabled by PCA and this usually occurs in an unsupervised manner. The S-

line was also used in this study which functions as statistical total correlation spectroscopy (STOCSY)[13].

Diagrammatic summary of the methods described above are presented in figure 1.

Results

Pharmacological analysis

C3-5 and C6 were observed to decrease uterine contractility (Fig. 2). Data analysis of C3-5 showed a reduction in the amplitude ($p = 0.05$), frequency and area under the curve of uterine contractions (Fig. 3). Similarly, data analysis of C6 showed a reduction in the amplitude, frequency and area under the curve ($p < 0.05$) of uterine contractions; however, the effect of frequency was less marked (Fig. 4).

HPLC-HRFTMS of Pheophytin and Pheophorbide compounds (C3-5 and C6)

HRFTMS experiments were analyzed using the multivariate analysis in SIMCA 14.0 and the bioinformatics software IPA as previously described[10]. Multivariate OPLS-DA plots showed distinct separations between the control (blue circles) and treated (red circles) groups of **C3-5** (Fig. S1) and **C6** (Fig. S2). Multivariate S-plots were also generated which represented every metabolite detected within the groups compared. Significantly, regulated metabolites and pathways were also displayed as blue in the control and red in the treated (Fig. 5 and 6). Cyclic adenosine monophosphate (cAMP), endocannabinoid and catecholamine signaling were upregulated while myoinositol (MI) was downregulated (Fig. 5 and 6) in both **C3-5** and **C6** treated groups. Other detected metabolites were listed in supplementary tables 1 and 2. These metabolites ranged from amino acids to lipids and included an increase in adenosine, cysteinyl dopamine, phosphatidyl ethanolamine, glycerolphosphate, phosphatidyl serine, triglycerides,

which were observed in the tissues and fluids while a decrease in noradrenaline, tyrosine, and phosphatidylinositol were observed in **C3-5** treated tissues and fluids (Supplementary Table 1). For **C6** treated groups, an increase in threonine and adenosine were observed in the tissues and fluids while a decrease in gamma amino butyric acid (GABA), glutamate, tyrosine, methionine, threonine and phenylalanine were detected in the tissues (Supplementary Table 2). Metabolites detected in the fluids included an increase in nicotinic acid, uridine 5'-diphospho-beta-L-threo-pentapyranosyl-4"-ulose (UDP-L-Ara4O), cysteinyldopamine, and ceramide while MI, sphingosine, phosphatidylinositol and phosphatidic acid were observed in the bath fluids to have been decreased (Supplementary Table 2).

The IPA bioinformatic software showed cell signaling, lipid metabolism, small molecule biochemistry, nucleic and amino acid metabolism as high-level functions associated with both **C3-5** and **C6** mechanisms (Fig. 7 and 8). Canonical pathways generated by IPA displayed MI signaling, cytidine diphosphate diacylglycerol (CDP-DAG) and serotonin pathways to be associated with the effect of both **C3-5** and **C6** on uterine contractility (Fig. 7 and 8). However, nicotinic acid, eicosanoid and GABA pathways were also found associated with the effect of **C6** on uterine contractility (Fig. 8).

^{1}H-NMR Analyses of C3-5 and C6

Multivariate OPLSDA showed distinct separations between the control and treated groups (Fig S3 and 4). For **C3-5** groups, correlation coefficient plots of the ^{1}H-NMR measurements showed Ras homolog gene family, member **(**RhoA), nucleic acid, and cAMP signaling to be detected in the control tissues and bath fluids (Fig. 9) while endocannabinoid and DAG signaling were detected in the treated tissues and bath fluids (Fig. 9). For the **C6** groups, the correlation coefficient plots of the ^{1}H-NMR measurements also indicated the presence of metabolites for endocannabinoids signaling in both control and treated groups (Fig. 10). GABA signaling was detected in the control while catecholamine and cAMP signaling were detected in the treated groups (Fig. 10). Other metabolites detected in **C3-4** treated groups included glutamine and tyrosine which were detected to have been upregulated in the uterine tissues while anandamide

(AEA), glutamate, aspartate, glutamine and phenylalanine were upregulated in the bath fluids (Table 2). Arachidonic acid (AA), AEA, MI, DAG, and adenosine triphosphate (ATP) were detected to have been downregulated in the uterine tissues, these were similarly observed in the bath fluids but in addition GABA, tyrosine, phenylalanine and threonine were downregulated (Table 2). In **C6**-treated groups, other metabolites detected were adenosine, glutamate, DAG, adenosine monophosphate, AEA, and aspartate, which were upregulated in the tissues while glutamine, ATP and GABA were detected as upregulated in the bath fluids. ATP and AA were observed to have been downregulated in the tissues and in the bath fluids and in addition MI, DAG, prostaglandin F_1, AEA, threonine, were downregulated in the bath fluids (Table 3).

Discussion

The chlorophyll compounds (**C3-5 and C6**) that affected the contraction of uterine tissue were observed to increase the metabolites for the cAMP pathway, as detected by both MS and NMR. An increase in myometrial cAMP concentration often results in inhibition of uterine contraction. The complete mechanism by which this is achieved appears to be still being elucidated[14], hence several mechanisms of cAMP-induced relaxation have been proposed. Increase cAMP has been reported to activate Na^+/Ca^{2+} pump and promote intracellular storage of Ca^{2+} [15]. Recent studies have also suggested that cAMP blocks signal transmission from Ras proteins to Raf-1 by interaction with protein kinase A this prevents mitogen activated protein kinase (MAPK) activation[16,17]. An inhibition of phosphoinositol (PI) turnover has also been suggested[18].

Myoinositol (MI) signaling was also detected to play a role in the effect of both compounds. For instance, MI signaling decreased upon **C3-5** and **C6** treatment. MI is an isomer of cyclohexane-hexol in eukaryotic cells and a precursor of the inositol phospholipids. In addition to the well documented pathways for both diacylglycerol (DAG) and IP_3 production, both acting as second messengers resulting [19–21], there are several other inositol phospholipid

pathways. These include the complex metabolism of IP_3[22], the metabolism of inositol polyphosphates[23], the novel inositol phospholipid pathway initiated by phosphatidylinositol 3-kinase which might be regulated by tyrosine kinases[24], and the synthesis of phosphatidylinositols (PtdIns) glycans[25]. The metabolism of inositol phosphate has been described as being extremely complex[20]. In summary, the metabolism of inositol results in the generation of several inositol phosphates the commonly known include: IP_3, inositol-1,3,4,5 phosphate (IP_4), inositol-5-phosphate, inositol-3,4,5,6-tetrakisphosphate and inositol-6-phosphate which have different biologic functions[21]. IP_4 has also been implicated in Ca^{2+} signaling[26,27]. Some studies have shown that IP_4 may exert both facilitatory and inhibitory feedback on calcium signaling as it inhibits IP_3 receptors at certain concentrations[28]. IP_4 has been reported to also inhibit IP_3 metabolism through IP_3 5-phosphatase thus facilitating the activation of store operated Ca^{2+} current (I_{CRAC}). IP_4 receptors are located on the nuclear membranes, activation of which results in calcium uptake and inhibition of IP_3. It has also been proposed that IP_4 calcium uptake might be mediated through the stimulation of calcium pumps [29]. From the foregoing, MI seems to generate several functional metabolites besides IP_3, which may play a role in smooth muscle relaxation through inhibition of IP_3 or calcium uptake. Based on the results from this study, it is therefore proposed that a decrease in IP_3 contributed in part to the pharmacological effect seen in this study of the chlorophyll compounds **C3-5** and **C6** on myometrial contraction**.**

An increase in gamma aminobutyric acid (GABA) signaling was observed with **C6** but not with **C3-5** where a decrease in GABA was observed. GABA and receptor binding sites of GABA have been shown to occur in the uterus of rat and rabbit[30]. GABA has been described to activate $GABA_B$ to elicit excitatory responses in the rabbit uterus[31] while activation of $GABA_A$ receptors in the uterus promotes inhibition of uterine contractility and has been proposed to play a role in the modulation of uterine contractility[30,31]. It has also been suggested

that the density of the GABA receptor type present in an organ may determine the response observed (while the prevailing hormonal environment occurs during pregnancy or during the estrous cycle) may determine the type and density of receptors present in the uterus [32–34].

Catecholamines (adrenaline and noradrenaline) have been reported to contribute to the regulation of myometrial contraction via interaction with the β- and α-adrenergic receptors present in the uterus[35]. The myometrial inhibitory effect observed upon activation of the β-adrenergic receptors occur through the upregulation of adenylyl cyclase[36] while myometrial stimulation is observed upon activation of α-adrenergic receptors through upregulation of the $G\alpha_{q/11}$ protein and phospholipase C stimulation[37,38]. In this study, catecholamine synthesis was decreased upon treatment of the uterus with the chlorophyll compounds (**C3-5** and **C6**). This observation suggested that catecholamines may not play any significant role in the effects of ficusphytins on uterine contraction. However, the effect of catecholamines in the regulation of spontaneous uterine contraction had to be decreased or inhibited in order for the effect of the compounds to be observed.

The endogenous cannabinoids (endocannabinoids) were also found to be perturbed upon treatment of the uterus with ficusphytins. Endocannabinoids were increased on **C3-5** treatment while a decrease was observed upon treatment of uterine tissues with **C6**. These endocannabinoids belong to a class of fatty amides. *N*-acylethanolamines (NAEs) are ethanolamides of long chain fatty acids and exist in various organisms including animals and plants[39,40]. Of these, anandamide (AEA) or *N*-arachidonoylethanolamide has attracted special interest because of its marked biological activities. NAEs are currently considered to be a class of endogenous lipid mediators[41,42]. Upon its identification in 1992 from porcine brain[43], AEA has so far been the most extensively characterized NAE, which functions as an endogenous ligand of cannabinoid receptors and transient receptor potential vanilloid 1 channels and actually exerts a variety of central and peripheral activities through these receptors[44]. Like the

pharmacologically active compounds in cannabis (*Cannabis sativa*), its effects is exerted through binding to and activating specific cannabinoid receptors (CBR) and along with 2-arachidonoyl-glycerol AEA[45]. Both cannabinoid receptors CB_1 and CB_2 are membrane-bound G-protein coupled receptors[46]. CB_1 is mainly found in the central nervous system[47] but has also been found in organs such as the heart, uterus, testis and small intestine, while the CB_2 receptor is found primarily in the spleen and other cells associated with immunochemical functions, as well as some peripheral cells[48]. Endocannabinoids have been found in the uterus with one of the highest levels detected in the mouse uterus[49] and has also been implicated in the regulation of the female reproductive cycle especially with the presence of cannabinoid receptors in the uterus[50]. There have been a couple of investigations into the effects of endocannabinoids on uterine contractions. However, there are also contradictory reports on the effect of endocannabinoids on uterine contractility. Dennedy *et al*. reported a relaxant effect of AEA on uterine contractility and demonstrated that this relaxant effect may be linked to a reduction in uterine prostaglandin synthesis[51]. In contrast, an increase in the force of spontaneous uterine contraction was reported for the endocannabinoids[52], which was purportedly due partly to a cannabinoid-induced production of PGE_2 and $PGF_{2\alpha}$ resulting in a decrease in the intracellular concentration of cAMP[53]. Similarly, some seemingly contradictory reports on the mechanisms of action of endocannabinoids exist. Some researchers report on a direct regulation of uterine contraction via binding with CB_1 and CB_2 receptors that preferentially couple to inhibitory $G_{\alpha i/o}$ proteins to inhibit adenylate cyclase activity, and consequently reduce intracellular cAMP levels [54]. Yet some researchers report on an observed increase in cAMP levels following CB_1 activation[55], suggesting a possible coupling to $G\alpha_s$ of the G protein receptors. Other signaling events, including increased activity of MAPK, inhibition of voltage-gated Ca^{2+} channels, activation of K^+ channels, and nitric oxide (NO) generation, have also been suggested to follow CB receptor subtypes activation under different conditions[53]. It may seem therefore, that

binding of endocannabinoids to their receptors can result in activation/regulation of a number of signal transduction pathways, which are dependent on the agonist involved and the factors in place upon binding. Briefly, binding can result in inhibition of Ca^{2+} channels; inhibition of adenylyl cyclase and corresponding decrease of cAMP-dependent protein kinase, which leads to decreased phosphorylation of the K^+ channels and regulation of ionic currents; activation of focal adhesion kinase and activation of MAPK cascades[56] that includes extracellular signal-regulated kinases (ERK) p38 MAPK cascades[57,58], as well as the stimulation of other intracellular pathways such as the phosphatidylinositol 3-kinase (PI_3K)/Akt (also known as Protein Kinase B (PKB)) pathway through CB_2[59]. Akt is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes[60]. Different subtypes of Akt exist but Akt_1 is involved in the PI_3K/AKT/mTOR pathway and other signaling pathways[61]. Activation of GPR55, ("CB_3" cannabinoid receptor), has been linked to: an increase in intracellular Ca^{2+} increase[62]; activation of the small GTPase proteins RhoA, Rac, and Cdc42 [63,64]; and ERK phosphorylation[65,66]. It has also been observed that muscarinic and glutamate receptors have allosteric sites for AEA binding[67]. It can therefore be seen that endocannabinoids exert a highly complex network of interactions[68], which may probably extend its role in modulating uterine contractions as well its role in the mechanism of uterine activity of the ficusphytins in this study.

Dopamine was also detected to contribute to the effects of *Ficus* fractions on uterine contractions. Uterine contraction increased on **C3-5** treatment. Dopamine has been reported to inhibit uterine contractions in the non-pregnant uterus via action on adrenergic receptors[69,70]. However, at early and term pregnancy, dopamine has been reported to stimulate myometrial contractility[71–73]. Dopamine can oftentimes exert different and opposing effects on organs or tissues and this observation has been associated with the species involved as well as the receptors present since dopamine is known to act on both dopaminergic and adrenergic

receptors[70]. Endogenous dopamine has been previously detected in the uterus[74]. Interestingly in this study, while catecholamine synthesis decreased, dopamine signaling increased. It was also observed that in each case where dopamine was increased, endocannabinoids signaling was also increased simultaneously. It is therefore possible that the increased dopamine detected in this study is a product of the AEA pathway. *N*-arachidonyldopamine (NADA) has been detected as an endogenous component of certain mammalian tissues[75]. NADA binds to the CB_1 receptor and transient receptor potential vanilloid type 1 (TRPV1) channels and regulates calcium mobilization[76]. Biosynthesis is believed to occur mainly by conjugation of dopamine with arachidonic acid, catalyzed by a fatty acid amide hydrolase (not via the CoA ester), although there are suggestions that some might be derived from arachidonoyltyrosine[77,78]. Studies have shown the inhibition of T-type Ca^{2+} channels by NADA[79] and sarcoendoplasmic reticulum calcium transport ATPase (SERCA) uncoupling[80]. Unfortunately, it would appear that not many studies have been done on the relationship between NADA and uterine contractility.

RhoA signaling was also detected in this study. A decrease was detected on treatment with **C3-5**. The role of the small G protein rhoA (a member of the Rho subfamily of the Ras superfamily of monomeric GTPases) in the regulation of myometrial contractility has recently received considerable attention. Small G protein rhoA has been described to be involved in agonist-induced Ca^{2+}-sensitization by acting downstream on receptor occupancy[81] as it has been shown to increase myosin light chain (MLC) phosphorylation at a constant Ca^{2+} concentration[82]. Inactivation of rhoA therefore would inhibit Ca^{2+}-sensitization. An effector molecule rho-associated kinase (ROK) appears to play a role in the Ca^{2+}-sensitizing effects of rhoA activation[83]. ROK inhibits the activity of myosin phosphatase[84,85] through the inhibition of myosin binding subunits[86]. Activation of certain receptors in the myometrium results in the translocation of rhoA from the cytoplasm to the cell membranes as was reported to occur with

muscarinic receptors[87]. RhoA and ROK have been shown to be involved in oxytocin-induced myometrial contractility[86]. Caveolins have also been shown to regulate the activities of rhoA and probably ROK[87]. Thus, a reduction in rhoA would result in the inhibition of myometrial contractility.

Extracellular signal-related kinases (ERK) signaling was detected in this study to have been increased on **C3-5** treatment of uterine smooth muscle but a decrease was observed with **C6**. The involvement of the ERK pathway has been proposed to play a role in the regulation of agonist-induced smooth muscle contraction, activation of which has been shown to stimulate contraction [88–91]. ERK also seems to be involved in spontaneous uterine contractility [92] (Li *et al.*, 2003). However, it has been proposed that the ERK pathway may not affect Ca^{2+} channels directly [93]. ERK pathway has been shown to be involved in OT-induced uterine contraction [14] as well as prostaglandin $F_{2\alpha}$ – induced uterine contraction[94] and has been proposed to exert its effect by phosphorylation of caldesmon (CAD)[88]. Therefore, activation of ERK would lead to an increase in contraction as exhibited with **C3-5** and a decrease in ERK would lead to inhibition of uterine contractility as observed with **C6**.

The idea of cross talk between signaling pathways is currently being investigated by several scientists particularly as relates to G-proteins and their second messenger pathways. For instance stimulation of the CB_1 cannabinoid receptor has been shown to lead to an increase in dopamine turnover[95,96] while the stimulation of D_2 receptors leads to an increase in the endocannbinoid AEA[97]. Activation of either CB_1 or D_2 receptors results in the inhibition of adenylyl cyclase, however simultaneous stimulation of both receptors appears to lead to the accumulation and increase in cAMP, and ERK receptor activation[98] suggesting the presence of associated signaling pathways regulated when both receptors are activated by agonists[98,99]. Increase in cAMP has also been reported to induce an increase in cGMP-dependent protein kinase which further potentiates relaxation of smooth muscles [100] and inhibits rhoA signaling

pathway [101,102]. Therefore, crosstalk between receptors may have played a role in the effects of pheophytins in this study considering the varying metabolites significantly regulated on treatment of the uterine tissues. A comprehensive list of significant metabolites detected by the NMR analysis is shown in supplementary table 3.

Conclusion

The cholorophyll compounds in fraction **C3-5**, acted primarily by its interaction with cAMP, dopamine, ERK1/2 and glutamate. Possible connection with other catecholamines, prostanoids, MI, RhoA and GABA was also suggested. While the chlorophyll compounds in fraction **C6** acted primarily by its interaction with cAMP, sphingosine, glutamate and GABA. Possible connection with catecholamines, endocannabinoids, ERK1/2, RhoA, DAG and MI was also postulated. An in-depth understanding of signal transduction pathways discovered from this study could therefore provide a significant aid in the design of compounds such as those isolated from *F. exasperata* and might prove useful in the treatment and prevention of uterine disorders.

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Declaration of Conflicting Interests

The author(s) declare no potential conflicts of interest with respect to this research, authorship, and/or article publication.

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Supplemental Material

The online supplementary figures and tables are available.

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Figure Legends

Figure 1. Flow chart depicting the pharmacological and metabolomic analysis processes.

Figure 2. Original recordings showing the effect of **C3-5** and **C6** on the isolated mouse uterus. Panel A shows **C3-5** on uterine contractions while panel B shows **C6** on uterine contractions.

Figure 3. Bar graphs showing the data analysis of **C3-5** on uterine contractility. **C3-5** inhibited the amplitude (A), and the frequency (B) of uterine contraction. An overall inhibitory activity was observed as shown in the area under the curve analysis (AUC) (C). n= 4 animals, *$p \leq 0.05$.

Figure 4. Bar graphs representing data analysis of **C6** on mouse uterine contractility. **C6** was shown to inhibit the amplitude (A) and shown to produce a slight inhibition of the frequency (B) of uterine contraction. Overall, an inhibitory effect of **C6** was observed as depicted in the area under the curve (AUC) (C). n= 4 animals, *$p \leq 0.05$.

Figure 5. OPLS-DA S plots of polar (A and B) and non-polar (C and D) **C3-5-**treated groups. A and B shows the S plots for treated uterine tissues, while C and D shows the S plots generated for the bath fluids. All metabolites detected are shown in green circles. Significantly, regulated metabolites of the control groups are presented in blue while those of the treated groups are presented in red with the corresponding *m/z* values. ⬣ = cAMP signaling; ▲= endocannabinoid signaling; ⬟ = amino acid (aa) metabolism;▼ = DAG signaling; ■ = catecholamine synthesis; ◆ = MI biosynthesis/signaling. n = 4 animals

Figure 6. OPLS-DA S plots of polar (A and B) and non-polar (C and D) **C6-**treated groups. A and B shows the S plots for treated uterine tissues, while C and D shows the S plots generated for the bath fluids. All metabolites detected are shown in green circles. Significantly, regulated metabolites of the control groups are presented in blue while those of the treated groups are presented in red with corresponding *m/z* values. ⬣ = cAMP signaling; ▲= endocannabinoid signaling; ▼ = DAG signaling; ■ = catecholamine synthesis; ◆ = MI biosynthesis/signaling; ★ = dopamine metabolism. n = 4 animals

Figure 7. Bar charts displaying top functions (A and B) and canonical pathways (C and D) extracted by IPA software for metabolites obtained from **C3-5**-treated uterine tissues and bath fluids. The high-level functional categories associated with **C3-5**- treated groups are displayed along the x-axis in A and B above while high-level canonical pathways are shown in C and D. The y-axis in A and B displays the -(log) significance. Taller bars in A are more significant

than the shorter bars. Functions are listed from most significant to least and the orange horizontal line in A and B denotes the cut-off for significance (p = 0.05).

Figure 8. Bar charts displaying top functions (A and B) and canonical pathways (C and D) extracted by IPA software for metabolites obtained from **C6**-treated uterine tissues and bath fluids. The high level functional categories associated with **C6**- treated groups are displayed along the x-axis in A and B above while high level canonical pathways are shown in C and D. The y-axis in A and B displays the -(log) significance. Taller bars in A are more significant than the shorter bars. Functions are listed from most significant to least and the orange horizontal line in A and B denotes the cut-off for significance (p = 0.05).

Figure 9 Correlation coefficient plots showing the distribution of metabolites in the NMR spectra of the polar (A and B) and non-polar **C3-5**-treated groups. Plot of NMR data for tissues are in A and B while plots for those associated with the bath fluids are in C and D. The blue columns represent the control groups and the red columns represent the treated groups. Significant metabolites have been highlighted and represented according to the corresponding pathways. ⬣ = cAMP signaling; ▲ = endocannabinoid signaling;▼ = DAG signaling/lipid metabolism;✦ = NA metabolism; ◆ = MI biosynthesis/signalling; ⯃ = RhoA signaling; ■ = catecholamine signaling; ⬟ = GABA signaling. These pathways were found to be strongly correlated with the activity of **C3-4**. n= 4 animals.

Figure 10. Correlation coefficient plots showing the distribution of metabolites in the NMR spectra of the polar (A and B) and non-polar **C6**-treated groups. Plot of NMR data for tissues are in A and B while plots for those associated with the bath fluids are in C and D. The blue columns represent the control groups and the red columns represent the treated groups. Significant metabolites have been highlighted and represented according to the corresponding pathways. ⬣ = cAMP signaling; ▲ = endocannabinoid signaling;▼ = DAG signaling/lipid metabolism;✦ = NA metabolism; ◆ = MI biosynthesis/signaling; ⯃ = RhoA signaling; ■ = catecholamine signaling; ⬟ = GABA signaling. These pathways were found to be strongly correlated with the activity of **C8**. n= 4 animals.

Table 1. Summary of Pathways and Metabolites detected by HR-FTLCMS on Effect of Pheophytins and Pheophorbides (C3-5 and C6) of *F. exasperata* on Uterine Contractility

FC= Fold change; **GABA** = gamma aminobutyric acid; **cAMP**= cyclic adenosine monophosphate; **MI**= myoinositol; **DAG** = Diacylglycerol; **PI**= Phosphatidylinositol; ***m/z*** = mass to charge ratio; **Rt** = Retention time; **nd** = not detected

Pathway	Metabolome	Adduct	*m/z*	Rt	C6		C3-5	
					Tissues	Fluids	Tissues	Fluids
GABA Receptor Signaling	GABA	$[M+H]^+$	118.0863	1.36	1.365	-1.9603	nd	nd
GABA Receptor Signaling	Glutamate	$[M+H]^+$	148.0606	1.37	-1.6216	nd	nd	nd
cAMP Metabolism	8-Azaadenosine	$[M+2Na]^{2+}$	157.0353	1.41	nd	nd	11433.3826	9785.7196
			157.0353	1.48	498.8266	1393.879	nd	nd
MI Biosynthesis	MI	$[M+Na]^+$	203.0527	1.57	nd	-1.498	nd	-1.3952
Catecholamine Synthesis	L-Tyrosine	$[M+2H+Na]^{3+}$	110.0201	3.66	1.47941	nd	nd	nd
			110.0201	4.55	nd	nd	nd	-1.7568
Endocannabinoid Metabolism	Oleoylethanolamide	$[M+H-2H_2O]^+$	304.2999	19.71	-24.8789	-1.2906	nd	nd
DAG Metabolism/Endocannabinoid Signaling	N- arachidonoyl amine	$[M+NH_4]^+$	504.3241	23.38	nd	nd	nd	7.3176
MI Biosynthesis	PI(17:1/22:2)	$[M+H-H_2O]^+$	885.5897	32.42	nd	nd	nd	-6.7596
MI Biosynthesis	PI(O-16:0/20:2)	$[M+H-2H_2O]^+$	813.5683	34.29	nd	-120.925	nd	nd

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Table 2. Summary of the Proposed Mechanism(s) of Action of C3-5 of *Ficus exasperata* Leaves Determined from Metabolomic Analysis

GABA = gamma aminobutyric acid**; cAMP** = cyclic adenosine monophosphate**; MI** = myoinositol**; DAG** = diacylglycerol**; ERK** = extracellular signa
egulated kinase**; RhoA** = Ras homolog gene family member A**; nd** = not detected**;** ↑ = increase**;** ↓ = decrease

Extract/ Fraction	Active Compound (s)	Uterine Smooth Muscle Activity	MS Metabolomic Pathways		NMR Metabolomic Pathways	
C3-5	Pheophytin a series	Inhibition/Contraction	**Uterine Tissues**	**Bath Fluids**	**Uterine Tissues**	**Bath Fluids**
			cAMP signaling↑	cAMP signaling↑	nd	cAMP signaling↑
			Dopamine signaling↑	nd	nd	nd
				MI signaling↓	MI signaling↓	nd
			Catecholamine synthesis↓	Catecholamine synthesis↓	Catecholamine synthesis↓	Catecholamine synthesis↓
			nd	Endocannabinoid signaling↑	Endocannabinoid signaling↑	Endocannabinoid signaling↑↓
			nd	nd	RhoA signaling↑↓	RhoA signaling↓
			Prostanoid signaling↓	Prostanoid signaling↓	Prostanoid signaling↓	Prostanoid signaling↓
			nd	nd	nd	nd
			nd	nd	DAG signaling↓	DAG signaling↓
			nd	nd	nd	GABA signaling↓
			nd	nd	ERK1/2 signaling↑	ERK1/2 signaling↑
			nd	nd	Glutamate signaling↑	Glutamate signaling↑

Table 3 Summary of the Proposed Mechanism(s) of Action of C6 of *Ficus exasperata* Leaves Determined from Metabolomic Analysis

GABA = gamma aminobutyric acid; **cAMP** = cyclic adenosine monophosphate; **MI** = myoinositol; **DAG** = diacylglycerol; **ERK** = extracellular signal regulated kinase; **RhoA** = Ras homolog gene family member A; **nd** = not detected; ↑ = increase; ↓ = decrease

Extract/ Fraction	Active Compound (s)	Uterine Smooth Muscle Activity	MS Metabolomic Pathways		NMR Metabolomic Pathways	
			Uterine Tissues	**Bath Fluids**	**Uterine Tissues**	**Bath Fluids**
C6	Pheophytin series	Inhibition	nd	Sphingosine signaling$^{\uparrow}$	nd	nd
			GABA signaling$^{\downarrow}$	nd	nd	GABA signaling$^{\uparrow}$
			cAMP signaling$^{\uparrow}$	cAMP signaling$^{\uparrow}$	nd	nd
			Catecholamine synthesis$^{\downarrow}$	Catecholamine synthesis$^{\downarrow}$	nd	Catecholamine synthesis$^{\downarrow}$
			Glutamate metabolism$^{\downarrow}$	nd	nd	nd
			Endocannabinoid signaling$^{\downarrow}$	Endocannabinoid signaling$^{\downarrow}$	Endocannabinoid signaling$^{\downarrow}$	Endocannabinoid signaling$^{\downarrow}$
			nd	MI signaling$^{\downarrow}$	nd	MI signaling$^{\downarrow}$
			nd	nd	nd	DAG signaling$^{\downarrow}$
			nd	nd	Glutamate signaling$^{\uparrow}$	Glutamate signaling$^{\uparrow}$
			nd	nd	ERK1/2 signalling$^{\downarrow}$	nd
			nd	nd	RhoA signaling$^{\downarrow}$	RhoA signaling$^{\downarrow}$