The Influence of *Vaccinium angustifolium* (Lowbush Blueberry) Leaf Extract on Trophoblast Biology

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ABSTRACT

Perturbations to extravillous trophoblast (EVT) cell migration and invasion are associated with the development of placenta-mediated diseases. Dietary polyphenols have been shown to influence cell migration and invasion in models of tumorigenesis and non-cancerous, healthy cells; however, never shown in EVT cells. We hypothesize that polyphenols present in *V. angustifolium* leaves will promote trophoblast migration and invasion through ERK and AKT activation. Using the HTR-8/SVneo cell line as a model for EVT cells, the leaf extract increased trophoblast migration and invasion, in an ERK- and AKT-independent manner, and had no effect on cell proliferation or viability. One major polyphenol of the leaf extract was identified and may be an active compound. We have demonstrated for the first time that *V. angustifolium* leaf extract increases EVT migration and invasion *in vitro*, thus further investigations examining potential therapeutic applications of this extract in the context of placenta-mediated diseases are warranted.

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
AGE	Advanced glycation end product
AKT	Protein kinase B
ANOVA	Analysis of variance
BSA	Bovine serum albumin
C_{max}	Maximum concentration
DCM	Dichloromethane
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-related kinase
EVT	Extravillous trophoblast
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3' phosphate dehydrogenase
^1H NMR	Proton nuclear magnetic resonance
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidise
IUGR	Intrauterine growth restriction
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
p-AKT	Phosphorylated AKT
p-ERK	Phosphorylated ERK
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PE	Pre-eclampsia
PI3K	Phosphoinositide 3-kinase
PTFE	Polytetrafluoro-ethylene
RAGE	AGE receptor
RT	Room temperature
SEM	Standard error of the mean
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sFlt-1	Soluble fms-like tyrosine kinase-1
STOX1	Storkhead box 1
TBST	Tris-buffered saline Tween-20
TIMP	Tissue inhibitor of metalloproteinase
T_{max}	Time to reach C_{max}

TNF	Tumour necrosis factor
uPA	Urokinase plasminogen activator
UPLC-MS-TOF	Ultra performance liquid chromatography-mass spectrometry-time of flight
VSMC	Vascular smooth muscle cell

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Finally, I would like to finish by sharing a conversation very dear to me. Julien, Jon, Dan, and I were speaking with Dr. Gruslin in her office and we were trying to express to her how amazing we thought she was. She graciously accepted our compliments but was quick to say, "You're only as good as the people you surround yourself with," and ended up complimenting each of us instead. At first I thought she was being her usual modest self, but now I know what she meant. I am so grateful to have met all of you and could not have asked for a better team. This thesis is dedicated in memory of you, Dr. Gruslin. You are deeply missed but forever in our hearts.

CHAPTER 1: INTRODUCTION

1.1 Overview of placental structure and function

The placenta is a vascular organ of fetal origin formed in the uterus during pregnancy. This vital organ is responsible for all nutrient, gas, and waste exchange between the fetal and maternal circulations and as such, is comprised of a maternal side and a fetal side. The decidua forms the maternal surface of the placenta, while the amnion, chorion, and umbilical cord form the fetal portion of the placenta. The placenta receives blood supply from both the maternal and fetal systems; however, these two circulations never come in direct contact with each other. As such, the placenta acts as a barrier and protects the fetus from being recognized by the mother as a foreign object. Other important roles of the placenta include the synthesis of important growth factors and hormones required to support pregnancy and fetal development.

The functional unit of the placenta is the chorionic villous tree, an intricately branched structure that is bathed in maternal blood, across which all maternal-fetal exchange takes place. These villous trees are comprised of fetal vasculature, stromal tissue, and a bi-layer of trophoblast cells, the primary cell type of the placenta. Trophoblast cells are derived from the trophectoderm layer of the blastocyst. In the human placenta, there are three morphologically and functionally distinct trophoblast populations: cytotrophoblast cells , syncytiotrophoblast cells, and extravillous trophoblast cells (EVTs). Cytotrophoblasts reside along the inner layer of the chorionic villous tree. The cytotrophoblasts act as a trophoblast progenitor cell, actively proliferating across gestation and terminally differentiating to give rise to syncytiotrophoblasts and EVTs. Syncytiotrophoblasts form a continuous, multi-nucleated layer of cells which line the outer surface of the chorionic villous tree. This important cellular layer is in direct contact with the maternal blood within the intervillous space thereby facilitating biochemical interactions between the mother and fetus, including the exchange of nutrients, gases and wastes, and the synthesis and secretion of hormones and growth factors. Conversely, EVTs are situated outside the chorionic villous tree structure and have migratory and invasive phenotypes. This cell type is responsible for invading the uterine wall and remodelling uterine vasculature to

optimize utero-placental blood flow throughout pregnancy. An illustration of the basic structure of the human placenta is represented in Figure 1.

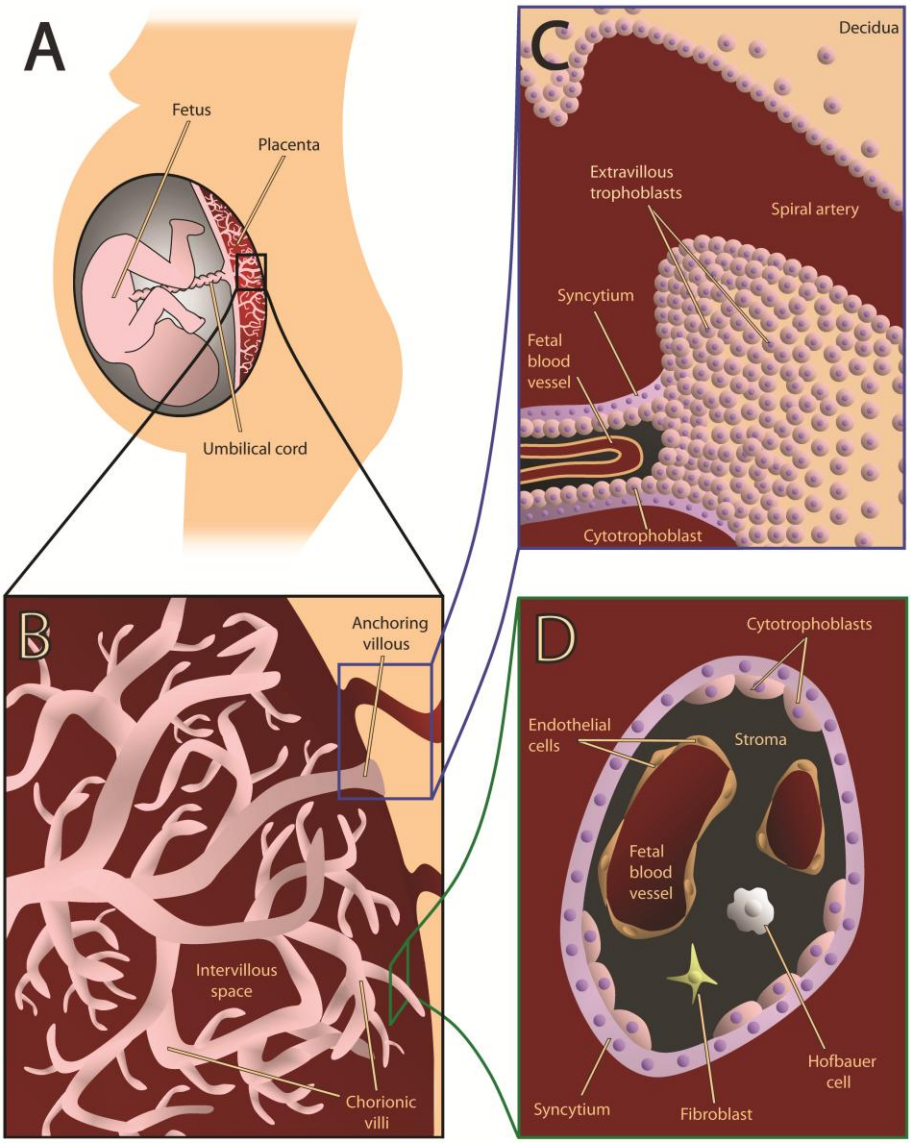

Figure 1. Basic structure of the human placenta. (**A**) The placenta and the developing fetus are connected by the umbilical cord. (**B**) The majority of chorionic villi are free-floating and bathed in maternal blood which fills the intervillous space. At the maternal-fetal interface, these villous trees facilitate all maternal-fetal exchange. (**C**) Approximately 30% of chorionic villi demonstrate a column of extravillous trophoblast cells (EVTs) at their tips and are called anchoring villi. Sufficient migration and invasion of EVTs from the tips of anchoring villi to the uterine decidua are necessary for proper placental anchorage and uterine spiral artery remodelling. (**D**) The chorionic villous is comprised of fetal vasculature, stromal tissue (containing fibroblasts and Hofbauer cells), and a bi-layer of trophoblast cells (cytotrophoblasts and syncytiotrophoblasts). (Figure prepared by Dr. Yockell-Lelièvre, unpublished).

1.2 Establishment of maternal-placental circulation

Sufficient EVT migration and invasion into the decidua is necessary for establishing the maternal-placental circulation via processes such as proper placental anchorage and uterine spiral artery remodelling. The majority of chorionic villi are free-floating within the intervillous space. However, approximately 30% of the chorionic villi (James *et al.*, 2006; Prossler *et al.*, 2014) demonstrate a column of EVTs at their tips which anchor the villous tree, and hence the placenta, to the uterine decidua. From these anchoring EVT cell columns, a population of EVT cells invade deep into the uterine wall and become actively involved in the remodelling of the uterine spiral arteries (Aplin *et al.*, 1998). The uterine spiral arteries are a unique population of tightly coiled blood vessels which provide blood flow to the endometrial lining of the uterus. In a non-pregnant state, these vessels demonstrate a narrow lumen (200 μm in diameter) and a defined layer of vascular smooth muscle cells (VSMCs) which are responsive to vasoactive compounds. In the context of a healthy pregnancy, remodelling of these blood vessels by EVTs leads to the replacement of the vascular endothelial lining by trophoblast cells, a dramatic increase in vessel lumen (up to 2 mm in diameter), and the removal of the overlying vascular smooth muscle cells (Whitley and Cartwright, 2010). These modifications of the uterine spiral arteries are required to support a constant delivery of oxygen and nutrients to the developing fetus.

EVT migration and invasion of the uterine wall occurs in two phases. The initial phase occurs during 8 to 10 weeks of gestation when EVTs migrate from the tips of anchoring villi to the lumen of the spiral arteries (Burton and Jauniaux, 2004). At this point, trophoblasts "plug" the distal ends of these vessels to prevent maternal blood flow into the placental intervillous space. This process creates a physiological state of hypoxia which favours optimal trophoblast cell differentiation (Burton *et al.*, 2003). From 10 to 12 weeks of pregnancy, these trophoblast cell plugs dissipate and a second phase of invasion begins (between 16 to 18 weeks of gestation) to initiate uterine spiral artery remodelling. Uterine spiral artery remodelling is a tightly regulated process requiring precise coordination between VSMCs, endothelial cells, and EVTs (Knöfler and Pollheimer, 2012). EVTs that reach the spiral arteries synthesize and secrete cytokines

and growth factors, including members of the tumour necrosis factor (TNF) family, which induce endothelial cell and VSMC apoptosis, and produce proteolytic enzymes which disrupt extracellular matrix proteins important in maintaining vessel integrity (Whitley and Cartwright, 2010). The apoptotic endothelial cells and VSMCs are removed by phagocytes and other cells with phagocytic activity, including trophoblasts, and the endothelium is temporarily replaced with a trophoblast cell layer. Other regulators of the remodelling process include maternal immune cells (e.g. uterine natural killer cells) and haemodynamic factors (e.g. blood flow) (Harris *et al.*, 2010).

1.3 Molecular regulation of trophoblast migration and invasion

A plethora of paracrine effectors (e.g. growth factors, chemokines, and cytokines secreted by maternal cells), autocrine factors, and cell signalling cascades have been shown to regulate trophoblast migration and invasion in different *in vitro* trophoblast model systems and in *in vivo* animal models (Knöfler and Pollheimer, 2012). These factors regulate downstream targets, including growth factor-dependent matrix metalloproteinases (MMPs; De Oliveira *et al.*, 2010; Zhang *et al.*, 2013) and urokinase plasminogen activators (uPAs; Graham, 1997). EVTs secrete MMPs and uPAs which leads to degradation of the extracellular matrix within the decidua. EVTs also produce respective inhibitors of these matrix degradation enzymes, including tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitors (PAIs). EVT production of enzymes with contrasting function is likely a mechanism to fine tune the degree of invasion within maternal tissue (Lala and Graham, 1990).

Our laboratory has previously reported that both the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways are important in regulating trophoblast migration and invasion (Qiu *et al.*, 2004a). The MAPK family includes a large group of protein kinases called extracellular signal-related kinases (ERKs) and are primarily activated by mitogenic stimuli (Kyriakis and Avruch, 2001). The PI3K signalling cascade is a major signalling component downstream of growth factor-activated tyrosine kinases and G-protein coupled receptors and leads to phosphorylation of AKT (also referred to as protein kinase B). Experimental studies have shown that

growth factor treatment of a human EVT cell line is accompanied by increased cell migration and increased phosphorylation of ERK and AKT (Chakraborty *et al.*, 2003; Qiu *et al.*, 2004a). Another study reported that growth factor treatment activates trophoblast secretion of MMP-9 in an ERK- and AKT-dependent manner and that chemical inhibitors of ERK and AKT phosphorylation in the presence of growth factors block the induction of MMP-9 (Qiu *et al.*, 2004b). These findings suggest that MAPK and PI3K signalling cascades are involved in regulating trophoblast invasion (Figure 2). For these reasons, the MAPK and PI3K cell signalling pathways were investigated in this study. Other proteins, including the insulin-like growth factor (IGF) family, focal adhesion kinase (FAK), Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT) have also been shown to be involved in trophoblast motility (Knöfler and Pollheimer, 2012).

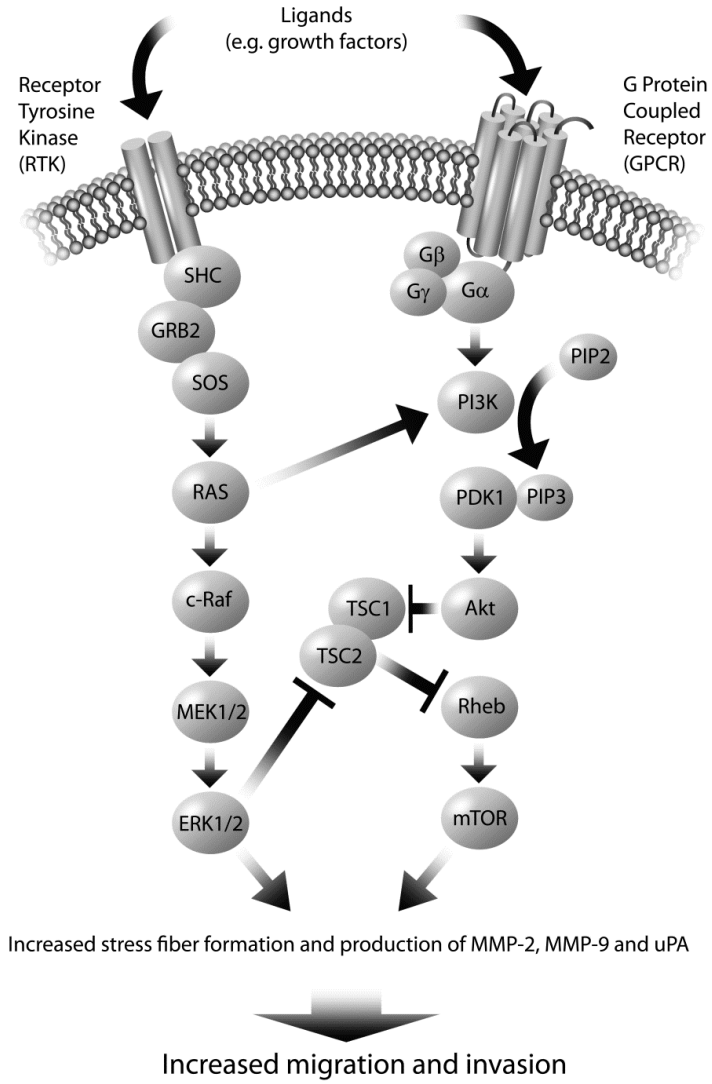

Figure 2. Schematic diagram of MAPK and PI3K signalling cascades. Ligands (e.g. growth factors) bind to receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs) thereby inducing receptor autophosphorylation and recruitment of various scaffolding proteins, respectively. The MAPK (ERK) and P13K pathways are activated through a series of phosphorylation events which leads to the transcription of target genes involved in regulating cellular events, including migration and invasion. (Figure prepared by Dr. Yockell-Lelièvre, unpublished).

SHC: Src homology 2 domain-containing adaptor protein; GRB2: growth factor receptor-bound protein-2; son of sevenless: SOS; RAS: rat sarcoma; c-Raf: cellular Raf; MEK: ERK kinase; ERK: extracellular signal-regulated kinase; G_{α}: G protein alpha subunit; G_{β}: G protein beta subunit; G_{γ}: G protein gamma subunit; PI3K: phosphoinositide 3-kinase; PIP2: phosphatidylinositol 4,5-biphosphate; PIP3: phosphatidylinositol (3,4,5)-triphosphate; PDK1: phosphoinositide-dependent kinase-1; Akt: protein kinase B; Rheb: Ras homolog enriched in brain; mTOR: mammalian target of rapamycin; TSC: tuberous sclerosis protein

1.4 Placenta-mediated diseases

Tight control over trophoblast function is required for successful pregnancy outcomes. Perturbations to trophoblast functions that are essential to placentation, such as trophoblast invasion and uterine spiral artery remodelling, are associated with placenta-mediated diseases, including pre-eclampsia (PE) and intrauterine growth restriction (IUGR). PE affects 3 to 8% of pregnancies and is a leading cause of maternal and fetal morbidity and mortality (Maynard *et al.*, 2008; Anderson *et al.*, 2012). Clinically, PE is characterized by new onset hypertension, proteinuria, and end organ damage from 20 weeks of gestation onwards. Furthermore, one in four cases of PE leads to IUGR (Anderson *et al.*, 2012), a pathological process impeding the growth trajectory of the fetus. Fetuses affected by IUGR are at an increased risk for pre-term delivery, stillbirth, and serious pre- and post-natal health complications that may determine the risk of disease in later life (Barker *et al.*, 1993; Kady and Gardosi, 2004).

Although the exact mechanistic cause of PE is unknown, evidence including that from uteroplacental bed biopsies suggests that insufficient trophoblast invasion and spiral artery remodelling play important roles in the etiology of this disorder (Bronsens *et al.*, 1972; Bronsens *et al.*, 1977). A study conducted by Kadyrov *et al.* (2006) compared trophoblast invasion and degree of spiral artery remodelling between pre-eclamptic and healthy pregnancies. Immunohistochemical analysis of the uterine wall in pre-eclamptic pregnancies demonstrated increased trophoblast apoptosis, shallow trophoblast and sub-optimal remodelling of the uterine spiral arteries. These uterine vessels exhibited a narrow vessel lumen and the maintenance of VSMC. Figure 3 compares trophoblast invasion before vasculature remodelling, and after remodelling in normal and pre-eclamptic pregnancies.

It is postulated that these poorly remodelled vessels, which retain their ability to respond to vasopressors in the maternal circulation, would provide decreased and fluctuating blood flow into the placenta, establishing localized areas of hypoxia-reoxygenation injury. In support of this hypothesis, numerous studies have associated PE with elevated levels of hypoxia and oxidative stress markers within the placenta, such as hypoxia-

inducible factor 1-alpha (Tal, 2012), protein carbonyls, and lipid peroxides (Myatt and Cui, 2004; Burton *et al.*, 2009). As a result of the hypoxic and oxidative stress insults, the ST of the pre-eclamptic placenta responds through increased production of cytokines and anti-angiogenic factors (Smarason *et al.*, 1993; Germain *et al.*, 2007). Furthermore, the ST layer demonstrates evidence of apoptosis and necrosis, and debris from this cellular layer is shed into the intervillous space and carried out into the maternal circulation (Goswami *et al.*, 2006). The transport of placenta-derived pro-inflammatory and anti-angiogenic factors into the maternal circulation is believed to initiate the maternal syndrome of PE, which includes an exaggerated maternal inflammatory response and a further increased production of pro-inflammatory cytokines (Madazli *et al.*, 2003; Redman and Sargent, 2003). Both oxidative stress and inflammation are thought to lead to endothelial cell damage and dysfunction which itself manifests in the many signs and symptoms of PE (Hung *et al.*, 2004). Overall, it is apparent that the pathogenesis of PE is complex and is unlikely due to a single factor, but rather a combination of interconnected elements.

Despite the common occurrence and severity of PE, there is currently no available cure for this disorder aside from delivery of the placenta. A limited number of minimally effective preventative therapies, such as low-dose aspirin and calcium supplements, are available for women at high risk (Duley *et al.*, 2006); however, these treatments only appear to be effective in very specific and narrow patient populations. Studies have examined the prophylactic use of high-dose vitamins C and E supplementation, as antioxidants to counteract the increased oxidative status in PE. However, the majority of randomized clinical trials conducted found this method to be ineffective at preventing PE (Rumbold *et al.*, 2005; Poston *et al.*, 2006; Roberts *et al.*, 2010) and in some instances were associated with an increased risk of adverse pregnancy outcomes (Xu *et al*, 2010). Because of these limited options, further preventative strategies for targeting placenta-mediated obstetrical complications need to be explored.

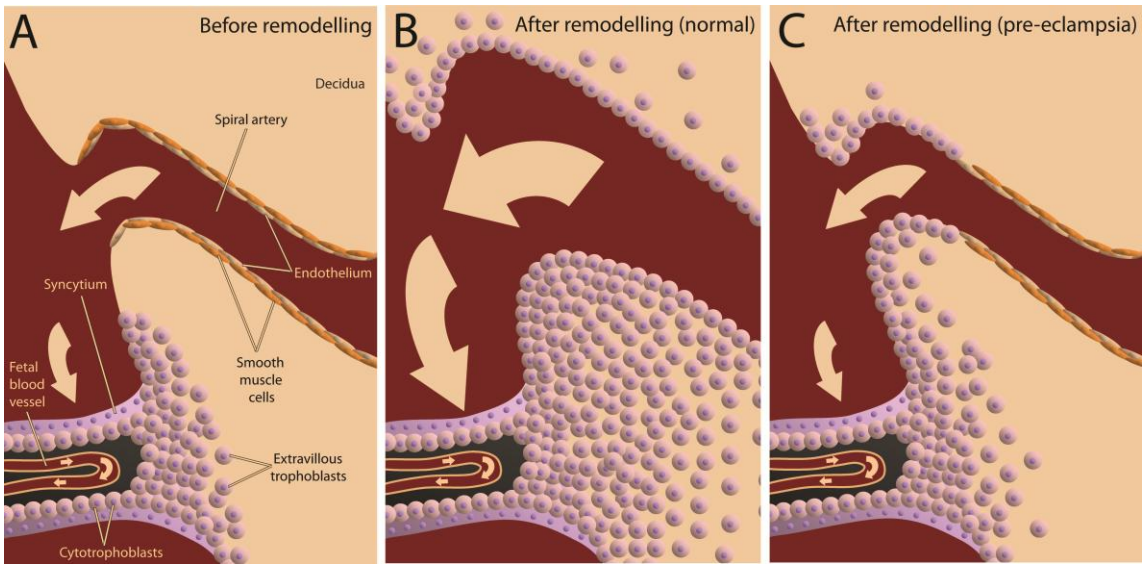

Figure 3. Comparison of trophoblast invasion in normal and in pre-eclamptic pregnancies. (**A**) Prior to the remodelling process, extravillous trophoblasts from the tips of the anchoring villi begin to invade into the decidua. (**B**) In normal pregnancies, trophoblasts invade deep into the decidua and replace the endothelial cells lining and the vascular smooth muscle cells surrounding the spiral arteries. This process results in the formation of dilated vessels with decreased vasoreactivity to help accommodate for the increase in blood flow (indicated with arrows) required by the developing fetus. (**C**) In pre-eclamptic pregnancies, trophoblast invasion is shallow and spiral artery remodelling is impaired. (Figure prepared by Dr. Yockell-Lelièvre, unpublished).

1.5 Models of EVT migration and invasion

As dicussed previously, poor EVT cell migration and invasion into the uterine decidua and maternal spiral arteries is associated with the development of placenta-mediated diseases, including PE. In order to gather a better understanding of the processes that regulate normal and pathological trophoblast migration and invasion, we rely on a limited number of *in vitro* models, including immortalized cell lines, primary EVT cells, and first trimester placental explants. The HTR-8/SVneo cell line is the most widely used human first trimester EVT cell line and is well-characterized in the literature (Graham *et al.*, 1993). Isolated primary human EVT cells, as well as *ex vivo* human explants can be collected from elective first trimester pregnancy terminations, and when cultured in the presence of Matrigel will also maintain their invasive EVT phenotype. These are the best models currently available, however these tissues can be difficult to obtain and have only a short window of viability in culture. Animal models of reduced trophoblast cell migration and invasion have been developed, particularly in the rat and mouse; however, differences in placental structure and the extent of EVT invasion between rodents and humans are some limitations with these animal models. Nonetheless, the use of *in vivo* animal models to complement *in vitro* cell culture and tissue culture studies is important to provide insights into the processes and factors that contribute to the development of placenta-mediated diseases.

1.6 Polyphenols

The term “polyphenol” (also known as phenolic) is used to define compounds exclusively derived from the shikimate/phenylpropanoid and/or the polyketide pathway, featuring more than one phenolic unit and deprived of nitrogen-based functionalities (Quideau *et al.*, 2011). Despite their diverse phytochemical structures, polyphenols can be classified into major groups (Figure 4) including: phenolic acids, stilbenes, lignans, and flavonoids which can be sub-categorized as flavanols, flavonols, flavones, isoflavones, flavanones, and anthocyanins. Polyphenols are the most abundant dietary antioxidants and constitute many plant food sources, including fruits, vegetables, chocolate, wine, coffee, and tea. Although the content of various polyphenols present in food sources varies, the general

distribution and approximate quantities of these compounds in common food items have been summarized (Table 1).

Upon ingestion, non-glycosylated polyphenols are absorbed in the small intestine by passive diffusion whereas glycosylated polyphenols are hydrolysed prior to being absorbed through the intestinal wall. Circulating polyphenols reach the liver and undergo phase I and II metabolism. Following phase I and phase II biotransformation, weakly conjugated polyphenols re-enter circulation and are excreted in the urine. Conversely, highly conjugated polyphenols are excreted in the bile and enter the large intestine where they can be processed by the microflora and then re-absorbed into circulation, or excreted in the feces. Although low levels of polyphenols have been detected in the placenta (Chu *et al.*, 2006; Arola-Arnal *et al*., 2013), the placental pharmacokinetic profile of polyphenols has not been investigated.

Natural polyphenols have garnered significant interest within the scientific community and public media due to emerging evidence which supports a role for polyphenols in the prevention of degenerative diseases, particularly cancer, cardiovascular disease, diabetes, and neurodegenerative disorders (Scalbert *et al.*, 2005). The health benefits of polyphenols have been traditionally attributed to their antioxidant properties (Jovanavic *et al.*, 1996; Brown *et al.*, 1998; Frei and Higdon, 2003). However, more recent evidence suggests that polyphenols can also regulate: (1) gene expression and enzyme activity of key targets related to oxidative stress and inflammation, such as cyclooxygenase (COX; Chan *et al.*, 1997; Williams *et al.*, 1999) and inducible nitric oxide synthase (iNOS; Chan *et al.*, 1997; Chen *et al.*, 2001; Hämäläinen *et al.*, 2007); and (2) cell migration and invasion through various cell signalling pathways, including those that regulate MMPs and TIMPs (Adams *et al.*, 2010; Déziel *et al.*, 2010). The majority of these studies have been conducted in models of chronic disease, thus further investigation is required to determine the relevance of these phenomena in the context of placenta-mediated diseases.

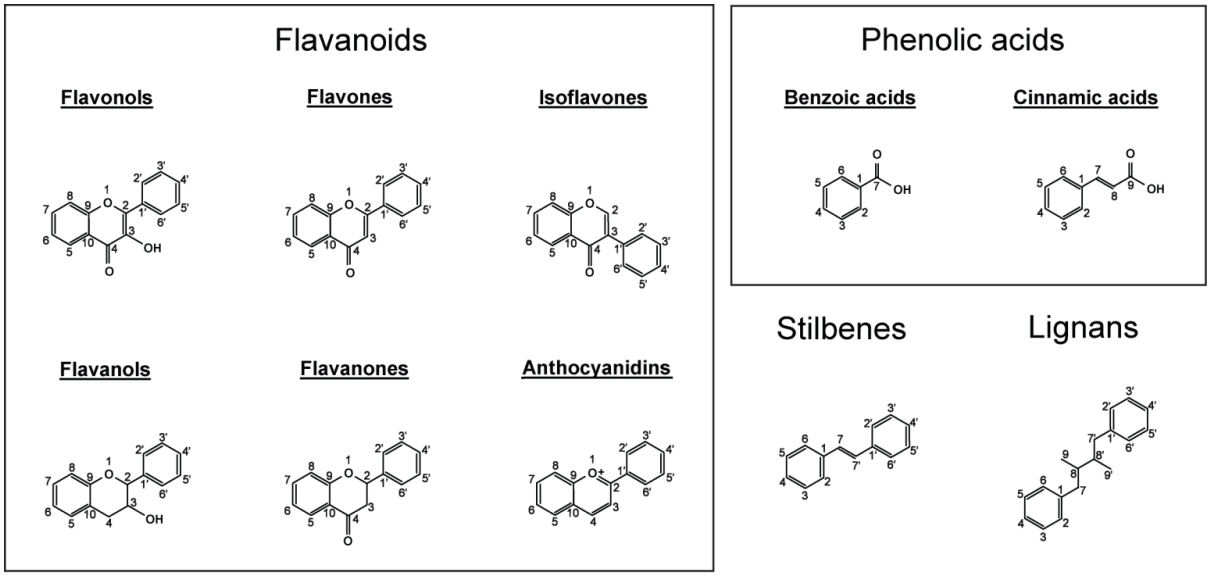

Figure 4. Generic structures of major polyphenol groups. (Figure prepared by Dr. Yockell-Lelièvre, unpublished).

Table 1. Phenolic acid and flavonoid content of selected foods (milligrams/100 g of fresh weight or 100mL of liquids)

Source	Phenolic acids		Flavonoids					
	Benzoic acids	Cinnamic acids	Flavanols	Flavonols	Anthocyanins	Flavones	Isoflavones	Flavanones
Fruit								
Blueberry	0.3-0.7[q]	200-220[l]	1-7[b]	3-16[l]	25-500[p]	0.8[f]	n.d.	0.00[j]
Raspberry	6-10[l]	2-3[l]	3.2-48[b]	n.d.	23-995[g]	n.d.	n.d.	n.d.
Strawberry	2-9[l]	1-3[l]	0.6-12.5[b]	1.5[g]; 1.9[f]	15-75[l]; 78.5-385[g]	0.00-0.03[j]	n.d.	1.8[f]
Vegetables								
Broccoli	15[m]		0.00[j]	0.4[h]; 4-10[l]	0.00[r]; 6[f]	0.8[h]	n.d.	0.00[j]
Celery	1.3[m]		0.00[j]	0.22[j]; 3.5[f]	0.00[r]	1.3[f]; 2-14[l]; 50[j]	n.d.	0.00[j]
Cereal grains								
Rice	20-38[d]		1.6-260[i]					
Beverages								
Coffee	n.d.	n.d.	0.08[e]	0.10[h]	n.d.	0.00[h]	n.d.	n.d.
Green tea	0.8-1.2[k]	n.d.	51.03-324.20[o]	2.81-4.77[j]	n.d.	n.d.	n.d.	n.d.
Red wine	2.2-3.4[p]	0.47-1.1[p]	11.08-18.36[p]	0.77-2.11[p]	19.27-152.98[n]	0.04-0.17[p]	n.d.	2.4[a]
Other								
Dark chocolate	n.d.	n.d.	53.49-108.6[e]	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. indicates that the value has not been determined

[a]Achilli *et al.*, 1993; [b]Arts *et al*., 2000a; [c]Arts *et al*., 2000b; [d]Dykes and Rooney, 2007; [e]Gu *et al*., 2006; [f]Harnly *et al.*, 2006; [g]Heinonen *et al*., 1998; [h]Hertog *et al*, 1993; [i]Huang and Ng, 2012; [j]Justesen *et al.*, 1998; [k]Lin *et al.*, 1998; [l]Manach *et al*., 2004; [m]Mattila and Hellström, 2007; [n]Nyman and Kumpulainen, 2001; [o]Price and Spitzer, 1993; [p]Rodriguez-Delgado *et al.*, 2002; [q]Schuster and Herrmann,1985; [r]Wu *et al*., 2006

1.7 *Vaccinium angustifolium* (lowbush blueberry)

Vaccinium is a genus of shrubs in the plant family Ericaceae, whose fruit products include blueberry, cranberry, and huckleberry. Species of *Vaccinium* are found in many areas around the globe but are mostly scattered across Southeast Asia, North America, and South and Central America (Song and Hancock, 2011; Ferrier, 2014). *V. angustifolium* is a species of blueberry native to eastern and central Canada and the northeastern United States (Brouillet *et al.*, 2006). Leaves from many *Vaccinium* species, including those from *V. angustifolium*, have been used for millennia as traditional medicines (e.g. antiemetics, cold remedies, and diaphoretics) in Bosnia, Herzegovina, and North America (Moerman, 2009; Ferrier, 2014). Currently, *V. angustifolium* is recommended as a natural health product for diabetes treatment in Canada (Martineau *et al.*, 2006). Furthermore, chemical analyses have shown that polyphenols, including phenolic acids and flavonoids, are major metabolites present in the leaf, stem, root, and fruit extracts of several *Vaccinium* species (Ek *et al.*, 2006; Harris *et al.*, 2007; Hokkanen *et al.*, 2009; Ferrier, 2014).

Evidence from epidemiological and intervention studies suggest that dietary intake of blueberries and other plant foods rich in polyphenols may decrease the risk of chronic diseases, including diabetes (Martineau *et al.*, 2006; Muraki *et al.*, 2013), cardiovascular disease (Basu *et al.*, 2010), and cancer (Johnson and Arjmandi, 2013). Although investigation into the underlying mechanisms responsible for the disease-preventing effects of blueberries is limited, studies from *in vitro* and *in vivo* models of cancer, cardiovascular disease, and diabetes provide evidence that extracts from *V. angustifolium* possess antioxidant (Riso *et al.*, 2013) and anti-inflammatory (Torri *et al.*, 2007; Vendrame *et al.*, 2013) properties. For instance, a study conducted by Riso *et al.* (2013) reported that regular consumption of a *V. angustifolium* fruit drink significantly reduced the levels of endogenous and hydrogen peroxide-induced DNA damage in human blood mononuclear cells. Furthermore, Vendrame *et al.* (2013) demonstrated a wild blueberry-enriched diet (8% *V. angustifolium*) decreased plasma concentrations of pro-inflammatory markers, including TNF-α, interleukin-6, and C-reactive protein, in the obese Zucker rat model of the metabolic syndrome. Extracts from *V. angustifolium* leaves

have been reported to have insulin-like properties by promoting glucose transport in differentiated C2C12 muscle cells and protect against glucose toxicity in PC12 neuronal cells (Martineau *et al.*, 2006). For diseases where oxidative stress, inflammation, and hyperglycemia play a role, these findings provide scientific evidence to help explain the proposed health benefits of blueberry consumption.

Blueberry phytochemicals, as well as those from other closely related species, have further been shown to influence cell migration and invasion both *in vitro* and in animal models. The majority of these studies focus on cell motility and invasion in the context of tumorigenesis. *Vaccinium* derived phytochemicals have been shown to suppress the growth and metastatic potential of cancer cells through a variety of cell signalling pathways (Matchett *et al.*, 2005; Adams *et al.*, 2010; Kausar *et al.*, 2012). Adams *et al*. (2010) reported that the fruit juice from *V. angustifolium* decreased MMP-9 activity and uPA secretion while increasing TIMP-1 and PAI-1 secretion in breast cancer cells via inhibition of the P13K pathway. Similarly, polyphenols from *V. macrocarpon* (cranberry) were found to inhibit MMP-2 and MMP-9 activity in prostate cancer cells by down-regulating AKT expression and increasing ERK phosphorylation (Déziel *et al.*, 2010).

In contrast to the results obtained from studies on tumorigenesis, *Vaccinium* extracts have further been demonstrated to increase cell migration in non-cancerous, healthy cells. For instance, Tulio *et al.* (2012) demonstrated that lowbush blueberry (*V. angustifolium*), strawberry, and cranberry extracts enhance human umbilical endothelial cell migration *in vitro* and this effect was associated with AKT activation. This same group also showed that the lowbush blueberry and strawberry extracts could attenuate experimentally-induced impairment of human umbilical endothelial cell migration (Chang *et al.*, 2014), although the mechanism responsible for this effect remains to be elucidated. In another study, topical treatment with phytochemicals from the oils of *V. macrocarpon* was shown to improve the rate of wound-healing in an excision wound model in rats (Nayak *et al.*, 2011). Despite the potential relevance of these phenomena in the context of PE, the effect of *V. angustifolium* on trophoblast biology and placentation has never been determined.

1.8 Rationale for investigation

Emerging evidence from clinical and epidemiological studies suggests that dietary polyphenols, including those found in *V. angustifolium*, play an important role in the prevention of several chronic diseases. These beneficial health claims are supported by experimental data which demonstrate the antioxidant and anti-inflammatory properties of *Vaccinium* extracts, as well as their ability to influence cell migration and invasion. Although these phenomena are particularly relevant in regards to placental development, the effects of *V. angustifolium* and its phytochemicals on trophoblast biology have not been investigated. Therefore, further study into the therapeutic potential of this plant extract for improved placentation in the context of placenta-mediated diseases is warranted. Furthermore, the ever-growing interest and public awareness surrounding the potential benefits of natural health products and polyphenols, in addition to their widespread availability and accessibility through nutritional supplements and fortified foods has led to increased consumption throughout gestation. Thus, understanding the implications of polyphenol intake on placental development is of utmost importance with respect to safe consumption during pregnancy.

1.9 Hypothesis and objectives

Hypothesis

Polyphenols present in *V. angustifolium* leaf extract will promote trophoblast migration and invasion. This effect will be mediated by activation of the MAPK and PI3K pathways through ERK and AKT phosphorylation, respectively.

Objectives

1. Assess the influence of *V. angustifolium* leaf extract on trophoblast biology (i.e. migration, invasion, proliferation, and cell viability).

2. Using series extraction, ultra performance liquid chromatography-mass spectrometry-time of flight (UPLC-MS-TOF), and proton nuclear magnetic resonance (^{1}H NMR), characterize the chemical compounds present in *V. angustifolium* leaf extract and identify the active components.

3. Determine if the effects of *V. angustifolium* leaf extract on trophoblast migration and invasion are associated with changes in ERK and AKT phosphorylation.

CHAPTER 2: MATERIALS AND METHODS

2.1 Collection and preparation of *V. angustifolium* crude leaf extract

V. angustifolium plant material was supplied and prepared by Dr. JT Arnason and Dr. J Ferrier (University of Ottawa, Ottawa, ON, Canada). *Vaccinium angustifolium* Aiton var. *laevifolium* House leaves were collected from Quyon, QC, Canada (latitude +45.59974, longitude -76.09649; University of Ottawa Herbarium Voucher #1591). Leaves were dried overnight at 37°C and stored at room temperature (RT). Dried leaf samples were ground using a Thomas Wiley Mill (Thomas Scientific, Swedesboro, NJ, USA) and passed through a 1 mm mesh prior to extraction. Plant material was extracted twice in 95% ethanol at RT for 24 hours per extraction. The recovered hydroethanolic extractions (hereinafter referred to as "crude extract") were pooled and dried using a Savant SpeedVac rotary evaporator (Thermo Scientific, Ottawa, ON, Canada) at RT for 24 hours. The crude extract was placed in a freezer for 2 hours at -20°C prior to lyophilization at -40°C for 24 hours.

In preparation for the cell culture experiments, a portion of the crude extract was reconstituted in dimethyl sulfoxide (DMSO), filtered through a 0.2 mm polytetrafluoro-ethylene (PTFE) filter (Chromatographic Specialties Inc., Brockville, ON, Canada), and stored at -80°C in aliquots. The remaining portion of the crude extract (i.e. not dissolved in DMSO) was stored at -20°C and subsequently subjected to a four-part series extraction (Section 2.3.1) and further characterized using ultra performance liquid chromatography-mass spectrometry-time of flight (UPLC-MS-TOF; Section 2.3.2), and proton nuclear magnetic resonance (^{1}H NMR; Section 2.3.3) analyses.

2.2 Determining the effects of *V. angustifolium* crude leaf extract on trophoblast biology

2.2.1 *Cell culture model*

The HTR-8/SVneo trophoblast cell line (provided by Dr. C Graham, Queen's University, Kingston, ON, Canada) was utilized for all cell culture experiments. This immortalized first trimester human extravillous trophoblast cell line demonstrates a proliferation,

migration, and invasion phenotype similar to primary extravillous trophoblast cells (Graham *et al.*, 1993). HTR-8/SVneo cells were cultured in RPMI 1640 medium (Thermo Scientific, Logan, UT, USA) containing 10% fetal bovine serum (FBS) and maintained at 37°C and 5% CO_2. Prior to conducting any experimental assays, cells were starved in serum-reduced RPMI 1640 medium supplemented with 0.5% FBS for 24 hours. Cells were harvested using trypsin EDTA (Corning, Manassas, VA, USA) and media containing 10% FBS was added to neutralize the trypsin. Cells were centrifuged at 1200 relative centrifugal force for 5 minutes and supernatant was discarded. The cell pellet was re-suspended using the appropriate medium for the assay. Unless stated otherwise, all treatments, including the control, were prepared in serum-free RPMI 1640 medium containing 0.2% DMSO.

2.2.2 *Migration and invasion assays*

Dose-response studies for both the migration (n=4) and invasion (n=4) assays were conducted in the presence of six different experimental treatments containing *V. angustifolium* crude leaf extract at concentrations ranging from 0 to $2x10^4$ ng/mL.

Trophoblast migration was assessed using a Boyden chamber assay. HTR-8/SVneo cells were re-suspended in the different crude leaf extract experimental treatments, made up in serum-free RPMI media. Cells were seeded at a density of $2.55x10^4$ cells (0.3 mL total volume) into the upper chamber of the Boyden assay (8.0 µm; VWR International, Mississauga, ON, Canada) in a 24-well Companion Plate (VWR International). In the lower chamber, 0.6 mL of corresponding experimental treatment, made up in serum-reduced RPMI media (1% FBS) was added. Following a 24 hour incubation period, the inserts were removed from the wells. Cells adhering to the surface of the membrane were fixed in 10% buffered formalin for 2 minutes at RT and then washed with phosphate buffered saline (PBS). Thereafter, cells were permeabilized using 100% methanol for 20 minutes and washed in PBS prior to staining with hematoxylin for 10 minutes at RT. After two washes in PBS, non-migrating cells which remained on the upper portion of the insert were removed with a cotton swab. Membranes were cut out with a small razor blade and mounted on microscope slides using Shandon Immu-Mount (Thermo

Scientific, Ottawa, ON, Canada). Images from four random fields of view (20X magnification) of each membrane were taken using the EVOS XL Core Imaging System (Life Technologies, Burlington, ON, Canada). Manual cell counts were performed using ImageJ software. The relative migration index for each treatment was reported as a percentage of total migrated cells normalized to the mean percentage of migrated cells in the untreated control group.

Trophoblast invasion was assessed using a modified Boyden chamber assay, with the chamber insert coated in Matrigel, a complex of extracellular matrix proteins. Prior to conducting the invasion assay, Matrigel-coated invasion inserts (24-well, 8 µm; BD Biosciences, Bedford, MA, USA) were removed from -20°C storage and allowed to come to RT. Warm serum-free RPMI media was added to the upper and lower chambers and the inserts were rehydrated for 2 hours in the incubator. After rehydration, the media was carefully removed. HTR-8/SVneo cells were re-suspended in the different crude leaf extract experimental treatments, made up in serum-free RPMI media. Cells were seeded at a density of $2.5x10^4$ cells (1 mL total volume) into the upper chamber of the Boyden insert. An additional 0.5 mL of the experimental treatment was added to the upper chamber. In the lower chamber, 0.75 mL of the corresponding experimental treatment, made up in serum-reduced RPMI media (5% FBS) was added. A FBS concentration gradient was established between the upper and lower chamber (0% FBS vs. 5% FBS) to promote trophoblast invasion from the upper chamber through the Matrigel-coated membrane into the lower chamber. Following a 48 hour incubation period, the invading cells were fixed, permeabilized, and stained, using the methodology described for the migration assay. Snapshots of the entire membrane were taken using the EVOS XL Core Imaging System at 4X magnification and assembled to obtain a single image using ImageJ software. All of the invading cells were counted manually using ImageJ software. The relative invasion index for each treatment was reported as a percentage of total invaded cells normalized to the mean percentage of invaded cells in the untreated control group.

2.2.3 *Proliferation assay*

Trophoblast proliferation was assessed using immunocytochemistry to stain for the nuclear proliferative marker Ki67. This assay was performed three independent times to examine the effects of *V. angustifolium* crude leaf extract (0 and 20 ng/mL) on HTR-8/SVneo cell proliferation. Cells were plated in 8-well Millicell EZ slides (Millipore Corp., Billerica, MA, USA) at a density of $1x10^4$ cells/well. Cells were grown in the different crude leaf extract experimental treatments, made up in complete media (10% FBS), for 24 hours. Cells were then processed for immunocytochemistry as follows.

Without removing the media, acid ethanol (90% ethanol, 5% acetic acid, 5% H_2O) was added to each well. After 5 minutes of incubation at RT, the media/fixative was aspirated and cells were fixed a second time in only acid ethanol for 30 minutes at 4°C. Next, cells were first incubated in 2 M HCl for 20 minutes, then 0.1 M NaB_4O_7 for 10 minutes, followed by 3% H_2O_2 for 10 minutes, all at RT. Slides were rinsed in PBS and placed in 0.01 M sodium citrate buffer (pH 6.0) prior to antigen retrieval using a Decloaking Chamber (Biocare Medical, Concord, CA, USA). In the chamber, slides were exposed to 125°C for 30 seconds and then cooled to 90°C for 10 seconds. Once the cycle was complete, slides were removed immediately from the chamber and cooled with distilled H_2O (dH_2O).

Two drops of Protein Block solution (Dako, Carpinteria, CA, USA) were added to each well and slides were left to incubate at RT for 30 minutes. Thereafter, cells were covered overnight with rabbit anti-human Ki67 primary antibody (1:300 diluted in PBS; Novus Biologicals, Oakville, ON, Canada) at 4°C in a humidity chamber. Slides were washed in PBS for 5 minutes (x3) after which they were incubated with a biotinylated goat anti-rabbit secondary antibody (K0675; Dako) for 1 hour at RT. Following another set of washes (5 minutes x3) in PBS, two drops of streptavidin-horseradish peroxidase (HRP; Dako) was added to each well and slides were incubated for 30 minutes at RT. Slides were thoroughly washed in PBS for 10 minutes (x4) prior to 3', 3'-diaminobenzidine (DAB; Sigma-Aldrich, Oakville, ON, Canada) staining for 10 seconds. Slides were

transferred to PBS for 5 minutes (x3) and then to dH_2O. Cells were counterstained in hematoxylin for 10 seconds and then rinsed several times with tap H_2O before they were dipped in acid ethanol (0.5% concentrated HCl) followed by 1% lithium carbonate solution. Slides were rinsed with tap H_2O before coverslips were applied using mounting media.

Images were taken at 20X magnification with the Aperio ScanScope (Leica Biosystems, St Louis, MO, USA) and ImageJ software was used to conduct manual cell counts of four random fields of view for each experimental treatment. Total cell numbers (in each field of view) were obtained from nuclear counts and all Ki67-immunopositive cells (in each field of view) were counted as proliferating cells. Cell proliferation was reported as a mean percentage of Ki67-positive cells compared to total cell number across all four fields of view.

2.2.4 *Cell viability assay*

Trophoblast viability was assessed using a trypan blue dye exclusion test. Live cells with intact membranes do not allow the trypan blue dye to be absorbed, whereas dead cells are stained blue. This assay was performed on HTR-8/SVneo cells three independent times following incubation with different experimental treatments of *V. angustifolium* crude leaf extract (0 to $2x10^5$ ng/mL). Cells were plated in a 12-well cell culture plate at a density of $8x10^4$ cells/well. Following a 24 hour incubation in the experimental treatment, the cells and their overlying media were collected and cellular viability was assessed using an automated trypan blue staining procedure on the Vi-CELL XR Cell Viability Analyzer (Beckman Coulter, Mississauga, ON, Canada). The number of trypan blue-negative cells (viable cells) was reported as a percentage of the total cell number (viable and non-viable cells).

2.3 Characterization of potential active components in *V. angustifolium* crude leaf extract

2.3.1 *Four-part series extraction*

To identify the active components of the crude extract, a four-part series extraction was performed to separate compounds based on their solubility in different solvents. The following solvents are listed based on their respective order of application (from lowest to highest level of polarity): hexane (least polar solvent), dichloromethane (DCM), acetonitrile (ACN), and water (H_2O; most polar solvent). First, one gram of the crude extract was fractioned using sonication for 30 minutes in 1.0 L of hexane. This process yielded two products: (1) hexane-soluble compounds present in the supernatant and (2) hexane-insoluble compounds remaining in the precipitate. The recovered extract containing hexane-soluble components was collected and then dried using a rotary evaporator (Yamato, Orangeburg, SC, USA). The sample was placed in a desiccator for 48 hours to remove any residual solvent followed by lyophilization at -40°C to remove any leftover water. After, the extract was weighed, dissolved in DMSO, filtered through a 0.2 mm PTFE filter, and stored at -80°C in aliquots. Hexane-insoluble compounds were dissolved in DCM (the next solvent in the series) and the extract collection procedure detailed above was performed. Sequentially, the same procedure was performed on the DCM-insoluble and ACN-insoluble precipitate using ACN and H_2O solvents, respectively. Figure 5 illustrates the series extraction protocol. Using the dried weight of each fraction, the abundance in the crude extract was calculated as a percentage of the total amount of initial plant material used. The percent abundance was then used to estimate the concentration of each fraction in the crude extract.

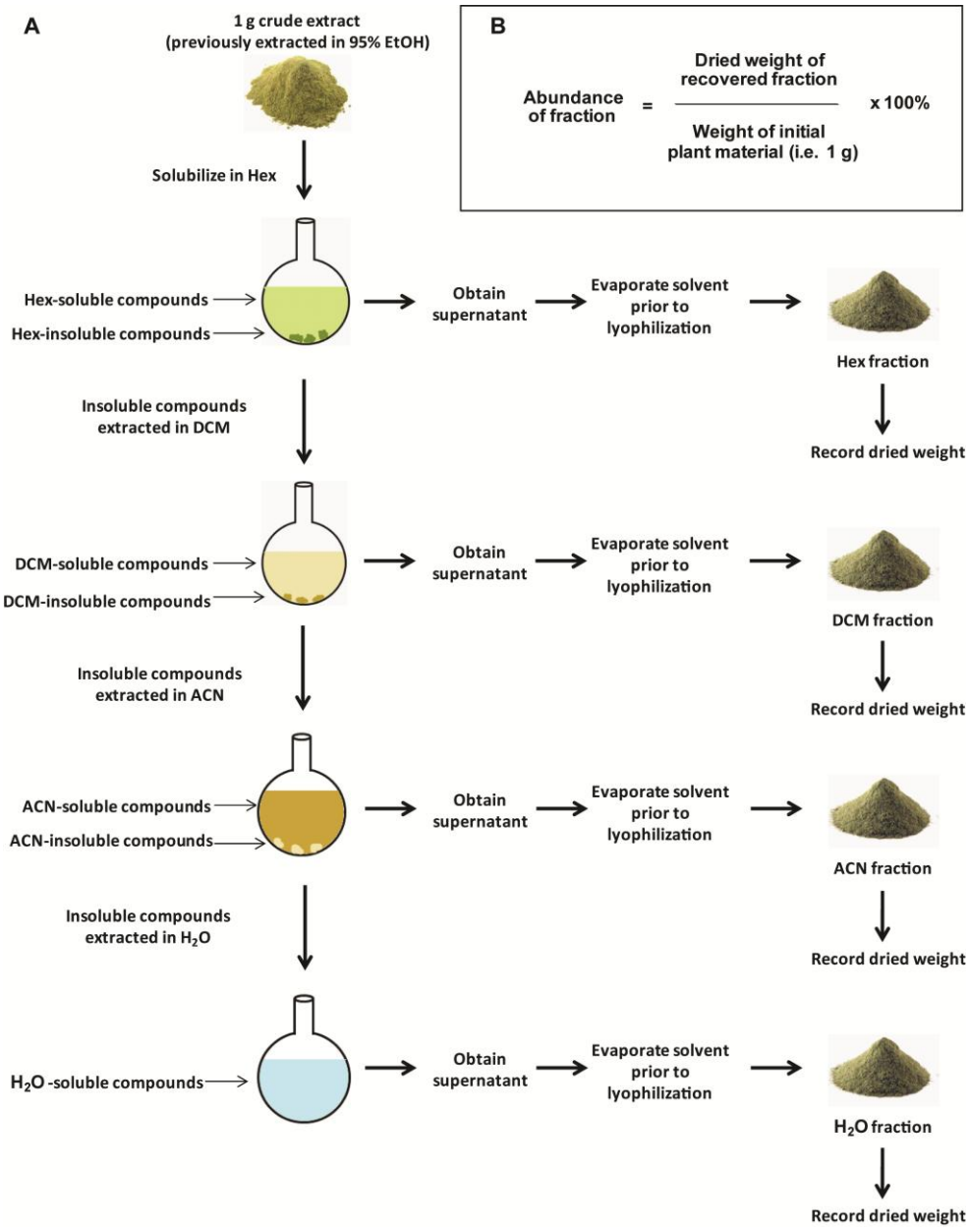

Figure 5. Four-part series extraction of *V. angustifolium* crude leaf extract. (**A**) To separate compounds in the crude extract with similar polarities, a four-step series extraction procedure was conducted using the following solvents listed in their respective order of application: Hexane (Hex), dichloromethane (DCM), acetonitrile (ACN), and water (H_2O). First, one gram of the crude leaf extract was dissolved in Hex. The supernatant containing Hex-soluble compounds was collected and the solvent was evaporated prior to lyophilization. The dried weight of the recovered extract was recorded. Hex-insoluble compounds present in the precipitate were dissolved in DCM and the extract collection process detailed above was repeated. Sequentially, the same procedure was performed on the DCM-insoluble and ACN-insoluble precipitate using ACN and H_2O solvents, respectively. (**B**) The dried weight of the recovered fraction was used to calculate its abundance.

2.3.2 *UPLC-MS-TOF analysis*

A limitation of using the four-part series extraction to characterize and identify potential active compounds within the crude extract is that some compounds may be soluble in more than one of the solvents used. As such, interpreting the effects of the individual fractions on trophoblast biology proved to be challenging. To address this issue, additional chemical characterization of *V. angustifolium* crude leaf extract was conducted in conjunction with Dr. J Ferrier using UPLC-MS-TOF. The crude extract was reconstituted in water: methanol (3:1) and the liquid sample was passed through a Waters ACQUITY UPLC column fitted with a SYNAPT G2 mass spectrometer (Waters Corporation, Milford, MA, USA). The UPLC column was used to separate compounds in the mixture based on polarity. A gradient elution was performed using two mobile phases: (a) water + 0.1% formic acid; and (b) ACN + 0.1% formic acid. Once a compound is eluted from the column, the MS apparatus ionizes it using a positive ion electrospray. This process generates charged molecules that are accelerated by an electric field and eventually reach a detector at a known distance where the flight time is recorded. Since the velocity of the molecule is dependent on the mass-to-charge ratio, the TOF measured can be converted to a mass and then compared to an online database (i.e. ChemSpider) of theoretical masses. Data was profiled with MarkerLynx software (Waters Corporation, Milford, MA, USA), a program used to detect major chromatographic peaks (limit of detection was 10/1 signal-to-noise ratio) and capture data of each fragment (i.e. retention time, ion intensity, and mass), to propose elemental compositions and identify major compounds.

2.3.3 *^{1}H NMR analysis*

Pilot studies were performed to assess the biological effects of three different UPLC-MS-TOF identified compounds, using the trophoblast migration and invasion assays described above. Based on the preliminary data obtained, it was postulated that two compounds identified in the crude leaf extract with biological activity were chlorogenic acid and hyperoside. Thus, the verification and identification of these two compounds in *V. angustifolium* crude leaf extract were performed in conjunction with Dr. J Ferrier using ^{1}H NMR.

^{1}H NMR uses an external applied magnetic field to alter the spin state of protons. Protons that align with the external magnetic field are in a lower energy state than protons that align against the external magnetic field. When protons flip between these two energy states, they emit electromagnetic signals whose frequencies are detected by the NMR spectrometer which then plots the signals on a graph of signal frequency versus signal intensity. Each proton produces a different signal depending on its chemical environment; thus, unlike UPLC-MS-TOF, ^{1}H NMR is able to determine a compound's unique chemical structure in addition to its chemical formula. As such, ^{1}H NMR was used to verify the identity of chlorogenic acid and hyperoside in the crude leaf extract. Briefly, 25 mg of the crude leaf extract was dissolved in 1.0 mL of deuterated DMSO (DMSO-d_6). From this solution, 600 µL was added to a 5 mm ^{1}H NMR tube with 1 µL of 300 mM DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid). ^{1}H NMR spectra were generated using a 600 MHz Bruker AVANCE III Spectrometer equipped with a 5 mm TCI CryoProbe. Standard 1D NOESY (Nuclear Overhauser effect spectroscopy) spectra were recorded. Assure-Raw Material Screening program (Bruker BioSpin, Billerica, MA, USA) was used to identify and quantify chlorogenic acid and hyperoside. Reference standards for chlorogenic acid (Sigma-Aldrich) and hyperoside (Extrasynthese, Genay, France) were used to generate calibration curves.

2.4 Determining the effects of isolated fractions and major components of *V. angustifolium* crude leaf extract on trophoblast biology

2.4.1 *Migration and invasion assays*

To begin characterizing the active components in *V. angustifolium* crude leaf extract, individual extraction fractions, isolated from the four-step series extraction (Section 2.3.1), were tested in the migration (n=3) and invasion (n=4) assays at concentrations that reflected their estimated concentrations in the 20 ng/mL crude leaf extract (Table 3). These assays were performed following the methodology described in Section 2.2.2.

Due to limitations associated in data-interpretation discussed above (Section 2.3.2), three pure compounds (chlorogenic acid, hyperoside, and myricetin) found in the crude leaf

extract, identified from the UPLC-MS-TOF analysis (Section 2.3.2), were also tested in dose-dependent migration (n=3) and invasion (n=4) assays. Chlorogenic acid, hyperoside, and myricetin (Extrasynthese, Genay, France) were first dissolved in DMSO and filtered through a PTFE filter prior to being serially diluted in serum-free RPMI media to generate the different experimental treatments. The concentrations of the compounds tested ranged from 0 to $2x10^4$ ng/mL. The migration and invasion assays were performed following the methodology described in Section 2.2.2.

2.4.2 *Proliferation and cell viability assay*

Trophoblast proliferation and viability were assessed using the protocols described in Section 2.2.3 (n=3) and Section 2.2.4 (n=3), respectively. Myricetin was not found to have biological activity in the migration and invasion assays and as such, was not included in the proliferation or cell viability assays. Hyperoside had no effect on trophoblast migration yet increased trophoblast invasion at a dose of 2000 ng/mL. This concentration was not expected to be physiologically relevant, and as a result hyperoside was not included in the proliferation or cell viability assays. Therefore, only the chlorogenic treatment was evaluated in the proliferation and cell viability assays.

2.5 Determining the effects of *V. angustifolium* crude leaf extract and chlorogenic acid on the activation of ERK and AKT signalling pathways

To determine potential intracellular signalling pathways altered in trophoblast cells following exposure to *V. angustifolium* crude leaf extract or chlorogenic acid, western blot studies specifically examining the phosphorylation status of ERK and AKT were conducted. This assay was performed three independent times. Cells were treated with *V. angustifolium* crude leaf extract (0 and 20 ng/mL) or chlorogenic acid (0 and 200 ng/mL) for 0, 5, 10, 15, 30, or 60 minutes. At the appropriate time points, the media was removed and the cells were rinsed with PBS. Cells were scraped from the bottom of the plate using a 25 cm cell scraper and centrifuged at $18x10^3$ rcf for 5 minutes at 4°C. Cells were resuspended in lysis buffer (containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 10 mM sodium pyrophosphate, 1.5 mM $MgCl_2$, 100 mM NaF, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 µg/mL aprotinin, and 1 mM sodium

orhovanadate) and left on ice for 1 hour. Protein concentrations of the cell lysates were determined using the *DC* (detergent compatible) colorimetric protein assay (Bio-Rad, Mississauga, ON, Canada) before samples were stored in aliquots at -20°C.

Protein samples were diluted in an equal volume of 2X loading buffer under reducing conditions. Twenty µg of protein was resolved using Tris-glycine gel (10% separating, 5% stacking) via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins in the gel were transferred onto a nitrocellulose membrane for 2 hours at 25 V. Membranes were blocked for 1 hour in blocking solution (Tris buffered saline with 0.05% Tween 20 [TBST] and 5% dehydrated non-fat milk). Membranes were then probed overnight at 4°C with primary antibody (directed against ERK, p-ERK, AKT, p-AKT, α-tubulin, or GAPDH; please see Table 2) diluted in blocking solution containing 0.02% sodium azide. The next day, membranes were washed for 10 minutes (x4) with TBST. After, membranes were probed for 1 hour at RT with either a goat anti-rabbit or goat anti-mouse HRP-conjugated secondary antibody (1:10,000 in blocking solution; Bio-Rad) and then washed for 10 minutes (x4) with TBST. Immunosignals were detected using the Amersham enhanced chemiluminescence detection kit (GE Healthcare Bio-Sciences Inc., Baie d'Urfe, QC, Canada). Band intensities were determined using GeneGnome XRQ and GeneSnap software (Syngene, Frederick, MD, USA). Total and phosphorylated protein levels were normalized to the loading control (α-tubulin used for ERK and p-ERK blot; GAPDH used for AKT and p-AKT blot) prior to determining changes in the level of phosphorylated protein (phosphorylated protein/total protein). Results were normalized to the untreated group at time zero.

2.6 Statistical analysis

GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA) was used to analyze the data. All data are presented as mean values $\pm$ standard error of the mean (SEM). All dose-response studies (i.e. migration, invasion, and viability assays) and time-course assays (i.e. ERK and AKT western blots) were analyzed with one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test. In cases where only two

experimental groups were compared (i.e. proliferation assay), a student *t*-test was performed. Statistical significant differences were inferred at a $p<0.05$.

Table 2. Primary antibodies used for Western blot analysis

	Antigen (Product #)	Species Raised Against	Species	Monoclonal or Polyclonal	Working Dilution	Manufacturer
(a)	α-tubulin (CP06)	Mouse	Mouse	Monoclonal	1:1000	EMD Millipore, Billerica, MA, USA
(b)	AKT (4691)	Mouse	Rabbit	Polyclonal	1:1000	Cell Signaling Technology, Danvers, MA, USA
(c)	ERK (4695)	Rat	Rabbit	Monoclonal	1:1000	Cell Signaling Technology, Danvers, MA, USA
(d)	GAPDH (3683)	Human	Rabbit	Monoclonal	1:5000	Cell Signaling Technology, Danvers, MA, USA
(g)	p-AKT (4060)	Human	Rabbit	Monoclonal	1:1000	Cell Signaling Technology, Danvers, MA, USA
(h)	p-ERK (4370)	Human	Rabbit	Monoclonal	1:1000	Cell Signaling Technology, Danvers, MA, USA

CHAPTER 3: RESULTS

3.1 *V. angustifolium* crude leaf extract preparation

Approximately 13.4 g of *V. angustifolium* leaves were harvested and processed, and 1360 mg of the crude extract was obtained. The percent yield was roughly 10%.

3.2 Effects of *V. angustifolium* crude leaf extract on trophoblast biology

3.2.1 *Effects of V. angustifolium crude leaf extract on trophoblast migration and invasion*

Dose-response studies using six concentrations of *V. angustifolium* crude leaf extract, ranging from 0 to $2x10^4$ ng/mL, were conducted to determine its effects on HTR-8/SVneo cell migration and invasion (Figure 6). At 20 ng/mL, the extract increased trophoblast migration and invasion by roughly 1.5- and 3-fold, respectively ($p<0.01$). Similar increases in migration and invasion were also observed at 200 ng/mL ($p<0.01$). Trophoblasts treated with $2x10^4$ ng/mL of the extract displayed a significant decrease in migratory ability ($p<0.01$); however, at this concentration, there was no effect on cell invasion. Doses of 2 and 2000 ng/mL did not significantly influence trophoblast migration or invasion.

3.2.2 *Effects of V. angustifolium crude leaf extract on trophoblast proliferation and cell viability*

To assess if the effects of the crude leaf extract on cell migration and invasion were attributed to changes in cell proliferation and/or cell viability, Ki67 immunoreactivity (Figure 7) and trypan blue dye exclusion assays (Figure 8) were performed, respectively. Results were compared between experimental treatment and untreated control groups. At 20 ng/mL, a concentration found to optimally promote trophoblast migration and invasion, the crude leaf extract had no effect on trophoblast cell proliferation or cell viability ($p>0.05$). However, the crude leaf extract significantly decreased trophoblast cell viability at $2x10^5$ ng/mL ($p<0.001$), indicating potential cytotoxicity at this higher concentration.

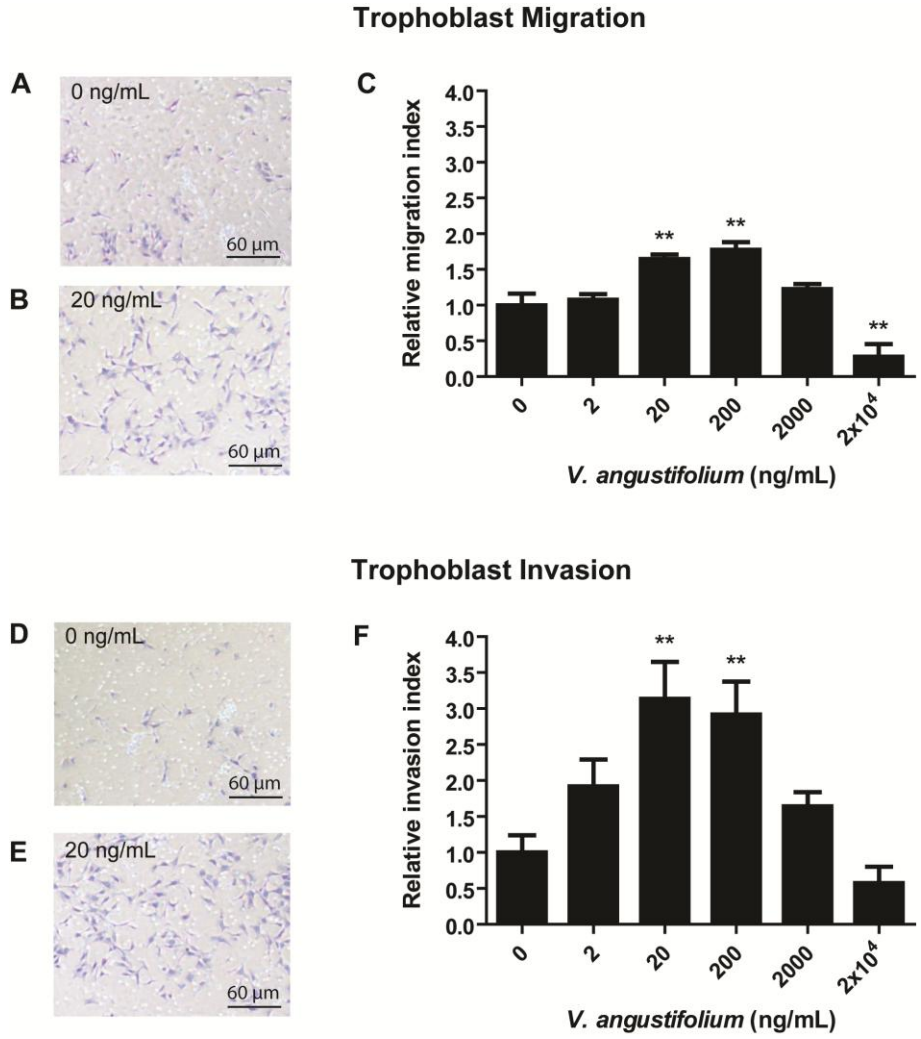

Figure 6. The effects of *V. angustifolium* crude leaf extract on trophoblast migration and invasion. Migration of HTR-8/SVneo cells across porous (8 µm) Boyden chamber membranes in the presence of increasing concentrations of crude leaf extract was assessed at 24 hours (**A-C**). Representative images of migrated cells treated with no extract (**A**) or 20 ng/mL crude leaf extract (**B**). The relative migration index was reported as a percentage of total migrated cells normalized to the mean percentage of migrated cells in the untreated control group (**C**). Cell counts were performed on four random fields of view (20X magnification) per experimental treatment. Invasion of HTR-8/SVneo cells across Matrigel-coated porous (8 µm) Boyden chamber membranes in the presence of increasing concentrations of crude leaf extract was assessed at 48 hours (**D-F**). Representative images of invaded cells treated with no extract (**D**) or 20 ng/mL crude leaf extract (**E**). The relative invasion index was reported as a percentage of total invaded cells normalized to the mean percentage of invaded cells in the untreated control group (**C**). Cells counts were performed for the entire membrane (4X magnification) per experimental treatment. All data are presented as mean ± SEM (n=4). Asterisks indicate a significant difference compared to the untreated control group. Data was analyzed by one-way ANOVA and Bonferroni *post hoc* test (**p<0.01).

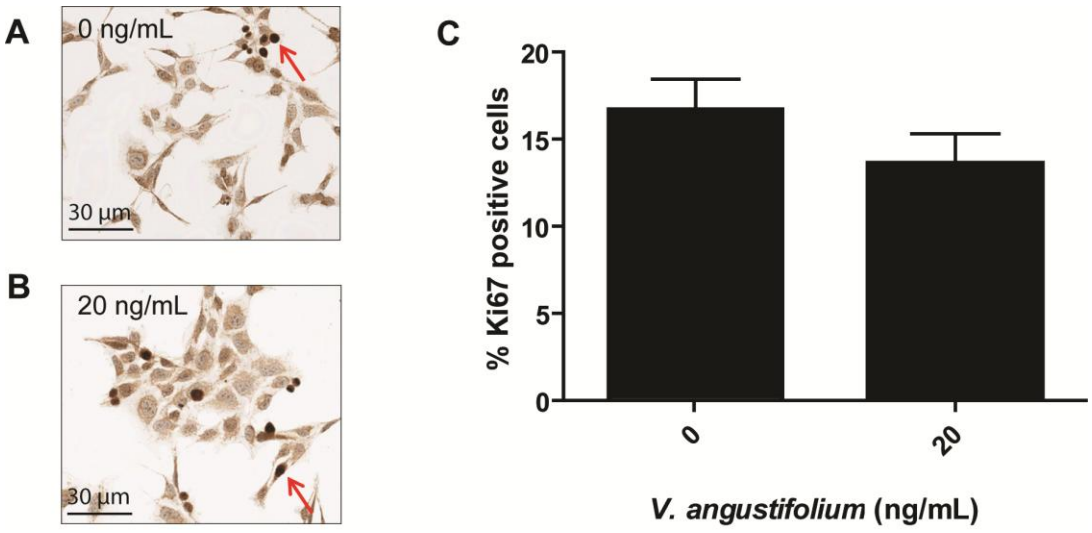

Figure 7. The effect of *V. angustifolium* crude leaf extract on trophoblast proliferation. HTR-8/SVneo cells were treated with 20 ng/mL of the crude leaf extract for 24 hours followed by Ki67 immunocytochemical staining. Representative images of untreated cells (**A**) or crude leaf extract treated cells (**B**). Proliferating cells were identified through nuclear immunoreactivity to Ki67 (red arrows). Total cell numbers were obtained from hematoxylin identified nuclear counts. The number of Ki67-positive cells was expressed as a percentage of total cell number (**C**) by performing cell counts in four random fields of view (20X magnification) per experimental treatment. All values are presented as mean $\pm$ SEM (n=3). Data was analyzed using a student *t*-test.

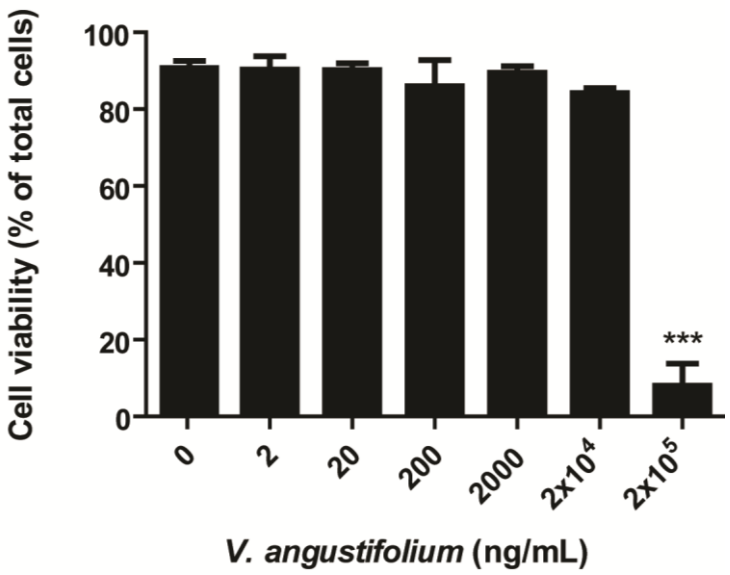

Figure 8. The effect of *V. angustifolium* crude leaf extract on trophoblast viability. HTR-8/SVneo cells were treated with different concentrations of crude leaf extract for 24 hours and cellular viability was determined using the trypan blue dye exclusion test. Cellular viability was expressed as a percentage of trypan blue-negative cells (viable cells) compared to total cell number (both viable and non-viable cells). All data are presented as mean $\pm$ SEM (n=3). Asterisks indicate a significant difference compared to the untreated control group. Data was analyzed by one-way ANOVA and Bonferroni *post hoc* test (***p<0.001).

3.3 Characterization of potential active components in *V. angustifolium* crude leaf extract

3.3.1 *Isolation of four distinct fractions of V. angustifolium crude leaf extract*

As a first attempt to narrow the search for active compounds within the crude leaf extract, a four-part series extraction was performed to separate groups of compounds (i.e. fractions) in the crude extract with similar chemical properties. One gram of the crude leaf extract was used as the starting material. Following sequential extractions in four different solvents (hexane, dichloromethane [DCM], acetonitrile [ACN], water [H_2O]), a cumulative yield of 0.88 grams (88%) of plant material was collected across all four isolated fractions. The abundance of each fraction in the original crude extract was estimated based on the mass of product obtained from each of the extractions. The compounds present in the hexane, DCM, ACN, and H_2O fractions were estimated to represent 21%, 6%, 46%, and 15% of the total compounds in the crude extract, respectively (Figure 9). There was no remaining precipitate following the final extraction with H_2O. Based on these abundances, the concentrations of each fraction in the 20 ng/mL crude extract treatment were estimated to be 4.2, 1.2, 9.2, and 3.0 ng/mL for the hexane, DCM, ACN, and H_2O fractions, respectively (Table 3).

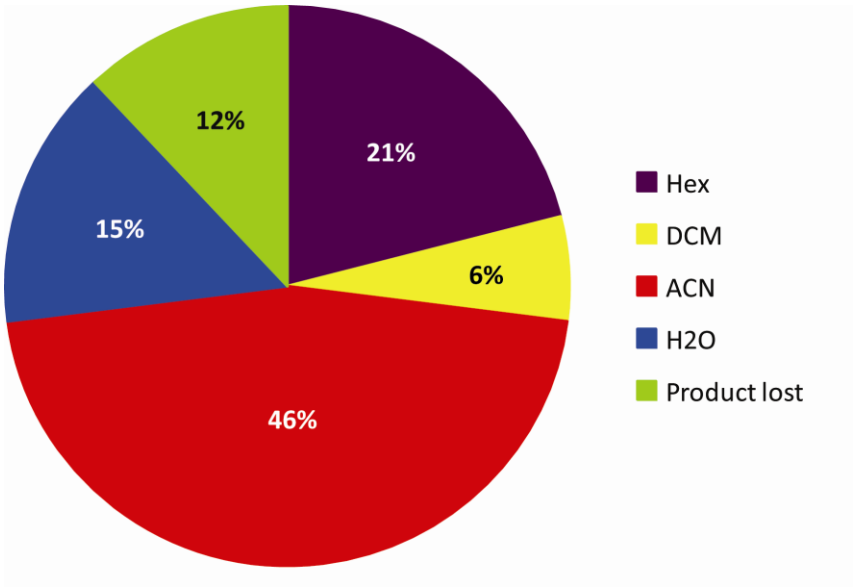

Figure 9. Abundances of four fractions obtained from a series extraction of *V. angustifolium* crude leaf extract. One gram of crude extract was fractioned using the following solvents with increasing polarity listed in their respective order of extraction: hexane (Hex), dichloromethane (DCM), acetonitrile (ACN), and water (H_2O). After each extraction, the solvent was evaporated and the extract was placed in a desiccator prior to lyophilization. The fraction was weighed and the amount of product obtained was used to calculate the abundance of the fraction as a percentage of the total yield. The total yield from the series extraction was 88%, thus 12% of the plant material was lost in the fractionation procedure.

Table 3. Estimated concentrations of individual fractions in *V. angustifolium* crude leaf extract. All concentrations were estimated for the 20 ng/mL crude extract treatment according to the abundance of each fraction. The fractions were obtained through a four-part series extraction using hexane (Hex), dichloromethane (DCM), acetonitrile (ACN), and water (H_2O).

Fraction	Abundance (%)	Estimated concentration in the 20 ng/mL crude leaf extract (ng/mL) [Abundance x 20 ng/mL]
Hex	21	4.2
DCM	6	1.2
ACN	46	9.2
H2O	15	3.0

3.3.2 *Identification of major components within V. angustifolium crude leaf extract using UPLC-MS-TOF*

Due to limitations in the interpretation of data collected using the leaf extract fractions described in Section 2.3.2, more sophisticated methods were required to further characterize the chemical compounds present in *V. angustifolium* crude leaf extract. Therefore, the major chemical compounds present within the crude leaf extract were further analyzed using ultra performance liquid chromatography-mass spectrometry-time of flight (UPLC-MS-TOF). Chromatographic peak data (i.e. retention time, ion intensity, and mass) for all major peaks was cross-referenced with data from the ChemSpider chemical database. Analysis of the elemental compositions of the major identified peaks suggests that polyphenols are major components of the crude extract (Figure 10, Table 4). The phenolic compounds identified thus far include chlorogenic acid (peak 1), myricetin (peak 2) and hyperoside (peak 3). Moreover, the chemical compositions of peaks 4 and 5 have also been characterized; however, their identities are still under investigation.

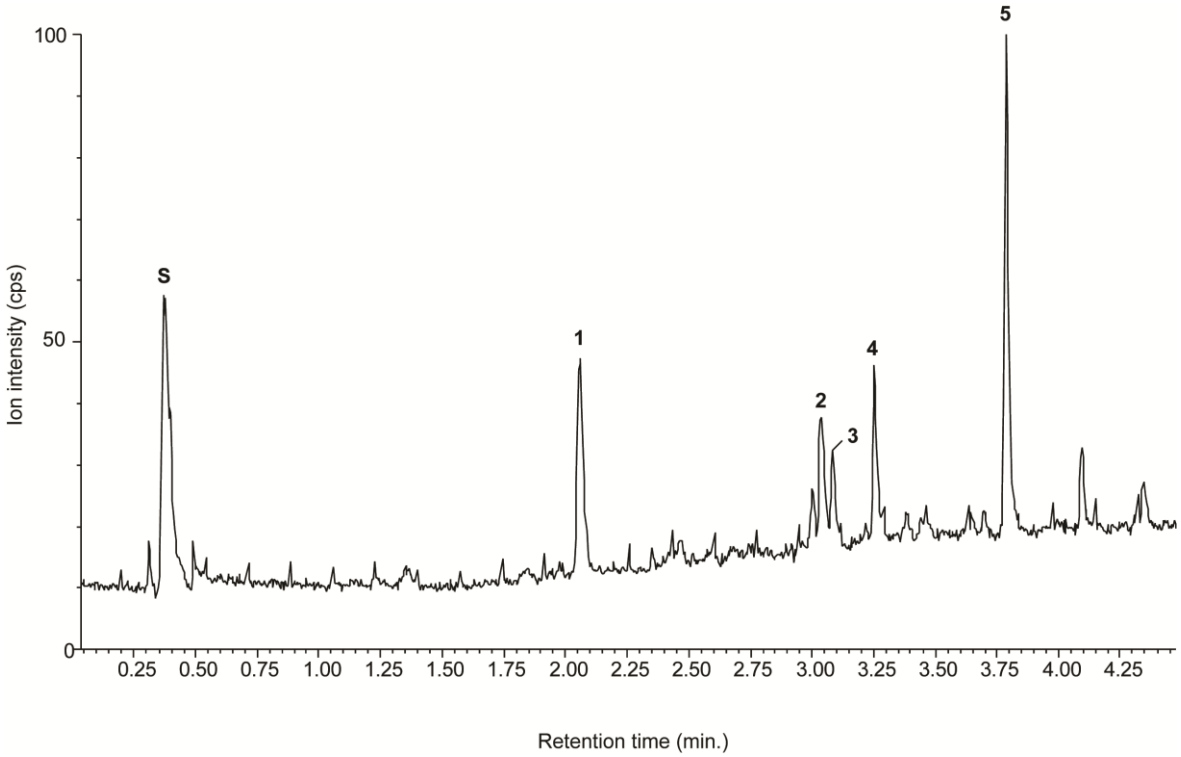

Figure 10. Ultra performance liquid chromatography-mass spectrometry-time of flight (UPLC-MS-QTOF) chromatogram for *V. angustifolium* crude leaf extract. Crude leaf extract was reconstituted in water: methanol (3:1) and analyzed using ultra performance liquid chromatography and mass spectrometry. The retention time of each peak is displayed in minutes and the signal intensity of the ions is measured as cycles per second (cps). Cross-reference of chromatographic data (i.e. retention time, ion intensity, and mass) with available chemical databases identified peaks 1, 2, and 3 as the phenolic compounds chlorogenic acid, myricetin, and hyperoside, respectively. The elemental compositions for peaks 4 and 5 have been determined; however, their identities are still under investigation. Peak S represents the solvent (i.e. water and acetonitrile, 0.1% formic acid). Figure adapted with permission from Ferrier *et al.*, unpublished.

Table 4. Identity of selected compounds from *V. angustifolium* crude leaf extract chromatogram. The elemental composition and identification of selected peaks in the chromatogram were determined using information provided by MarkerLynx software and the online ChemSpider chemical databases. Asterisks indicate that the identity of the compound has been verified in the crude extract using proton nuclear magnetic resonance. The abbreviation n.d. indicates that the information has not yet been determined. Table adapted with permission from Ferrier *et al.*, unpublished.

Peak #	Elemental composition	Identification	Class
1	$C_{16}H_{19}O_9$	Chlorogenic acid*	Phenolic acid-derivative
2	$C_{21}H_{21}O_{12}$	Myricetin	Flavonoid
3	$C_{21}H_{21}O_{12}$	Hyperoside*	Flavonoid
4	$C_{27}H_{31}O_{15}$	n.d.	n.d.
5	$C_{40}H_{71}O_4$	n.d.	n.d.

3.3.3 *Quantification of chlorogenic acid and hyperoside in V. angustifolium crude leaf extract using ^{1}H NMR*

Proton nuclear magnetic resonance (^{1}H NMR) was used to verify the identities and quantify the concentrations of two compounds initially identified within the crude leaf extract using UPLC-MS-TOF and postulated to demonstrate biological activity; chlorogenic acid (peak 1, Figure 10) and hyperoside (peak 3, Figure 10). Quantification via ^{1}H NMR determined that chlorogenic acid and hyperoside account for 7.14% and 2.27% of the total crude extract, respectively. Using these determined abundances, the concentrations of chlorogenic acid and hyperoside in the 20 ng/mL crude extract treatment were calculated to be approximately 1.43 and 0.45 ng/mL, respectively (Table 5).

Table 5. Validation and quantification of chlorogenic acid and hyperoside in *V. angustifolium* crude leaf extract using proton nuclear magnetic resonance (^{1}H NMR). Crude extract was reconstituted in deuterated DMSO and analyzed using ^{1}H NMR. Reference standards and Assure-RMS (Raw Material Screening) software were used to identify and quantify chlorogenic acid and hyperoside in the crude leaf extract sample. The concentration of each compound is reported as a percentage of the total extract. Based on this abundance, the concentration of chlorogenic acid and hyperoside in the 20 ng/mL crude extract treatment was calculated. Table adapted with permission from Ferrier, 2014.

Compound	Abundance in the crude extract (%)	Concentration in the 20 ng/mL crude extract (ng/mL) [Abundance x 20 ng/mL]
Chlorogenic acid	7.14	1.43
Hyperoside	2.27	0.45

3.4 Effects of isolated extraction fractions and major components of *V. angustifolium* crude leaf extract on trophoblast biology

3.4.1 *Effects of extraction fractions on trophoblast migration and invasion*

In a first attempt to narrow down the active components present in the crude leaf extract capable of promoting trophoblast migration and invasion, the isolated serial solvent fractions (Section 3.3.1) were tested for their ability to promote trophoblast migration and/or invasion (Figure 11) using the Boyden chamber assay. The hexane, DCM, and ACN fractions increased trophoblast migration approximately 2.5-fold compared to the untreated control group ($p<0.05$). Moreover, the hexane and ACN treatments increased trophoblast migration to a greater extent than the 20 ng/mL crude leaf extract treatment ($p<0.05$). Only the hexane fraction was shown to significantly increase trophoblast invasion by approximately 3.5-fold; a similar effect seen with the 20 ng/mL leaf crude extract treatment. Conversely, trophoblast cells exposed to the the ACN and H_2O fractions were notably less invasive than those treated with the original crude leaf extract.

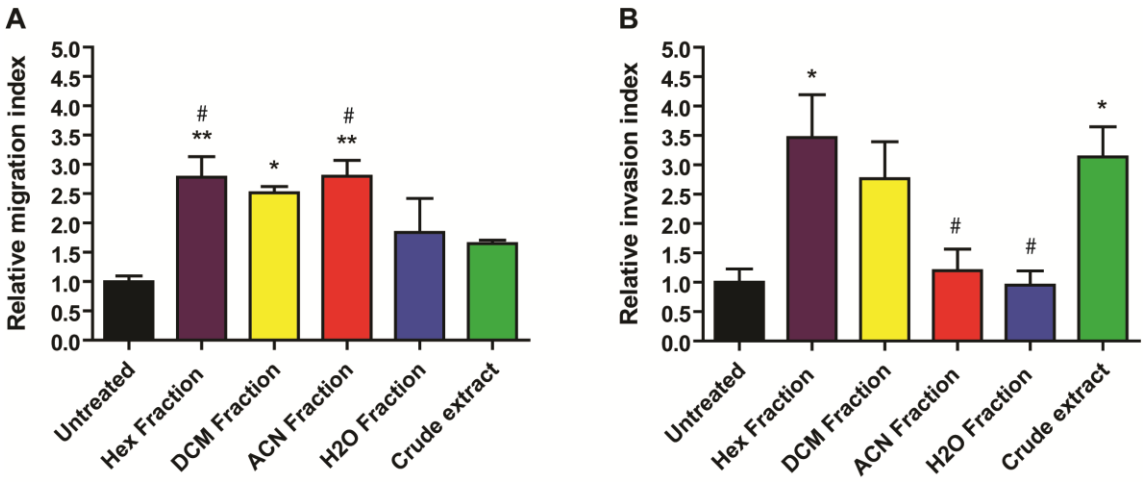

Figure 11. The effects of *V. angustifolium* leaf extract fractions on trophoblast migration and invasion. A four-part series extraction of the crude extract was performed using hexane (Hex), dichloromethane (DCM), acetonitrile (ACN), and water (H_2O). The concentration of each fraction tested was reflective of that estimated in the 20 ng/mL crude leaf extract. The ability of each fraction to promote trophoblast migration **(A)** and invasion **(B)** was assessed using a Boyden chamber migration assay (n=3) and a Matrigel-coated Boyden chamber invasion assay (n=4), respectively. In both cases, the effects of the treatment with each individual fraction were compared to both untreated controls and treatment with the original 20 ng/mL crude leaf extract. The number of migrated or invaded cells were obtained from nuclear counts and normalized to the untreated control group. All data are presented as mean $\pm$ SEM. Significant differences are indicated with the symbols * (data compared to the untreated group) and # (data compared to the crude extract). Data was analyzed by one-way ANOVA and Bonferroni *post hoc* test (*p<0.05, **p<0.01, #p<0.05).

3.4.2 *Effects of selected polyphenols present in V. angustifolium crude leaf extract (chlorogenic acid, myricetin, and hyperoside) on trophoblast migration and invasion*

Chlorogenic acid, myricetin, and hyperoside were identified to be major components in the crude leaf extract using UPLC-MS-TOF (Section 3.3.2). As such, the ability of each compound to promote trophoblast migration and invasion was assessed individually using a Boyden chamber migration assay and a Matrigel-coated Boyden chamber invasion assay, respectively (Figure 12). Dose-response studies using five concentrations ranging from 0 to 2000 ng/mL were performed for each of the three compounds. At 200 ng/mL chlorogenic acid was found to significantly increase both trophoblast cell migration and invasion by roughly 5- and 4-fold, respectively ($p<0.01$). The other doses of chlorogenic acid did not significantly affect trophoblast migration or invasion; however, there appeared to be a dose-dependent response. Myricetin treatment had no effect on cell migration or invasion at any of the concentrations tested, while hyperoside increased trophoblast invasion only at 2000 ng/mL ($p<0.01$). As myricetin and hyperoside did not appear to have a significant biological effect on trophoblast cell migration and invasion at experimentally relevant concentrations, they were not included in further studies of trophoblast cell proliferation and viability.

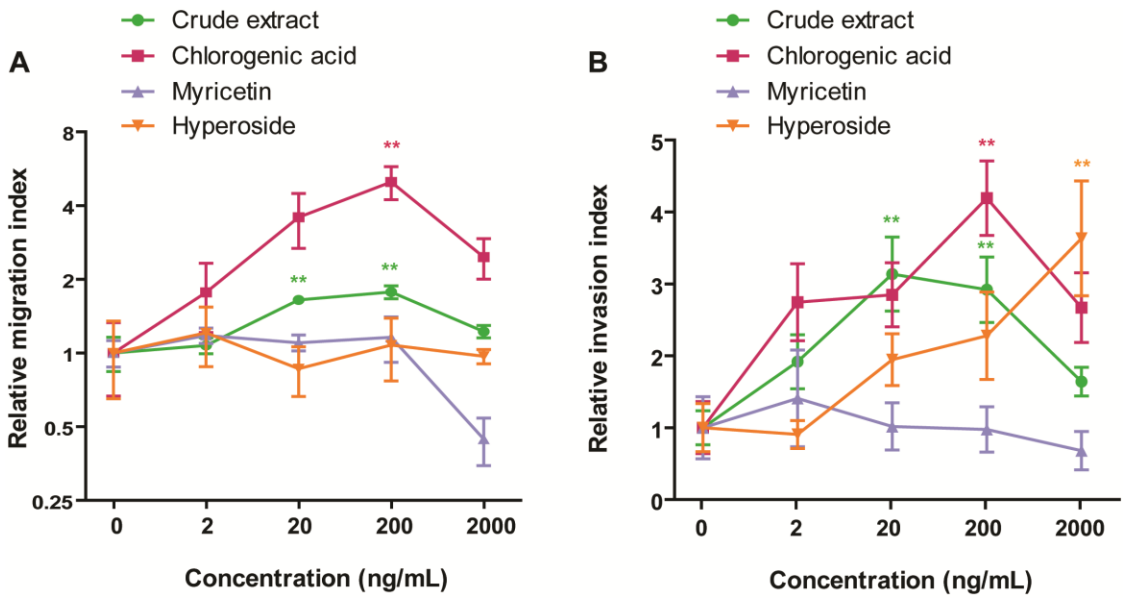

Figure 12. The effects of selected polyphenols in *V. angustifolium* crude leaf extract on trophoblast migration and invasion. HTR-8/SVneo cells were treated with the crude leaf extract, chlorogenic acid, myricetin, or hyperoside. Migration of trophoblast cells across porous (8 µm) Boyden chamber membranes in the presence of increasing concentrations of each experimental treatment was assessed at 24 hours (**A**; n=3). Invasion of trophoblast cells across Matrigel-coated porous (8 µm) Boyden chamber membranes in the presence of each experimental treatment was assessed at 48 hours (**B**; n=4). Number of migrated and invaded cells were obtained from nuclear counts and normalized to the untreated control group. All data are presented as mean ± SEM. Asterisks indicate a significant difference compared to the untreated control group (reference value of 1). Data was analyzed by one-way ANOVA and Bonferroni *post hoc* test (**p<0.01).

3.4.3 *Effects of chlorogenic acid on trophoblast cell proliferation and viability*

To assess if the effects of chlorogenic acid on cell migration and invasion were attributed to changes in cell proliferation and/or cell viability, Ki67 immunocytochemical expression levels and trypan blue dye exclusion assays were performed, respectively. Results were compared between experimental treatment and untreated control groups. At 200 ng/mL, a concentration found to significantly increase trophoblast migration and invasion, chlorogenic acid had no effect on trophblast cell proliferation ($p>0.05$, Figure 13) or cell viability ($p>0.05$, Figure 14). However, chlorogenic acid significantly decreased trophoblast cell viability at $2x10^5$ ng/mL ($p<0.001$), indicating potential cytotoxicity at this higher concentration.

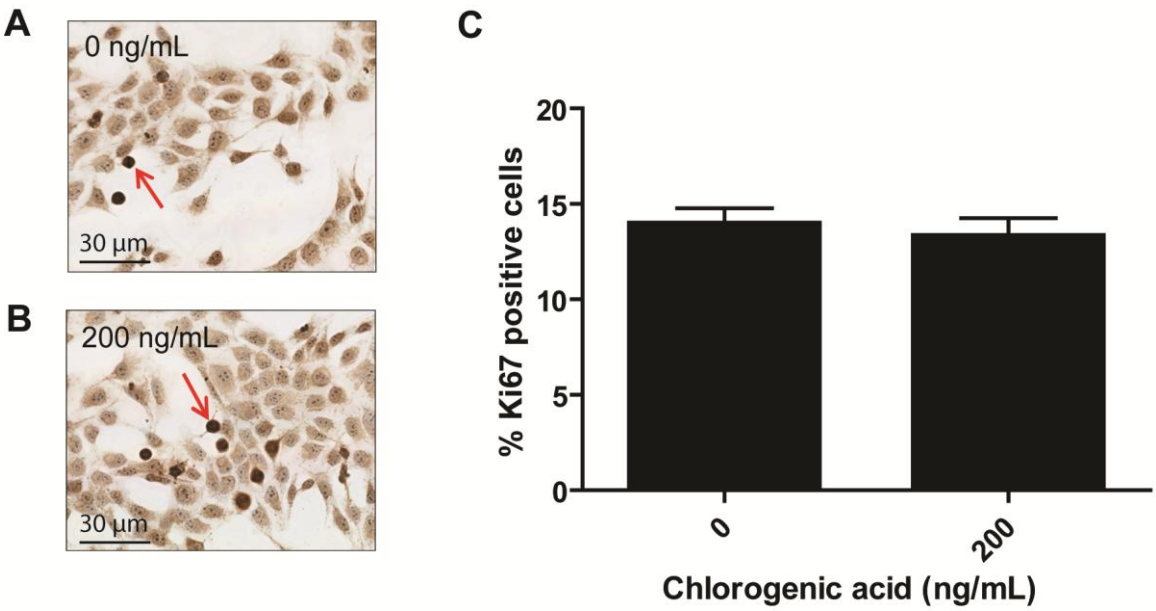

Figure 13. The effect of chlorogenic acid on trophoblast proliferation. HTR-8/SVneo cells were treated with 200 ng/mL chlorogenic acid for 24 hours followed by Ki67 immuncytochemical staining. Representative images of untreated cells (**A**) or chlorogenic acid treated cells (**B**). Proliferating cells were identified through nuclear immunoreactivity to Ki67 (red arrows). Total cell numbers were obtained from hematoxylin identified nuclear counts. The number of Ki67-positive cells was expressed as a percentage of total cell number (**C**) by performing cell counts in four random fields of view (20X magnification) per experimental treatment. All values are presented as mean ± SEM (n=3). Data was analyzed using a student *t*-test.

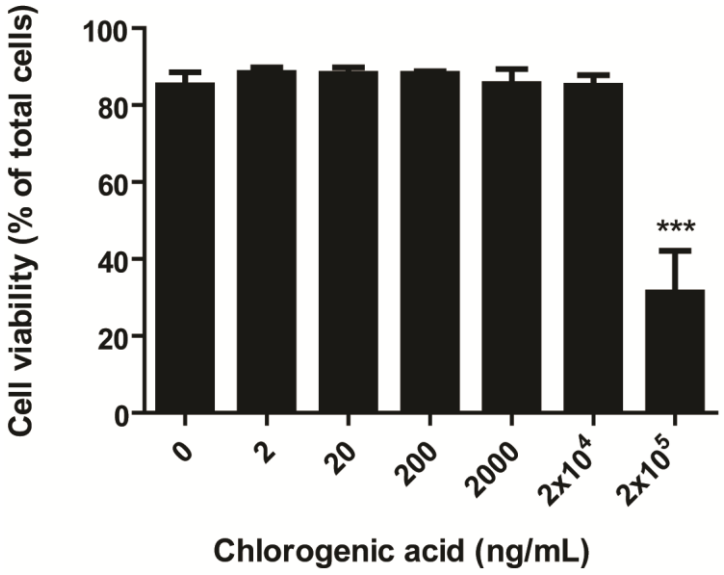

Figure 14. The effect of chlorogenic acid on trophoblast viability. HTR-8/SVneo cells were treated with chlorogenic acid for 24 hours and cellular viability was determined using the trypan blue dye exclusion test. Cellular viability was expressed as percentage of trypan blue-negative cells (viable cells) compared to total cell number (both viable and non-viable cells). All data are presented as mean ± SEM (n=3). Asterisks indicate a significant difference compared to the untreated control group. Data was analyzed by one-way ANOVA and Bonferroni *post hoc* test (***$p<0.001$).

3.5 Effects of *V. angustifolium* crude leaf extract and chlorogenic acid on ERK and AKT phosphorylation

To determine if the effects of the crude leaf extract on trophoblast migration and invasion were associated with changes in ERK and AKT phosphorylation, HTR-8/SVneo cells were treated with either 0 or 20 ng/mL of the crude extract and cell lysates were obtained after 0, 5, 10, 15, 30, and 60 minutes of exposure. Similarly, the possible involvement of ERK and AKT phosphorylation to explain the effects of chlorogenic acid on cell migration and invasion were investigated by treating trophoblasts with either 0 or 200 ng/mL of chlorogenic acid prior to obtaining cell lysates at the same time points listed above. Results from western blot analyses suggest that *V. angustifolium* leaf extract does not affect ERK or AKT phosphorylation (Figure 15). Treatment with the extract may have slightly increased levels of phosphorylated-AKT around 15 minutes of exposure but this effect was not statistically significant. Likewise, chlorogenic acid was not shown to influence ERK or AKT phosphorylation (Figure 16). Levels of phosphorylated-ERK appeared to increase around 2-fold at 30 minutes and then decline at 60 minutes; however, this change was not statistically significant. Therefore, the crude extract and chlorogenic acid likely increased trophoblast migration and invasion in an ERK- and AKT-independent manner.

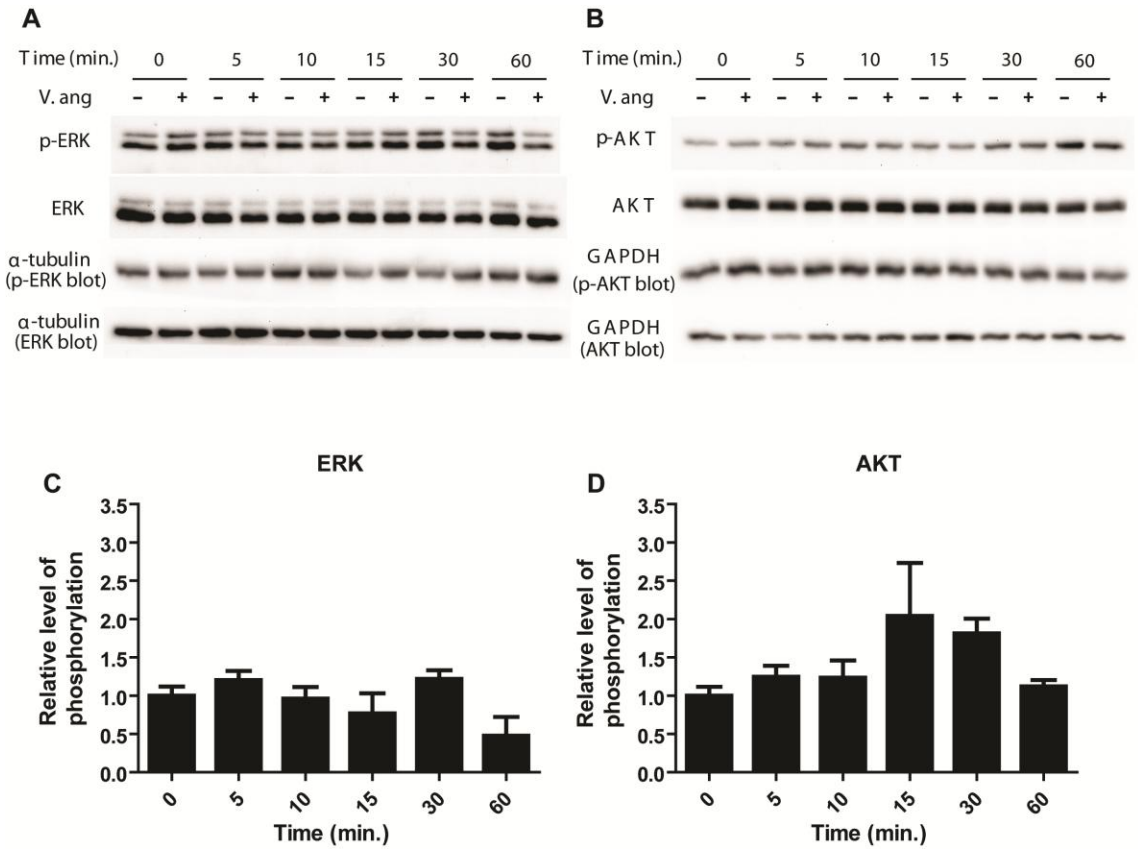

Figure 15. The effects of *V. angustifolium* crude leaf extract on ERK and AKT phosphorylation. HTR-8/SVneo cells were treated with (+) or without (-) 20 ng/mL of *V. angustifolium* (V. ang) crude leaf extract for 0, 5, 10, 15, 30, and 60 minutes (min.), and cell lysates were obtained. Changes in the phosphorylation status of ERK (**A, C**) and AKT (**B, D**) were assessed over time by western blot. Phosphorylated and total protein levels were normalized to the loading control (i.e. α-tubulin or GAPDH) prior to determining the ratio of phosphorylated protein to total protein (i.e. p-ERK/total ERK or p-AKT/total AKT). Results were normalized to the untreated control group at time zero. All values are presented as mean ± SEM (n=3). Data was analyzed by one-way ANOVA and Bonferroni *post hoc* test.

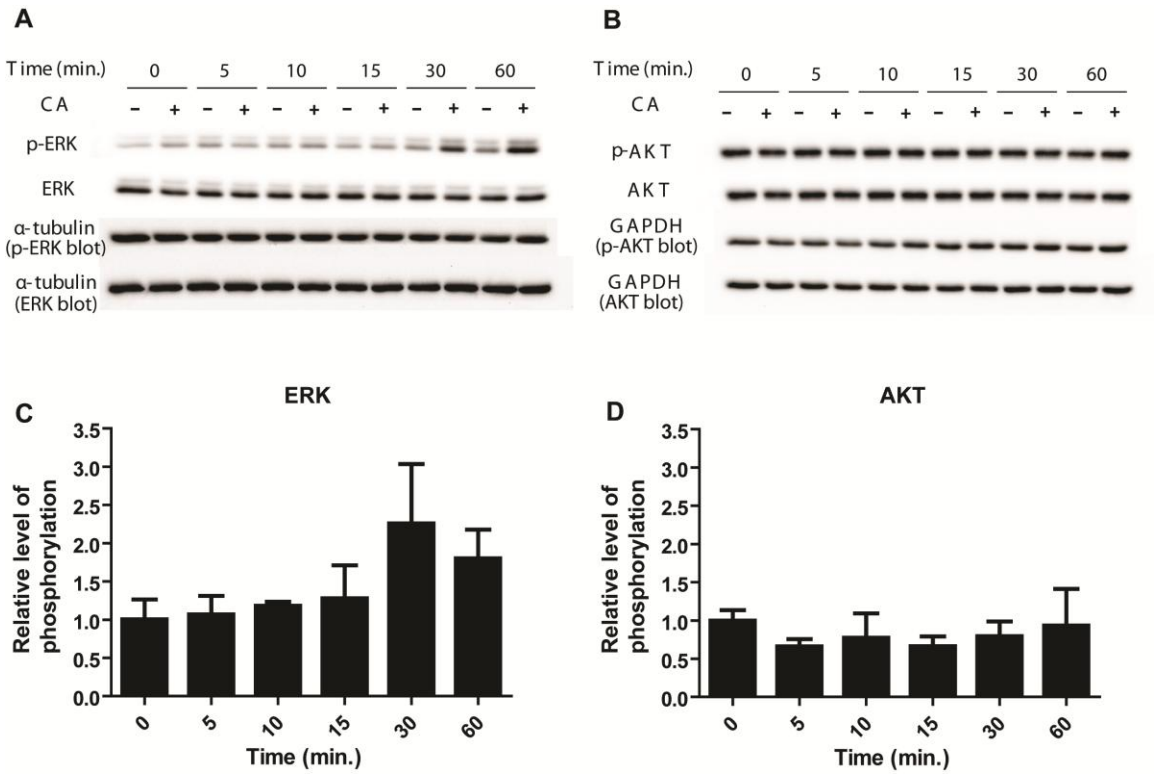

Figure 16. The effects of chlorogenic acid on ERK and AKT phosphorylation. HTR-8/SVneo cells were treated with (+) or without (-) 200 ng/mL of chlorogenic acid (CA) for 0, 5, 10, 15, 30, and 60 minutes (min.), and cell lysates were obtained. Changes in the phosphorylation status of ERK (**A, C**) and AKT (**B, D**) were assessed over time by western blot. Phosphorylated and total protein levels were normalized to the loading control (i.e. α-tubulin or GAPDH) prior to determining the ratio of phosphorylated protein to total protein (i.e. p-ERK/total ERK or p-AKT/total AKT). Results were normalized to the untreated control group at time zero. All values are presented as mean ± SEM (n=3). Data was analyzed by one-way ANOVA and Bonferroni *post hoc* test.

CHAPTER 4: DISCUSSION & CONCLUSIONS

Vaccinium extracts, including those from *V. angustifolium*, are a rich source of polyphenols and have been shown to influence cell migration and invasion using: *in vitro* models of cancer metastasis (Matchett *et al.*, 2005; Adams *et al.*, 2010), *in vitro* models of endothelial cell function (Tulio *et al.*, 2012; Chang *et al.*, 2014), and animal models of wound healing (Nayak *et al.*, 2011). In this study, the influence of *V. angustifolium* leaf extract on extravillous trophoblast (EVT) migration and invasion was investigated. The polyphenols present in *V. angustifolium* leaf extract were postulated to increase trophoblast migration and invasion through activation of the MAPK and PI3K cell signalling pathways.

4.1 *V. angustifolium* crude leaf extract

To determine the influence of *V. angustifolium* crude leaf extract on trophoblast migration and invasion, dose-response studies were conducted using the *in vitro* Boyden chamber assay. Concentrations of the crude leaf extract used in these series of experiments ranged from no extract (0 ng/mL), to those that were estimated to be physiologically relevant (2-200 ng/mL), and those which surpassed what would be expected *in vivo* ($\geq$ 2000 ng/ml). These values were estimated based on a collective review of 97 studies which summarized the bioavailability of 18 major polyphenols, namely phenolic acids and flavonoids (Manach *et al.*, 2005). In these studies, adults were administered a single dose of polyphenol in the form of a pure compound, plant extract, or whole food/beverage. Although the circulating concentration of *V. angustifolium* crude leaf extract has never been examined in humans or animal models, polyphenols are likely to be major constituents of the extract, and therefore, the bioavailability of these compounds were used to estimate the values of physiologically relevant doses.

We have demonstrated for the first time that lowbush blueberry leaf extract increases HTR-8/SVneo trophoblast cell migration and invasion, independent of changes in cell proliferation and cell viability. Furthermore, this effect appears to be dose-dependent, with the most significant increase in trophoblast migration and invasion observed following treatment with physiologically relevant concentrations of 20 and 200 ng/mL

crude leaf extract. The results presented demonstrate the potential for *V. angustifolium* to regulate EVT migration and invasion, both crucial processes required for placental development and proper establishment of the uteroplacental circulation (Cross *et al.*, 2002). Inadequate EVT migration and invasion are associated with poor placentation and a reduction in the number and depth of remodelled spiral arteries in the uterine wall. Both of these trophoblast pathologies have been described in cases of pre-eclampsia (PE) and intrauterine growth restriction (IUGR) (Bronsens *et al.*, 1972; Bronsens *et al.*, 1977), and are considered to play important roles in the pathophysiology of these disorders (Khong *et al.*, 1986). The findings from our study provide preliminary evidence that *V. angustifolium* crude leaf extract may prove to be a beneficial nutraceutical during the very early stages of pregnancy to promote EVT migration and invasion, possibly preventing obstetrical complications associated with impairments in these processes. Indeed, further studies investigating the safety of *V. angustifolium* consumption is required; however, its long history as a traditional medicine (Moerman, 2009; Ferrier, 2014) and current use as a natural health product in Canada (Martineau *et al.*, 2006) makes this plant a promising candidate. According the World Health Organization (WHO), up to 80% of the population in developing countries relies on traditional medicine for their primary healthcare needs (WHO, 2002). This number is expected to increase steadily as the development of adverse effects and microbial resistance to chemically synthesized drugs increases, and the discovery of safe and effective natural alternatives with less adverse effects continues to garner interest within the general population and scientific community (Sasidharan *et al.*, 2010).

Data from our trypan blue dye exclusion assay suggests that there is a wide range of concentrations at which the leaf extract does not affect trophoblast cell viability. In fact, *V. angustifolium* leaf extract has been shown to have cytoprotective effects against high glucose level-mediated cell apoptosis in rat adrenal medulla cells (Martineau *et al.*, 2006). Hyperglycemia is known to promote reactive oxygen species accumulation and in turn, promote cellular damage (Floretino *et al.*, 2013). This is of direct relevance to the potential prophylactic and/or therapeutic use of this extract in instances of pathological placentation since activation of oxidative stress pathways is associated with the

pathophysiology of placenta-mediated diseases such as PE (Myatt and Cui, 2004; Burton *et al.*, 2009; Tal, 2012). As such, the ability of *V. angustifolium* crude crude leaf extract to not only improve placental development through promotion of trophoblast migration and invasion, but also have the potential to protect the placenta against the cytotoxic effects of oxidative stress is an interesting consideration.

Hyperglycemia is also known to accelerate the formation of advanced glycation end products (AGEs). AGEs are a heterogenous group of compounds known to cause oxidative damage and inflammation by binding with the receptor RAGE (Singh *et al.*, 2001), and may play a role in the pathogenesis of PE (Chekir *et al.*, 2006). A study conducted by Chekir *et al.* (2006) reported that serum from pre-eclamptic women contained a significantly higher concentration of AGEs compared to samples obtained from healthy pregnant women. This group also found that the expression levels of AGEs and RAGE were elevated in pre-eclamptic placentae compared to healthy controls and that this accumulation was associated with an up-regulation of oxidative stress markers in the placenta. Results from other studies suggest that AGEs promote abnormal production of soluble fms-like tyrosine kinase, an important anti-angiogenic factor believed to contribute to the PE syndrome (Maynard *et al.*, 2003), in HTR-8/SVneo cells (Huang *et al.*, 2013), and induce secretion of chemokines and apoptosis in trophoblasts isolated from first trimester chorionic villi (Konishi *et al*., 2004). Interestingly, *V. angustifolium* leaf extract is a potent inhibitor of AGE formation *in vitro* (McIntyre *et al.*, 2009; Ferrier *et al*., 2012), thus future studies examining the potential of this extract to attenuate AGE production during pregnancy could have novel implications.

The decision to use the *V. angustifolium* leaf extract rather than the blueberry fruit was based on a variety of factors. Firstly, blueberry leaf contains significantly higher concentrations of phenolic compounds compared to the fruit tissue itself, and demonstrates a greater antioxidant capacity than the fruit, as determined by its ability to absorb oxygen free radicals (Prior *et al.*, 1998; Ehlenfeldt and Prior, 2001). Since oxidative stress is associated with a broad spectrum of placental pathologies, including PE and IUGR, the greater antioxidant content in the leaves was considered a benefit. That

being said, there is controversy surrounding the use of antioxidant supplementation for the prevention of placental dysfunction, particularly as it relates to the obstetrical complication of PE. Several large scale randomized controlled trials have attempted to demonstrate a protective effect of antioxidant vitamins C and E against the development of PE; however, these studies were largely unsuccessful and in some instances increased adverse fetal health outcomes (Spinnato *et al.*, 2008; Roberts *et al.*, 2010). These disappointing results can likely be explained in part by an unbalanced administration of vitamins and/or trace elements in these patients (Al-Gubory *et al.*, 2010). Furthermore, it is probable that at the super-physiological concentrations used in these studies, vitamins C and E instead acted as pro-oxidants (Rietjens *et al.*, 2002; Poston *et al.*, 2006), which may explain the adverse effects seen with their usage. As such, alternative approaches which capitalize on the natural abundance of antioxidant phytonutrients in fruits and vegetables, such as a blueberry leaf tea, may prove to be a superior method in promoting appropriate placentation. However, more research on the requirements of maternal antioxidant micronutrients for normal fetal growth and development is required and limited at present.

The second reason for selecting the leaf extract, over the use of the fruit itself, is that the leaf contains significantly less sugar than the fruit. This consideration is particularly relevant for women with pre-existing diabetes or women who may develop gestational diabetes. Pre-existing maternal diabetes and gestational diabetes are known risk factors for both PE (Östlund *et al.*, 2004; Catov *et al.*, 2007) and IUGR (Stratton *et al.*, 1995). It is estimated that both PE and IUGR are twice as common in diabetic pregnancies compared with normal pregnancies (Garner *et al.*, 1990; Figueras and Gardosi, 2011). Therefore, a significant portion of the patient population for which this treatment may prove beneficial will demonstrate these co-morbidities and thus, tight regulation of sugar intake will be required.

4.2 Principal components of *V. angustifolium* crude leaf extract

The second research objective of this project was to identify the biologically active compound(s) present in the crude leaf extract which were responsible for the increase in

trophoblast migration and invasion observed. The complexity and biodiversity of plants is reflected in the leaf extracts, with the identification of hundreds to even thousands of different chemical components (Sasidharan *et al.*, 2011). This makes the identification of bioactive compounds in these extracts a significant challenge. To tackle the complex nature of this objective, we first decided to separate the phytochemicals in the crude extract based on their polarities by performing a four-part series fractionation, using hexane, dichloromethane (DCM), acetonitrile (ACN), and water (H_2O). Each of these fractions was then individually tested for potential effects on trophoblast migration and invasion *in vitro*. Hexane is a non-polar solvent and therefore, best dissolves non-polar compounds, including glycerol esters of fatty acids, coloured hydrocarbons, and terpenes with few polar bonds. DCM and ACN are more polar solvents and typically dissolve many organic compounds with low to medium polarity, including flavonoids and essential oils. Water is a highly polar solvent and will solubilise polar compounds, such as carbohydrates and their conjugate, amino acids, small peptides, and small carboxylic acids.

Our results show that the hexane, DCM, and ACN fractions increased trophoblast migration; however, only the hexane fraction increased trophoblast invasion. Conversely, the H_2O fraction did not affect trophoblast migration or invasion. In the migration assay, the individual hexane and ACN fractions were found to increase trophoblast migration to a greater extent than the crude leaf extract. This effect may be explained if the DCM and/or H_2O fractions contain compounds which act as antagonists to the active compounds found in the hexane and ACN fractions responsible for the increases seen in cell migration. In the invasion assay, the hexane fraction caused a similar fold increase in trophoblast invasion compared to that of the crude leaf extract, while the DCM fraction had no effect and the ACN and H_2O fractions actually decreased trophoblast invasion compared to the crude leaf extract experimental treatment. It is possible that the ACN and H_2O extractions pulled out a large subset of compounds that inhibit trophoblast invasion, which may explain the effects seen in this assay.

Although cellular migration and invasion are related functions and regulated by similar mechanisms, these two processes each play important and independent physiological roles within the body. In the field of experimental cell biology, these two activities have been clearly defined and several assays have been developed to examine these processes *in vitro*. Kramer *et al.* (2012) define migration as the directed movement of cells on 2D surfaces, such as basal membranes, extracellular matrix fibers, or plastic plates, without any disruption to the fiber network, whereas invasion is the movement of cells through a 3D matrix, accompanied by a restructuring of the extracellular matrix. Since the hexane fraction was able to increase both trophoblast migration and invasion, this finding would suggest that hexane-soluble compounds present in the crude leaf extract were able to activate both migration and invasion cell signalling pathways. Conversely, the compounds soluble in DCM and ACN may have specifically been able to restructure the trophoblast cell cytoskeleton, permitting migration, without affecting the secretion of matrix degradation enzymes necessary for invasion. While migration of EVTs is important, invasion of EVTs is the critical physiological function which allows these cells to establish the utero-placental circulation. As such, the invasion assay results are of utmost importance. Further studies are required to generate and compare the chemical profiles for each of the individual fractions in order to identify which compounds in the crude leaf extract promote and/or inhibit trophoblast migration and/or invasion.

There are, however, limitations to the interpretation of these results. First, since the series extraction was performed using *V. angustifolium* crude leaf extract originally extracted with 95% ethanol, it is possible that the compounds isolated with the subsequent hexane extraction only represent a subset of the hexane-soluble compounds in the *V. angustifolium* leaf. Additionally, since the series extraction uses four solvents, one after the next, compounds that are soluble in more than one solvent may have been extracted to a greater extent, or to its entirety, in the solvent that was first applied. Thus, it is difficult to conclude that compounds only soluble in hexane are responsible for the biological effects seen on trophoblast cells. Furthermore, within the isolated hexane fraction there are still likely hundreds of different compounds, and while this approach may have narrowed the search using chemical polarity, it does not specifically identify the active

compound(s). For these reasons, more sophisticated means were sought out to characterize and identify bioactive compounds in the *V. angustifolium* crude leaf extract.

Consequently, the next step of this project was to characterize the major chemical constituents of the blueberry leaf extract using UPLC-MS-TOF methodology. The advantage of using this methodology is that specific compounds with abundant presence within the leaf extract can be identified by cross-referencing the chemical profiles generated (i.e. retention time, ion intensity, and mass) with ChemSpider, a robust online chemical database. Through this analysis, it was demonstrated that the phenolic compounds chlorogenic acid, myricetin, and hyperoside are major constituents of the leaf extract. Chlorogenic acid and hyperoside have been previously identified and quantified in *V. angustifolium* leaf extracts through a variety of analytical techniques (Harris *et al.*, 2007; McIntyre *et al.*, 2009; Hicks *et al.* 2012). To our knowledge, we are the first to report that myricetin is also present in *V. angustifolium* leaf extract, thus further validation studies are necessary to confirm this finding. However, myricetin has been described in many different types of edible berries (Häkkinen *et al.*, 1999), including blueberries and cranberries, and therefore, is it not surprising that it is present in the leaf as well.

Upon identification, each of the three polyphenols was assessed for its ability to influence trophoblast migration and/or invasion using *in vitro* assays. The results demonstrate that only chlorogenic acid significantly increased both trophoblast migration and invasion and therefore, may be one of the active compounds present in the crude leaf extract. Quantification of the amount of chlorogenic acid in the crude leaf extract, using ^{1}H NMR methodology, indicated an abundance of 7%, or 1.43 ng/mL (in the 20 ng/mL crude leaf extract formulation). However, the concentration of purified chlorogenic acid found to significantly increase trophoblast migration and invasion was 200 ng/mL, a concentration approximately 100-times higher. This discrepancy may indicate that there are other compounds present in the extract that act in synergy with chlorogenic acid to promote trophoblast migration and invasion; or that other components in the crude leaf extract may promote uptake of chlorogenic acid allowing for biological activity at lower

concentrations. Nevertheless, 2 ng/mL of purified chlorogenic acid (i.e. the dose of chlorogenic acid used in the migration and invasion assays that was most reflective of the concentration of chlorogenic acid found in the 20 ng/mL crude leaf extract) produced similar fold increases in migration and invasion seen with the 20 ng/mL crude leaf extract treatment, and therefore supports our hypothesis that this compound may be one of the active polyphenols present in the crude leaf extract.

Chlorogenic acid is an ester formed between caffeic acid and quinic acid. It is one of the major phenolic compounds identified in coffee beans and stone fruits (e.g. cherries, plums, and peaches), and found in low to moderate quantities in green and black tea leaf, pome fruits (e.g. apples and pears), berry fruits, grapes and wine, citrus fruits, and vegetables (Clifford, 1999). Depending on the type of brew and roast, a 200 mL cup of coffee can contain between 20 mg to 675 mg of chlorogenic acid (Clifford and Walker, 1987), thus regular coffee drinkers can easily consume an excess of 1 g per day (Clifford, 1999). Although there is no available data for the content of chlorogenic acid in blueberry leaf tea brew, our results suggest that approximately 70 µg of chlorogenic acid is present in 1 mg of *V. angustifolium* leaf extract (~7% abundance; Section 3.3.3). Harris *et al*. (2007) reported similar results, indicated that approximately 100 µg of chlorogenic acid is present in 1 mg of leaf extract. Although a cup of coffee is likely to contain higher quantities of chlorogenic acid than a blueberry leaf tea brew, coffee consumption during pregnancy may have adverse effects on fetal, neonatal, and maternal health in certain subpopulations. For instance, maternal coffee consumption during pregnancy may increase the risk of childhood acute leukemia (Cheng *et al.*, 2014) and the incidence of small for gestational age births (Hollins, 2013; Hoyt *et al*., 2014; Sengpiel *et al.*, 2013). These effects may be attributed to the high caffeine content in coffee (Jahanfar and Jaafar, 2013; Sengpiel *et al.*, 2013); however, the birth weight of babies born to women who consumed caffeinated versus decaffeinated coffee was not significantly different (Bech *et al.*, 2007). Thus, caffeine may not be the only agent in coffee responsible for the proposed adverse effects during pregnancy. As a result, if chlorogenic acid is responsible for the effects seen on trophoblast migration and invasion, then the blueberry leaf extract may be a better source than coffee.

It is important to note that the concentration at which chlorogenic acid displayed its most significant effect on trophoblast cell migration and invasion can be achieved *in vivo* through dietary intervention. Using high performance liquid chromatography (HPLC), Monteiro *et al.* (2007) assessed the plasma concentrations of chlorogenic acid in six healthy subjects at various time points following coffee consumption. The mean maximum plasma concentration of chlorogenic acid was roughly 1700 ng/mL, although it should be noted that this value varied greatly amongst subjects (± 900 ng/mL). Nevertheless, the effective dose of chlorogenic seen in the trophoblast migration and invasion assays falls within this spectrum of pharmacokinetic parameters. Moreover, the time to reach the maximum plasma concentration was around two hours (± one hour) after which plasma levels of chlorogenic acid began to decrease. Thus, regular consumption of the extract would be required to maintain therapeutic concentrations in blood.

4.3 Mechanistic studies

The third research objective of the current research project was to determine the mechanism by which *V. angustifolium* crude leaf extract and chlorogenic acid increased trophoblast migration and invasion. MAPK and PI3K cell signalling pathways are known to play key roles in HTR-8/SVneo cell migration and invasion (Qiu *et al.*, 2004a; Qiu *et al.*, 2004b). Furthermore, polyphenols, including chlorogenic acid, have been shown in other systems to affect the phosphorylation of ERK and AKT (Matchett *et al.*, 2005; Adams *et al.*, 2010; Déziel *et al.*, 2010; Kausar *et al.*, 2012; Tulio *et al.*, 2012), two important signalling proteins within the MAPK and PI3K cascades. Therefore, it was hypothesized that the crude leaf extract and chlorogenic acid increased trophoblast cell migration and invasion in an ERK- and/or AKT-dependent manner. Results from the Western blot experiments suggest that neither the crude leaf extract nor chlorogenic acid influenced ERK or AKT phosphorylation, and therefore, the effects of these treatments seen on trophoblast migration and invasion occurred through an ERK- and AKT-independent mechanism. Further analyses need to now be conducted to characterize thecellular receptors and cell signalling cascades that are activated in trophoblasts upon

exposure to *V. angustifolium* crude leaf extract. The same mechanistic studies should also be performed upon treating trophoblasts with chlorogenic acid. If chlorogenic acid activates the same signalling pathways as the crude extract, then this would further support our hypothesis that chlorogenic acid may be one of the active components in the extract. A few potential candidates worth investigating include: (1) the nuclear factor-kappaB (NF-κB) pathway; (2) signal transduction by FAK; (3) the epidermal growth factor receptor (EGFR) system; and (4) MMP-2 and MMP-9. These pathways have been clearly documented to play a role in regulating trophoblast migration and invasion (Jokhi *et al.*, 1994; Li *et al.*, 2010; Knöfler and Pollheimer, 2012), and may represent molecular targets of polyphenols as demonstrated in other models (Higashi *et al.*, 2005; Matchett *et al.*, 2005; Romier *et al.*, 2008; Pastore *et al.*, 2012).

4.4 Study limitations

HTR-8/SVneo cells were selected as the *in vitro* culture model to determine the effects of *V. angustifolium* crude leaf extract and its phytochemicals on EVT function. This culture model is a well-established immortalized human EVT cell line that displays phenotypic properties similar to primary EVTs, including morphology, secretion of matrix degrading enzymes, and ability to migrate and invade (Graham *et al.*, 1993). This particular model was chosen due to its homogenous characteristics and prolonged *in vitro* lifespan, both of which are advantageous for achieving reproducible results and conducting experiments with ease. Despite these advantages and similarities, cell lines are never the exact same as primary cells and this limitation should be considered when extrapolating experimental data to *in vivo* conditions. Using primary cells isolated from first trimester placental villi would allow for better *in vivo* interpretation of results; however, various factors such as first trimester placental sample availability and heterogeneous patient populations make this experimental design significantly more challenging. However, with the generation of the promising results presented, the replication of these experiments using a primary EVT culture model is now warranted.

Since the variety of the *V. angustifolium* leaf used for this project was harvested in the wild, it is possible that environmental factors, including climatic and geographic region,

season, and soil, could impact the phytochemical properties of the plant (Rieger *et al.*, 2008; McIntyre *et al.*, 2009). Different processing methods, including those related to the drying and extraction of the leaf material, are also known to influence an extract's phytochemical content (Chakraborty *et al.*, 2010). Therefore, strict adherence to the standard operating procedures for the collection and extraction of the plant material is imperative. Conversely, some factors, such as temperature and rainfall, are variable and impossible to control in a natural setting, but are still extremely important since they can impact the quality of the soil and therefore, the chemical content of plants (Jayanthy *et al.*, 2013). Although the plant material used for this study was obtained from a single population at one specific time point, future projects may require additional collection of this plant. As such, these factors should be considered if the bioactivity of other *V. angustifolium* batches is tested in future studies.

4.5 Future directions

In addition to confirming the presented results in a primary EVT cell culture model, *in vivo* animal studies should also be completed to further investigate the effects of *V. angustifolium* crude leaf extract on placentation and pregnancy outcomes. A widely held assumption in the general public is that a health product made of natural substances is always safe for consumption (Ipsos-Reid, 2010), and further that 'more is always better'. Therefore, understanding the influence of maternal consumption of these widely available and used agents on reproductive health is imperative. One approach is to administer different concentrations of the crude leaf extract as a tea infusion to healthy animals (i.e. mice or rats) throughout gestation. Maternal and perinatal health outcomes (e.g. side effects, weight loss, litter size, fetal resorption sites, fetal birth weight, developmental delay, congenital malformations, and post-natal mortality) could be monitored to determine a safe therapeutic concentration window. Different concentrations of the pure isolated compounds identified in the crude leaf extract could also be tested in this model to determine the safety of polyphenol consumption. Based on the results from our *in vitro* trophoblast cell viability assays, we do not expect the crude leaf extract or chlorogenic acid experimental treatments to have serious adverse effects on placentation as long as the dose does not exceed $2x10^4$ ng/mL. Some studies have highlighted potential hazards

of certain polyphenols, namely curcumin (Chen and Chan 2012) and genistein (Jefferson *et al.*, 2002), on female mice fertility and sexual development; however, whether the concentrations used in these animal studies are relevant to human intake and whether these polyphenols are present in *V. angustifolium* crude leaf extract remains to be elucidated. As such, thorough safety trials for both the crude leaf extract and its principal components in healthy pregnant animal models are required.

Subsequent experiments employing an animal model of PE, such as STOX1-overexpressing mice (Doridot *et al.*, 2013) or BPH/5 mice (Davisson *et al.*, 2002), could be performed to elucidate the prophylactic and/or therapeutic potential of the crude leaf extract and/or its isolated components against the development of PE-like symptoms. At the onset of pregnancy, different concentrations of the crude leaf extract and/or its isolated components could be administered as a tea infusion or dissolved in the drinking water, respectively. These mice could be monitored throughout gestation to determine whether the treatment was effective at preventing or limiting clinical characteristics of PE. These measurements would include maternal blood pressure monitoring, via either tail-cuff plesthmography or telemetry, and assessment of proteinuria, through measurement of the albumin/creatinine ratio in the urine. It is hypothesized that the crude leaf extract experimental treatment will prevent the onset of hypertension in these mice as this plant has been previously shown to improve vascular tone in spontaneously hypertensive rats (Kristo *et al.*, 2010). While the effects of *V. angustifolium* have never been studied in the context of kidney function, a closely related species named *V. myrtillus* (bilberry) has been shown to have protective effects on potassium bromate-induced kidney damage in mice (Bao *et al.*, 2008). Thus, *V. angustifolium* may also protect against the kidney damage (proteinuria) seen in mouse models of PE.

This experimental model could further be used to assess whether *V. angustifolium* leaf extract, or its bioactive components, could influence the release of anti-angiogenic compounds by the dysfunctional pre-eclamptic placenta. Blood samples could be collected from the treated mothers and used to determine the concentration of angiogenic factors such as soluble fms-like tyrosine kinase-1 (sFlt-1), a widely studied biomarker of

PE (De Vivo *et al.*, 2008). Since *V. angustifolium* crude leaf extract is known to be a potent inhibitor of AGE formation (McIntyre *et al*., 2009; Ferrier *et al*., 2012), and AGEs have been reported to trigger sFlt-1 production in EVTs (Huang *et al.*, 2013), it is postulated that mice treated with the crude leaf extract (or its active components) will display lower plasma levels of sFlt-1. Finally, an advantage of this experimental treatment model includes the ability to collect placenta tissue at different stages of gestation to determine the effects of *V. angustifolium* treatment on critical aspects of placentation, including the extent of trophoblast invasion of the uterine wall and remodelling of uterine spiral arteries. Collectively, these experiments will help determine if peri-conceptional and/or early pregnancy treatment with *V. angustifolium* crude leaf extract and its phytochemicals could prevent the symptoms and pathological placentation associated with the PE disorder.

Since the bioactivity of a treatment is dependent on its bioavailability and the extent to which it is absorbed, distributed, and metabolised within, and eliminated from the body (i.e. its pharmacokinetics), studies directed towards understanding these parameters within the context of pregnancy are required to determine its safety and efficacy. Researchers have investigated polyphenol pharmacokinetics in adult subjects by measuring plasma and urine concentrations of known metabolites following single-dose administration of the pure compound or food/beverage of interest (Scalbert and Williamson, 2000; Manach *et al.* 2004; Manach *et al*., 2005). Similar studies have also been conducted in animal models, where in addition to plasma and urine collection, organ tissue distribution of the compounds of interest can be examined (Chu *et al.*, 2006; Arola-Arnal *et al*., 2013). In this vein, a comprehensive analysis of the pharmacokinetics and bioavailability of the *V. angustifolium* crude leaf extract, and its major components, should be conducted using an *in vivo* pregnant animal model. Pregnant mice could be fed the crude leaf extract tea brew or the isolated compounds dissolved in the drinking water. At different time points (e.g. 0, 0.5, 1, 2, 3, 5, 8, 10, 12, 16, 20, and 24 hours after administration) blood, urine, and tissue (e.g. placenta and fetus) samples could be collected and polyphenol concentrations could be analyzed using UPLC-MS-TOF. This data would allow us to determine the maximum concentration (C_{max}), the maximum

concentration time (T_{max}), and the area under the curve (AUC) to compare the absorption and disposition of various polyphenols in maternal plasma, placenta, and fetus, as well as polyphenol elimination in the urine.

Due to the heterogeneity of metabolic and absorption pathways, we expect large deviations between pharmacokinetic measurements for each animal. Nevertheless, based on the data compiled from other studies (Manach *et al.*, 2005), our general assumption is that the C_{max} for most of the polyphenols present in the crude leaf extract will rarely exceed 1 uM in the plasma and that this value will be reached 1 to 2 hours after ingestion. We also expect the C_{max} to be higher in the maternal plasma compared to that of the placenta and fetus, and that the placenta will have a greater C_{max} than the fetus, as it is the barrier between the maternal and fetal systems. These pharmacokinetic studies will help determine an appropriate dose and dosing interval for the crude leaf extract treatment and also help identify metabolites of the crude leaf extract in the plasma, urine, and tissue samples collected from the treated animals. Purified forms of these metabolites could then be tested using *in vitro* cell culture models and *in vivo* animal models to determine their effects on trophoblast biology.

Overall, the data obtained from these proposed animal studies would help guide efforts to conduct randomised controlled trials aimed at determining the safety and efficacy of a *V. angustifolium* crude leaf extract treatment in healthy, non-pregnant population. Once the immediate and long-term side effects and outcomes are thoroughly evaluated in the healthy, non-pregnant cohort, the crude leaf extract, or its isolated phytochemicals, could be administered to an obstetrical population to determine if the treatment is able to decrease the incidence of placenta-mediated diseases, including PE and IUGR. These studies should employ large prospective cohorts, with adequate power and sample sizes to detect change in the primary outcome, and be randomized, double-blind, and placebo-controlled to provide a more comprehensive, bias-free understanding of the biological effects of *V. angustifolium* crude leaf extract on placental development, health, and function.

4.6 Conclusions

The effects of *V. angustifolium* crude leaf extract on trophoblast biology were investigated using the HTR-8/SVneo human EVT cell culture model. Our *in vitro* studies demonstrated that *V. angustifolium* crude leaf extract promotes trophoblast migration and invasion, independent of changes in cell proliferation of viability. Importantly, these effects of the crude leaf extract were observed at physiologically relevant concentrations. While it was hypothesized that activation of MAPK and PI3K cell signalling pathways were responsible for these findings, measurements of the phosphorylation status of ERK and AKT, respectively, indicate that these signalling pathways are likely not involved. Chemical analyses identified polyphenols as the major constituents of the crude leaf extract; more specifically, chlorogenic acid is present in high concentrations in this extract. Further, *in vitro* studies confirmed that purified chlorogenic acid is also capable of enhancing trophoblast migration and invasion to a similar extent of that seen with the crude leaf extract. As such, chlorogenic acid may be one of the bioactive polyphenols in the crude leaf extract. Overall, the results from this project suggest that *V. angustifolium* leaf extract and/or its isolated phytochemicals may have therapeutic applications for the prevention of placenta-mediated diseases, such as PE, where trophoblast invasion is impaired. Therefore, the next logical steps would be to further investigate the mechanism by which the active compounds increase trophoblast migration and invasion and assess the safety and efficacy of the treatment using animal models and randomised controlled trials.

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