An in vitro study of the biological actions of a Chinese Herbal Formula on selected haematological and vascular processes related to endometrial receptivity

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July 2017

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The thesis is submitted in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy of the University of Portsmouth

i. ABSTRACT

Chinese herbal medicines are used by women across the world to treat infertility in natural and assisted reproductive cycles. Randomised control trials and systematic reviews have shown the clinical efficacy of these medicines, but there remains a paucity of information as to the mechanisms by which they exert their effect. The herbal formula in this study, Gui Shao Di Huang Wan comprises eight herbs and is used to treat infertility. It is a base formula from which practitioners create a personalised medicine. The observed fertility benefits may be due to improvements in the receptivity of the endometrium, and a key aspect of this is haematology. The blood is a concept where the western and Chinese perspectives on fertility converge. Aqueous extracts of the whole formula and the individual herbs were used. Prothrombin time and activated partial thromboplastin time were used to evaluate time to clot formation. An MTT assay was used to look at proliferation of human umbilical vein endothelial cells. A 2D differentiation assay was performed on matrigel using human umbilical vein endothelial cells and human uterine microvascular endothelial cells. Lysates of Ishikawa endometrial cells exposed to the herb extracts were used in immunoblotting to explore changes in oestrogen and progesterone receptor expression.

Haemostasis, proliferation and differentiation of endothelial cells and expression of steroid receptor proteins on an endometrial cell line have all been shown to be affected by the herb extracts in *in vitro* assays modelling these functions. Prothrombin time ($Welch's\ F\ (8,14.57) = 50.21,\ p < 0.0005$) and activated partial thromboplastin time ($Welch's\ F\ (8,13.77) = 38.79,\ p < 0.0005$) were both increased. The whole formula led to an increase in proliferation at concentrations between 0.68 and 5.47 μg/mL at 48 and 72 hours.

Metrics of tubule number, length, junctions, and mesh size were increased in endothelial cells. All parameters of Human Umbilical Vein Endothelial Cells were statistically significantly increased by the whole formula but not the individual herbs. The Human Uterine Microvascular Endothelial Cell mesh was increased by all herb extracts, total length, and segment number by Radix Paeoniae Alba, Sclerotium Poria

Cocos, Radix Rehmanniae Preparata, Rhizoma Dioscoreae, Fructus Corni, and Rhizoma Alismatis, total length and junction number for Fructus Corni
Modifications to expression of ERα and PRβ were observed. Downregulation of Estrogen receptor alpha after 24 hour exposure to the herbs for Radix Paeoniae Alba, Radix Angelicae Sinensis, Sclerotium Poria Cocos, and Cortex Moutan. Progesterone receptor beta was upregulated by treatment with Radix Paeoniae Alba, Radix Angelicae Sinensis, Sclerotium Poria Cocos and Radix Rehmanniae Preparata after 48 hours exposure to the herb extract.
trials currently available."

This study adds a new perspective to the limited evidence available through clinical trials which examine Chinese herbal medicines and their potential for use in treating female infertility. The observed effects of the herbs may be mediated by the influence on the identified haematological and vascular processes related to endometrial receptivity.

ii. CONTENTS

iii. DECLARATION

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

Word Count: 43652

(excluding tables and supplementary information)

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vi. List of Abbreviations and Acronyms

aPTT	activated Partial Thromboplastin Time
ALAS	5-Aminolevulinate Synthase
ART	Assisted Reproductive Therapies
βA	Beta Actin
BCL	B-Cell Lymphoma
BSA	Bovine Serum Albumin
CHM	Chinese Herbal Medicine
CI	Confidence Interval
CYP	Cytochrome P450
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
ERα	Oestrogen Receptor alpha
ERβ	Oestrogen Receptor beta
FBS	Foetal Bovine Serum
FSH	Follicle Stimulating Hormone
G-CSF	Granulocyte-colony stimulating factor
GnHCL	Guanidine hydrochloride
GSDW	Gui Shao Di Huang Wan
HFEA	Human Fertilisation and Embryology Authority
HUVEC	Human Umbilical Endothelial Cells
HUtMEC	Human Uterine Microvascular Endothelial Cells
IVF	*In vitro* fertilisation
kDa	Kilodalton
LIF	Leukaemia Inhibitory Factor
mPRα	Membrane Progesterone Receptor Alpha
mRNA	Messenger Ribonucleic Acid
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NAT	N-acetyltransferase
NFE2L2	Nuclear Factor Erythroid 2 Related factor
PAGE	Polyacrylamide gel electrophoresis
PAQR	Progestin and AdipoQ Receptor
PBS	Phosphate Buffered Saline
PRα	Progesterone Receptor alpha
PRβ	Progesterone Receptor beta
PT	Prothrombin Time
Rf	Retention factor
SD	Standard Deviation
TCM	Traditional Chinese Medicine
TLC	Thin Layer Chromatography
TT	Thrombin Time
TXA	Thromboxane A
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

vii. LIST OF TRADITIONAL CHINESE MEDICINE TERMS

Taken from "The web that has no weaver" (1). Capital letters are used in the text to denote a term which has specific meaning in Chinese medicine. Where the word is in use in the English language it will generally have a different meaning from the scientific understanding of it.

Traditional Chinese Medicine (TCM) is a term that encompasses several branches of medicine including Chinese Herbal Medicine (CHM), acupuncture, and dietary therapy.

Yin and Yang – the concept originated from observation of the Yin or shady side of the mountain and Yang the sunny side. Everything has a Yin and Yang aspect, Yin and Yang create each other, control each other, and transform into one another. Yin is conserving and nourishing, Yang is dynamic and moving.

Qi – often translated as energy or primordial matter. It is the common thread between all things, mineral to man. It is the source of all movement and accompanies all movement. There are many categories of Qi and its movements which affect health. Deficient Qi – when there is insufficient Qi of to perform the required functions. Spleen Qi deficiency, the Spleen Qi is unable to perform all the functions associated with the Spleen. Stagnant Qi – Qi does not move through the body leading to aches, pains, failure to digest food or to move Blood.

Blood – Blood is the compliment to Qi. It originates from the transformation of food via the Spleen, is propelled through the Lungs to receive Qi from air and becomes Blood which is able to nourish the body and organs. Blood may also be deficient or stagnant.

Heart – the Heart stores the Shen and responsible for consciousness and volition. It ensures appropriate behaviour and responses.

Kidney – the Kidney stores essence, governs birth, growth, and reproduction. Kidney function declines with age.

Spleen – The organ most closely associated with Qi but also closely related to Blood. It transforms and transports and its failure leads to a lack of nourishment.

Liver – the Liver is most closely connected with Blood which it is said to store, but ensures the smooth movement of Qi.

Shen – the spirit, a fundamental that defines humans. It is mind, consciousness, self-awareness.

Damp – is a pathological manifestation of healthy Yin, it accumulates and stagnates, can create heat, and often originates from disharmony of the Spleen.

Heat - is a pathological manifestation of the healthy Yang of the body, empty heat from a lack of Yin.

viii. ACKNOWLEDGEMENTS

To my husband who supported me though this whole process and listened patiently to the tribulations. To my daughter who was the inspiration to finish it, and who gave up some of our playtimes and bedtimes for me to do so.

For all the women who struggle to become mothers.

With thanks to Roz Gibbs without whom none of this would have been possible, to John Young for his sage advice on blotting, to Alan Cooper for sharing such techniques as the "patented" flask tap for HUVECs, to Asmita Sautreau for the heads up on heparin, and to Helen Fillmore for her wisdom and passionate interest in my work.

ix. DISSEMINATION

The author has published work in the journal of the Register of Chinese Herbal Medicine and has presented work at the conference of the Register of Chinese Herbal Medicine.

“Never give up on a dream just because of the time it will take to accomplish it. The time will pass anyway.”

Earl Nightingale

“You may have to fight a battle more than once to win it.”

Margaret Thatcher

1 Introduction

1.1 Why investigate Chinese herbal medicine and its influence on fertility?

There is a need for improvements in the assistance that can be offered to infertile couples whether they are trying to conceive naturally or using Assisted Reproductive Techniques (ART) such as *In Vitro* Fertilisation (IVF). In 2014 in the UK 52,288 women had a total of 67,708 cycles of IVF (2). There is little research into how Chinese herbs can be of assistance, particularly produced by researchers in the West, but what exists is positive, showing that herbs can be clinically effective (3, 4). Research into pharmacological mechanisms that underlie these positive effects is almost exclusively undertaken in China and published in Chinese. This lack of accessible evidence creates an appropriate reserve in biomedical practitioners when asked to recommend herbal medicines or permit them alongside conventional treatments. This is in part due to lack of information on how they work and so their likely interactions with pharmaceutical medicines.

In the United Kingdom (UK) both the supply and the practice of herbal medicine remains without statutory regulation. European Union (EU) legislation, which passed into law in 2004 and whose transitionary period ended in 2011, overrode the 1968 Medicines Act under which legal directive herbalists had previously practiced (5). This change to the way in which herbalists were permitted to practice led to many years of discussion and government reports, predominantly concerned with the safety of supply and practice. Although not the central purpose of the discussions, inevitably questions of efficacy are raised. Concerns have been expressed in debate that

statutory regulation might infer efficacy of herbal medicines, as well as ensuring the safe supply of herbs by qualified practitioners (6). The practice of herbal medicine would benefit from research generated in the west to assess possible efficacy of herbal medicine to back up arguments around regulation.

One of the two research studies cited above on Chinese Herbal Medicine (CHM) and fertility (4), was conducted in Australia; it was a meta-analysis of existing studies covering 1851 women with a range of fertility disorders. These studies all used pregnancy as the primary outcome. They found that pregnancy was three and a half times more likely using Chinese herbs and pharmaceuticals over four months than pharmaceuticals alone. The second study measured markers of endometrial receptivity in women receiving herbal treatment for whom IVF had previously failed. All six measured markers of endometrial receptivity were significantly improved and there was a 34% pregnancy outcome (3).

IVF is both expensive and invasive and should not be considered as one of the first solutions to infertility either for the benefit of the individual, or in the UK, for the National Health Service (NHS). There are many factors that affect fertility; age, weight, diet, and lifestyle, all of which have their effect on the endocrine system, immunity, oocyte quality, and uterine environment (7). IVF is generally successful in oocyte retrieval, fertilisation and the production of embryos for transfer, but has only an average 30% live birth rate (8). When oocyte quality is apparently acceptable then failures may be attributable to the implantation environment. One aspect of the receptivity of the endometrium to implantation is uterine perfusion and blood vessel development (9). Haematology was chosen for this study as a meeting point of western and Chinese thought, both sharing the understanding that sufficient blood supply is essential for preparing the uterus for embryo implantation and for maintenance of a pregnancy.

The Traditional Chinese Medicine (TCM) understanding of Blood or “xue" includes, but is not limited to, that of blood circulating within the vessels and delivering Qi (which in this context could equate to hormones, oxygen, glucose etc.) to the cells.

Several herbal formulae have been studied in China for their effects on endometrial receptivity, expression of oestrogen receptors, and angiogenesis promoting factors. A Chinese herbal formula called Zhu Yun[1] has been shown to improve endometrial receptivity in induced implantation dysfunction in mice. It is thought to reinforce the expression of endometrial Leukaemia Inhibitory Factor (LIF) and integrin β3 which are adhesion molecules active in the secretory phase of the menstrual cycle to promote implantation of the blastocyst (10). Also looking at expression of LIF, another formula called Bu Shen An Tai, showed an increased expression of LIF mRNA in the endometrium of mice with embryo implantation dysfunction (11). A third formula Jian Tai Ye has been shown to increase endometrial receptivity by increasing oestrogen receptor proteins and mRNA in mice with embryo implantation dysfunction (12). Er Zhi Tian Gui was found to reduce the need for administered gonadotropins, increase deoxyribonucleic acid (DNA) methyltransferase protein expression in the endometrium, increase oocyte number and increase pregnancy rate in infertile women diagnosed with Kidney Yin deficiency who were undergoing IVF (13).

Most of the studies on Chinese herbal medicine and fertility including those investigating endometrial receptivity and angiogenesis have been conducted in China, and few are translated for publication in English. There has been little research in relation to fertility and the biological effects of Gui Shao Di Huang Wan (GSDW)[2], the formula chosen for this investigation. There are many studies in the literature for a range of conditions on the related formulae Liu Wei Di Huang Wan. This research will be discussed in more detail in Section 1.7. The studies here expand upon the Chinese medical theory actions of "nourishing Blood and Yin[3]" thereby supporting the rationale of this study.

There is also very limited research undertaken on whole formulae here or in China. The trend is for the reductionist analysis of individual compounds. Pharmacological

1 Details of the herbs that make up this formula can be found in Table 1-4
2 Details of the herbs that make up this formula can be found in Table 1-1
3 Chinese medicine terms are capitalised to distinguish them from their western use, explanations of their meaning can be found in vii

research tends to be reductionist, searching for compounds which in isolation may be modified and patented to provide a marketable drug, but techniques and attitudes are changing (14). Recognition of the importance of polypharmacy, polyvalence and the developing -omics techniques all provide a framework to investigate health and drugs using an interdependent, systems biology approach (Section 5.12.1). This resonates with the premise of researching Chinese herbs as part of formula instead of the pharmaceutical route of breaking it down to seek the specific "active" compound.

Whilst a reductionist approach can provide information on the mechanisms of the therapeutic effect, it does not provide information on how the whole herbs or components within it can act, let alone the whole formula. Radix Panax Ginseng (Panax ginseng C.A. Mey) has multiple compounds which show opposing effects on vascular pathophysiology (15), any extracted individual compound would give different results to that of using the whole herb. Just as there is interaction between the compounds within a herb, so too between the herbs in a formula. Gui Shao Di Huang Wan contains eight herbs and a current review of literature finds 289 identified individual compounds (Appendix III). Some of the compounds have only been isolated and characterised, others have been investigated for their roles in a range of physiological functions.

In clinical practice, herbs are mainly prescribed as a formula and it is important that approaches are developed to evaluate the actions of the herbs in the way they are prescribed. The evidence base needs to reflect clinical practice. Investigating whether polyvalence can be detected between the herbs in a formula will help to inform its future use as a medicine, determining whether an individual compound or herb should be used in preference to a whole formula or whether the whole provides more than the parts.

The proposed research takes the Chinese herbal formula Gui Shao Di Huang Wan, which is commonly modified for prescription in the UK for fertility, and seeks to elucidate some of the mechanisms by which it has been seen to produce positive effects on endometrial receptivity and pregnancy rates seen in clinical studies (3, 4,

16). Established *in vitro* techniques are used to investigate the effect on haemostasis, angiogenesis, and ovarian steroid receptor expression to inform our understanding of their mechanisms of action. This is vital for the public in receiving herbal treatment and other healthcare professionals in providing concurrent conventional care.

Studying a Chinese herbal formula within a western paradigm requires some difficult parallels to be drawn. The principles of the two systems are diverse and the background to the investigation in this study necessitates enquiry into both realms of thinking.

1.2 Natural products and the -omics

Pharmaceutical drugs can be lifesaving and their development a testimony to the ingenuity of researchers. They can also be ineffective and subject to side effects. They can be used incorrectly, and may be unavailable or unaffordable to large proportion of the world population. Herbal medicines are also subject to these problems and are largely unregulated which leads to issues with quality. Pharmaceutical medicines require approval for use following trials of safety and efficacy but the majority of herbal medicines have not been scrutinised in this fashion.

There is a long history of the drug discovery from the plant sources. Only since the industrial revolution has the approach of isolating pure compounds with the intent of having more active and safer drugs been prevalent. Of the two hundred and fifty two drugs considered essential by the World Health Organisation, 11% are exclusively of plant origin, and many more are derived from natural plant precursors (17). Diosgenin is an extract of Rhizoma Dioscoreae (*Dioscoreae opposita* Thunb.), one of the herbs in Gui Shao Di Huang Wan, and was used as the precursor to many of the common steroids in use today such as cortisone and progesterone. Of fourteen drugs used for chemotherapy in 2001, five drugs originate from plants (18). Natural products are the origin for the vast majority of the pharmaceutical drugs introduced over the last fifty years. Despite the focus on biotechnology synthesis, genetic engineering and meta-genomic approaches to product development, natural products remain a rich source for novel antibiotics, oncology and other therapeutic compounds (19).

Plants used as medicine may be dried or fresh, whole or parts of plants, alone or in combinations. Extractions for internal use are typically prepared with water, and used as decoction, granules, powder, pills, or capsules. They may be prepared in alcohol as tinctures, in sugars as syrups, or for external application. Purification of plant materials can be used to isolate specific compounds which are precursors of therapeutic pharmaceutical drugs such as diosgenin from Rhizoma Dioscoreae, or be the therapeutic compound itself such as morphine (17). It has been shown repeatedly that the entire extract of a plant can have a greater effect than an isolated constituent. In his paper on -omic technologies in phytotherapy, Ulrich (20) describes 16 medicinal plants for which this has been shown to be the case including several Chinese medicines. *Gingko biloba* L. affects platelet aggregation, two gingkosides interact synergistically in a dose dependent manner; Radix Glycyrrhizae *(Glycyrrhiza glabra L.)* is both anti-inflammatory and amplifies the activity of hydrocortisone. Rhizoma Paris Polyphylla (*Paris polyphylla* Sm.) contains saponin glycosides and is used to treat abnormal uterine bleeding. Twenty two glycosides were purified and were shown to be contractile agonists for the uterus and to work synergistically (21).

A formula called Sheng Mai San was examined as a whole formula, as a single herb Fructus Schisandrae *(Schisandra arisanensis* Hayata), which is one of the constituent herbs, and an isolated compound from it called schizandrin. Three of the lignans found in *Fructus Schisandrae* were quantified in this experiment. It was found that higher levels were present in the decoction of the three herb formula Sheng Mai San than when it was decocted alone. The presence of the two additional herbs in the decoction process increased liberation of the compounds into the water. The schizandrin, a compound present in all three preparations, was detected in rat plasma. It was found that the absorption levels were increased, as was time to elimination, when administered as a three herb formula in comparison to administration as an isolated compound or as a single herb (22).

Fan et al. (23) summarises the challenges of developing a western drug from a TCM formula. There are many compounds in each herb and the synergy and antagonism

between them is largely unknown. Individual compounds may be less potent that the total extract, compounds may be pro-drugs, active only after metabolism, and the actions tend to be slower than that of pharmaceuticals (23). Plant extracts containing a variety of bioactive compounds have the possibility of being within themselves a combination therapy, targeting multiple systems which is not the case with single compound based drugs. It will require research and embracing new methodologies to characterise the herbs and assess their therapeutic potential.

1.3 East to west, bridging the gap

The theory of Chinese medicine like Chinese philosophy is less reductionist and more focussed on whole world observation. Western medicine over the past few centuries has become increasingly micro focussed from organism to organ, cell to organelle, to the binding of specific molecules to cellular receptors, and the expression of DNA. Isolation of single protein targets has provided many drugs; particularly effective for the treatment of acute disease, but these strategies when used over long periods for chronic conditions may lead to epigenetic changes which can cause serious side effects (24).

Identifying molecular targets is a critical step in the validation of a biological effect but the complexity of Chinese herbal medicines makes this a challenging approach (25). Focus on the details can lead to neglect of an appreciation for the whole. This is where Chinese medicine theory takes the opposite view, the effects on whole system are prioritised over the detail at a cellular level. Greel (26) suggests that western medicine in its use of systems biology is moving back from the reductionist investigations towards a more systematic view. Pharmacological investigation using the -omics technologies and systems biology approach applied to traditional products may be used to validate the traditional use of a herb or formula without threatening the philosophy of formulae and polyvalent prescribing. New approaches are needed for drug discovery as fewer and fewer new pharmaceutical drugs are making it to market each year (26). There is a crisis in areas such as antibiotics, and Chinese herbs are a huge potential resource for these substances; a first trial for Chinese herbs for

recurrent urinary tract infection treatment by general practitioners in the NHS has begun (27).

1.4 History and context of the formula Gui Shao Di Huang Wan

Chinese medicine has a long history of treating fertility and first discussions of it are found in the classic text the Shan Han Lun (Treatise on Febrile Disease) from 200CE. Gui Shao Di Huang Wan (GSDW), Dang Gui and Peony Rehmanniae pill. is an ancient formula based on one called Liu Wei Di Huang Wan, the Six Gentleman's decoction, which treats Yin deficiency, and was first recorded in Qian Yi's work Xiao Er Yao Zheng Zhi Jue (The Correct Execution of Paediatric Medicinals & Patterns) which was published in 1119CE. The addition of Radix Angelicae Sinensis and Radix Paeoniae Alba to make the formula Gui Shao Di Huang Wan provides additional support for the Blood, so creating a Yin and Blood tonic. There are many formulae that could have been chosen for this study; many herbs that are considered to be of therapeutic use for fertility. According to Traditional Chinese Medicine (TCM) theory the main causes of infertility are Kidney Yin deficiency, Liver and Blood stasis (28). Gui Shao Di Huang Wan is often used in practice and it comes up frequently as a guiding formula throughout a leading book on treating infertility with Chinese medicine by Jane Lyttleton (29). It is also one of the key formulae that were used in the study looking at markers of endometrial receptivity. Patients were treated using a four week, four formula phased approach; in week two an unmodified Gui Shao Di Huang Wan was used and in weeks three and four, six of the eight ingredients were used in a modified formula. Fifty patients, who had two previous failed IVF cycles, received treatment with herbs. All six physiological parameters on ultrasound known to affect endometrial receptivity were improved, and 34% of patients conceived (3).

Traditionally Chinese herbs are prescribed in a formula, this is typically two to twelve herbs simmered together in a "tea". Occasionally a herb will be used alone but this is unusual. This combining of herbs is used to address the multifactorial nature of most illness, both in origins and in manifestations. In addition, it is generally considered that some herbs will potentiate efficacy or ameliorate toxicity. A definition of synergy

by Evans (30) suggests that synergy is frequently misapplied to describe potentiation and attenuation of therapeutic effects which would more accurately be described as polyvalence. Yang et al. (31) states that the term synergistic may be applied to a broader range of interactions such as regulation of the same or different pathways, regulation of enzymes and transporters in metabolic processes, overcoming resistance pathways in microbes and cancers, or by eliminating adverse effects.

Compounds which may not contribute directly to the therapeutic effects may bind to those that do and potentiate their actions through increasing availability, transport into the cell, or across the blood brain barrier. Many herbal medicines contain terpenoids which have a strong affinity for cell membranes so can permeate cells to exert their effects (32). Rhizoma Coptis (Coptis *chinensis* Franch.) has been shown to have stronger anti hyperglycaemic actions in its whole form than just the alkaloid fraction (33). Also, in this same plant is 5′-methoxyhydnocarpin, an amphipathic weak acid which has no antimicrobial action. In combination with berberine, which is antimicrobial and is present in the same plant, it effectively disables the bacterial resistance mechanism potentiating the antimicrobial effect of the berberine (34). Epigallocatechin gallate present in green tea enhances the therapeutic effect of temozolomide in treating glioblastoma by crossing the blood brain barrier and causing chemo sensitisation (35).

1.5 Fertility and Traditional Chinese Medicine

In 2010 a review of herbal medicine in IVF treatment found that there were no RCT's on the use of Chinese herbs and no conclude there is no evidence to support their use (36). Since this review there have been several clinical studies which have shown positive results with the use of Chinese herbal treatment used as an adjunct to IVF, and with natural conception One of the most recent is a trial where patients with previous failed IVF cycles had either three months of Chinese herbal treatment and then an IVF cycle, or the control which was a three month wait and then IVF treatment. The Chinese herbs treatment group had significantly higher pregnancy rate, higher numbers of fertilised oocytes and lower numbers of biochemical

pregnancies (28). A review of 21 RCT's using a range of herbal formulae was undertaken and although a number of limitations were identified such as no blinding, small sample sizes they do all show increased pregnancy rate (37).

A survey was undertaken to assess levels of Chinese herbal medicine use by patients at fertility clinics; in the UK it was 10%, Australia 8% and the USA 18% (4) and this is despite opposition from many clinic doctors and a poor understanding of the effects of herbal medicines. In an Irish IVF unit, a study showed that 48% of patients had used herbal medicines and 38% of those in the 3 months prior to treatments and the concern raised by the study was that none had discussed this with their IVF physician (38).

A meta-analysis using stringent selection criteria, within a Cochrane framework were completed in 2011 (39) of eight randomised control trials with 1005 women that showed that there was a pregnancy rate of 50% over four months using Chinese herbal medicine compared to 30% with IVF alone. An updated meta-analysis in 2015 (4) reviewed forty randomised control trials with 4200 women and suggested that in the treatment of female infertility, Chinese herbal medicine alongside pharmaceutical treatments, including IVF, increased pregnancy rate from 30 to 60% over a three to six month period. Further analysis of this data was performed looking at the pregnancy rate for women treated with Chinese herbs alone. Data was from only seven studies, but they were high quality and included 616 women, there was a 49% pregnancy rate.

A meta-analysis undertaken in the UK of studies examining the use of Chinese herbal medicines for support during IVF cycles showed a significantly increased clinical pregnancy rate (40), a subset of the studies showed an increase in implantation rate. Only in one study were herbs continued post conception, this study involved 81 women and did show a decreased miscarriage rate. The conclusions of the authors were that the methodologies of the studies were poor, results were subject to high risk of bias, and that the significant differences found in the meta-analysis were

unlikely to be accurate. In the US a retrospective analysis was performed using records of 1231 IVF patients and they found IVF with a whole system IVF which included acupuncture and Chinese herbal medicines specific to the patient was associated with greater odds of live birth in both donor and own gamete cycles (41).

One clinical trial undertaken in the UK, by a Chinese herbal specialist, looked at influence of herbs on six parameters affecting endometrial receptivity using 3D ultrasound and blood tests (3). The measures were serum Follicle Stimulating Hormone (FSH) and progesterone levels, thickness of endometrium and sub endometrial uterine perfusion (three measures). Participants were women with at least two previous IVF failures as its test group who all received treatment, the control groups were normal healthy women without fertility problems. The control group were not trying to achieve pregnancy but were treated and all other parameters measured. The control group were randomised and double blinded as to whether they received treatment, the test group were not. The treatment duration was three menstrual cycles and the treatment group showed significant improvements in all six measures and had a 34% pregnancy outcome. The control group who were treated showed no changes in uterine perfusion or hormone levels (3). This is of particular interest when considering the debated premise of adaptogenic herbs, which are able to return an organism to a state of balance rather than having a specific inhibitory or potentiating effect (42).

A Japanese study used a seven Chinese herb combination with women aged 38.5±0.7 with previous failed intracytoplasmic sperm injection (ICSI) assisted IVF cycles and found improvements in oocyte development and pregnancy rate (43). A prospective RCT in 2014 of 432 patients under 42 years old were given IVF plus Chinese herbs or the control without and found that the endometrium was thicker, and the number of high quality embryos was higher as was the biochemical pregnancy rate. This did not translate into a statistically significant higher birth rate (44).

Despite a growing number of studies that indicate possible advantages to treatment with Chinese herbs for fertility or alongside IVF, there are methodological concerns of these studies including low numbers of patients, lack of blinding, diverse methodologies, and treatment protocols, all of which make it difficult to draw firm conclusions. Patients fail to discuss the use of these therapies to their clinicians and a clinician lack the research to advise their patients on adjunctive use.

1.6 Traditional Chinese Medicine actions of Gui Shao Di Huang Wan

The formula in this study is a 'Yin and Blood tonic' (Section vii for an explanation of TCM terms). Liu Wei Di Huang is a classic Yin tonic formula commonly modified to Gui Shao Di Huang Wan which makes it a Yin and Blood tonic. Yin and Blood are key to female fertility. In order for ovarian follicles to develop the Yin "needs to be strong", especially in the first half of the menstrual cycle which is under influence of FSH. During this time, the follicles secrete oestrogen which promotes the development of the endometrium which, according to Chinese medicine theory needs to be nourished by Blood. Once Yin has reached its peak an oocyte is released and the follicle transforms to become the corpus luteum, which releases progesterone the hormone associated with Yang. Disorders of fertility more specific related to the Yang include early luteal failure, polycystic ovary syndrome and hypothyroidism. Although Yang is not directly treated with this formula, Yin and Yang are interrelated and transform into one another; treating the Yin improves the Yang. A poor follicle creates less oestrogen, this causes lower inhibin so that FSH is raised. The duration of the development phase of the follicle is correspondingly reduced (45). High FSH leads to abnormal FSH to LH ratios, and both this and the decreased inhibin, impairs the corpus luteum and serum progesterone (46). A classic Yang tonic for fertility shares many of the same herbs as the Gui Shao Di Huang Wan with additional warming herbs added to increase the Yang. Adequate uterine perfusion must continue under the influence of progesterone to maintain the endometrium and for it to continue to become optimally receptive during the implantation window (47). Gui Shao Di Huang Wan is not a personalised formula as would be used in a clinical situation, but is a suitable base formula for many types of female infertility.

In TCM theory organs involved with the creation and storage of Yin and Blood include the Kidney, Liver, and Spleen. The Kidneys store the Jing, which is the origin of the Yin and reproductive energy. The Liver both stores and moves the Blood and the Spleen governs the transformation of nutrients which are used for the production of Blood. The original formula Liu Wei Di Huang Wan is composed of two sets of three herbs. The first set are the three tonics which have their primary action on the Kidney, Liver and Spleen, the organs responsible for producing Yin and Blood. The second set of three herbs drain Damp and Heat which are common pathologies associated with Yin and Blood deficiency (29). A TCM formula is structured to have a chief, deputy, assistants and sometimes an envoy. The chief is the primary herb and defines the purpose of the formula. Additional herbs will work together with the chief to strengthen the main effect but will have their own effects, prevent against unwanted effects or to guide the direction of the formula. Table 1-1 lists the eight herbs in Gui Shao Di Huang Wan. Radix Rehmanniae Preparata tonifies Yin and Blood of the Liver and Kidney and is the chief herb of this formula. The first of the deputies is Fructus Corni, it is an astringent but also tonifies the Liver and Kidney. It is an astringent in that it retains Jing and body fluids which nourish the Yin. The second deputy is Rhizoma Dioscoreae which is a Qi tonic and nourishes the Yin of the Kidney and Spleen. Deficiency of the Kidneys often results in poor water transformation leading to an accumulation of Damp, which can lead to Heat and the assistants treat this. Sclerotium Poria Cocos drains Damp as well as being a gentle Spleen tonic. Rhizoma Alismatis clears Damp as well as Heat, and Cortex Moutan clears deficient Heat. Radix Angelicae Sinensis and Radix Paeoniae Alba are both primarily Blood tonics. Angelicae Sinensis tonifies the Blood of the Heart and Liver whilst also moving Blood stasis. Paeoniae Alba tonifies the Blood of the Liver and preserves the Yin. Table 1-1 presents the names of the eight herbs in the formula under investigation, as well as its purpose within the formula.

Table 1-1 The names and functions of herbs in the formula Gui Shao Di Huang Wan

Pharmaceutical name (48)	Scientific name	Plant Family	Chinese	Pinyin	Role of herb in the formula (49)
Radix Rehmanniae Preparata	*Rehmannia glutinosa* (Gaertner) De Candolle	Orobanchaceae	熟地黄	Shu Di Huang	Kidney Yin and Blood tonic
Fructus Corni	*Cornus officinalis* Siebold & Zuccarini	Cornaceae	山茱萸	Shan Zhu Yu	Kidney and Liver Yin tonic, astringent
Rhizoma Dioscoreae	Dioscorea oppositifolia Carolus Linnaeus	Dioscoreaceae	山药	Shan Yao	Kidney and Spleen Yin tonic
Sclerotium Poria Cocos	Wolfiporia extensa (Peck) Ginns	Polyporaceae	茯苓	Fu Ling	Spleen tonic, clear damp
Rhizoma Alismatis	*Alisma plantago-aquatica* subsp. orientale (Sam.) Sam	Alismataceae	泽泻	Ze Xie	Drain damp, clear heat
Cortex Moutan	*Paeonia ostii* T. Hong & J. X. Zhang	Paeoniaceae	牡丹皮	Mu Dan Pi	Clears deficient heat
Radix Angelicae Sinensis	Angelica sinensis (Oliv.) Diels	Apiaceae	当归	Dang Gui	Blood tonic, moves Blood stasis
Radix Paeoniae Alba	Paeonia lactiflora Pallas	Paeoniaceae	白芍	Bai Shao	Blood and Yin tonic

1.7 Review of literature on the formula Liu Wei Di Huang Wan

A number of studies have been undertaken examining Liu Wei Di Huang Wan, the antecedent formula to the one used in this study. From a review of titles alone it appears that papers written in Chinese journals are extensive. Where the title of the paper appeared particularly pertinent, the papers were obtained and translated, but for the most part papers reviewed are those for which the full text in English was available. The following demonstrates some of systems influenced by the formula and a few of the many mechanisms by which it may treat disease.

1.7.1 Administration

Some of the studies provide valuable information about the use of the formula. It was shown to have no adverse effects on rats at 200mg/kg/day (50). The length of time these herbs are decocted may be important as it was shown that mineral content varied according to decoction time (51). One of the measured effects of the formula was modification of caffeine metabolism by altering enzymes cytochrome P450(CYP)1A2, CYP2A6 and N-acetyltransferase (NAT)2. There are many prescribed

drugs whose metabolism would be affected by these pathways including the antibiotic Ciprofloxin and the anti-depressant Fluvoxamine. This demonstrates the potential for interactions with other drugs administered in combination with the formula (52). The formula must be prepared correctly, and care must be taken during prescribing to understand the potential interactions with other medications.

Synergy has been demonstrated within Liu Wei Di Huang Wan. In one study three compounds, 5-hydroxymethyl-2-furoic acid, loganin, and paeonol, were used as plasma markers of absorption. It concluded that the pharmacokinetics of these three compounds within the formula led to more rapid absorption and slower elimination than administration of each individual herb (53).

Si Wu Tang contains three of the same Blood tonifying herbs as GSDW (Table 1-1, p14) with the addition of Rhizoma Ligustici Chuanxiong, a study shows how these herbs stimulate haemopoiesis by promoting erythropoietin and granulocyte-colony stimulating factor (G-CSF) gene expression (54). A study using microarrays to analyse gene expression induced by this formula has shown that it upregulates nuclear factor erythroid 2 related factor (NFE2L2), signalling a cytoprotective pathway resulting in increased antioxidant response. This is not the system targeted by oestradiol or ferulic acid, but another mechanism. It was also demonstrated that the gene expression induced by Si Wu Tang closely aligned to that obtained from oestradiol treated cells, which is consistent with the use of this formula to treat women's reproductive health (55). Eight pairs of Chinese herbs, including an ethanolic extraction of Radix Angelicae Sinensis and Radix Paeoniae Alba, were tested for their antioxidant properties, all of the pairs when decocted together were shown to have greater radical scavenging abilities than the sum of the individual herbs (56).

A study examined the pharmacokinetics of three triterpene acids in Sclerotium Poria Cocos following oral administration to rats. The formula Gui Zhi Fu Ling Wan which contains Poria was seen to have more effective absorption and slower elimination of the triterpenes when administered within the formula than the Poria extract alone (57).

1.7.2 Neuronal

Cognitive enhancement and reversal of induced amnesic behaviour was seen in rodents after administration of Liu Wei Di Huang Wan (58). The formula was able to modulate neuronal and synaptic function in rats (59), and improve synaptic plasticity in a cell model (60). Both functions suggested a mechanism for the cognition enhancing effect. It also has positive effects on demyelination in experimental autoimmune encephalitis in mice (61).

1.7.3 Haematology

Microcirculatory blood flow was measured using spectral analysis of skin surface laser doppler at the Kidney acupuncture point Tai Xi. It was shown that in patients, following administration of Liu Wei Di Huang Wan, microcirculation was increased (62). Bone marrow suppressed mice showed improvements in progenitor cell proliferation following administration of Liu Wei Di Huang Wan when measured using flow cytometry (63).

1.7.4 Endocrine

Insulin sensitivity in rats was increased and insulin resistance was delayed with administration of the formula (64). Plasma glucose was reduced in a dose dependent manner even when there was insulin resistance (65). In a Cochrane review of Chinese herbs and glucose metabolism, impaired glucose tolerance and fasting glucose were both improved with Liu Wei Di Huang Wan. However, there were concerns about the methodologies. There was the lack of data on the herbs, risk of bias, and no account taken of likely reversion to normal glucose considered in the studies (66).

Liu Wei Di Huang Wan used with antihypertensive drugs was more effective in reducing blood pressure and associated symptoms than antihypertensive drugs alone in an analysis of 6 trials (67).

Menopausal symptoms in women either through natural decline or following surgery were given Liu Wei Di Huang Wan for one year, showed improvements in FSH, LH

and E2 levels (68). Liu Wei Di Huang Wan granules were shown to improve a range of markers of bone quality in ovariectomised rats (69).

1.7.5 Genetic

Looking for the geno-protective effects of Liu Wei Di Huang Wan in human cells in vitro by using the COMET (single-cell gel electrophoresis) assay, it was found that the whole formula did not protect DNA from breaking under hydrogen peroxide mediated stress but Cortex Moutan and Rhizoma Dioscoreae individually did (70).

It is a somewhat disparate group of studies that have been found on Liu Wei Di Huang Wan. In TCM, Liu Wei Di Huang Wan is seen as a Yin nourishing formula; it is frequently used to treat older people and particularly older women. It is not prescribed for age, or for women, but when a diagnosis of Yin deficiency is made at any age. Yin deficiency is most common in the very young, and the old. The incidence of hypertension, insulin resistance, cognitive decline, and errors in DNA transcription all increase with advancing age. Many of these studies are the first of their kind, but they do employ standard assays and techniques.

1.8 Fertility, the endometrium, and uterus

Infertility is an increasing problem worldwide. Infertility can have many identifiable physical causes such as blocked fallopian tubes, polycystic ovaries, endometriosis or fibroids but in 24% of cases the cause is idiopathic (8). IVF has provided window into infertility; historically research into how a system works frequently begins by looking at dysfunction. IVF allows us to examine many of the usually concealed steps of the conception process. In the US five million couples are affected by infertility (71), and many perceive it as a stigmatising condition. Technology has provided interventions for those who can pay, but for many of these it simply extends the crisis and turns a private pain into a public one (71). Couples experiencing infertility talk of their sadness, depression, anger, confusion, desperation, hurt, embarrassment and humiliation (72). It is argued that the medicalisation of infertility leads to a disregard for the emotional response (73), and that therapies such as TCM, which are able to

acknowledge and support the emotional, as well as physical, needs may be vital to a successful outcome. Statistically, the limited number of herbal medicine studies so far, show similar success rates to IVF intervention (3, 4, 40) and the financial burden is significantly less. The NHS suggests a private IVF cycle costs in the region of £5000 (74), it is in the authors experience that treatment with herbal medicines, taken over a 3 months period is likely to cost in the region of £1000. The emotional burden of infertility and IVF is often overlooked in conventional treatment and is exacerbated by the medications (73).

Increasingly we are seeing women trying to conceive later in life, so age and reduced oocyte quality play a role. The use of IVF has increased in the thirty years since its inception. In 1991, 14,057 women underwent IVF, and in 2011 this number had risen to 48,147 (2). Success rates have not improved for the majority of clinics remaining at around 30% (2). This is thought to be largely due to the implantation environment, an incredibly complex process which is poorly understood. The complete picture of the changes that must occur in the uterus for implantation to occur are unknown. The newest technique for assessing endometrial receptivity uses RNA sequencing from a uterine biopsy to assess the levels of expression of 236 genes which may be up or downregulated during the implantation window (75).

One of the disadvantages to the IVF stimulation phase is supra physiological oestradiol concentrations which causes a pro-coagulable state. This is likely to be detrimental to the implanting embryo (76). It has been shown the extracts from Chinese herbs improve endometrial receptivity in mice that have been subjected to ovarian stimulation. In a mouse model of the stimulation phase of IVF, it was shown that the Chinese herbs reversed the downregulation of LIF and integrin β3 subunit which contribute to poor endometrial receptivity (10). To address the pro-coagulable state induced by IVF treatment, fertility clinics may use pharmacological interventions such as aspirin, enoxaparin, and sildenafil, which are used despite an inconclusive evidence base (77, 78). This provides an opportunity for further research on Chinese herbs to assess possible effects on haematological parameters.

In his book entitled “The endometrium”, Aplin (79) describes in detail the angiogenic, apoptic and immune processes that occur in the endometrium during the four phases of the female cycle.

The uterus is comprised of three main layers the perimetrium, myometrium and endometrium. The endometrium has two distinct layers the basilis, which is permanent, and the functionalis which forms and sheds cyclically.
Figure 1.1 shows how the blood vessels emerge from the arcuate vessels dividing and penetrating the layers of the uterus. The two uterine cell types used in the assays of this paper are shown in this figure for reference.

1st Phase:

The early proliferative phase follows the shedding of the functionalis during menstruation so repair must be initiated from the baslilis. Under the influence of oestrogen, mediated by insulin like growth factors, epidermal growth factor and transforming growth factors endometrial glands begin to transform. During this time, the endometrium is hostile. Endometrial arteries develop from the radial artery at the endometrial-myometrial border (Figure 1.1).

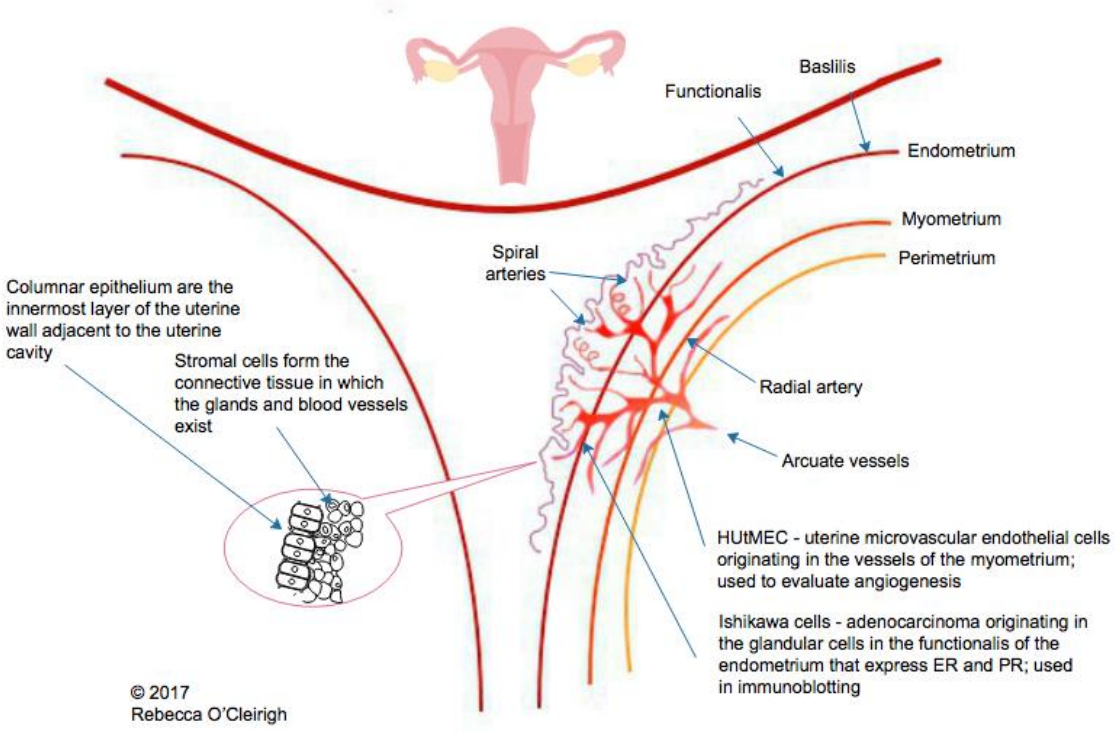


Figure 1.1 Structure of the uterus and endometrium
showing three distinct layers and the penetrating blood vessels. The cell types of the endometrium are described as reference for the cell types used in the assays in this paper.

2nd Phase:

New blood vessels develop from existing mature vessels in several different processes. Vasculogenesis, when the vascular plexus forms angioblasts from which emerge primitive blood vessels. Arteriogenesis, when there is both creation of new arterial vessels but also enlargement of collaterals and lymphangiogensis, the formation of new lymph vessels. Many hormones, growth factors and cytokines have an effect on angiogenesis but the most well-known are the Vascular Endothelial Growth Factor (VEGF) family. VEGF also regulates lymphangiogensis. Oestrogen driven angiogenesis in the mid proliferative phase causes elongation of endometrial vessels. Oestrogen is considered to be a promoter of angiogenesis but it may also be antiangiogenic, it acts directly on the endometrial vasculature and indirectly on other cell types.

3rd Phase:

After ovulation follows the secretory phase when, under the influence of progesterone, there is growth of the spiral arteries. During this progesterone phase, apical cellular protrusions occur in the endometrium called pinopods. These are thought to be involved in implantation as they are only present for a short period during the implantation window. Expression of apoptosis related genes B-cell lymphoma 2 (Bcl2) and BCL2 Associated X (Bax) are likely regulated by ovarian steroid hormones and are involved in remodelling as well as cell destruction. The Bcl2/Bax ratio alters in favour of apoptosis as the cycle progresses. Lymphomyeloid cells contribute to remodelling and at their most abundant may be 40% of the cellular content of the tissues. Eosinophils, macrophages, neutrophils, uterine natural killer cells, dendritic B cells, T cells and mast cells are all present. Decidualisation occurs 10 days after the rise in progesterone levels, when fibroblast cells become more like epithelial cells, and well known markers such as relaxin, prolactin, insulin like growth factor binding protein-1 are raised. Once pregnancy is established these decidualised cells persist and become uniform.

4th Phase:

Menstruation is due to a loss of steroidal support. Since decidualisation is a non reversible differentiation, these cells must be shed if no implantation occurs. Withdrawal of progesterone leads to inflammation. Proinflammatory and vasoactive mediators lead to tissue destruction and shedding; prostaglandin F2 alpha, cyclooxygenase-2 and endothelin 1 are all involved.

The blood supply enters the uterus through a branched structure and the vessel size decreases as it passes through the myometrium and into the endometrium. These vessels are transformed into the spiral arteries of the secretory phase which persist into pregnancy and are present in both basilis and functionalis (80). The spiral arteries are a unique blood vessel found only in the uterus and are highly sensitive to the ovarian steroids. Haemodynamics have a significant effect on endothelial cells, and their interaction with the smooth muscles cells in the remodelling of the spiral arteries (81). It is the decidualisation of the spiral arteries occurring prior to

trophoblast invasion, which facilitates implantation and necessitates menstruation in the absence of the fertilised oocyte (82).

Implantation of an embryo in the endometrium is a key step in establishing a pregnancy. It is possible only during the implantation window, which occurs 6 to 10 days after ovulation. This is when the embryo reaches attachment competence and the endometrium also reaches a receptive state. A complex molecular dialogue then occurs between embryo and endometrium (83). The endometrium is only receptive in these 4-5 days when the blastocyst can anchor to the basal lamina, and stromal extracellular matrix (84). Comparing gene expression in the endometrium in the receptive window, 172 genes were upregulated and 20 downregulated in comparison to the pre receptive state (85). Ovarian stimulation in IVF treatment is considered to be detrimental to luteal phase function, it creates an altered endocrine environment with dyssynchronous glandular and stromal differentiation. Pinopods, integrins and LIF are all prematurely expressed and this is likely to contribute to IVF failures (86).

Adequate perfusion in the endometrium at embryo transfer is associated with high pregnancy success in IVF (87). Endometrial perfusion was seen to have greater predictive value of pregnancy following IVF than endometrial thickness or morphology (88). The high oestradiol associated with many follicles in IVF creates a pro-coagulable state which reduces uterine perfusion (76). Significant correlations with successful IVF embryo transfers were shown between a lower pulsatility index of the uterine artery on day 14, and increased oestrogen receptor protein (analysed from biopsy). This correlation was not seen with increased endometrial thickness. It was also observed that prolongation of unopposed oestrogen in downregulated IVF cycles, where a gonadotrophin releasing hormone agonist is used prior to commencement of stimulation, also results in a lower pulsatility index (89).The endometrial processes investigated through *in vitro* assays in this study, and the known influences of the herbs in Gui Shao Di Huang Wan, are expanded upon in the following sections; haemostasis (Section 1.9, p23), angiogenesis (Section 1.10, p31) and the steroid receptors (Section 1.11, p37).

1.9 Endometrial haemostasis and effects of GSDW component herbs

1.9.1 Haemostasis and the endometrium

Haemostasis is the process by which blood is kept as a fluid, and how bleeding is arrested after damage to a blood vessel. If a vessel is damaged, collagen in the vessel wall is exposed and promotes clot formation (Figure 1.2, p25). Von Willebrand factor present in the blood binds to collagen and to the platelets which then aggregate. Platelets alone are not robust enough to maintain a clot against arterial pressure and so a series of reactions leads to the creation of thrombin, which converts soluble protein into insoluble fibrinogen. This forms a mesh which imparts structural integrity to the aggregation of platelets. Inhibitors exist to modulate this process and shut down the coagulation cascade once the bleeding is stopped. Once the vessel has been repaired the clot is broken down by plasmin in a similar process to thrombin formation of the clot but with different activators and inhibitors (90).

There are a number of *in vitro* assays used to examine the haemostatic process. Prothrombin Time (PT) is considered to measure the time to clot formation via the extrinsic pathway (Figure 1.2) and detects changes relating to factors II, V, VII, X or fibrinogen. The intrinsic pathway measured by the Activated Partial Thromboplastin Time (aPTT) may be due to modifications of factors II, V, VII, IX, X, XI, XII, or fibrinogen. These changes may be due to such alterations in the levels or availability of these factors, to drugs, vitamin K and autoantibodies. Thrombin Time (TT) assesses any influence of the conversion of fibrinogen to fibrin only (90).

Endometrial bleeding affects the capillary and vascular structures of the functionalis, which comprises a layer of endothelial cells wrapped in pericytes on a basement membrane. Coagulation in the endometrium is different from the rest of the body as the process is under the regulation of the ovarian steroids (81). Normal menstrual bleeding is the result of enzymatic auto digestion of the functionalis. The proteolytic enzyme plasmin is thought to be responsible for preventing the thrombi typical in

blood exposed to the collagen of damaged vessels. Lysosomes containing proteolytic enzymes are present in epithelial, stromal, and endothelial cells. During the secretory phase progesterone stabilises lysosomal membranes, but as the corpus luteum disintegrates, this support is withdrawn and the lytic enzymes are released (91).

Factor VII binds to tissue factor, which mediates haemostasis via thrombin generation, and plasminogen activator inhibits fibrinolysis, both of which are regulated by progesterone (92). Successful implantation requires the trophoblast to penetrate maternal uterine circulation whilst the endometrium retains haemostasis.

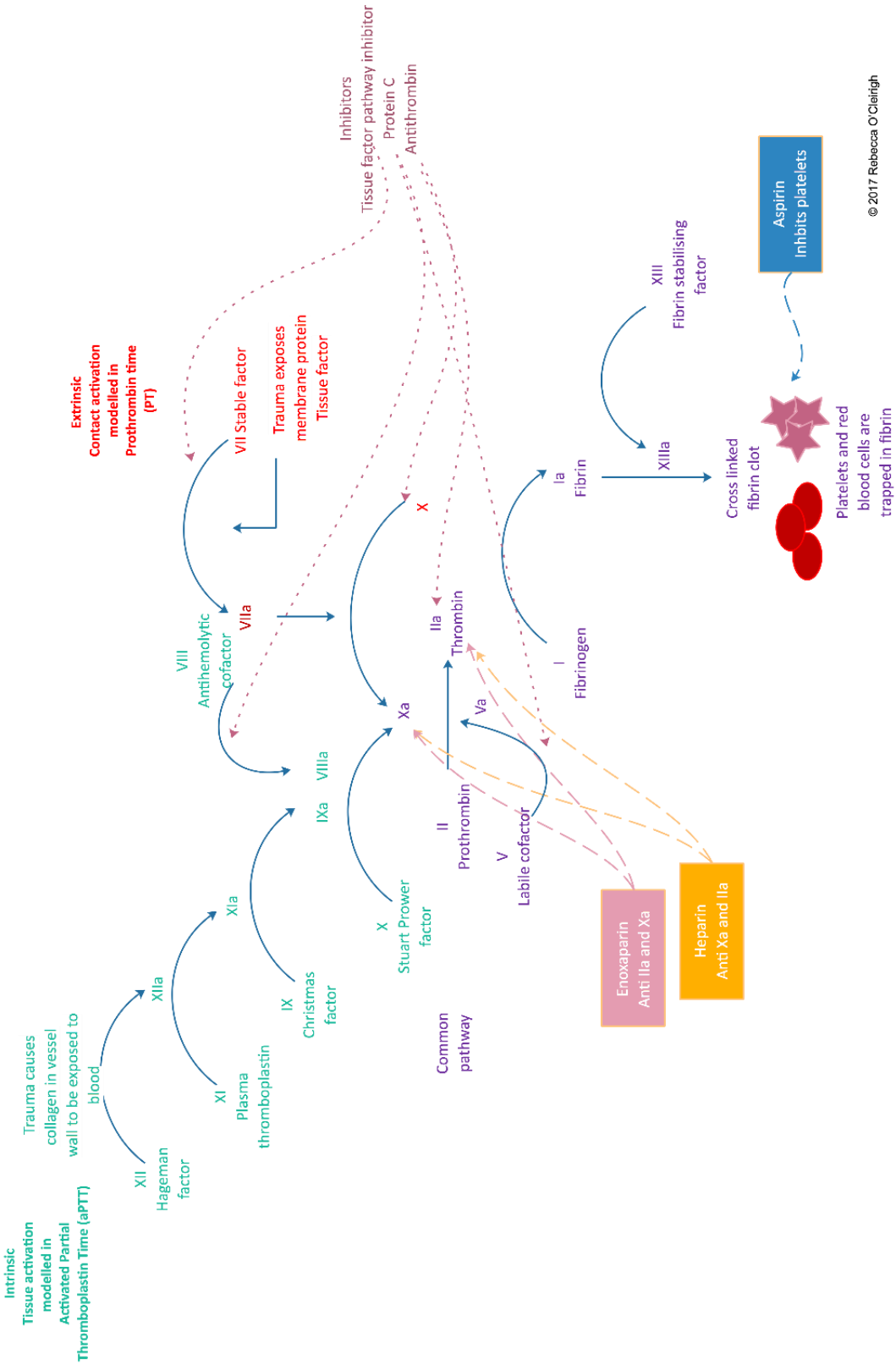


Figure 1.2 The cascade model of haemostasis
showing the fundamental activations and conversion leading to cessation of bleeding. This is presented as two pathways, intrinsic and extrinsic, which in vivo occur simultaneously but are used to evaluate clotting disorders and anti-coagulants in vitro by the aPTT and PT tests.

A condition known as antiphospholipid syndrome is when the immune system creates antibodies against a range of phospholipids involved in the haemostatic cascade. It is known to cause problems in pregnancy such as implantation failures in IVF, early pregnancy loss, placental insufficiency, and pre-eclampsia (90). Although the pathogenic mechanisms of this syndrome are not fully understood, aspirin, and enoxaparin are used for their treatment. Enoxaparin is a low molecular weight heparin. Heparin has been suggested to inhibit the binding of phospholipids with antibodies (93). Both heparin and enoxaparin bind to antithrombin III inactivating factors Xa and IIa. Heparin has a greater inhibition on factor Xa and enoxaparin on factor IIa (94). Therapeutically heparin is less often used as it binds with plasma proteins, platelets, macrophages, and endothelial cells which enoxaparin does not (95). Aspirin inhibits platelet aggregation which may prevent hyper coagulation once implantation has occurred (96). A Cochrane review in 2005 examined studies including combinations of therapies; aspirin, heparin, enoxaparin, prednisone, and intravenous immunoglobulin. This review concluded that use of the combination of enoxaparin and aspirin may reduce pregnancy loss by 54% (97). Contradictory opinions have been published since (77, 78).

Inflammation and haemostasis are closely interlinked. Inflammation repairs tissue damage due to infection, haemostasis damage to vessels. The mediators of inflammation increase platelet activation, impair anti-coagulant pathways, supress fibrinolysis and downregulate tissue factor (98). In GSDW 34 of the compounds do have anti-inflammatory functions although these links are not explored here.

1.9.2 The effects of GSDW herbs on haemostasis

A summary of the actions of the herbs within the formula can be found in Table 1-2, p31, but here they are discussed in more detail with references to the literature.

1.9.2.1 Radix Paeoniae Alba and Cortex Moutan

A study into the ethanolic whole herb extract of Radix Paeoniae Alba has been shown to extend the aPTT, possibly by inhibition of thrombin and fibrin polymerisation, and endogenous blood coagulation (99, 100). Within Radix Paeoniae Alba and Cortex Moutan, which are both peonies, are a range of compounds which are thought to mediate the improved blood circulation and inhibit platelet aggregation. This includes paeonol, paeoniflorin, benzoylpaeoniflorin, and benzoyloxypaeoniflorin In addition, methylgallate, catechin, paeoniflorigenone, galloylpaeoniflorin, and daucosterol may contribute to these actions (101).

Paeonol and a three synthesized derivatives of paeonol were tested in an automated platelet aggregation analyser, and have been shown to inhibit platelet aggregation (102). Paeonol has a potent vasodilatory effect, it attenuates intracellular Ca^{2+} by impairing the Ca^{2+} channels in vascular smooth muscle, and thereby interrupting the excitation-contraction coupling process (103). Both vasodilatory and anti-inflammatory effects were found for the 1,2,3,4,6-penta-*O*-galloyl-β-*D*-glucose which is present in Paeoniae. It is thought to dilate smooth muscle and suppress inflammation via endothelium dependent nitric oxide / cyclic guanosine monophosphate signalling (104).

Paeoniflorin has a series of anti-thrombotic effects which have been shown *in vitro* in HUVECs. It increases tissue plasminogen activator, an enzyme, and so increases fibrinolysis. In platelets, it has been shown to decreases thromboxane A2 (TXA) production and so reduces platelet aggregation and inhibits production of new platelets. (105). Two acetophones 2,5-dihydroxy-4-methoxyacetophenone and 2,5-dihydroxy-4-methylacetophenones were shown to inhibit the aggregation of platelets

in vivo in rabbits, and these were found to be more potent than the major compound paeonol (106).

1.9.2.2 Radix Angelicae Sinensis

Radix Angelicae Sinensis, used in a 5:1 ratio with Radix Astragali in a formula known as Dang Gui Bu Xue Tang, has long been used to "tonify the Blood" in TCM. A study of the effects of these herbs traditionally decocted in combination showed that Hep3B cells, exposed to the formula, demonstrate that it regulates erythropoietin mRNA expression, and secretion of erythropoietin protein (107). Erythropoietin stimulates the production of erythrocytes. Angelicae Sinensis has been shown to be anti-thrombotic in a rat model of *in vivo* thrombosis, significantly increasing prothrombin time and TT (108). Polysaccharides of Radix Angelicae Sinensis have also been shown to have both haematopoietic and thrombopoietic actions. Peripheral blood cells from a mouse model of thrombocytopaenia, treated with the polysaccharides of Radix Angelicae Sinensis, showed increased recovery of erythrocytes, leukocytes, and platelet numbers (109).

(Z)-ligustilide, in Radix Angelicae Sinensis, is a monomeric phthalide that has been investigated for its neuroprotective effects in ischaemia. Part of this protection may be due to its inhibition of platelet aggregation thus preventing thrombosis. The effects do not appear to be mediated through the intrinsic or extrinsic pathways as time to clot formation was not prolonged when looking at PT or aPTT (110). *N*-butylphthalide has antiplatelet and antithrombotic effects when administered to mice with collagen and epinephrine induced thromboembolism (111). Radix Angelicae Sinensis contains *p*-cresol a phenolic compound which reduces thrombosis by inhibiting platelet aggregation, reactive oxygen species production, extracellular signal-regulated kinase /p38 activation and TXA2 production (112). Oleic acid is a fatty acid in Radix Angelicae Sinensis found in the membranes of erythrocytes (113). It may serve as an intracellular messenger in human platelets (114). α-pinene found in Radix Angelicae Sinensis has been shown to be antithrombin and anti platelet aggregatory (115).

Radix Angelicae Sinensis extract was fractioned and the parts investigated for their anticoagulant activity. It was found that the petroleum ether soluble fraction had the highest anti-coagulant activity on TT using rat plasma. They identified 26 compounds in this fraction and found 38 metabolites of these *in vivo*. Additional compounds identified as anticoagulant include senkyunolide I, senkyunolide D and senkyunolide F (116).

Compounds in Radix Angelia Sinensis do have serotoninergic actions, binding to 5-HT7 receptors *in vitro* (117) and this receptor affects haemodynamics by acting as a vaso-relaxant (118). Platelets do have serotonin binding sites and serotonin is involved in platelet aggregation (119).

1.9.2.3 Radix Rehmanniae Preparata

Radix Rehmanniae Preparata was found to contain a *bis*-furan derivative 1,5-*bis*(5-methoxymethyl)furan-2-yl-penta-1,4-dien-3-one which inhibits *in vitro* platelet aggregation (120). Salidroside which is also present, has been shown to protect human erythrocytes from H_2O_2 induced haemolysis *in vitro* (121). Catalpol which is present in Radix Rehmanniae, administered to a rat stroke model was shown to upregulate erythropoietin expression, when sections of brain tissue were probed (122).

1.9.2.4 Rhizoma Dioscoreae

Diosgenin is a steroidal saponin found in Rhizoma Dioscoreae. This compound has been isolated from other plants and found to have antithrombotic effects, it produces elongated PT, aPTT, TT, and inhibition of platelet aggregation *in vitro*. *In vivo* inferior vena cava ligation in rats showed inhibition of thrombus formation. Increases in *in vivo* tail bleeding and time to clot formation were shown in mice.(123).

1.9.2.5 Fructus Corni

Fructus Corni contains eugenol and elemicin (124) and these have been shown to have anti platelet action through inhibition of TXA2, TXAB2, and of cyclooxygenase2 in a manner similar to that of aspirin (125).

No evidence of research into the haemostatic properties of Sclerotium Poria Cocos and Rhizoma Alismatis was found.

In Radix Paeoniae Alba and Cortex Moutan are a range of compounds which inhibit platelet aggregation, modify Ca^{2+} channels to vasodilate smooth muscle cells, and increase fibrinolysis. Radix Angelicae Sinensis regulates erythropoietin, is anti thrombotic, haematopoietic and thrombopoietic. In Radix Rehmanniae compounds inhibit platelet aggregation, protect against oxidative stress, and upregulate erythrocyte production. Rhizoma Dioscoreae is antithrombotic and thrombolytic.

Overall, the combination of herbs in the formula GSDW would be expected to reduce platelet aggregation, more rapidly break down clots, increase production of erythrocytes and be vasodilatory. Twenty compounds within five of the eight herbs in GSDW have been researched for their mechanisms of influence on haemostasis both *in vitro* and *in vivo*. It is expected that the whole formula would have a significant effect on blood clot formation and on breaking down clots which form. Table 1-2 summarises the effects on haemostasis of the herbs or compounds within them. It is important to note when looking at any compounds whether they are soluble in water and therefore extracted from the plant material in an aqueous decoction. This also influences whether they will be bioavailable once ingested. _Solubility of organic compounds is dependent on their polar group e.g. hydroxyl and amine function groups and the extent to which they interact with water. Saponin type entities in herbs can behaving like surfactants solubilising the more lipophilic compounds. Listed in Table 1-2 and Table 1-3 are the predicted solubilities based on the structure of the molecule (126) at pH 7.2 and 25°C with those closest to 0 being the most soluble and -10 being least soluble in water (127). These are approximations based on the chemical structure and will vary from expected due to the pH of the solution,

temperature, and other compounds present during extraction. Z-ligustilide for example has been considered to be insoluble in H_2O, but despite having low solubility it has been detected by HPLC in aqueous extraction of Radix Angelicae Sinensis (128).

Table 1-2 Compounds known to occur in the herbs included in the formula which influence on haematology

Herb	Compound / Herb	Aqueous solubility LogS	Action
Radix Angelicae Sinensis	Whole herb		Antithrombotic, haematopoietic and thrombopoietic
	With Radix Astragali		Regulate erythropoietin
	(z)-ligustilide	-2.58	Inhibits platelet aggregation
	p-cresol	-0.67	Inhibits platelet aggregation, ROS production, ERK/p38 activation and TXA_2 production
	Oleic acid	-6.37	Intracellular messenger in human platelets
	α-pinene	-2.94	Antithrombin and anti platelet aggregatory
	*N-b*utylphthalide	-3.46	Antiplatelet and antithrombotic
Radix Paeoniae Alba and Cortex Moutan	Whole herb		Inhibition of thrombin and fibrin polymerisation and endogenous blood coagulation
	Benzoylpaeoniflorin Benzoyloxypaeoniflorin Methylgallate Catechin Paeoniflorigenone, Galloylpaeoniflorin Daucosterol	-2.97 -2.93 -1.65 -2.65 -1.85 -2.74 -5.31	Improve blood circulation, inhibit platelet aggregation
	Paeonol	-1.75	Inhibit platelet aggregation, vasodilatory
	Paeoniflorin	-1.96	Inhibit platelet aggregation, reduce production of platelets, increase fibrinolysis.
	1,2,3,4,6-penta-*O*-galloyl-β-*D*-glucose	-3.14	Vasodilatory and anti-inflammatory
	2,5-dihydroxy-4-methoxyacetophenone	-1.68	Inhibits platelet aggregation
Sclerotium Poria Cocos	n/a		
Radix Rehmanniae Preparata	1,5-*bis*(5-methoxymethyl) furan-2-yl-penta-1,4-dien-3-one	-4.33	Inhibit platelet aggregation
	Salidroside	-1.36	Protects erythrocytes
	Catalpol	-0.19	Upregulates erythropoietin expression
Rhizoma Dioscoreae	Diosgenin	-5.88	Antithrombotic, thrombolytic, inhibits platelet aggregation, increases time to clot formation
Fructus Corni	Eugenol Elemicin	-2.06 -3.16	Inhibit platelet aggregation
Rhizoma Alismatis	n/a		

1.10 Endometrial angiogenesis and effects of GSDHW component herbs

1.10.1 The endometrium and angiogenesis

New blood vessel formation such as that seen in the endometrium is uncommon in the adult body. The arcuate arteries in the myometrium end in arterioles, which pass into the basalis of the endometrium where they are initially straight, but become spiralled as the cycle progresses (129). Vasculogenesis is the creation of new vessels from precursor cells, and angiogenesis is the process of new vessels forming from the existing network by elongation, sprouting, or intussusception. These are multi step processes which include proliferation and migration of endothelial cells through the extracellular matrix under the influence of multiple regulators and growth factors (130).

There has been debate over the proliferation of endothelial cells in the endometrium, an early study states that the number of vessels does not change through the cycle, but this study was looking at the endometrium as a whole (131). Another study suggests that significant proliferation occurs at all stages of the menstrual cycle in humans (132). It has been found that the number of vessels does not change dramatically but the proportion surrounded by vascular smooth muscle cells does (133) and also that little changes in the basalis but there is proliferation in the functionalis (134).

The cycling endometrium is a highly angiogenic tissue and defects in this process could to contribute to implantation failure and recurrent miscarriage. Angiogenesis in the functionalis during the proliferative phase, occurs through elongation. In the late proliferative and early secretory phase the complex branched capillary sub epithelial network occurs through intussusception from the vascular stumps that remain in the basal layer of endometrium following menstruation (135). Intussusception is described as the process of increasing capillary complexity through the insertion of trans-capillary pillars which divide the existing vessel. Conventional sprouting angiogenesis is where a new vessel is formed, often extending across gaps in the vasculature. Intussusception permits rapid expansion of the capillary network (136).

Migration of endothelial cells occurs predominantly post menstruation but there is a mid to late proliferative rise in migratory activity and some evidence of migration mid secretory which may be associated with spiral artery development. As each of these occur under variable prevailing hormonal influences the mechanisms are likely to be different (137). Migration of endothelial cells is in part regulated by sheer stresses influenced by the blood flow (138). A representation of these interactions can be seen in Figure 1.3.

Vascular endothelial growth factor (VEGF) mediates endometrial angiogenesis and is in part controlled by the oestrogen receptors, Oestrogen Receptor alpha (ERα) and Oestrogen Receptor beta (ERβ) (88). Oestrogen and progesterone regulate uterine vascular function upregulating VEGF and fibroblast growth factor2 messenger ribonucleic acid (mRNA) and protein expression (89). Endothelial cell migration, viability, tube formation and haematopoietic functions are all in part regulated by VEGFR2 and integrin αvβ3 (139). In the proliferative phase, endothelial cells express VEGFR2, but after ovulation these receptor levels decrease and VEGFR1 increases. When VEGF binds to VEGFR1 it causes endometrial cells to migrate whereas VEGFR2 leads to proliferation (140).

In vitro assays for the evaluation of angiogenesis have commonly been performed with HUVECs which are easy to obtain. But, since angiogenesis often involves the micro vasculature rather than the macro vasculature, these are not an ideal model (141), it is better to use the specific tissue type where possible. The characteristics of HUVECs and placental microvascular endothelial cells were shown to have phenotypical and physiological differences (142).

Oestrogen and progesterone both upregulate angiogenesis, but these receptors are lost on HUVECs after six passages (143). Oestrogen and progesterone receptors affect vasculature in the endometrium and receptors have been demonstrated on endothelial and smooth muscle cells. Oestrogen has been shown to increase the density of the microvasculature, permeability, and vasodilation of endothelial cells. Increase in vascular density has been shown in response to progesterone (144).

1.10.2 The effects on GSDW herbs on angiogenesis

1.10.2.1 Radix Angelicae Sinensis

Effects of an aqueous Radix Angelicae Sinensis extract on angiogenesis showed that HUVEC proliferation was increased, as was migration in the wound healing assay, and mean tubule length in the differentiation assay on matrigel. The extract was also used to demonstrate changes in angiogenesis *in vivo* in zebra fish. All results indicated the extract promotes angiogenesis and that these effects are due to p38 and c-Jun N-terminal Kinase (JNK) 1/2 phosphorylation. P38 and JNK 1/2 are mitogen activated kinases which are involved in VEGF signalling (145).

N-Butylphthalide derived from the volatile oils of Radix Angelicae was shown to inhibit angiogenesis in *in vitro* matrigel assays and *in vivo* capillary sprouting of mouse aortic rings (146).

The angiogenesis activity was seen in an aqueous extract and the anti-angiogenic in the volatile oils. This pattern of opposite influences on angiogenesis, in compounds present within a single herb, was also seen in also Radix Ginseng (15).

Ferulic acid is present in Radix Angelicae Sinensis and has been shown to induce significant angiogenesis in HUVECs, and to promote the formation of microvascular networks *in vivo*. Ferulic acid is hypothesised to modulate VEGF, platelet derived growth factor and hypoxic inducible factor 1-α (147). It has also been shown to promote proliferation of endothelial cells through upregulating cyclin D1 and VEGF in a dose dependent manner (148).

1.10.2.2 Cortex Moutan

Cortex Moutan contains quercetin, which is also present in many plant foods. Quercetin is seen *in vitro* to inhibit cell viability, expression of vascular endothelial growth factor receptor 2 and angiogenic tube formation (149). Quercetin inhibits tubule formation by directly inhibiting p300 histone acetyltransferase which

suppresses activation of the cyclooxygenase-2 pathway which stimulates angiogenic growth factors such as VEGF and B-cell lymphoma 2 (BCL) (150).

1.10.2.3 Radix Rehmanniae Preparata

Radix Rehmanniae Preparata aqueous extract was shown to promote angiogenesis in diabetic wound healing, although the exact compounds involved were not investigated (151). Catalpol in Radix Rehmanniae Preparata has been shown to increase brain angiogenesis, probably through its effects on erythropoietin and vascular endothelial growth factor (122). It was also shown to stimulate human mammary endothelial cells to increase tubule length, sprout formation and cell migration (152). Rehmanniae contains campesterol which has been isolated from *Chrysanthemum coronarium* L. and shown to inhibit angiogenesis and disrupt neovascularization in chick chorio-allantoic membranes (153).

1.10.2.4 Rhizoma Dioscoreae

A polysaccharide from Rhizoma Dioscoreae was isolated and shown to promote proliferation of human endometrial epithelial cells. This is attributed to the decrease in levels of BCL2-Associated X proteins which promote cell death and the increase in anti-apoptic BCL-2 protein leading to reduced cell death and a higher number of epithelial cells (154).

1.10.2.5 Fructus Corni

An ethanol extract of Fructus Corni was shown to be inhibitory to angiogenesis in HUVECs. Immunoblotting shows that it has the effect of suppressing vascular endothelial cadherin which is a component of the endothelial cell junctions and protein kinase B which regulates angiogenesis via VEGF (155). Cornel iroid glycosides from Fructus Corni have been shown to promote neurogenesis and angiogenesis after induced cerebral ischaemia in rats. There was upregulation of VEGF seen in the cortex of the rats (156).

No evidence of research into the angiogenic properties of Radix Paeoniae Alba, Sclerotium Poria Cocos, and Rhizoma Alismatis were found.

Across the herbs in GSDW are a range of pro and anti angiogenic compounds and the effects of any compound are not quantified. It is not clear from the research what the overall effect of the formula is likely to be. Table 1-3 summarises the known angiogenic promoting or inhibiting properties of the herbs and compounds discussed above.

Table 1-3 Herbs and compounds in GSDW known to have angiogenic promoting or inhibiting properties

Herb	Inhibits angiogenesis	Aqueous solubility Log S predictions	Promotes angiogenesis	Aqueous solubility Log S predictions
Radix Paeoniae Alba	n/a			
Radix Angelicae Sinensis	*N*-Butylidene phthalide	-3.11	Whole herb Ferulic acid *N*-Butylphthalide	 -2.33 -3.46
Sclerotium Poria Cocos	n/a			
Cortex Moutan	Quercetin	-3.06		
Radix Rehmanniae Preparata	Campesterol	-7.26	Whole herb Catalpol	 -0.19
Rhizoma Dioscoreae			Single polysaccharide promotes proliferation	unknown
Fructus Corni			Cornel iroid glycosides upregulate VEGF	unknown
Rhizoma Alismatis	n/a			

1.11 Endometrial ovarian steroid receptors and effects of GDSW component herbs

1.11.1 Endometrial ovarian steroid receptors

Endometrial blood vessels are unique in their regulation by oestrogen and progesterone, and the paracrine mediation of these actions on vascular and other cells of the endometrium. Regulatory mechanisms are not well understood and many factors have been identified but are likely to have zone and cell type specific regulatory function (157).

Both ERα and ERβ, membrane Progesterone Receptor alpha (mPRα) and Progesterone Receptor beta (PRβ), are all expressed in the epithelial, stromal and vascular cells (84). At a simplistic level, rising oestrogen levels in the secretory phase predominantly enhances endometrial cell proliferation, while progesterone in the secretory phase leads to differentiation. However ERα is thought to be necessary for downregulation of PR, and PR in stroma are required to inhibit oestrogen stimulated proliferation (158). The interactions between the hormones and receptors are complex, and this is compounded when the interactions of angiogenesis and blood flow are added, as is illustrated in Figure 1.3. All these factors influence one another, they do not occur in isolation.

In an examination of the glandular, stromal, and vascular compartments of the endometrium over an entire cycle, it has been shown that ERα is more predominant than ERβ, but both vary through the cycle. In the proliferative phase, glandular, stromal, and vascular compartments express ERα and ERβ, but ERα is more predominant. ERβ staining peaks on the periovulatory days in epithelial cells and ERα in mid proliferative and periovulatory phases. In the secretory phase this expression of ERα is reduced as is glandular ERβ. ERα drops and ERβ increases in vascular cells, and in the smooth muscles of the spiral arteries in the late secretory phase (159). It

has been proposed that ERβ may be needed for uterine perfusion over the long term in response to oestrogen during pregnancy, and ERα for the dynamic vasodilation in remodelling (160). ERα is involved in the dynamic control of actin arrangement and cell migration through recruitment of focal adhesion kinase which regulates the connections of the cytoskeleton of a cell to the extracellular matrix (161).

ERα increases in response to oestrogen in the proliferative phase and is diminished in response to progesterone in the secretory phase; so the endometrium is resistant to oestrogen in the implantation window. This decline in ERα is thought to be an important effect, signalling receptivity to begin. Inappropriately high expression of ERα is seen in reproductive disorders such as luteal phase defects, endometriosis, and polycystic ovarian syndrome (162).

Progesterone selectively down regulates PR expression and is important in establishing endometrial receptivity. It may be that abnormal persistence of the PR is a contributor to infertility. It has been suggested that PRβ may remain whilst PRα declines (163). Progesterone *in vivo* shows anti proliferative effects on microvasculature and on decidual endothelial cells (92). Serum progesterone facilitates pinopod formation (164) and luminal closing, which facilitates blastocyst apposition (165).

PRs regulate many genes for growth factors, transcription factors, and morphogens (165). The receptor is present exclusively in uterine blood vessels and selectively in uterine veins and lymphatic vessels, it is also present in the smooth muscle cells of the uterine arteries but not in the endothelium (166).

The increase in vascular permeability that makes the endometrium receptive to implantation is controlled by PRs. This increased permeability is vital for the proteins contained in the blood to pass into the interstitial space to support the developing blastocyst (167). PRs also co-ordinate the molecular changes in the stroma, matrix, and vasculature that govern decidualisation (168) and lead to the establishment of a pregnancy. Progesterone receptors in the endometrium are increased in pregnancy

and are key to maintaining the endometrium (139). Smooth muscle cells of the spiral arteries have high levels of PRs, suggesting it modulates blood during pregnancy (169).

Loss of progesterone signalling has been implicated in endometriosis when PRs are reduced, especially PRβ. Loss of PRβ in the stroma of the endometrium leads to abnormal interactions between the epithelium and the stroma and causes epithelial cell defects (140).

Rapid responses to progesterone initiated at the cell surface have long been described, but only recently been characterised as due to the presence of membrane progesterone receptors. These have been shown to exist in three forms; alpha, beta, and gamma, and belong to the seven-transmembrane progesterone adiponectin Q receptor family (93). Membrane PRs are highly tissue specific and mPRα is the predominant form in reproductive tissues (170). It has been implicated in regulation of progesterone function in the uterus in humans and in gonadotrophin releasing hormone secretions in rats (171). Membrane bound receptors are present in the human cervix, and upper and lower myometrium (172). It is suggested the membrane bound progesterone ratios may be important in triggering labour (141).

As with progesterone receptors there are membrane bound oestrogen receptors such as G protein-coupled oestrogen receptor 1 which appear to be regulated by the nuclear oestrogen and progesterone receptors (173).

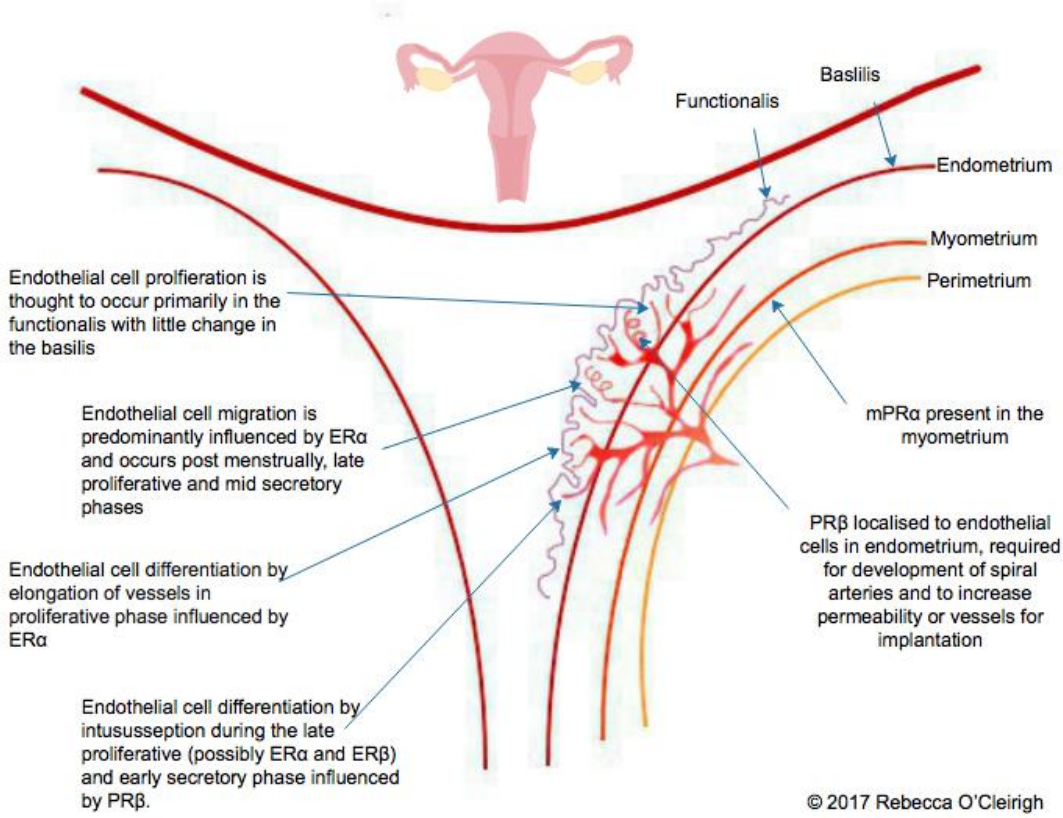


Figure 1.3 Angiogenesis in endothelial cells and actions of oestrogen and progesterone receptors in the endometrium.

1.11.2 The effects of GSDW herbs on endometrial ovarian steroid receptors

1.11.2.1 Radix Angelicae Sinensis

Administration of an aqueous extract of Radix Angelicae Sinensis led to an increase in ERα and ERβ, but not all activities could be attributed to these receptors as the response pattern was different to that of oestradiol (174). Dang Gui Bu Xue Tang aqueous extract containing Radix Angelicae Sinensis was shown to phosphorylate ERα at Ser 167, a different location on the ER to that of oestrogen which binds predominantly at Ser 118 (175). Radix Angelicae Sinensis contains a flavonoid, calysosin, with a similar structure to oestradiol and has been suggested to impart its oestrogenic action in human mammary epithelial carcinoma cells *in vitro* (176).

An ethanolic extract of this herb was also shown to have anti-progesterone activities in a progesterone receptor-positive human mammary adenocarcinoma cell line MCF-7, using a transfected progesterone response element-driven luciferase reporter gene bioassay (177).

1.11.2.2 Rhizoma Dioscoreae

The same anti-progesterone activities were seen for an ethanolic extract of Rhizoma Dioscoreae MCF-7 (177). In another study, an extract of Rhizoma Dioscoreae showed that PR was upregulated in MCF-7 breast cancer cells and ERα was downregulated (178). A third study showed that ERβ was upregulated by oestrogen stimulating proteins isolated from an aqueous extract of Dioscoreae but ERα was not (179).

It is expected that ERα and β will be upregulated by Radix Angelicae Sinensis and that PRα and β may be downregulated. The outcomes for Rhizoma Dioscoreae is more unpredictable with both downregulation and no changes of ERα seen, upregulation of ERβ, upregulation of PR with anti-progesterone activities. For these studies, the details of the compounds within the extracts used in the studies are not included, making an understanding of the conflicting results difficult.

1.12 Research into TCM formulae used in treating infertility

A number of studies have been undertaken almost exclusively in China, many more than those discussed here, but those that will be discussed here are considered to be of higher quality and the most pertinent to this investigation. The studies presented used a range of different formulae (11 in total) and Table 1-4, p44 lists the herbs which are present in each of the formulae discussed.

The formulae all treat the Kidneys some focussing on Yin, Yang, or both and they all nourish and move Blood. Table 1-4, p44 shows the herbs which are common to the formula used in this study and the herbs which are different. Radix Angelicae Sinensis is in 6 of the 11 formulae and Radix Rehmanniae is in 3 of the 11, but there are 44

unique herbs in the 11 formulae. The herbs and formulae that could have been chosen to study are extensive.

The "correct" way to administer a Chinese herbal medicine is to make an individualised prescription according to the patient not the disorder. There is a saying in Chinese medicine 'Tong Bing Yi Zhi, Yi Bing Tong Zhi', which means same disease different treatment, different disease same treatment. In essence, it means each person and disease must be diagnosed and treated in their own way. The concept of personalised medicine is emerging into mainstream healthcare with evidence and discussion of how genomics might be part of NHS care in the UK, in particular within the context targeted cancer care, prevention of disease, and screening (180).

Bu Shen An Tai up regulates endometrial leukaemia inhibitory factor (LIF) expression, which increases cyclooxygenase that enhances vascular permeability and angiogenesis in mice with an embryonic implantation dysfunction (11). This formula also regulates endometrial thickness, micro vessel density and number of pinopods during the implantation window in mice (181).

Bu Shen Yu Gong was tested in rats and seen to increase levels of oestrogen receptors, increase the number of follicles, weight of the uterus, and serum oestradiol levels (182).

Er Zhi Tian Gui increases DNA methyltransferase 1 expression in the endometrium of infertile women during the implantation phase, which may lead to greater receptivity (13). In mice it is thought to improve oocyte quality by elevating insulin like growth factor 1R mRNA in ovarian granular cells (183). The expression of integrin β3 and its ligand osteopontin in the endometrium were normalised in a mouse model of IVF using ovarian stimulation (184).

Gui Zhi Fu Ling Wan leads to a regression of endometriotic implants in rats by immunological regulation (185).

Jian Tai Ye formula was administered to mice with an induced endometrial dysfunction and analysis of the endometrial oestrogen receptors and mRNA showed that both were up regulated (12).

Nu Zhen Yun Yu was shown to improve the blood supply to the ovaries and the uterus as seen on Doppler in humans (186).

Yi Wei Ning was administered in a rat model of endometriosis and shown to inhibit synthesis and secretion of tumour necrosis factor-alpha, interleukin (IL) -6 and IL8, molecules which may contribute to infertility (187).

You Gui Wan was administered to rats following an ovariectomy. Combined administration of the herbs and oestrogen supplementation prevented the atrophy of the vaginal fold and blood vessels in the lamina propria, without causing endometrial hyperplasia. It did not increase oestrogen levels, but upregulated ERα and ERβ receptors (188).

Zhu Yun aqueous extract used in a rat model has been used to improve endometrial receptivity and counteract the dysfunction seen as a result of the ovarian stimulation used in IVF cycles. It was shown to reverse the high expression of endometrial LIF and integrin β3 (10).

Zi Shen Yu Tai has been shown to improve implantation rates in women undergoing IVF treatment. This formula has also been shown in a mouse model to ameliorate the early endometrial maturation associated with the stimulation drugs used in IVF cycles and improves receptivity by up regulating homeobox A10 (189). It has been shown to have a significant effect on threatened abortion in rats by increasing levels of oestradiol and progesterone, and regulating the Type 1 T helper /Type 2 T helper balance (190).

Most of these studies are using mouse or rat models to examine the influence of the formulae. This incorporates both the complexity of the whole organism and of the

whole formula, whilst looking for specific effects. There are however substantial differences between different species in their menstrual cycles, fertilisation and implantation environments (191). Mice and rats reproduce quickly, are mammalian and so share many reproductive features. Additionally, obtaining ethical approval to work with mice and rats is easier than on human women with infertility.

Table 1-4 shows the herbs which are contained each of the formulae discussed above, for the Latin nomenclature of the herbs see Appendix II. This provides an insight into the range of herbs and formulae that are investigated for their effects on fertility. There are both overlaps and disparities.

Table 1-4 The herbs present in the formulae discussed with an effect on endometrial receptivity

Formula	TCM actions	Herbs in formula different to Gui Shao Di Huang Wan	Herbs in formula in common with Gui Shao Di Huang Wan
Gui Shao Di Huang Wan	Tonify Kidney Yin and Blood		Radix Rehmanniae, Fructus Corni, Rhizoma Dioscoreae, Sclerotium Poria Cocos, Rhizoma Alismatis, Cortex Moutan, Radix Angelicae Sinensis, Radix Paeoniae Alba
Bu Shen An Tai	Tonify Spleen and warm Kidney	Semen Cuscutae, Radix Dipsaci, Ramulus Loranthi, Radix Astragali seu Hedysari Radix Salviae Miltiorrhizae	Radix Angelicae Sinensis
Bu Shen Yu Gong	Tonify Kidney Yin and Yang, nourish Blood	Semen Cuscutae, Cortex Eucommia, Fluoritum, Placenta Hominis, Cornu Cervi, Radix Salviae Miltiorrhizae	Radix Rehmaniae, Fructus Corni
Er Zhi Tian Gui	Tonify Yin and Blood	Fructus Ligustri Lucidi, Herba Eclipta Alba, Fructus Lycii, Semen Cuscutae, Radix Ligusticum wallichii, Rhizoma cyperi, Radix Glycyrrhizae Preparata	Angelicae Sinensis, Radix Paeoniae Alba, Radix Rehmaniae
Gui Zhi Fu Ling Wan	Warm Kidney, move Blood and dispel stasis	Ramulus Cinnamomi Cassiae, Semen Pruni Persicae	Sclerotium Poria Cocos, Cortex Moutan, Radix Paeoniae Lactiflorae
Jian Tai Ye	Tonify yin, move and tonify Blood	Ramulus Loranthis, Radix Salviae Miltiorrhizae, Rhizoma Ligustici Chuanxiong	Radix Angelicae Sinensis
Nu Zhen Yun Yu	Tonify Kidney Yin and Yang, nourish Blood	Semen Cuscutae, Fructus Ligustrum, Radix Salviae Miltiorrhizae, Fructus Lycii, Radix Bupleuri, Fructus Rubi, Radix Glycyrrhizae Preparata	Radix Angelicae Sinensis, Radix Paeoniae Lactiflorae
Yi Wei Ning	Move Blood	Rhizoma Corydalis, Rhizoma Curcumae, Semen Persicae, Radix Paeoniae Rubra, Flos Carthami, Radix Salviae Miltiorrhizae, Carapax et Plastrum testudines	Radix Angelicae Sinensis
You Gui Wan	Tonify Kidney Yang, nourish Blood.	Cortex Eucommiae, Semen Custcutae, Fructus Lycii, Cervi Cornus, Cortex Cinnamomi, Radix Aconiti Preparata	Radix Angelicae Sinensis, Radix Dioscoreae Oppsitae, Radix Rehmaniae
Zhu Yun	Warm Kidney Yang	Herba Epimedii, Radix Morindae Officinalis, Semen Cuscutae, Cortex Eucommia	
Zi Shen Yu Tai	Tonify Kidney Yin and Yang, nourish Blood and Qi	Semen Cuscutae, Fructus Amomi, Radix Ginseng, Herba Taxilli, Colla Corii asini, Radix Polygoni Multiflori, Folium Artemisiae Argyi, Radix Morinda officinalis, Rhizoma Atractylodis Macrocephalae, Radix Codonopsis, Cornu cervi, Fructus Lycii, Radix Dipsaci, Cortex Eucommiae	Radix Rehmanniae

1.13 Summary

Chinese herbs have a long history of use and of success in treating fertility. A number of studies on various formulae looking at fertility have been undertaken predominantly in China, and these suggest that some of the pharmacological mechanisms for the success in assisting fertility can be elucidated. Research into the individual herbs and compounds of Gui Shao Di Huang Wang would suggest that finding differences to haematology, angiogenesis and ovarian steroid receptor expression is likely. Figure 1.4 summaries these potential areas of influence on haematology in the endometrium through the menstrual cycle.

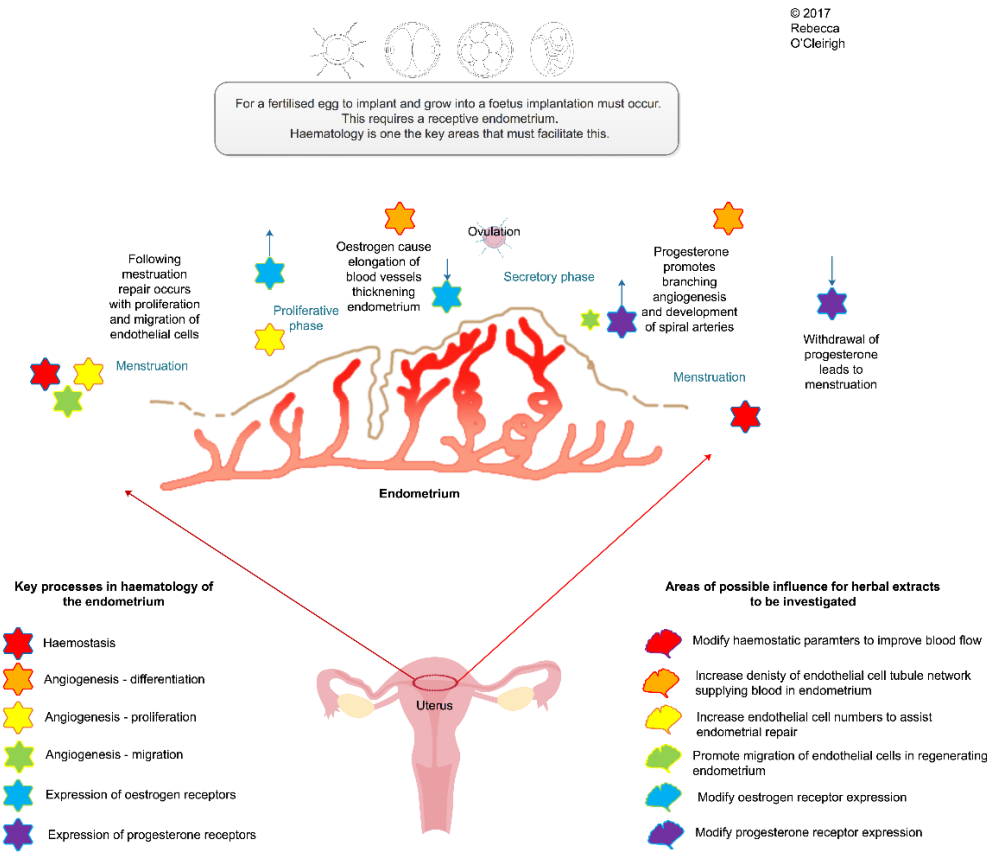


Figure 1.4 An overview of the haematological processes taking place in the endometrium and areas of possible influence for herb extracts to be investigated.

Traditional indications suggest that understanding of the mechanisms of action seen in clinical trials may be found in the ways in which these herbs can influence the blood in the uterus through haemostasis and angiogenesis. Gui Shao Di Huang Wan is an appropriate formula to study due to its use in a clinical trial, in clinical treatment of infertility and presence as a core formula in TCM texts on infertility. Examination of the details of the endometrial changes through the menstrual cycle show that angiogenesis and haemostasis are important aspects of a receptive endometrium and that both of these are influenced by the expression of ovarian steroid receptors.

The use of formulae can be seen as confounding to analysis by western methods and it is likely that systems biology, integrated with the reductionist approach, will lead to greater understanding of the effects of Chinese herbs. Pharmacokinetics shows us that the presence of a compound in a formula does not define if and where those compounds can act in the body. The examination of the effects of GSDW in endometrial receptivity through literature, theory, clinical trials, and bioassays each bring their own contributions to providing answers to the question of how this formula can improve measures of endometrial receptivity.

1.13.1 Aims and hypotheses

To explore the underlying biological mechanisms by which the clinically observed effects of a Chinese herbal formula may improve endometrial receptivity.

H1 Gui Shao Di Huang Wan will increase parameters of uterine perfusion and angiogenesis in endothelial cell proliferation, migration, and differentiation.

H2 Gui Shao Di Huang Wan will modify oestrogen and progesterone receptor expression.

H3 The influence of the extract whole formula Gui Shao Di Huang Wan is greater than the individual herb extracts.

2 Materials and Methods

2.1 Extraction procedures for individual herbs and whole formula

In practice, a Chinese herbal formula is prescribed on an individualised basis following a consultation with a trained practitioner of Chinese herbal medicine. This will typically mean modifying a classic formula; adding or removing herbs to tailor it to the specific patient's symptoms and presentation. The classic formula before any modifications are made is what is meant by a "base" formula. The formula in this study was chosen as it was the base formula used in a clinical study. The study employed ultrasound imaging, testing of serum FSH, and serum progesterone to investigate the influence of herbal medicines on endometrial receptivity (3). It is also the base formula recommended in one of the leading textbooks on infertility (29). This formula is used extensively as the base formula by the practitioners at The London Acupuncture Clinic. This is an acupuncture and herbal clinic in Harley Street, London which specialises in treating infertility and where the author of this work was in practice for 10 years.

Herbs were obtained from Avicenna, a herbal supplier based in Hove, UK. This company is part of the approved suppliers scheme run by the professional body the Register of Chinese Herbal Medicine. Samples were submitted for macroscopic identification and species validation to the medical botanist at Kew Gardens Jodrell laboratory (192). They were verified as being consistent with the authenticated samples held for reference at Kew (Appendix I).

Each herb extract was prepared individually, and as part of a whole formula, as shown in Table 2-1. This was to enable the investigation of individual contributions to any effects, interactions between the herbs, and the effects of the formula as a whole.

Table 2-1 Herbs and their proportions in the formula GSDW, scientific names are given in Table 1-1

Latin	Pinyin	% composition in formula	grams in decoction
Radix Rehmanniae Preparata	Shu Di Huang	25	8
Fructus Corni	Shan Zhu Yu	12	4
Rhizoma Dioscoreae	Shan Yao	12	4
Sclerotium Poria Cocos	Fu Ling	9	3
Rhizoma Alismatis	Ze Xie	9	3
Cortex Moutan	Mu Dan Pi	9	3
Radix Angelicae Sinensis	Dang Gui	12	4
Radix Paeoniae Alba	Bai Shao	12	4

2.1.1 Patient decoction

This is the form used in all experiments except for the thin layer chromatography analysis. The herb, in grams as listed in Table 2-1, was soaked for 12h in 100mL distilled water. They were then brought to the boil using a hot plate (Fisher Scientific / 11-600-49SH) and simmered for 20 min.

The herb extracts were first filtered with a filter paper to remove the plant pieces and were then allowed to cool before undergoing centrifugation at 3500 rpm (2383g Heraeus Labofuge 400) for 10 min to create a pellet of fine plant residue. The supernatant was removed and passed through a 22µm syringe driven filter (Millex) in a Biological Class II laminar airflow cabinet into a sterile universal container (Thermo scientific) and stored at 4 °C until required. All samples were sterilised as this is necessary for cell culture work.

2.1.2 Water based extract

The process was identical to the patient extraction, but the herbs were crushed using a pestle and mortar prior to boiling, and were not soaked for 12 hours.

2.1.3 Ethanol extraction

Herbs were ground in a pestle and mortar, then added to 100mL of ethanol in flasks that were placed in a water bath at 80°C for 20 min. These then underwent centrifugation and were filter sterilised as above.

2.1.4 Data for comparative mg/mL for extracted herbs

1 mL of each of the herb extracts were evaporated and the dry weight of the dissolved compounds was recorded, as is shown in Table 2-2, in order to calculate mg/mL to facilitate comparison between assays. Of the assays in this study one utilises human blood plasma and the others utilise cells of three different types HUVECs, HUtMECs and Ishikawa. These are all seeded at different densities and assays are tested with varying quantities of herbs or dilution factors which are stated with the results reported for each of the assays.

Table 2-2 Concentration in mg/mL of herb extracts determined from dry weights

		mg/mL								
Preparation method	**Used in assays**	**GSDW**	**Radix Paeoniae Alba**	**Radix Angelicae Sinensis**	**Sclerotium Poria Cocos**	**Cortex Moutan**	**Radix Rehmanniae Preparata**	**Rhizoma Dioscoreae**	**Fructus Corni**	**Rhizoma Alismatis**
patient	PT and APTT	0.1053	0.0251	0.0532	0.011	0.0674	0.0451	0.0387	0.0733	0.017
patient	TLC	0.133	0.0064	0.0208	0.0014	0.0072	0.0571	0.0065	0.0225	0.0064
H_2O	TLC	0.0851	0.0091	0.0426	0.0032	0.015	0.0048	0.0319	0.0904	0.0172
ethanol	TLC	0.0234	0.0011	0.0061	0.001	0.0037	0.0029	0.0145	0.0017	0.0022
patient	Angiogenesis, MTT, migration and immunoblotting	0.0867	0.0365	0.0404	0.0021	0.0126	0.1204	0.0176	0.0358	0.0092

2.2 Qualitative examination of the herb extraction methods by thin layer chromatographic analysis

Many studies investigating the action of herbal medicines use ethanol based extractions. The extraction method used for Chinese herbal medicines will significantly alter the compounds extracted and the ratios in which they are extracted, and hence potentially the biological effect (193, 194). In order to maintain clinical relevance for these investigations, herbs were prepared as they are for administration to a patient. To qualitatively illustrate these differences according to the extraction method, thin layer chromatography was performed on three variants of the herbal preparation as described in 2.1

2.2.1 Method

Herb and formula samples produced as described previously in Section 2.1 were applied to silica G coated plates that were produced in house. Using glass capillary tubes, samples were applied in rows 2 cm up from the base of the plate and at 1.5 cm intervals. Herb extracts were applied to the silica of the chromatography plate and each application allowed to dry before this was repeated until the herb extracts formed a visible spot on the plate.

Once the applied samples were dry the plates were placed in chromatography tanks to develop the chromatograms. The mobile phase for the two water-based extractions was chloroform 75 mL and methanol 25 mL. The mobile phase for the ethanol-based extraction was chloroform 90 mL and methanol 10 mL. Plates were allowed to air dry before analysis.

The dried plates were analysed under two UV light wavelengths, 254 and 365nm. The compounds on the chromatograms were then derivatized by spraying until saturated with a solution containing 88% (v/v) methanol, 2% (v/v) anisaldehyde and 10% (v/v) sulphuric acid, followed by heating for 15 min at 100°C. Photographs were taken to record any detected compounds and any variation between the extraction methods.

2.3 Haemostasis assays PT and aPTT

Fresh plasma for these assays was obtained from healthy, male volunteers. Males were used so that ovarian steroids from cycling females would not add an uncontrolled variable to the results as oestrogen has been shown in cause a pro coagulable state. Ethical approval for the obtaining the samples is in Appendix IV.

The KC10 (Amelung) is an automated clot detector as seen in Figure 2.1. It contains reaction chambers which are pre-warmed to 37°C and loaded with 10 cuvettes, each containing a steel ball. The cuvettes are rotated by the drive shafts and the reagents are added. When there is only liquid in the KC10 the steel ball remains stationary and its presence is detected by the magnetic sensor.

Reagents are added and once the final reagent, the $CaCl_2$ (100µL, 25 mM) is added a timer is manually started. When a clot has formed, the steel ball is moved as the cuvette rotates. The magnetic sensor detects that the steel ball is no longer present and the timer stops.

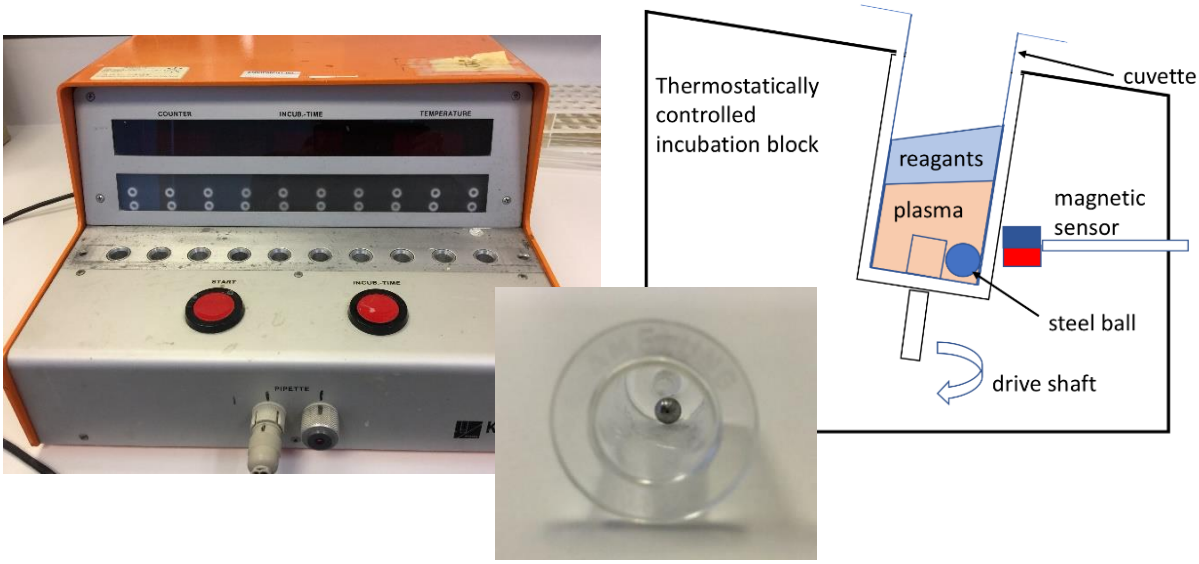


Figure 2.1 The KC10 Automated clot detector
is used to semi automatically detect the time to clot formation, a steel ball in a cuvette is rotated and the reagents and plasma added. At clot formation, the steel ball is moved and the magnetic sensor detects the missing ball to trigger the timer to stop.

To each assay 0 - 0.04 mL of GSDW and 0 - 0.04 mL dH_20, or 0.02mL of the individual herb extracts were added to the plasma as shown in Table 2-3 to examine the anti-thrombotic actions of the herb extracts over a range of concentrations in the PT and aPTT assays.

Table 2-3 Herb to dH_20 volumes used in PT and aPTT assays

Herb extract added in mL	dH_20 mL	Dilution of herb extract in dH_20	Dilution of herb extract in dH_20 and plasma
0.000	0.040	No herb	0 in 100
0.005	0.035	12.5 in 100	5 in 100
0.010	0.030	25 in 100	10 in 100
0.015	0.025	37.5 in 100	15 in 100
0.020	0.020	50 in 100	20 in 100
0.025	0.015	62.5 in 100	25 in 100
0.030	0.010	75 in 100	30 in 100
0.035	0.005	87.5 in 100	35 in 100
0.040	0.000	No dilution	40 in 100

2.3.1 Prothrombin time (PT)

This assay was performed according to the protocol provided by Diagnostic Reagents Ltd. Freeze Dried Rabbit Brain Thromboplastin (Diagen FRBT10) was made up with 5mL dH_20 and 0.1mL of this was added to the cuvettes in the KC10 and left to warm for 3 min. 0.06mL of fresh plasma from volunteers was added and 0.4mL of the herb/dH_20 mixture added (Table 2-3). All reagents were allowed to warm to 37°C in the cuvette for a further minute before 0.01mL of $CaCl_2$ (100µL, 25 mM) prewarmed to 37°C was added. The time to clot formation in seconds was then detected by the KC10 and recorded. Normal lab range is 11-15 s.

2.3.2 Activated partial thromboplastin time (aPTT)

This assay was performed according to the protocol provided by Diagnostic Reagents Ltd. 0.06mL fresh plasma and the 0.04mL herb/H_2O mixture (Table 2-3) was added to the cuvette in the KC10. Platelet substitute (Bell and Alton BAPS040) made up with 5mL dH_2O and 0.1mL of this was added to the cuvette and warmed for 1 minute. 0.1mL of liquid kaolin (5mg/mL in saline solution) was added and the mixture was left to warm for a further 2 min and 0.1mL warmed $CaCl_2$ (100µL, 25 mM) was added. The time to clot formation in seconds was then detected by the KC10 and recorded. Normal lab range is 25-35 s.

Heparin was used to compare the size of the effects of the herb extracts. The therapeutic dose of heparin was taken as 10,000 iU/5L of blood *in vivo* and so heparin was added to the assays at 10 iU/mL which for the 1 iU dose was 0.75mg/mL for the PT and 1.0mg/mL for the aPTT. A 10x dilution was also used as tissue levels of heparin will not be as high as blood levels *in vivo* (195).

2.4 Cell culture techniques

2.4.1 Initiation of proliferating cultures

Cells were removed from liquid nitrogen and placed on dry ice whilst the lid was loosened to release pressure from the vial and the cryovial was then placed with the bottom half in a water bath at 37°C. It was swirled until only a small piece of ice was left. The cells were immediately pipetted into 25cm² culture flasks. The flasks used were lysine coated cell culture flasks (Thermo scientific Nucleon delta EasYFlask 25cm²). Flasks were filled with 5mL of pre warmed media (as listed in Table 2-4) and swirled to distribute evenly. The flasks were left undisturbed in an incubator at 37°C and 5% CO_2 (Nuaire, DH Autoflow) for 24 h to allow the cells to adhere. Media was changed every 48 hours and cells passaged when they were considered confluent as recommended by the supplier (Table 2-4). This table also describes the supplier recommended media, supplements, and specific recommendations for cell culture. Phenol red free media was used as phenol red has been shown to be weakly oestrogenic (196).

Table 2-4 Cells used and their cultivation specifics

Cell type	Supplier	Medium	Supplements	Seeding density	Considered confluent	Ratio for splitting cells
Human Umbilical Vein Endothelial Cells (HUVEC)	Caltag ZHC-2301	Cellworks Human Large Vessel Endothelial Cell Basal Medium KC1015	Cellworks Human Large Vessel Endothelial Cell Growth Supplement KC1016 Cellworks Antibiotic Supplement (Gentamycin/Amphotericin B) KC1019	2,500 cells/cm^2	60-80%	1:2
Human Uterine Microvascular Endothelial Cells (HUtMEC)	2bScientific	Promo cell Endothelial Cell Basal Medium MV2 (prf)	+ Foetal Calf Serum, Epidermal Growth Factor, Basic Fibroblast Growth Factor, Insulin-like Growth Factor, Vascular Endothelial Growth Factor 165, Ascorbic Acid, Hydrocortisone	10,000-20,000 cells/cm^2	>70%	1:2
Ishikawa	Sigma Aldrich 99040201 (from the European collection of authenticated cell cultures)	Ishikawa Culture Medium MEM	+ 2mM Glutamine + 1% v/v Non-Essential Amino Acids (NEAA) + 5% v/v Foetal Bovine Serum (FBS)	20,000-30,000 cells/cm^2	70-80%	1:5

2.4.2 Splitting cells

At the recommended confluence (Table 2-4) the cells were passaged. Media was removed and cells were washed in PBS (Gibco 1x pH 7.4 CaCl, MgCl) to remove residual media and then disaggregated using 1mL TrypLE Express (Gibco). Cells were then incubated with TrypLE Express until they were observed under a microscope to have become detached from the base of the flask, typically 5-10 min. Once disaggregated, the actions of TrypLE Express were neutralised by the addition of 3 mL fresh media. The suspension was split across flasks according to the ratio in (Table 2-4, p57) and the total volume of media/flask increased to 5mL by the addition of fresh media.

2.4.3 Neutralising and counting cells

If the cells were to be used in an experiment following passage, 3mL of media was added to neutralise the TrypLE Express. Following centrifugation for 5 min at 1500 rpm (438 *x g* Heraeus Labofuge 400), the supernatant was removed and cells were resuspended in 5mL fresh media.

Once re-suspended the cells were then counted manually using a haemocytometer (C-Chip, Neubauer improved DHC-N01). 10µl of the cell suspension was added to haemocytometer and, under a light microscope, the cells in the four outer corners of nine large squares were counted (Figure 2.2). The mean of the four large squares was taken as the cell count. The final concentration of the cells was then adjusted by centrifuging, counting, and re-suspending in media to obtain the required cell count for the experiment to be performed.

Figure 2.2 Diagram of C-Chip, Neubauer improved DHC-N01 haemocytometer
used for counting cells to obtain the correct seeding density for the experiments.

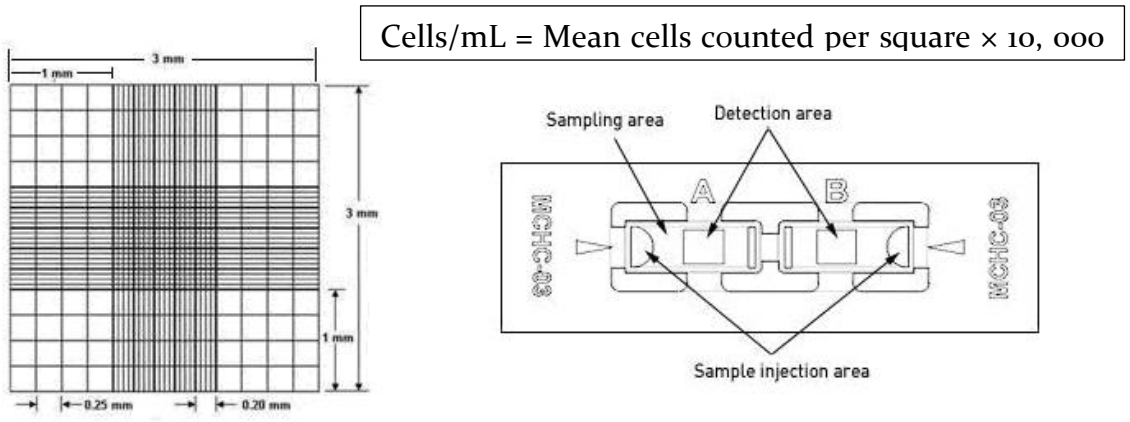


Image courtesy of http://www.incyto.com/product/product02_detail.php

When HUVEC and HUtMEC media is supplied, it comes with additional growth factors and antibiotic supplements in separate containers to be added when ready for use, these are required to sustain growth in primary cell lines. In order to assess the effects on the herb extracts on angiogenesis method development indicated that experiments needed to be conducted with growth factor free media, as the growth

factor supplements induce angiogenic activity and mask the influence of the herbs. Thus, media for use in setting up the experiments was kept without any additional supplements and only small amounts of either bovine serum albumin (BSA) (1% v/v) for migration experiments, or foetal calf serum (FCS) (0.1% v/v) for the angiogenesis which were added to the culture media for that cell type. When cells were trypsinised for use in angiogenesis experiments, TrypLE Express was first neutralised with media with growth factors, then cells were centrifuged (as detailed above) and resuspended in growth factor free media with either BSA/FCS. This was repeated twice to remove any remaining growth factors prior to counting and adjusting to the correct seeding density.

For the MTT, and for the protein extraction for immunoblotting assays the cells were re-suspended in the media containing growth supplements as these experiments were performed over 24-72 hours. Without growth factor supplements cell survival was reduced to between 12 and 24 h.

2.5 Proliferation assays using MTT and MultiTox methods

The MTT assay gives a spectrophotometric measure of the mitochondrial activity of the cells which is considered to be correlated with cell viability. The mitochondria in viable cells cleave the tetrazolium ring of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, Sigma Aldrich, UK) reagent to yield purple formazan crystals. The crystals are then solubilised in dimethyl sulphoxide (DMSO, Sigma Aldrich, UK) to yield a purple coloured solution the intensity of which, as determined spectrophotometrically, is considered to be proportional to viability (197).

2.5.1 Method

Day 1:

This assay was performed with both HUVECs and Ishikawa cells. The HUtMEC did not reach a sufficient number to perform this assay. HUVECs were resuspended in media to 20 x 10^4 /mL and the Ishikawa cells in media to 7.5 x10^4/mL. The cells were added to 96 well plates (Thermo scientific 167006 Nunclon delta surface) at 100µl per well in the central 60 wells (Figure 2.3) 100µL of PBS was added to the outside wells to reduce evaporation in the central wells. Plates were then incubated at 37°C and 5% CO_2 (Nuaire, DH Autoflow) overnight to allow the cells to adhere.

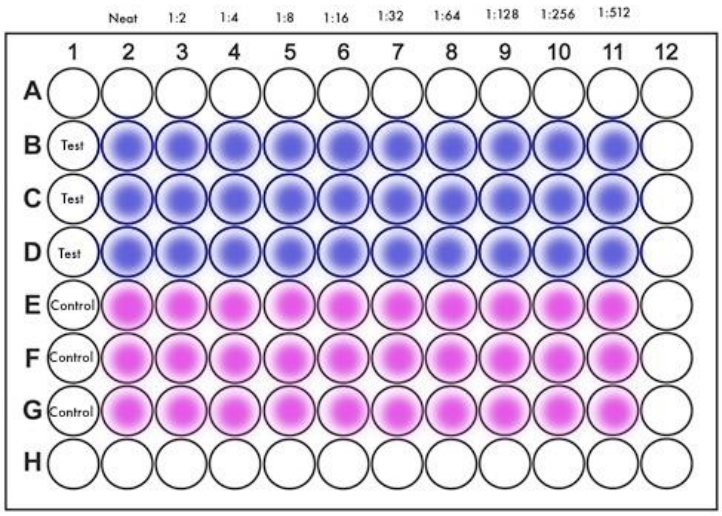


Figure 2.3 96 well plate plan for MTT assay
Cells suspended in media were added to wells coloured pink/purple and PBS to uncoloured wells. In experiments, serial dilutions of test herb extracts were added to wells coloured blue and herb free controls with dH20 only, to wells coloured pink.

Day 2:

100µL of the neat herb extract (prepared as described in Section 2.1.1) was put into wells 2B-D and 100µL of dH_20 control in 2E-G. Using a multichannel pipette, a serial double dilution of the herb extracts/control were prepared across columns 3 – 11 inclusive. During dilution, solutions were mixed by evacuating and refilling the pipette three times at each dilution until row 11, when 100µL was removed and discarded so that all wells contained the same volume. This was replicated on three plates which were then incubated for three time periods of 24, 48 and 72 h.

GSDW used was at a dry weight equivalent concentration of 0.0867mg/mL so the actual final dry weight equivalent of the herb extract added in serial dilution is shown in Table 2-5.

Table 2-5 µg dry weight equivalents of the herb extract added

	µg in 100µL	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**	**1:245**	**1:512**
GSDW	8.670	4.335	2.167	1.084	0.542	0.271	0.135	0.068	0.034	0.017

Day 3, 4 or 5:

A 0.5% (w/v) stock solution of MTT was prepared by dissolving 0.1mg of MTT was in 20mL of distilled water. Following the required incubation period of 24, 48 or 72 h and immediately prior to use, the MTT stock solution was diluted 1 in 5 with cell culture media and 50µL added to each well of the plate that contained cells. The plate was kept in an incubator at 37°C and 5% CO_2 (Nuaire, DH Autoflow) for 2-4h until purple crystals were visible in the wells.

The media, herb extract, and PBS were all removed from the wells by tipping the plate and then blotting on an absorbent towel. 100µL of DMSO was added to each well to solubilise the formazan crystals. This was assisted by agitating the plate on a plate mixer (Heidolph, Titramax 100) for 2 mins.

The absorbance of the resulting solutions was measured using a plate reader (Biotech Powerwave 340) and the KC Junior software set at a wavelength of 570nm as is described in Sigma Aldrich MTT protocol.

MTT data is written by KC Junior software into an Excel file (Table 2-6). The number represents quantification of the 560nm wavelength absorbance of the dissolved formazan crystals in DMSO.

Table 2-6 Raw data colour metric quantification output from KC Junior plate reader

Dilution from neat GSDW	1.000	0.500	0.250	0.125	0.063	0.031	0.016	0.008	0.004	0.002
Test well	0.048	0.069	0.094	0.101	0.261	0.288	0.312	0.302	0.286	0.332
Test well	0.047	0.048	0.085	0.093	0.235	0.282	0.307	0.322	0.293	0.319
Test well	0.045	0.056	0.087	0.109	0.226	0.303	0.333	0.301	0.275	0.234
Control well	0.238	0.262	0.223	0.27	0.254	0.248	0.27	0.25	0.262	0.257
Control well	0.242	0.282	0.268	0.232	0.247	0.239	0.215	0.216	0.253	0.269
Control well	0.239	0.258	0.253	0.254	0.256	0.241	0.224	0.248	0.261	0.295

The calculation of viability takes the mean of each of the three test wells and the mean of each (Equation 1) of the three controls wells and a percentage viability is calculated as presented in Table 2-7.

Equation 1 viability calculation

$$\frac{mean\ absorbance\ of\ three\ test\ wells}{mean\ absorbance\ of\ three\ control\ wells} \times 100 = \%\ survival\ of\ test\ over\ control$$

Table 2-7 Calculation of % viability from the raw data

Dilution from neat GSDW	**1.000**	**0.500**	**0.250**	**0.125**	**0.063**	**0.031**	**0.016**	**0.008**	**0.004**	**0.002**
µg/mL	86.70	43.35	21.68	10.84	5.42	2.71	1.35	0.68	0.34	0.17
Absorbance Test	0.047	0.058	0.089	0.101	0.241	0.291	0.317	0.308	0.285	0.295
Absorbance Control	0.240	0.267	0.248	0.252	0.252	0.243	0.236	0.238	0.259	0.274
% Viability	19	22	36	40	95	120	134	130	110	108

2.5.2 MultiTox-Glo Multiplex Cytotoxicity Assay

100µL of HUVECs (20 x 10^4 /mL) were plated into 96 well plates (Greiner 96 F-bottom) as described above for the MTT assay (Section 2.5.1, p60) and treated with either GSDW/dH2O in serial dilution (Table 2-5, p61) or dH2O only, and incubated for 48 h.

The media, herb extract, and dH2O in were all removed from the wells by tipping the plate and blotting on an absorbent towel. Wells were washed by adding 100µL of HUVEC media and then agitated on a plate mixer (Heidolph, Titramax 100) for 1 min and then repeated. 50µL of fresh HUVEC media was then added to each well.

Reagents were prepared according to the protocol provided for the MultiTox-Glo Multiplex Cytotoxicity Assay (Promega G9270). 50µl of GF-AFC, the live cell reagent, was added to all wells and mixed on plate mixer for 2 min. The cells were incubated at 37°C and 5% CO_2 for 60 min, protected from light by wrapping in foil.

The resulting live-cell fluorescence was measured using a multimode fluorescence plate reader (Polar Star Omega) equipped with filter sets for excitation ~400nm and emission ~520nm. The plate was returned to the incubator for a further 30 min and live-cell fluorescence was repeated as documentation states that a longer incubation may improve the assay sensitivity.

After the second measure of live cell fluorescence, 25µl of AAF-Glo™ reagent (to detect non-viable cells) was added to all wells and mixed on plate mixer for a further 2 min. The plate was kept at room temperature for 15 min protected from light by wrapping in foil. The resulting dead-cell luminescence was measured using a multimode plate reader (Polar Star Omega).

2.6 Differentiation assays on matrigel using HUVECs and HUtMECs

An influence on angiogenesis may be one of the ways in which the haematology of the endometrium is affected by the Chinese herbs. Angiogenic processes can be investigated though several different bioassays. *In vivo* these processes occur together and are interrelated but to try and determine how the herb extracts may be influencing this activity it is broken down into three distinct processes; proliferation, migration, and differentiation. The first of these bioassays examines differentiation, the process of endothelial cells forming new vessels which will become the new vessels through which blood is carried through the tissues.

2.6.1 Protocol development

The development of an appropriate protocol for evaluation of the HUVEC differentiation was an extensive process. Initially matrigel with growth factors was used as was cell culture media with growth factors. No clear patterns of differentiation according to the test conditions was found. Variables such a seeding density, passage of cells, age of herb extracts were all evaluated for contributory effects. Matrigel with no growth factors (GF-) was used but this failed to show any consistent differences in the test conditions. The cells were weaned off growth factors in the culture flasks but this led to poor differentiation. Media without the commercial growth factors with the addition of 0.1% (v/v) FBS and the GF-matrigel did show differences. The cells were kept in media with growth factors until the final pipetting into the wells of the µ-slides. The cells viability once all growth factors were removed was only 12-18 h, whereas with the growth factors they were able to survive to 36 h.

2.6.2 Final Method

Growth factor deficient phenol red free matrigel (Corning) was defrosted on ice at 2-5°C for 12 h prior to use. Ibidi µ-slides were used as these have a lower well for containing the matrigel which prevents a meniscus forming in the gel (Figure 2.4). The surface on which the cells differentiate provides a flat focal plane for the microscope to enable clear photographs of the differentiated cells for analysis.

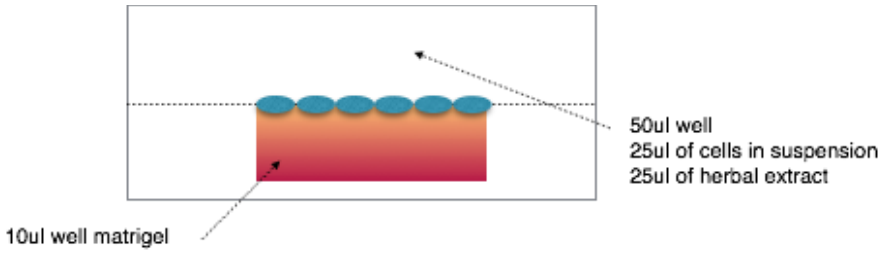


Figure 2.4 Diagram of the Ibidi µ-slides
showing the lower well containing matrigel and the cells adhering to the matrigel for differentiation on a flat plane to enable good focus of the entire field for the photographs used for analysis.

An 18 well µ-slide (Ibidi) was placed on ice and a reverse phase pipette used to pipette 10µL of neat Matrigel into each well. The slides were placed into an incubator at 37°C and 5% CO_2 (Nuaire, DH Autoflow) for 1h to allow the gel to form a matrix.

Cells were grown to 80% confluence and dissociated from the flask and suspended in media (Section 2.4.3, p58). Following centrifugation, they were re-suspended in the media containing 0.1% FBS (v/v) (Gibco 10270106) but no supplied growth supplements or antibiotics. The cells were and added to the µ-slide at a concentration of 3 x 10^5 /mL and 25µL per well. The slides were then incubated for 1h to allow the cells to adhere to the matrigel.

The herb extracts as prepared in Section 12.1.1 for use with the HUVECs were then diluted 1:100 with 0.1% FBS (v/v) HUVEC media, so as to produce a final dilution of 1:200 when added to the µ-slide. 25µL of the herb extract was added see in Table 2-8, p67. VEGF and suramin were used as positive and negative controls for growth respectively.

The herb extracts prepared as for the HUtMECs were then diluted 1:250 with 0.1% FBS (v/v) HUtMEC media, so as to produce a final dilution of 1:500 when added to the µ-slide. Controls here were media with and without growth supplements added. 25µL of the herb extract was added in (Table 2-8, p67) VEGF and suramin were used as positive and negative controls for growth (described below).

Controls of VEGF and suramin used in the HUVEC assays were obtained from the angiogenesis assay control kit (Cellworks ZHA-1300) and used according to the manufacturer's guidance. Suramin was an effective inhibitor of differentiation, however, VEGF did not show significant increases in differentiation. In the HUtMEC assay media with supplements was used as the positive control and the supplement reduced media alone was used as the negative control.

Table 2-8 Concentrations of herb extract added to each well for HUVECs and HUtMECs, determined from the dry weights of the herbal decoctions prepared.

µg/mL dry weight equivalent of extract added	GSDW 1:500	Radix Paeoniae Alba	Radix Angelicae Sinensis	Sclerotium Poria Cocos	Cortex Moutan	Radix Rehmanniae Preparata	Rhizoma Dioscoreae	Fructus Corni	Rhizoma Alismatis
HUVECs 25ul added at 1:100	0.0217	0.00913	0.0101	0.00053	0.00315	0.0301	0.0044	0.0089	0.0023
HUtMECs 25ul added at 1:500	0.0043	0.0018	0.0020	0.00011	0.00063	0.0060	0.0009	0.0018	0.0005

The µ-slides were incubated before being photographed on an inverted microscope. HUVECs were photographed on an inverted microscope (Zeiss Axioscope A1) coupled to digital camera (Zeiss Axiocam 503) at 5X and photographed at 6h and 12h but tubules were significantly degraded by 18 h. The microscope used for the HUtMECs (Nikon eclipse 80i) was coupled to a digital camera (Nikon digital sight DS-Fi2) at 4X magnification and slides were viewed at 6, 12 and 18 h. Marked differentiation had not occurred at 12 h so the slides were photographed at 18 h.

Six repeats were made of each condition for HUVECs and HUtMECs. Data analysis was performed using the angiogenesis analyser add on to ImageJ (v1.49).

2.7 Migration assay using culture inserts

Migration is the third aspect of angiogenesis, and migration assays were performed with both HUVECs and HUtMECs. The ability of endothelial cells to migrate can be assessed in a number of different assays but the assay selected was a basic migration assay. Boyden chambers can be used to assess chemotaxis, would healing assays assess the rate of migration of cells. The wound healing assay is where a scratch is made with a pipette tip or similar tool in a layer of confluent cells. The migration of the cells across the scratch is recorded and rate of migration across the "wound" and can be measured (138). A modification of this assay was used, the 2 well culture insert (Ibidi) see Figure 2.5. According to the manufacturer these have higher repeatability than scratch assays as the "wound" in this case is typically a more consistent gap across which the cells must migrate making measurements more reliable. This method of creating the "wound" does not damage cells can which then release molecules that can inhibit migration (198).

Picture courtesy of
ibid.com/applications/
wound-healing-and-migration/

Figure 2.5 Ibidi 2 well culture insert used to obtain a clean "wound" across which the migration of cells can be measured accurately.

Cells at 80% confluence were dissociated from the flask using TrypLE Express (Section 2.4.2, p57). Following centrifugation at 1500 rpm (438 *x g* Heraeus Labofuge 400) for 5 min, the supernatant media was removed cells were and resuspended in

fresh media containing BSA 1% (v/v) to a final concentration of 8 x 10^5 cells/mL. Using sterile tweezers, culture inserts were placed into each well of a 6 well plate (Corning tissue culture plate) and 70µL of the cell suspension was dispensed into each chamber of the insert, a total of 140µL cell suspension per insert.

Plates were then incubated in a humidified atmosphere at 37°C and 5% CO_2 for 24 h to allow the cells to adhere to the base of the plates. Once the cells had formed a confluent layer, the inserts were removed using sterile tweezers. The adhered cells were washed with sterile PBS (Gibco, pH 7.4) to remove any loose cells before the herb extract was added at dilution of 1 in 100, 500 and 1000 for GSDW and 1 in 200 for the individual herb extracts. Table 2-2, p51 shows the equivalent concentrations mg/mL based on the dry weights of the extracts. Dilutions were prepared in media without the standard supplements but with 1% (v/v) BSA (Fisher Chemical, Microbiological grade BPE9700-10.0) and were added at 5mL per well.

Live cell imaging was performed using a Zeiss Axiovert 200M (inverted) microscope (Carl Zeiss, Welwyn Garden City, Herts, UK) contained in an incubator (37 °C, 5% CO_2, humid atmosphere). Phase images (camera model C4742-80-12AG; Hamamatsu Photonics, Hamamatsu, Japan) were acquired every 15 min for 24 h with a 5x objective using Volocity software (V6.1.1, Perkin-Elmer, Waltham, MA, USA). Time-lapse movies (.avi) were compiled from the images, using the same software, at 10 fps.

Images were analysed using ImageJ (v1.49) MRI wound healing tool which is used to calculate the area of space, in pixels, between the two areas of cells at the start of the experiment and then as the gap is reduced as the cells migrate.

2.8 Immunoblotting

The herbal formula could influence the receptivity of the endometrium by altering the expression of hormone receptors in endometrial cells, as discussed in Section 1.11.1. In order to analyse the possible influence of the herb extracts on selected receptors expressed on an endometrial cell line, Ishikawa cells (Sigma Aldrich 99040201) were exposed to the herb extract before being lysed to extract total cell proteins. Lysates were frozen (Nuaire, -86°C Ultralow Glacier) whilst waiting for analysis.

Immunoblotting was used for comparative analysis of selected proteins in the extracts. Proteins are separated according to molecular weight using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS- PAGE). The proteins are then transferred to a Polyvinylidene Difluoride (PVDF) membrane where they can be probed using specific antibodies.

Ishikawa cells were used in this series of experiments. These are a clone of a well differentiated human endometrial adenocarcinoma cell line, the cells originated from the glandular cells of the epithelium and express both oestrogen and progesterone receptors (199).

Oestrogen receptors have so far been seen in five forms, three membrane bound receptors and two nuclear. The two nuclear forms are the most widely understood and are the ones which are investigated here. Progesterone receptors are also found as two nuclear forms PRα and PRβ. These are very similar in structure, transcribed from the same gene with a different 165 amino acid terminal sequence on the beta isoform. Membrane progesterone receptors come in three isoforms: alpha (mPRα), beta and gamma. MPRα was investigated as the only one of the three cited as having uterine function (171).

2.8.1 Method for obtaining the protein extracts from Ishikawa cells

Day 1:

Ishikawa cells were cultured to 80% confluence (Table 2-4, p57) and dissociated from the flasks as described in Section 2.1.1 and re-suspended in media (Gibco Minimum Essential Medium) containing 5% (v/v) FCS (details in Table 2-4, p57) and seeded into 6 well plates (Corning costar 3516 culture cluster flat bottom plate) at 3mL cell suspension containing $1x10^5$ cells/mL. These were incubated overnight in a humidified atmosphere at 37°C and 5% CO_2 (Nuaire, DH Autoflow).

Day 2:

On the second day 1.5mL of the media was removed and media with herb extract or media only control was added. The herb extracts were diluted 1:50 with media for a final concentration in the wells of 1:100. Table 2-2, p51 presents the equivalent herb concentrations (mg/mL), as determined from their dry weight. Cells were incubated for either 24 or 48 h.

Day 3 or 4:

Once removed from the incubator the plates were placed on ice. Media was removed from the wells into sterile Eppendorf tubes and, following centrifugation (Sigma Aldrich, Eppendorf mini spin) at 12,000 rpm (9660 x *g*) for 1 min the supernatant was removed and discarded. This was repeated for all media and for all wells to ensure that any cells not adhered were collected. The resulting cell pellets were then washed in ice cold PBS before being kept on ice.

The adhered cells were washed with 3mL of ice cold PBS which was then discarded. 100µL of Pierce RIPA buffer was added to each well, and cells removed using sterile cell scrapers. The resulting cell suspensions were then added to the respective cell pellets on ice. In a cold laboratory (4°C) all Eppendorf tubes were agitated using a vortex mixer (Scientific Industries, Vortex Genie 2) for 30 seconds each, repeated for 30 min. They then underwent centrifugation for 1 minute at 12,000 rpm and the

resulting supernatants were placed into clean sterile Eppendorf tubes for storage at -80˚C until analysis.

2.8.2 Quantification of proteins obtained from Ishikawa cells using the Pierce BCA Assay

To ensure equal loading of proteins on the SDS PAGE gel the extracted cellular proteins were quantified using a BCA protein assay (Pierce, 23225) according to the manufacturer's instructions. Samples were loaded onto a Thermo scientific 167006 Nunclon delta surface 96 well plate. Measurements of the provided reference samples and the extracted proteins were compared using a colour-metric assessment of the protein quantity at 562nm using a Biotech plate reader and KC Junior software. The absorbance of the standards was then plotted onto standard curve (Appendix VIII) and the protein concentration of the samples could then be determined.

2.8.3 Preparation of acrylamide gels

A Bio-Rad Mini-PROTEAN Tetra Cell hand cast electrophoresis system was used as shown in Figure 2.6.

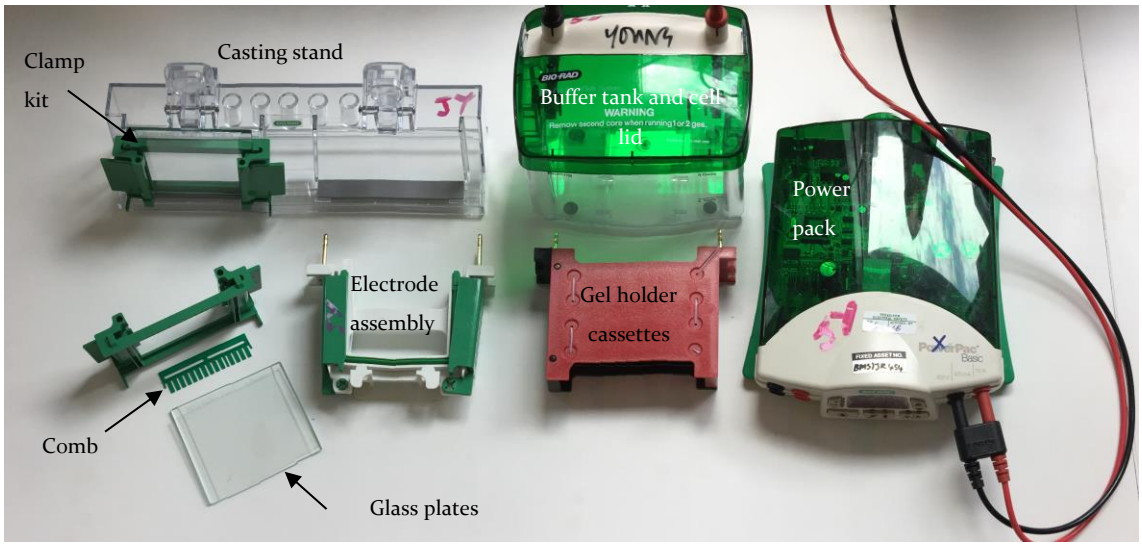


Figure 2.6 Photograph of Bio Rad mini protean tetra cell hand cast equipment used to prepare the gels and perform electrophoresis.

A 12.5% resolving gel was prepared according to a modification of the Laemmli protocol (200) as detailed in Table 2-9 and loaded into the glass plates held securely in the clamp kit in the casting frame and allowed to polymerise. A gel of 12.5% is recommended by Abcam for proteins of 10-70 kilodalton (kDa) which was an appropriate range for the proteins of interest (201). Recipes for all of the solutions made up in the lab are in Appendix VI, as are the reference codes for any purchased reagents.

Resolving 1 gel	quantity
dH2O	2.45mL
40% acrylamide	2.25mL
1.5M Tris HCl	1.9mL
10% SDS	75µl
10% APS	75µl
TEMED	3µl

Stacking 1 gel	quantity
dH2O	2.85mL
40% acrylamide	0.55mL
0 .5M Tris HCl	1.25µl
10% SDS	50µl
10% APS	50µl
TEMED	5µl

Table 2-9 Resolving and stacking gel preparation

Once the resolving gel had polymerised, a 5% stacking gel was prepared see Table 2-9 and added on top of the resolving gel. A 12 well comb was immediately inserted into the stacking gel which was then allowed to polymerise.

2.9 Electrophoresis of Samples

Protein extracts prepared from Ishikawa cells as described in Section 2.8.1, were thawed. 20µg of the protein sample was loaded in each lane (calculated in Excel spreadsheet appendix VIII). Laemmli loading buffer (Appendix VII) was added to the samples to make up to a final volume of 20µl and the samples were then placed into a heat block (Techne, Dri-Block) at 90˚C for 10 mins.

The comb was removed from the polymerised gel and the gel and plates were secured in the electrophoresis tank. The electrophoresis tank was filled with running buffer (Appendix VII) and wells were flushed with running buffer before the samples were loaded. 20µl of sample were added along with the reference protein at their recommended loading concentrations (Table 2-11, p77) and the protein ladders (Abcam Ultra prism ladder ab116027).

Samples were electrophoresed at 60V for 30 min and then 150V for ~60 min until the dye front could be seen approaching the bottom of the gel. After electrophoresis, the glass plates were removed and gels were soaked in transfer buffer prior to transfer of proteins onto PVDF membranes.

2.9.1 Electroblotting for Protein Transfer

PVDF membrane was placed into methanol for 10s then equilibrated in transfer buffer for 10 mins, a "transfer sandwich" was then constructed (Figure 2.7).

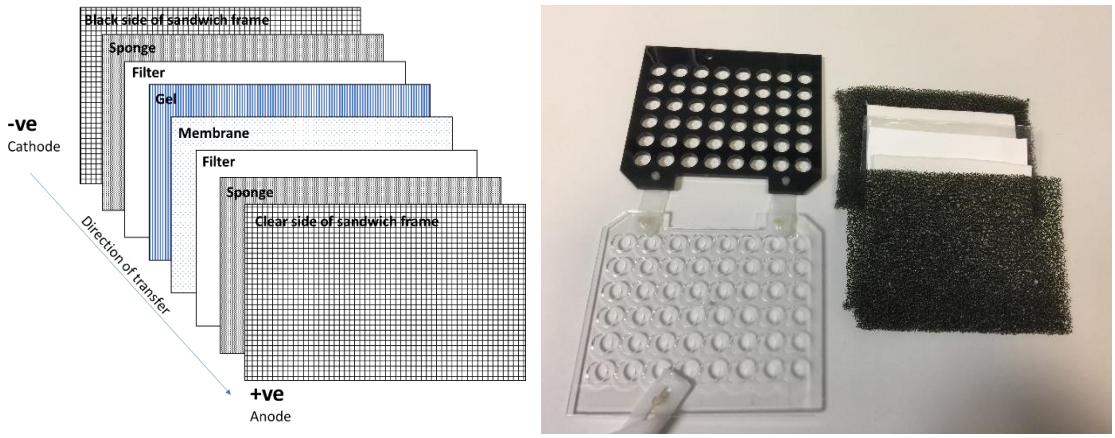


Figure 2.7 Construction of the transfer sandwich
Layers of sponge, filter, gel, and membrane are created in order to transfer the separated proteins from the gel onto a membrane for probing with antibodies.

Prior to completion of the "transfer sandwich", any air bubbles between the gel and membrane were removed using a roller to ensure a good contact between the gel and the membrane. The "transfer sandwich" was then placed into a tank filled with transfer buffer. A stirrer and an ice pack were placed inside the tank. The tank was then left overnight in a cold laboratory (4°C) on a stirrer plate and a current of 30V was applied to transfer proteins. To check for any protein loss, experiments were performed where a second membrane was placed behind the first to ensure that proteins were not passing through the membrane with an overnight transfer, proteins were not detected on the second membrane.

2.9.2 Protein detection

Following transfer, PVDF membranes were removed from the "transfer sandwich", washed in distilled water and then blocked with 5% (w/v) skimmed milk powder in PBS for 1 h at room temperature, with constant agitation using a roller set at 35 rpm. They were then placed into 3mL of 5% (w/v) skimmed milk powder in PBS containing the primary antibody typically at a concentration of 1:1000 (Table 2-10). These were left for 1 h at room temperature, with constant agitation using a roller set at 35 rpm. The primary anybody (Table 2-10) was then removed and the membrane washed three times in 5mL of PBS-T before being returned to the rollers for 10 min in 30mL of PBS-T, this process repeated a further two times. The secondary antibody was added to 4mL of 5% (w/v) skimmed milk powder in PBS, typically at a concentration of 1: 2000 (Table 2-10) and the membrane incubated with the secondary antibody for 1 h at room temperature with constant agitation using a roller set at 35 rpm. The secondary antibody was removed and the membrane washed with PBS-T as described above. Molecular weights (kDa) of the antibodies used are indicated on a protein ladder diagram (Figure 2.8).

For analysis, the membrane was placed on an acetate sheet and 800µL of Luminata Forte HRP substrate (Merck Millipore, BLUF0500) was added. Analysis was performed on a ChemiDoc MP imaging system (Bio-Rad) using ImageLab 6.0 software and auto detection of optimal resolution time. ImageLab software allows the user to quantify the relative saturation of the bands detected on a single blot for comparison.

Table 2-10 Antibody reference table

Antibody	ref	antibody	2nd AB	concentration provided	Concentration for WB	kDa data from manufacturers datasheet
Loading controls						
Anti-beta actin antibody	ab8226	mouse monoclonal	GxM	100 µg at 1 mg/mL	1/500 - 1/10000	42
Anti GAPDH antibody	ab8245	mouse monoclonal	GxM	100 µg at 2 mg/mL	1/500 - 1/10000	Observed band 37, predicted band 40
Anti-cyclophilin	ab74173	donkey	DxM	100 µL at 1 mg/mL	1/1000 - 1/2000	23
Anti-Alas1 antibody (ALAS1)	ab84962	rabbit polyclonal	GxR	100ul at 0.5mg/mL	1µg/mL	Observed band 65, predicted band 71
Anti-Alas1 antibody Mitochondrial Marker (ALAS2)	ab154860	rabbit monoclonal	GxR	100 µL at 0.445 - 0.63 mg/mL	1/1000 - 1/10000	71
Oestrogen						
Anti-Estrogen receptor alpha antibody (ERα)	ab108398	rabbit monoclonal	GxR	100 µL at 1.228 mg/mL	1/1000 - 1/10000	66
Anti-Estrogen Receptor beta antibody (ERβ1)	ab3577	rabbit polyclonal	GxR	50 µg at 1 mg/mL	1 - 2 µg/mL	~55
Anti-Estrogen Receptor beta antibody (ERβ2)	ab133467	rabbit monoclonal	GxR	100 µL at 0.346 - 0.437 mg/mL batch dependent	1 - 2 µg/mL	59
Progesterone						
Anti -Membrane Progestin Receptor Alpha (mPRα)	ab75508	rabbit polyclonal	GxR	100ug at 1mg/mL	1/500-1/1000	~40
Anti progestin receptor beta antibody (PRβ)	ab46535	rabbit polyclonal	GxR	not specified	"assay dependent" 1ul/mL has worked	~40

The kDa reported in the manufacturers datasheets are in some cases reported as a range, an approximate value, and at other times at a specific kDa. Variations in the detected protein can occur due to modifications of the protein such as glycosylation and phosphorylation altering the predicted versus observed molecular weight. The charge of the molecule may be altered changing its migration rate. Cleavage or degradation of the protein, splice variants and multimers can all modify the molecular weight. Reference proteins were used to verify that the correct receptor proteins were being identified (Table 2-11).

Table 2-11 Reference proteins used on blots to verify the identity of the detected bands

Receptor	Reference proteins	Loading volume	Outcome
Oestrogen receptor alpha	Recombinant Human Estrogen Receptor alpha protein ab82606	0.5 µL	Receptor verified
Oestrogen receptor beta	Recombinant Human Estrogen Receptor beta protein ab114181	up to 6µL	Never detected
Progestin receptor alpha	Recombinant Human mPRα protein ab165766	3µL	Protein detected but receptor not seen in lysates
Progesterone receptor beta	Recombinant Human Progestin Receptor Beta protein ab164739	3µL	Receptor verified

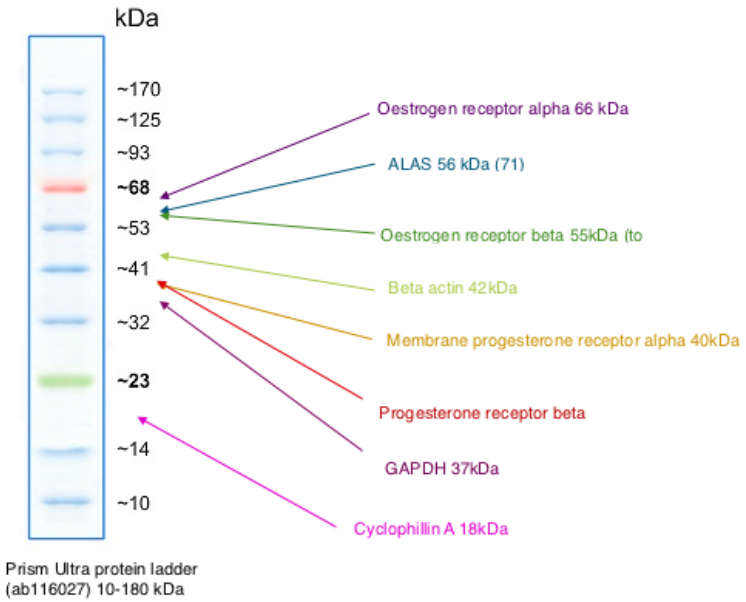


Figure 2.8 Molecular weights of antibodies used to probe membranes for ERα, ERβ, mPRα, PRβ, and the loading control proteins used.

2.10 Statistical analyses

The data for the TLC, migration and the receptor expression were not quantitively analysed.

Data for the haemostasis, proliferation and differentiation was analysed using a T-test for difference between GSDW and control and a one way ANOVA was used for differences between the individual herbs if the assumptions for outliers, normal distribution and homogeneity of variance were met. In this case the Tukey post hoc tests were used.

In several cases the homogeneity of variance was not met and so a Welch's ANOVA was performed with Games Howell post hoc tests.

Where data failed to meet the assumptions for parametric testing a Mann Whitney U and Kruskal Wallis were used with Dunn's post hoc tests.

Analysis was performed using SPSS v22, graphs were produced in Graphpad Prism 6.

3 Herbal Extraction Methods

The extraction methods for analysis of herbal medicines effects *in vivo* should be congruent with preparation methods used for patient treatment

3.1 Introduction

When east meets west in herbal research an early conflict in methodology arises. Pharmaceutical extraction methods for herbal products may involve grinding or crushing plant materials to a fine and uniform texture and the use of a soxhlet extractor with solvents such as ethanol and methanol. Hydroalchoholic solvent mixtures are considered optimal for investigation. They have an expanded polarity range so that more compounds are extracted. More advanced techniques such as microwave assisted extraction, ultrasonic extraction or supercritical fluid extraction may be employed (202).

Amino acids can be separated by TLC, for identification they are usually hydrolysed prior to separation. They are generally soluble in water but some require alcohol(203). Amino acids in GSDW include phenylalanine in Radix Paeoniae Alba, histidine in Sclerotium Poria Cocos and uracil in Radix Angelicae Sinensis and Radix Rehmanniae Preparata.

Phenols, of which phenolic acids are a sub group, are the most common secondary metabolite (204). Phenols include tannins, flavonoids, and phenolic acids. Flavonoids include quercetin present in Cortex Moutan, and paeonin in Radix Paeoniae Alba. Tannins including gallotannin, tellimagrandin I and II are found in Fructus Corni and 1-O-Galloyl pedunculagin in Radix Paeoniae Alba. Extraction of high molecular weight phenols will require aqueous acetone and low molecular weight with ethanol, there is no universal solvent (205).

Phenolic acids are aromatic secondary metabolites and have a wide variety of functions in the plants, location in the plant and therapeutic functions. They have excellent ability to scavenge free radicals, they are immune stimulants, anti-cancer, antimicrobial, and sedative amongst a range of other functions (203). Ferulic acid in Radix Angelicae Sinensis and gallic acid in Radix Paeoniae Alba, Cortex Moutan, and Fructus Corni are phenolic. Many of these acids are precursors to more complex compounds and are typically extracted in alcohol.

Terpenes are ubiquitous in plants and present in all of the eight herbs including the triterpenes, monoterpenes, phytosterols, glycoside saponins and these compounds are known to have many biological functions in the plant and have many uses therapeutically for humans. Two very well known terpenes, that have been isolated from Chinese herbs and used as a pharmaceutical compound, are the antimalarial compound artemisinin and the anticancer drug Taxol (206). Chloroform and methanol on silica plates are an appropriate method for the extraction of this class of compounds and can be observed under UV as black quenching spots. The number of sugars can be as high as 10 and these determine the polarity of the compounds so a huge range is possible.

Carbohydrates include the polysaccharides some of which are the energy storage for plants others are structural. These molecules are soluble in water but bind strongly to silica and so a highly polar mobile phase must be used. Methanol and chloroform the mobile phase used are highly polar (203).

When herbal decoctions are prescribed to a patient in a clinical setting one traditional way of preparing prescribed herbs is to place the raw herbs in water for 12 h. It is then brought to the boil, simmered for 20 min, and strained, retaining the fluids for therapeutic consumption.

Prior to the biological and functional assays, three extraction protocols were explored; ethanol, water with a pharmaceutical technique and water as a patient would prepare

it when prescribed clinically. Extracts from these methods were compared using Thin Layer Chromatography (TLC) (Figure 3-1). Extracts were prepared (Section 2.1., p48) in ethanol and water (ground and whole dried plants, the latter with prior soaking). The method for the TLC is described in Section 2.2. The adsorbent used in these chromatograms is silica which is not a strong adsorbent. It was used with a combination of a very polar solvent, methanol, and the moderately polar chloroform. All plates were sprayed with an anisaldehyde 2% (v/v), sulphuric acid 10% (v/v) in methanol reagent spray. This is a spray used for all plates in a chromatographic study of Chinese herbs (207-210).

3.2 Results

Differences between the patient and water based extractions were minimal. Differences between the patient and ethanol were pronounced. There were many compounds seen in the patient or water based decoction which are not seen in this ethanol extraction. In additional there were a few compounds not seen in the patient or water based extraction which were visualised in the ethanol extraction.

The protocols were not optimised for specific herb extracts or compounds. A simple technique was used which was the same for all herb samples with the exception of the different proportion of methanol to chloroform in the mobile phases. Three methods used to extract compounds were visualised using TLC. Qualitative differences are presented in photographs collated in Figure 3.1, p83 with circles to reference specific differences. It could be seen that differences exist in the profile of compounds from these extractions.

In the UV 365 nm images that the circle 1 shows a fluorescing compound was present in both patient and water extractions with a very low (retention factor) *Rf*. The *Rf* is a measure of how far the compound has been eluted and this may be affected by the thickness of the silica on the plate, the depth of the mobile phase, temperature, and sample size so even with near identical conditions the *Rf* value may not be consistent. In the patient decoction, the compound appeared less bright

than in the water extraction. This may be due to unequal loading of the extracts and cannot be assumed to represent different levels of compounds in the extracts themselves. They were applied until a mark was visible not by applying a measured quantity. Variances in mg/mL have been seen between herbs extracted at different times using the same process (Table 2-2, p51). Differences in loading as well as differences in concentration of the herb extracts mean that no quantitative evaluation is possible, only a qualitive presence or absence of the compound. The sample loading position on the ethanol plate (circle 1) is present but no compound was carried up the plate.

There are two highly fluorescing compounds at the upper limit of the solvent front (circles 2 and 3) in the ethanol extraction of Radix Angelicae Sinensis also seen in GSDW that were not present in the patient and water based extractions.

There were additionally compounds visualised in UV 254 nm (circles 6-10) where compounds with at least two conjugated double bonds appear dark, as they quench the fluorescence. The differences are pronounced. Circle 10 does appear to be slightly different between the patient and water based extraction and a compound (circle 11) is absent from the patient decoction. It may be that the different appearance, a longer dark smudge (circle 10), may be the same compound but the *Rf* is varying as it is on a different plate. The only way to confirm the presence is using a known compound on the plate with the unknown for comparison. *(z)*-ligustilide will form a noticeable band under UV 254 and it may be this that has been visualised (circle 10) for Radix Angelicae (207).

Terpenes and steroids will show a red colour after anisaldehyde and sulfuric acid spray under UV 365 nm (203) For Sclerotium Poria Cocos (207) the main triterpenoic acids with a single carboxyl group are expected to be seen higher up the chromatogram and poricoic acid with two carboxyl groups to be lower but this was with a diethyl ether extraction. Little was visualised in these chromatograms for Poria except in the ethanolic extraction where slight fluorescence could be seen where the herb extract was applied in circle 4.

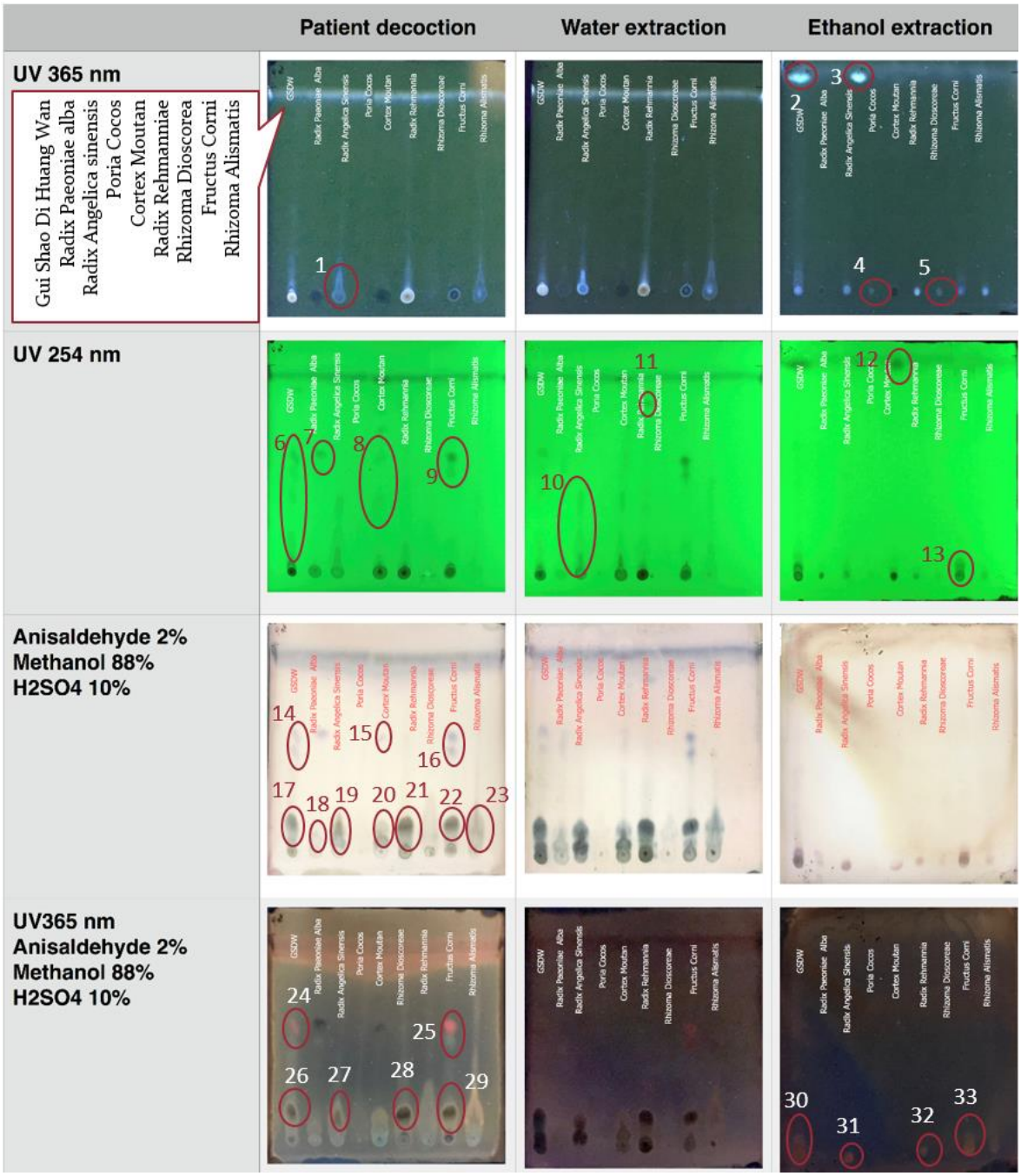


Figure 3.1 Thin Layer Chromatography of three forms of herb extraction for comparison
Patient decoction which represents the methods used in a therapeutic setting, a water based extraction of ground herbs and an ethanol extraction of ground herbs. All plates are set up in the same order as shown in the enlarged text. The most prominent differences are highlighted with a red circle and numbers used for reference in the descriptive text.

Fructus Corni will show a clear band of loganin and other triterpenoids as a pink band when a methanolic extract is used and an anisaldehyde spray at UV 365nm (210). This noticeable pink spot is also present in these chromatograms (circles 24 and 25). There is also a distinct grey spot below the pink which may be due to the gallotannins or gallocysides.

Radix Paeoniae Alba will show a strong band for the monoterpene glycoside paeoniflorin with a vanillin sulphuric acid reagent spray under visible light (207). It may be that this is seen in the smudge (circle 18). An optimised TLC method for extraction of paeoniflorin uses methanol extraction under reflux to collect any volatiles before drying and reconstituting with acetone (211).

Rhizoma Dioscoreae diosgenin and other steroidal saponins would be expected to be seen in an ethanolic extraction after anisaldehyde spray with UV 365nm (209). This was using n-butanol mobile phase for separation. In these chromatograms, there was a fluorescing spot seen at UV 365nm before the anisaldehyde spray (circle 5). There were faint smudges present after the anisaldehyde spray in the patient and water extractions. These could be the steroidal saponins.

Iridoids are frequently soluble in water but they are frequently unstable compounds. Some iridoids will quench UV 254nm but the majority are not directly detectable. Acids in the developing sprays tends to degrade iridoid aglycones and show as violet blue or grey on TLC in visible or UV 365nm. Anisaldehyde can be used for visualisation of iridoids. (203). Radix Rehmanniae will show fluorescence across the *Rf* range under UV 365 (208) and this was seen for the patient and water extractions. In visible light with the vanillin reagent it may be possible to see the iridoids, glycosides and the rehmanniosides. These are not differentiated in this chromatogram but a very dense spot is present for this herb (circles 21 and 28).

Rhizoma Alismatis (209) will typically show alisol-B and alisol-B monoacetate in an methanolic extraction with anisaldehyde spray under visible light. Dark spots are present at the bottom of the chromatogram (circle 23) with anisaldehyde in visible light for the patient and water extractions but only a very faint dark spot is present in the ethanolic extraction which is also visible under UV 365nm.

In summary four compounds which fluoresce under UV 365nm are present in the alcoholic extraction were not seen in the water based (circles 2-5). All of these were

at the extremes of the mobile front, two not moved from where they were applied, and two at the very distal edge of the front. The aqueous extract of the herbs did appear to contain some organic compounds and their extraction in water may be due to their small size or that they have hydrophilic functional groups such as alcohol, thiol, or ester.

UV 254nm showed quenching of UV from compounds (circle 6-11) which are present in the water based extractions but not the ethanol. Two double bonded compounds which quench the UV present in the ethanolic extraction were not seen in the water based (circles 12 and 13). It was in the anisaldehyde and sulphuric acid reagent sprayed chromatograms that the biggest differences are observed between the ethanol and water based extractions (circle 14-23). Differences between the patient decoction and water extraction were minor. Under UV, and with the reagent spray, (circles 24-29) are highlighting the differences already seen (circle 14-17) but show the pink and grey colours of the compounds in Fructus Corni which may be the triterpenes and gallotannins. Colours are present (circles 30-34) in the herbs of the ethanolic extraction under these conditions which are absent in the patient and water based extraction.

3.3 Discussion

Very specialised extraction protocols are used for identification of the herbs as stated in the People's Republic of China (PRC) pharmacopeia, but here a single process was applied to all herbs in an extraction. The mobile phase contained the same solvents and the reagent spray used was the same. Without reference compounds, which are difficult to obtain and costly, it is not possible to identify compounds. This does not necessarily detract from the value of comparing the extraction techniques using a fingerprint of the herbs. Differences could be seen even though not identified as specific compounds or quantified.

According to the PRC pharmacopeia (48) verification for herb requires the following processes for identification of the eight herbs in Gui Shao Di Huang Wan. These are performed on silica G plates which use a gypsum binder or for Rhizoma Alismatis Silica H, which does not have a binder. Reference drugs are prepared as for the powdered herb, as described in herb extraction column in Table 3-1. The procedures are complex and very specific for the extraction of a single reference compound, which was not the objective in this case. It would be interesting to verify the presence of these compounds, but these identified compounds are not specifically those which are suggested to confer the therapeutic actions.

Table 3-1 Summary of the process for identification of the eight herbs of GSDW from the PRC Pharmacopeia

Herb	Herb extraction	Mobile phase	Spray reagent	Reference compound
Radix Paeoniae Alba	Ethanol ultrasonicate, filter, dry and dilute with ethanol	chloroform, ethyl acetate, methanol, formic acid 40:5:10:0.2	vanillin 5% in sulfuric acid, heat	Paeoniflorin, blue purple spot
Radix Paeoniae Alba	Ethanol ultrasonicate, filter, dry and dilute with ethanol	cyclohexane and ethyl acetate 3:1	Ferric chloride 5% in ethanol and hydrochloric acid, heat until band appears	paeonol
Radix Angelicae Sinensis	Sodium bicarbonate solution, adjust to pH2, extract with ether, ultrasonicate, filter, dry and dilute with methanol	Cyclohexane, dichloromethane, ethyl acetate, formic acid 4:1:1:0.1	n/a UV 365nm	ferulic acid *(z)*-ligustilide
Radix Angelicae Sinensis	Ether ultrasonicate, filter, dry and dilute with, ethanol	n-hexane and ethyl acetate 4:1	n/a UV 365nm	reference drug (unspecified)
Sclerotium Poria Cocos	Ether ultrasonicate, filter, dry and dilute with, methanol	toluene, ethyl acetate and formic acid 20:5:0.5	vanillin in sulfuric acid 4:1 heat to 105°C	reference drug (unspecified)
Cortex Moutan	Ether no ultrasonicate, acetone	cyclohexane, ethyl acetate, glacial ethyl acetate 4:1:0.1	vanillin in sulfuric acid 1:10 heat to 105°C	paeonol
Radix Rehmanniae Preparata	Methanol, ultrasonicate, filter, dry, dissolve in water, extract in n-butanol, dry, dissolve in methanol	ethyl acetate, methanol, and formic acid	Soak plate in 2,2-diphenyl-1-picrylhydrazyl in dehydrated ethanol air dry	verbascoside
Fructus Corni	Ethyl acetate, ultrasonicate, filter, dry, dissolve in ursolic acid	toluene, ethyl acetate and formic acid 20:4:0.5	10% sulfuric acid in ethanol and heat to 105°C	reference drug (unspecified) violet red in daylight, orange fluorescent in UV 365nm
Fructus Corni	Ethyl acetate, ultrasonicate, filter, dry, dissolve in ursolic acid	Ethyl acetate, ethanol, glacial acetic acid 50:10:1	5% vanillin in sulfuric acid and heat to 105°C	loganin violet red spot
Rhizoma Dioscoreae	Dichloromethane, heat for 2 hours, filter, dry, dissolve in dichloromethane	ethyl acetate, methanol, and ammonia 9:1:0.5	phosphomolybdic acid 10% in ethanol and heat to 105°C	reference drug (unspecified)
Rhizoma Alismatis	Ethyl acetate, ultrasonicate, apply filtrate to alumina column, elute with 10mL ethyl acetate, collect elutes, evaporate, dissolve residue in 1mL of ethyl acetate	cyclohexane and ethyl acetate 1:1	silicotungstic acid 5% in ethanol	23-acetate alisol-B

Paeonol from the peonies is one of several “active” phenols including paeoniflorin, benzoylpaeoniflorin, and benzoyloxypaeoniflorin, methylgallate, catechin, paeoniflorigenone, galloylpaeoniflorin, and daucosterol may also be important in the effects on haemostasis (101). Verbascoside from Radix Rehmanniae is a glycoside which is antioxidant, hepatoprotective and anti-inflammatory (212). Loganin from Fructus Corni is an iridoid glycoside and promotes differentiation and inhibit apoptosis (213). Rhizoma Alismatis is identified by 23-acetate alisol-B a triterpene which is antimicrobial (214). The Silica G type plates were used for this analysis but the specific extraction methods for the herbs were not and neither were the mobile phases. Vanillin was not used but a related compound anisaldehyde was. The specific reference compounds were not purchased and so identification could not be made and for three of the herbs the reference drug was not specified but described only as the reference drug.

3.4 Conclusion

An ethanolic extraction used for the assays would not be representative of the predominant clinical use of the herbs. Tinctures are an ethanolic extraction which is part of the tradition of TCM but not the most commonly used in the west. Many of the studies on TCM herbs including those cited in this work use techniques to optimise extraction of compounds. Whilst this has value in exploring the full range of compounds present in plant material is does not represent the medicinal product in use. This has been explored more fully in a previous work by the author using HPLC to compare concentrated powders, raw herb decoction and tinctures for a specific herb (215). The patient style extraction was therefore used in all subsequent assays in order to most closely relate to the clinical used of the formula.

4 Effects of Gui Shao Di Huang Wan and its individual herb extracts on uterine perfusion and endothelial function.

4.1 Haemostasis

Gui Shao Di Huang Wan shows a dose dependent increase in time to clot formation, and evidence of polyvalent interaction between the component herbs in *in vitro* haemostasis assays

4.1.1 Introduction

Good uterine vascular perfusion in the endometrium is vital to implantation of the embryo to facilitate natural conception as well as being associated with successful outcome of IVF cycles (216). Uterine perfusion is reduced in women with infertility (187), and high pulsatility index is due to uterine impedance (217) and is correlated with poor reproductive outcome (218). In many assisted reproductive technologies clexane and aspirin are used as anticoagulants to improve endometrial receptivity (96). Anticoagulants reduce the pulsatility index in the uterine arteries (219). Anticoagulants can be used to overcome increased coagulability as a result of the hyper oestrogenic state induced by supra physiological levels of FSH. Anticoagulants are also used when hypercoagulability is due to anti-phospholipid syndrome which can lead to infertility, IVF failures and recurrent miscarriage (96). The failure rate for assisted conception is circa 70% even when a visually acceptable embryo is transferred (8) and therefore any intervention that improves the receptivity of the endometrium is of high value. 3.5 million people in the UK are struggling to conceive (2). If infertility is caused by poor endometrial receptivity, then it may be that IVF can exacerbate this pre-existing state rather than being the solution to infertility.

There is some evidence to support the use of Chinese herb extracts for improving fertility and Gui Shao Di Huang Wan is widely used as a base formula. Despite the clinical evidence supporting the use of Chinese herbs (3, 4, 28, 39, 40), there is little research to date which explains the mechanisms of action. In order to investigate the effects on uterine perfusion two assays which model the clotting processes (Figure 1.2) were used.

The Prothrombin Time (PT) and activated Partial Thromboplastin Time (aPTT) are *in vitro* assays which are tests of haemostasis representing the models of the extrinsic and intrinsic coagulation pathways respectively (Section 1.9, p23). These pathways are an artificially segregated sequence of events describing the *in vitro* activity of plasma. *In vivo* the extrinsic and intrinsic pathways are interrelated activities with feedback loops, inhibitors, cofactors, interaction with blood flow, vessel vasoconstriction, and interaction with endothelium (220). These pathways and tests do however remain valuable tools for assessing substances for their anti-coagulant activity (221).

The following section presents the results obtained when the herb extracts, prepared as detailed in Section 2.1.1, were tested using the PT and aPTT assays. Methods are described in full in Section 2.3, p53.

4.1.2 Results

4.1.2.1 Increasing concentration of GSDW on prothrombin time

Results indicate that GSDW increases the PT in a dose dependent manner. The PT assay tests the extrinsic pathway, results shown in Figure 4.1 show the gradual increase in time to clot formation as the volume of GSDW increases relative to dH_2O.

Concentrations for which the time to clot formation is significantly higher are indicated by *. This is for volumes above 0.015mL GSDW. The mean time ± Standard Deviation (SD) at 0.04mL was 112 s ±36.64 (n-5), compared to the next highest 0.035mL which was 42.84 s ±2.96. The time to clot increases with increasing dosage

of GSDW to 0.04mL, where very high variance (SD) impacts on the reliability of measurements.

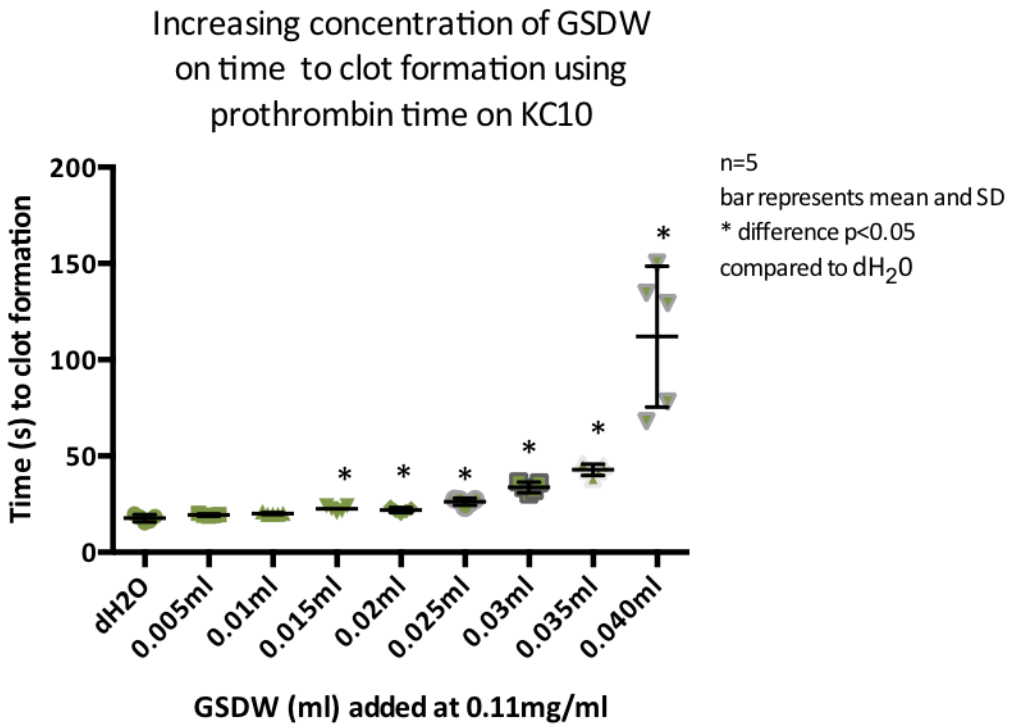


Figure 4.1 Time(s) to clot formation in PT assay with increasing concentrations of GSDW.
Data is presented as mean±SD, GSDW 0 (17.68 s ±1.85), 0.05 (19.38 s ±0.57), 0.10 (20.12 s ±0.52), 0.15 (22.58 s ±1.52), 0.20 (21.86 s ±1.32), 0.25 (26.22 s ±1.81), 0.30 (33.70 s ±2.71), 0.35 (42.84 s ±2.96), 0.040 (112.0 s ±36.64). A Welch's ANOVA was used to analyse differences between group and those which were significantly different to the control of dH2O are marked with an *.

Time to clot formation in PT assay was significantly different Welch's F (8,14.57) =50.21, p<0.0005.

Games Howell post hoc analysis revealed that the increases were significant as shown in Table 4-1, values stated are those where $p<0.05$. Above 0.015mL of GSDW added to the assay, the increase in time to clot formation was significant.

Table 4-1 Summary of significant ($p<0.05$) pairwise comparisons of PT with increasing concentration of GSDW from Games-Howell post hoc tests

mL herb	0	0.005	0.010	0.015	0.020	0.025	0.030
0							
0.005							
0.010							
0.015	0.025						
0.020	0.048						
0.025	0.001	0.006	0.010	0.099	0.036		
0.030	0.000	0.002	0.002	0.002	0.002	0.016	
0.035	0.000	0.000	0.000	0.000	0.000	0.000	0.013
0.040	0.039	0.042	0.043	0.047	0.046		

4.1.2.2 Increasing concentration of GSDW on activated partial thromboplastin time (aPTT)

Results indicate that GSDW increases the aPTT in a dose dependent manner. This assay tests the intrinsic pathway, results shown in Figure 4.2 shows the gradual increase in time to clot formation as the volume of GSDW relative to dH_2O is increased in the assay (n=5).

The graph shows those concentrations for which the time to clot formation is significantly different indicated by * and this is for volumes between 0.015mL and 0.030mL. Above these levels the time to clot formation becomes highly variable.

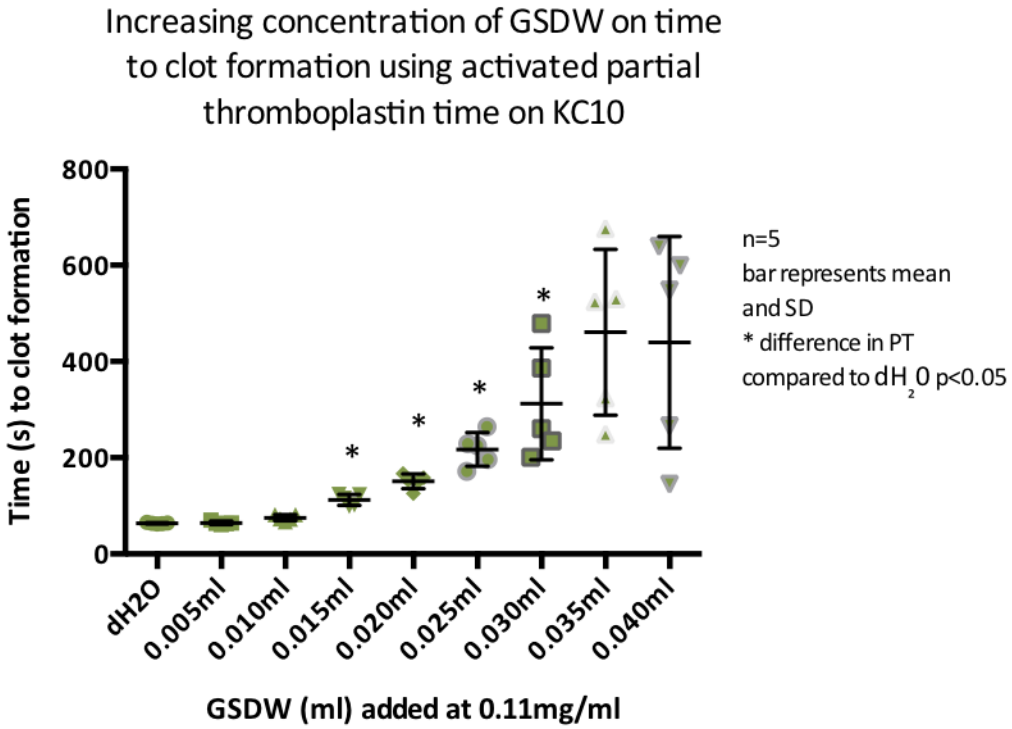


Figure 4.2 Time (s) to clot formation in aPTT assay with increasing concentrations of GSDW.
Data is presented as mean ± standard deviation, GSDW 0 (63.74 s ±1.19), 0.005 (64.56 s ±3.5515), 0.010 (75.28 s ±6.37), 0.015 (112.52 s ±11.47), 0.020 (150.9 s ±15.51), 0.025 (217.16 s ±34.96), 0.030 (312.28 s ±116.31), 0.0350 (465.46 s ±174.94), 0.040 (439 s ±220.50). A Welch's ANOVA was used to analyse differences between group and those which were significantly different to the control of dH2O are marked with an *

Time to clot formation in aPTT assay was significantly different Welch's F (8,13.77) =38.79, p<0.0005.

Games Howell post hoc analysis revealed that the increases were significant as shown in Table 4-2, values stated are those where $p<0.05$.

Table 4-2 Summary of significant ($p<0.05$) pairwise comparisons of aPTT from Games-Howell post hoc tests

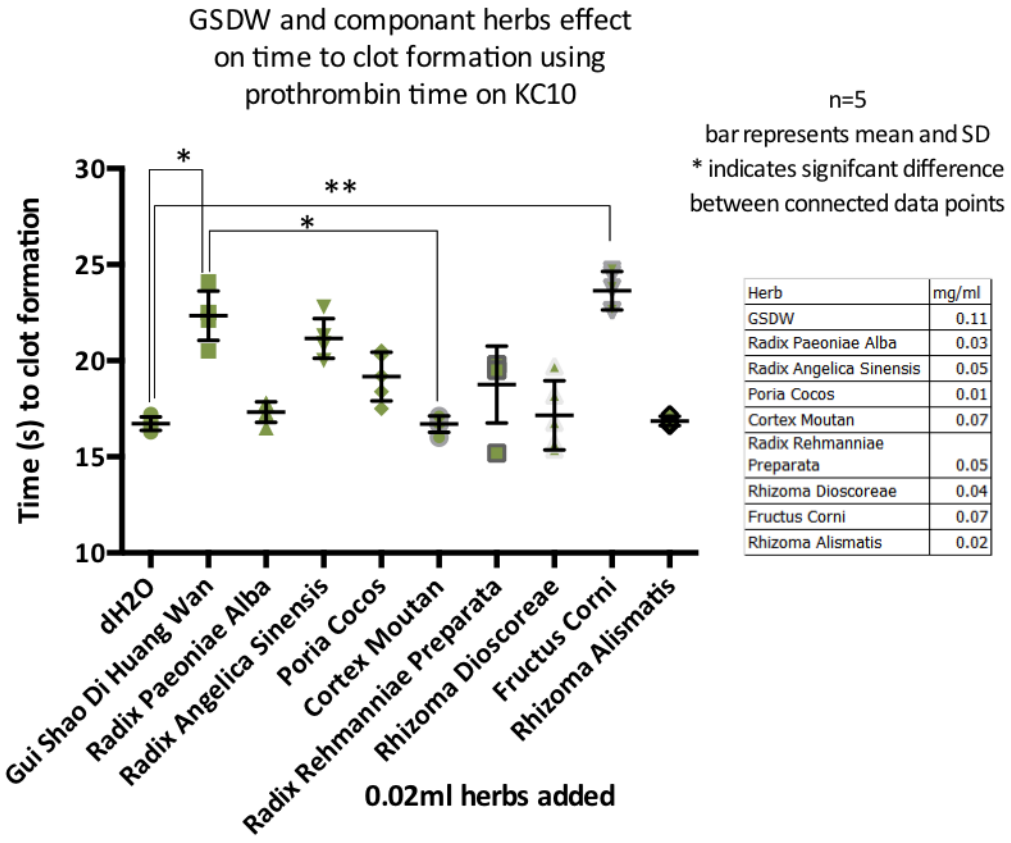
mL herb	0	0.005	0.010	0.015
0				
0.005				
0.010				
0.015	0.005	0.004	0.007	
0.020	0.002	0.001	0.001	0.031
0.025	0.005	0.005	0.006	0.015
0.030	0.066	0.067		

The extrinsic pathway tested by the PT, and the intrinsic pathway tested using the aPTT assay both show a pattern of increase in coagulation time as the volume of the herbal formula increases, but standard deviations show that this difference becomes less consistent at higher concentrations.

Having established that the formula did significantly increase coagulation time over a no herb control of dH_2O, and that the effect was seen in both pathways, each herb was tested individually. This was to see if the changes could be attributed to a particular herb. The volume added was 0.02 mL chosen as the midpoint of the concentrations tested for the whole formula and at a concentration where the herb extracts showed a significant change from no herb control but were still showing consistent results in the time to clot formation.

4.1.2.3 Effects of the individual herb extracts on prothrombin time (PT)

Results shown in Figure 4.3 shows that for GSDW and for Fructus Corni the difference in PT was significantly higher than for a dH_2O control only, and that there was a difference between GSDW and Cortex Moutan. Data here did not meet the assumptions for parametric testing and so a Kruskal Wallis was used. Complete pairwise comparisons from Dunn's post hoc can be found in Appendix XII.



Herb	mg/ml
GSDW	0.11
Radix Paeoniae Alba	0.03
Radix Angelica Sinensis	0.05
Poria Cocos	0.01
Cortex Moutan	0.07
Radix Rehmanniae Preparata	0.05
Rhizoma Dioscoreae	0.04
Fructus Corni	0.07
Rhizoma Alismatis	0.02

Figure 4.3 Time (s) to clot formation for individual herb extract using prothrombin assay.
Mann Whitney U was used as the assumptions for a parametric test were not met. It showed GSDW (mean rank=8) was statistically higher than for dH2O (mean rank=3), U=25, z=-2.62, p=0.008. Kruskal Wallis showed significant differences between individual herb extracts $\chi2(9) = 38.11$, $p < 0.0005$. Dunn's post hoc analysis revealed statistically significant differences between dH2O (11.40) and GSDW (42.10) (p=0.039); dH2O (11.40) and Fructus Corni (47.00) (p=0.005); GSDW (42.10) and Cortex Moutan (11.90) (p=0.047).

4.1.2.4 Effects of the individual herb extract on activated partial thromboplastin time

aPTT with the individual herb extracts (Figure 4.4) show that for GSDW and for Fructus Corni the difference in time to clot formation was significantly higher than for a dH_2O control, and that GSDW was significantly higher than all of the contributory herbs.

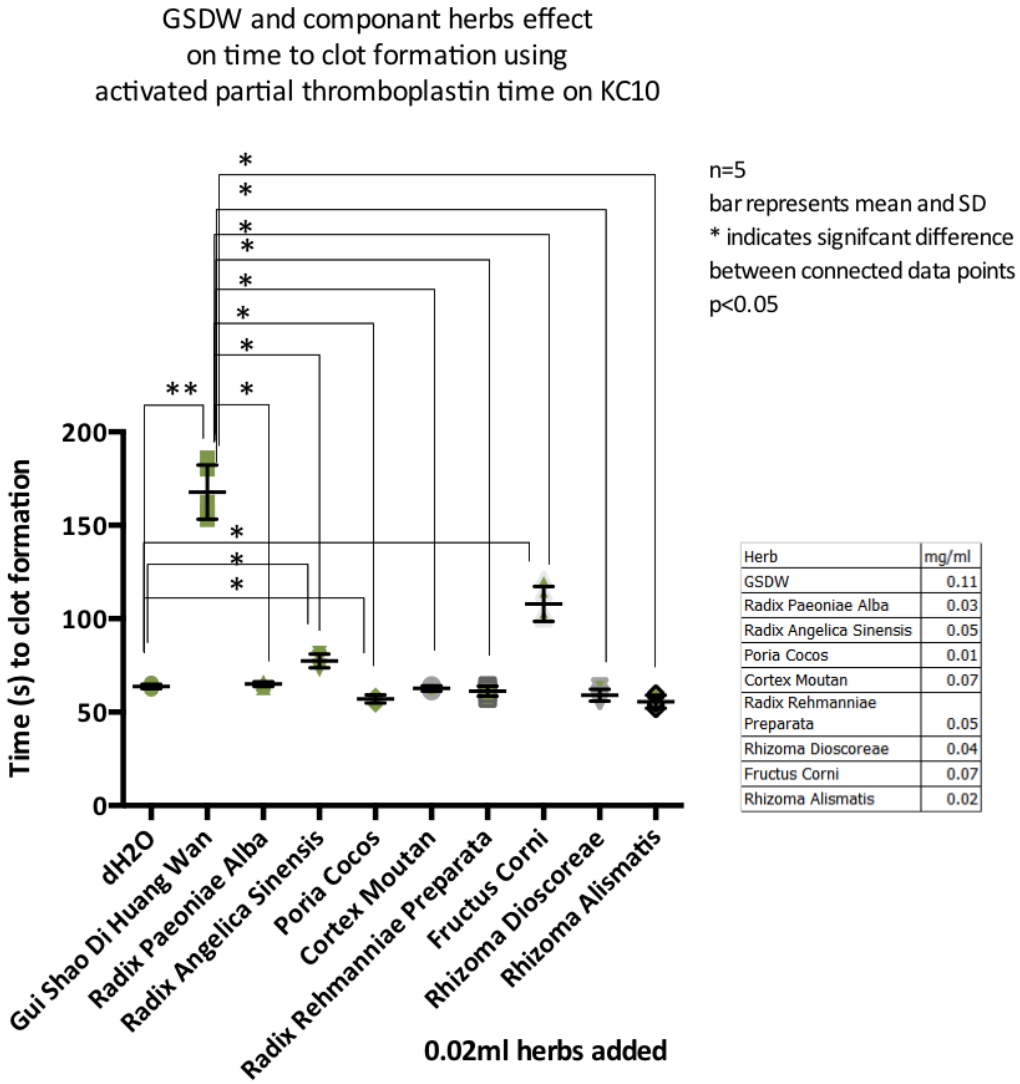


Figure 4.4 Time (s) to clot formation for individual herb extract using activated partial thromboplastin time assay.
T-Tests showed was a statistically significant difference between media and GSDW for aPTT, data is shown here as mean±SD, dH2O (63.74 s ±1.19), Gui Shao Di Huang Wan (167.74 s ±14.52) t (8) =15.94, p<0.0001 the effects size Cohen's D is 10.1. Radix Paeoniae Alba (65 s ±1.19), Radix Angelicae Sinensis (77.4 s ±3.69), Sclerotium Poria Cocos (57.1 s ±2.15), Cortex Moutan (62.68 s ±1.45), Radix Rehmanniae Preparata (62.68 s ±1.45), Rhizoma Dioscoreae (61.2 s ±2.68), Fructus Corni (107.86 s ±9.32), Rhizoma Alismatis (59.12 s

±3.19). A Welch's ANOVA was used to analyse differences between group and those which were significantly different to the control of dH2O are marked with an *

Activated thromboplastin times for the individual herb extracts did significantly change time to clot formation, Welch's $F(9, 16.135) = 42.64$, $p < .0005$.

Games-Howell post hoc analysis revealed significant differences between dH2o and the individual herbs and significant differences also existed between GSDW and every other herb as is shown in Table 4-3.

Table 4-3 Games Howell post hoc tests for individual herb aPTT

Control/formula	Herb extract	Mean difference	95% CI	p
dH2O	GSDW	-104.0	-139.78 to -68.22	0.001
dH2O	Radix Angelicae Sinensis	-13.66	-22.37 to -4.94	0.008
dH2O	Sclerotium Poria Cocos	6.64	1.67 to 11.61	0.012
dH2O	Fructus Corni	-44.12	-66.96 to -21.28	0.004
GSDW	dH2O	104.0	68.22 to 139.78	0.001
GSDW	Radix Paeoniae Alba	102.74	66.96 to 138.52	0.001
GSDW	Radix Angelicae Sinensis	90.34	55.57 to 125.11	0.001
GSDW	Sclerotium Poria Cocos	110.64	75.17 to 146.11	0.001
GSDW	Radix Rehmanniae	105.06	69.35 to 140.77	0.001
GSDW	Rhizoma Dioscoreae	106.54	71.29 to 141.79	0.001
GSDW	Fructus Corni	59.88	26.00 to 93.76	0.002
GSDW	Rhizoma Alismatis	108.62	73.61 to 143.63	0.001

There were further significant differences between the individual herb extracts, but these are not directly useful in answering the hypotheses here. The full table of all post hoc tests can be seen in Appendix XIII.

4.1.2.5 Comparison of the effect of Gui Shao Di Huang Wan with Heparin

To compare the effects of GSDW with a commonly used pharmaceutical anticoagulant medication the assays were repeated. The whole formula heparin and heparin PT and aPTT were performed and time to clot formation was compared. The therapeutic dose of heparin was assumed to be 10,000 iU/5L (v/v) of blood *in vivo* and so was used at an equivalent concentration in the assay and at 10x dilution. The dilution was used because heparin has a very short half-life of only 90 min, and binds to a range of plasma proteins (195) and so a direct therapeutic dose is unlikely to represent *in vivo* plasma concentrations (222). Heparin binds to antithrombin in the plasma and so inactivates thrombin and factor Xa which are both on the common pathway and so will inhibit both assays (94).

4.1.2.5.1 PT Heparin

Figure 4.5 shows PT for GDSW as compared to the effect with a 1U dose of heparin calculated for this assay to be 0.075mL of heparin. Heparin at this dose and GSDW both have a significant difference (GSDW $p<0.005$, Heparin $p<0.005$) over dH_2O but the difference between them is not significant ($p=1.0$).

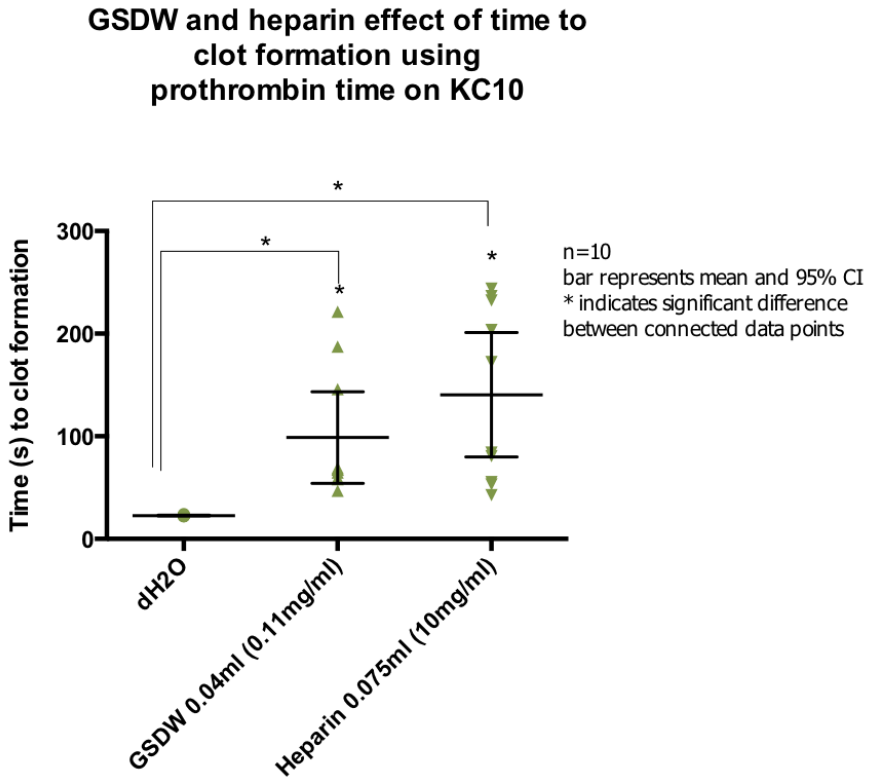


Figure 4.5 Time (s) to clot formation for GSDW and heparin using prothrombin time assay.
Kruskal Wallis showed PT was significantly different $\chi2(4)$ 41.27, $p < .0005$. Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons using adjusted p values, data shows ranks and demonstrates statistically significant differences between dH2O (7.85) and GSDW 0.04mL (39.20) ($p<0.005$); dH2O (7.85) and heparin 0.075mL (41.80) ($p<0.005$) but not between GSDW 0.04mL (39.20) and heparin 0.075mL (41.80) ($p=1.000$).

4.1.2.5.2 aPTT Heparin

The previous assay used heparin at 1U dose but in the assay no clot formed after 15 min. In this assay, GSDW was compared to the effect with a 1/10U dose of heparin calculated for this assay to be 0.001mL Figure 4.6 shows aPTT for GDSW as of heparin. Heparin at this dose and GSDW both have a significant difference over dH_2O (GSDW $p<0.005$, Heparin $p<0.005$, but the difference between them is not significant($p=1.0$).

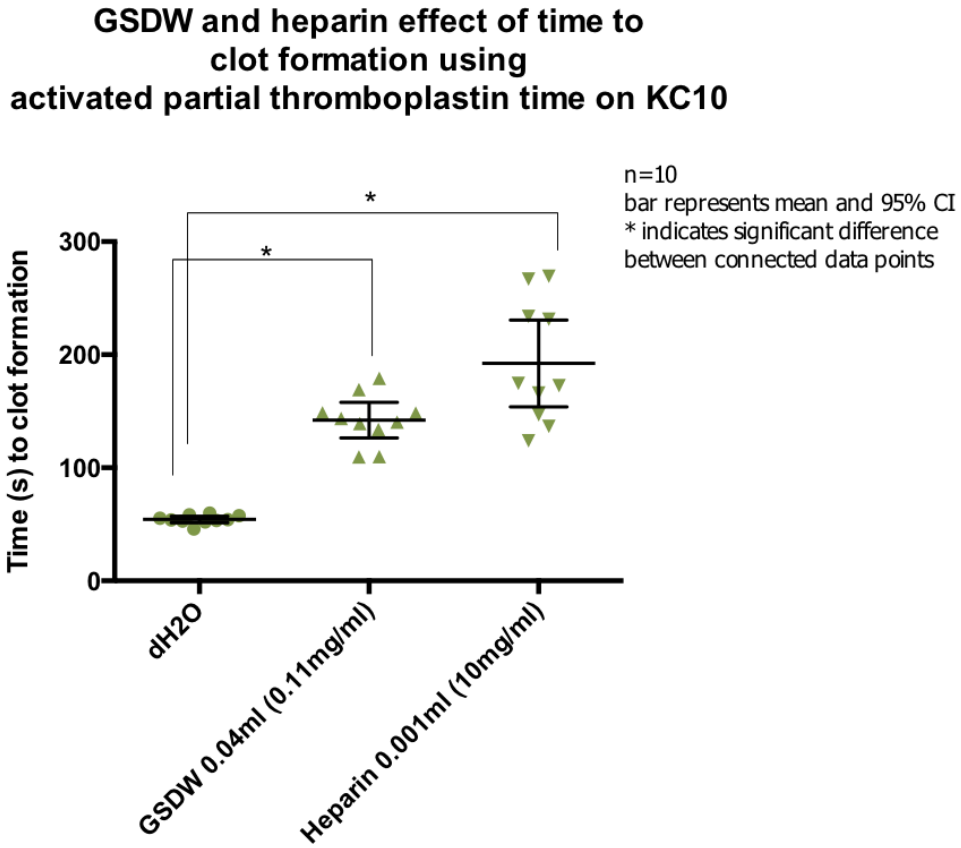


Figure 4.6 Time (s) to clot formation for GSDW and heparin using activated partial thromboplastin time assay.
Kruskal Wallis showed that aPTT time were significantly different $\chi2(3)$ 32.25, $p < 0.005$. Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons using adjusted p values, data shows ranks demonstrates statistically significant differences between dH2O (5.50) and GSDW 0.04 (16.50) ($p<0.005$); dH2O (5.50) and heparin 0.075mL (33.10) ($p<0.005$) but not between GSDW 0.04mL (16.50) and heparin 0.075mL (33.10) ($p=1.000$).

The graphs show the results for the PT for the 1 iU dose, 1/10 iU was tested and a small effect was seen, similar to a 0.02mL dose of GSDW (mean±SD 1/10 iU heparin 26.33±5.26, GSDW 0.02mL 29.91±0.96, dH2O 22.71±0.57). A 1/10 iU equivalent only is shown for the aPTT as a 1 iU heparin did not form a clot within 15 min.

4.1.3 Discussion

GSDW increases time to clot formation in both the PT and aPTT assays, and it does so in a dose dependent manner which is statistically significant ($p<0.05$) at volumes above 0.015mL added to the assays. The effect on PT appears to be similar for both GSDW and Fructus Corni suggesting the effects originate from that herb alone. A derivative of Fructus Corni containing malic acid, succinic acid and citric acid has been shown to be anti-thrombotic but this is suggested to be mediated by interference in ADP activated signalling in platelets and regulation of endothelial function (223) none of which are tested in these assays. Fructus Corni is a peony and contains many of the same compounds Radix Paeoniae Alba which has been shown to have antithrombin effects, but no effect was seen in Radix Paeoniae Alba (100).

In the aPTT assay, GSDW, Radix Angelicae Sinensis and Fructus Corni all showed a significant increase in time to coagulation over the dH_2O control. H1 Gui Shao Di Huang Wan will increase parameters of uterine perfusion and angiogenesis in endothelial cell proliferation, migration, and differentiation and H3 the influence of the extract whole formula Gui Shao Di Huang Wan is greater than the individual herb extracts are accepted the time to clot formation is increased and the different herbs have different contributions. Radix Angelicae Sinensis does contain *(z)*-ligustilide which has been shown to inhibit clot formation and platelet aggregation (110) but in another study time to clot formation via the PT and aPTT assays were not extended with *(z)*-ligustilide alone. *N*-butylphthalide is also found in Radix Angelicae Sinensis and has been show to increase coagulation times; the mechanism of its anti-thrombotic and anti-platelet activity is related to its regulation of cAMP level and 5-

HT release (111), these effects will not lead to the extended clotting times observed. The compound α-pinene (115) found in Radix Angelicae Sinensis has been shown to be weakly antithrombin and ferulic acid (224) it has been shown to inhibit tissue factor. Fructus Corni is closely related to Radix Paeoniae Alba which has antithrombin effects.

Many of the anticoagulant effects seen in herb extracts are due to their effects on inhibition of platelet activation and aggregation, by antithrombin activity, or by inhibition of tissue factor (225).

4.1.3.1 Polyvalence

The mean aPTT of GSDW was 167.74 s, Radix Angelicae Sinensis was 77.4 s and Fructus Corni 107.86 s. If clot formation in dH_2O control is 63.74 s then effect of Radix Angelicae Sinensis over control is 13.66 s and for Fructus Corni is 44.12 s, the combined effect is 55.78 s assuming the effects are additive. The increase in time to clot formation for GSDW over dH_2O is 104 which suggests that the effects is not merely a cumulative effect of the two herbs. N=5 repeats in the analysis but time to clot formation of 150-250 s was seen in 25 repeats over the course of the experiments. This suggests that some combination of the compounds that are present are causing an effect greater than the individual contributions or possibly a compound not present in the individual herb extracts is being created when the herbs are decocted together.

4.1.3.2 Morphology of the clots

The clots that were formed in the PT and aPTT assays were visually different are the concentration of GSDW increased. They appeared dark, which is consistent with the appearance of the herb extract, but also shrunken. It was possible that a mechanical interference could be causing the changes and so the extracts were ultra-filtered, but the changes remained. Change in clot structure could be due to alteration in the production of fibrin. To microscopically examine fibrin morphology, the clots were fixed in formaldehyde 37% (v/v) overnight before being placed in cytoblock cassettes

for dehydration. They were run through a tissue processor (Citadel 2000); placed in 70% ethanol, 90% ethanol, 100% ethanol and then xylene. Once completed the samples were embedded in paraffin wax and cut into sections 5µm thick using a microtome. These were floated in a water bath and mounted on slides which were initially dried on a hot plate, then air dried overnight. The slides were dewaxed by placing in xylene for five min, then rehydrated in 100% alcohol for one minute, then 95% alcohol for 30s, 70% alcohol for 30s, 50% alcohol for 30s and then distilled water for 30s. Sections were then stained using Martius, Scarlet and Blue (MSB) stain which is considered to be a reliable stain for fibrin which should appear red, fresh fibrin can be yellow and old fibrin blue. Sections were then dehydrated and placed under a coverslip mounted with DPX.

Sections were then examined and photographed under the microscope (Figure 4.7). Predominantly the tissue in the sections were stained blue with small areas of red on some, but not on all of the slides. It was expected that the tissue being composed of recent fibrin clots would be red. Nothing was elucidated of the structure of the fibrin partly because the stains showed no consistent changes and because the sections of the clot were very friable and damaged in the microtome.

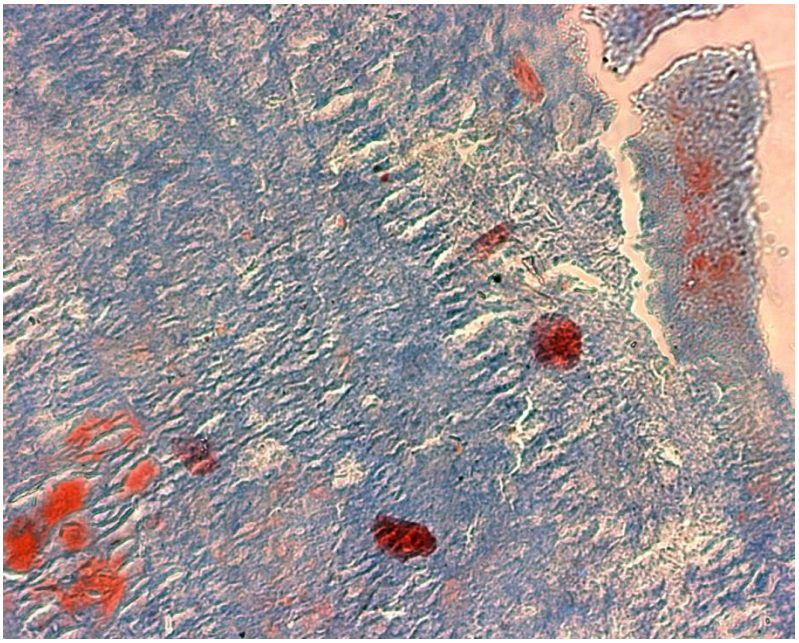


Figure 4.7 Image of clot stained with Martius scarlet blue from an aPTT clot with 20µL Radix Rehmanniae added to the plasma in the assay. Image taken using a 20X objective.

Fibrin creates a mesh of cross linked fibres that form the structure of the clot. It is converted from fibrinogen as a result of thrombin mediated release of fibrinopeptides. These two substances have overlapping roles in the formation of a clot. The mechanical properties of fibrin are affected by its structure at the level of the molecule, fibre, and fibre network. These can be affected by genetics as well as environment. Anticoagulants such as heparin affect fibrin characteristics through reduced thrombin generation (226).

Fibrin clot structure can be altered by procoagulant stimuli, fibrinogen (227) and prothrombin concentration (228). Factor XIIa modulates the structure of the clot independent of any changes in thrombin by binding to the N-terminus of fibrinogen (229). If Factor XIIa were affected by the herb extracts greater changes in clot morphology would be seen in the aPTT assay. The changes in fibrin can alter its stability and resistance to lysis (230).

The photos in Figure 4.8 show a possible difference in the structure of the fibrin in the clot, and a difference in the photos of the fluids retrieved from around the clot. When the clot forms abnormally the fluids are not bound into the fibrin mesh and are apparent as shown in Figure 4.9. The photographs of the clots were highly inconsistent and the stains did not highlight fibrin so this laborious process was only completed on two clots for each condition, and for each assay. There are observable changes and this may indicate a direction for future work.

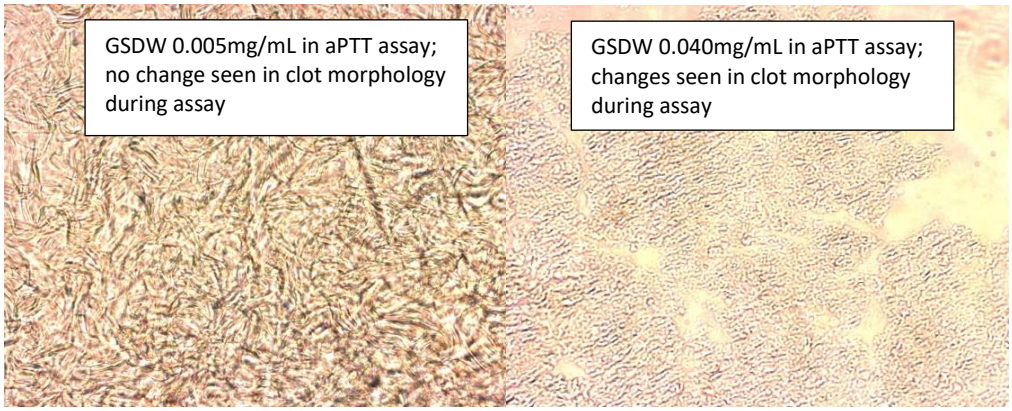


Figure 4.8 Photographs of slices of the clots with 0.005mg/mL and 0.040mg/mL GSDW in the aPTT assay. Image taken using a 20X objective.

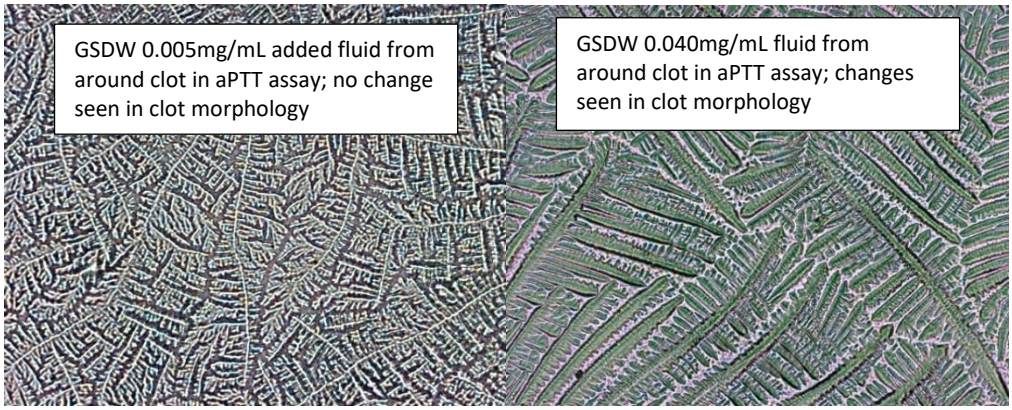


Figure 4.9 photographs of the fluid which remains around the formed clot in aPTT assay. Image taken using a 20X objective.

An undergraduate student at the University of Portsmouth, Queenie Szeto, attempted to investigate this further testing the tensile strength of the clots formed. Thrombi were tied at either end with fine suture thread and tied to a force transducer in a tissue bath and subjected to pull at either end. This was achieved with clots produced with no dilution of plasma, but when herb extract was substituted in the assay, the resultant clot was too unstable to connect to the force transducer and disintegrated in the process. There are methods for the visco-elastic analysis of fibrin at the molecular, fibre and clot level using laser or magnetic tweezers, torsion pendulum, thromboelastography which would provide more information about the changes seen in the clot morphology (231).

The structure of clots formed *in vitro* are not representative of clots formed *in vivo* as they tend to lack the alignment of fibrin seen in the architecture of the *in vivo* clots. This is better assessed with an electron microscope than a light microscope (232).

Platelets are produced in in bone marrow from megakaryocytes which are the fragmented and enter the blood stream. They are activated by a number of pathways including contact with collagen which bind to the platelet and they are stabilised in the forming fibrin mesh. Bound platelets activate further platelets as by releasing TXA2, adenosine triphosphate and thrombin, and by local tissue factor release (233). Platelets also have a function in regulation of inflammation binding to neutrophils and interacting with leukocytes (233).

Many plant compounds decrease platelet activation and aggregation through a variety of mechanisms (225). The general mechanism by which flavonoids have been shown to inhibit platelet aggregation is through their effects on the arachidonic pathway, which facilitates its conversion to TXA, which amplifies platelet activation and aggregation (234). This is true of Radix Paeoniae Alba and Cortex Moutan which contain paeonol, thought to inhibit conversion of TXA (235). Herbs in GSDW have been shown to affect platelet aggregation but that does not provide any information as to the mechanisms by which PT and aPTT are being elongated in these assays.

Coumarins which are present in Radix Angelicae Sinensis have been shown to inhibit vitamin K dependent carboxylation. The amino acid γ-carboxyglutamate residues are required for the functioning of procoagulant prothrombin, and factors VII, IX, and X and for the anticoagulants such as Protein C (236). Ferulic acid which is present in Radix Angelicae Sinensis is inhibitory to tissue factor which activates blood clotting via factor VII (224).

Oxypaeoniflorin in Radix Paeonia Alba and Cortex Moutan have been shown to increase PT time, catechin and benzoylpaeoniflorin in Paeonia have been shown to increase aPTT time (101). Paeonol, gallic acid, catechin and benzoyloxypaeoniflorin also present in both has been shown to increase TT.

Pharmaceutical research remains focussed on factor Xa inhibitors as the inhibition of other factors is less direct than influencing factor X and so more difficult to manage. Factor X also has no known other functions. Direct inhibition of a number of synthetic and natural compounds are being investigated as are indirect inhibitors such as the heparan polysaccharides that promote antithrombin (237). aPTT can be elongated by inhibition of factors VIII, XII, XI, IX, thrombin inhibitors, promotion of Protein C or S which inactivate Factors Va and VIIIa. Elongation of aPTT occurs with administered heparin which acts as physiological heperans do. They inhibit coagulation by activating antithrombin (AT) which inhibits the serine proteases thrombin (IIa), factor Xa and to a lesser extent factor XIa, and factor IXa (238). Elongation of PT may be due to inhibition of tissue factor or factor VII. Any effect on prothrombin (II), fibrinogen (I), factors X, V or XIII will affect both PT and aPTT.

4.1.4 Conclusion

Although it has been shown that both in this study and other research that elongation of time to clot formation is extended (Figure 4.10, p108) the mechanisms by which it exerts its influence are not clear. Radix Paeonia Alba and Cortex Moutan, contain compounds shown to inhibit thrombin and fibrin which would affect both pathways but the effects are not seen in either assay for either of these herb extracts alone. The most significant effects were seen for Fructus Corni for which only mild anti platelet activity was found in the literature. Radix Angelicae Sinensis was shown to extend both assays and literature was found supporting inhibition of tissue factor by ferulic acid which would influence PT and to be anti-thrombin which would affect both pathways. It does not seem likely that the elongation effects of GSDW are due to this alone as the whole formula shows greater effects than this herb. The herbs do have effects on platelet aggregation and on inflammation which will contribute to their promotion of blood flow *in vivo* but a further influence is evidenced by these assays.

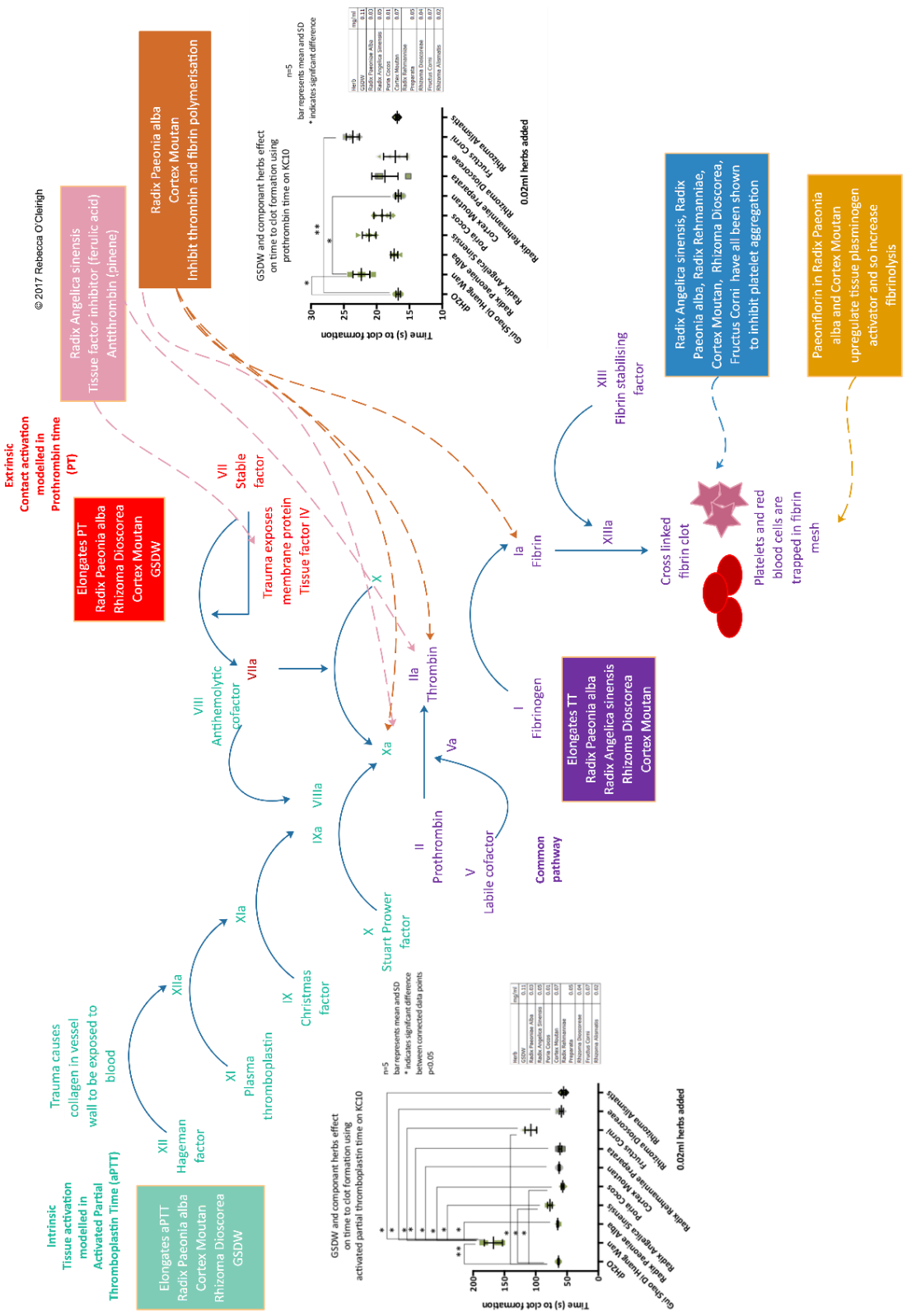


Figure 4.10 Summary of effects of the herb extracts on the clotting cascade seen in the aPTT and PT assays and any expected actions as seen in the literature reviewed.

4.2 Angiogenesis: Proliferation

Gui Shao Di Huang Wan may promote endometrial receptivity increasing endometrial thickness by increasing proliferation of endothelial cells

4.2.1 Introduction

Regeneration of the endometrium occurs following menstruation, the surface of the endometrium is re-epithelialised beginning on the second day of bleeding and completing at day 5. This occurs through the migration and proliferation of epithelial cells accompanied by changes in the supporting endothelial cells (239). Proliferation in the endometrium leads to the thickening of the mucosa into distinct zones with different vascularity, which may be due to the differing functions of the basilis and functionalis. During this proliferative phase oestrogen receptors numbers increase (239).

The MTT assay is commonly accepted to be a measure of proliferation, but specifically measures cell viability (240). It is possible that there is no increase in the number of cells but an upregulation of their mitochondrial activity. It is generally considered in most cell populations that total mitochondrial activity is related to the number of viable cells (241) and so is indicative of a proliferative effect. A second assay, a MultiTox assay was also performed which measures live cell protease activity.

96 well plates were seeded with HUVECs, a serial dilution of the whole formula GSDW was added to half of the wells, leaving half as control. Cell viability was calculated using either an increase in the colourimetric density using the MTT assays, or using MultiTox Glo regents that measure live cell luminescence and dead cell fluorescence. The full methodology for these assays can be found in Section 2.4, p60.

4.2.2 Results

4.2.2.1 MTT relative viability data 24, 48, and 72 hours

HUVECs at 24, 48, and 72 hours were exposed to serial dilutions of the neat GSDW starting at a concentration of 86.70 µg/mL. There was a clear cytotoxic effect at higher concentrations of the extract (86.70 – 10.84 µg/mL) and the effect on endothelial cell proliferation was dose dependent. Between 10.84 µg/mL and 5.42 µg/mL GSDW, circa 100% viability was reached. Test and control values were approximately equal at all three time points; % viability is expressed as mean±SD and at 5.42 µg/mL was 24 h (109.17±9.70), 48 h, (114.83±16.31), 72 h (109.50±13.44). The analysis presented in Figure 4.11 shows the level of cytotoxicity of the herb.

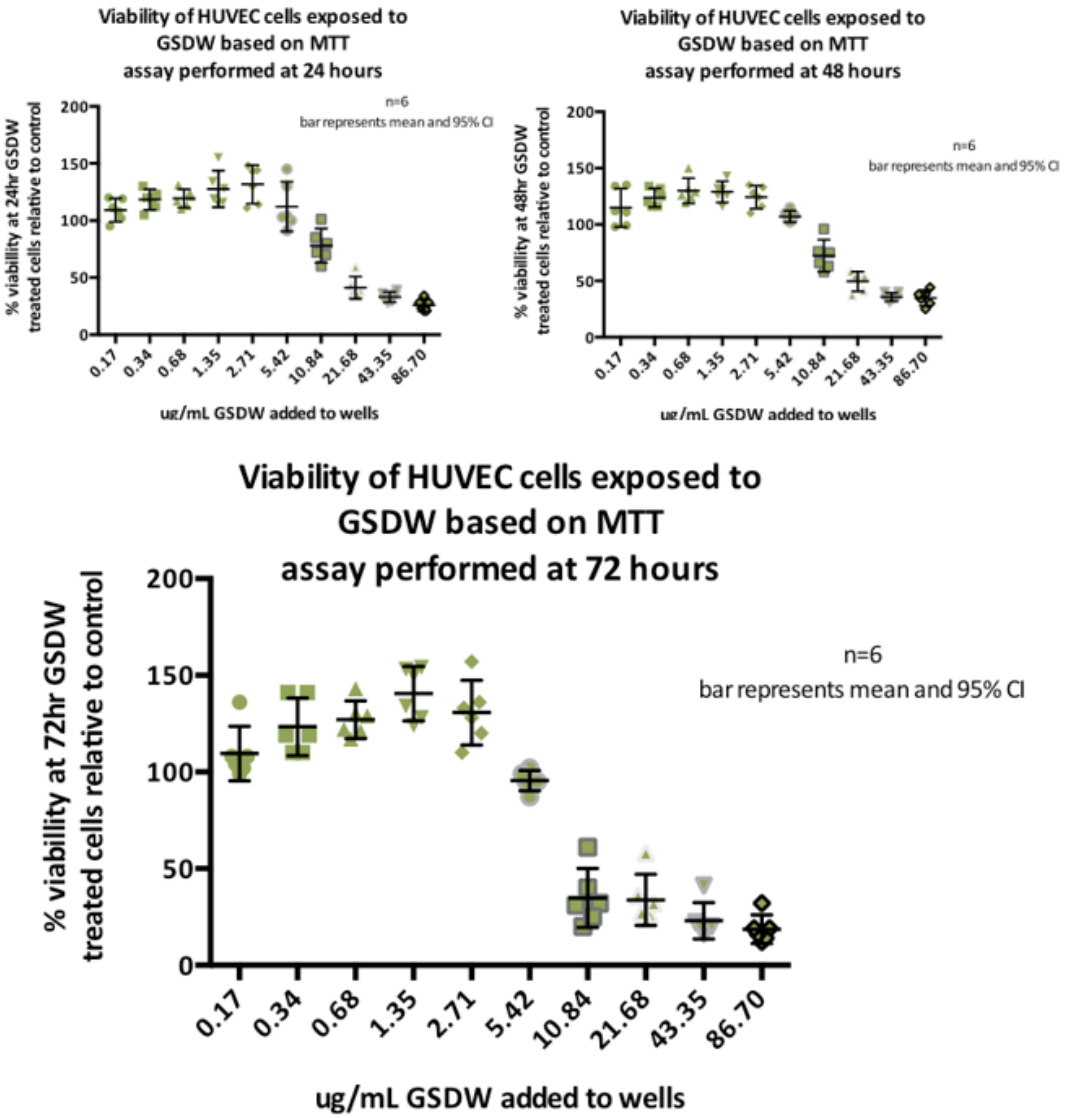


Figure 4.11 Viability data for HUVECs at 24, 48 and 72 h.
Used to assess the level at which the herb extracts were no longer cytotoxic. Data is displayed as mean±SD at 5.42 µg/mL viability reached parity between test and control, 24 h 112.17±20.70, 48 h 107.17±4.83, and 72 h is 95.50±5.05. At higher concentrations viability was reduced.

4.2.2.2 MTT IC50 data 24, 48, and 72 hours

Figure 4.12 displays the IC50 which is a standard measure of the half inhibitory concentration of a drug. In this case it displays the half measure between 100% survival and cell death. In these experiments it was considered of greater value to establish the levels at which the herbs were not cytotoxic for further cell assays, as well as investigating the proliferative effects seen.

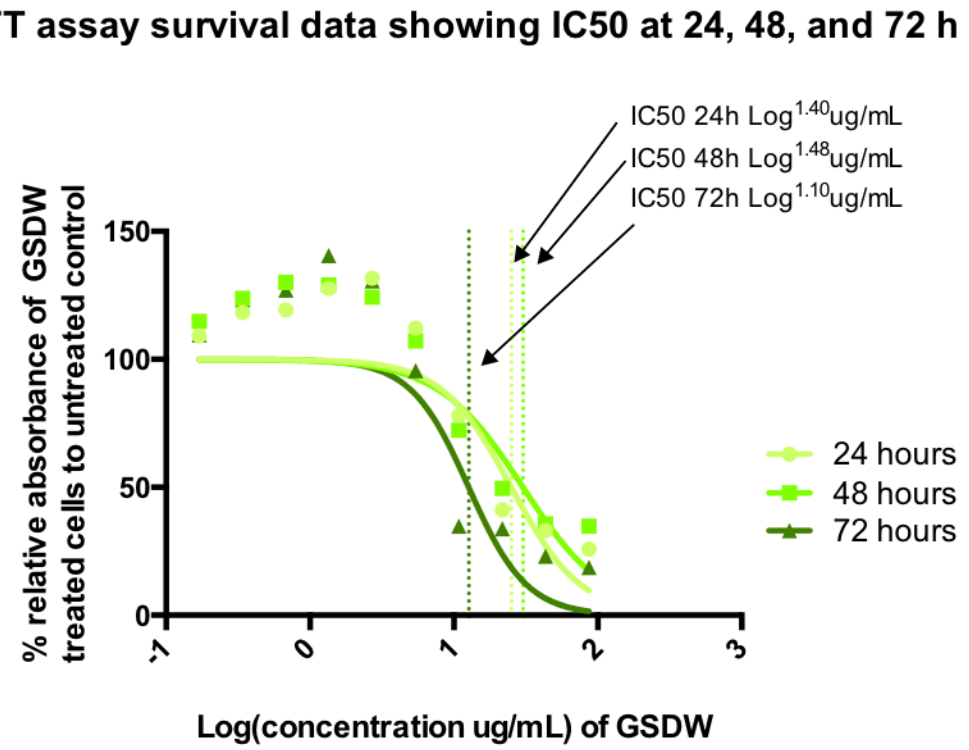


Figure 4.12 shows the IC50 values for the three time scales used.
The IC50 doses are at 24h 25.1 µg/mL CI 11.89-52.79 µg/mL, 48h 30.2µg/mL CI 12.34-73.87 µg/mL, and 72h 12.7 µg/mL 5.45-29.43 µg/mL.

At concentrations below 5.42 µg/mL % cell viability appeared to increase above 100% suggesting a proliferative effect. This slight increase was still seen at the 9th dilution $\frac{1}{512}$ which is 0.017 µg/mL on a single plate. In order to investigate to what dilution this effect was present, the experiment was performed extending the serial dilution across two plates to a final dilution of $\frac{1}{524288}$ or 0.00017 µg/mL. The slight increase in % viability was not observed in any well on the second plate (data not presented). To examine the increase in % viability a subset of the data was considered.

4.2.2.3 MTT 24, 48, and 72 hours excluding cytotoxic doses

A one-way ANOVA (either ANOVA with Tukey post hoc tests, or Welch's ANOVA with Games Howell post hoc tests) was performed on the subset of data where herb concentrations were below 5.42 µg/mL is shown in Figure 4.13. As discussed above this is because an increase in % viability was observed. An ANOVA wad used to determine whether this increase was statistically significant.

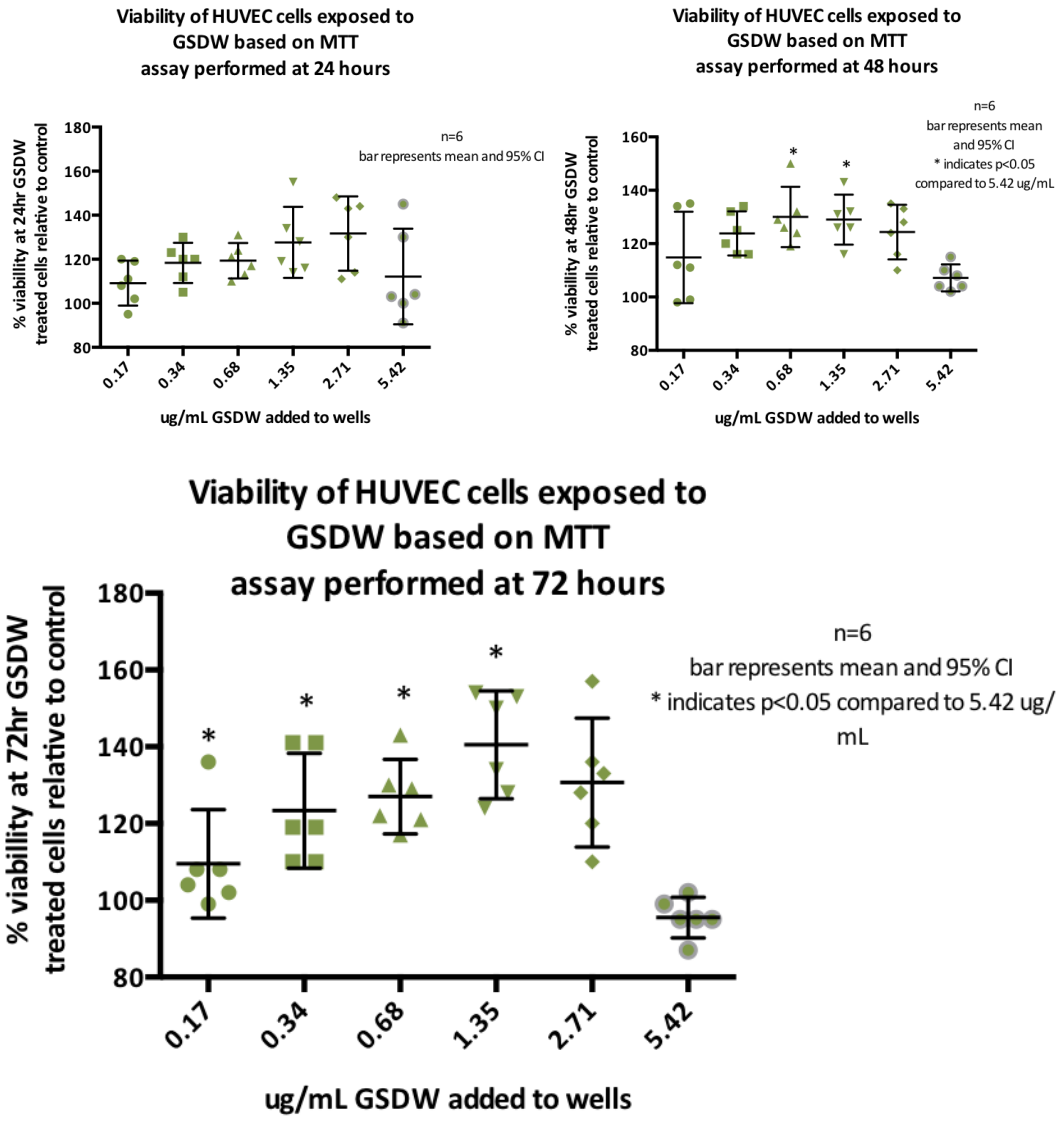


Figure 4.13 Subset of viability data for HUVEC MTT assay cells at 24, 48 and 72 h.
Data is described here as mean±SD. 24 h; 0.17 µg/mL (109.17±9.70), 0.34 µg/mL (118.33±8.73), 0.68 µg/mL (119.33±7.66), 1.35 µg/mL (127.67±15.40), 2.71 µg/mL (131.67±16.06), 5.42 µg/mL (112.17±20.70). 48 h; 0.17µg/mL (114.83±16.31), 0.34µg/mL (123.83±7.86), 0.68 µg/mL (130±10.75), 1.35 µg/mL (129±8.90), 2.71 µg/mL (124.33±9.77), 5.42 µg/mL GSDW (107.17±4.83). 72 h; 0.17 µg/mL (109.50±13.44), 0.34 µg/mL (123.33±14.26), 0.68 µg/mL (127±9.27), 1.35 µg/mL (140.50±13.41), 2.71 µg/mL (1130.67±15.97), 5.42 µg/mL (95.50±5.50).

Results of the ANOVA:

At 24 h, the differences were not statistically significantly different according to the concentration of herb added Welch's F (5, 13.77) = 2.066, p=0.132

At 48 h, differences between herb concentrations were statistically significant F (5, 30) = 4.398, p = 0.004, effect size is η^2= 0.4

At 72 h, differences between herb concentrations were statistically significant F (5, 30) = 10.039, p <.0005, effect size is η^2= 0.63

Tukey's post hoc analysis showed that at 48 h viability increased as compared to 5.42µg/mL taken as 100% viability when GSDW was added at concentrations of 0.68 µg/mL (95%CI 4.67 to 40.10) and 1.35 µg/mL (95%CI -.99 to 35.33).

At 72 h, the difference in viability was significant between 5.42µg/mL and concentrations between 0.34µg/mL and 2.71 µg/mL. 0.34 µg/mL (95%CI -49.70 to -5.96.), 0.68 µg/mL (95%CI -53.37 to -9.63), 1.34 µg/mL (95%CI -66.97 to -23.13), 2.71µg/mL (95%CI -57.04 to -13.30).

The group means showed a statistically significantly difference ($p < 0.05$) at 48 and 72 h, indicating that viability of HUVECs at 48 and 72 h is increased by the presence of GSDW added to the cell media at concentrations of between 0.34 to 2.71 µg/mL. H1 Gui Shao Di Huang Wan will increase parameters of uterine perfusion and angiogenesis in endothelial cell proliferation, migration, and differentiation can be accepted in relation to this assay, GSDW does increase viability.

4.2.2.4 MultiTox-Glo Multiplex cytotoxicity assay

The MultiTox-Glo assay was only performed on a single plate, with each dilution tested in triplicate, (n=3 each experimental condition, and n=3 for control). The purpose was to indicate whether the results observed in the MTT assay described above were due to proliferation, rather than due to an increase in mitochondrial activity. The MultiTox Glo assay measures live and dead cell protease activity (Figure 4.14). The GF-AFC reagent enters intact cells and is only cleaved to become fluorescent within a live cell by its protease and so fluorescence is proportional to viable cell number. The dead cell reagent AAF only becomes luminescent in contact with dead cell protease released when cells membranes have lost integrity, and so is proportional to dead cell number.

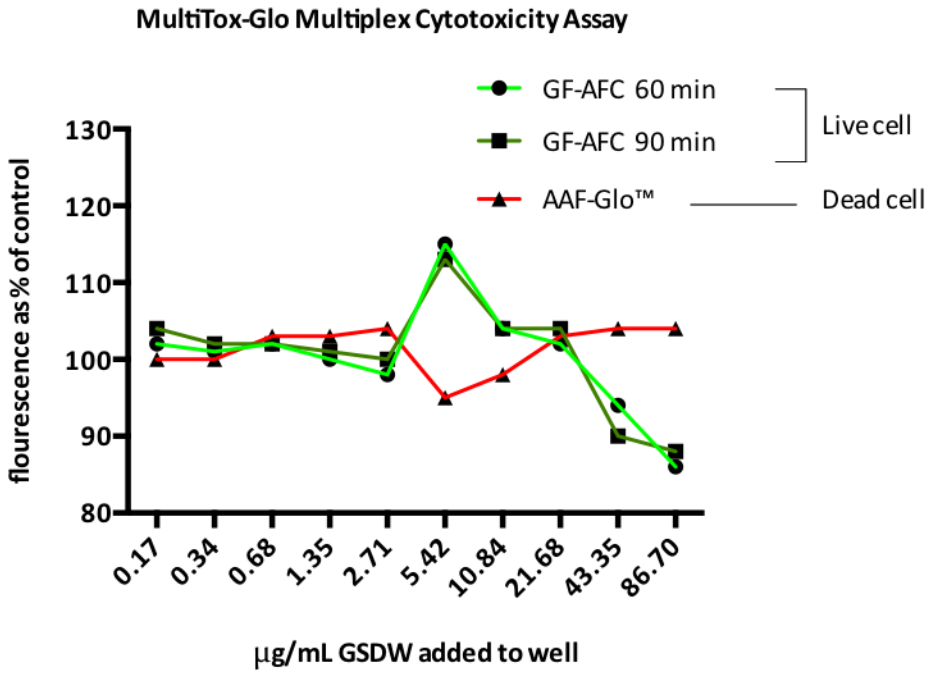


Figure 4.14 Results of the MultiTox-Glo cytotoxicity assay indicating the number of live cells at 60 and 90 min (test sensitivity potentially changes) versus dead cells as concentration of GSDW increases. There is a distinct peak at 5.42µg/mL in live cell protease detected and a corresponding dead cell protease increase corroborating the proliferative effect of GSDW.

The observed patterns of increase in live cell numbers combined with a decrease in protease activity resulting from cell death suggests this herb extract is having a proliferative effect at between 2.71 and 5.47 μg/mL. This range overlaps with the MTT assays but the peak is at the same concentration as was seen to show parity with survival in the MTT assays.

4.2.3 Discussion

Addition of GSDW to endothelial cells resulted in the promotion of cell proliferation as seen in the MTT assay between 48 h from 0.68 to 1.35 μg/mL and at 72 h from 0.34 to 2.71 μg/mL. The MTT assays are a relatively simple and affordable assay to use for large numbers of wells. They are reliable but can indicate increases in mitochondrial activity within the cell as well as increase in cell numbers. The MTT was not performed using the commercial serum free media as the duration of the cell viability did not reach the first investigated time point. Repeating the assay using cell count and an early time point with serum reduced media could yield further valuable information and this could be performed with each individual herb extract.

By combining the results of the MTT with the MultiTox Glo assay, a more expensive assay requiring more sophisticated laboratory equipment, it was possible to indicate the effects of GSDW are proliferative rather than due to mitochondrial activity. The MultiTox showed a proliferative peak between 2.71 and 5.42 μg/mL. It is not clear why the MultiTox Glo assay peak is at a higher concentration, it may be that because this assay was run later in the year and so the herbs were less potent than when the first assays were run with the MTT.

There is debate over when in the cycle and in which parts of the endometrium proliferation of endothelial cells occurs. It is generally considered to be highest after menstruation when epithelial cells proliferate to repopulate the functionalis after menstruation (79). This is true of the epithelial cells (242, 243) and this proliferation, measured as thickness of the functionalis on ultrasound, has been correlated with

increased embryo implantation (244). It has been suggested that the number of vessels in the endometrium doesn't change (131) does change (132) and that there is little change in the endothelial cells in the basilis but changes in the functionalis (134). This last study seems logical, proliferation is required in order to provide vascularity to the regenerating epithelial functionalis post menstruation. However, the suggested mechanism of intussusception, for the rapid creation of new vessels in the endometrium, does not require the extensive proliferation of endothelial cells that sprouting angiogenesis does (245).

Radix Angelicae Sinensis whole extract (mechanism for extraction not specified) (145) and ferulic acid (147) which it contains have been shown to increase proliferation in HUVECs. Rhizoma Dioscoreae has been shown to have a proliferative on endometrial epithelial cells (154) but endothelial cells were not studied. No other evidence of proliferative activity has been shown for any of the herbs. The MTT requires very high numbers of cells and so it was not repeated for each herb individually but this would provide valuable additional information.

4.2.4 Conclusions

GSDW does promote proliferation of endothelial cells at concentration of between 0.68 and 5.47 µg/mL at 48 and 72 hours and this is likely to be due proliferative effects on cells and not due to upregulation of mitochondrial activity. Increases in vascularity and thickness of the endometrium, and therefore receptivity could be due to the influence of GSDW.

4.3 Angiogenesis: Migration

Effects of Gui Shao Di Huang Wan and its individual herb extracts on HUVEC and HUtMEC migration

4.3.1 Introduction

Migration of endothelial cells is one of the three main processes explored in relation to angiogenesis although little research on the migration in the endometrium has been published and very little on the herbs in GSDW. Migration can be in response to specific ligands pro or anti-angiogenic or relating to mechanotaxic stimulus from the sheer stress encountered at the periphery of developing blood vessels (138).

For this version of the wound healing assay HUVECs or HUtMECs are seeded in the two well ibidi migration insert in a 6 well plate and left to adhere overnight. Inserts are removed and the GSDW or individual herb extract is added in dilute media with no manufacturer supplied supplement only 1% (v/v) BSA with the appropriate HUVEC or HUtMEC media (Table 2-4, p57). These were incubated and images were taken every 15 min for 24 h, the full methodology can be found in Section 2.7, p68.

4.3.2 Results

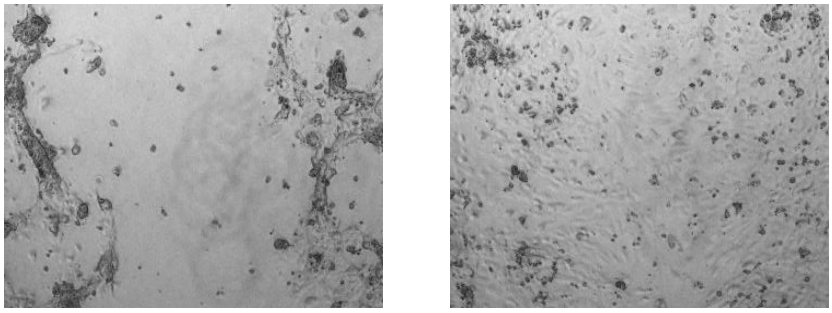
4.3.2.1 HUVEC migration experiment GSDW

These are single wells for each experimental condition and therefore not subject to statistical analyses and have not been repeated, they are single instances and as such are not conclusive but do indicate that here would be value in repeating the assays although insufficient cells were available to do this.

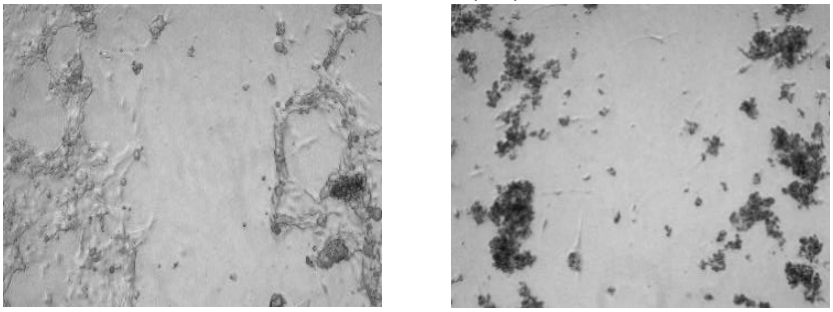
The HUVECs with media containing the commercial supplements (for details see Table 2-4) did migrate (Figure 4.15). The HUVECs with media and only 1% (v/v) BSA added showed minimal migration. With GSDW 0.017 µg/mL there was no evidence

of migration but an increase in the central gap may suggest cytotoxicity, inhibition of proliferation or non adhesion. At the most dilute GSDW 0.0087 µg/mL there was evidence of migration, but not to the extent of that observed with media containing commercial supplements but still greater than that of media containing 1% (v/v) BSA.

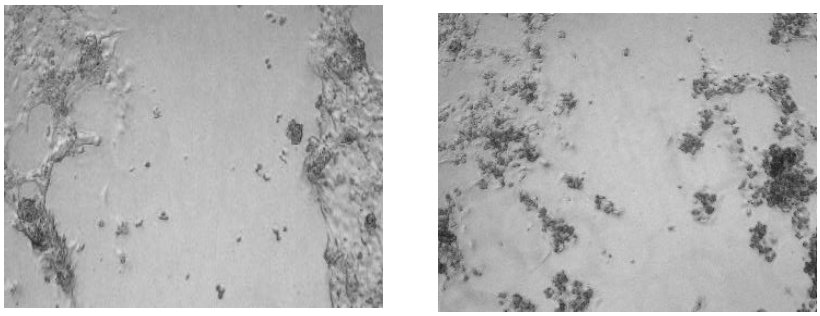
a. Media with commercial supplements



b. Media 1% (v/v) BSA



c. GSDW 0.017 µg/mL



d. GSDW 0.0087 µg/mL

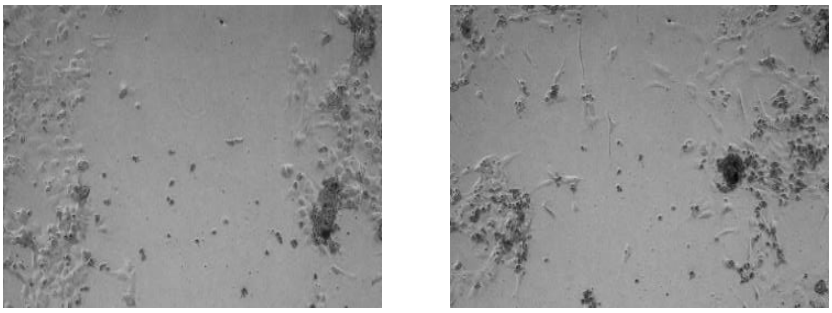


Figure 4.15 HUVEC migration in presence of different concentrations of GSDW (c, d), and media with 1% BSA (v/v) (b) and commercial supplements (a). 1st image is at 1 h and the 2nd is at 12 h, media with commercial supplements (a) showed migration, with only 1% (v/v) BSA in media (b) slight migration is observed. At GSDW 0.0087 µg/mL in media with 1 % BSA (d) some migration is observed, at higher concentrations of GSDW (c) none is seen. Image taken using a 5X objective.

Table 4-4 lists the results of the analysis using ImageJ wound healing tool an hour after the assay was set up and again at 12 h. The third row is a calculation of the difference between them. In Figure 4.16 the graph shows how GSDW at 0.017 µg/mL leads to an increase in the area between the cells either side of the "wound" which may be due to cytotoxicity of the herbs at this concentration. The other three conditions all show migration, for the media with commercial supplements this was the gap almost entirely closing but for media with only BSA this was a very small change. GSDW 0.0087 µg/mL in media 1% (v/v) BSA showed a greater closure of the wound that without GSDW in the same media, but not as much as the media with full supplements.

Table 4-4 Analysis using ImageJ MRI Wound Healing Tool to calculate area in pixels of the gap between cells on either side of the insert

	Media with commercial supplements	Media 1% (v/v) BSA	GSDW 0.017 µg/mL in media 1% (v/v) BSA	GSDW 0.0087 µg/mL in media 1% (v/v) BSA
Area of gap at 1 h (pixels)	710022	600221	561026	669050
Area of gap 12 h (pixels)	174656	543586	779748	571932
Difference between 1 and 12 h	535366	56635	-218722	97118

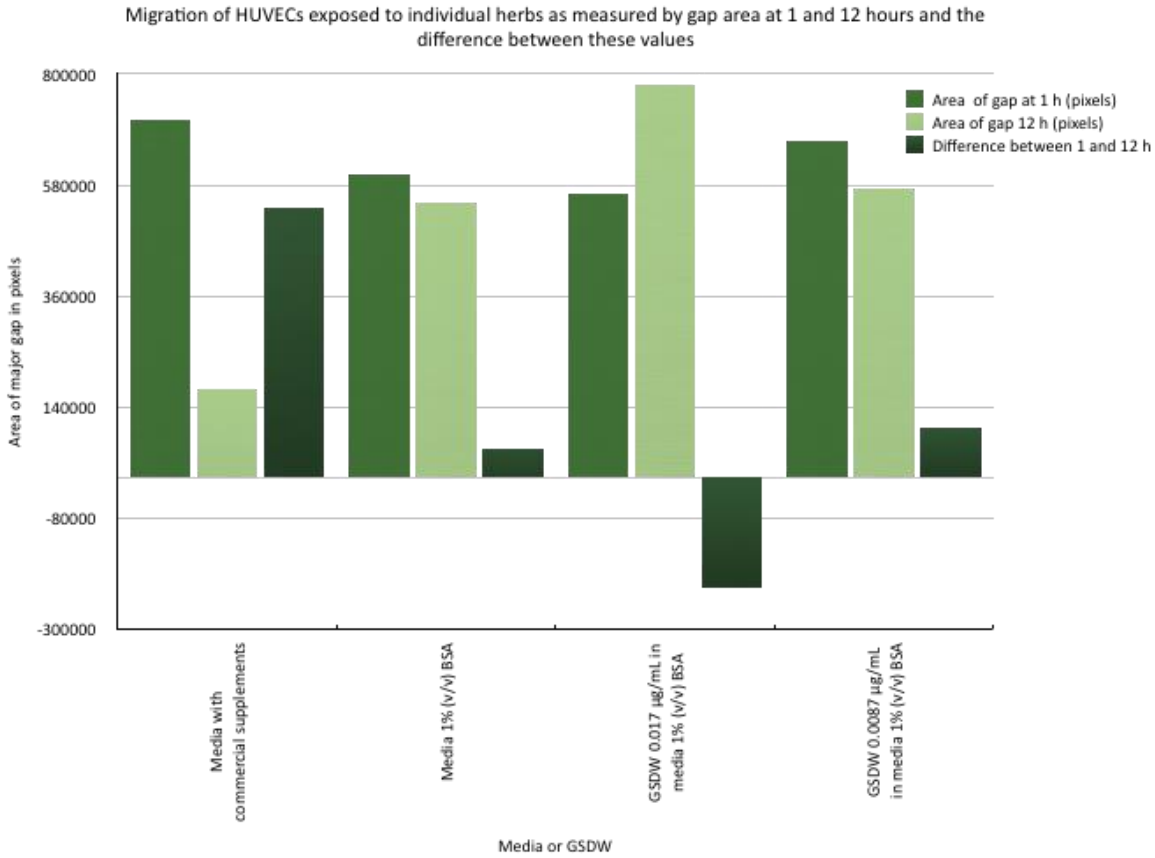
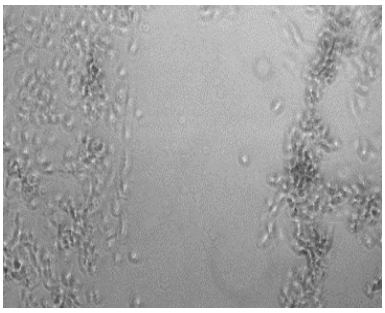
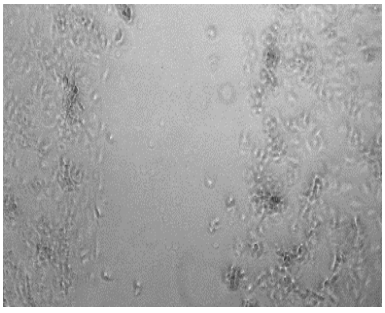


Figure 4.16 Graph showing the results of the wound healing tool analysis for the HUVEC migration with two different concentrations of GSDW in media with BSA 1% (v/v), the media with BSA 1% (v/v), and commercial supplements. The 1st bar represents the gap at 1 h after the assay had been set up, the 2nd is the gap after 12 h and the third bar is the difference between them showing whether the gap had increased (negative value for difference) as in GSDW 0.017 µg/mL in media 1% (v/v) BSA or a decrease in the gap suggesting migration as in media with commercial supplements, media 1% (v/v) BSA, and GSDW 0.0087 µg/mL in media 1% (v/v) BSA.

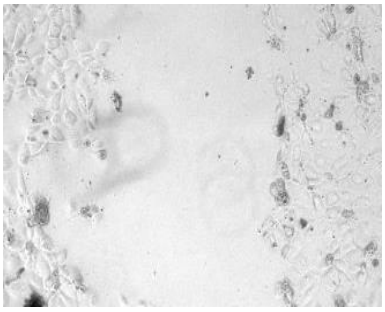
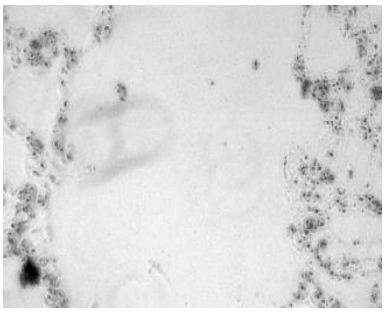
4.3.2.2 Individual herb extracts HUVEC migration experiment

The individual herb extracts were tested to see if any differences could be observed between them in their effects on endothelial cell migration. This was set up with media, whole formula and each of the individual herbs extracts at 1 in 200 dilutions (see Table 2-2, p51 for mg/mL). The photographs in Figure 4.17 show the gap 1 h after the assay was set up and again at 12 h. The wound gap has been reduced in the presence of Sclerotium Poria Cocos, Radix Rehmanniae, Fructus Corni, and Rhizoma Alismatis suggesting that migration across the wound has occurred.

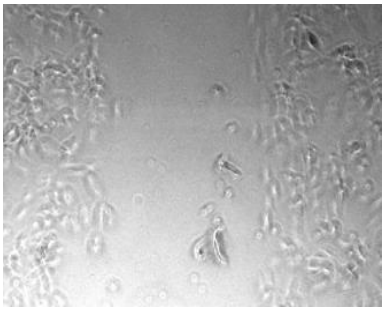
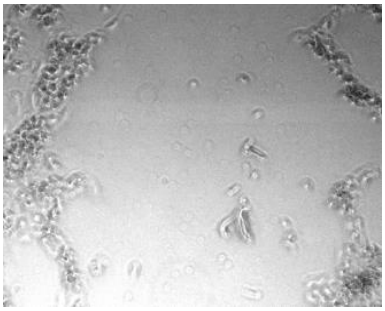
a. Media with 1% (v/v) BSA

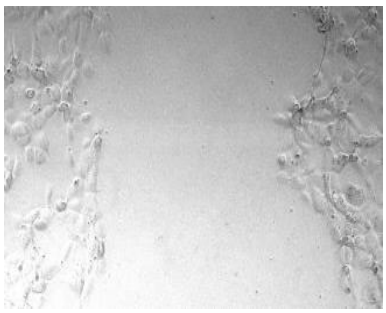
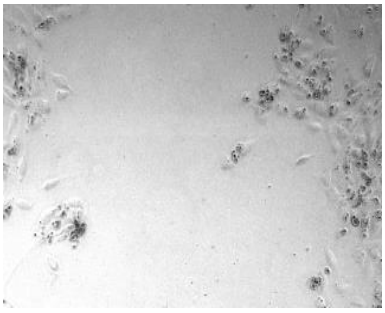
b. GSDW 0.04335 µg/mL in media with 1% (v/v) BSA

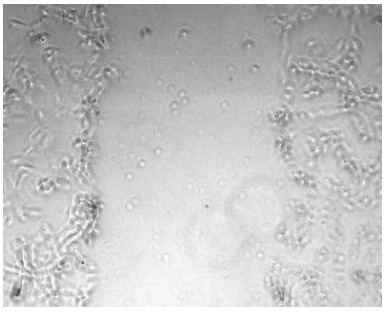
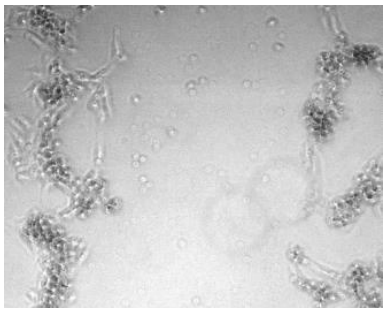
c. Radix Paeoniae Alba 0.01825 µg/mL in media with 1% (v/v) BSA

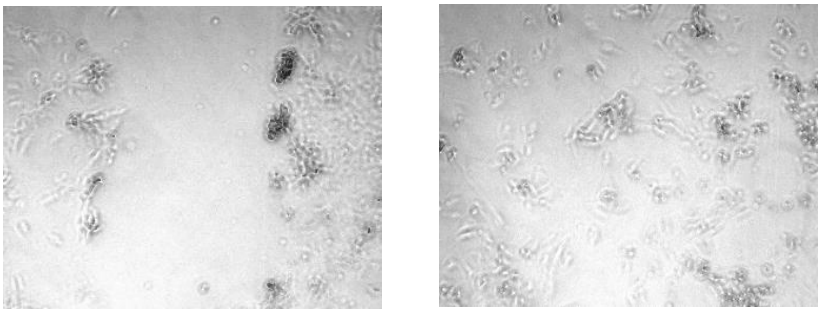
d. Radix Angelicae Sinensis 0.0202 µg/mL in media with 1% (v/v) BSA

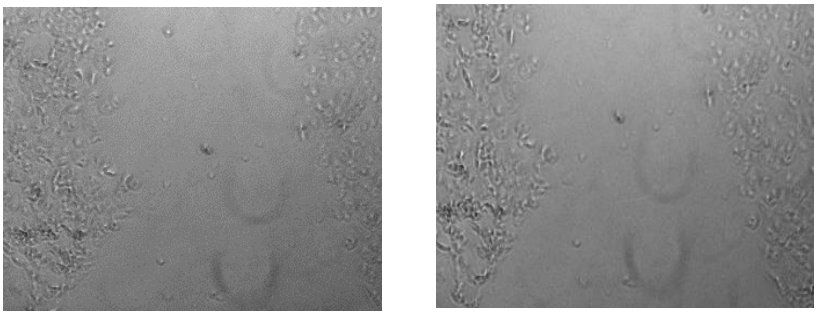
e. Sclerotium Poria Cocos 0.00105 µg/mL in media with 1% (v/v) BSA

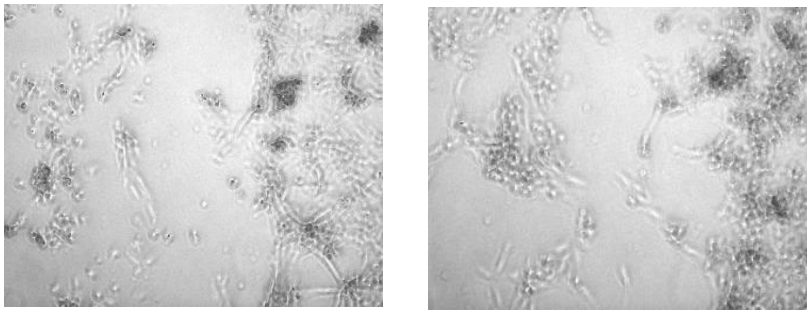
f. Cortex Moutan 0.0063 µg/mL in media with 1% (v/v) BSA



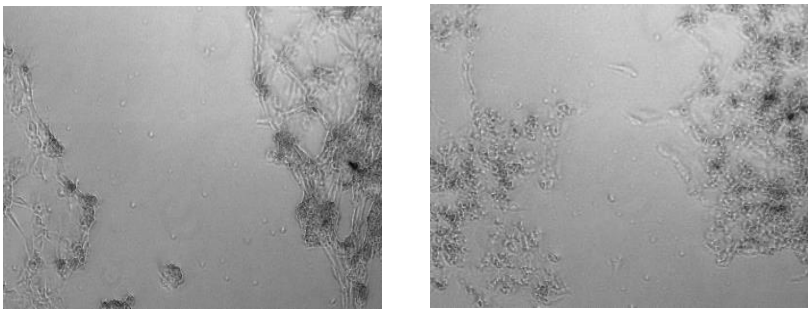
g. Radix Rehmanniae preparata 0.0602 µg/mL in media with 1% (v/v) BSA



h. Rhizoma Dioscoreae 0.0088 µg/mL in media with 1% (v/v) BSA



i. Fructus Corni 0.0179 µg/mL in media with 1% (v/v) BSA



j. Rhizoma Alismatis 0.0046 µg/mL in media with 1% (v/v) BSA

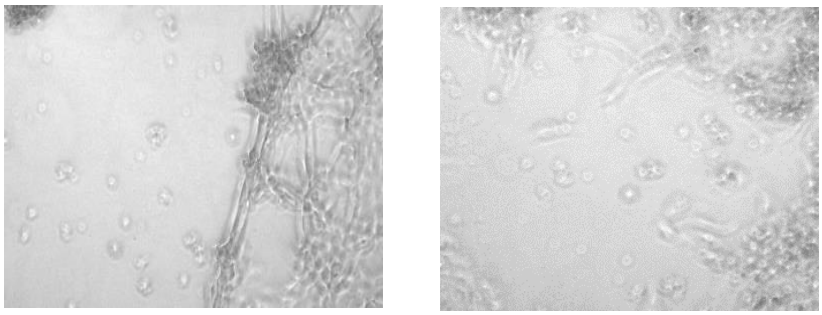


Figure 4.17 HUVECs tested with individual herb extracts added to the media; first image is at 1 h, the second is at 12 h

Table 4-5 lists the results of the analysis using ImageJ wound healing tool an hour after the assay was set up and again at 12 h. The third row is a calculation of the difference between them. In Figure 4.17 the graph shows more clearly than the images how in the presence of some of the herb extracts in media with BSA 1% (v/v) the cell free gap increased rather than decreased. This was for media with BSA (a), GSDW 0.04335 µg/mL (b), Radix Paeoniae Alba (c), Radix Angelicae Sinensis (d), Cortex Moutan (f), and Rhizoma Dioscoreae (h). The gap between areas of cells had reduced for Sclerotium Poria Cocos (e), Radix Rehmanniae (g), Fructus Corni (i), and Rhizoma Alismatis (j) suggesting migration has occurred. This most pronounced for Rhizoma Alismatis (j).

Table 4-5 Analysis using ImageJ MRI Wound Healing Tool to calculate area in pixels of the gap between cells from either side of the insert for individual herb extracts

	Media with 1% (v/V) BSA	GSDW	Radix Paeoniae Alba	Radix Angelicae Sinensis	Sclerotium Poria Cocos	Cortex Moutan	Radix Rehmanniae	Rhizoma Dioscoreae	Fructus Corni	Rhizoma Alismatis
Area of gap at 1 h	522318	466481	489132	564727	771630	437207	573613	533768	497595	481106
Area of gap 12 h	590133	545014	722898	660756	647731	610419	440953	544113	398208	288275
Difference	-67815	-78533	-233766	-96029	123899	-173212	132660	-10345	99387	192831

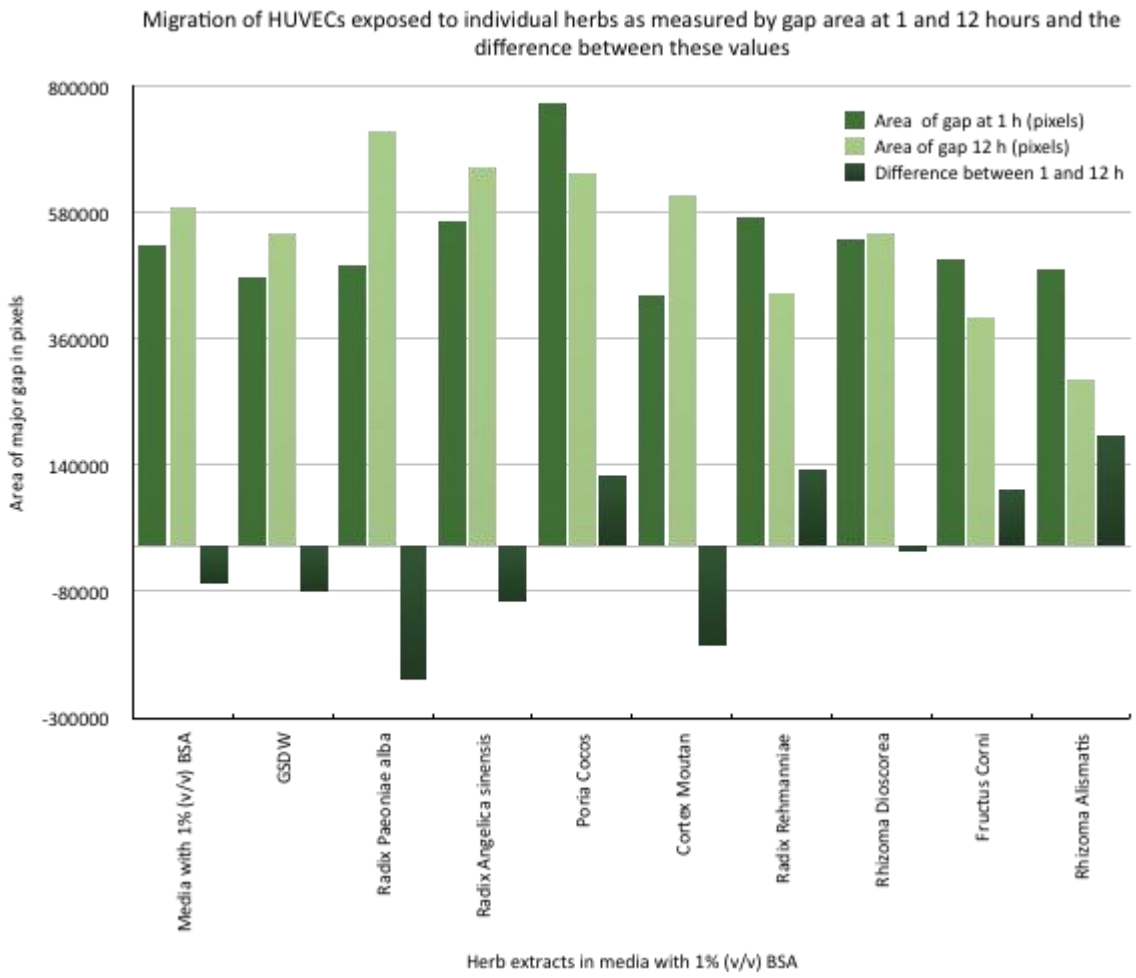


Figure 4.18 Graph showing the wound healing tool analysis for the individual herb extracts
The 1st bar is the gap at 1 h after the assay had been set up, the 2nd is the gap after 12 h and the third bar is the difference between them showing whether the gap had increased (negative value for difference) suggesting cell death as in media only, GSDW. Radix Paeoniae Alba, Radix Angelicae Sinensis, Cortex Moutan, and Rhizoma Dioscoreae. A positive difference suggests cells have migrated across the wound as in Sclerotium Poria Cocos, Radix Rehmanniae, Fructus Corni, and Rhizoma Alismatis.

4.3.3 Discussion

Although preliminary results suggest that there is an effect on migration, future studies including optimisation experiments in this area are warranted. This effect is seen in media with minimal supplementation of 1% BSA (v/v) containing GSDW 0.0087 µg/mL (Figure 4.15) and with Sclerotium Poria Cocos, Radix Rehmanniae, Fructus Corni, and Rhizoma Alismatis (Figure 4.17). Only single wells of the experimental conditions were used in these experiments and due to poor boundaries, the analysis is not very reliable. It depends upon detecting a clear area without cells at an early time point and then repeating this measure with the cells assumed to have

migrated across the gap. In many cases here those boundaries were poor to start with and cell death occurred so they became even less distinct.

The assay was initially performed with 9th passage HUVECs to obtain the high numbers required to produce sufficient replicates but these failed to migrate (data not shown), which has been shown with high passage cells (246). It was not presumed that the GSDW was inhibiting proliferation as cells in media with only 1% BSA without GSDW failed to migrate either. In the subsequent experiments low (4^{th}-6^{th}) passage HUVECs were used, but this meant cell numbers were also low and so replicates were limited.

The assay was inconclusive due to methodological issues, insufficient low passage cells of the HUVECs were obtained, the cells did not adhere well and were not confluent. With higher numbers, it might have been possible to experiment with higher seeding densities, which may have then formed a more confluent layer from which migration could be measured. Lysine coated TC plates were used and it is possible that different well coatings such as collagen, laminin or even a matrigel layer could support adhesion. The cells did not adhere well but due to the serum reduced media were not expected to thrive and proliferate if left for a further 12-24 h. It may be that the cells need to remain in media with serum whilst they form a confluent layer which can be removed and replaced with the serum free rather than serum starved from seeding as is recommended (247).

Despite the poor results obtained with the HUVECs the assay was also performed using the HUtMECs. The cells were low passage, 4th. Cells were on the whole better adhered than the HUVECs and showed reasonably defined boundaries. However, with the exception of the media with commercial supplements all demonstrated the presence of a contaminating agent which was not identified (Figure 4.19). Investigations into the possible contaminant were undertaken including culturing the media, culturing the remaining cells, seeking advice from specialists from images on the contaminant. The herb extracts were all used in both the HUVEC and HUtMEC experiments as they so the source of the infection was either in the cells themselves,

but suppressed in the media with supplements, which does contain antibiotics, or was present in the HUtMEC media without supplements. Only one vial of the HUtMECs were purchased which stopped proliferating and became quiescent at passage 5, which may have been due to the contaminant although this was not seen to appear in the cells or in the reduced supplement media when tested. Due to cost issues and technical problems encountered with the HUtMEC cells, experiments using these cells were not continued further.

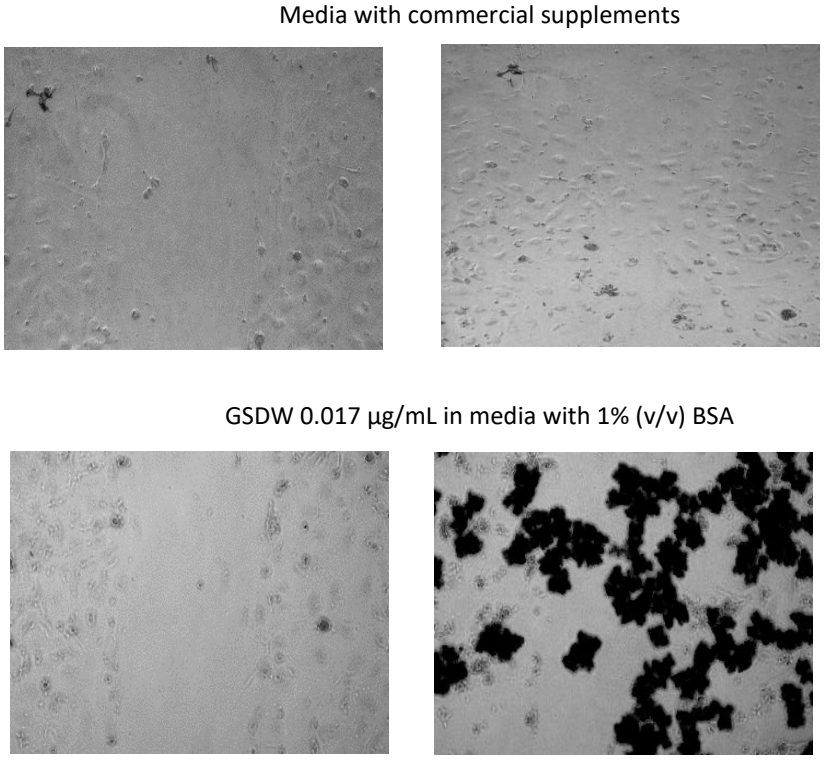


Figure 4.19 HUtMECs showing contaminant first image of each pair is at 1 h, second is at 12 h. The media with commercial supplements shows that the cells were able to thrive and migrate.

There is limited published work in this area however one study has demonstrated that an aqueous extract of Radix Rehmanniae can promote the migration of human mammary endothelial cells (152), which is in keeping with the preliminary observation made here. In contrast, Radix Angelicae Sinensis has been shown to stimulate migration in HUVECs (145), an observation that was not supported in this work. Rhizoma Alismatis and Sclerotium Poria Cocos were surprising herb extracts to show an effect on endothelial cell migration, no research could be found in the

literature than might indicate this type of activity. It also does not correspond with an expected TCM activity.

4.3.4 Conclusions

With this level of information, the H_0 is retained in relation to this assay awaiting further research. There is very limited suggestive evidence that the whole formula at 0.087 μg/mL and Sclerotium Poria Cocos, Radix Rehmanniae, Fructus Corni, and Rhizoma Alismatis might promote endothelial cell migration but insufficient repeats meant that no conclusive evidence is provided. Optimisation of the protocol is required, as are high numbers of low passage cells.

4.4 Angiogenesis: Differentiation

Measurements of HUVEC differentiation into pseudo tubules are modified by Gui Shao Di Huang Wan; HUVECs show greater effects with composite than individual herb extracts

4.4.1 Introduction

Differentiation of endothelial cells occurs in the endometrium during initial repair in the early proliferative phase, in the mid proliferative phase, and during the formation of the spiral arteries during the early secretory phase (79). Rising oestrogen levels in the secretory phase predominantly enhances endometrial cell proliferation but some differentiation does occur. Progesterone in the secretory phase promotes differentiation (84). Subendometrial vascularity is reduced in women with infertility (248) as is uterine perfusion (249). Increased uterine perfusion and vascular permeability enhances nutrient transport to the embryo (250) and facilitates the embryo maternal signalling which is necessary for successful implantation (251). Haemostasis is in part controlled by the endothelium (252). Changes to the uterus leading to greater vascularity and uterine perfusion could improve endometrial receptivity.

HUVECs and HUtMECs had the media with commercial supplements removed (2.4.3, p58) and were suspended in media containing 0.1% (v/v) FBS. They were seeded onto an ibidi µ-slide which has a 10 µL well containing growth factor deficient matrigel. This provides a meniscus free surface for visualising tubule growth. GSDW or the individual herb extracts are added and the cells incubated for 12-18 hours before being photographed. Details of the method are found in Section 2.6.2, p66. The method of analysis required as much protocol development as the assay itself (p200).

4.4.2 Method to assess differentiation

Assessment of angiogenesis according to Staton, Lewis et al. (253) is by four key variables; total tubule length, number of tubules, average tubule length and tubule area (which may increase or decrease with increased angiogenesis). Khoo, Micklem et al. (254) suggest branching points, tubule area and average tubule length. Studies differ as to which are considered the optimum metrics for analysis and some authors even suggest multiple measures need to be taken into account in each analysis. Wang, Pearson at al. (255) in an experiment looking at thrombin showed that it stimulates vascular smooth muscle proliferation and VEGF expression, but reduces numbers of vascular tubules and branching of new vascular structures; they counted number of junctions, number of tubules and tubule length. Rossiter et al. (256) in their study showing that honey promotes angiogenesis, looked at tubule length, branching and density. It is not clear which are the best metrics to select or what these represent *in vivo* and this is discussed further in Section 5.8. The software used for analysis was ImageJ's angiogenesis analyser. It has a vast array of metrics which are output by the analyser (Appendix V). Four metrics including number of tubules, number of junctions, tubule length, and area were chosen for this analysis incorporating the range of metrics discussed in the literature (253-256). These parameters are explained in detail in Figure 4.20.

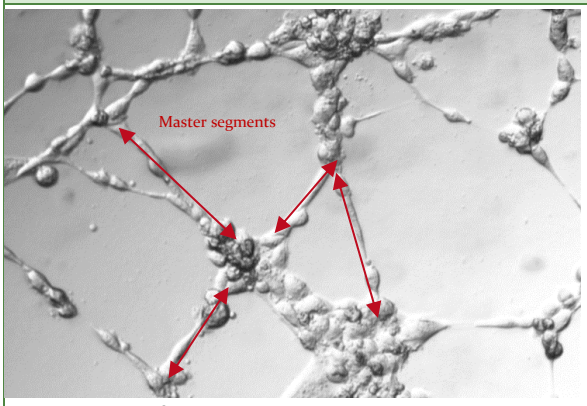
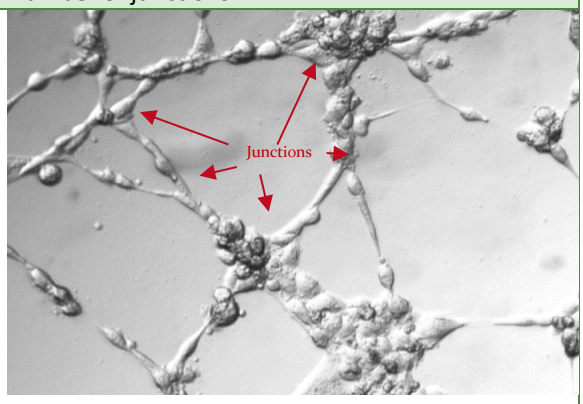
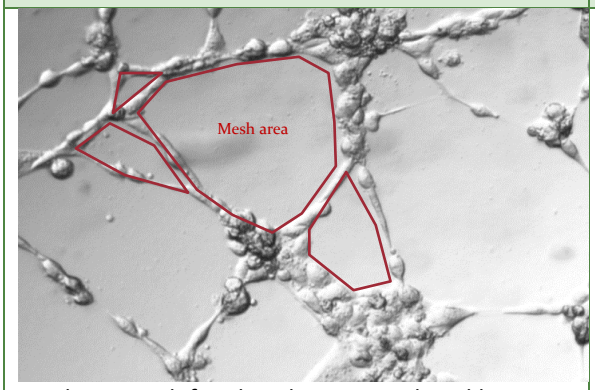
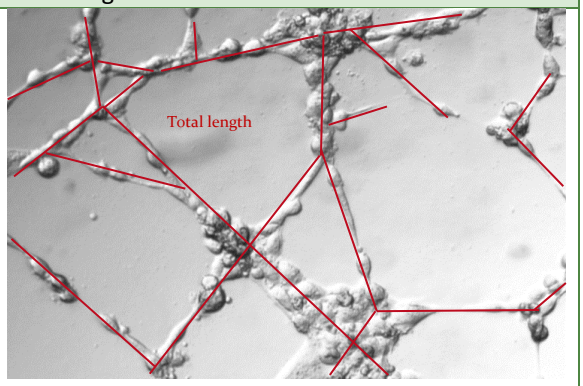
Number of master segments	Number of junctions
	
Segments defined as master segments which are tubules which have junctions at either end.	Junctions are defined by the software as a pixel with three neighbours.
Total mesh area	**Total length**
	
Mesh area is defined as the area enclosed by segments.	Total length is the length of every tubule, segments, branch, and isolated branch whether delimited by a junction or not.

Figure 4.20 Photographs of HUVEC differentiation with overlay and description of the parameters used for assessing the tubule formation.

As endothelial cells adhere to the matrigel basement membrane they begin to differentiate and to form pseudo tubules. This protein matrix emulates the extracellular environment endothelial cells encounter *in vivo*. They grow and branch forming junctions which are considered to mimic the process of forming new blood vessels *in vivo* through sprouting angiogenesis. The following photographs are taken of the network of tubules formed by the cells on the basement membrane at 12 h for the HUVECs, 18 h for the HUVEC (Figure 4.21). These were observed to be the optimal times showing formation of tubules and prior to their disintegration.

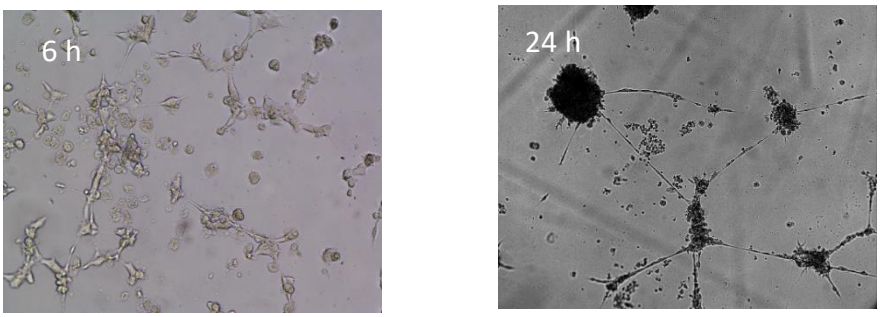


Figure 4.21 HUVEC cell differentiation at 6 h and 24 h
At 6 h the cells are beginning to differentiate and at 24 h they have differentiated but cell death is occurring and the tubules are now beginning to disintegrate. Image taken using a 5X objective.

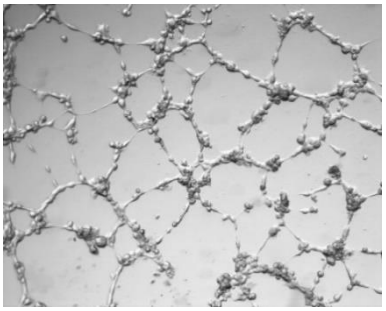
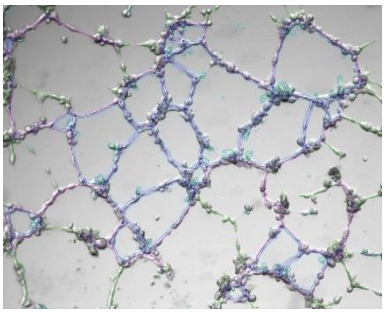
4.4.3 HUVEC Results

The first image in each set of two is the photo taken through the microscope, a Zeiss Axioscope A1 coupled to a camera, Zeiss Axiocam 503 at 5X magnification. The second image is the tubule map created by the ImageJ, from which the tubule metrics are calculated. The experiment was performed in triplicate for each experimental condition in two independent experiments i.e. n=6 for the analysis.

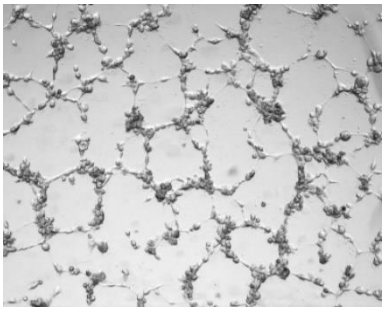
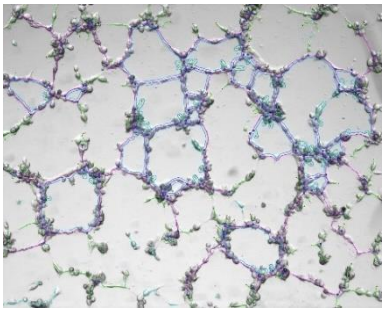
4.4.3.1 Representative photographs of tubule development

A single representative photograph and ImageJ overlay of the tubule analysis from the of the images of six repeats of each condition analysed is shown Figure 4.22.

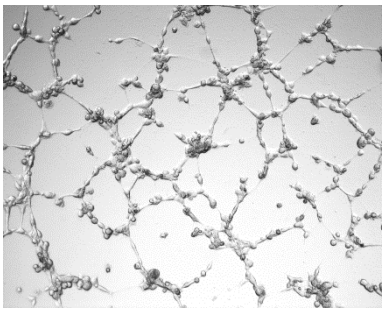
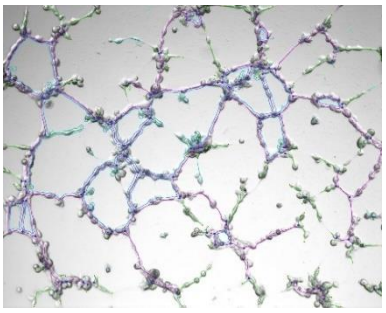
Media with 0.1% (v/v) FBS

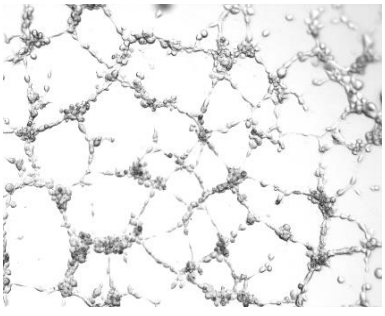
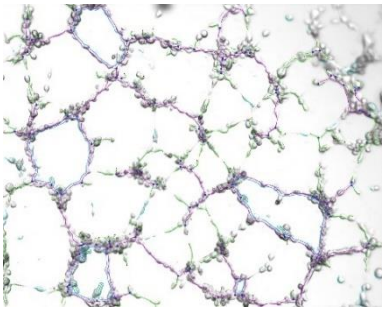
GSDW 0.0434 µg/mL in media with 0.1% (v/v) FBS

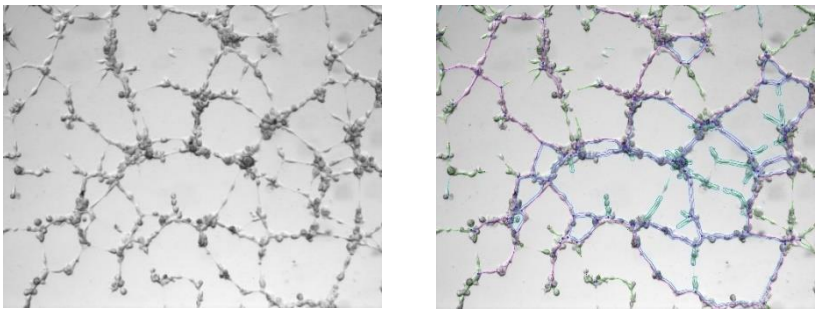
Radix Paeoniae Alba 0.0183 µg/mL in media with 0.1% (v/v) FBS

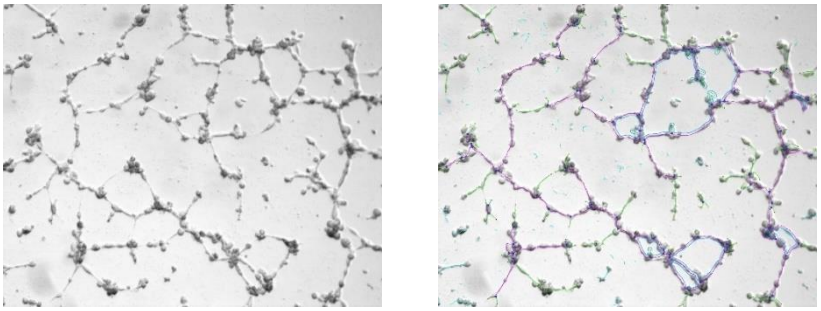
Radix Angelicae Sinensis 0.0202 µg/mL in media with 0.1% (v/v) FBS

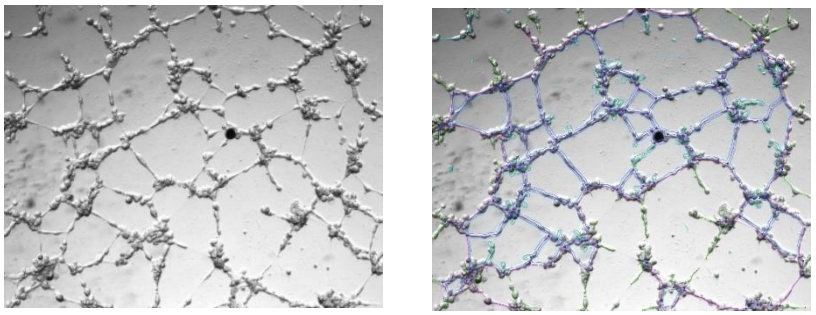
Sclerotium Poria Cocos 0.0011 µg/mL in media with 0.1% (v/v) FBS



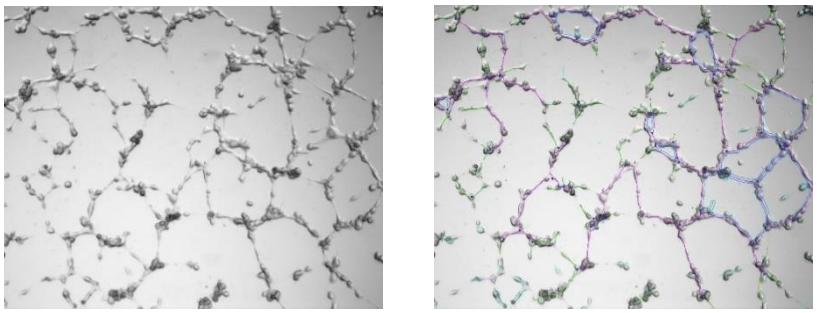
Cortex Moutan 0.0063 µg/mL in media with 0.1% (v/v) FBS



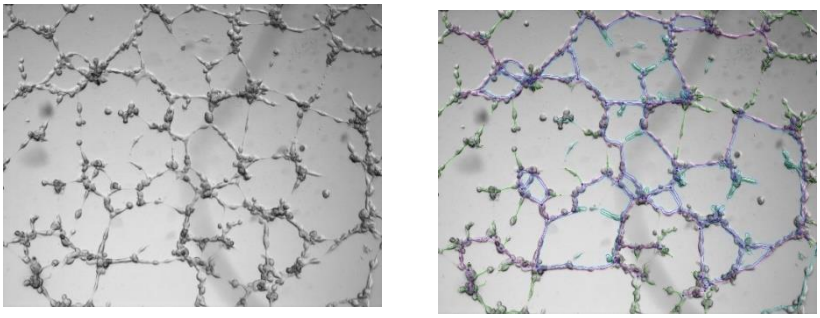
Radix Rehmanniae Preparata 0.0602 µg/mL in media with 0.1% (v/v) FBS



Rhizoma Dioscoreae 0.0088 µg/mL in media with 0.1% (v/v) FBS



Fructus Corni 0.0179 µg/mL in media with 0.1% (v/v) FBS



Rhizoma Alismatis 0.0046 µg/mL in media with 0.1% (v/v) FBS

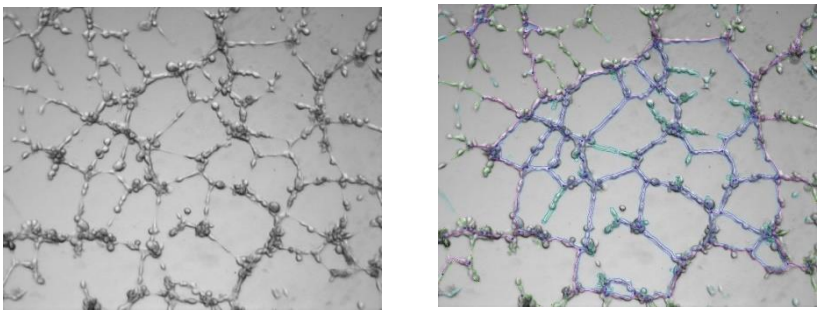


Figure 4.22 Representative photographs of 1 of 6 of the analysed images of HUVEC differentiation after 12 h in the 10 experimental conditions, with and without the ImageJ angiogenesis analyser overlay. Image taken using a 5X objective.

4.4.3.2 HUVEC Parameters

Parameters examined in differentiating HUVECs on GF- matrigel in 0.1% (v/v) FBS media displayed as scatter dot plots are shown in Figure 4.23.

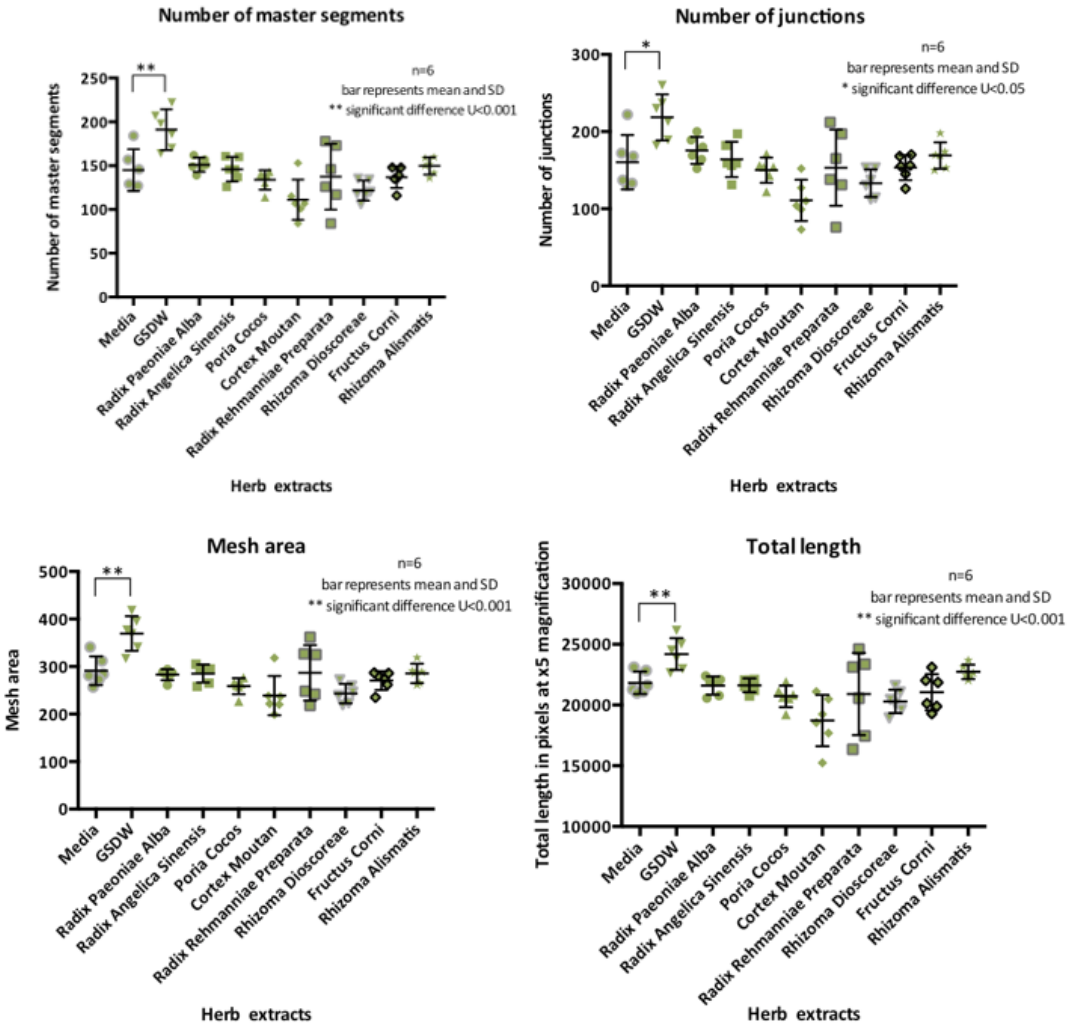


Figure 4.23 HUVEC parameters measured displayed as scatter dot plot with mean and SD.
Details of herb extract mg/mL added can be found in Table 2-8, p67. T-tests showed statistically significant differences between media and GSDW number of segments 46.34±13.00 (t (10) =3.57, p=0.005), d=2.05, number of junctions 58.17±18.83 (t (10) =3.09, p=0.011) d=1.78, mesh area 78.17± 19.32(t (10) =4.05, p=0.002), d=2.33 and total length 653±910.79 (t (10) =3.62, p=0.005), d=2.09. ANOVA performed on the individual herb extracts showed number of segments was significantly different Welch's F (8, 18.63) = 4.82, p=0.002, number of junctions Welch's F (8,18.68) =3.91, p=0.007, mesh area Welch's F (8,18.56) =3.31, p=0.016 and total length Welch's F (8,18.58) =5.151, p<0.002.

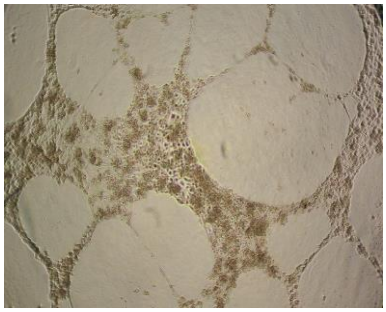
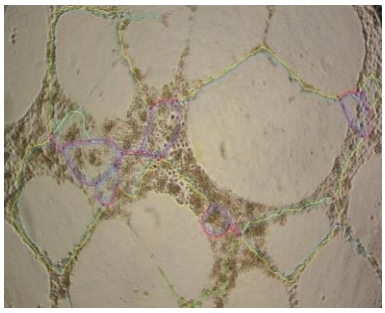
The group means were statistically significantly different ($p < 0.05$), differences do exist in parameters of HUVEC differentiation with the GSDW over media. H1 Gui Shao Di Huang Wan will increase parameters of uterine perfusion and angiogenesis in endothelial cell proliferation, migration, and differentiation is accepted, there are differences between media and GSDW, and H3 the influence of the extract whole formula Gui Shao Di Huang Wan is greater than the individual herb extracts is rejected for this assay as no significant differences were seen between media and any of the individual herb extracts.

4.4.4 HUtMEC Results

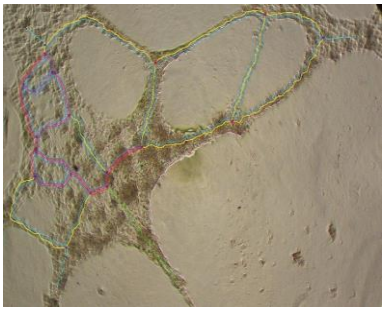
4.4.4.1 Representative photographs of tubule development

The first of each of the following sets of two images are taken through a microscope, Nikon eclipse 80i coupled to a camera, Nikon digital sight DS-Fi2 at 4X. The second image is the tubule map created by the ImageJ, from which the tubule metrics are calculated. This experiment was performed in triplicate for each experimental condition in two experiments i.e. n=6 for the analysis. Images were taken 18 h after the herb extracts had been added as at 12 h the tubules were still forming.

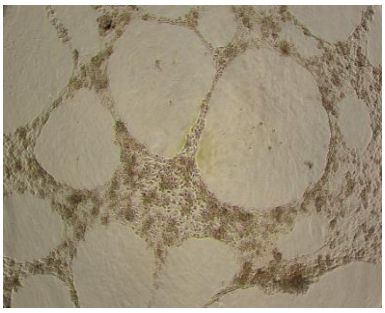
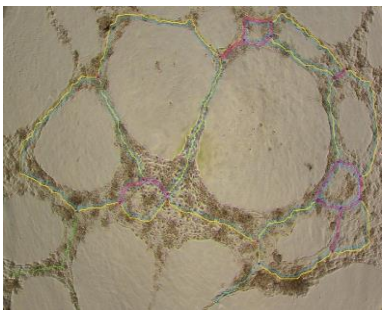
Media with commercial supplements

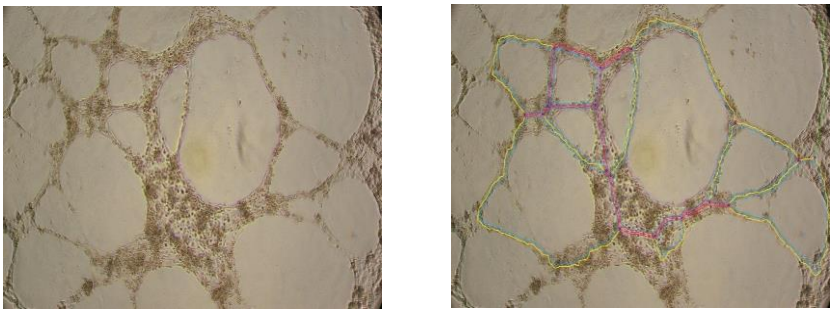
Media with 0.1% (v/v) FBS

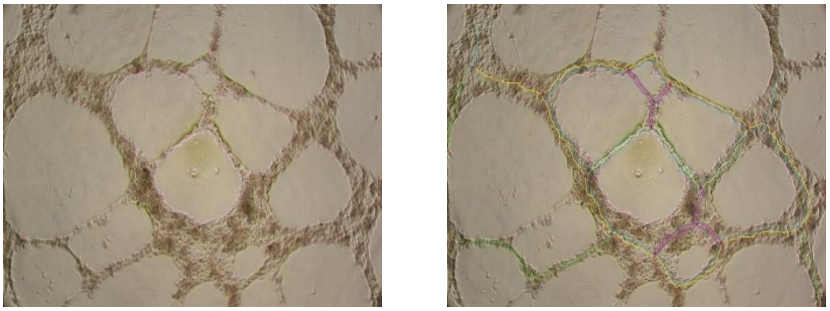
GSDW 0.0173 µg/mL in media with 0.1% (v/v) FBS

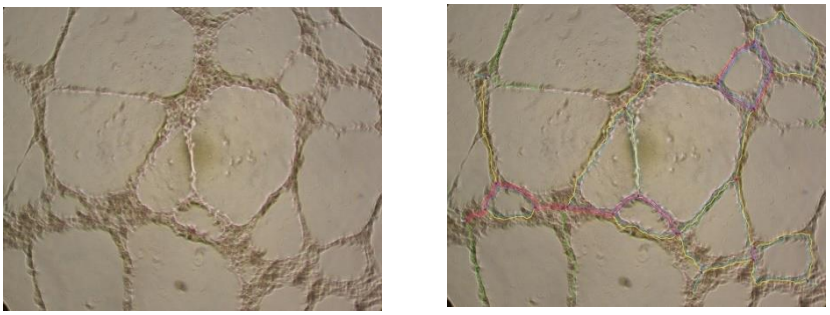
Radix Paeoniae Alba 0.0073 µg/mL in media with 0.1% (v/v) FBS



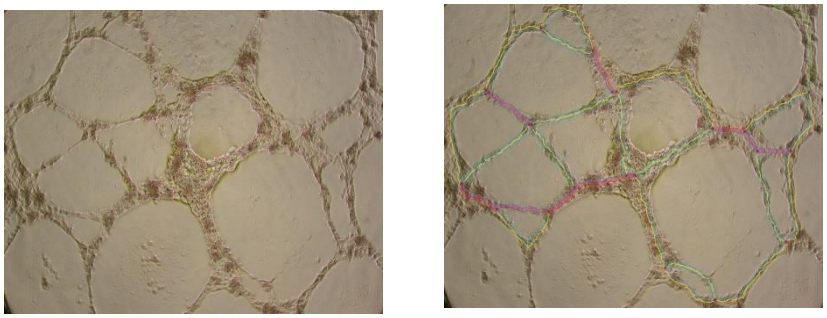
Radix Angelicae Sinensis 0.0081 µg/mL in media with 0.1% (v/v) FBS



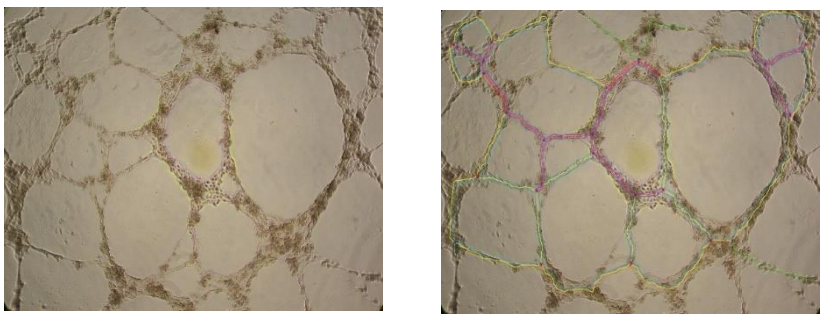
Sclerotium Poria Cocos 0.0004 µg/mL in media with 0.1% (v/v) FBS



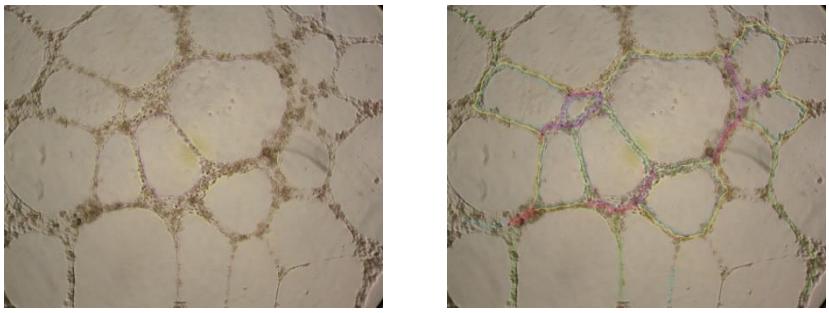
Cortex Moutan 0.0025 µg/mL in media with 0.1% (v/v) FBS



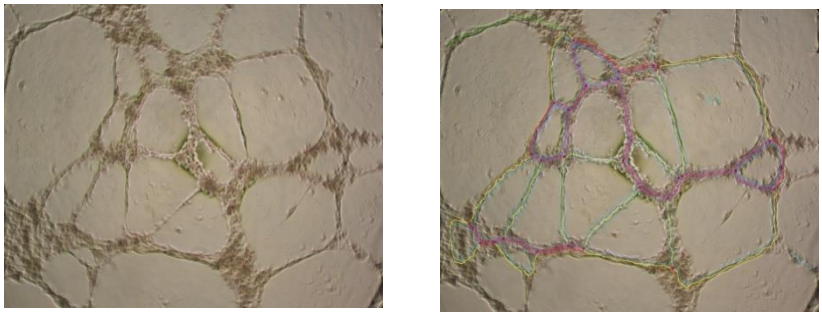
Radix Rehmanniae Preparata 0.0241 µg/mL in media with 0.1% (v/v) FBS



Rhizoma Dioscoreae 0.0035 µg/mL in media with 0.1% (v/v) FBS



Fructus Corni 0.0072 µg/mL in media with 0.1% (v/v) FBS



Rhizoma Alismatis 0.0018 µg/mL in media with 0.1% (v/v) FBS

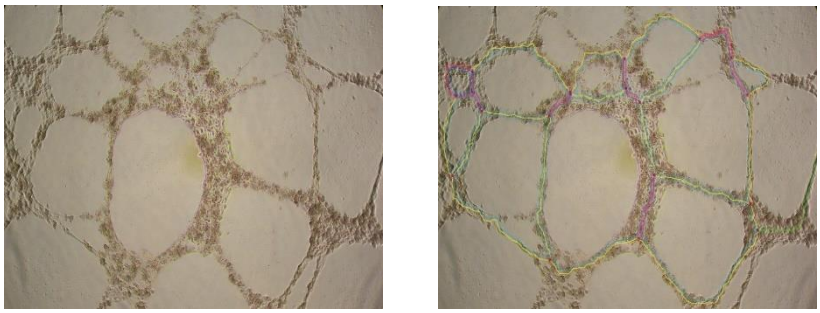


Figure 4.24 Representative photographs of 1 of 6 analysed images of HUtMEC differentiation after 18 h in the 10 experimental conditions with and without the ImageJ angiogenesis analyser overlay. Image taken using a 4X objective.

The HUtMEC cell line produced sufficient cells to run this assay twice and the migration once before becoming quiescent at passage 5. No optimisation of the protocol specific to this cell type was undertaken. The experiment was performed with the herb extracts at a 1:500 dilution in media with 0.1% (v/v) FBS. A more conservative concentration was used as cytotoxicity levels had not been established with this cell type. The experiment was performed with controls of media, with and without supplements.

Parameters examined in differentiating HUtMECs on GF- matrigel in media with 0.1% (v/v) FBS and herb extracts are displayed as scatter dot plots are shown in Figure 4.25.

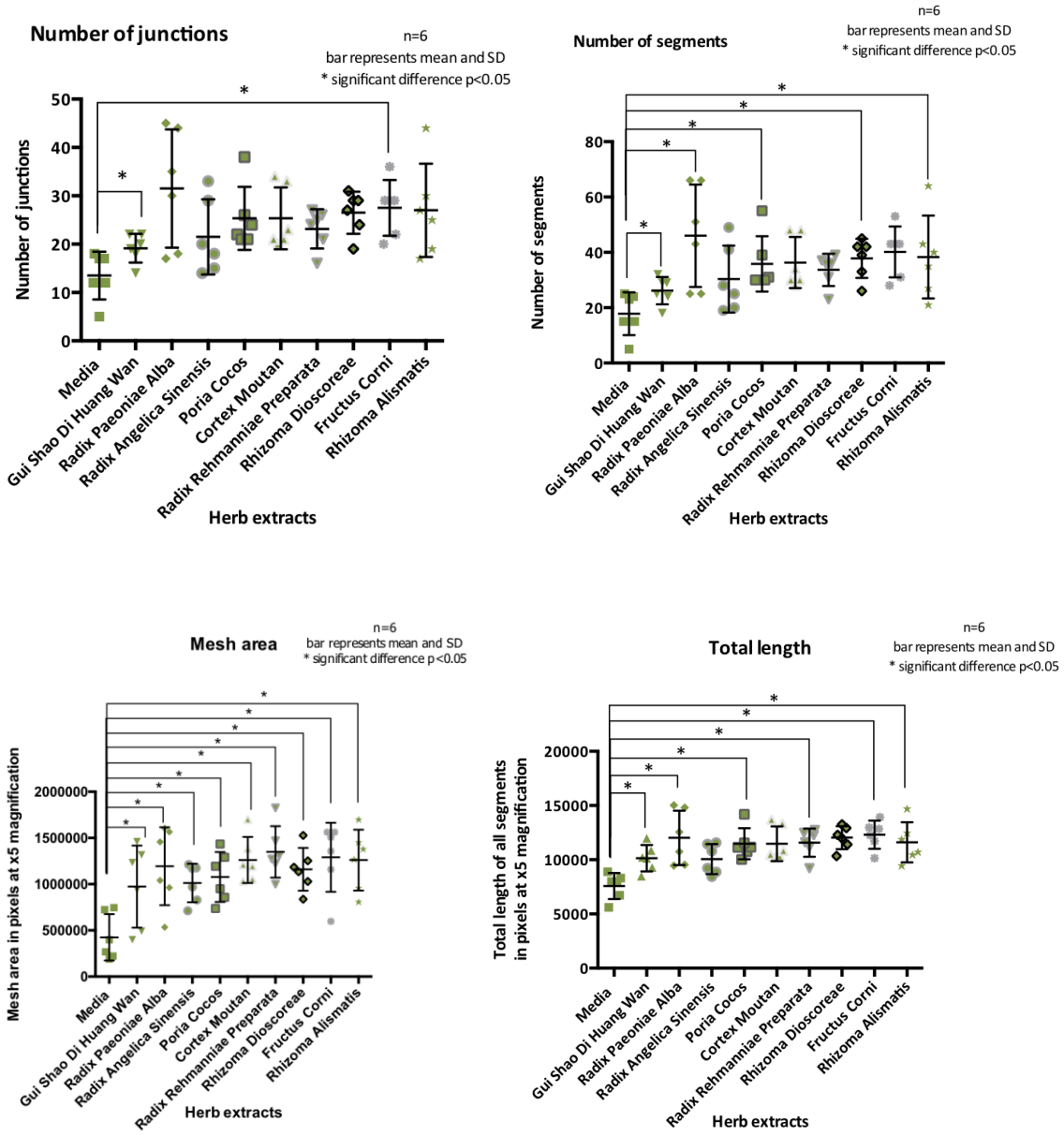


Figure 4.25 HUtMEC parameters measured displayed as scatter dot plot with mean and SD.
Details of herb extract mg/mL added can be found in Table 2-8, p67. T-Tests showed a statistically significant difference between media and GSDW for all parameters. Data presented here as mean±SD and the effects size is Cohen's D; number of segments 4± 1.63 (t (10) =2.46, p=0.034), d=1.42, number of junctions 5.67±2.36 (t (10) = 2.41, p=0.037 d=1.39, mesh area 549304± 208247 (t (10) =3.67, p=0.004), d=1.52 and total length 2562±697 (t (10) =2.67, p=0.004), d=2.12.
An ANOVA was performed to test differences between individual herb extracts, number of segments F (8, 45) = 6.54, p<0.0005, effect size is η2=0.54; number of junction's F (8,45) =3.41, p=0.004, effect size is η2=3.77; mesh area F (8,45) =5.24, p<0.0005, effect size is η2=0.48 and total length F (8,45) =6.48, p<0.0005, effect size is η2=0.54.

Tukey post hoc tests showed where these differences between media and the eight herb extracts were and are displayed in Table 4-6.

Table 4-6 Tukey post hoc tests for HUtMEC differentiation

HUtMEC differentiation parameter	Herb extract	Mean difference	Sig.	95% CI lower	95% CI upper
Number of junctions	Fructus Corni	-14.00	0.049	-27.96	-0.04
Number of segments	Radix Paeoniae Alba	-5.83	0.041	-11.52	-0.14
	Sclerotium Poria Cocos	-6.17	0.025	-11.86	-0.48
	Rhizoma Dioscoreae	-6.50	0.015	-12.19	-0.81
	Rhizoma Alismatis	-6.50	0.015	-12.19	-0.81
Total length	Radix Paeoniae Alba	-4440.33	0.000	-7354.31	-1526.36
	Sclerotium Poria Cocos	-3887.67	0.002	-6801.64	-973.69
	Radix Rehmanniae Preparata	-3991.83	0.002	-6905.81	-1077.86
	Rhizoma Dioscoreae	-4452.17	0.000	-7366.14	-1538.19
	Fructus Corni	-4725.00	0.000	-7638.98	-1811.02
	Rhizoma Alismatis	-4022.33	0.001	-6936.31	-1108.36
Total mesh area	Radix Paeoniae Alba	-779772.17	0.001	-1343355.8	-216188.53
	Radix Angelicae Sinensis	-587414.83	0.035	-1150998.47	-23831.2
	Sclerotium Poria Cocos	-653197.33	0.013	-1216780.97	-89613.7
	Cortex Moutan	-837147.67	0.000	-1400731.3	-273564.03
	Radix Rehmanniae Preparata	-924884.33	0.000	-1488467.97	-361300.7
	Rhizoma Dioscoreae	-736179.00	0.003	-1299762.63	-172595.37
	Fructus Corni	-866539.83	0.000	-1430123.47	-302956.2
	Rhizoma Alismatis	-836029.67	0.001	-1399613.3	-272446.03

The group means were statistically significantly different ($p < 0.05$) so it is shown that differences do exist in all measured parameters of HUtMEC differentiation with the formula GSDW over media only and for some of the individual herb extracts.

4.4.5 Discussion

Four different measures of differentiation were taken but they are not completely independent of one another; higher numbers of junctions and segments will also tend towards higher mesh area for example. All measured parameters of HUVEC differentiation were significantly upregulated by GSDW but none of the individual herb extracts had a significant effect. It may be that calculations were underpowered for an ANOVA with n=6 for each condition and that greater repetition would show more differences. In HUtMECs differences between GSDW and media 0.1% FBS were

significant but here there were also differences in mesh area for all herb extracts, total length for all herb extracts except Radix Angelicae Sinensis, Sclerotium Poria Cocos and Rhizoma Dioscoreae. There was an increase in number of junctions and segments for Fructus Corni. Segment number and length was increased Radix Paeoniae Alba, Sclerotium Poria Cocos and Rhizoma Dioscoreae and Rhizoma Alismatis. H1 Gui Shao Di Huang Wan will increase parameters of uterine perfusion and angiogenesis in endothelial cell proliferation, migration, and differentiation and H3 the influence of the extract whole formula Gui Shao Di Huang Wan is greater than the individual herb extracts can be accepted for this assay.

The HUVECs showed a different pattern of response to the herb extracts to the HUtMECs. A lower concentration of the herb extract was used for the HUtMECs than the HUVECs. This was a precaution because no cytotoxicity data had been established for this cell type. The whole formula GSDW is at a much higher mg/mL as it contains all eight herbs. It seems unlikely that the failure to show differences in the individual herbs for the HUVECs was because concentrations were too high compared with the HUtMECs, where difference were seen. If inhibition of differentiation was occurring it should be more pronounced in the whole formula than for any individual component.

The statistically significant results may not represent the full possible effects of the herb extracts. The variability in measured parameters between wells of the same experimental condition could mask changes that are occurring due to the low number of samples. It is recommended that n≥3, so the use of 6 repetitions is above the minimum recommended (253). In the first year of performing the differentiation assays using media with supplements it was observed that the differences within the experimental conditions were as great as between them. Two wells may have been set up moments apart, with the same cell suspension, media, and matrigel and yet show very different metrics of differentiation (Figure 4.26, p146). Extensive method development was undertaken to reducing this variability but it is still present. Bioassays are known to be prone to high levels of variability, an entire paper

describing research dedicated to reducing this variability were able to reduce it by 85% (257).

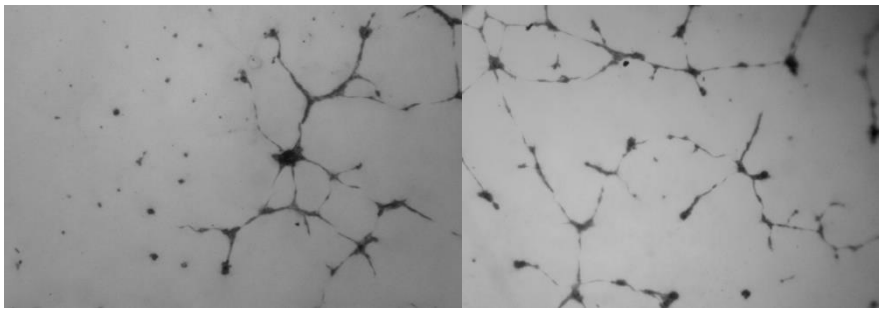


Figure 4.26 Two photographs of HUVEC differentiation after exposure to GSDW in neighbouring wells set up moments apart showing how two wells set up almost identically can produce very dissimilar differentiation metrics. Image taken using a 4X objective.

Measures of tubule differentiation are complex and drawing parallels to their meaning *in utero* is not straightforward. The endometrium contains endothelial cells in a wide range of tissue types which are morphologically dynamic in response to hormonal changes (258). The phenotype of a vessel at the section proximal to the myometrium will be different from that in the functionalis and in the functionalis it will be different in the proliferative and secretory phases. The assay was under normoxic conditions but the endometrium is a hypoxic environment and hypoxia regulates VEGF (259). It is possibly to mimic a hypoxic environment *in vitro* and eliminate one further *in vitro* variable. Oestrogen's influence on angiogenesis does appear to be present under normoxic conditions (260).

Mean tubule length in HUVECs has been previously shown to increase with aqueous Radix Angelicae Sinensis (145) but sprouting was inhibited in mouse aortic ring with the volatile oils of Radix Angelicae Sinensis (146). This herb extract was not seen to increase significantly any parameters of differentiation in either cell type except for mesh area in the HUtMECs which was seen for every herb extract. Quercetin in Cortex Moutan has been shown to be inhibitory to tube formation (150) but no inhibition was observed in the assays conducted in this study. Radix Rehmanniae has been shown to increase tubule length, sprout formation and cell migration (152). In the current study, this herb was seen to increase total length of segments and mesh area in the HUtMECs but not in the HUVECs. The literature would suggest that an aqueous extract of Radix Angelicae might show increase in differentiation but this

was not observed, Radix Rehmanniae would be expected to increase length and junctions however only length increases were observed here. Increased number of segments and length were seen for Radix Paeoniae Alba or Sclerotium Poria Cocos for which no references were found in the literature relating to angiogenesis. Fructus Corni also showed increases in number of junctions in the HUtMECs and it has been shown that the cornel iroid glycosides upregulate VEGF (155), this herb and Radix Paeonia Alba are both peonies and share many of the same compounds including glycosides. Sclerotium Poria Cocos is an interesting herb to upregulate differentiation as most research performed has been in relation to the immune stimulating properties of the polysaccharides (261-263).

There are a wide range of factors that influence differentiation, multiple regulators and growth factors including VEGF, vascular endothelial cadherin, plasminogen activators, angiopoietin's, fibroblast growth factor, and matrix metalloproteinase (130). It is possible that for the HUVECs, individual parts of the whole formula each have a small influence on different aspects of the regulation of the whole process which in combination make a significant difference, this has been shown in the Liu Wei Di Huang Wan (53) and in combinations of some of the herbs in GSDW (54, 56). In the HUtMECs the influence of some of the individual herb extracts appeared to be greater than the whole. Radix Paeonia Alba would appear to have a greater effect on number of segments than GSDW although this was not significant due to the high variance in the number of segments with four of the six images showing far greater numbers and 2 of them very similar numbers; this was also true of junction number. It is possible that there are both pro and anti angiogenic influences within the herbs which would mean that the sum of herbs in GSDW was less than the parts. It is also important to note that no overall downregulation was seen for any individual herb, all increased measures of differentiation.

4.4.6 Conclusion

All parameters of HUVEC and HUtMEC differentiation were significantly increased by GSDW. The HUVEC differentiation parameters were not increased by any of the individual herb extracts. The HUtMEC mesh was increased by all herb extracts, total length, and segment number by Radix Paeoniae Alba, Sclerotium Poria Cocos, Radix Rehmanniae Preparata, Rhizoma Dioscoreae, Fructus Corni, and Rhizoma Alismatis, total length and junction number for Fructus Corni.

4.5 Immunoblotting for oestrogen and progesterone receptor expression

Downregulation of ERα and upregulation PRβ in Ishikawa cells observed following administration of Gui Shao Di Huang Wan

4.5.1 Introduction

The endometrium becomes receptive to implantation due to controlling influences of oestrogen and progesterone receptors (264). Oestrogen acts through uterine and stromal ERα with some involvement of ERβ. Oestrogen increases epithelial cell proliferation and differentiation through stromal ERα and both stromal and epithelial ERβ (265). Progesterone receptors work with oestrogen to promote stromal cell proliferation and inhibit oestrogen induced epithelial cells proliferation (266). Progesterone promotes differentiation post ovulation and decidualisation post implantation (168). It is also responsible for increased vascular permeability pre implantation (167) and modulating uterine perfusion during pregnancy (169).

The Ishikawa epithelial cell line was chosen to examine the effects of the herb extracts on expression of receptor proteins using immunoblotting. Ishikawa cells are clones of an human endometrial adenocarcinoma. This is a cancer originating in the epithelial glandular structures. They have been shown to express both oestrogen and progesterone receptors (199) making them a valuable cell line for the study of hormonally regulated processes in the endometrial epithelium (267).

These cells were exposed to GSDW and the herb extracts for 24 and 48 hours and the lysates of the cells in each of these experimental conditions were then probed using immunoblotting for changes to the ovarian steroid receptors ERα, ERβ, mPRα (which was not detected, no data is presented), and PRβ. The full methodology is presented in Section 2.8, p70.

4.5.2 Method of analysis

For each image auto exposure was used to obtain optimal saturation for the images. Lanes were manually configured and bands typically were autodetected successfully. Images of the ladders were also taken and merged images were used to verify molecular weights for all blots (Figure 4.27). A custom standard was set up in ImageLab for the Ultraprism ladder. ImageLab subtracts background automatically using a rolling disc background subtraction which can be checked and amended as required, automated settings were always found to be acceptable.

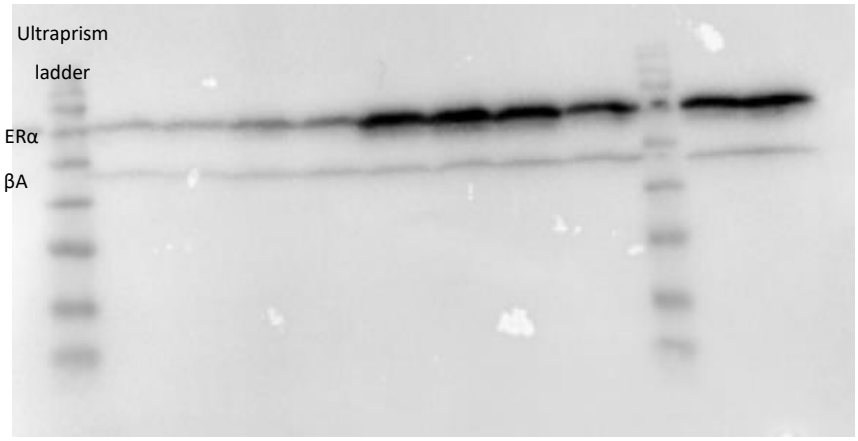


Figure 4.27 shows a ERα 48 hour blot with ladder merged with Beta Actin (βA) and the Ultraprism ladder. This was completed for each image to verify that the bands detected are close to the expected molecular weight.

Accurate quantitation of proteins requires that the signal intensity is proportional to the volume loaded. This can be verified using a serial dilution of the lysate. The ChemiDoc camera has a larger linear range than traditional film based detection and the auto expose in the ImageLab software detects the optimal exposure without saturated pixels.

Intensities of the bands are a measure of the abundance of the protein and are obtained by the software from captured images and exported to an Excel spreadsheet. Data was analysed quantitatively in three ways:

1. Relative abundance compared to media only control. Relative intensities are also calculated by the software following user selection of a reference band in this case the media only condition for the Ishikawa cell lysates.
2. Ratio/normalisation to β actin loading control. The normalisation to the β actin loading control was performed as described in Equation 2 and Equation 3.

Equation 2 calculate normalisation ratio

$$\frac{intensity\ of\ band\ of\ protein\ of\ interest\ for\ media}{intensity\ of\ band\ of\ loading\ control\ for\ media} = normalisation\ ratio$$

And applying the normalisation ratio to all subsequent lanes as in Equation 3

Equation 3 normalise all lanes

$$\frac{intensity\ of\ band\ of\ protein\ of\ interest}{intensity\ of\ band\ of\ loading\ control} * normalisation\ ratio = normalised\ intensity$$

3. Mean absolute values/volumes of intensity. Presentation of the mean absolute volumes of intensity enables concurrent changes in receptor and loading control band intensities to be evaluated and compared for each test condition.

Due to the use of relative not absolute values as the key variable for comparison, i.e. that of the relative abundance to media only control, and normalisation to the βA loading control, standard statistical analyses could not be performed. No variance exists in the data to which the other samples have been normalised therefore an ANOVA is not appropriate. Absolute volumes were analysed but again the statistics were not appropriate given the high variability between blots.

Displayed in the following results are three blots for each of the three receptors at 24 and 48 hours of exposure to the herb extracts. N=3 for each condition, each blot was performed on lysates originating from different wells, they are not three repeats on the same lysate. The individual blots in each Figure are numbered, for example, in Figure 4.26 they are ERα 24-1, ERα 24-2, ERα 24-3 respectively.

4.5.3 Results

4.5.3.1 Oestrogen Receptor alpha (ERα) expression in Ishikawa cells after 24 hours incubated with the herb extracts

The blots in Figure 4.28 show ERα and βA in three separate lysates prepared from Ishikawa cells after 24 h exposure to the herb extracts where the proteins have been transferred to a PVDF membrane. The first probe was for ERα, then the membrane was stripped using a guanidine hydrochloride (GnHCL) buffer (Appendix VII) and a second probe was performed for βA. ERα purified protein as the positive control was detected in the blots and at the same kDa as the proteins in the samples.

In the blot ERα 24-1 (Figure 4.28) increases in the intensity of the ERα bands are observed following treatment with Radix Paeonia Alba and Rhizoma Alismatis. The βA band is more intense in the media only, Radix Angelicae Sinensis, Sclerotium Poria Cocos and Rhizoma Alismatis treated samples. This would suggest the increases in ERα levels are not due to variability in loading as, a higher ERα with correspondingly higher βA would be expected if protein levels were higher in a particular lane. For the blot ERα 24-2, ERα levels are increased in GSDW, Sclerotium Poria Cocos and Rhizoma Alismatis treated samples. ERα expression is increased in Rhizoma Alismatis treated samples in blots ERα 24-1 and 2, but not in blot ERα 24-3. βA bands are more intense in Radix Paeonia Alba and Sclerotium Poria Cocos treated samples in blot ERα 24-2. In blot ERα 24-3, Sclerotium Poria Cocos and Fructus Corni treated samples produce more intense ERα bands but for βA, intensity is greatest in GSDW and Radix Angelicae Sinensis treated samples. Thus, from a visual inspection of the blot, it appears that no consistent patterns in protein expression emerge. Figure 4.29 shows the results of the quantitative analysis of the blots.

Oestrogen receptor alpha 24 hours

Full blots can be seen in the appendices in Figure 8.1, Figure 8.2, Figure 8.3, Figure 8.4, and Figure 8.5. ERα was detected at ~68kDa which is close to the expected 66kDa in the manufacturers data sheet.

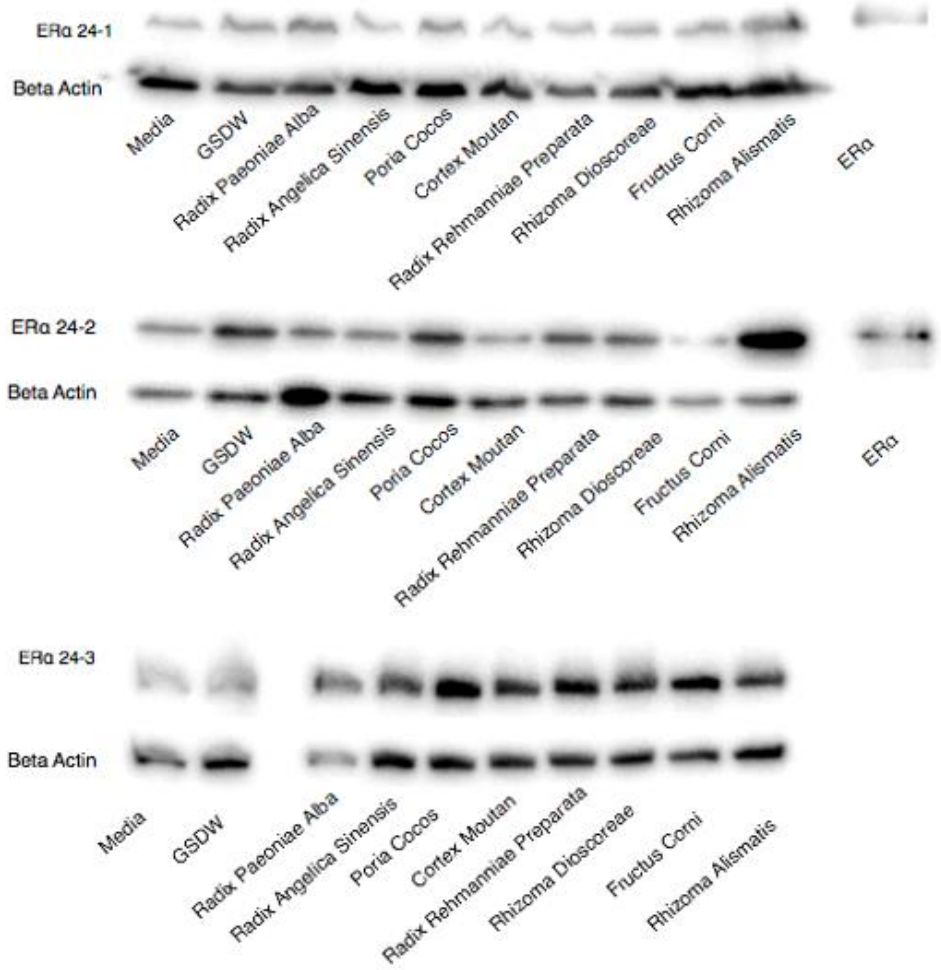


Figure 4.28 Oestrogen receptor alpha expression from lysates of three different wells of Ishikawa cells that have been exposed to herb extracts for 24 h showing few consistent changes in receptor expression between the blots.

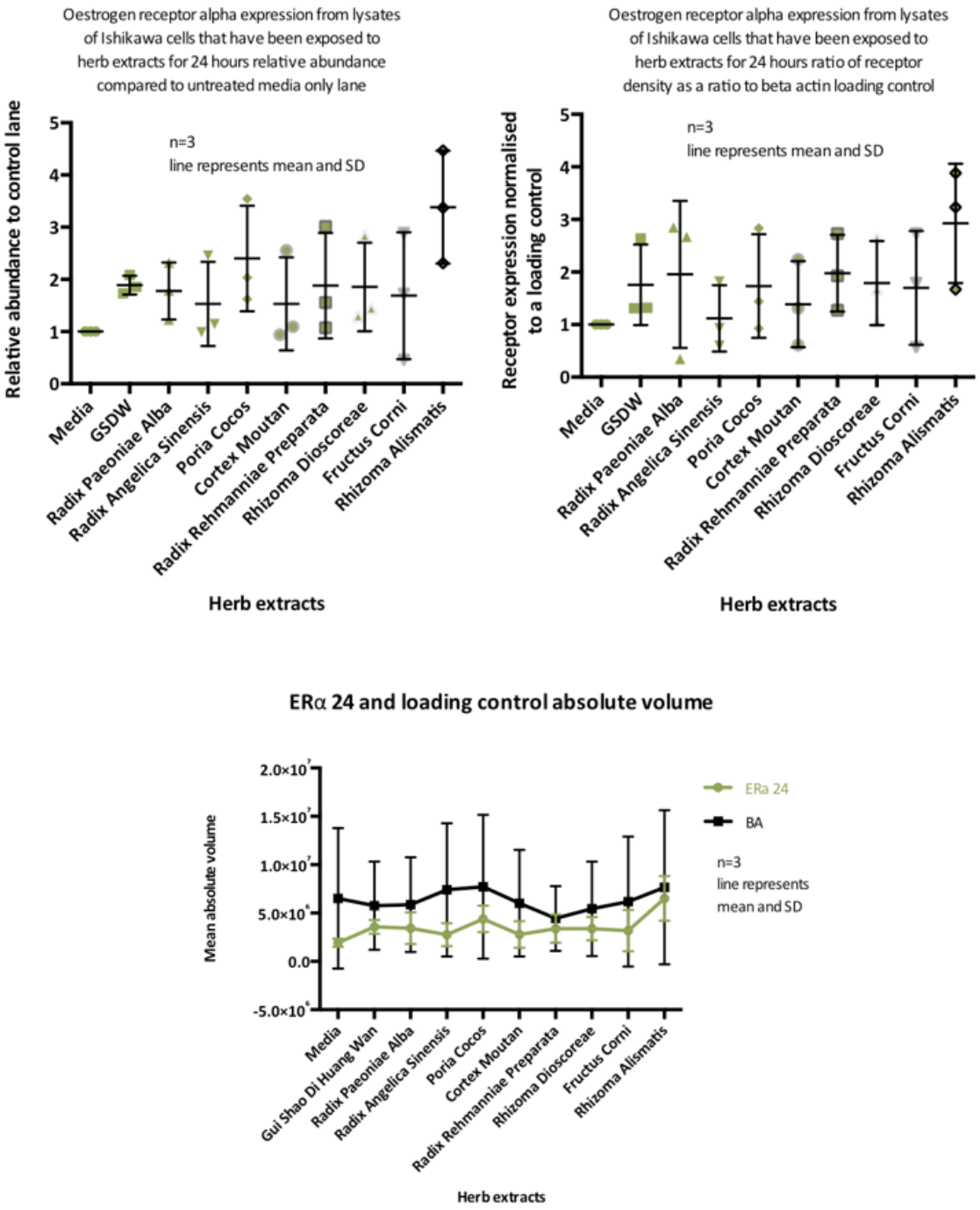


Figure 4.29 Oestrogen receptor alpha expression at 24 h
Displayed as relative abundance compared to a media control lane, ERα normalised to βA loading control and absolute volumes for ERα and βA. The first two graphs show that at 24 h ERα appears upregulated and the in absolute volume ERα is more consistently higher and BA lower than in other bands.

In the both the graphs showing relative abundance to media only control and normalisation to βA loading control (Figure 4.29) it would appear there is upregulation of ERα at 24 h following treatment with GSDW. In the analysis of relative abundance to media only control, it appears that Sclerotium Poria Cocos and Rhizoma Alismatis both upregulate ERα but this is less consistent when normalised to the βA loading control. When analysing the data according to the mean absolute volumes, again there is no evidence of consistent upregulation of ERα. The mean absolute volumes for βA are reasonably consistent but the variance between the individual data points is much higher than for the ERα suggesting that βA is not functioning as an effective loading control here.

4.5.3.2 Oestrogen Receptor alpha (ERα) expression in Ishikawa cells after 48 hours incubated with the herb extracts

The blots presented in Figure 4.30 show ERα and βA protein in three separate Ishikawa cell lysates prepared after 48 h exposure to the herb extracts where the proteins have been transferred to a PVDF membrane. The first probe was for ERα then the membrane was re-probed without stripping for βA. Quantitative analysis of the blots are presented in Figure 4.31

The blots in Figure 4.30, ERα 48-1, 2 and 3 look similar to a blot where stripping is poor (Figure 4.42, p179) but these blots were not stripped before re-probing. In all blots, the intensity of bands in the media only samples are higher for both ERα and βA than for the lysates obtained following treatment with GSDW, Radix Paeonia Alba, and Radix Angelicae Sinensis. For samples treated with Rhizoma Dioscoreae and Fructus Corni, the ERα bands are more intense on all three blots. Blot ERα 48-3 does not show the same high intensity ERα band for Sclerotium Poria Cocos treated samples that is seen in the other two blots. There does appear to be some downregulation of the ERα in particular in response to the Blood nourishing herbs Radix Paeonia Alba, Radix Angelicae Sinensis, Cortex Moutan and Radix Rehmanniae. The variance in ERα 48 and βA for several of the herbs would, in a single blot appear to be due to errors of loading but seen in three separate blots is more likely to indicate a variation in the levels of the detected proteins.

Oestrogen receptor alpha 48 hours

Full blots can be found in the appendices in Figure 8.6, Figure 8.7,Figure 8.8, and Figure 8.9. ERα was detected at ~68kDa which is close to the expected 66kDa in the manufacturers data sheet.

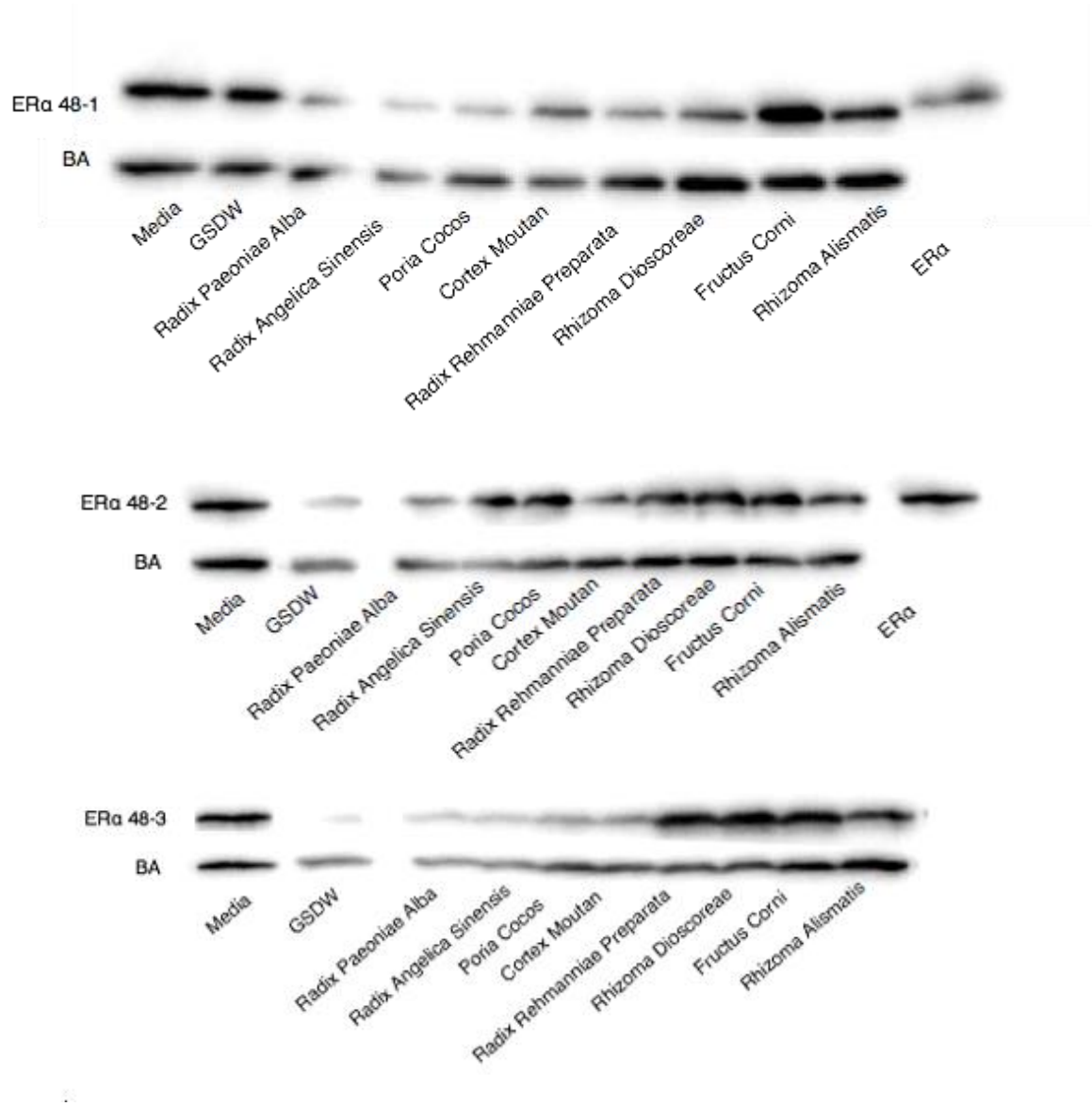


Figure 4.30 Oestrogen receptor alpha expression from lysates of three different wells of Ishikawa cells that have been exposed to herb extracts for 48 h. There is marked downregulation in all three blots of ERα at 48 h for GSDW, Radix Paeoniae Alba, Radix Angelicae Sinensis, Cortex Moutan, and Radix Rehmanniae and predominantly correspondingly lower βA.

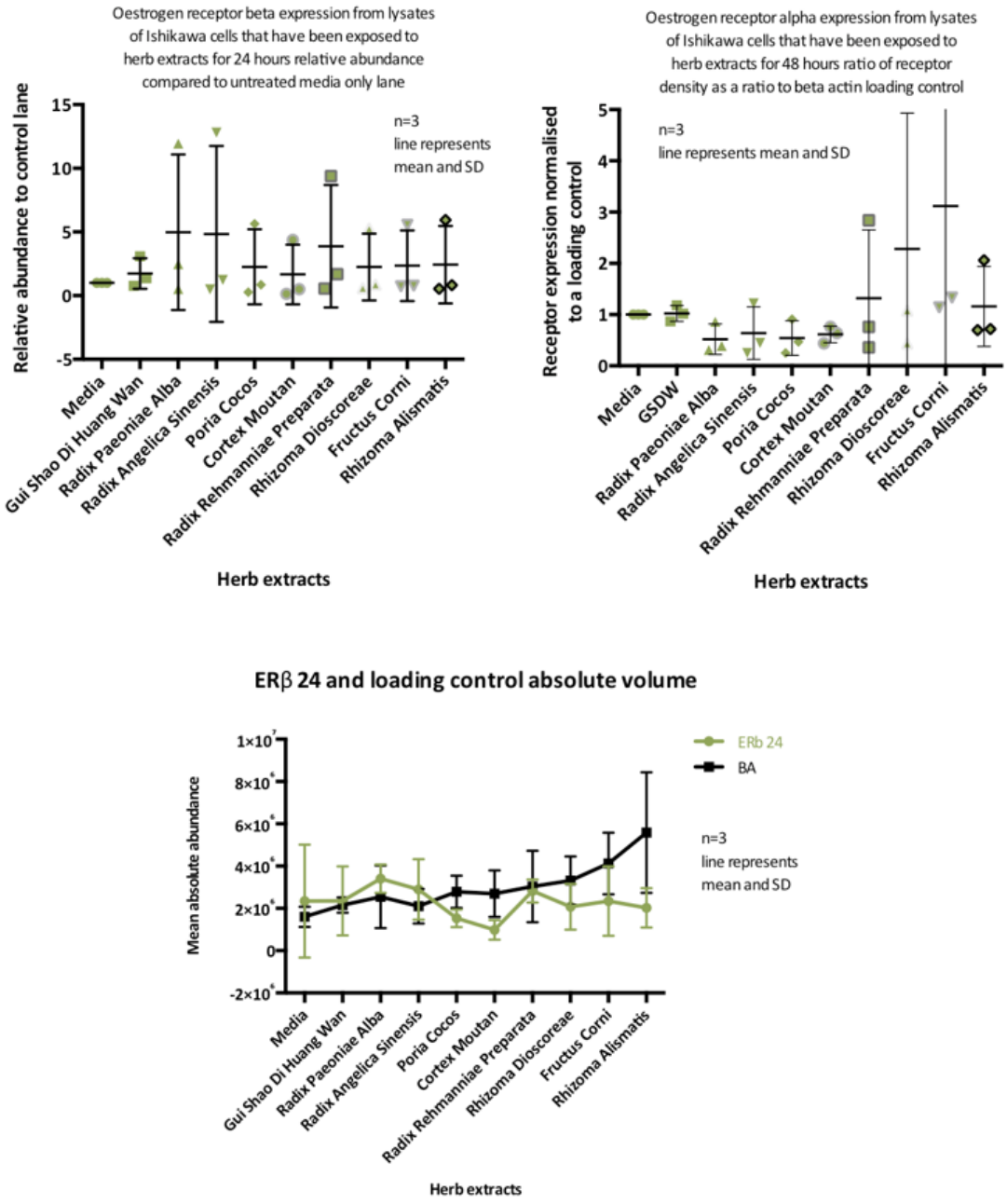


Figure 4.31 Oestrogen receptor alpha expression at 48 h
Displayed as relative abundance compared to a media control lane, ERα normalised to βA loading control and absolute volumes for ERα and βA. Downregulation of ERα is consistent in all three graphs for Radix Angelicae Sinensis, Radix Paeoniae Alba, Sclerotium Poria Cocos, Cortex Moutan, and Radix Rehmanniae.

At 48 h, the pattern appears to be of downregulation of ERα as shown in both the analyses of relative abundance to media only control and normalisation ratio to βA loading control (Figure 4.31). Fructus Corni treatment appears not to have downregulated ERα in the relative abundance to media only control analysis, unlike all of the other herb extracts. However, variation of ERα expression is high when normalised to the βA loading control, and the absolute volumes would also suggest that Fructus Corni is not affecting the expression of ERα since βA remains consistently lower in absolute volume. The results of ERα expression when normalised to βA loading control also become highly variable for Radix Rehmanniae, Rhizoma Dioscoreae and Fructus Corni treated samples. The absolute volumes show the same pattern of overall downregulation of ERα at 48 h and particularly following treatment with Radix Paeoniae Alba, Radix Angelicae Sinensis, Cortex Moutan and Radix Rehmanniae.

4.5.3.3 Oestrogen Receptor beta (ERβ) expression in Ishikawa cells after 24 hours incubated with the herb extracts

The blots (ERβ 24-1, -2 and -3) in Figure 4.32 show protein expression in three separate Ishikawa cell lysates prepared after 24 h exposure to the herb extracts where the proteins have been transferred to a PVDF membrane. The first probe was for ERβ then the membrane was stripped using a GnHCL buffer (Appendix VII) then a second probe for βA was used. Quantitative analyses of the blots are presented in Figure 4.33.

Visual examination of the blots shows that, on blot ERβ 24-1, a very faint ERβ band in the media only sample is seen which is dissimilar to the other two blots where the band intensity of ERβ in this sample is more intense. This may be due to an error in loading, however the intensity of the βA band in ERβ 24-1 is not dissimilar to that seen in blot ERβ 24-2 for the media only sample. This will lead to an error in the quantitative analysis of the blots by relative abundance to the media only control and normalisation ratio to βA loading control. Blots ERβ 24-1 and 2 both show intense ERβ bands for Radix Angelicae Sinensis treated samples but in Blot ERβ 24-3 the opposite is true, there is an intense band for βA and a faint band for ERβ for the Radix Angelicae Sinensis treated band and for Radix Rehmanniae treated samples the ERβ signal is more intense, but βA intensity is very variable between the three blots. No consistent pattern of expression is seen across the blots for both media only or GSDW treated lysates.

The ERβ purified receptor protein (positive control) was not detected on any blot despite using two different ERβ antibodies (ab3577 and ab133467). The band at 55~59kDa was detected in all samples and with both antibodies so it is considered to be the ERβ receptor that is being detected.

Oestrogen receptor beta 24 hours

Full blots can be seen in the appendices in Figure 8.11, Figure 8.12, Figure 8.13, Figure 8.14, and Figure 8.15. ERβ was detected at ~62 kDa which is close to the expected 55~59 kDa in the manufacturers data sheet.

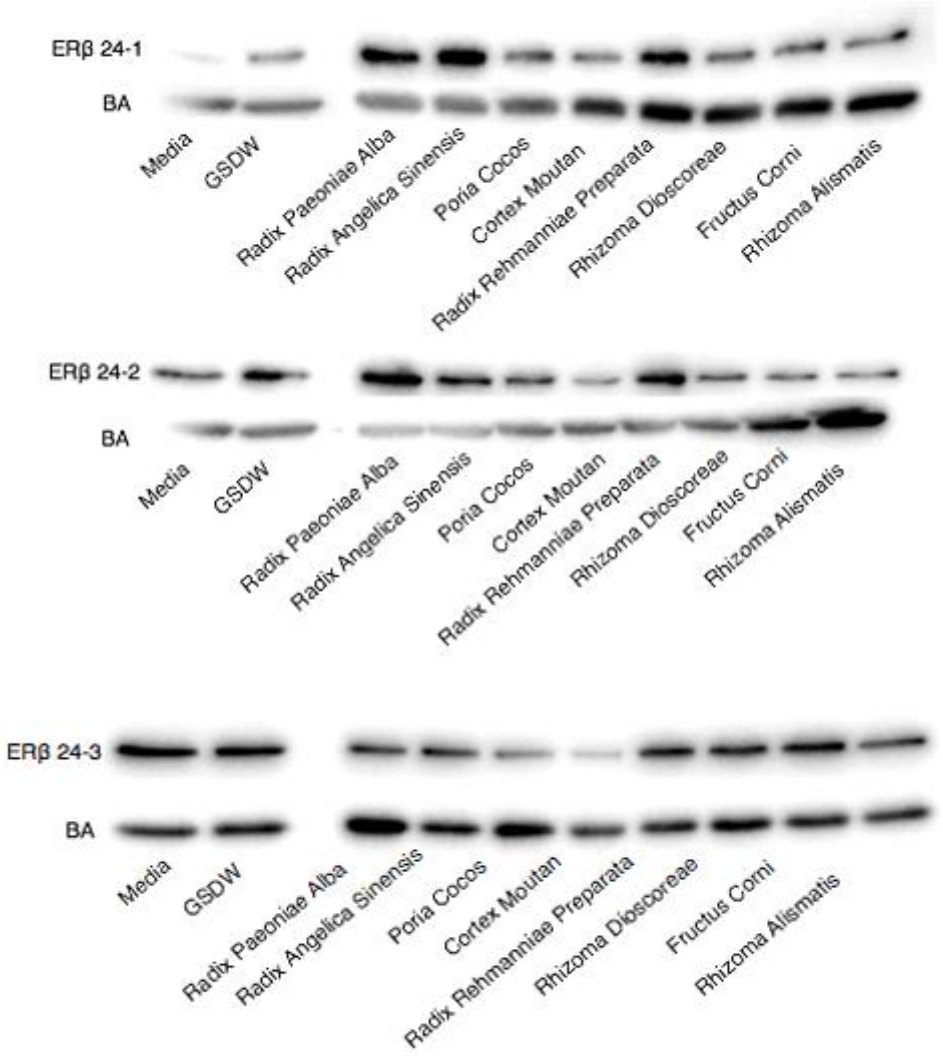


Figure 4.32 Oestrogen receptor beta (ab3577) expression from lysates of three different wells of Ishikawa cells that have been exposed to herb extracts for 24 h.
No consistent changes are seen between experimental conditions across the three blots.

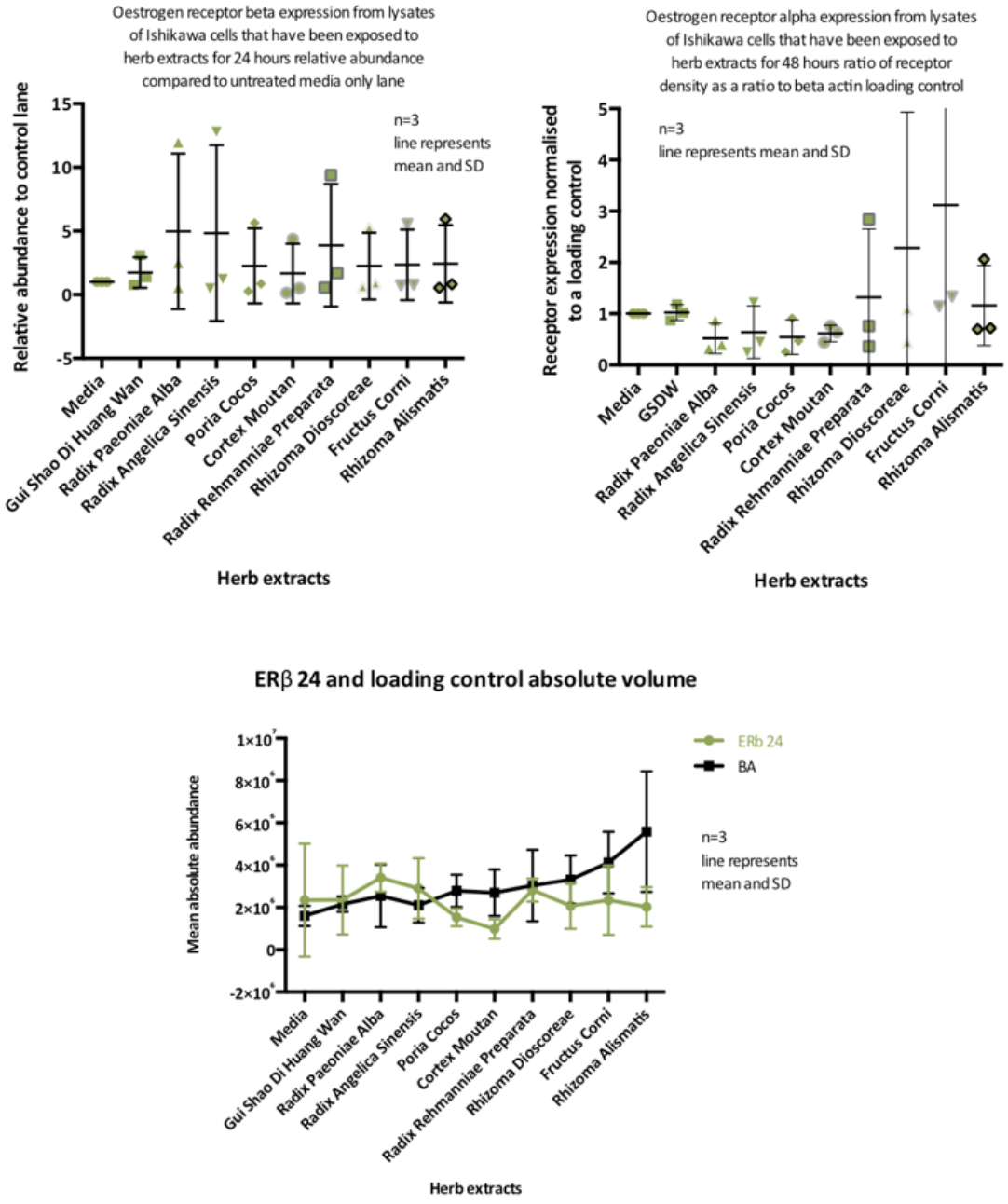


Figure 4.33 Oestrogen receptor beta expression at 24 h
Displayed as relative abundance compared to a media control lane, ERβ normalised to βA loading control and absolute volumes for ERβ and βA. Changes between experimental conditions are not consistent.

Changes in ERβ expression in Ishikawa cells following treatment with herb extracts are small and inconsistent at 24 h. This is the cases for all three analyses, relative abundance to media only control, the normalised ration to βA loading control, and the absolute volumes (Figure 4.33). Observations of the less intense ERβ band on the blots for Cortex Moutan treated lysates are also seen in the ERβ reduced absolute volume, but this is not observed in the relative abundance to media only control or normalised ratio to βA loading control plots. The intense ERβ bands for Radix Rehmanniae treated samples do show as one of the higher points in the plot of mean absolute volumes but again this is not seen in the other two plots. As previously discussed the variance in band intensity in the media only lane for all three blots means that neither of these methods of normalising can be considered to be accurate. Absolute volumes of βA increase across the blot for ERβ 24-1 and -2 but not for -3 and so the variability of the absolute volume measurements becomes very high. Thus, little can be deduced with any certainty from the analyses of these blots.

4.5.3.4 Oestrogen Receptor beta (ERβ) expression in Ishikawa cells after 48 hours incubated with the herb extracts

The blots ERβ 48-1, -2, and – 3 (Figure 4.34) show ERβ and βA expression in three separate lysates prepared after 48 h exposure of Ishikawa cells to the herb extracts, where the proteins have been transferred to a PVDF membrane. The first probe used was for ERβ, then the membrane was stripped using a GnHCL buffer (Appendix VII) and a second probe for βA applied. Quantitative analyses of the blots by three methods are presented in Figure 4.35.

Visual examination of the blots in Figure 4.34 show few consistent changes in protein expression following treatment. ERβ 48-1 and 2 are more alike with fainter bands for ERβ observed in samples following treatment with Radix Rehmanniae, Rhizoma Dioscoreae, Fructus Corni, and Rhizoma Alismatis. Blot ERβ 48-3 has a faint ERβ band for samples treated with Fructus Corni but not for the other three herbs. The βA band in blots ERβ 48-1 and 2 is relatively consistent in intensity across the blots. Blot ERβ48-1 shows a faint βA band for Cortex Moutan treated samples and blot ERβ 48-3 shows quite variable intensity in βA bands.

Oestrogen receptor beta 48 hours

Full blots can be seen in the appendices in Figure 8.10, Figure 8.11, Figure 8.12, Figure 8.13, Figure 8.14, and Figure 8.15. ERβ was detected at ~62 kDa which is close to the expected 55~59 kDa in the manufacturers data sheet.

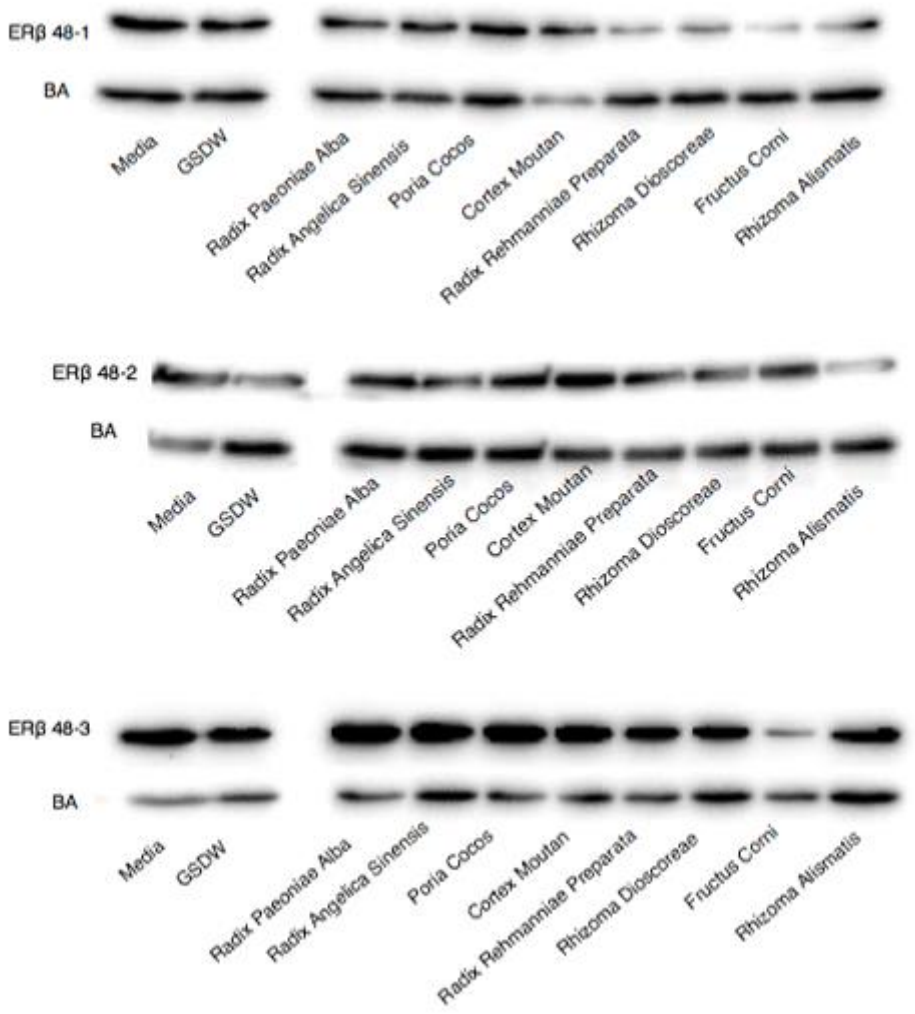
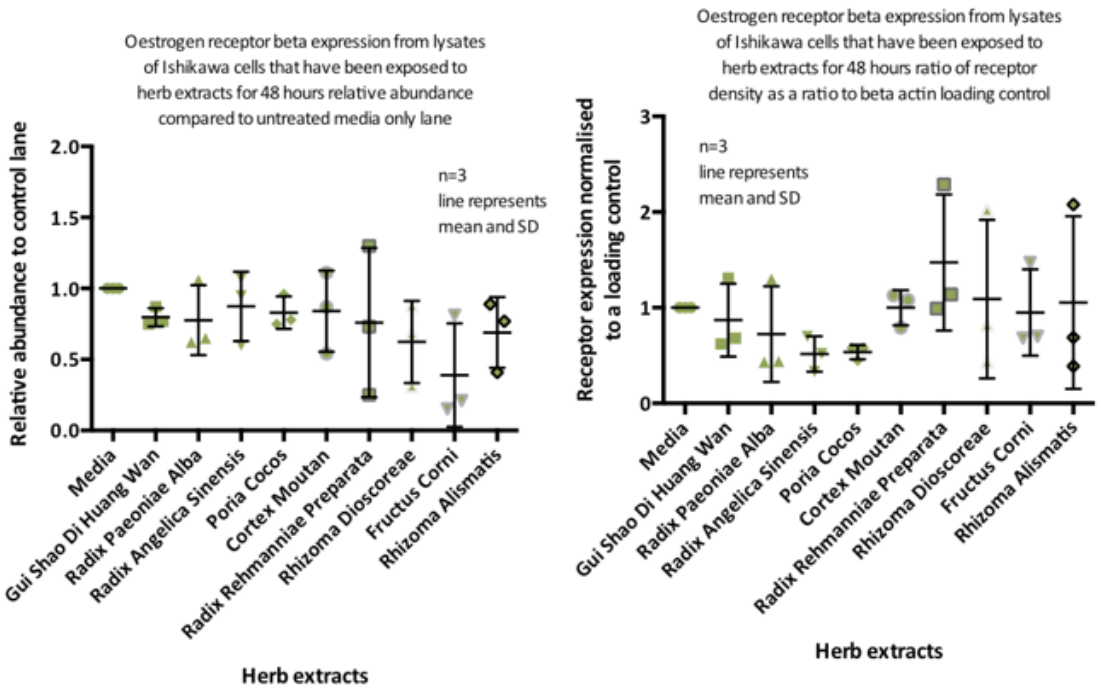


Figure 4.34 Oestrogen receptor beta expression from lysates of three different wells of Ishikawa cells that have been exposed to herb extracts for 48 h. Changes observed are not consistent between experimental conditions across the three blots.



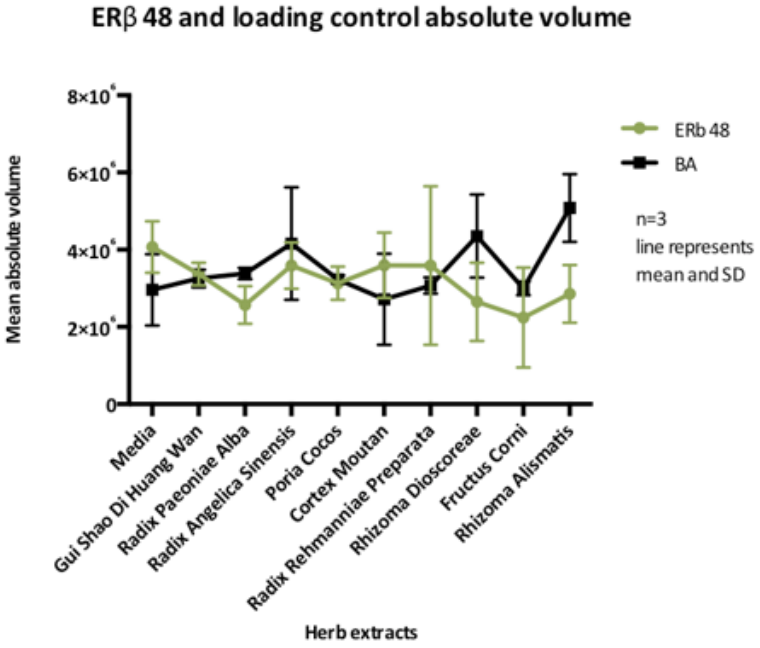


Figure 4.35 Oestrogen receptor beta expression at 48 h
Displayed as relative abundance compared to a media control lane, ERβ normalised to βA loading control and absolute volumes for ERβ and βA. Absolute volumes would suggest a downregulation of ERβ at 48 h but the high variability seen in the βA loading control that does not parallel the changes of ERβ makes it difficult to draw any conclusions.

There are inconsistencies in the quantitative analyses of the three blots (Figure 4.35) but there is a possible slight downregulation of ERβ following Radix Paeoniae Alba and Radix Angelicae Sinensis treatment. In both the analysis of relative abundance to media only control and the normalised ratio to βA loading control, the same patterns of expression are seen, but there are differences in the blot ERβ 48-3 compared to blots ERβ 48-1 and -2 and this is represented by the highest data points in the normalised ratio to βA loading control graph. On this blot, the bands for ERβ are more intense and bands for βA fainter than on the other two blots. As a result, little can be concluded with certainty about the effects of the herbs extracts at 24 or 48 hours on expression of ERβ in Ishikawa cells.

4.5.3.5 Progesterone Receptor beta (PRβ) expression in Ishikawa cells after 24 hours incubated with the herb extracts

The blots in Figure 4.36 show protein expression in three separate Ishikawa cell lysates prepared after 24 h exposure to the herb extracts where the proteins have been transferred to a PVDF membrane. The first probe was for PRβ then the membrane was re-probed without stripping for βA. Quantitative analyses of the blots by three methods are presented in Figure 4.37. The PRβ recombinant protein was detected and can be seen in PRβ 24-3.

Visual examination of the blots indicates that the PRβ band in the media only lysate on blot PRβ 24-1 is very faint, on blot PRβ 24-2 it is visible and on blot PRβ 24-3 it is relatively intense. The βA band is of similar intensity on all three blots for the media only lysate samples. This variation will again disrupt any calculation based on the relative abundance to the media only control, or normalisation ratio to the βA loading control. The PRβ band for GSDW treated samples is generally more intense than PRβ bands for other samples, and more so on blots PRβ 24-2 and 3. The band for βA is also more intense in Radix Paeoniae Alba and Sclerotium Poria Cocos treated samples on blot PRβ 24-3 and for Radix Angelicae Sinensis and Sclerotium Poria Cocos treated samples on blot PRβ 24-2. On blot PRβ 24-1 the βA band is marginally more intense for Sclerotium Poria Cocos treated samples, however it is of fairly even intensity across all samples on this blot.

Progesterone receptor beta 24 hours

Full blots can be seen in the appendices in Figure 8.22, Figure 8.23, Figure 8.24, and Figure 8.25. PRβ was detected at ~52 kDa which is higher than the expect ~40 kDa in the manufacturers data sheet.

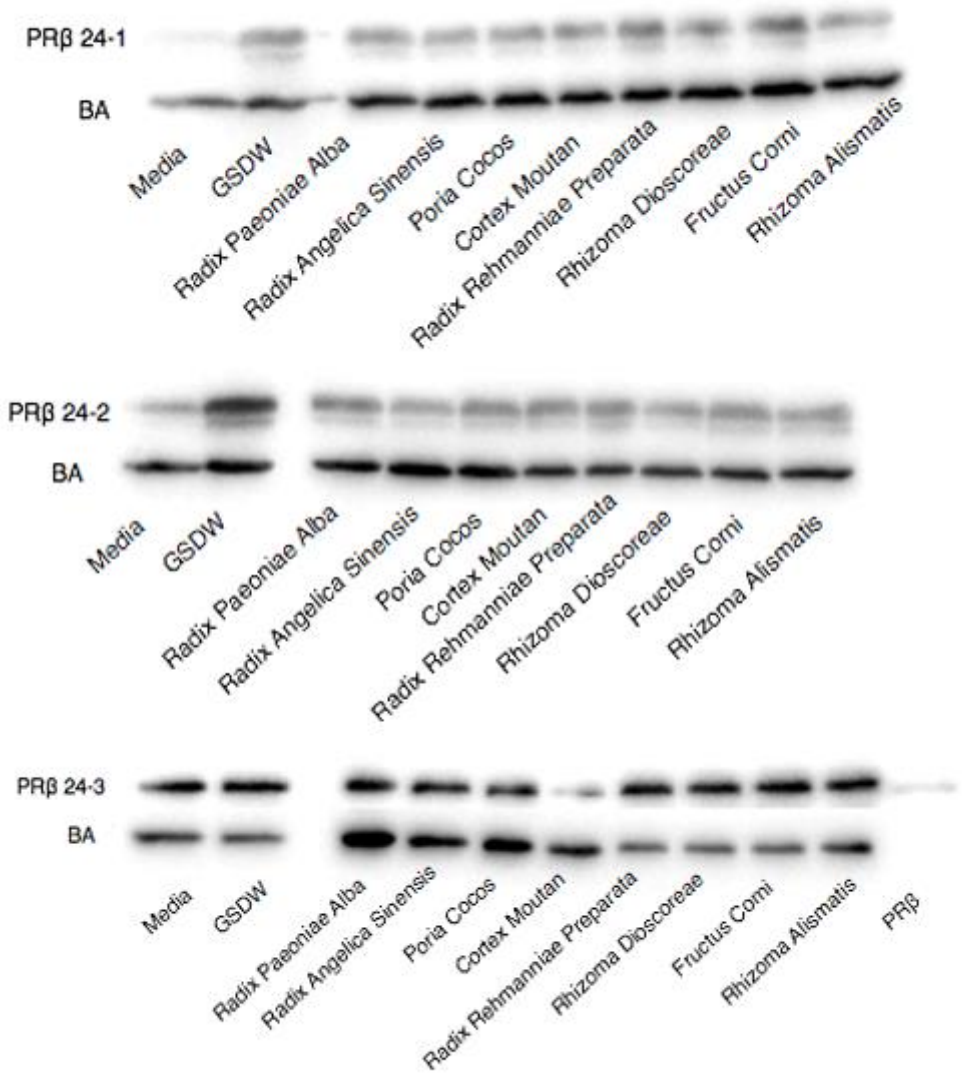
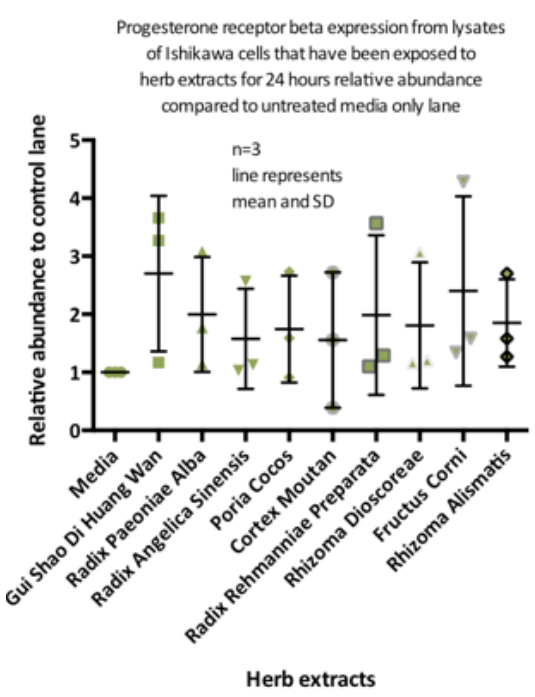
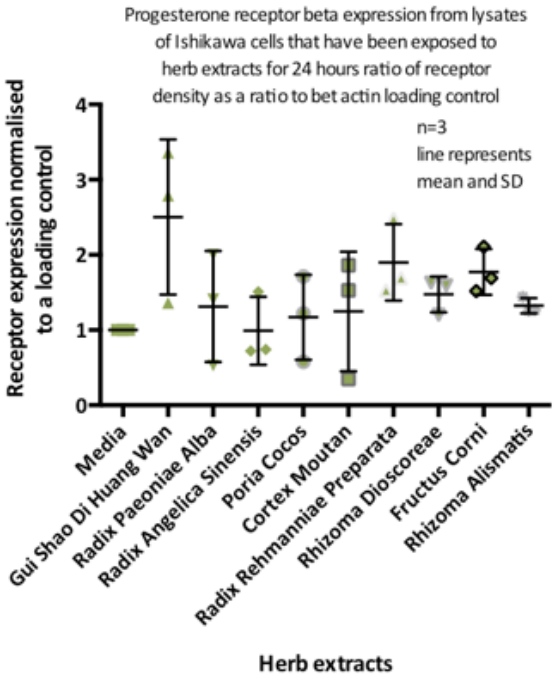


Figure 4.36 Progesterone receptor beta expression from lysates of three different wells of Ishikawa cells that have been exposed to herb extracts for 24 h.
Upregulation of PRβ at 24 h for GSDW can be seen in these blots but other changes are not consistent between blots.





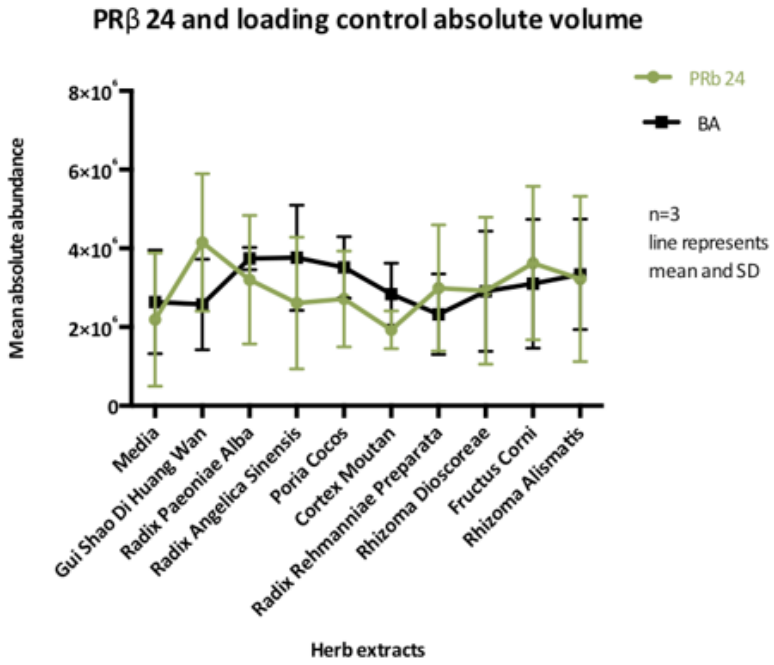


Figure 4.37 Progesterone receptor beta expression at 24 h
Displayed as relative abundance compared to a media control lane, PRβ normalised to βA loading control and absolute volumes for PRβ and βA. These graphs also show that whichever analysis is used normalised to media lane, loading control or absolute volume there does appear to be upregulation of PRβ.

Quantitative analysis of the blots indicates that PRβ at 24 h shows a clear upregulation in GSDW treated samples in both the relative abundance to media only control and normalisation ratio to βA loading control graphs. Changes in expression are less clear for Radix Rehmanniae, Rhizoma Dioscoreae, Fructus Corni and Rhizoma Alismatis treated samples where there is some upregulation when analysed by the normalisation ration to βA loading control but when analysed by the relative abundance to the media only control there is a lot of variability. As observed from visual examination of the blots, the variation in the intensity of the PRβ band in media only samples make both of these measures unreliable. The pattern of variation in expression of protein is quite different in the absolute volumes of PRβ and βA suggesting there is an effect of the herb extract on one or both of the proteins. The most clear and consistent change seen from all three methods of analysis is the upregulation of PRβ expression following treatment of Ishikawa cells with GSDW.

4.5.3.6 Progesterone Receptor beta (PRβ) expression in Ishikawa cells after 48 h incubated with the herb extracts

The blots PRβ 48-1, -2, and -3 (Figure 4.38) show three separate Ishikawa cell lysates prepared after 48 h exposure to the herb extracts where the proteins have been transferred to a PVDF membrane. The first probe was for PRβ then the membrane was re-probed without stripping for βA. Quantitative analyses of the blots by three methods are presented in Figure 4.39. The background staining on these blots is quite high as a relatively long exposure time was required for detection of the bands although this was more pronounced on the blots for the 48 h incubation than those for the 24 h incubation.

Visual examination of the blots Figure 4.38 do indicate that there are relatively consistent changes in the intensity of the bands for PRβ in all samples. The intensity of the bands for βA and PRβ in the media only samples were consistent meaning that the analyses of relative abundance to media only control and normalisation ratio to βA loading control are likely to be reliable. In all three blots, PRβ bands were visibly more intense for Radix Paeoniae Alba, Radix Angelicae Sinensis, Sclerotium Poria Cocos, and Radix Rehmanniae treated samples. The intensities of the βA bands were also relatively consistent across all blots with the exception of a slight increased intensity for Radix Rehmanniae treated samples in blot PRβ 48-2.

Progesterone receptor beta 48 hours

Full blots can be seen in the appendices in Figure 8.26, Figure 8.27, Figure 8.28, Figure 8.29, Figure 8.30, and Figure 8.31. PRβ was detected at ~52 kDa which is higher than the expect ~40 kDa in the manufacturers data sheet.

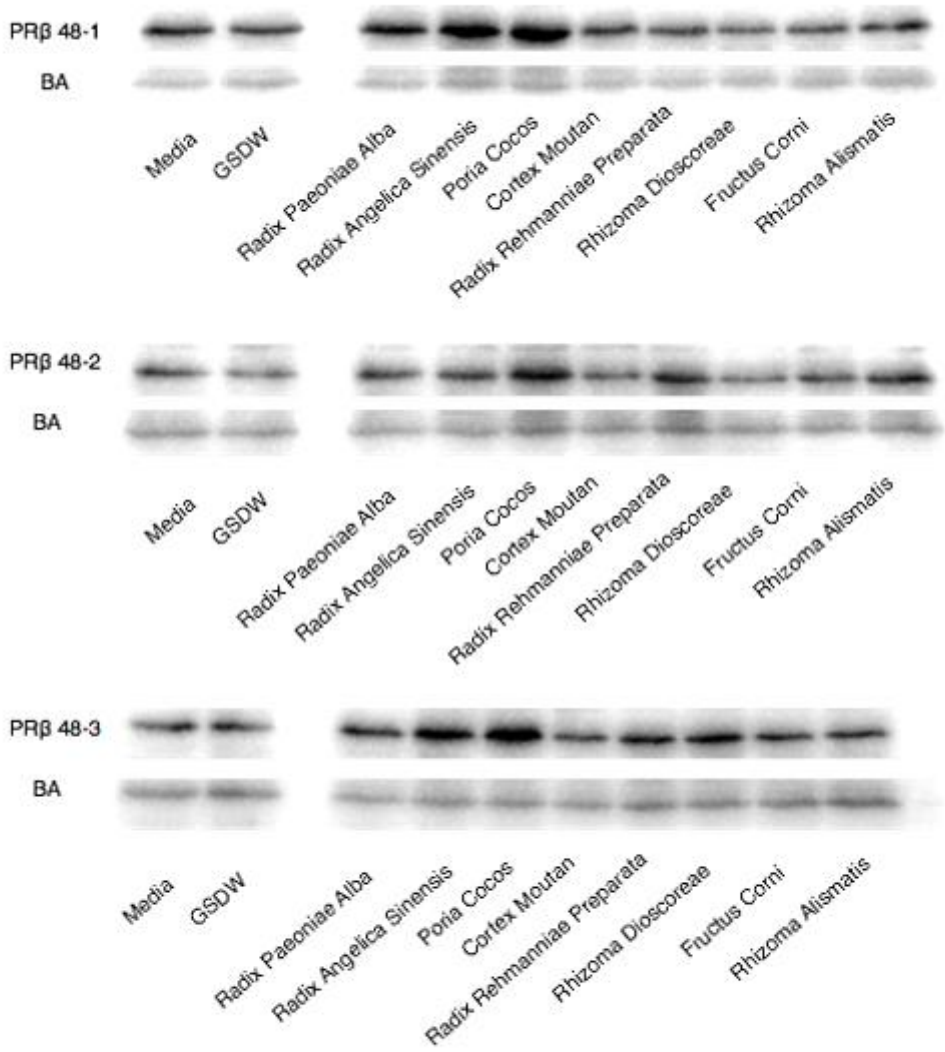


Figure 4.38 Progesterone receptor beta expression from lysates of three different wells of Ishikawa cells that have been exposed to herb extracts for 48 h. Upregulation of PRβ can be seen in Radix Angelicae Sinensis, Radix Paeoniae Alba, Cortex Moutan, and Radix Rehmanniae treated lysates.

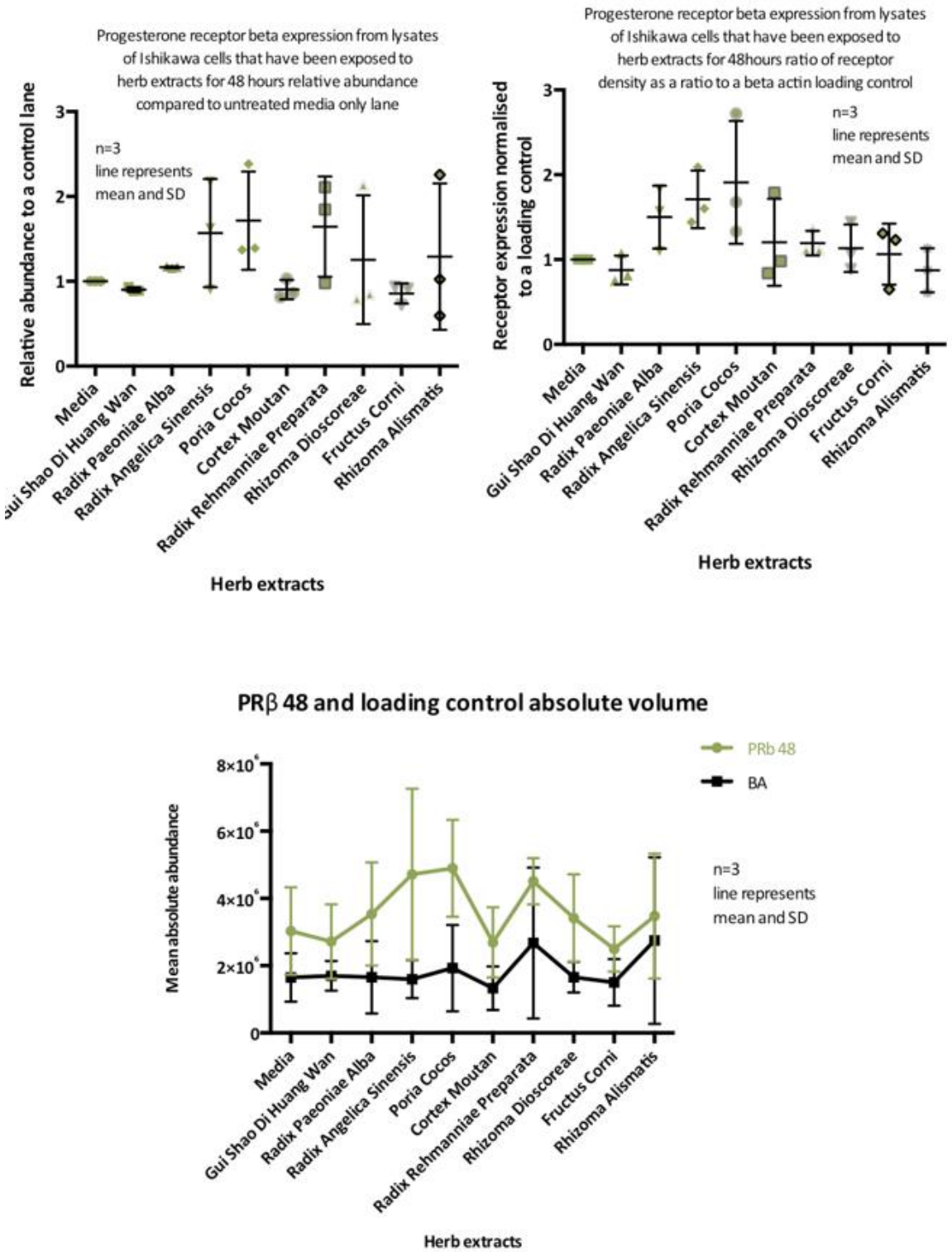


Figure 4.39 Progesterone receptor beta expression at 48 h
Displayed as relative abundance compared to a media control lane, PRβ normalised to βA loading control and absolute volumes for PRβ and βA.

From the quantitative analysis of blots, in both the relative abundance to media only control and normalisation ratio to βA loading control graphs the patterns of change were similar, in that PRβ is upregulated following Radix Paeonia Alba, Radix Angelicae Sinensis, Sclerotium Poria Cocos, and Radix Rehmanniae treatment. This was also shown in the analysis of absolute volumes. The absolute volumes for βA in this assay also remained fairly constant and mirrored the change in PRβ intensities but to a lesser extent. These results give a clear pattern of expression which is consistent across the three blots and using each of the three methods of quantitative analysis of the data and so it is with relative confidence it can be suggested that Radix Paeoniae Alba, Radix Angelicae Sinensis, Sclerotium Poria Cocos, and Radix Rehmanniae upregulate the expression of PRβ in Ishikawa cells after 48h incubation. It is interesting that the observed possible increase in PRβ following GSDW treatment for 24 h is not seen here in the quantitative analysis. This is similar to the results of ERα expression where there is possible upregulation after GSDW treatment for 24 h and likely downregulation after GSDW treatment for 48 h.

4.5.4 Discussion

Ideally all immunoblotting is analysed in reference to what is known as a loading control which is expected to be expressed at constant levels in the cell type. This is in order to correct for any inconsistencies in loading volume not accounted for by the calculations performed using the Pierce colorimetric assessment of protein levels.

βA was used as a loading control as seen in the blots in the results but not with complete confidence that it is an appropriate loading control (268). This was observed in the results of the blots as well as through research. It was seen to vary in level across experimental condition but at times increasing with a corresponding decrease in target proteins or decreasing with increases in target proteins Figure 4.40.

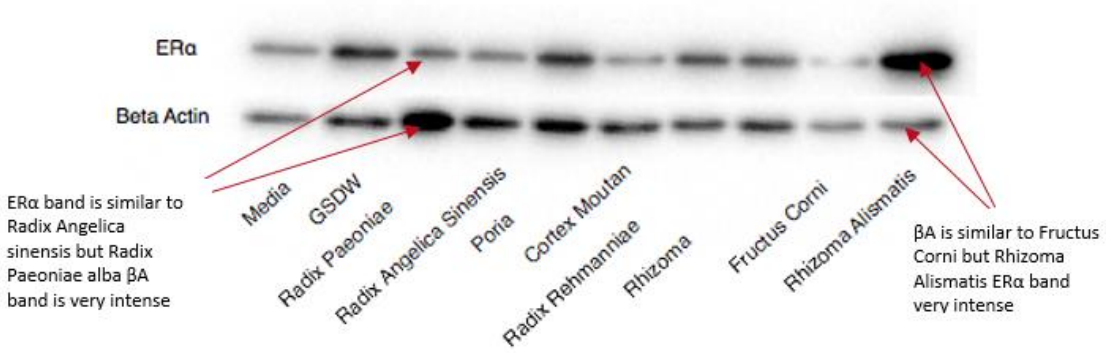


Figure 4.40 Inconsistent abundance of βA compared to ERα does not support loading errors as the main cause of variation and that any changes in βA are not due to experimental conditions

This is not conclusive but it is possible changes in βA levels may be due to the treatment not loading volumes. βA is involved in proliferation, differentiation, and migration, which all occur in the epithelium and under the influence of the sex steroids and so are likely affected by the herb extracts. Actin is a major subunit of tubules, re-epithelialization in the early proliferative phase requires significant alterations in the organization of the actin cytoskeleton (138).

It is also considered inappropriate when high levels of protein are required to probe for low abundance proteins (187). Beta actin bands were seen to be over saturated if probed simultaneously at an exposure that allowed the protein of interest to be visualised. Overexposure of the image leads to underestimates of protein levels (Figure 4.41).

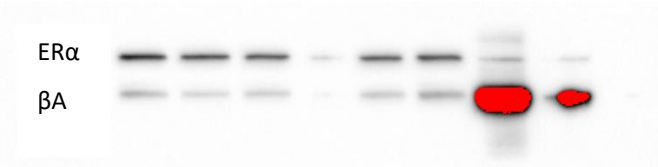


Figure 4.41 Early blot with ERα and βA probed simultaneously show that the band is highly over exposed and therefore protein levels would be underestimated.

Beta actin at 42 kDa was also considered to be a poor choice as it was theoretically too close for simultaneous detection for progesterone receptors (Figure 2.8, p77). In practice PRβ was detected at a higher kDa (molecular weight) than stated and the identity of the bands were confirmed with a sample of the purified receptor.

Stripping and re-probing was performed but mild stripping such as the Abcam mild stripping buffer (Appendix VII for all stripping buffer recipes) was ineffective at removing the primary antibody. A harsh stripping agent containing GnHCL 557.046 mg/mL was seen to remove proteins inconsistently throwing doubt on the accuracy of second and third probes. It has been shown that a substantial proportion of the proteins can be lost in stripping (269). Stripping is not always even, as seen in Figure 4.42, a blot of ERβ and βA. The second probe with βA would in isolation suggest very poor loading but in combination with the first of ERβ suggests poor stripping. In Figure 4.42, it is very clear that there has been uneven stripping. It is not clear to what extent this affects other blots. Later blots were probed for the protein of interest with the antibody for the loading control without stripping to eliminate this uncertainty.

Figure 4.42 Blot showing ERβ first probe followed by harsh stripping and a second probe with BA.
The lower row of βA bands shows loss of proteins from Radix Angelicae Sinensis to Radix Rehmanniae which does not correspond with the first blot of ERβ.

Cyclophilin is another commonly used loading control used and was also tested and appears to be affected by the treatment (Figure 4.43). Cyclophilin A is an important protein expressed at the maternofoetal interface, it regulates Th1 and Th2 balance and increases during pro-inflammatory diseases (270). All of these are functions that may be altered by a treatment shown to improve the receptivity of the endometrium.

The graph in Figure 4.43 shows the variation by blot rather than mean and SD to illustrate that there are some changes which appear to be consistent between blots performed with different lysates and at different times. There is low abundance cyclophilin in lysates from of Radix Rehmanniae Preparata and Rhizoma Alismatis treated cells which are consistent between all four blots and unlikely to be due to errors of loading.

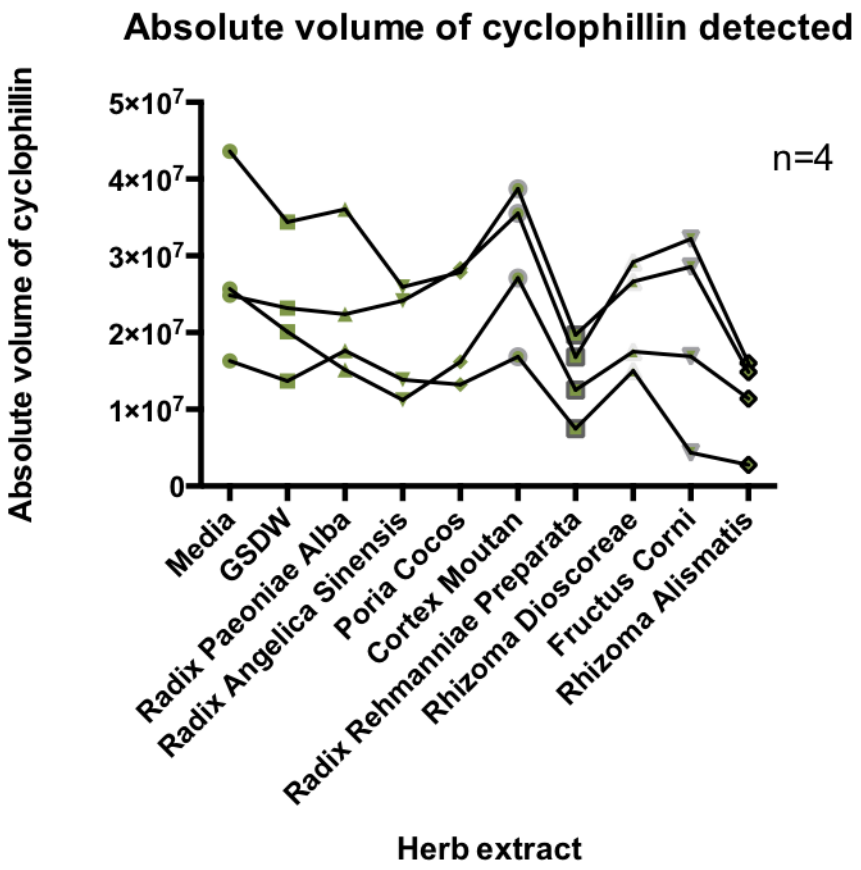


Figure 4.43 Cyclophilin absolute volume in four blots shows a consistent pattern of variation across the blots indicating that the protein may well be influenced by the experimental conditions and is therefore not an effective loading control.

Another loading control protein GAPDH is frequently used but research suggests it is upregulated in response to oestrogen receptors which are also likely to be affected by the applied treatments (271) and so is an unsuitable candidate. It was tested, and like cyclophilin appeared to vary consistently with the herb extracts.

Research on other suitable loading control proteins suggested the 5-aminolevulinate synthase (ALAS). In one study looking at gene expression profiles in response to oestradiol, the researchers used a loading control gene selection kit by Roche and determined this to be appropriate for Ishikawa cells in response to hormonal test conditions (272). This was using RT-PCR, looking at gene expression, not protein expression. ALAS was expected to be expressed in endometrial adenocarcinoma cells as shown in the human tissue atlas (273). The ALAS proteins are expected to be detected at 56-71 kDa which overlaps with both of the oestrogen receptors at 55-59kDa for ERβ and 67kDa for ERα. Stripping and re probing would be required for

this protein. The ALAS was probed for but not detected in any of the samples. Troubleshooting included probing only for this protein with no prior stripping, varying transfer time, two different variants of the antibody, longer incubation time, varying quantities of both antibodies but it was never detected.

The lysates could have been loaded onto a gel and an antibody used to detect a protein in the samples in a series of dilutions thereby manipulating the concentration of the protein loaded. This could have been used to verify that loading techniques were robust.

A measure of total protein is generally considered to be a more reliable method for normalisation and so Ponceau red was applied (274). This was unsuccessful possibly due to low overall concentrations of protein 20µg/mL in the samples obtained from the Ishikawa cells. It is a relatively low sensitivity, Ponceau red staining of total proteins (40 to 80µg) in heart homogenates show that stains were linear between 40 to 60 µg of total protein (275). In order to have all experimental variables on the same blot the 12 well combs were used which meant in total 20µL could be loaded in each well. Initially an 8 well comb was used with larger wells and more protein was loaded. It was found that further inconsistencies for analysis were introduced when comparing across two blots as seen in Figure 4.3. Ideally all of the blots would be repeated using the stain free total protein normalisation (Bio-Rad) gels.

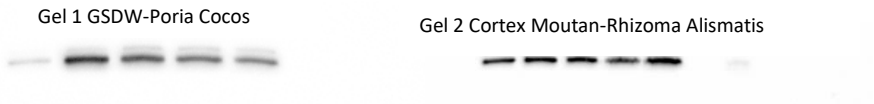


Figure 4.44 Initially an 8 well comb was used and the samples spread across two gels.
Comparison of the blots with an effective loading control would be possible but in the absence of this densitometry cannot be used across multiple blots and exposures.

Despite the concerns about normalisation of the blots to βA the overall pattern of regulation of the ERα and PRβ is consistent. This was true whether normalised to the media only control and therefore not taking into account errors of loading, or whether using βA as a loading control which may be subject to variations due to influence of the treatment on actin expression. It may be that with total protein

normalisation influence at 24 h with ERα would be informative and for ERβ, but the results obtained here are inconclusive. H2 Gui Shao Di Huang Wan will modify oestrogen and progesterone receptor expression is rejected in the absence of statistical data.

The literature would suggest that Radix Angelicae Sinensis can upregulate ERα and β (174) and downregulate PRβ (177). In fact, the observation made in this study was the opposite, there was downregulation of ERα and upregulation of PRβ expression following treatment with Radix Angelicae Sinensis.

The effects for Radix Angelicae Sinensis are interesting and perhaps indicate that there are compounds within the herb capable of both up and downregulating the receptors. It is often considered to be an adaptogen, although there is debate over whether that is a useful term (42). It, like ginseng with its pro and anti angiogenic properties (15), may have the ability to influence the steroid receptors differently according to disease or time in the cycle. It is prescribed for a very wide variety of menstrual problems.

Rhizoma Dioscoreae was the only other herb for which any published research was found and this was for both no effect (179) and downregulation (178) of ERα and upregulation of PR (178) and anti progesterone effects (177). No consistent changes were seen in either direction for any of the receptors following treatment with this herb.

4.5.5 Conclusion

Due to a lack of confidence in either using a loading control or using a media only control lane to normalise the protein of interest, only when both of these methods showed the same changes has it been reported here. It is of interest that βA appears to be variable in some blots but not others. Since βA is important in some functions

regulated by the steroid receptors it may be that there is an interaction occurring that modifies the expression of βA, possibly through VEGF (96, 97).

ERα at 24 h did show an upregulation in receptor expression compared to a media only lane and in some blots when compared to βA but this was not as consistent. ERα at 48 h showed a trend towards downregulation for Radix Paeoniae Alba, Radix Angelicae Sinensis, Sclerotium Poria Cocos, and Cortex Moutan treatments. The pattern for the other herb extracts is less clear due to high variability between the blots. Fructus Corni is noticeable for its lack of effects, levels of ERα and βA were consistent with those of the media only control for ERα and βA.

ERβ expression after 24 h of herb treatment showed no particular changes and this is confounded by the band for ERβ in the media only samples being very different for each of the three blots whilst the band for βA in the media only samples remained relatively consistent. At 48 h, there were again few consistent changes. One blot ERβ 48-3 showed very different patterns of band intensity than the other two. The variability seen in values for the normalisation ratio to βA loading control for ERβ expression undermines confidence in the normalised values, the variability in βA intensity was greater than for ERβ at 48 h and these changes did not appear to correspond to loading.

PRβ expression at 24 h showed an increase for GSDW treated cells but with inconsistent changes for the individual herb extracts. Here, as for ERβ 24 h, the PRβ band in the media only sample was very different between the three blots whilst the βA band intensities remained more consistent undermining confidence in the normalised values. After 48 h incubation, the expression of PRβ in GSDW treated cells showed little change. There was however consistent upregulation in Radix Paeoniae Alba, Radix Angelicae Sinensis, Sclerotium Poria Cocos and Radix Rehmanniae Preparata treated cells.

It appears that GSDW does modify the expression of endometrial steroid receptors and the individual herbs of the formula GSDW will vary in the contributions to any changes seen in receptor protein expression.

5 Discussion

5.1 Background

Infertility affects a huge number of women and their partners. It leads to psychological distress and creates a financial burden on state and individual. In 24% of cases infertility is idiopathic (2) and in this situation a typical progression will be rapidly to the most expensive and invasive solution which is IVF. Success rates are only around 30% (2) even once eggs have been collected, fertilised and embryos returned to the uterus. The quality of the embryo is a key factor, but so is the receptivity of the endometrium to implantation. The pharmacological treatments used in IVF to improve the implantation environment in relation to the quality of the endometrium are limited to aspirin, clexane and sildenafil citrate (276). Treatments targeting the intra uterine immune environment include prednisolone, dexamethasone, intralipids and intravenous immunoglobulin (277-281). These treatments can be expensive and experimental, with limited and controversial research. There is a need for more options to improve the outcome for those diagnosed with idiopathic infertility. This applies whether couples want to try to conceive naturally, or choose the route of assisted reproduction. Herbal medicines are generally more affordable than the pharmaceutical options, but despite some positive clinical trials (40) (3) (4) herbal medicines have gained no widespread respect within the medical community.

This research aims to investigate some of the mechanisms by which the positive effects seen in some clinical trials of herbal medicine (3, 4, 16) are mediated. The contribution of Chinese herbs to fertility has been demonstrated to improve sperm quality (282), oocyte quality (283), and to regulate the immune system (284) but this investigation looks only at the haematology of the endometrium. Because this investigation bridges two contradictory paradigms an area of common understanding was chosen, that of the blood of the uterus being vital to conception.

The trials by Dr Wing (3) demonstrated changes in the vascularity and blood flow of the uterus in women treated with Chinese herbal treatment using GSDW as measured by 3D doppler. These changes occur within the complex environment of the constant remodelling of the endometrium influenced by the immune and hormonal systems. In order to look for particular mechanisms by which these changes are mediated the complexities were reduced. *In vitro* techniques were used to examine specific effects of the herb extracts on aspects of the haematology of the endometrium.

The receptivity of the endometrium is determined by both uterus and blastocyst, autocrine, paracrine and juxtracrine factors at hormonal, cellular and molecular levels (192). There is a complex cascade of molecules involved in endometrial receptivity including integrins, growth factors, selectins, cytokines, mucins and transcription factors but it is the steroid hormones that direct this system (266).

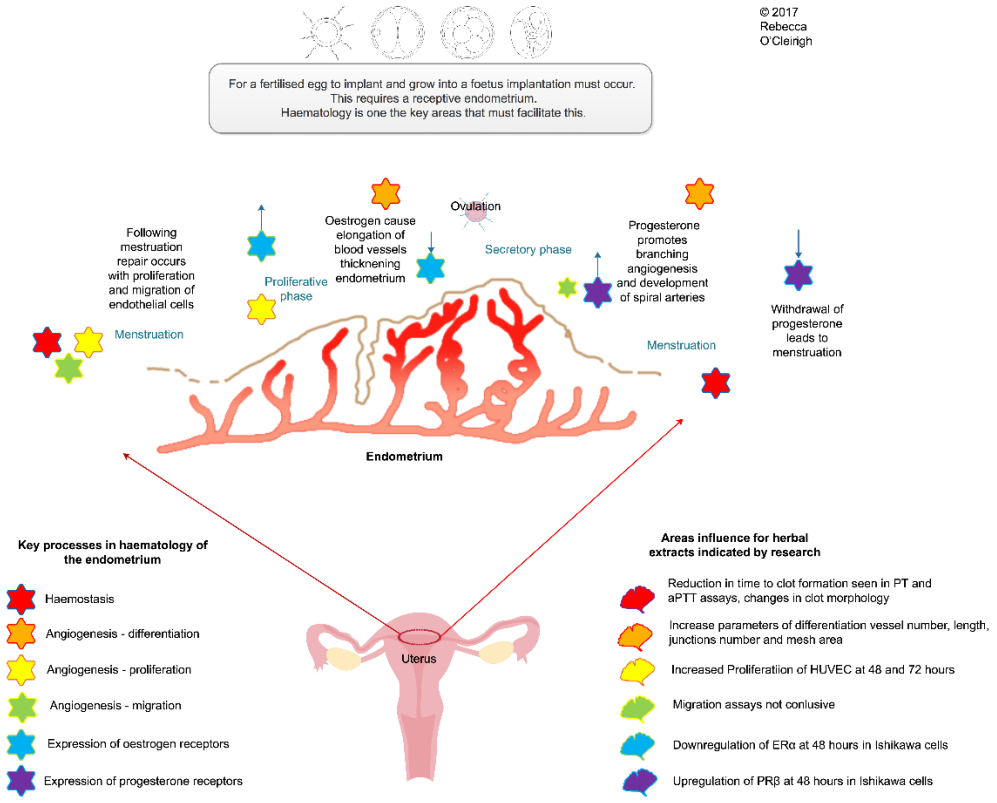


Figure 5.1 Summary of GSDW actions on haematology of the endometrium.
Haemostasis, proliferation, and expression of ovarian steroid receptors have all been observed in the assays.

The assays performed indicate that the herb extracts are influencing the haematology of the endometrium in that they increased time to clot formation in tests of the extrinsic and intrinsic pathways, increasing proliferation of endothelial cells, and increasing parameters of endothelial cell differentiation. These functions are in part regulated by the ovarian steroid hormones and the expression of these receptors are also seen to be modified.

The modifications in these parameters will be more or less appropriate dependent on time in the menstrual cycle. Differentiation of vessels would not be appropriate in the menstrual phase and nor would upregulation of progesterone receptors; proliferation would be inappropriate in the secretory phase as would upregulation of oestrogen receptors.

In a clinical setting, the herb extracts given will be varied through the cycle. A formula like GSDW would be modified with herbs added and removed dependent on the time of the cycle and proportions of herbs would also be altered. The herbs taken by a woman, the compounds absorbed and the metabolites formed which reach the uterus in what relative doses may vary significantly from what is added to cells *in vitro*.

5.2 The herbs

All grown natural products, including herbs, are subject to significant variation due to growing location, year, season of harvest, genotype, chemotype and phenotype of the source materials (285). Chinese herbs are subject to additional issues of quality, contamination, and substitution. The herbs obtained were from an RCHM approved supplier in the UK and were visually tested for authenticity at the Jodrell Laboratory in Kew by their botanist for TCM materials (192).

Standardisation and identification of herbal products is currently based on quantification of one or two “active” substances. The -omic techniques may allow the standardisation using a fingerprint in the same way plant materials are now being accurately identified regardless of processing. There remain the challenges of working

with plant materials whose ingredients and concentration of compounds vary according to climatic growing conditions and time of harvest and it is likely an expressions profile will require concentration ranges required for mechanism of action (32).

Extraction of the herbal materials although a repeatable process cannot be done with the same source materials and variation is expected. All extracted herbs were reduced to their dry matter in order to calculate a mg/mL value which can be seen in Table 2-2, p51. In order to minimise variation that might result from natural variation in the herbs themselves, replicates of an experiment were completed with a single extraction of the herbs and all the cellular assays used one extraction. TLC was a separate set of extractions, as were the haemostasis assays. In the method development, other extracts were used as herbs were not considered to be stable and active, over the five year course of the research. Experiments were conducted during method development of the differentiation assay where extracts were made up monthly, stored and tested together to see if herb age was influencing differentiation. No differences were seen over the course of 3 months (data not presented) but at this stage of experimentation consistent differences were not detected in the assay. It would be valuable to assess the stability of compound profile and levels in the herb extracts looking at 7 °C, -18 °C and -80°C storage

Variability is an inherent problem in the use of natural products which might be overcome in a number of ways, but each have their own limitations. It is possible to modify the volumes used so that mg/mL remains constant in the assay but the key hypothesis concerns the use of the whole formula which cannot be decocted together and then mg/mL varied for all of the 8 herbs contained within it. The eight individual herbs could be varied, but then they would not represent the parts of the whole under investigation. Extraction of the active compounds is a common approach (110) but identifying the compounds that are active is a flawed approach. Compounds have been shown not to act in isolation, and an apparently insignificant compound can make a difference to the function of the whole (34).

This variation is also the reality of treating in a clinical setting. The TLC experiments was performed because of the frequency with which alcohol (99), acetone (13) or other extraction methods are used in published research. These extraction methods are used dependent on the molecules to be recovered. The purpose of this investigation was not to maximise recovery of compounds but to emulate the process by which herbs are extracted for consumption by a patient, and therefore to evaluate the possible biological mechanisms of action of the herbal extract.

5.3 Drug administration

When a person consumes a herbal medicine, there will be food in their digestive tract, they will each have a unique microbiome that modifies absorption, their own metabolome, liver enzymes, and body volume. Pharmacokinetic studies show that ingestion of a substance is not the same as absorption and a compound may be modified during absorption. Compounds may be absorbed best in the acid environment of the stomach; there is absorption into the blood through the small intestine, and although there are few apparent absorption sites in the rectum, it too can be a very effective route of administration. There can be 60 types of bacteria in the colon which catalyse drug reactions. Initial metabolism can occur in the intestines such as oxidation, reduction, hydrolysis as well as glucorination, sulphation, *N*-acetylation, methylation, gluthianone and glycine conjugation. Once through the gut wall it must then pass through the liver where substances are subject to first pass hepatic metabolism which modifies many compounds. Food changes the absorption of drugs and distribution will depend on absorption, permeability, diffusion, perfusion and tissue binding (286).

One of the limitations of using a cellular assay is that the cells in the experiments are presented with the whole extract, not a selection of compounds which may have been altered when absorbed through the gut or in hepatic metabolism as would occur in a patient. Only a series of tissue assays at different time points after the consumption of the herbs would indicate the actual compounds present in the target tissues. This is not feasible in patients, but metabolomics may be able to provide an approximation

of this information. This could be through blood samples or urinary markers to see what had been absorbed and metabolised and at what levels. This will not indicate what is present in the blood in the uterus, but it would show what has been absorbed and metabolised and how this compares to the administered decoction. In rats Radix Angelicae Sinensis new compounds have been identified as metabolites not present in the original herb (116).

Systems biology assesses the interactions of environment and behaviour through gene control much as Traditional Chinese Medicine theory allows for the effects of environment and emotion (287). A paradigm shift from the "one drug fits all" mindset may occur with the use of pharmacogenomics leading to the creation of personalised medicines (288). This mirrors the TCM view of a medicine needing to be specific to a patient based on their individual characteristics and expression of symptoms.

5.4 Complexity, synergy, and polyvalence

The interaction between compounds in herbs and herbs in formula are at the heart of Chinese medicine and theory. The interconnectedness of the organism and each of its biological processes is now widely accepted in western medicine as is evidenced by whole fields of research in systems biology and specialist fields such as psychoneuroimmunology. Treating a complex organism with a complex medicine makes the interpretation of outcomes within the scientific method challenging. But not sufficiently so that those therapeutics should be overlooked or reduced to a single pharmaceutical compound. Those substances are simpler to manufacture and to test. They often have more easily measurable influence, and have their place but their own problems. Deaths from adverse reactions to prescribed drugs is estimated in Europe at 200,000/year (289), it is the 4th leading cause of death. It is considered by herbalists that it is the complexity of a herb and the way it acts on multiple systems that make them so useful. This is seen in Radix Ginseng which contains adaptogenic ginsenosides (290). The planned polypharmacy within a formula tries to predict any possible negative impact of any herb and ameliorate them at the point of administering the medication. Radix Glycyrrhizae is added to many formulae to

ameliorate potential toxicity The molecular mechanism for this, in relation to one very toxic herb Radix Aconiti, has been shown to be due to the creation of a complex between liquritin and aconitine, binding the free aconitine (291). The definition of synergy depends on context. It has been considered to be as a literal as the definition of working together, to needing to be proven by a mathematical model such as the isobolograms (292). This requires dose response curves rather than the evidence of herbs working in combination to produce an effect greater than the sum of the individual parts.

The assays performed here did not include dose response except for the proliferation assay and this was performed for the whole formula only. This could be repeated with each of the individual herb extracts and a dose response curve generated to see is there is an increased response in proliferation. However, a very large number of cells is required to perform these assays. Insufficient HUtMECs were ever obtained to performed the MTT for the whole formula. The angiogenesis assays were performed with each individual herb but due to the variation between wells it is likely that a high number of repetitions for each condition would be required to create a dose response curve. With sufficient cells, it could be repeated with 20 repeats of 10 experimental conditions for 10 concentrations; a total of 2000 wells but this was not within the scope of this study.

A more general term, less subject to controversy is polyvalence. This is used to describe different compounds that influence the same disease process by altering metabolism, excretion, absorption and distribution (293). It is likely that GSDW has an effect on the steroid receptors in promoting angiogenesis, but the increased perfusion of less coagulable blood assists in the sheer stresses than promote endothelial cell proliferation and differentiation (294). The increase in blood perfusion will likely lead to increased delivery of systemic circulating sex steroids to the receptors that are upregulated by the herb extracts.

In the aPTT assay was there evidence that points to an effect greater than the individual parts and in the differentiation assays with the HUVECs where the whole formula showed effects not present in any one individual herb extract.

5.5 Haemostasis

The assays examining haemostasis through PT and aPTT are useful tests of coagulation but are not accurate representations of thrombus formation *in vivo*. Nor do they show how the herbal products are interacting in the cascade to extend the duration of coagulation. There are 21 key proteins in the coagulation cascade (Figure 4.10, p108) and 289 compounds in the herbs (Appendix III), which could be affecting any of these in any combination. They could be competing for their binding site, blocking receptors, affecting calcium availability or blocking co-factor availability.

The prothrombin time will indicate whether the compounds inhibit factors II, V, VII, X, fibrinogen or thrombin and the activated partial thromboplastin time will indicate whether the compounds inhibit factors II, V, VIII, IX, X, XII and high molecular weight kininogen (90). These two tests do not account for effects that might be seen *in vivo* interacting with endothelial cells, collagen and surrounding tissues or the sheer stresses of blood flow. It does not factor in the blood flow changes due to interaction with platelets or with mediators of inflammation as discussed in Section 4.1.3.

Fructus Corni increased the PT and the aPTT and although it does contain anti thrombotic compounds these would not extend clotting time. Radix Paeoniae Alba has anti thrombin effects which would extend PT time although this no extending of clotting time was observed in the assays for Radix Paeoniae Alba. These two herbs are closely related peonies and have many of their compounds in common.

In the aPTT Radix Angelicae Sinensis extended clotting time. Compounds in Radix Angelicae Sinensis *(z)*-ligustilide (110) and *N*-butylphthalide (111) inhibit clot formation and platelet aggregation so these are not the mechanisms acting here. α-

pinene is found and Radix Angelicae Sinensis and has been shown to be weakly antithrombin (115). Ferulic acid in Radix Angelicae Sinensis and inhibits tissue factor (224).

The assays did show that there was an increase in time to clot formation in both pathways and that this was dose dependent. The effect seen was comparable in PT to an estimation of the therapeutic dose of heparin. In the aPTT assay heparin at the same dose did not form a clot in the assay but it did at a 1/10th dilution.

The changes observed in clot morphology warrant further investigation because of the visual differences noted when performing the PT and aPTT. Clot structure was examined and did show microscopic differences but staining did not demonstrate this to be clearly due to fibrin. Fibrin is key in providing the tensile strength of the clot, but tests of tensile strength could not be completed as the clot was friable. Altering fibrin structure may be the entire effect of the herb extracts on the clotting cascade, or it may just be one of several effects. The conversion of fibrinogen to fibrin under the influence of thrombin is common to both pathways but the changes in clot morphology were more pronounced in the aPTT assays than the PT. It may be that the time to clot formation being longer in the aPTT assay meant the changes in fibrin structure were compounded (230).

Fibrin morphology does change in pregnancy becoming 1.3x stiffer than non pregnant and more resistant to lysis (295). Friable fibrin, such as that seen in the clots under the influence of the herb extracts, tends to be stiffer (296). The outer cells of the blastocyst are the trophoblast, which will form the placenta. During invasion of the endometrium, at around day 9, these cells create a fibrin plug enclosing the blastocyst within the endometrial layer (297). The trophoblast is then embedded in a matrix of fibrin (298) as the spiral arteries decidualise. This is the process where the spiral arteries become fibrinoid as elastic tissue and smooth muscle is destroyed. This facilitates the invading trophoblast, plugs form in the arteries shielding it from maternal circulation and allowing it to develop in an environment where the blood flow is high and blood pressure low (299). It is thought that abnormal uterine

perfusion can lead to problems in pregnancy such as intrauterine growth retardation and pre-eclampsia (80) but these failures are set up during the conception cycle as failures of decidualisation (300). Changes in fibrin could affect the receptivity of the endometrium by facilitating implantation through the remodelling of the spiral arteries.

Balanced haemostasis is essential for the commencement and cessation of menstrual bleeding (301). Much of the research on haemostasis in the endometrium relates to the pathologies of abnormal uterine bleeding and suggests an imbalance of clotting factors. In this situation herb extracts that are anti-coagulant would be detrimental. Clinically it is more common for infertility patients to have short duration of bleeding, increased incidence of clots in the menstrual blood, and painful periods in which case the balance may be shifted towards a pro coagulable state that would benefit from the anti-coagulant function.

Coagulation is influenced by oestrogen and progesterone. Oestrogen is a vasodilator and increases uterine perfusion in the vascular bed of the myometrium and endometrium (302). It induces vascular relaxation by stimulating release of endothelium-derived vasodilatory substances and by acting directly on the vascular muscle (303). The greater the ratio of oestrogen to progesterone, the greater the quantity of blood flowing through the endometrium as the progesterone counteracts the vasodilatory response to oestrogen (298). Women with subfertility have significantly poorer measures of endometrial uterine perfusion and vascularity (304).

5.6 Cell types

Human Umbilical Vein Endothelial Cells (HUVEC) were chosen as a relatively robust, stable, affordable primary cell line which is well suited and extensively used for the chosen *in vitro* techniques of investigating angiogenesis. The HUVECs are macrovascular endothelial cells and so they are not directly comparable to the uterine endothelial cells which are microvascular. Endothelial cells are not heterogenous (140), at different parts of the vasculature they are responsible for pro and anti-coagulant functions, inflammation, angiogenesis, permeability, and vessel tone. It is their heterogeneity that permits this range of responses (305). HUVECs can be used from cryopreservation for between 5 and 10 passages before they begin to lose their organ specific phenotype and this results in decreased migration and differentiation (246).

Human Uterine Microvascular Endothelial Cells (HUtMEC) were also used for the assays as these are cells of specific interest. Obtained from the myometrium these cells are known to modulate adhesion molecule expression, show increased proliferation, cell survival and angiogenic activity in response to the ovarian steroids (306). This is also a primary cell line but is expensive, and more difficult to work with. The intention was to optimise the assays with the HUVECs and repeat with the HUtMECs. This proved to be difficult and the cell line produced enough cells to perform the differentiation twice and the migration assays only once. The cells then became quiescent at their fifth passage, proliferation slowed so it was not possible to obtain the required numbers of cells to perform further assays. Performing the proliferation assays with this cell type would be beneficial. The heterogeneity of the endothelial cells was seen in the results obtained in the differentiation assays where the HUVECs showed different responses to the herb extracts than the HUtMECs.

The Ishikawa epithelial cell line was used to examine the effects of the herb extracts on expression of receptor proteins using immunoblotting. Ishikawa cells are clones of an human endometrial adenocarcinoma and they have been shown to express both oestrogen and progesterone receptors (199).. There are other cell lines such as the

HEC-1A which is appropriate to investigate the post-menopausal endometrium. Ishikawa cells were chosen as the most appropriate cell line to examine the effects of the herb extracts on steroid receptors in the premenopausal endometrium (307) and this was used for the analysis of effects of the herb extracts on the expression of the steroids receptor proteins using immunoblotting.

The primary cells lines do alter each passage and have a very short usable time for the assays. The HUtMECs should, according to the supplier (2bScientific), have been able to reach their 10th passage but this was not achieved. Ishikawa cells are stable, proliferate rapidly, and as they transformed they theoretically have no passage limit. It has however been shown that they will transform into undifferentiation cells after long term culture, but long term was not defined (199).

An MTT assay was performed with the Ishikawa cells to establish a cytotoxic level of the herb extracts. GSDW was inhibitory to proliferation in concentrations over 0.035µg/mL in the Ishikawa cells and never showed any proliferative effects (data not shown). The HUVECs were not inhibited below 5.42µg/mL and showed a proliferative effect at lower doses. It is interesting that the primary cell line proliferated in response to the herbs and a cancerous cell line did not, and that the Ishikawa were inhibited from growing at a much lower dose than the HUVECs. Radix Angelicae Sinensis has been shown to be more toxic to breast and cervical cancer cell lines than primary cell lines and this is attributed to its anti-mutagenic actions (308).

All *in vitro* cellular assays, even with the primary cell lines are in an artificial environment, without the normal stimulation of their environment and neighbouring cells, different epigenetic changes will occur. Endothelial cells forming structures will behave differently in contact with pericytes and smooth muscles cells and subject to the sheer stress of vascular flow (141).

A non cancer cell line would be more representative and normal endometrial cells can be cultured from patient derived samples or bought as endometrial tissue blocks commercially. Research can be performed on biopsied cells but these are

phenotypically unstable and do not grow well. Few cell lines were available to purchase other than cancer cell lines, such as the Ishikawa's which are epithelial-like but there are transformed human endometrial, epithelial, and stromal cells. Repeating the assays with a wider range of cells types might yield further interesting results that would show effects in a wider representation of the uterine tissues involved in receptivity.

In vitro cell assays have significant limitations and cannot be taken as direct evidence that the same behaviour would exist in the specific cell type *in vivo.* When looking at therapeutic effects it may be necessary to be able to model the actual pathology of the disease to see true effects. Would the effects of the herb extracts on haemostasis be greater in patients with antiphospholipid syndrome? If the cells of the sub fertile endometrium were cultured would they be more responsive to treatment? In the research using normal women changes were not significant after administration of herbs in a normally fertile control group (3). These assays do allow investigation of specific functions outside the uncontrolled variables of the organism and so have their place in a diverse portfolio of evidence. In this case it enabled the investigation of the observed clinical effects of administering a herb extract, to examine the pharmacological mechanisms by which it may be exerting its effect.

5.7 Proliferation

It is generally considered that after menstruation, the cells of the endometrium proliferate and migrate in order to repopulate the shed functionalis. Proliferative activity with GSDW was observed within a narrow range (Section 4.2.4, p117). The herb extract was able to promote cell proliferation at 48 h from 0.68 to 1.35 µg/mL and at 72 h from 0.34 to 2.71 µg/mL. The individual herbs extracts were not assessed as very high numbers of herbs were required for each assay to be repeated at three time points. Measuring proliferation using cell count would reduce the requirement for such high numbers of cells.

A second assay looking at the protease activity of live cells would also suggest that this effect is due to the proliferative effects on the endothelial cells rather than an upregulation of their mitochondrial activity. Proliferation of epithelial cells leads to a thickening of the endometrium, as measured using ultrasound or 3D doppler which is correlated with endometrial receptivity (242, 243) although this measure is debated. Some researchers show that 3D doppler and uterine perfusion are considered more useful measures (309).

There are different opinions as to whether there are peaks in endothelial cell proliferation. There is variance between the different regions of the endometrium and there is proliferation of epithelial cells (310). If epithelial cells were to proliferate without concurrent proliferation of endothelial cells a thickening of the endometrium with poor uterine perfusion could occur, which would have poor prognosis for implantation.

5.8 Differentiation

The blood flows through the uterine arteries into the arcuate arteries, the radial arteries and into the basal arteries. In the myometrium, the basal arteries supply the endometrium from where regeneration occurs post menstruation, and from which the spiral arteries emerge which characterise the luteal phase endometrium (209).

Endothelial cell morphology will be determined by the specific tissue in which is forms (208). Endothelial cells in the umbilical vein (HUVEC) may be continuous and they are polygonal because fluid exchange occurs at the placenta not in the veins themselves. The uterine HUtMECs are likely to be fenestrated as the myometrium where the cells originate is a tissue where fluid exchange is occurring (252). Although HUVECs are a useful model they are not the same as the cells that are involved in implantation. The HUtMECs are microvascular cells from the myometrium, which are one of the multiple tissue types with which an implanting blastocyst will interact. It will first come into contact the luminal epithelium, displacing this to invade the basal lamina and into the stroma (311). The myometrium where HUtMECs originate is below the stroma. The myometrium is not the functionalis, the part which is remodelled through the cycle. It is not the tissue most dynamically responsive to the sex steroids, nor is it part of the regenerative basal layer. It is however, a closer tissue type to those intimately involved in implantation than HUVECs, and the vascularity of the myometrium will affect the downstream uterine perfusion and may be sufficiently similar to be a useful model.

In the differentiation experiments performed with the HUVECs all four measured parameters of differentiation, number of segments, number of junctions, length of tubules and mesh area were increased significantly by the whole formula GSDW but no significant change was seen in any individual herb extract (Section 4.4.6, p148). When tested the HUtMECs again showed this significant difference between media and GSDW but also for some of the individual herb extracts for total length and mesh area. This difference in effect was not surprising given the evidence of heterogeneity of the endothelial cells investigated.

The upregulation of the tubules *in vitro* suggests that there are compounds within the herb extracts that are promoting differentiation but the comparison to a growth factor deficient media for growth is artificial since there would be growth factors present *in vivo*. It is not possible to establish whether the herb extracts are having a beneficial effect when the cells are in an environment optimised for their survival. When put under stress it is possible to evaluate possible beneficial properties of the herbs. *In vivo* the herbs are not used in healthy women only when a disease process is present and the cells are no longer in their optimal environment. It is unclear exactly how these metrics of endothelial cell differentiation translate into changes in the endometrium. Is an increase in length advantageous? Does it lead to greater penetration of the myometrium? Increase tubule available for intussusception? Translating the metrics of tubule development to function of the vessels *in vivo* is not direct. The formation of a network it is argued is not the same process as a functional lumen and therefore blood containing vessel (136). It is debated whether a functional lumen is present in the tubules formed by endothelial cells on a basement matrix. A functional lumen is in part stimulated to form by blood flow (312).

The matrigel assay is a simplistic assay and it is possible to extend this investigation through the use of endometrial endothelial cells from biopsy, the use of multiple basement matrix, endothelial mural co culture, organ culture and *in vivo* angiogenesis assays (141). The current research using this assay is in itself subject to inconsistency. It is rare to find the specifics of the measurement techniques used, from where does the length of a tubule being measured begin? At the centre point of the cells? Where the projection reaches a consistent pixel width? Automated techniques have a consistent algorithm but typically papers describe that image manipulation is required for the images to be of sufficient quality for automated analysis. The details of how precisely measures are taken are part of the intellectual property of the developers and are not readily available. An extensive comparison of the outcomes of analysis of the same image processed through Wimasis, ImageJ and Angiosys at the start of the investigation showed that each gave different results. The use of automated software analysis eliminates human error and introduces computer ones.

A human is able to distinguish between a clump of cells and a mesh but a computer may not. Or it may be optimised so that the clump of cells is ignored but then also overlooks a small but clear mesh. Human analysis is prone to bias, which the computer is not.

The most consistent and accurate analysis was found to be from a microscope and camera, obtained by the laboratory towards the end of the research period, which captures extremely high resolution, clear photographs. These were not subjected to analysis through the three different software and manual analysis, as some early images were. They were seen to produce accurate tubule maps, when compared visually to the original photographs, and these can both be seen in Section 4.4.2, p135.

The best understood mechanisms for development of new vessels is by sprouting. A new vessel is formed as an outgrowth from an existing vessel. The mechanism seen in the endometrium and the developing embryo is known as intussusception. This is where new vessels are formed when an existing vessel divides its lumen in two. Proliferation of endothelial cells as measured by integrin $\alpha V\beta 3$. This adhesion molecule is required for the survival and maturation of blood vessels (310). It was seen to occur within the existing endometrial vessels, supporting the theory that intussusception is the most important mechanisms for new vessel growth in the endometrium (310). This mechanism is not mimicked in the differentiation assay on matrigel but may be induced in the chick chorio-allantoic membrane assay (313). Vessel length may be a useful comparison to the elongation seen as a result of the influence of oestrogen. Junctions have several different types which are not all related to sprouting and do occur in intussusceptive differentiation. Different junction types are another difference between micro and macrovascular endothelial cells (314).

5.9 Migration

Preliminary migration assays were performed. These assays were not optimised and not replicated, due to resourcing and issues with the cell lines, so it was not possible to reach any firm conclusions. The assays performed with the HUVECs show that there may be differences in the whole formula at 0.087 µg/mL and in the individual herb extracts of Sclerotium Poria Cocos, Radix Rehmanniae, Fructus Corni, and Rhizoma Alismatis but these were a single well, single experiments. In addition, the poor adhesion of the HUVECs meant the analysis using this method or coverage of the denuded area of cells is not very accurate.

The assay was not repeated due to the difficulties of obtaining sufficient cells to complete the desired replicates. Timing of an appropriate number and passage of the cells to correspond with availability of the time lapse facilities proved another significant hurdle. The results seen warrant further investigation. Migration is an important factor in the regenerating endometrium and this repair ensures the continuity of the entire endometrial surface to support the development of the functionalis reducing non-receptive areas for implantation.

Migration assays, in particular this wound healing type have a number of limitations the use of the ibidi insert overcomes many of the commonly cited ones. Using the scratch assay can lead to inconsistent wounding and inhibition of migration from molecules released from damaged cells (141). Migration occurs in response to multiple ligands and sheer stress across a basement membrane, these variables are not simulated in these assays. The effects of proliferation also need to be considered since these processes occur together (141).

Other *in vitro* assays would not account for these variables either. To model these would require *in vitro* /*in vivo* assays such as the aortic ring assay, or the *in vivo* zebra fish assays (253).

5.10 Steroid receptors

The expression of four receptors were analysed, three were intracellular ERα, ERβ and PRβ. The fourth was a membrane bound receptor mPRα which was never detected. On consultation with the supplier of the antibody (Abcam) it was suggested that a beta-mercaptoethanol free (i.e. non denaturing) Laemmli loading buffer should be used (Appendix VII). When this was used the lysates remained viscous. Only 6 of the 10 samples were successfully loaded into the wells in the gel, and the protein was still not detected. It may also be due to the protein extraction protocol that mPRα was not detected. Although RIPA is a suitable lysis buffer for membrane bound receptors there are other protocols which may have been more suitable. Most membrane receptors are integral and extraction requires disruption of the lipid bilayer using a non-ionic detergent such as Triton X-100 or CHAPS can be used to retain the proteins shape during extraction (315). SDS which is found in RIPA buffer in known to denature membrane bound proteins (316). Low expression of the mPRα protein in mammalian cells has been a significant challenge to the investigation of its actions (171). In order to obtain greater volume the protein was transfected into yeast and isolated with mechanical lysis (317) so it may be that concentrations are too low and it is not a problem with antibody or methodology.

Reference proteins were purchased and MPRα protein could be identified so the blotting protocol and antibody were appropriate. Reference proteins for ERα and PRβ were used to verify the antibody. For ERβ the reference protein was never identified despite using two antibodies, increased incubation times and increasing volumes of the reference protein well above the recommended loading volume.

Ishikawa cells were incubated for 24 and 48 h with the herb extracts and it was at 48 h that most of the consistent changes were seen. It is likely that there was a downregulation in ERα by Radix Paeoniae Alba, Radix Angelicae Sinensis, Sclerotium Poria Cocos, and Cortex Moutan and upregulation of PRβ for GSDW at 24 h and Radix Paeoniae Alba, Radix Angelicae Sinensis, Sclerotium Poria Cocos, and Radix Rehmanniae Preparata at 48 h. The only literature found relating to expression of the

steroid receptors was for Rhizoma Dioscoreae which was not observed to modify expression and Radix Angelicae Sinensis. The opposite effects to that seen in the literature were observed here with downregulation of ERα and upregulation of PRβ (182).

It would appear that the steroid receptors ERα and PRβ are affected by the treatment but whether this is advantageous to receptivity or not would depend on the stage of the cycle. Downregulation of ERα would be beneficially early in the cycle and again from the time of ovulation and through implantation but needs to be upregulated for migration and proliferation during the secretory phase (149). Upregulation of PRβ would support the changes required for differentiation of the vessels (84), implantation (167) and maintenance (169) of the pregnancy but would be inhibitory to the migration and differentiation during the middle part of the secretory phase.

In specific disease processes these actions may be of benefit. Downregulation of ERα may be appropriate when treating luteal phase defects and polycystic ovarian syndrome (135). Upregulation of PRβ could be appropriate in endometriosis (140).

The observations here are without the influence of circulating sex steroids, in a very discrete cell type, in one part of a complex of tissues, and in a transformed endometrial cancer cell line. Circulating sex steroids may significantly modify observed influences. Oestrogenic effects might be expected since Rhizoma Dioscoreae contains a steroidal glycosides diosgenin known to have oestrogenic effects (318) and this could lead to changes in oestrogen receptor expression although diosgenin has been shown not to affect ER expression in rat uterus (319). Oestrogen receptors can be upregulated or downregulated by oestrogen dependent on their co factors (320). The receptors themselves are interdependent and any pre-existing disease state, as well as time of cycle, will change the appropriateness of and upregulation or downregulation of receptors in a cycling endometrium.

It can be said that there is an influence on the steroid receptors which could contribute to the changes seen in receptivity of the endometrium in clinical studies

of this formula. The influences observed here would be most beneficial in the ovulatory and post ovulatory phase when ERα is downregulated and PRβ is upregulated. Radix Paeoniae, Radix Angelicae Sinensis, Sclerotium Poria Cocos all showed these effects on ERα and PRβ. Cortex Moutan additionally downregulated ERα and Rhizoma Rehmanniae upregulated PRβ. GSDW showed upregulation of PRβ at 24 h. The whole formula did not show any other consistent changes.

5.11 The whole and the parts

A summary (Table 5-1) is presented of the assays, the outcomes, and a recap of the evidence from the introduction, Figure 5.2, p208 shows a pictorial summary. There is some congruence between the evidence in the literature and that presented here but also discordance. Radix Paeoniae Alba, Fructus Corni, Rhizoma Alismatis stand out as herbs seen to have effect in the assays but are poorly represented in the literature as is GSDW. Radix Angelicae Sinensis is well researched and the evidence here is much the same as has been seen in previous studies (107, 145, 176, 321-323). Rhizoma Dioscoreae was the opposite, much was expected (123, 154, 177, 179) but little shown in these assays.

GSDW was chosen as one formula amongst many that could have been selected. As seen in the Section 1.12 each research study uses a different formula. This is typical of the method of treating in Chinese medicine; a formula is selected for the patient not the disease and then modified for them specifically. It is frequently observed in teaching, that when multiple students are presented with a case they will choose different base formulae. They then modify them for the patient to end up with very similar final herbs. GSDW has been used in clinical research, recommended in core texts on the subject and in the authors clinic and personal practice. The conclusions reached for this specific formula are not intended to be definitive answer for treating infertility but signposts to a deeper understanding of the potential effects of this medicine.

Table 5-1 Summary of herb functions and literature review

Herb extract	Research outcomes from this study			Pharmacological research from literature		
	Haemostasis	Angiogenesis	Steroid receptors	Haemostasis Table 1-2, p14	Angiogenesis Table 1-3, p36	Steroid receptors 1.11.2
Gui Shao Di Huang Wan	Increase PT and aPTT	Differentiation HUVECs all parameters and HUtMECs all parameters Proliferation Migration	Upregulate PRβ 24 h			
Radix Paeoniae Alba		Differentiation HUtMECs segments, mesh area, total length	Upregulate PRβ 48 h	Yes		
Radix Angelicae Sinensis	Increase aPTT	Differentiation HUtMECs mesh	Downregulate ERα 48 h Upregulate PRβ 48 h	Yes	Yes	Yes
Sclerotium Poria Cocos		Differentiation HUtMECs segment, mesh, total length Migration	Downregulate ERα 48 h Upregulate PRβ 48 h		Yes	
Cortex Moutan		Differentiation HUtMECs mesh	Downregulate ERα 48 h	Yes	Yes	
Radix Rehmanniae Preparata		Differentiation HUtMECs mesh, total length Migration	Upregulate PRβ 48 h	Yes	Yes	
Fructus Corni	Increase aPTT Increase PT	Differentiation HUtMECs junctions Migration			Yes	
Rhizoma Dioscoreae		Differentiation HUtMECs mesh		Yes		Yes
Rhizoma Alismatis		Differentiation HUtMECs segment, mesh, total length Migration				

In the haemostasis assays, there was a dose dependent increase in time to clot formation in both PT and aPTT. When compared to heparin a 1U and 1/10th U were used. The pharmacokinetics of heparin with a short half-life of 90 min and non selective binding (195) mean that the plasma concentration is unlikely to be at 1U for long. PT had a similar effect as a 1U and for the aPTT to a 1/10th, the 1U did not form a clot within 15 min.

In the PT assay the whole formula GSDW had a significant increase over control and this increase was similar to that of Fructus Corni so this herb may convey the full effects seen in the formula. The aPTT was increased by the whole formula and this effect appears to be greater than the individual herbs which might be attributable to polyvalent activity. Some interaction is occurring between the herbs that create an prolonging of time to clot formation that is not seen in any of the individual herb extracts. Two herbs, Radix Angelicae Sinensis and Fructus Corni showed an increase

in time to clot formation, but a simple addition of the effect of those two over control was not equal to that of the whole formula. Research would suggest that Radix Angelicae Sinensis would be anti-thrombotic and time to clot formation in both assays was increased. In TCM terms it is a mild blood mover and so this action concurs with tradition.

A greater effect was observed with Fructus Corni for which no research suggesting such action was found. It is an astringent in TCM theory, it contains the fluid and is used for excessive bleeding which does not concur with antithrombotic action.

When looking at angiogenesis the proliferative effects were only explored in whole formula. In differentiation assays the HUVECs showed greater effects for all parameters for GSDW than the control but the individual herb extracts had a mean effect both higher and lower than control although no others showed significant differences. In the HUtMECs the situation was different as might be expected in heterogenous cells. The effects of GSDW was significantly greater than for control but not as great as for some of the individual herb extracts.

In the migration assays, there were preliminary results that suggest further work might yield results but resourcing of cells and of time lapse prevented this being undertaken. The GSDW did show an effect at a low dose 0.00087 mg/mL
and with the individual herb extracts Radix Rehmanniae Preparata, Fructus Corni and Rhizoma Alismatis.

Oestrogen and progesterone expression were affected by the whole formula and by the individual herb extracts. Fewer changes were observed in the whole formula than in the other assays and this would be consistent with the upregulation by some herb extracts and downregulation by others. In such a complex and interrelated system as the hormonal regulation of angiogenesis conclusions are difficult to draw from these studies except that receptor expression is modified by the herb extracts.

GSDW does have demonstrable effects on measures of haematology that would provide rationale for the positive effect seen in clinical trials as is summarised in Figure 5.2. There was increased time to clot formation, increased vascular density in angiogenesis, and modification of steroid receptors. Increases in uterine perfusion presented here are linked to the uterus but ovarian function also requires good blood flow and so results might be generalisable to oocyte and corpus luteum development. It should be used with caution with other anti-thrombotic medications as it may have an additive effect but it has potential to be used as an antithrombotic in IVF to treat the pro-coagulable state induced by supra physiological oestradiol levels, where uterine perfusion is poor, or with recurrent implantation failures.

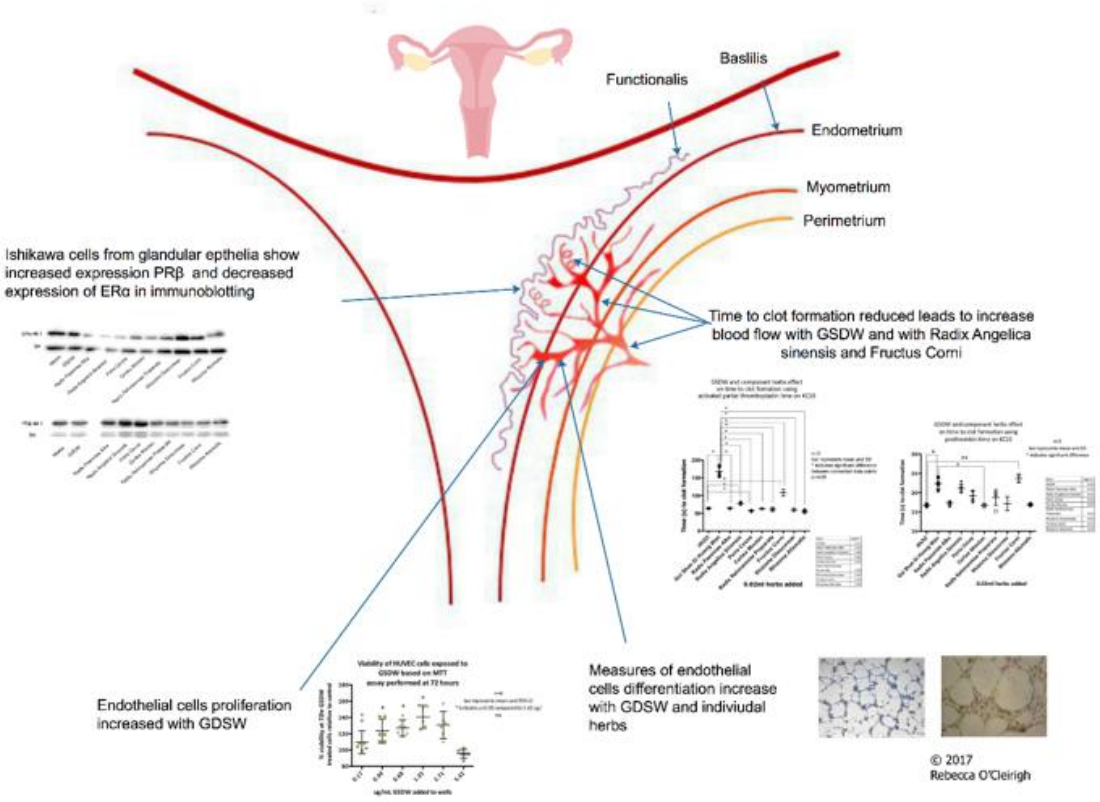


Figure 5.2 Summary of research presented on the effects of GSDW on the haematology of the endometrium in the PT, aPTT, MTT, differentiation, and immunoblotting assays.

A compound is complex, a herb complete with all its compounds is both variable and complex, the cells and organism, the patient and their disease are all variable and complex. A reductionist approach to research disregards the complexity at every level

of the natural environment in which we exist. Even if an effect is seen *in vitro* it may not be *in vivo* and vice versa but this does not make the approach worthless. It too has a place, but as a one piece of evidence not a definitive answer.

The Chinese philosophical thinkers look at the complexity of the universe and us as a microcosm of that. They look for patterns and commonalities at a higher level and try to observe similarities and trends. Plant medicines exist because of experimentation and systematic observation of effects. Conventional science looks at the nuance of a cell's behaviour to understand the organism and its disease, but should this be considered "higher evidence"? Approaching the question of the usefulness of the herbs from the opposite poles of a clinical trial as well as *in vitro* cellular assay can lead to greater confidence in the outcomes of both where they concur. By combining the use of herbal formulae, the science behind them and the science of the reproductive technologies, it might be possible to harness the best of both systems in order to meet their shared goal of offering women with fertility problems a hope of becoming mothers.

5.12 Future work

5.12.1 Systems biology and the -omics

Systems biology is an emerging method of investigation which utilises novel techniques such as metabolomics and metabonomics that allow for the analysis of an entire system. This is possible using techniques such as nuclear magnetic resonance spectroscopy, liquid, and gas chromatography mass spectrometry to identify variance in a wide range of biomarkers. TCM faces severe challenges due to a lack of scientific and technical research restricting development across the world (324). However, TCM is getting more popular and has advantages in early intervention, combination therapy and personalised medicine and as these are backed up by evidence from the new -omics technologies they may gain wider acceptance. Systems biology and specifically metabolomics is the ultimate phenotyping and provides a methodology for the examination of complex interventions on complex systems. This creates a bridge between TCM and molecular pharmacology leading to the possibility of a science based practice of herbal medicine (325). Genomics, proteomics, metabolomics are technologies which over the long term may economise the evidence of efficacy without the active compound principle; the outputs of these technologies raises questions over the appropriateness of the reductionist style of investigation (20).

Zhang et al. (326) describes how metabolomics represents the metabolite complement of an integrated living system and can be used to analyse the responses to both endogenous and exogenous factors. This top down strategy of metabolomics coincides with the holistic thinking of Chinese medicine. According to metabonomics (the analysis of drug responses through metabolomic profiling) there are identifiable metabolic patterns of disease and these can be correlated with TCM diagnoses (327) of syndromes such as that of Liver Qi stagnation (328) and Kidney Yang deficiency (329).

Herbalomics has been described as a system for studying the pharmacological effects, mechanisms, safety, and compatibility of Chinese medicines using a systems biology approach. The strategy of herbalomics is to analyse the plant for as many compounds as possible using chromatographic techniques. These plants can then be tested for bioactivity in cell lines, animal models, or humans using body fluids such as saliva, urine, and blood. Then a technique known as multivariate data analysis enables the visualisation and interpretation of patterns between compounds and bioactivity (325).

A study using metabolomics has cross referenced specific metabolic patterns that relate to the syndromes of TCM theory. The study looked at the formula Liu Wei Di Huang Wan which is the base formula from which GSDW is created. A rat model of Kidney Yin deficiency was induced using thyroxine and reserpine. This study used liquid chromatography to analyse the urine of rats that had induced Kidney Yin deficiency that were untreated, treated using the herbal formula as well as normal controls. They were able to characterise the metabolite changes from healthy rats to the induced Kidney Yin deficiency state and then to the treated rats. Subsequently they identified twenty differentiating metabolites using an orthogonal projection to latent structures discriminate analysis (330).

TCM modulates functional units within the body and in thinking about disease and especially developing drugs from TCM herbs this complexity needs to be respected. A model called instance based mutual information was proposed by Li et al. (331) to map relationships between herbs, genes and disease. Exploring relationships between herbs within formulae, in particular looking at angiogenesis, they found that Radix Angelicae Sinensis, Radix Paeoniae Alba and Radix Rehmanniae Preparata are pro angiogenic. Also, that the biological activities of the six herbs of Liu Wei Di Huang Wan are connected closely with genes in cancer pathways and neuro-endocrine-immune pathway, which are the diseases frequently treated with this formula.
Identification of molecular mechanisms is going to be a key step in validating the use of herbal products. Another advantage of the -omics analyses are that they can be performed in human studies looking at metabolites in urine (25), post administration

of the formula in a fashion consistent with its clinical use. This study does not use the -omics technologies but they have great potential for future work. The richness of the data produced by these techniques means mining for meaning is often advanced by understanding where to seek within the data, and these bioassays can provide that data. This study does address complexity at the formula level by analysing the whole and each individual herb in each of the assays to identify any polyvalent interactions.

There is scope within this area of research to answer many more questions. These are just a few of the areas that are most directly connected to continuing to answer the questions posed here as to how the receptivity of the endometrium is improved by Chinese herbal medicines.

Herbs and administration:
Metabolomic studies of urine or blood samples in women after administration of herbs could be used to examine bioavailability and metabolites of the herbs. This would give far greater insight into the likely compounds eliciting the effects seen and possibly narrow a search for the mechanisms. Modifying the formula to be more tailored to Blood stasis and see the effects on the haemostasis or to be more Yang nourishing and to look for effects on PR expression for example.

Haemostasis:
The origins of the changes in the clot morphology have not been found and nor have the mechanisms for the extension of the clotting times. Fractions of the extracts could be used to investigate which compounds are causing the elongation. Methods for examining the fibrin structure such as laser or magnetic tweezers, torsion pendulum, or thromboelastography could be used. Platelet aggregometry could also be performed. Although the research is substantial that the individual herbs will alter platelet aggregation it would be interesting to test the whole formula.

Proliferation:
Exploring proliferation of HUVECs using the individual herbs using the MTT assay, cell counts or the MultiTox Glo assay. Repeating these with GSDW and each herb

extract with the HUtMECs and with other uterine cell types including epithelial cells, culture endometrial cells from biopsy both healthy and diseased.

Migration:

Only preliminary data was obtained for the migration assays and this assay requires optimising, and then repeating with HUtMEC using GSDW and each herb extract. *In vivo* assays of cell migration could then be explored if the results were promising. Migration of epithelial cells could also be explored.

Immunoblotting:

Using total protein normalisation to verify all the results found here. Exploring extraction methods for receptor bound mPRα and the for the G protein-coupled oestrogen receptor. Looking at expression in cultured endometrial cells from biopsy form healthy and infertile women. Looking at receptor expression in epithelial cells. Probing for a wider range of molecules involved in implantation such as VEGF, integrin $\alpha V\beta 3$ and MMP's

6 Conclusions

Studies exist demonstrating positive outcomes of studies both as RCT's, retrospective analyses and meta-analyses of the impact of Chinese herbs on fertility both natural and alongside IVF. The methodologies of these studies and the numbers of participants have been criticised but they contribute to a growing body of evidence about the use of Chinese herbs. Studies such as these have not been undertaken in the west. This study is also unusual in its approach of analysing both the whole formula and components parts on the processes that underlie changes that have been observed on the endometrium.

In vitro cell assays have their limitations, but in light of a clinically observed improvement in endometrial uterine perfusion and the small number of studies indicating evidence of increased pregnancy rates this research indicates mechanisms by which the effects of Gui Shao Di Huang Wan may be mediated. The herbs are suggested to have an anticoagulant action leading to improved uterine perfusion. There is increased proliferation of endothelial cells and increased differentiation shown in tubule numbers, junction number, length and mesh area suggesting improvements in the vascularity of the endometrial tissues. Modification of the oestrogen and progesterone receptors may also contribute to the increased receptivity. These actions have the potential to lead to the observed increases endometrial receptivity, conception, and pregnancy. All of the effects are interlinked *in vivo* with uterine perfusion stimulating tubule development, flow of blood delivering sex steroids to the receptors, the receptors stimulating proliferation and differentiation. In two of the assays the combination of herbs within the formula was seen to have a greater effect than the herbs individually. This suggests a polyvalent activity that might be lost if, instead of the formula, a herb or compound was defined as the "active". This study offers a pharmacological perspective on key mechanisms from which further research into possible effects of Chinese herbal medicines and their use in infertility can be examined, contributing a different perspective to the limited clinical trials currently available.

7 References

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8 Appendices

I. KEW IDENTIFICATION OF SAMPLES

Name of sample provided by Rebecca O'Cleirigh	Macroscopic authentication provided by Christine Leon, RBG Kew 15/7/2016
Bai Shao	**good match with Kew's authentic samples of Bai Shao (i.e. sliced root of *Paeonia lactiflora* Pall.).**
Dang Gui	**good match with Kew's authentic samples of Dang Gui (i.e. longitudinally sliced root of *Angelicae Sinensis* (Oliv.) Diels ('head, body and tail').**
Fu Ling	**good match with Kew's authentic samples of Fu Ling (i.e. *Wolfiporia extensa* (Peck) Ginns [synonym: *Sclerotium Poria Cocos* (Schw.) Wolf]).**
Mu Dan Pi	**good match with Kew's authentic samples of Mu Dan Pi (i.e. transversely sliced root-bark of *Paeonia suffruticosa* Andrews or *Paeonia ostii* T. Hong & J.X. Zhang) - both are official.**
Shu Di	**good match with Kew's authentic samples of Shu Di Huang (i.e. root of *Rehmanniae glutinosa* (Gaertn.) Libosch. ex Fisch. & C.A. Meyer processed with yellow-rice wine).**
Shan Yao	**good match with Kew's authentic samples with Kew's authentic samples of Shan Yao (i.e. sliced rhizome of *Dioscoreae polystachya* Turcz. [synonym: *D. batatas* Decne.] structure, taste, and fracture seem consistent.**
Shan Zhu Yu	**good match with Kew's authentic samples of Jiu Shan Zhu Yu (i.e. fruit of *Cornus officinalis* Siebold & Zuccarini wine).**
Ze Xie	**good match with Kew's authentic samples of Ze Xie (i.e. sliced rhizome of *Alisma orientale* (Sam.) Juz.)**

II. HERBS REFERRED TO IN THIS STUDY WITH PINYIN, SCIENTIFIC NAME, AND AUTHORITY

Latin	Pin Yin	Scientific name and authority
Carapax et Plastrum testudines	Gui Ban	Tortoise shell
Colla Corii asini	E Jiao	Donkey hide gelatine
Cornu cervi	Lu Jiao	Deer antler
Cortex Cinnamomi	Rou Gui	Cinnamomum cassia (L.) J. Presl
Cortex Eucommiae	Du Zhong	Eucommia ulmoides Oliv.
Cortex Moutan	Mu Dan Pi	*Paeonia ostii* T. Hong & J. X. Zhang
Flos Carthami	Hong Hua	Carthamus tinctorius L.
Fluoritum	Zi Shi Ying	Fluorite
Folium Artemisiae argyi	Ai Ye	*Artemisia argyi* H. Lév. & Vaniot
Fructus Amomi	Sha Ren	Aframomum angustifolium (Sonn.) K. Schum.
Fructus Corni	Shan Zhu Yu	*Cornus officinalis* Siebold & Zuccarini[1]
Fructus Ligustri Lucidi	Nu Zhen Zi	Ligustrum lucidum W.T. Aiton
Fructus Lycii	Gou Qi Zi	Lycium barbarum L.
Fructus Rubi	Fu Pen Zi	Rubus Chingii Hu
Herba Eclipta Alba	Han Lian Cao	Eclipta prostrata (L.) L.
Herba Epimedii	Yin Yang Huo	Epimedium brevicornu Maxim
Herba Taxilli	Sang Ji Sheng	Taxillus chinensis (DC.) Danser
Placenta hominis	Zi He Che	Human placenta
Sclerotium Poria Cocos	Fu Ling	Wolfiporia extensa (Peck) Ginns
Radix Aconiti Preparata	Zhi Fu Zi	Aconitum carmichaelii Debeaux
Radix Angelicae Sinensis	Dang Gui	Angelicae Sinensis (Oliv.) Diels
Radix Astragali seu Hedysari	Huang Qi	Astragalus propinquus Schischkin
Radix Bupleuri	Chai Hu	Bupleurum chinense DC
Radix Codonopsis	Dang Shen	*Codonopsis affinis* Hook.f. & Thomson
Radix Dipsaci	Xu Duan	Dipsacus inermis Wall.
Radix Ginseng	Ren Shen	Panax ginseng C.A. Mey
Radix Glycyrrhizae Preparata	Zhi Gan Cao	Glycyrrhiza glabra L.
Radix Ligusticum wallichii	Chuan Xiong	Ligusticum striatum DC
Radix Morindae Officinalis	Bai Ji Tian	Morinda officinalis F.C. How
Radix Paeoniae Alba	Bai Shao	Paeonia lactiflora Pall.
Radix Paeoniae Rubra	Chi Shao	Paeonia lactiflora or veitchii Pall
Radix Polygoni Multiflori	He Shou Wu	Reynoutria multiflora (Thunb.) Moldenke
Radix Rehmanniae Preparata	Shu Di Huang	*Rehmannia glutinosa* (Gaertner) Liboschitz ex Fischer & C. A. Meyer
Radix Salviae miltiorrhizae	Dan Shen	Salvia miltiorrhiza Bunge
Ramulus Cinnamomi	Gui Zhu	Cinnamomum cassia (L.) J. Presl
Ramulus Loranthi	Sang Ji Sheng	Ramulus Loranthi Seu Visci
Rhizoma Alismatis	Ze Xie	*Alisma orientale* (Samuelsson) Juzepczuk
Rhizoma Atractylodis Macrocephalae	Bai Zhu	Atractylodes macrocephala Koidz.
Rhizoma Corydalis	Yan Hu Suo	*Corydalis yanhusuo* Y.H. Chou & Chun C. Hsu) W.T. Wang ex Z.Y. Su & C.Y. Wu
Rhizoma cyperi	Xiang Fu	Cyperus rotundus L.
Rhizoma Dioscoreae	Shan Yao	Dioscoreae polystachya Turczaninow
Semen Cassiae	Jue Ming Zi	*Senna obtusifolia* (L.) H.S. Irwin & Barneby
Semen Cuscutae	Tu Si Zi	Cuscuta chinensis Lam
Semen Pruni Persicae	Tao Ren	Prunus persica (L.) Batsch

III. LIST OF COMPOUNDS PRESENT IN THE 8 HERBS OF GUI SHAO DI HUANG WAN

Compound	Herb
(24R)-α-methyl cholest-8(14)-enol	Rhizoma Dioscoreae
(24R)-α-methyl cholestanol	Rhizoma Dioscoreae
(24S)-β-methyl cholest-8(14)-enol	Rhizoma Dioscoreae
(E)-4-(5-(hydroxymethyl) furan-2-yl) but-3-en-2-one	Radix Rehmanniae Preparata
(E)-4-(5-(methoxymethyl) furan-2-yl) but-3-en-2-one	Radix Rehmanniae Preparata
1-O-Galloyl pedunculagin	Radix Paeoniae Alba
1,2,3-tri-*O*-galloyl-*β*-*D*-glucose	Fructus Corni
1,2,3,4,6-penta-o-galloyl glucose	Cortex Moutan
1,2,3,4,6-penta-o-galloyl-beta-*D*-glucose	Radix Paeoniae Alba
1,2,3,6-tetra-o-galloyl-*β*-*D*-glucose	Fructus Corni
1,2,6-tri-*O*-galloyl-*β*-*D*-glucose	Fructus Corni
1,5-bis(5-methoxymethyl) furan-2-yl-penta-1,4-dien-3-en-2-one	Radix Rehmanniae Preparata
11-alpha, 12-alpha-epoxy-3*β*,23 dihydroxy-30-norolean-20(29)-en-13 *β* -olode	Radix Paeoniae Alba
11 alpha, 12alpha-epoxy-3 *β*,23dihudroxolean-28,13 *β* olide	Radix Paeoniae Alba
13-methyl tetradecanoic acid	Radix Paeoniae Alba
2-methyl-dodecane-5-one	Radix Angelicae Sinensis
2-Phenylethyl glycosides	Radix Rehmanniae Preparata
2,3-di-O-galloyl-*β*-*D*-glucose	Fructus Corni
2,3-dicresol	Radix Angelicae Sinensis
2,3-dihydroxy-4-methoxyacetophenone	Cortex Moutan
2,3-dimethylphenol	Radix Angelicae Sinensis
2,4-dihydroxyacetophenone	Radix Angelicae Sinensis
2'-acetylacteoside	Radix Rehmanniae Preparata
24-trien-21-oic acid	Sclerotium Poria Cocos
3-hydroxy-4-methoxyacetophenone	Cortex Moutan
3,4-dihydroxy-β-phenethyl-*O*-*α*-*L*- rhamnopyranosyl-(1 -> 3)-*O*-*β*-*D*-galactopyranosyl-(1 -> 6)-4-*O*-caffeoyl-*β*-*D*-glucopyranoside	Radix Rehmanniae Preparata
3,4-seco-lanostanes	Sclerotium Poria Cocos
3,5-dihydroxybenzoic acid	Fructus Corni
30-norhederagenin	Radix Paeoniae Alba
3B-hydroxy olean-12-en-28-al	Radix Paeoniae Alba
3B-p-hydroxybenzoyl	Sclerotium Poria Cocos
3β-hydoxylanosta-7,9(11),24-trien-21-oic acid	Sclerotium Poria Cocos
3β-p-hydroxybenzoyldehydrotumulosic acid	Sclerotium Poria Cocos
4-ethylresorcinol	Radix Angelicae Sinensis
4-Methoxy-1,2-benzodioxole	Fructus Corni
4-terpineol	Fructus Corni
5-hydroxy aeginetic acid	Radix Rehmanniae Preparata
5-Hydroxymethylfurfural	Radix Rehmanniae Preparata
5-methyl furfural	Fructus Corni
6-*O*-E-feruloylajugol	Radix Rehmanniae Preparata
6-*O*-p-coumaroylajugol	Radix Rehmanniae Preparata
6-*O* -sec-hydroxyaeginetoyl ajugol	Radix Rehmanniae Preparata
6-*O* -sec-hydroxybenzoyl ajugol	Radix Rehmanniae Preparata
6-*O*-vanilloylajugol	Radix Rehmanniae Preparata
6-*O*-Z-feruloylajugol	Radix Rehmanniae Preparata
7-*O*-methyl morronoside	Fructus Corni
8-epiloganic acid	Radix Rehmanniae Preparata
abscisin	Rhizoma Dioscoreae
acetoside	Radix Rehmanniae Preparata
acetylcatalpol	Radix Rehmanniae Preparata
acoradiene	Radix Angelicae Sinensis
adenine	Sclerotium Poria Cocos
adenine	Radix Angelicae Sinensis
adenosine	Radix Rehmanniae Preparata
aeginetic acid	Radix Rehmanniae Preparata
ajugol	Radix Rehmanniae Preparata
ajugoside	Radix Rehmanniae Preparata
albiflorin	Radix Paeoniae Alba
alismaketone C 23 acetate	Rhizoma Alismatis
alisman SI and PII	Rhizoma Alismatis

alismol	Rhizoma Alismatis
alismoxide	Rhizoma Alismatis
alisol A	Rhizoma Alismatis
alisol A 24 acetate	Rhizoma Alismatis
alisol B	Rhizoma Alismatis
alisol B 23 acetate	Rhizoma Alismatis
alisol C	Rhizoma Alismatis
alisol C 23acetate	Rhizoma Alismatis
alisol D	Rhizoma Alismatis
alisol E	Rhizoma Alismatis
allantoin	Rhizoma Dioscoreae
alloocimene	Radix Angelicae Sinensis
α-pinene	Fructus Corni
anethol	Fructus Corni
angelica ketone	Radix Angelicae Sinensis
angelicide	Radix Angelicae Sinensis
angelicin	Radix Angelicae Sinensis
anisic acid (methoxybenzoic acid)	Radix Angelicae Sinensis
anthraquinone	Rhizoma Alismatis
apiopaeonoside	Cortex Moutan
arachidic acid	Radix Rehmanniae Preparata
astragalin	Radix Paeoniae Alba
aucubin	Radix Rehmanniae Preparata
azelaic acid	Radix Angelicae Sinensis
β Sitosterol	Cortex Moutan
β Sitosterol	Fructus Corni
β Sitosterol	Radix Angelicae Sinensis
β Sitosterol	Radix Paeoniae Alba
β Sitosterol	Radix Paeoniae Alba
β Sitosterol	Radix Rehmanniae Preparata
β Sitosterol	Rhizoma Alismatis
β Sitosterol	Rhizoma Dioscoreae
β -amyrin acetate	Sclerotium Poria Cocos
β -bisabolene	Radix Angelicae Sinensis
β -ocimene-X	Radix Angelicae Sinensis
β -pinene	Fructus Corni
β -sitosterol *β* -*D*- glucopyranoside	Cortex Moutan
β -sitosterol *D* -glucoside	Radix Angelicae Sinensis
batatasin I	Rhizoma Dioscoreae
batatasin IV	Rhizoma Dioscoreae
behenic acid (docosanoic acid)	Radix Rehmanniae Preparata
benzoic acid	Cortex Moutan
benzoic acid	Radix Paeoniae Alba
benzoyloxypaeoniflorin	Cortex Moutan
benzoylpaeoniflorin	Cortex Moutan
benzoylpaeoniflorin	Radix Paeoniae Alba
benzyl cinnamate	Fructus Corni
benzyl ethyl alcohol	Fructus Corni
beta glucan	Sclerotium Poria Cocos
betulinic acid	Radix Paeoniae Alba
bicycloelemene	Radix Angelicae Sinensis
biotin (vitamin H, coenzyme R, B7)	Rhizoma Alismatis
brefeldin A	Radix Angelicae Sinensis
caffeic acid stearyl ester	Cortex Moutan
calyosin	Radix Angelicae Sinensiss
campesterol	Radix Rehmanniae Preparata
camphene	Fructus Corni
camphene	Radix Angelicae Sinensis
camphoric acid	Radix Angelicae Sinensis
capric acid (decanoic acid)	Radix Rehmanniae Preparata
caprylic acid	Sclerotium Poria Cocos
caprylic acid (octanoic acid)	Radix Rehmanniae Preparata
carvacrol	Radix Angelicae Sinensis
catalpol	Radix Rehmanniae Preparata
celebroside	Radix Rehmanniae Preparata
chamigrene	Radix Angelicae Sinensis
cholestanol	Rhizoma Dioscoreae
cholesterol	Rhizoma Dioscoreae
choline	Sclerotium Poria Cocos
choline	Radix Angelicae Sinensis

choline	**Rhizoma Dioscoreae**
cinnamic acid	**Radix Rehmanniae Preparata**
cis-2,5- dicarboxylic acid	**Fructus Corni**
cistanoside A	**Radix Rehmanniae Preparata**
cistanoside F	**Radix Rehmanniae Preparata**
clerosterol	**Rhizoma Dioscoreae**
cnidilide	**Radix Angelicae Sinensis**
cornin	**Fructus Corni**
cornuside	**Fructus Corni**
cornusiins A, B, C, G,	**Fructus Corni**
cresol	**Radix Angelicae Sinensis**
cuparene	**Radix Angelicae Sinensis**
***D*-arabinose**	**Radix Rehmanniae Preparata**
***D* -catechin**	**Radix Paeoniae Alba**
***D* -galactose**	**Radix Angelicae Sinensis**
***D* -galacturonic acid**	**Radix Angelicae Sinensis**
***D* -galacturonic acid**	**Radix Rehmanniae Preparata**
***D* -glucosamine**	**Radix Rehmanniae Preparata**
***D* -glucose**	**Radix Angelicae Sinensis**
***D* -mannitol**	**Radix Rehmanniae Preparata**
***D* -rhamnose**	**Radix Rehmanniae Preparata**
danmelittoside	**Radix Rehmanniae Preparata**
daturic acid (heptadecanoic acid)	**Radix Rehmanniae Preparata**
daucosterol	**Radix Angelicae Sinensis**
daucosterol	**Radix Paeoniae Alba**
daucosterol	**Radix Rehmanniae Preparata**
dehydroeburicoic acid	**Sclerotium Poria Cocos**
dehydrologanin	**Fructus Corni**
dehydromorroniaglycone	**Fructus Corni**
dehydrotrametenolic acid	**Sclerotium Poria Cocos**
deltonin	**Rhizoma Dioscoreae**
deoxypaeonisuffrone	**Cortex Moutan**
dihydrocatalpol	**Radix Rehmanniae Preparata**
dihydroxy-*β*-ionone	**Radix Rehmanniae Preparata**
dihyrophthalic anhydride	**Radix Angelicae Sinensis**
dihyropinosylvin	**Rhizoma Dioscoreae**
dimethyl camphorate	**Radix Angelicae Sinensis**
dimethyl phthalate	**Radix Angelicae Sinensis**
dimethyl sebacate	**Radix Angelicae Sinensis**
dioscin	**Rhizoma Dioscoreae**
diosgenin	**Rhizoma Dioscoreae**
diosgenin dioglucoside	**Rhizoma Dioscoreae**
dodecanol	**Radix Angelicae Sinensis**
dopamine (3,4-dihydroxyphenyl ethyl amine)	**Rhizoma Dioscoreae**
eburicoic acid	**Sclerotium Poria Cocos**
echinacoside	**Radix Rehmanniae Preparata**
elemicin	**Fructus Corni**
epialisol A	**Rhizoma Alismatis**
ergosterol	**Sclerotium Poria Cocos**
ethyl-*α*-*D*-galactoside	**Radix Rehmanniae Preparata**
ethylphenol	**Radix Angelicae Sinensis**
eugeniin	**Radix Paeoniae Alba**
eugenol methyl ether	**Fructus Corni**
ferulic acid	**Radix Angelicae Sinensis**
folinic acid	**Radix Angelicae Sinensis**
formyltetrahydrofolate	**Rhizoma Alismatis**
forsythiaside	**Radix Rehmanniae Preparata**
furfural	**Fructus Corni**
furfural	**Rhizoma Alismatis**
gallic acid	**Cortex Moutan**
gallic acid	**Fructus Corni**
gallic acid	**Radix Paeoniae Alba**
gallotanin	**Radix Paeoniae Alba**
galloyloxypaeoniflorin	**Cortex Moutan**
gemin *D*	**Fructus Corni**
geniposide	**Radix Rehmanniae Preparata**
glucosamine	**Radix Rehmanniae Preparata**
glutinoside	**Radix Rehmanniae Preparata**
gracillin	**Rhizoma Dioscoreae**
guaiacol	**Radix Angelicae Sinensis**

hederagenin	**Radix Paeoniae Alba**
heneicosanoic acid	**Radix Rehmanniae Preparata**
histidine	**Sclerotium Poria Cocos**
isoacetoside	**Radix Rehmanniae Preparata**
isoamyl alcohol	**Fructus Corni**
isobutanol	**Fructus Corni**
isobutyl alcohol	**Fructus Corni**
isocnidilide	**Radix Angelicae Sinensis**
isoeugenol	**Radix Angelicae Sinensis**
isofucosterol	**Rhizoma Dioscoreae**
isoterchebin	**Fructus Corni**
jiocarotenoside A1, A2,	**Radix Rehmanniae Preparata**
jiofuran	**Radix Rehmanniae Preparata**
jioglutins A-E,	**Radix Rehmanniae Preparata**
jioglutosides A, B.	**Radix Rehmanniae Preparata**
l-rhamnose	**Radix Rehmanniae Preparata**
lactiflorin	**Radix Paeoniae Alba**
lathosterol	**Rhizoma Dioscoreae**
lauric acid (dodecanoic acid)	**Sclerotium Poria Cocos**
lauric acid (dodecanoic acid)	**Radix Rehmanniae Preparata**
leonurine	**Radix Rehmanniae Preparata**
***(z)*-ligustilide**	**Radix Angelicae Sinensis**
limonene	**Fructus Corni**
linoleic acid	**Fructus Corni**
linoleic acid	**Radix Angelicae Sinensis**
linoleic acid	**Radix Rehmanniae Preparata**
loganin	**Fructus Corni**
malic acid	**Fructus Corni**
mannan	**Rhizoma Dioscoreae**
mannotriose	**Radix Rehmanniae Preparata**
margaric acid (Heptadecanoic acid)	**Radix Rehmanniae Preparata**
martynoside	**Radix Rehmanniae Preparata**
melittoside	**Radix Rehmanniae Preparata**
methyl gallate	**Radix Paeoniae Alba**
mioporosidegenin	**Radix Rehmanniae Preparata**
morroniside	**Fructus Corni**
mudanoside B	**Cortex Moutan**
mudanpinoic acid A	**Cortex Moutan**
mudanpiosides A	**Cortex Moutan**
mudanpiosides B	**Cortex Moutan**
mudanpiosides C	**Cortex Moutan**
mudanpiosides E	**Cortex Moutan**
mudanpiosides F	**Cortex Moutan**
myrcene	**Radix Angelicae Sinensis**
myristic acid	**Radix Angelicae Sinensis**
myristic acid	**Radix Rehmanniae Preparata**
n-butylphthalide	**Radix Angelicae Sinensis**
n-nonadecanoic acid	**Radix Rehmanniae Preparata**
n-tetracosanoic acid	**Radix Angelicae Sinensis**
n-valerophenone-O-carboxylic acid	**Radix Angelicae Sinensis**
nicotinic acid	**Radix Angelicae Sinensis**
nonanoic acid	**Radix Rehmanniae Preparata**
oleanic acid	**Cortex Moutan**
oleanic acid	**Fructus Corni**
oleanic acid	**Radix Paeoniae Alba**
oleic acid	**Radix Angelicae Sinensis**
orientalols A-E (Sulfoorientalols)	**Rhizoma Alismatis**
oxypaeoniflorin	**Cortex Moutan**
oxypaeoniflorin	**Radix Paeoniae Alba**
p-cymene	**Fructus Corni**
pachyman	**Sclerotium Poria Cocos**
pachymaran	**Sclerotium Poria Cocos**
pachymic acid	**Sclerotium Poria Cocos**
paeoniflorigenone	**Radix Paeoniae Alba**
paeoniflorin	**Cortex Moutan**
paeoniflorin	**Radix Paeoniae Alba**
paeonilactone A	**Radix Paeoniae Alba**
paeonin	**Radix Paeoniae Alba**
paeonisothujone	**Cortex Moutan**
paeonisuffral	**Cortex Moutan**

paeonisuffron	**Cortex Moutan**
paeonol	**Cortex Moutan**
paeonol	**Radix Paeoniae Alba**
paeonoside	**Cortex Moutan**
palmitic acid	**Sclerotium Poria Cocos**
palmitic acid (hexadecanoic acid)	**Fructus Corni**
palmitic acid (hexadecanoic acid)	**Radix Angelicae Sinensis**
palmitic acid (hexadecanoic acid)	**Radix Rehmanniae Preparata**
palmitoleic acid	**Radix Rehmanniae Preparata**
pedunculagin	**Radix Paeoniae Alba**
pentadecanoic acid	**Radix Rehmanniae Preparata**
peonolide	**Cortex Moutan**
phenethyl alcohol	**Fructus Corni**
phenol	**Radix Angelicae Sinensis**
phenylacetic acid	**Radix Rehmanniae Preparata**
phenylalanine	**Radix Paeoniae Alba**
phthalic anhydride	**Radix Angelicae Sinensis**
phytic acid	**Rhizoma Dioscoreae**
pinicolic acid	**Sclerotium Poria Cocos**
plantamajoside	**Radix Rehmanniae Preparata**
polyporenic acid C	**Sclerotium Poria Cocos**
poriaic acid A, B, C	**Sclerotium Poria Cocos**
poricoic acid A, B, D, AM	**Sclerotium Poria Cocos**
protogracillin	**Rhizoma Dioscoreae**
pulegone	**Fructus Corni**
pyrethrin II	**Radix Paeoniae Alba**
quercetin	**Cortex Moutan**
raffinose	**Radix Rehmanniae Preparata**
rehmaglutins A, B, C, D,	**Radix Rehmanniae Preparata**
rehmaionosides A	**Radix Rehmanniae Preparata**
rehmaionosides B	**Radix Rehmanniae Preparata**
rehmaionosides C	**Radix Rehmanniae Preparata**
rehmannans A, B, C,	**Radix Rehmanniae Preparata**
rehmannans SA	**Radix Rehmanniae Preparata**
rehmannans SB	**Radix Rehmanniae Preparata**
rehmanniosides A, B, C, D,	**Radix Rehmanniae Preparata**
rehmapicrogenin	**Radix Rehmanniae Preparata**
resacetophenone	**Cortex Moutan**
rhamnose	**Radix Angelicae Sinensis**
salidroside	**Radix Rehmanniae Preparata**
scopoletin	**Radix Angelicae Sinensis**
sebacic acid	**Radix Angelicae Sinensis**
sec-hydroxyaeginetic acid	**Radix Rehmanniae Preparata**
stachyose	**Radix Rehmanniae Preparata**
stearic acid	**Radix Angelicae Sinensis**
stearic acid	**Radix Rehmanniae Preparata**
stigmasterol	**Radix Angelicae Sinensis**
stigmasterol	**Radix Rehmanniae Preparata**
stigmasterol	**Rhizoma Alismatis**
Stigmasterol	**Rhizoma Dioscoreae**
stigmasterol-β-*D*-glucoside	**Radix Angelicae Sinensis**
succinic acid	**Radix Angelicae Sinensis**
succinic acid	**Radix Rehmanniae Preparata**
suffructicosa A-E	**Cortex Moutan**
sulfoorientalols A-D	**Rhizoma Alismatis**
sweroside	**Fructus Corni**
tartaric acid	**Fructus Corni**
tellimagrandin I	**Fructus Corni**
tellimagrandin II	**Fructus Corni**
tetrahydrofuran	**Fructus Corni**
trametenolic acid	**Sclerotium Poria Cocos**
trans-*β*-farnesene	**Radix Angelicae Sinensis**
trans-linalool oxide	**Fructus Corni**
tumulosic acid	**Sclerotium Poria Cocos**
undecanoic acid	**Sclerotium Poria Cocos**
uracil	**Radix Angelicae Sinensis**
uracil	**Radix Rehmanniae Preparata**
ursolic acid	**Fructus Corni**
vanillic acid	**Radix Angelicae Sinensis**
vanillin	**Radix Angelicae Sinensis**

verbascose	**Radix Rehmanniae Preparata**
verbenalin	**Fructus Corni**
vitamin A	**Fructus Corni**
vitamin A	**Radix Angelicae Sinensis**
vitamin B12	**Radix Angelicae Sinensis**
vitamin B12	**Rhizoma Alismatis**
y-elemene	**Radix Angelicae Sinensis**
α-cedrene	**Radix Angelicae Sinensis**
α-pinene	**Radix Angelicae Sinensis**
β-phellandrene	**Radix Angelicae Sinensis**
ϒ-aminobutyric acid	**Radix Rehmanniae Preparata**

IV. ETHICS APPROVAL

Science Faculty Ethics Committee
Science Faculty Office
University of Portsmouth
St Michael's Building
White Swan Road
PORTSMOUTH
PO1 2DT

023 9284 3379
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19 April 2017

Miss Rebecca O'Cleirigh
School of Pharmacy and Biomedical Science
University of Portsmouth

rebecca.ocleirigh@port.ac.uk

FAVOURABLE ETHICAL OPINION – FOLLOWING RESUBMISSION

Study Title: Searching for synergy with a TCM formula: Gui Shao Di Huang Wan Exploring possible mechanisms of action in relation to endometrial receptivity.

Reference Number: SFEC 2017-037

Date Resubmitted: 10 April 2017

Thank you for resubmitting your application to the Science Faculty Ethics Committee (SEFC) for ethical review in accordance with current procedures, for making the requested changes following the first SFEC review, and for the clarifications provided.

I am pleased to inform you that SFEC was content to grant a favourable ethical opinion of the above research on the basis described in the submitted documents listed at Annex A, and subject to standard general conditions (*See Annex B).*

Please note that the favourable opinion of SFEC does not grant permission or approval to undertake the research. Management permission or approval must be obtained from any host organisation, including the University of Portsmouth or supervisor, prior to the start of the study.

Wishing you every success in your research

Dr Jim House
Chair, Science Faculty Ethics Committee

Annexes

A - Documents reviewed
B - After ethical review - Guidance for researchers

V. IMAGE J DOCUMENTATION FOR ANALYSIS ANALYZER ADD IN

« Image Name » : name of the recorded analyzed image (initial name plus the suffix « -tr »).
« Analysed area »: area of the image or the user selection concerned by the analysis.
« Nb extrem. »: number of extremities in the analyzed area.
« Nb nodes »: number of nodes in the analyzed area.
« Nb Junctions »: number of junctions in the analyzed area.
« Nb master junction »: number of master junctions in the analyzed area.
« Nb master segments »: number of master segments in the analyzed area.
« Tot. master segments length »: sum of the length of the detected master segments in the analyzed area.
« Nb meshes »: number of meshes in the analyzed area.
« Tot.meshes area »: sum of mesh areas detected in the analyzed area.
« Nb pieces »: sum of number of segments, isolated elements and branches detected in the analyzed area.
« Nb segments »: number of segments in the analyzed area.
« Nb branches »: number of branches in the analyzed area.
« Nb isol. seg. »: number of isolated elements in the analyzed area.
« Tot. length »: sum of length of segments, isolated elements and branches in the analyzed area.
« Tot. branching length »: sum of length of the trees composed from segments and branches in the analyzed area.
« Tot. segments length »: sum of length of the segments in the analyzed area.
« Tot. branches length »: sum of length of the branches in the analyzed area.
« Tot. isol. branches length »: sum of length of the isolated elements in the analyzed area.
« Branching interval »: mean distance separating two branches in the trees in the analyzed area. (Tot. segments length / Nb branches).
« Mesh index »: mean distance separating two master junctions in the trees in the analyzed area. (Tot. master segments length / Nb master segments).
« Mean Mesh Size »: mean mesh size in the analyzed area.

VI. MATERIALS LIST

Herb preparation
Millex syringe driven filter units PES 22µm express

Haemostasis
Bell and Alton platelet substitute BAPS040
Diagen Calcium rabbit brain thromboplastin CBRT000/001
Diagen Freeze Dried Rabbit Brain Thromboplastin FRBT010

Cells and media
Thermo scientific Nucleon delta EasYFlask 25cm²
Neubauer C-Chip Haemocytometer DHC-N01
Gibco Phosphate Buffered Saline Sterile 100010-031
Gibco Type Express 12604-013

Human Umbilical Vein Cells (HUVEC)
Caltag Cellworks Early Passage Human Umbilical Vein Endothelial Cells ZHC-2301
Cellworks Human Large Vessel Endothelial Cell Basal Medium KC1015
Cellworks Human Large Vessel Endothelial Cell Growth Supplement KC1016
Cellworks Antibiotic Supplement (Gentamycin/Amphotericin B) KC1019

Human Uterine Microvascular Endothelial Cells (HUtMEC)
Human Uterine Microvascular Endothelial Cells purchased from 2bScientific
Promo cell Endothelial Cell Basal Medium MV2 (prf)

Ishikawa
Sigma-Aldrich Ishikawa Cell line human endometrial adenocarcinoma 9904021 purchased from Sigma Aldrich 99040201 originating from the European collection of authenticated cell cultures
Ishikawa Culture Medium MEM + 2mM Glutamine + 1% Non-Essential Amino Acids (NEAA) + 5% Foetal Bovine Serum (FBS)

Angiogenesis
Cellworks angiogenesis assay control kit ZHA-1300
Ibidi u-slide angiogenesis 18 well
Ibidi u-slide angiogenesis 96 well
Corning matrigel matrix basement membrane growth factor reduced 356231
Gilson Micron positive displacement pipette

Migration
Ibidi Culture-Insert 2 Well 81176

Protein extraction
Pierce RIPA buffer 89900

Immunoblotting
Fisher Bis acrylamide 37 5:1 40%
Marvel Skimmed milk powder
Fisher Tween 20 BP337-500
Fisher Tris base BP 152-1
Fisher Sodium dodecyl sulphate S/5200/53
Sigma Ammonium persulfate A3678-100G
Sigma Aldrich 2-Mercaptoethanol M6250
Fisher NP40 Alternative 4920 16
Sigma TEMED T-9281

Fisher Methanol M/4000/PC17
Millipore Luminata Forte Western HRP Substrate
Sigma Phosphate Buffered Saline P4417
Fisher Guanidine Hydrochloride BP178-500
Gibco 1M Tris HCL 15568-025
Sigma Aldrich Sodium hydroxide S7770

VII. SOLUTION RECIPES FOR IMMUNOBLOTTING

Laemmli loading buffer
9.5mL pure H2O
2.5mL 0.5M Tris HCL pH 6.8
2.0mL Glycerol
4.0mL 10% SDS (omitted for non-reducing)
1.0mL 2-beta Mercaptoethanol
1.0mL 1.5% Bromophenol blue

10 x Running buffer
25g SDS
75.5g Tris
360g glycine
make up to 2.5l in dH20

1 x Transfer buffer
28.8 g glycine
400 mL methanol
6.06 g Tris base
Add dH20 to get 2 liters

Ammonium persulfate (APS) liquid from powder
2g + 18mL dH20—makes 20mL
APS: freeze 100 ul aliquots

Sodium dodecyl sulphate (SDS) liquid from powder
2g + 18mL dH20—makes 20mL
SDS: store at room temp

Abcam mild stripping buffer
15 g glycine
1 g SDS
10 mL Tween20
Adjust pH to 2.2
Bring volume up to 1 L with ultrapure water.
GnHCL Stripping buffer

Abcam harsh stripping buffer
20 mL SDS 10%
12.5 mL Tris HCl pH 6.8 0.5M
67.5 mL ultra-pure water
Add 0.8 mL ß-mercaptoethanol

GnHCL Very harsh stripping buffer
57.32g GnHCL powder
0.2mL 0.2% NP40
0.7mL 0.1M B-mercaptoptoethanol
2mL 1M TrisHCL
Adjust pH to 7.5
Make up to 100mL with dH2O

TBS
6.05g 50Mm Tris
8.76g NaCl
800mL dH2O

Adjust pH to 7.6 with 1M HCL
Make up volume to 1L and store at 4°C

VIII. BCA CALCULATIONS

Sample of the spreadsheet used to calculate protein levels in the samples from Pierce BCA assay

Herbs at 1:100 dilution

loading vol (ul)

protein conc (ug/ml)	absorbance	sample ID	calc'd pr conc	absorbance	dilution	calc'd pr conc (ug/ml)	calc'd pr conc (ug/ul)	vol for 10ug (ul)	sample buffer required (ul)	20	sample buffer required (ul)
0	0.1195	Media	4,278.20	1.981	1	4,278.20	**4.28**	2.34	17.66	4.67	15.325
25	0.14525	GSDW	2,244.20	0.964	1	2,244.20	**2.24**	4.46	15.54	8.91	11.088
125	0.2105	BS	4,254.20	1.969	1	4,254.20	**4.25**	2.35	17.65	4.70	15.299
250	0.29575	DG	3,836.20	1.76	1	3,836.20	**3.84**	2.61	17.39	5.21	14.787
500	0.37725	FL	3,952.20	1.818	1	3,952.20	**3.95**	2.53	17.47	5.06	14.940
750	0.548	MDP	3,648.20	1.666	1	3,648.20	**3.65**	2.74	17.26	5.48	14.518
1000	0.69325	SD	4,086.20	1.885	1	4,086.20	**4.09**	2.45	17.55	4.89	15.105
1500	0.87775	SY	4,198.20	1.941	1	4,198.20	**4.20**	2.38	17.62	4.76	15.236
2000	1.09975	SZY	4,274.20	1.979	1	4,274.20	**4.27**	2.34	17.66	4.68	15.321
		ZX	4,332.20	2.008	1	4,332.20	**4.33**	2.31	17.69	4.62	15.383

slope 0.0005
intercept -0.1581

Instructions
1 Enter absorbance values for STANDARDS
2 (Plot will automatically update. Change range for a high r-sq values.)
3 Enter SLOPE (y) and INTERCEPT(the minus number after) values from the fitted line.
4 Enter details of sample ID and their absorbance values
5 Were any of the samples diluted (when measurements taken at the Nanodrop)
6 Tailor loading volume

IX. HAEMOSTASIS RAW DATA

Test	GSDW added ul	Repeat	Time
PT	**0.4**	**1**	**67.9**
PT	**0.4**	**2**	**129.3**
PT	**0.4**	**3**	**78**
PT	**0.4**	**4**	**150.2**
PT	**0.4**	**5**	**134.6**
PT	**0.35**	**1**	**44.9**
PT	**0.35**	**2**	**42.5**
PT	**0.35**	**3**	**42.3**
PT	**0.35**	**4**	**38.4**
PT	**0.35**	**5**	**46.1**
PT	**0.3**	**1**	**33.6**
PT	**0.3**	**2**	**36.6**
PT	**0.3**	**3**	**30.2**
PT	**0.3**	**4**	**32**
PT	**0.3**	**5**	**36.1**
PT	**0.25**	**1**	**27.4**
PT	**0.25**	**2**	**27.6**
PT	**0.25**	**3**	**25.6**
PT	**0.25**	**4**	**27.2**
PT	**0.25**	**5**	**23.3**
PT	**0.2**	**1**	**23.5**
PT	**0.2**	**2**	**21.5**
PT	**0.2**	**3**	**21.4**
PT	**0.2**	**4**	**22.8**
PT	**0.2**	**5**	**20.1**
PT	**0.15**	**1**	**21.6**
PT	**0.15**	**2**	**24**
PT	**0.15**	**3**	**24.1**
PT	**0.15**	**4**	**22.6**
PT	**0.15**	**5**	**20.6**
PT	**0.1**	**1**	**20.2**
PT	**0.1**	**2**	**19.8**
PT	**0.1**	**3**	**21**
PT	**0.1**	**4**	**19.8**
PT	**0.1**	**5**	**19.8**
PT	**0.05**	**1**	**19.5**
PT	**0.05**	**2**	**20.3**
PT	**0.05**	**3**	**18.8**
PT	**0.05**	**4**	**19.1**
PT	**0.05**	**5**	**19.2**
PT	**0**	**1**	**18.4**
PT	**0**	**2**	**18.5**
PT	**0**	**3**	**19.9**
PT	**0**	**4**	**16.3**
PT	**0**	**5**	**15.3**

Test	GSDW added ul	Repeat	Time
APTT	**0.4**	**1**	**598.8**
APTT	**0.4**	**2**	**145.3**
APTT	**0.4**	**3**	**266.1**
APTT	**0.4**	**4**	**639.1**
APTT	**0.4**	**5**	**548.2**
APTT	**0.35**	**1**	**530.3**
APTT	**0.35**	**2**	**248.7**
APTT	**0.35**	**3**	**324**
APTT	**0.35**	**4**	**676.1**
APTT	**0.35**	**5**	**548.2**
APTT	**0.3**	**1**	**478.7**
APTT	**0.3**	**2**	**386**
APTT	**0.3**	**3**	**260.3**
APTT	**0.3**	**4**	**235.2**
APTT	**0.3**	**5**	**201.2**
APTT	**0.25**	**1**	**196**
APTT	**0.25**	**2**	**225.1**
APTT	**0.25**	**3**	**171.9**
APTT	**0.25**	**4**	**264**
APTT	**0.25**	**5**	**228.8**
APTT	**0.2**	**1**	**152.7**
APTT	**0.2**	**2**	**151.1**
APTT	**0.2**	**3**	**125.5**
APTT	**0.2**	**4**	**167.1**
APTT	**0.2**	**5**	**158.1**
APTT	**0.15**	**1**	**101.7**
APTT	**0.15**	**2**	**123.4**
APTT	**0.15**	**3**	**115.6**
APTT	**0.15**	**4**	**122.7**
APTT	**0.15**	**5**	**99.2**
APTT	**0.1**	**1**	**81.8**
APTT	**0.1**	**2**	**67.1**
APTT	**0.1**	**3**	**81.7**
APTT	**0.1**	**4**	**73.3**
APTT	**0.1**	**5**	**72.5**
APTT	**0.05**	**1**	**70.5**
APTT	**0.05**	**2**	**64.6**
APTT	**0.05**	**3**	**63.2**
APTT	**0.05**	**4**	**61.1**
APTT	**0.05**	**5**	**63.4**
APTT	**0**	**1**	**65.5**
APTT	**0**	**2**	**63.3**
APTT	**0**	**3**	**63.4**
APTT	**0**	**4**	**62.3**
APTT	**0**	**5**	**64.2**

GSDW	Hours	Test or Control	Absorbance
0.00002	24	1	0.2236667
0.00002	24	1	0.28
0.00002	24	1	0.1856667
0.00002	24	1	0.2556667
0.00002	24	2	0.1856667
0.00002	24	2	0.2896667
0.00002	24	2	0.167
0.00002	24	2	0.268
0.00003	24	1	0.2223333
0.00003	24	1	0.318
0.00003	24	1	0.27
0.00003	24	1	0.286
0.00003	24	2	0.225
0.00003	24	2	0.33
0.00003	24	2	0.207
0.00003	24	2	0.2386667
0.00007	24	1	0.2003333
0.00007	24	1	0.3593333
0.00007	24	1	0.2746667
0.00007	24	1	0.2886667
0.00007	24	2	0.1923333
0.00007	24	2	0.3253333
0.00007	24	2	0.21
0.00007	24	2	0.2543333
0.00014	24	1	0.2493333
0.00014	24	1	0.2823333
0.00014	24	1	0.3283333
0.00014	24	1	0.2923333
0.00014	24	2	0.1973333
0.00014	24	2	0.2523333
0.00014	24	2	0.212
0.00014	24	2	0.2513333
0.00027	24	1	0.3246667
0.00027	24	1	0.3966667
0.00027	24	1	0.3363333
0.00027	24	1	0.3133333
0.00027	24	2	0.2176667
0.00027	24	2	0.3876667
0.00027	24	2	0.2273333
0.00027	24	2	0.2743333
0.00055	24	1	0.235
0.00055	24	1	0.331
0.00055	24	1	0.2913333
0.00055	24	1	0.272
0.00055	24	2	0.2476667
0.00055	24	2	0.3173333
0.00055	24	2	0.224
0.00055	24	2	0.2646667
0.00109	24	1	0.07333333
0.00109	24	1	0.2073333
0.00109	24	1	0.1923333
0.00109	24	1	0.2086667
0.00109	24	2	0.2843333
0.00109	24	2	0.37
0.00109	24	2	0.225
0.00109	24	2	0.2616667
0.00218	24	1	0.062
0.00218	24	1	0.065
0.00218	24	1	0.082
0.00218	24	1	0.09633333
0.00218	24	2	0.24
0.00218	24	2	0.3493333
0.00218	24	2	0.2166667
0.00218	24	2	0.2726667
0.00437	24	1	0.036
0.00437	24	1	0.048
0.00437	24	1	0.05333333
0.00437	24	1	0.08366667
0.00437	24	2	0.2486667
0.00437	24	2	0.3026667
0.00437	24	2	0.1896667
0.00437	24	2	0.2726667
0.00874	24	1	0.057
0.00874	24	1	0.02
0.00874	24	1	0.03333333
0.00874	24	1	0.068
0.00874	24	2	0.2783333
0.00874	24	2	0.239
0.00874	24	2	0.1603333
0.00874	24	2	0.2426667
0.00002	48	1	0.3363333
0.00002	48	1	0.343
0.00002	48	1	0.3296667
0.00002	48	2	0.2533333
0.00002	48	2	0.2
0.00002	48	2	0.223
0.00003	48	1	0.358
0.00003	48	1	0.3523333
0.00003	48	1	0.319
0.00003	48	2	0.2613333
0.00003	48	2	0.205
0.00003	48	2	0.2083333
0.00007	48	1	0.3463333
0.00007	48	1	0.342
0.00007	48	1	0.3296667
0.00007	48	2	0.27
0.00007	48	2	0.2236667
0.00007	48	2	0.1923333
0.00014	48	1	0.3246667
0.00014	48	1	0.3346667
0.00014	48	1	0.343
0.00014	48	2	0.2566667
0.00014	48	2	0.2053333
0.00014	48	2	0.186
0.00027	48	1	0.3213333
0.00027	48	1	0.3176667
0.00027	48	1	0.3116667
0.00027	48	2	0.225
0.00027	48	2	0.24
0.00027	48	2	0.1926667
0.00055	48	1	0.2793333
0.00055	48	1	0.2926667
0.00055	48	1	0.284
0.00055	48	2	0.2236667

0.00055	48	2	0.2353333
0.00055	48	2	0.1916667
0.00109	48	1	0.21
0.00109	48	1	0.183
0.00109	48	1	0.1946667
0.00109	48	2	0.204
0.00109	48	2	0.2313333
0.00109	48	2	0.1916667
0.00218	48	1	0.091
0.00218	48	1	0.088
0.00218	48	1	0.084
0.00218	48	2	0.244
0.00218	48	2	0.2373333
0.00218	48	2	0.1916667
0.00437	48	1	0.104
0.00437	48	1	0.07833333
0.00437	48	1	0.08266667
0.00437	48	2	0.214
0.00437	48	2	0.2086667
0.00437	48	2	0.1633333
0.00874	48	1	0.07033333
0.00874	48	1	0.07566667
0.00874	48	1	0.072
0.00874	48	2	0.187
0.00874	48	2	0.16
0.00874	48	2	0.136
0.00002	72	1	0.3006667
0.00002	72	1	0.2676667
0.00002	72	1	0.2286667
0.00002	72	2	0.303
0.00002	72	2	0.2613333
0.00002	72	2	0.2203333
0.00003	72	1	0.2123333
0.00003	72	1	0.2743333
0.00003	72	1	0.2593333
0.00003	72	2	0.151
0.00003	72	2	0.231
0.00003	72	2	0.2186667
0.00007	72	1	0.2236667
0.00007	72	1	0.298
0.00007	72	1	0.2513333
0.00007	72	2	0.183
0.00007	72	2	0.231
0.00007	72	2	0.2076667
0.00014	72	1	0.256
0.00014	72	1	0.216
0.00014	72	1	0.2743333
0.00014	72	2	0.166
0.00014	72	2	0.1413333
0.00014	72	2	0.215
0.00027	72	1	0.2803333
0.00027	72	1	0.2263333
0.00027	72	1	0.2916667
0.00027	72	2	0.2106667
0.00027	72	2	0.144
0.00027	72	2	0.2146667
0.00055	72	1	0.215
0.00055	72	1	0.145
0.00055	72	1	0.2086667
0.00055	72	2	0.2176667
0.00055	72	2	0.1666667
0.00055	72	2	0.2186667
0.00109	72	1	0.05433333
0.00109	72	1	0.05166667
0.00109	72	1	0.04233333
0.00109	72	2	0.2153333
0.00109	72	2	0.162
0.00109	72	2	0.2106667
0.00218	72	1	0.052
0.00218	72	1	0.05266667
0.00218	72	1	0.048

0.00218	72	2	0.1923333
0.00218	72	2	0.1636667
0.00218	72	2	0.202
0.00437	72	1	0.03733333
0.00437	72	1	0.03333333
0.00437	72	1	0.02933333
0.00437	72	2	0.1833333
0.00437	72	2	0.1696667
0.00437	72	2	0.1816667
0.00874	72	1	0.025
0.00874	72	1	0.02066667
0.00874	72	1	0.022
0.00874	72	2	0.1336667
0.00874	72	2	0.1323333
0.00874	72	2	0.1586667

X. ANGIOGENESIS RAW DATA

HUVEC

Image Name	Nb Junctions	Nb master segments	Tot.meshes area	Tot. lenght
12hr BS 1-tr	**151**	**99**	**604787**	**21897**
12hr BS 2-tr	**149**	**101**	**874629**	**21909**
12hr BS 3-tr	**157**	**108**	**611015**	**22384**
12hr BS 4-tr	**139**	**84**	**471170**	**20580**
12hr BS 5-tr	**162**	**132**	**709484**	**22094**
12hr BS 6-tr	**149**	**86**	**757713**	**20773**
12hr DG 1-tr	**146**	**80**	**304810**	**22066**
12hr DG 2-tr	**161**	**99**	**1176575**	**22111**
12hr DG 3-tr	**160**	**114**	**325960**	**21537**
12hr DG 4-tr	**137**	**91**	**912826**	**20743**
12hr DG 5-tr	**146**	**80**	**304810**	**22066**
12hr DG 6-tr	**126**	**58**	**759144**	**21211**
12hr FL 1-tr	**143**	**94**	**516391**	**20536**
12hr FL 2-tr	**138**	**77**	**750046**	**21137**
12hr FL 3-tr	**138**	**76**	**891020**	**21930**
12hr FL 4-tr	**140**	**84**	**959405**	**20847**
12hr FL 5-tr	**114**	**52**	**241280**	**19220**
12hr FL 6-tr	**130**	**68**	**541931**	**20655**
12hr GSDW 1-tr	**207**	**127**	**800049**	**25032**
12hr GSDW 2-tr	**198**	**126**	**333175**	**23873**
12hr GSDW 3-tr	**222**	**150**	**742404**	**26140**
12hr GSDW 4-tr	**186**	**111**	**824230**	**24501**
12hr GSDW 5-tr	**170**	**96**	**739592**	**22646**
12hr GSDW 6-tr	**164**	**99**	**399231**	**22978**
12hr MDP 1-tr	**153**	**72**	**440544**	**21100**
12hr MDP 2-tr	**84**	**39**	**58271**	**15239**
12hr MDP 3-tr	**115**	**71**	**394000**	**20498**
12hr MDP 4-tr	**107**	**44**	**132776**	**18566**
12hr MDP 5-tr	**107**	**54**	**251386**	**19241**
12hr MDP 6-tr	**101**	**43**	**92850**	**17693**
12hr Media 1-tr	**126**	**68**	**384164**	**21299**
12hr Media 2-tr	**157**	**83**	**928511**	**22846**
12hr Media 3-tr	**127**	**74**	**1053787**	**21088**
12hr Media 4-tr	**184**	**123**	**930875**	**23112**
12hr Media 5-tr	**129**	**65**	**398357**	**20992**
12hr Media 6-tr	**146**	**79**	**1031346**	**21632**
12hr SD 1-tr	**173**	**109**	**317257**	**23125**
12hr SD 2-tr	**117**	**68**	**251142**	**17464**
12hr SD 3-tr	**126**	**75**	**292816**	**20556**
12hr SD 4-tr	**84**	**31**	**22537**	**16350**
12hr SD 5-tr	**178**	**120**	**1298754**	**24610**
12hr SD 6-tr	**146**	**100**	**267445**	**23358**
12hr Sura 1-tr	**111**	**59**	**67740**	**17429**
12hr Sura 2-tr	**103**	**50**	**170995**	**14765**
12hr Sura 3-tr	**98**	**42**	**92795**	**15450**
12hr Sura 4-tr	**91**	**37**	**35621**	**12446**
12hr Sura 5-tr	**103**	**32**	**23825**	**15068**
12hr Sura 6-tr	**90**	**43**	**40694**	**14901**
12hr SY 1-tr	**106**	**61**	**292395**	**18926**
12hr SY 2-tr	**134**	**86**	**1065953**	**21590**
12hr SY 3-tr	**118**	**72**	**1021118**	**19622**
12hr SY 4-tr	**120**	**76**	**1020311**	**20155**
12hr SY 5-tr	**135**	**84**	**823407**	**21058**
12hr SY 6-tr	**117**	**46**	**129703**	**20440**

12hr SZY 1-tr	**135**	**91**	**431722**	**19296**
12hr SZY 2-tr	**139**	**92**	**1224699**	**23114**
12hr SZY 3-tr	**135**	**75**	**342920**	**22028**
12hr SZY 4-tr	**116**	**62**	**864936**	**20127**
12hr SZY 5-tr	**148**	**95**	**1145438**	**21863**
12hr SZY 6-tr	**148**	**99**	**612491**	**19889**
12hr VEGF 1-tr	**129**	**80**	**478753**	**22058**
12hr VEGF 2-tr	**149**	**82**	**430507**	**21281**
12hr VEGF 3-tr	**143**	**85**	**502480**	**21159**
12hr VEGF 4-tr	**112**	**49**	**339313**	**20360**
12hr VEGF 5-tr	**121**	**66**	**792342**	**20600**
12hr VEGF 6-tr	**176**	**119**	**573784**	**21939**
12hr ZX 1-tr	**142**	**80**	**1033967**	**22651**
12hr ZX 2-tr	**159**	**135**	**1457279**	**23700**
12hr ZX 3-tr	**136**	**80**	**876752**	**22422**
12hr ZX 4-tr	**152**	**94**	**1047043**	**22446**
12hr ZX 5-tr	**150**	**103**	**752324**	**22056**
12hr ZX 6-tr	**160**	**90**	**761952**	**23148**

HUtMEC

Image Name	Nb Junctions	Nb master segments	Tot.meshes area	Tot. length
160708 GF- 21hr Media with supplements 1	**25**	**6**	**337072**	**8730**
160708 GF- 21hr Media with supplements 2	**10**	**6**	**32982**	**7421**
160708 GF- 21hr Media with supplements 3	**26**	**10**	**1271666**	**11415**
160708 GF- 21hr Media with supplements 4	**20**	**14**	**1187320**	**10121**
160708 GF- 21hr Media with supplements 5	**12**	**3**	**492258**	**8617**
160708 GF- 21hr Media with supplements 6	**9**	**5**	**716358**	**8319**
160708 GF- 21hr Media with FBS 0.1% 1	**12**	**6**	**724102**	**7992**
160708 GF- 21hr Media with FBS 0.1% 2	**17**	**6**	**399131**	**8890**
160708 GF- 21hr Media with FBS 0.1% 3	**12**	**6**	**267043**	**7973**
160708 GF- 21hr Media with FBS 0.1% 4	**17**	**9**	**188423**	**6727**
160708 GF- 21hr Media with FBS 0.1% 5	**18**	**8**	**745662**	**8310**
160708 GF- 21hr Media with FBS 0.1% 6	**5**	**2**	**222584**	**5604**
160708 GF- 21hr GSDW 1000 with FBS 0.1% 1	**33**	**11**	**981886**	**11014**
160708 GF- 21hr GSDW 1000 with FBS 0.1% 2	**29**	**9**	**525856**	**9910**
160708 GF- 21hr GSDW 1000 with FBS 0.1% 3	**42**	**6**	**949327**	**12935**
160708 GF- 21hr GSDW 1000 with FBS 0.1% 4	**21**	**5**	**494372**	**8448**
160708 GF- 21hr GSDW 1000 with FBS 0.1% 5	**19**	**9**	**867764**	**9900**
160708 GF- 21hr GSDW 1000 with FBS 0.1% 6	**21**	**8**	**637442**	**10623**
160708 GF- 21hr GSDW 500 with FBS 0.1% 1	**22**	**11**	**939171**	**10201**
160708 GF- 21hr GSDW 500 with FBS 0.1% 2	**14**	**6**	**398498**	**8469**
160708 GF- 21hr GSDW 500 with FBS 0.1% 3	**19**	**10**	**1234346**	**11970**
160708 GF- 21hr GSDW 500 with FBS 0.1% 4	**20**	**13**	**1317068**	**10118**
160708 GF- 21hr GSDW 500 with FBS 0.1% 5	**22**	**14**	**1460716**	**10837**
160708 GF- 21hr GSDW 500 with FBS 0.1% 6	**18**	**7**	**492973**	**9274**
160708 GF- 21hr BS 500 with FBS 0.1% 2	**30**	**14**	**1457064**	**12559**
160708 GF- 21hr BS 500 with FBS 0.1% 3	**17**	**10**	**1040071**	**9462**
160708 GF- 21hr BS 500 with FBS 0.1% 4	**18**	**7**	**534474**	**9540**
160708 GF- 21hr BS 500 with FBS 0.1% 5	**35**	**12**	**962243**	**10743**
160708 GF- 21hr BS 500 with FBS 0.1% 6	**75**	**14**	**1666225**	**18118**

160708 GF- 21hr BS 500 with FBS 0.1% 1	44	14	1565501	14807
160708 GF- 21hr DG 500 with FBS 0.1% 1	**14**	**12**	**969303**	**9222**
160708 GF- 21hr DG 500 with FBS 0.1% 2	**20**	**10**	**1176511**	**8873**
160708 GF- 21hr DG 500 with FBS 0.1% 3	**33**	**8**	**1212770**	**11436**
160708 GF- 21hr DG 500 with FBS 0.1% 4	**18**	**12**	**1170224**	**10810**
160708 GF- 21hr DG 500 with FBS 0.1% 5	**15**	**11**	**713184**	**8441**
160708 GF- 21hr DG 500 with FBS 0.1% 6	**29**	**10**	**829442**	**11574**
160708 GF- 21hr FL 500 with FBS 0.1% 1	**22**	**10**	**951388**	**10738**
160708 GF- 21hr FL 500 with FBS 0.1% 2	**24**	**12**	**737579**	**11153**
160708 GF- 21hr FL 500 with FBS 0.1% 3	**21**	**10**	**858289**	**10032**
160708 GF- 21hr FL 500 with FBS 0.1% 4	**21**	**15**	**1194127**	**11141**
160708 GF- 21hr FL 500 with FBS 0.1% 5	**26**	**12**	**1290502**	**11558**
160708 GF- 21hr FL 500 with FBS 0.1% 6	**38**	**15**	**1434244**	**14200**
160708 GF- 21hr MDP 500 with FBS 0.1% 1	**33**	**13**	**1700544**	**13693**
160708 GF- 21hr MDP 500 with FBS 0.1% 2	**20**	**12**	**1052968**	**10319**
160708 GF- 21hr MDP 500 with FBS 0.1% 3	**23**	**11**	**1209291**	**10179**
160708 GF- 21hr MDP 500 with FBS 0.1% 4	**21**	**18**	**1191863**	**10822**
160708 GF- 21hr MDP 500 with FBS 0.1% 5	**34**	**14**	**1382531**	**13332**
160708 GF- 21hr MDP 500 with FBS 0.1% 6	**21**	**10**	**1032634**	**10474**
160708 GF- 21hr SD 500 with FBS 0.1% 1	**16**	**10**	**999147**	**9205**
160708 GF- 21hr SD 500 with FBS 0.1% 2	**26**	**17**	**1469371**	**12483**
160708 GF- 21hr SD 500 with FBS 0.1% 3	**27**	**16**	**1270088**	**12258**
160708 GF- 21hr SD 500 with FBS 0.1% 4	**21**	**14**	**1223873**	**11191**
160708 GF- 21hr SD 500 with FBS 0.1% 5	**24**	**18**	**1824129**	**12721**
160708 GF- 21hr SD 500 with FBS 0.1% 6	**25**	**16**	**1309643**	**11589**
160708 GF- 21hr SY 500 with FBS 0.1% 1	**19**	**10**	**836902**	**10340**
160708 GF- 21hr SY 500 with FBS 0.1% 2	**29**	**10**	**1253253**	**12289**
160708 GF- 21hr SY 500 with FBS 0.1% 3	**29**	**17**	**1527741**	**13254**
160708 GF- 21hr SY 500 with FBS 0.1% 4	**31**	**16**	**1183334**	**12933**
160708 GF- 21hr SY 500 with FBS 0.1% 5	**27**	**14**	**1134200**	**11980**
160708 GF- 21hr SY 500 with FBS 0.1% 6	**24**	**9**	**1028589**	**11413**
160708 GF- 21hr SZY 500 with FBS 0.1% 1	**22**	**17**	**1562879**	**12264**
160708 GF- 21hr SZY 500 with FBS 0.1% 2	**20**	**10**	**597154**	**10146**
160708 GF- 21hr SZY 500 with FBS 0.1% 3	**36**	**20**	**1562082**	**13921**
160708 GF- 21hr SZY 500 with FBS 0.1% 4	**29**	**13**	**1357063**	**12842**
160708 GF- 21hr SZY 500 with FBS 0.1% 5	**29**	**14**	**1159215**	**11701**
160708 GF- 21hr SZY 500 with FBS 0.1% 6	**29**	**19**	**1507791**	**12972**
160708 GF- 21hr ZX 500 with FBS 0.1% 1	**25**	**12**	**806986**	**10708**
160708 GF- 21hr ZX 500 with FBS 0.1% 2	**27**	**16**	**1699361**	**12464**
160708 GF- 21hr ZX 500 with FBS 0.1% 3	**30**	**13**	**1267552**	**11867**
160708 GF- 21hr ZX 500 with FBS 0.1% 4	**17**	**8**	**957505**	**9440**
160708 GF- 21hr ZX 500 with FBS 0.1% 5	**44**	**11**	**1453083**	**14688**
160708 GF- 21hr ZX 500 with FBS 0.1% 6	**19**	**16**	**1378636**	**10463**

XI. IMMUNOBLOTTING RAW DATA

ERα 24

1	ERα 24	βA	Ratio	Normalised ratio	Relative to media
Media	**2405844**	**14736540**	**0.16**	**1**	**1.00**
GUI SHAO DI HUANG WAN	**4310644**	**10623698**	**2.49**	**15.5625**	**1.86**
RADIX PAEONIAE ALBA	**5243088**	**11481662**	**0.18**	**1.125**	**2.32**
RADIX ANGELICA SINENSIS	**2234438**	**15125786**	**0.8**	**5**	**0.99**
PORIA COCOS	**3801406**	**16176030**	**0.29**	**1.8125**	**1.62**
CORTEX MOUTAN	**2480526**	**12163002**	**0.7**	**4.375**	**1.10**
RADIX REHMANNIAE PREPARATA	**2457422**	**7889180**	**0.45**	**2.8125**	**1.07**
RHIZOMA DIOSCOREAE	**2981594**	**10854168**	**0.62**	**3.875**	**1.30**
FRUCTUS CORNI	**3952494**	**13713516**	**0.47**	**2.9375**	**1.72**
RHIZOMA ALISMATIS	**8155158**	**16559698**	**1.05**	**6.5625**	**3.37**
2					
Media	**1684158**	**996606**	**1.69**	**1**	**1.00**
GUI SHAO DI HUANG WAN	**3511200**	**1575954**	**2.23**	**1.318416701**	**2.08**
RADIX PAEONIAE ALBA	**2069277**	**3548952**	**0.58**	**0.345031837**	**1.23**
RADIX ANGELICA SINENSIS	**1925427**	**1901664**	**1.01**	**0.599147756**	**1.14**
PORIA COCOS	**3429279**	**2187774**	**1.57**	**0.927557899**	**2.04**
CORTEX MOUTAN	**1580649**	**1534518**	**1.03**	**0.609542678**	**0.94**
RADIX REHMANNIAE PREPARATA	**2620884**	**1218654**	**2.15**	**1.272647258**	**1.56**
RHIZOMA DIOSCOREAE	**2424009**	**1365066**	**1.78**	**1.050802853**	**1.44**
FRUCTUS CORNI	**764988**	**794682**	**0.96**	**0.56964188**	**0.45**
RHIZOMA ALISMATIS	**7523292**	**1147644**	**6.56**	**3.879193047**	**4.47**
3					
Media	**1677662**	**3826116**	**0.44**	**1**	**1**
GUI SHAO DI HUANG WAN	**2901395**	**5068404**	**0.57**	**1.31**	**1.729428**
RADIX PAEONIAE ALBA	**2997687**	**2561944**	**1.17**	**2.67**	**1.786824**
RADIX ANGELICA SINENSIS	**4123380**	**5174370**	**0.80**	**1.82**	**2.457813**
PORIA COCOS	**5945727**	**4784526**	**1.24**	**2.83**	**3.544055**
CORTEX MOUTAN	**4289098**	**4358466**	**0.98**	**2.24**	**2.556592**
RADIX REHMANNIAE PREPARATA	**5056641**	**4225716**	**1.20**	**2.73**	**3.0141**
RHIZOMA DIOSCOREAE	**4748252**	**4095990**	**1.16**	**2.64**	**2.830279**
FRUCTUS CORNI	**4842321**	**4049342**	**1.20**	**2.73**	**2.886351**
RHIZOMA ALISMATIS	**3873188**	**5286880**	**0.73**	**1.67**	**2.308682**

ERα 48

1	ERα 48	βA	Ratio	Normalised ratio	Relative to media
Media	**3493217**	**2778906**	**1.26**	**1**	**1.00**
GUI SHAO DI HUANG WAN	**2363049**	**1842064**	**1.28**	**1.02**	**0.68**
RADIX PAEONIAE ALBA	**691429**	**1752522**	**0.39**	**0.31**	**0.20**
RADIX ANGELICA SINENSIS	**343710**	**1080792**	**0.32**	**0.25**	**0.10**
PORIA COCOS	**569677**	**1796130**	**0.32**	**0.25**	**0.16**
CORTEX MOUTAN	**1370641**	**1441104**	**0.95**	**0.76**	**0.39**
RADIX REHMANNIAE PREPARATA	**1064475**	**2356416**	**0.45**	**0.36**	**0.30**
RHIZOMA DIOSCOREAE	**1905339**	**3409480**	**0.56**	**0.44**	**0.55**
FRUCTUS CORNI	**4247146**	**2542768**	**1.67**	**1.33**	**1.22**
RHIZOMA ALISMATIS	**2569921**	**2828628**	**0.91**	**0.72**	**0.74**
2					
Media	**7074570**	**2555369**	**2.77**	**1**	**1.00**
GUI SHAO DI HUANG WAN	**1197173**	**2385831**	**0.5**	**0.18**	**0.17**
RADIX PAEONIAE ALBA	**2006739**	**1733028**	**1.16**	**0.42**	**0.28**
RADIX ANGELICA SINENSIS	**4289481**	**2945475**	**1.46**	**0.53**	**0.61**
PORIA COCOS	**5566449**	**3294866**	**1.69**	**0.61**	**0.79**
CORTEX MOUTAN	**3048822**	**3903170**	**0.78**	**0.28**	**0.43**
RADIX REHMANNIAE PREPARATA	**5690265**	**4073239**	**1.4**	**0.5**	**0.80**
RHIZOMA DIOSCOREAE	**6668193**	**3609144**	**1.85**	**0.67**	**0.94**
FRUCTUS CORNI	**6437256**	**3597380**	**1.79**	**0.65**	**0.91**
RHIZOMA ALISMATIS	**4575804**	**5684058**	**0.81**	**0.29**	**0.65**
3					
Media	**6530223**	**3737431**	**1.75**	**1**	**1.00**
GUI SHAO DI HUANG WAN	**3323727**	**1610466**	**2.06**	**1.18**	**0.51**
RADIX PAEONIAE ALBA	**2345011**	**1565175**	**1.5**	**0.86**	**0.36**
RADIX ANGELICA SINENSIS	**3360441**	**1577560**	**2.13**	**1.22**	**0.51**
PORIA COCOS	**3859422**	**2437080**	**1.58**	**0.91**	**0.59**
CORTEX MOUTAN	**2461640**	**2215260**	**1.11**	**0.64**	**0.38**
RADIX REHMANNIAE PREPARATA	**3146193**	**2355340**	**1.34**	**0.76**	**0.48**
RHIZOMA DIOSCOREAE	**4907694**	**2605920**	**1.88**	**1.08**	**0.75**
FRUCTUS CORNI	**6674822**	**3351400**	**1.99**	**1.14**	**1.02**
RHIZOMA ALISMATIS	**5213964**	**4286540**	**1.22**	**0.7**	**0.80**

ERβ 24

1	ERβ 24	βA	Ratio	Normalised ratio	Relative to media
Media	**347016**	**1208952**	**0.29**	**1**	**1.00**
GUI SHAO DI HUANG WAN	**1070555**	**1753642**	**0.61**	**2.13**	**3.09**
RADIX PAEONIAE ALBA	**4145405**	**2561562**	**1.62**	**5.64**	**11.95**
RADIX ANGELICA SINENSIS	**4446646**	**2660310**	**1.67**	**5.82**	**12.81**
PORIA COCOS	**1958767**	**3338154**	**0.59**	**2.04**	**5.64**
CORTEX MOUTAN	**1515573**	**3949434**	**0.38**	**1.34**	**4.37**
RADIX REHMANNIAE PREPARATA	**3258481**	**4980116**	**0.65**	**2.28**	**9.39**
RHIZOMA DIOSCOREAE	**1831125**	**4630262**	**0.4**	**1.38**	**5.28**
FRUCTUS CORNI	**1927664**	**4989894**	**0.39**	**1.35**	**5.55**
RHIZOMA ALISMATIS	**2064720**	**6944328**	**0.3**	**1.04**	**5.95**
2					
Media	**1311362**	**1460178**	**0.9**	**1**	**1.00**
GUI SHAO DI HUANG WAN	**1795104**	**2258631**	**0.79**	**0.88**	**1.37**
RADIX PAEONIAE ALBA	**3240925**	**1062666**	**3.05**	**3.4**	**2.47**
RADIX ANGELICA SINENSIS	**1628460**	**1164108**	**1.4**	**1.56**	**1.24**
PORIA COCOS	**1128096**	**1919918**	**0.59**	**0.65**	**0.86**
CORTEX MOUTAN	**669114**	**2247520**	**0.3**	**0.33**	**0.51**
RADIX REHMANNIAE PREPARATA	**2215188**	**1900382**	**1.17**	**1.3**	**1.69**
RHIZOMA DIOSCOREAE	**1140660**	**2603898**	**0.44**	**0.49**	**0.87**
FRUCTUS CORNI	**960280**	**4937240**	**0.19**	**0.22**	**0.73**
RHIZOMA ALISMATIS	**1073512**	**7502456**	**0.14**	**0.16**	**0.82**
3					
Media	**3683960**	**2429030**	**1.52**	**1**	**1.00**
GUI SHAO DI HUANG WAN	**3199760**	**3119789**	**1.03**	**0.68**	**0.75**
RADIX PAEONIAE ALBA	**2288660**	**3468078**	**0.66**	**0.44**	**1.06**
RADIX ANGELICA SINENSIS	**3938500**	**5000407**	**0.79**	**0.52**	**0.95**
PORIA COCOS	**2883020**	**3292491**	**0.88**	**0.58**	**0.96**
CORTEX MOUTAN	**4084380**	**3396690**	**1.2**	**0.79**	**0.87**
RADIX REHMANNIAE PREPARATA	**4775060**	**3194388**	**1.49**	**0.99**	**0.73**
RHIZOMA DIOSCOREAE	**3232820**	**4974233**	**0.65**	**0.43**	**0.68**
FRUCTUS CORNI	**2988900**	**2897463**	**1.03**	**0.68**	**0.21**
RHIZOMA ALISMATIS	**3289680**	**5587873**	**0.59**	**0.39**	**0.77**

ERβ 48

1	ERβ 48	βA	Ratio	Normalised ratio	Relative to media
Media	**4838027**	**4029025**	**1.2**	**1**	**1**
GUI SHAO DI HUANG WAN	**3712082**	**3521300**	**1.05**	**0.88**	**0.767272**
RADIX PAEONIAE ALBA	**3129000**	**3210563**	**0.97**	**0.81**	**0.646751**
RADIX ANGELICA SINENSIS	**2900132**	**2474568**	**1.17**	**0.98**	**0.599445**
PORIA COCOS	**3631659**	**3111714**	**1.17**	**0.97**	**0.750649**
CORTEX MOUTAN	**2621315**	**1357740**	**1.93**	**1.61**	**0.541815**
RADIX REHMANNIAE PREPARATA	**1221603**	**2829060**	**0.43**	**0.36**	**0.2525**
RHIZOMA DIOSCOREAE	**1478728**	**3106962**	**0.48**	**0.4**	**0.305647**
FRUCTUS CORNI	**746810**	**3202032**	**0.23**	**0.19**	**0.154363**
RHIZOMA ALISMATIS	**1995239**	**4067080**	**0.49**	**0.41**	**0.412408**
2					
Media	**3683960**	**2929407**	**1.26**	**1**	**1**
GUI SHAO DI HUANG WAN	**3199760**	**4087120**	**0.78**	**0.62**	**0.868565**
RADIX PAEONIAE ALBA	**2288660**	**4262422**	**0.54**	**0.43**	**0.62125**
RADIX ANGELICA SINENSIS	**3938500**	**4475700**	**0.88**	**0.7**	**1.069094**
PORIA COCOS	**2883020**	**3919120**	**0.74**	**0.58**	**0.782587**
CORTEX MOUTAN	**4084380**	**3019455**	**1.35**	**1.08**	**1.108693**
RADIX REHMANNIAE PREPARATA	**4775060**	**3324060**	**1.44**	**1.14**	**1.296176**
RHIZOMA DIOSCOREAE	**3232820**	**3142736**	**1.03**	**0.82**	**0.877539**
FRUCTUS CORNI	**2988900**	**3376353**	**0.89**	**0.7**	**0.811328**
RHIZOMA ALISMATIS	**3289680**	**3802240**	**0.87**	**0.69**	**0.892974**
3					
Media	**3683960**	**2429030**	**1.52**	**1**	**1**
GUI SHAO DI HUANG WAN	**3199760**	**3119789**	**1.03**	**0.68**	**0.750303**
RADIX PAEONIAE ALBA	**2288660**	**3468078**	**0.66**	**0.44**	**1.064833**
RADIX ANGELICA SINENSIS	**3938500**	**5000407**	**0.79**	**0.52**	**0.949828**
PORIA COCOS	**2883020**	**3292491**	**0.88**	**0.58**	**0.960325**
CORTEX MOUTAN	**4084380**	**3396690**	**1.2**	**0.79**	**0.868447**
RADIX REHMANNIAE PREPARATA	**4775060**	**3194388**	**1.49**	**0.99**	**0.733558**
RHIZOMA DIOSCOREAE	**3232820**	**4974233**	**0.65**	**0.43**	**0.678695**
FRUCTUS CORNI	**2988900**	**2897463**	**1.03**	**0.68**	**0.210855**
RHIZOMA ALISMATIS	**3289680**	**5587873**	**0.59**	**0.39**	**0.770334**

PRβ 24

1	PRβ	βA	Ratio	Normalised ratio	Relative to media
Media	**582192**	**2270450**	**0.26**	**1**	**1**
GUI SHAO DI HUANG WAN	**2133164**	**2476760**	**0.86**	**3.36**	**3.664021**
RADIX PAEONIAE ALBA	**1790280**	**3490940**	**0.51**	**2**	**3.075068**
RADIX ANGELICA SINENSIS	**1495662**	**3851190**	**0.39**	**1.51**	**2.569018**
PORIA COCOS	**1592154**	**3621096**	**0.44**	**1.71**	**2.734758**
CORTEX MOUTAN	**1582972**	**3312127**	**0.48**	**1.86**	**2.718986**
RADIX REHMANNIAE PREPARATA	**2076703**	**3267043**	**0.64**	**2.48**	**3.567041**
RHIZOMA DIOSCOREAE	**1782140**	**4255220**	**0.42**	**1.63**	**3.061086**
FRUCTUS CORNI	**2491880**	**4608700**	**0.54**	**2.11**	**4.280169**
RHIZOMA ALISMATIS	**1570690**	**4254976**	**0.37**	**1.44**	**2.69789**
2					
Media	**2023920**	**4100730**	**0.49**	**1**	**1**
GUI SHAO DI HUANG WAN	**5198382**	**3771831**	**1.38**	**2.79**	**3.269301**
RADIX PAEONIAE ALBA	**2826296**	**4050134**	**0.7**	**1.41**	**1.774698**
RADIX ANGELICA SINENSIS	**1804740**	**5051718**	**0.36**	**0.72**	**1.132598**
PORIA COCOS	**2541420**	**4235940**	**0.6**	**1.22**	**1.59467**
CORTEX MOUTAN	**2477920**	**3274220**	**0.76**	**1.53**	**1.556257**
RADIX REHMANNIAE PREPARATA	**2053984**	**2469360**	**0.83**	**1.69**	**1.291766**
RHIZOMA DIOSCOREAE	**1911080**	**3217960**	**0.59**	**1.2**	**1.199993**
FRUCTUS CORNI	**2511160**	**3336980**	**0.75**	**1.52**	**1.579287**
RHIZOMA ALISMATIS	**2508288**	**4038840**	**0.62**	**1.26**	**1.577481**
3					
Media	**3958042**	**1546133**	**2.56**	**1**	**1**
GUI SHAO DI HUANG WAN	**5123096**	**1472720**	**3.48**	**1.36**	**1.167674**
RADIX PAEONIAE ALBA	**4989432**	**3682115**	**1.36**	**0.53**	**1.137209**
RADIX ANGELICA SINENSIS	**4533606**	**2377348**	**1.91**	**0.74**	**1.033315**
PORIA COCOS	**4007740**	**2686663**	**1.49**	**0.58**	**0.913458**
CORTEX MOUTAN	**1726868**	**1931115**	**0.89**	**0.35**	**0.393594**
RADIX REHMANNIAE PREPARATA	**4845742**	**1239691**	**3.91**	**1.53**	**1.104458**
RHIZOMA DIOSCOREAE	**5079404**	**1249619**	**4.06**	**1.59**	**1.157715**
FRUCTUS CORNI	**5875452**	**1361581**	**4.32**	**1.69**	**1.339153**
RHIZOMA ALISMATIS	**5590640**	**1724429**	**3.24**	**1.27**	**1.274238**

PRβ 48

1	PRβ	βA	Normalised ratio	Relative to media
Media	**4512784**	**1202026**	**1**	**1**
GUI SHAO DI HUANG WAN	**3984942**	**1414530**	**0.75**	**0.883034**
RADIX PAEONIAE ALBA	**5294400**	**1279450**	**1.1**	**1.1732**
RADIX ANGELICA SINENSIS	**7338072**	**1224993**	**1.6**	**1.626063**
PORIA COCOS	**6280764**	**1261428**	**1.33**	**1.391771**
CORTEX MOUTAN	**3896739**	**1236963**	**0.84**	**0.863489**
RADIX REHMANNIAE PREPARATA	**4418379**	**1063209**	**1.11**	**0.979081**
RHIZOMA DIOSCOREAE	**3815574**	**1135806**	**0.89**	**0.845503**
FRUCTUS CORNI	**3255315**	**1337574**	**0.65**	**0.721354**
RHIZOMA ALISMATIS	**2678616**	**1160892**	**0.61**	**0.593562**
2				
Media	**2482245**	**1536489**	**1**	**1**
GUI SHAO DI HUANG WAN	**2204950**	**1275625**	**1.07**	**0.888289**
RADIX PAEONIAE ALBA	**2868300**	**1133310**	**1.57**	**1.155527**
RADIX ANGELICA SINENSIS	**2244585**	**962157**	**1.44**	**0.904256**
PORIA COCOS	**3403974**	**1253868**	**1.68**	**1.371329**
CORTEX MOUTAN	**2019780**	**1279383**	**0.98**	**0.813691**
RADIX REHMANNIAE PREPARATA	**5237148**	**2916627**	**1.11**	**2.109843**
RHIZOMA DIOSCOREAE	**1956864**	**1135344**	**1.07**	**0.788344**
FRUCTUS CORNI	**2268147**	**1143702**	**1.23**	**0.913748**
RHIZOMA ALISMATIS	**5600637**	**3056970**	**1.13**	**2.256279**
3				
Media	**2097025**	**1272600**	**1**	**1**
GUI SHAO DI HUANG WAN	**1960659**	**1469745**	**0.81**	**0.934972**
RADIX PAEONIAE ALBA	**2453180**	**815143**	**1.83**	**1.169838**
RADIX ANGELICA SINENSIS	**4560050**	**1325082**	**2.09**	**2.174533**
PORIA COCOS	**5000200**	**1115160**	**2.72**	**2.384426**
CORTEX MOUTAN	**2163060**	**732240**	**1.79**	**1.03149**
RADIX REHMANNIAE PREPARATA	**3870480**	**1722020**	**1.36**	**1.8457**
RHIZOMA DIOSCOREAE	**4468120**	**1879880**	**1.44**	**2.130695**
FRUCTUS CORNI	**1965440**	**910840**	**1.31**	**0.937252**
RHIZOMA ALISMATIS	**2147375**	**1484050**	**0.88**	**1.02401**

XII. PT POST HOC TESTS (DUNN'S) FOR INDIVIDUAL HERB EXTRACTS

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
dH2O-Cortex Moutan	.500	9.214	.054	.957	1.000
dH2O-Rhizoma Alismatis	2.300	9.214	.250	.803	1.000
dH2O-Rhizoma Dioscoreae	3.700	9.214	.402	.688	1.000
dH2O-Radix Paeoniae Alba	8.100	9.214	.879	.379	1.000
dH2O-Radix Rehmanniae Preparata	13.600	9.214	1.476	.140	1.000
dH2O-Poria Cocos	18.500	9.214	2.008	.045	1.000
dH2O-Radix Angelica Sinensis	28.000	9.214	3.039	.002	.107
dH2O-Gui Shao Di Huang Wan	30.700	9.214	3.332	.001	.039
dH2O-Fructus Corni	35.600	9.214	3.863	.000	.005
Cortex Moutan-Rhizoma Alismatis	1.800	9.214	.195	.845	1.000
Cortex Moutan-Rhizoma Dioscoreae	3.200	9.214	.347	.728	1.000
Cortex Moutan-Radix Paeoniae Alba	7.600	9.214	.825	.409	1.000
Cortex Moutan-Radix Rehmanniae Preparata	13.100	9.214	1.422	.155	1.000
Cortex Moutan-Poria Cocos	18.000	9.214	1.953	.051	1.000
Cortex Moutan-Radix Angelica Sinensis	27.500	9.214	2.984	.003	.128
Cortex Moutan-Gui Shao Di Huang Wan	30.200	9.214	3.277	.001	.047
Cortex Moutan-Fructus Corni	35.100	9.214	3.809	.000	.006
Rhizoma Alismatis-Rhizoma Dioscoreae	1.400	9.214	.152	.879	1.000
Rhizoma Alismatis-Radix Paeoniae Alba	5.800	9.214	.629	.529	1.000
Rhizoma Alismatis-Radix Rehmanniae Preparata	11.300	9.214	1.226	.220	1.000
Rhizoma Alismatis-Poria Cocos	16.200	9.214	1.758	.079	1.000
Rhizoma Alismatis-Radix Angelica Sinensis	25.700	9.214	2.789	.005	.238
Rhizoma Alismatis-Gui Shao Di Huang Wan	28.400	9.214	3.082	.002	.092
Rhizoma Alismatis-Fructus Corni	33.300	9.214	3.614	.000	.014
Rhizoma Dioscoreae-Radix Paeoniae Alba	4.400	9.214	.478	.633	1.000
Rhizoma Dioscoreae-Radix Rehmanniae Preparata	9.900	9.214	1.074	.283	1.000
Rhizoma Dioscoreae-Poria Cocos	14.800	9.214	1.606	.108	1.000
Rhizoma Dioscoreae-Radix Angelica Sinensis	24.300	9.214	2.637	.008	.376
Rhizoma Dioscoreae-Gui Shao Di Huang Wan	27.000	9.214	2.930	.003	.152
Rhizoma Dioscoreae-Fructus Corni	31.900	9.214	3.462	.001	.024
Radix Paeoniae Alba-Radix Rehmanniae Preparata	5.500	9.214	.597	.551	1.000
Radix Paeoniae Alba-Poria Cocos	10.400	9.214	1.129	.259	1.000
Radix Paeoniae Alba-Radix Angelica Sinensis	19.900	9.214	2.160	.031	1.000
Radix Paeoniae Alba-Gui Shao Di Huang Wan	22.600	9.214	2.453	.014	.638
Radix Paeoniae Alba-Fructus Corni	27.500	9.214	2.984	.003	.128
Radix Rehmanniae Preparata-Poria Cocos	4.900	9.214	.532	.595	1.000
Radix Rehmanniae Preparata-Radix Angelica Sinensis	14.400	9.214	1.563	.118	1.000
Radix Rehmanniae Preparata-Gui Shao Di Huang Wan	17.100	9.214	1.856	.063	1.000
Radix Rehmanniae Preparata-Fructus Corni	22.000	9.214	2.388	.017	.763
Poria Cocos-Radix Angelica Sinensis	9.500	9.214	1.031	.303	1.000
Poria Cocos-Gui Shao Di Huang Wan	12.200	9.214	1.324	.186	1.000
Poria Cocos-Fructus Corni	17.100	9.214	1.856	.063	1.000
Radix Angelica Sinensis-Gui Shao Di Huang Wan	2.700	9.214	.293	.770	1.000
Radix Angelica Sinensis-Fructus Corni	7.600	9.214	.825	.409	1.000
Gui Shao Di Huang Wan-Fructus Corni	4.900	9.214	.532	.595	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

XIII. APTT POST HOC TESTS FOR INDIVIDUAL HERB EXTRACTS

Games Howell post hoc tests						
(I) Herb	(J) Herb	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
dH2O	Gui Shao Di Huang Wan	-104.000000*	6.514461	.001	-139.78146	-68.21854
	Radix Paeoniae Alba	-1.260000	.754718	.787	-4.41839	1.89839
	Radix Angelicae Sinensis	-13.660000*	1.734532	.008	-22.37793	-4.94207
	Sclerotium Poria Cocos	6.640000*	1.098453	.012	1.67230	11.60770
	Cortex Moutan	1.060000	.838451	.937	-2.48429	4.60429
	Radix Rehmanniae Preparata	1.060000	.838451	.937	-2.48429	4.60429
	Rhizoma Dioscoreae	2.540000	1.313621	.659	-3.68901	8.76901
	Fructus Corni	-44.120000*	4.202523	.004	-66.96486	-21.27514
	Rhizoma Alismatis	4.620000	1.522498	.260	-2.84567	12.08567
Gui Shao Di Huang Wan	dH2O	104.000000*	6.514461	.001	68.21854	139.78146
	Radix Paeoniae Alba	102.740000*	6.514492	.001	66.95875	138.52125
	Radix Angelicae Sinensis	90.340000*	6.699075	.001	55.57126	125.10874
	Sclerotium Poria Cocos	110.640000*	6.563200	.001	75.16658	146.11342
	Cortex Moutan	105.060000*	6.524722	.001	69.34605	140.77395
	Radix Rehmanniae Preparata	105.060000*	6.524722	.001	69.34605	140.77395
	Rhizoma Dioscoreae	106.540000*	6.602621	.001	71.29333	141.78667
	Fructus Corni	59.880000*	7.715582	.002	25.99897	93.76103
	Rhizoma Alismatis	108.620000*	6.647330	.001	73.60786	143.63214
Radix Paeoniae Alba	dH2O	1.260000	.754718	.787	-1.89839	4.41839
	Gui Shao Di Huang Wan	-102.740000*	6.514492	.001	-138.52125	-66.95875
	Radix Angelicae Sinensis	-12.400000*	1.734647	.012	-21.11756	-3.68244
	Sclerotium Poria Cocos	7.900000*	1.098636	.005	2.93222	12.86778
	Cortex Moutan	2.320000	.838689	.287	-1.22504	5.86504
	Radix Rehmanniae Preparata	2.320000	.838689	.287	-1.22504	5.86504
	Rhizoma Dioscoreae	3.800000	1.313773	.286	-2.42883	10.02883
	Fructus Corni	-42.860000*	4.202571	.005	-65.70456	-20.01544
	Rhizoma Alismatis	5.880000	1.522629	.122	-1.58536	13.34536
Radix Angelicae Sinensis	dH2O	13.660000*	1.734532	.008	4.94207	22.37793
	Gui Shao Di Huang Wan	-90.340000*	6.699075	.001	-125.10874	-55.57126
	Radix Paeoniae Alba	12.400000*	1.734647	.012	3.68244	21.11756
	Sclerotium Poria Cocos	20.300000*	1.909450	.000	11.74780	28.85220
	Cortex Moutan	14.720000*	1.772682	.005	6.10217	23.33783
	Radix Rehmanniae Preparata	14.720000*	1.772682	.005	6.10217	23.33783
	Rhizoma Dioscoreae	16.200000*	2.040833	.001	7.43124	24.96876
	Fructus Corni	-30.460000*	4.483369	.012	-52.21353	-8.70647
	Rhizoma Alismatis	18.280000*	2.181146	.001	9.09863	27.46137
Sclerotium Poria Cocos	dH2O	-6.640000*	1.098453	.012	-11.60770	-1.67230
	Gui Shao Di Huang Wan	-110.640000*	6.563200	.001	-146.11342	-75.16658
	Radix Paeoniae Alba	-7.900000*	1.098636	.005	-12.86778	-2.93222
	Radix Angelicae Sinensis	-20.300000*	1.909450	.000	-28.85220	-11.74780
	Cortex Moutan	-5.580000*	1.157756	.030	-10.61886	-.54114
	Radix Rehmanniae Preparata	-5.580000*	1.157756	.030	-10.61886	-.54114
	Rhizoma Dioscoreae	-4.100000	1.537205	.322	-10.61978	2.41978
	Fructus Corni	-50.760000*	4.277686	.002	-73.19128	-28.32872
	Rhizoma Alismatis	-2.020000	1.719128	.955	-9.50265	5.46265

Cortex Moutan	dH2O	-1.060000	.838451	.937	-4.60429	2.48429
	Gui Shao Di Huang Wan	-105.060000*	6.524722	.001	-140.77395	-69.34605
	Radix Paeoniae Alba	-2.320000	.838689	.287	-5.86504	1.22504
	Radix Angelicae Sinensis	-14.720000*	1.772682	.005	-23.33783	-6.10217
	Sclerotium Poria Cocos	5.580000*	1.157756	.030	.54114	10.61886
	Radix Rehmanniae Preparata	.000000	.914768	1.000	-3.82817	3.82817
	Rhizoma Dioscoreae	1.480000	1.363598	.969	-4.72710	7.68710
	Fructus Corni	-45.180000*	4.218412	.004	-67.92844	-22.43156
	Rhizoma Alismatis	3.560000	1.565822	.504	-3.83348	10.95348
Radix Rehmanniae Preparata	dH2O	-1.060000	.838451	.937	-4.60429	2.48429
	Gui Shao Di Huang Wan	-105.060000*	6.524722	.001	-140.77395	-69.34605
	Radix Paeoniae Alba	-2.320000	.838689	.287	-5.86504	1.22504
	Radix Angelicae Sinensis	-14.720000*	1.772682	.005	-23.33783	-6.10217
	Sclerotium Poria Cocos	5.580000*	1.157756	.030	.54114	10.61886
	Cortex Moutan	.000000	.914768	1.000	-3.82817	3.82817
	Rhizoma Dioscoreae	1.480000	1.363598	.969	-4.72710	7.68710
	Fructus Corni	-45.180000*	4.218412	.004	-67.92844	-22.43156
	Rhizoma Alismatis	3.560000	1.565822	.504	-3.83348	10.95348
Rhizoma Dioscoreae	dH2O	-2.540000	1.313621	.659	-8.76901	3.68901
	Gui Shao Di Huang Wan	-106.540000*	6.602621	.001	-141.78667	-71.29333
	Radix Paeoniae Alba	-3.800000	1.313773	.286	-10.02883	2.42883
	Radix Angelicae Sinensis	-16.200000*	2.040833	.001	-24.96876	-7.43124
	Sclerotium Poria Cocos	4.100000	1.537205	.322	-2.41978	10.61978
	Cortex Moutan	-1.480000	1.363598	.969	-7.68710	4.72710
	Radix Rehmanniae Preparata	-1.480000	1.363598	.969	-7.68710	4.72710
	Fructus Corni	-46.660000*	4.337926	.002	-68.83080	-24.48920
	Rhizoma Alismatis	2.080000	1.863974	.968	-5.78372	9.94372
Fructus Corni	dH2O	44.120000*	4.202523	.004	21.27514	66.96486
	Gui Shao Di Huang Wan	-59.880000*	7.715582	.002	-93.76103	-25.99897
	Radix Paeoniae Alba	42.860000*	4.202571	.005	20.01544	65.70456
	Radix Angelicae Sinensis	30.460000*	4.483369	.012	8.70647	52.21353
	Sclerotium Poria Cocos	50.760000*	4.277686	.002	28.32872	73.19128
	Cortex Moutan	45.180000*	4.218412	.004	22.43156	67.92844
	Radix Rehmanniae Preparata	45.180000*	4.218412	.004	22.43156	67.92844
	Rhizoma Dioscoreae	46.660000*	4.337926	.002	24.48920	68.83080
	Rhizoma Alismatis	48.740000*	4.405678	.002	26.79747	70.68253
Rhizoma Alismatis	dH2O	-4.620000	1.522498	.260	-12.08567	2.84567
	Gui Shao Di Huang Wan	-108.620000*	6.647330	.001	-143.63214	-73.60786
	Radix Paeoniae Alba	-5.880000	1.522629	.122	-13.34536	1.58536
	Radix Angelicae Sinensis	-18.280000*	2.181146	.001	-27.46137	-9.09863
	Sclerotium Poria Cocos	2.020000	1.719128	.955	-5.46265	9.50265
	Cortex Moutan	-3.560000	1.565822	.504	-10.95348	3.83348
	Radix Rehmanniae Preparata	-3.560000	1.565822	.504	-10.95348	3.83348
	Rhizoma Dioscoreae	-2.080000	1.863974	.968	-9.94372	5.78372
	Fructus Corni	-48.740000*	4.405678	.002	-70.68253	-26.79747

*. The mean difference is significant at the 0.05 level.

XIV. COMPLETE BLOT DATA

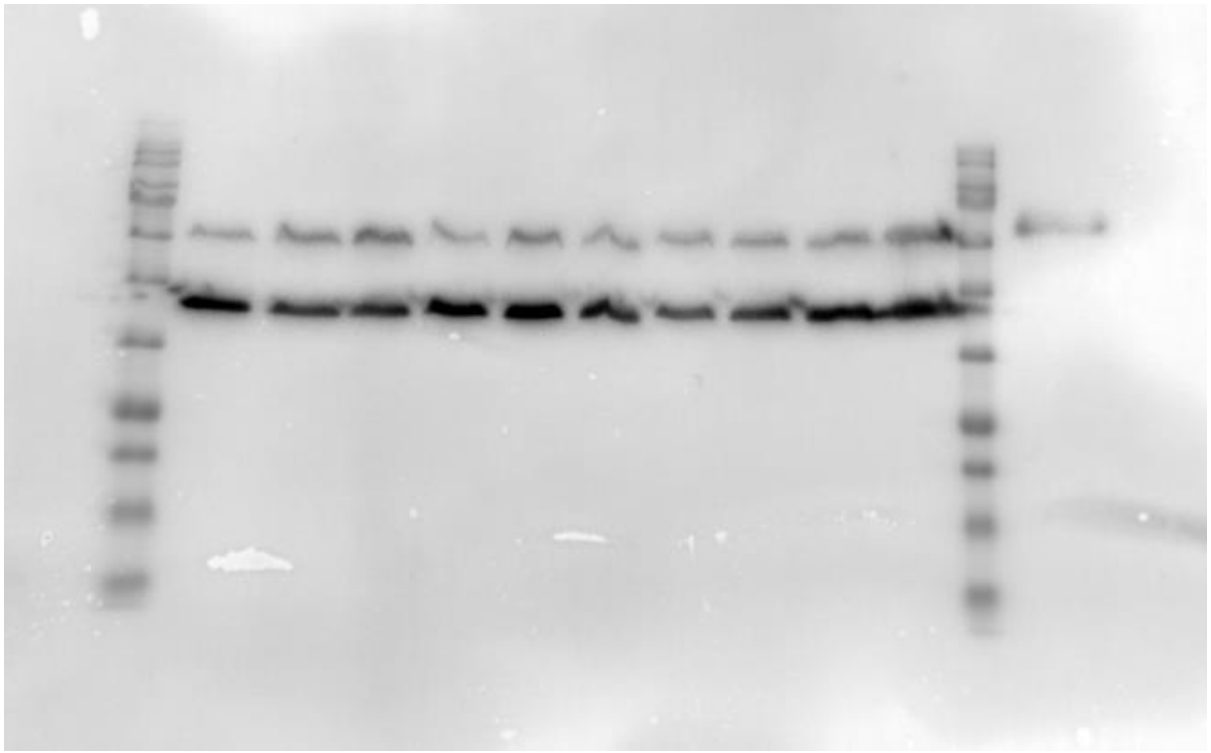

Figure 8.1 ERα24-1 ERα and BA

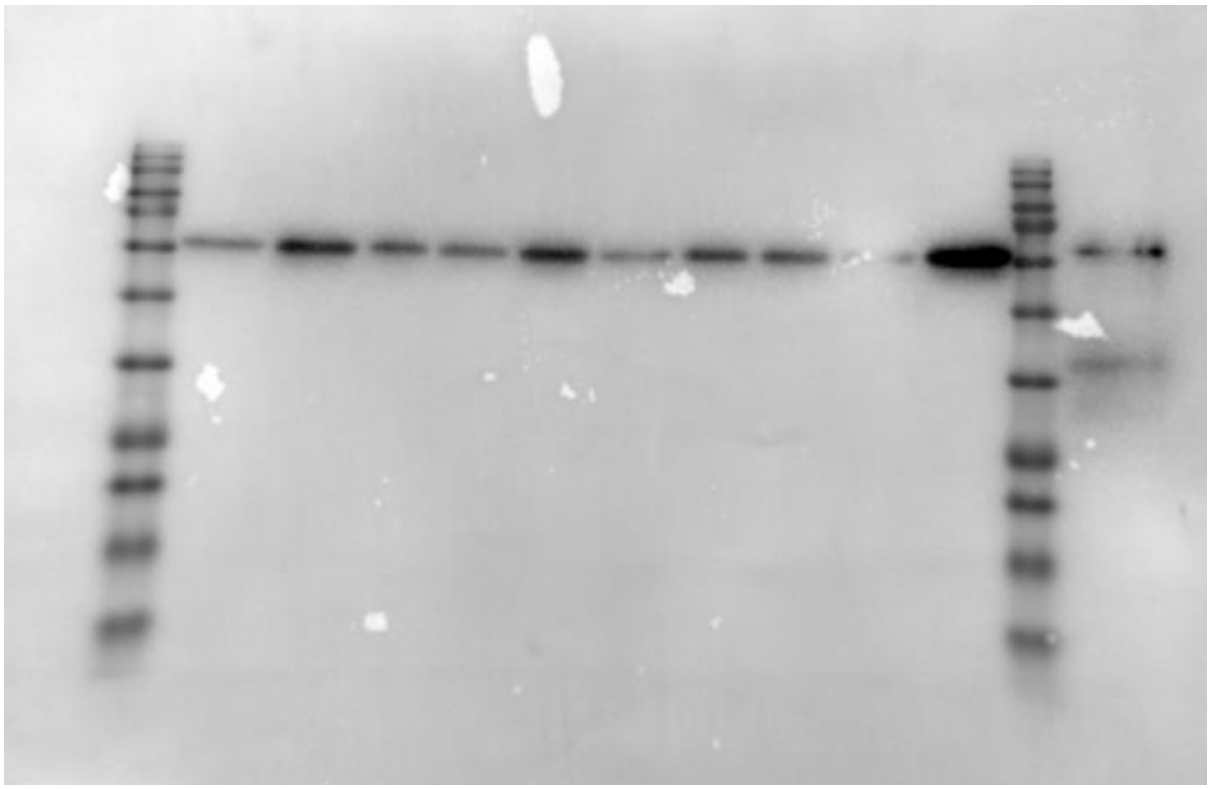

Figure 8.2 ERα24-2 ERα

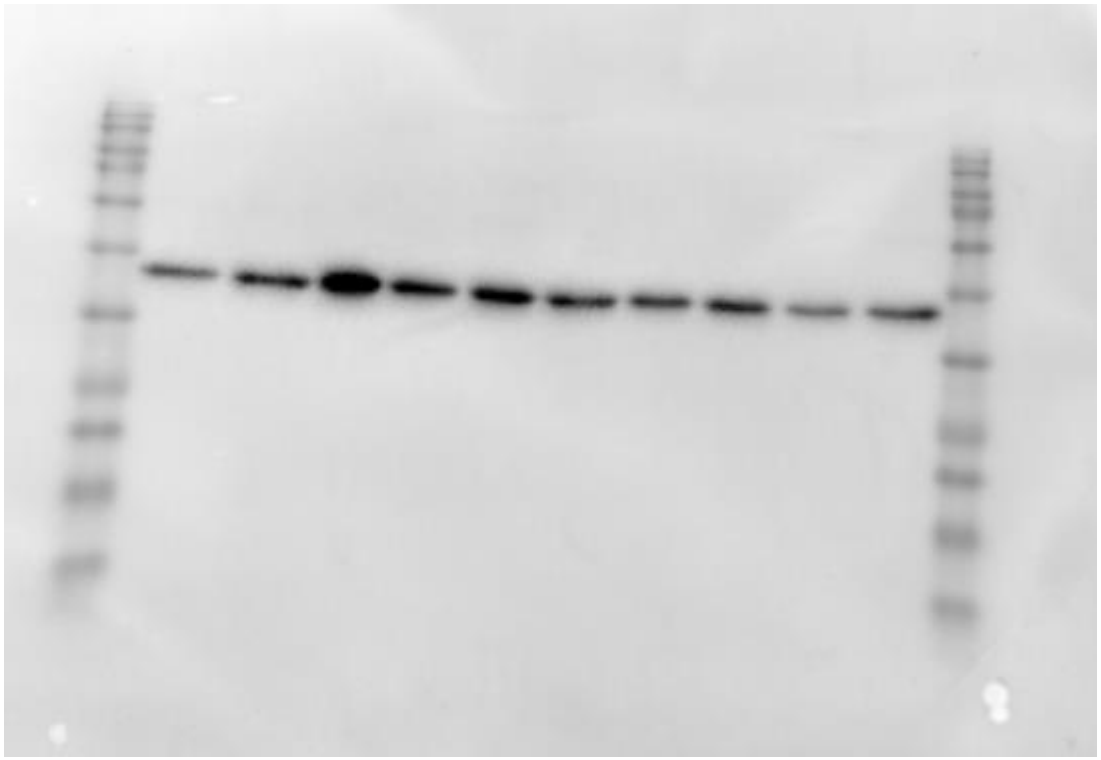

Figure 8.3 ERα24-2 BA

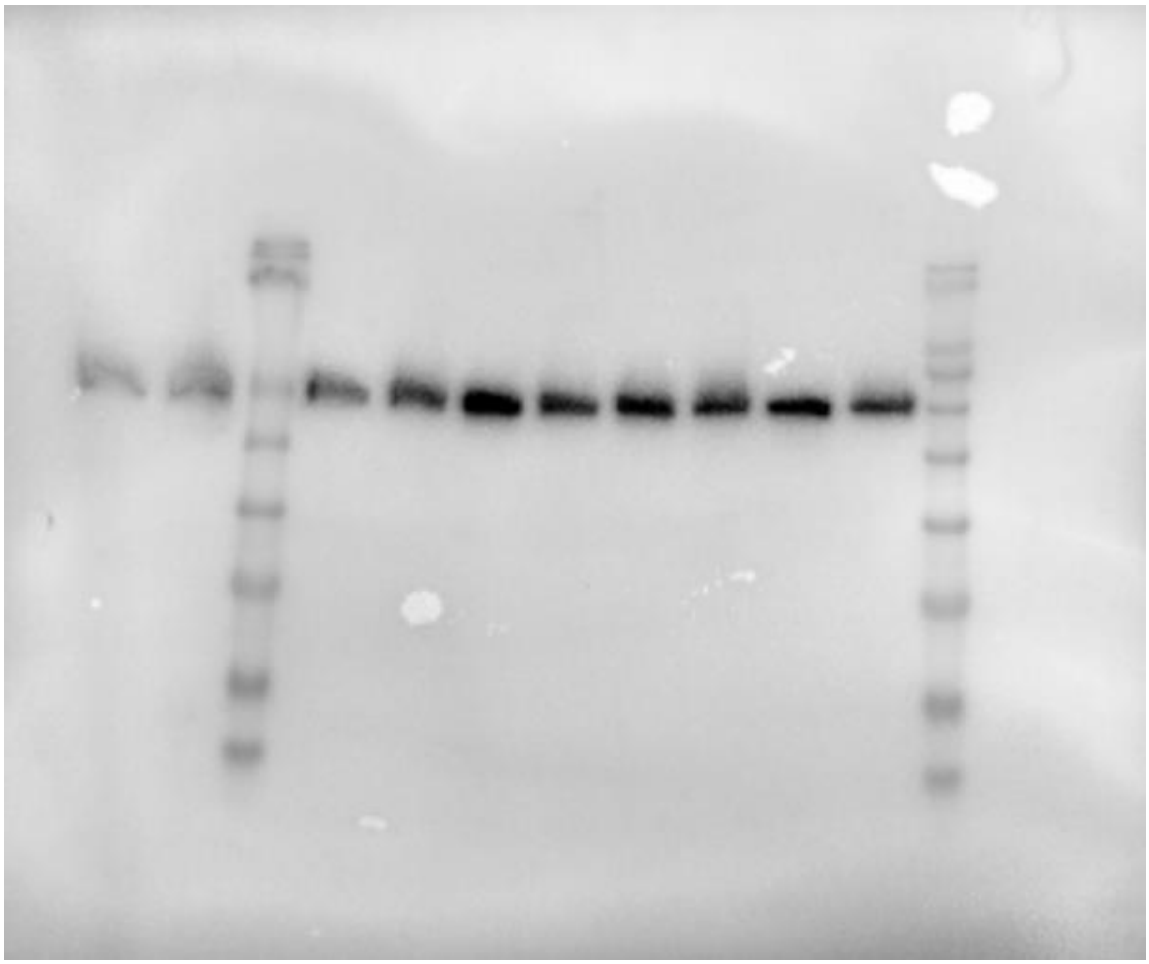

Figure 8.4 ERα24-3 ERα

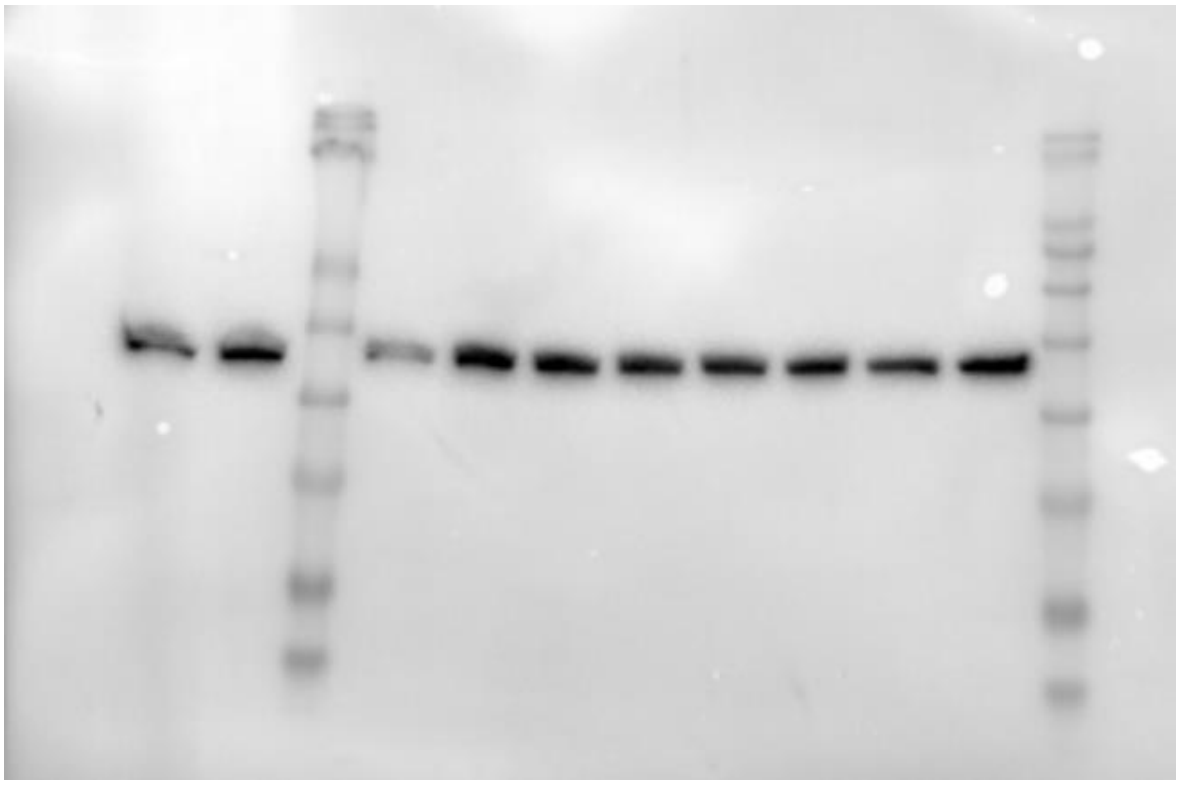

Figure 8.5 ERα24-3 BA

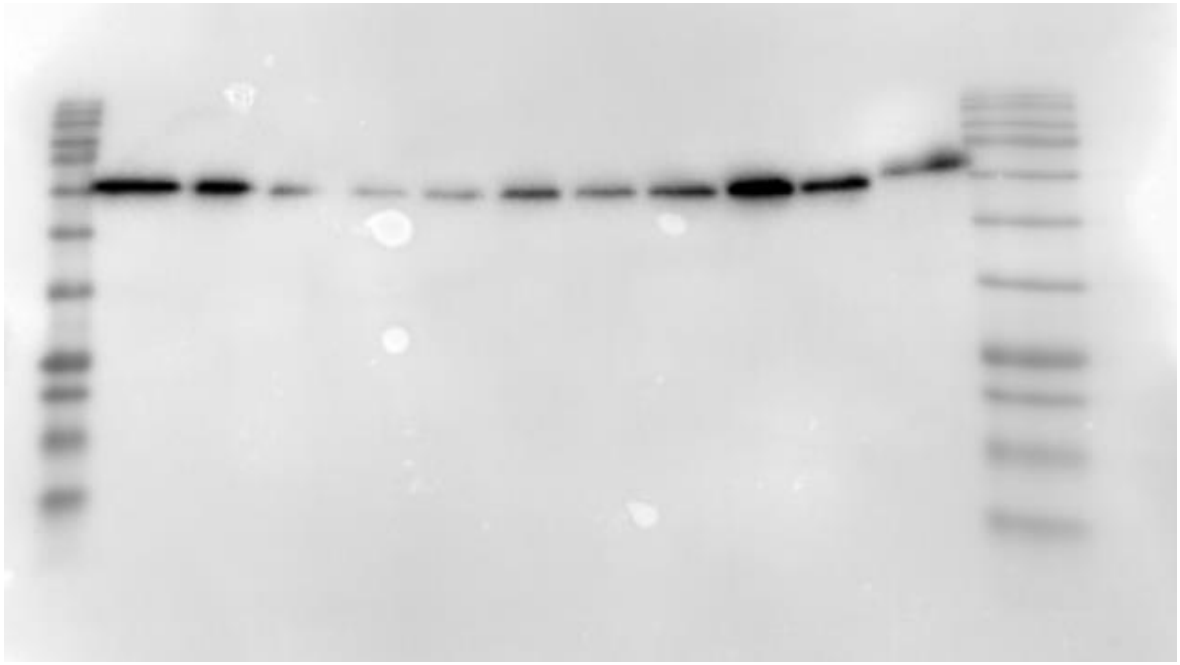

Figure 8.6 ERα48-1 ERα

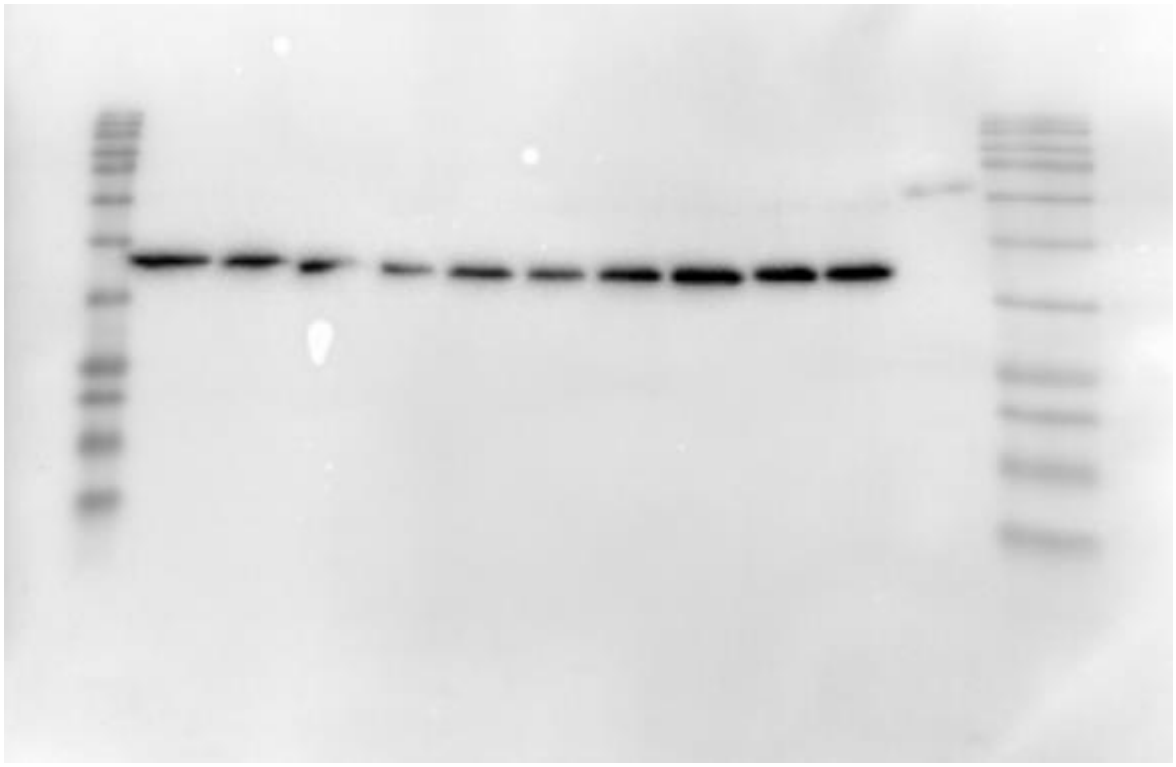

Figure 8.7 ERα48-1 BA

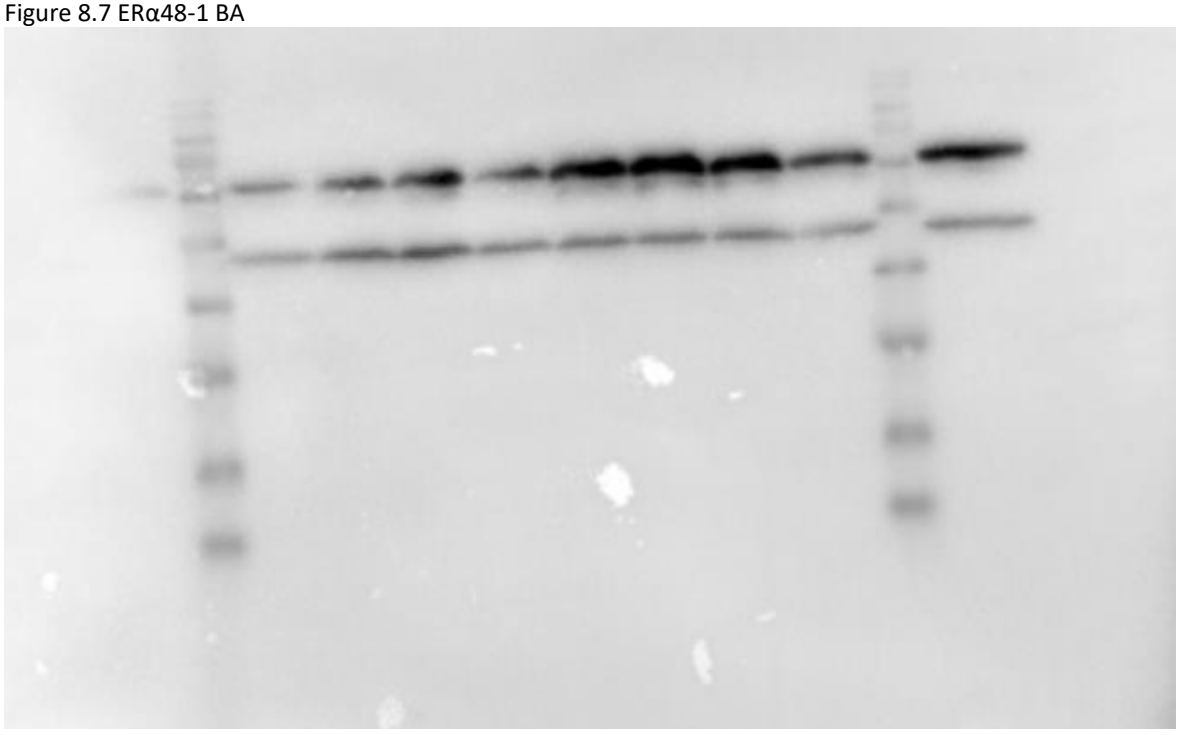

Figure 8.8 ERα48-2 ERα and BA

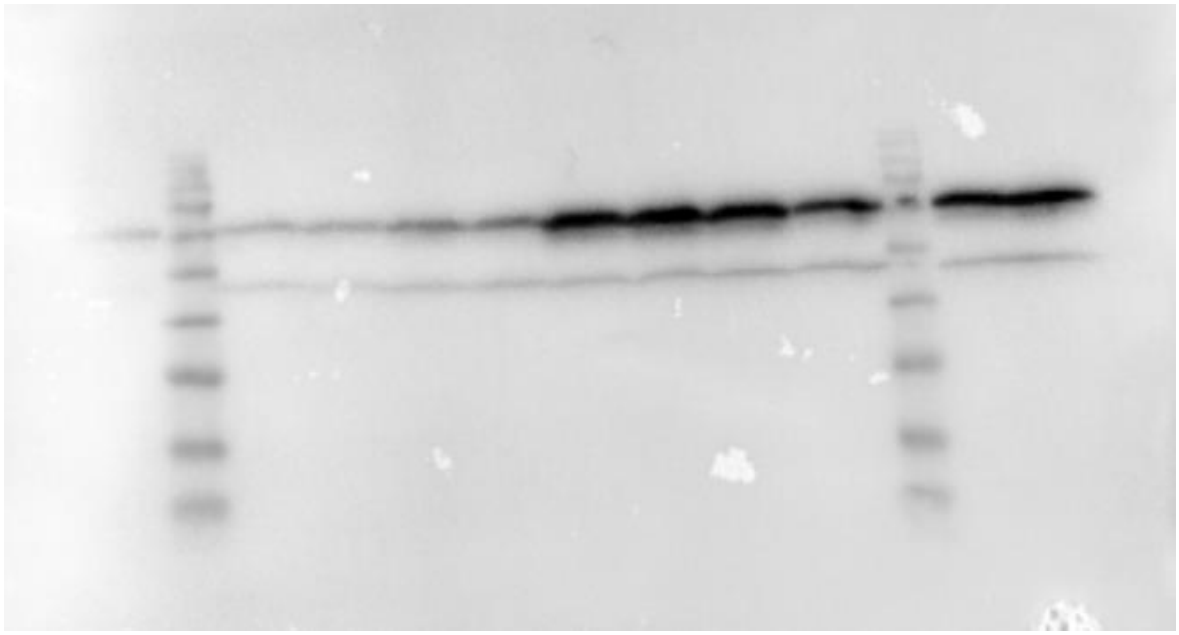

Figure 8.9 ERα48-3 ERα and BA

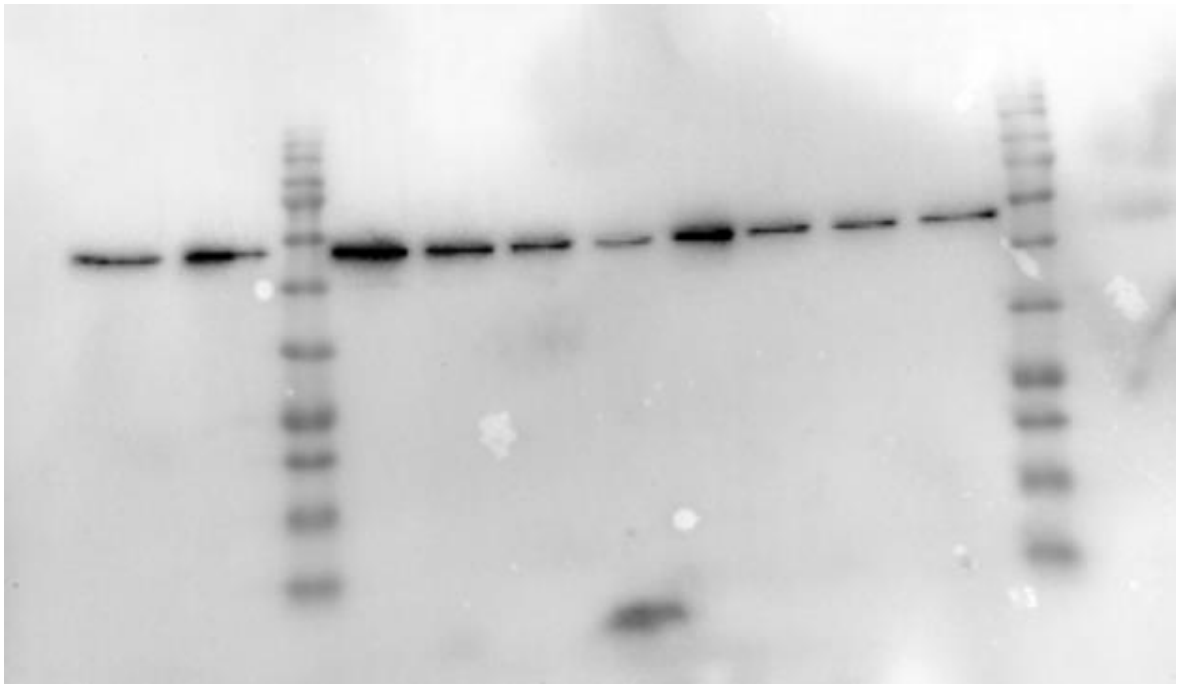

Figure 8.10 ERβ 24-1 ERβ

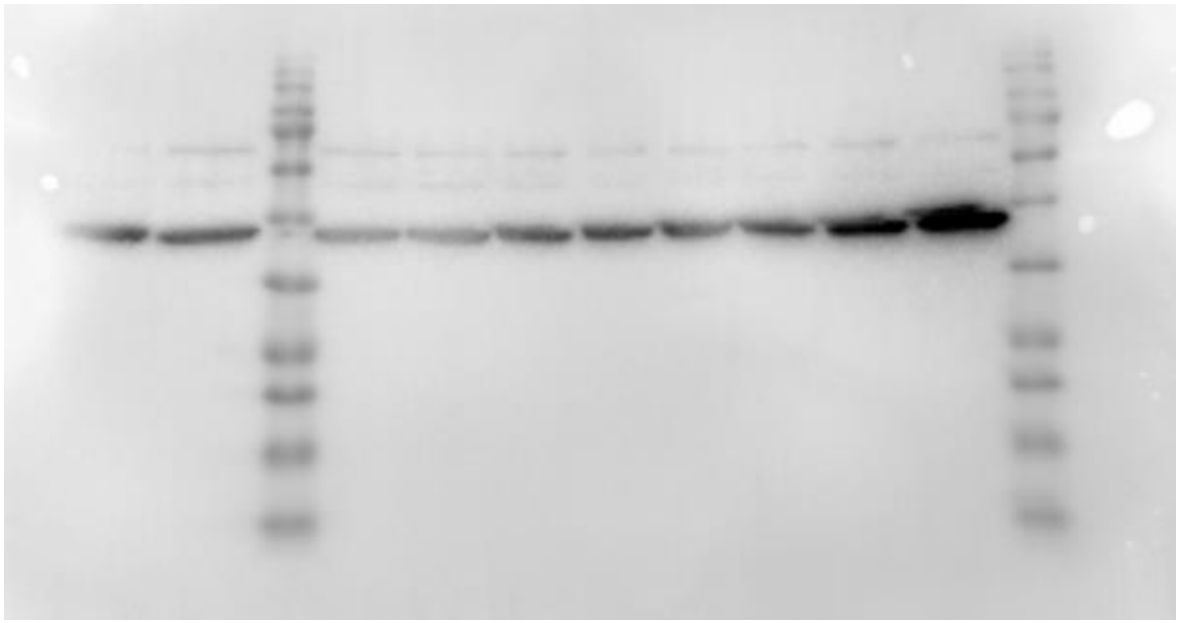

Figure 8.11 ERβ 24-1 BA

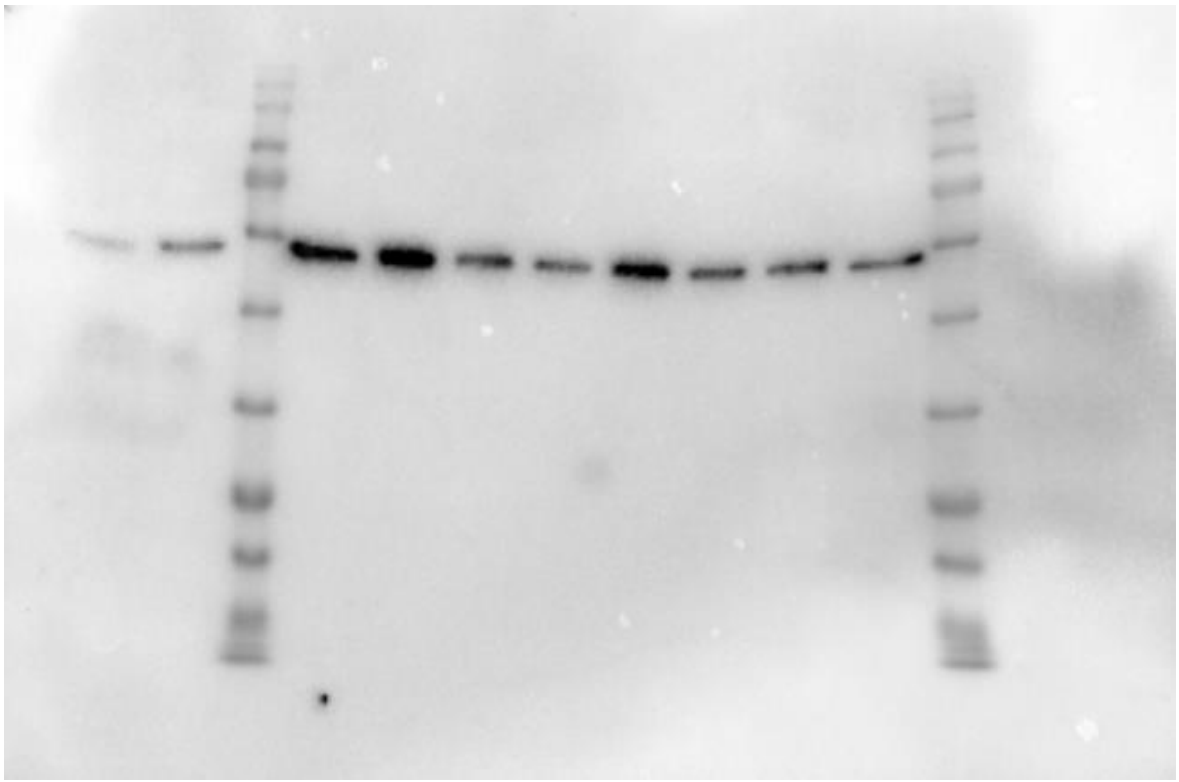

Figure 8.12 ERβ 24-2 ERβ

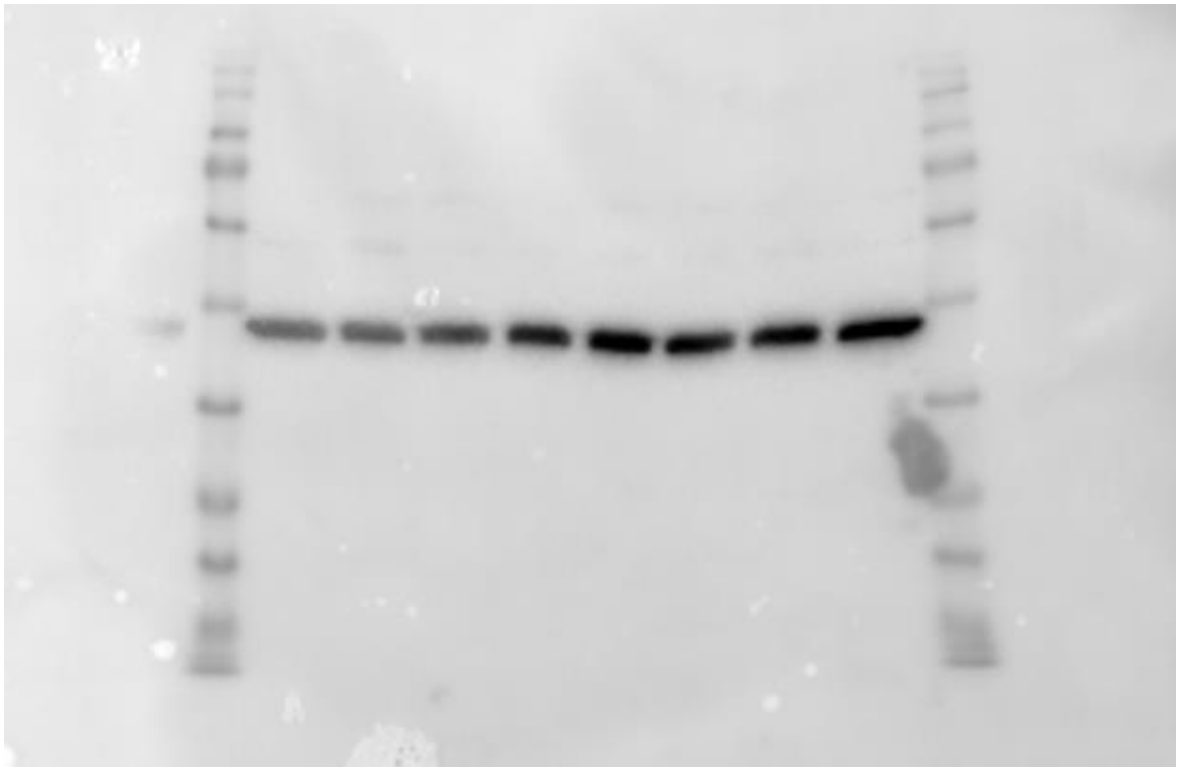

Figure 8.13 ERβ 24-2 BA

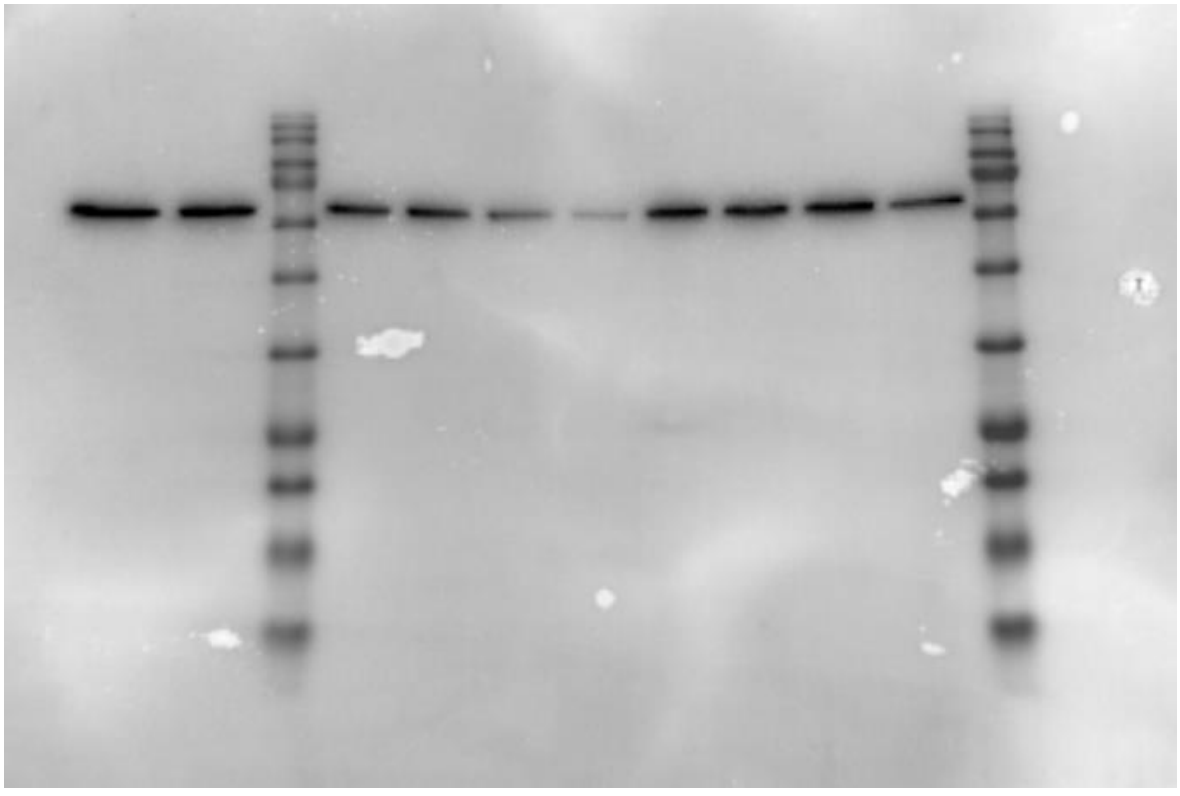

Figure 8.14 ERβ 24-3 ERβ

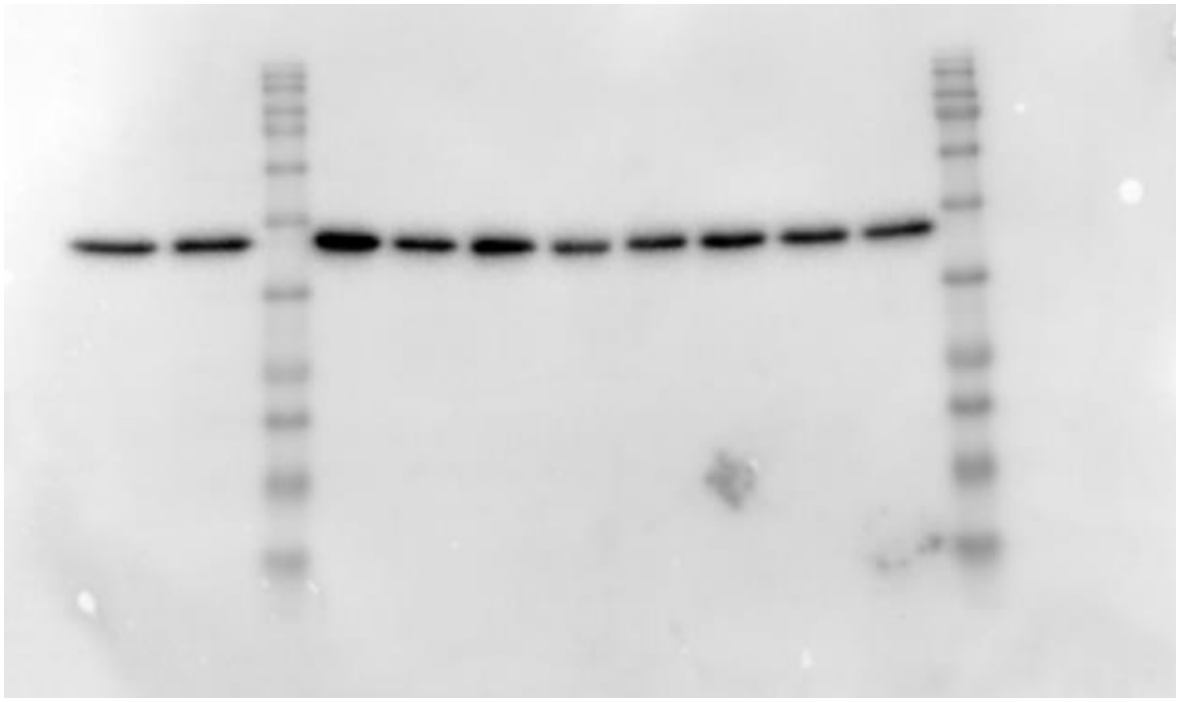

Figure 8.15 ERβ 24-3 BA

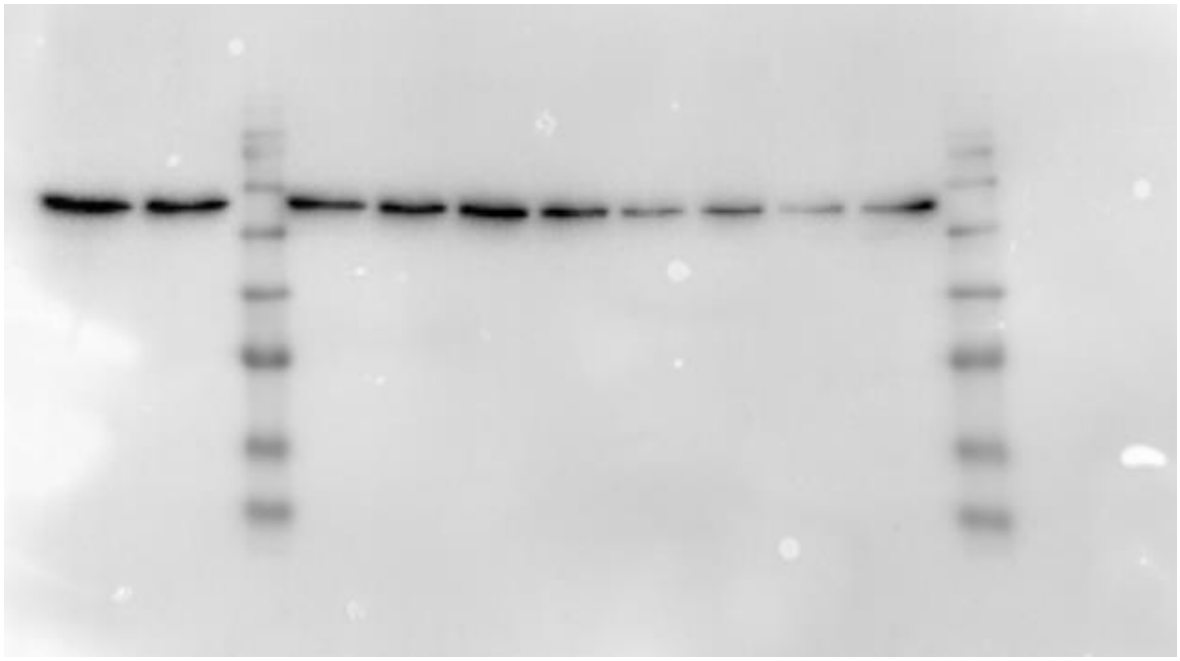

Figure 8.16 ERβ 48-1 ERβ

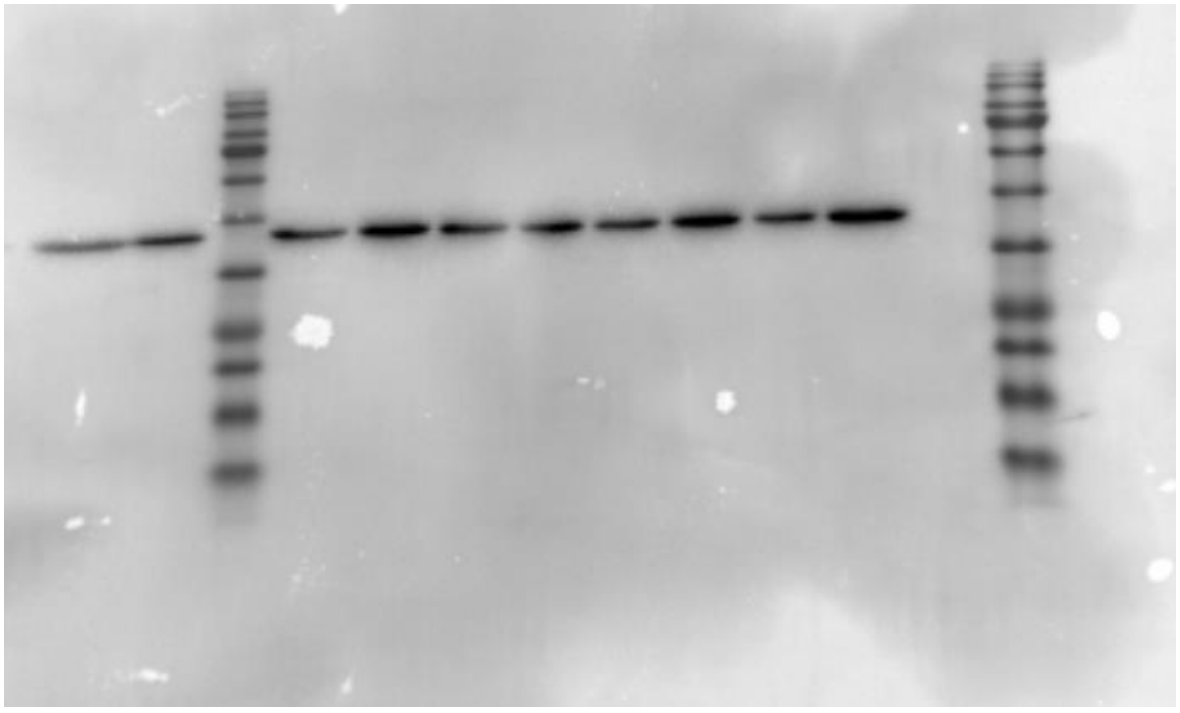

Figure 8.17 ERβ 48-1 BA

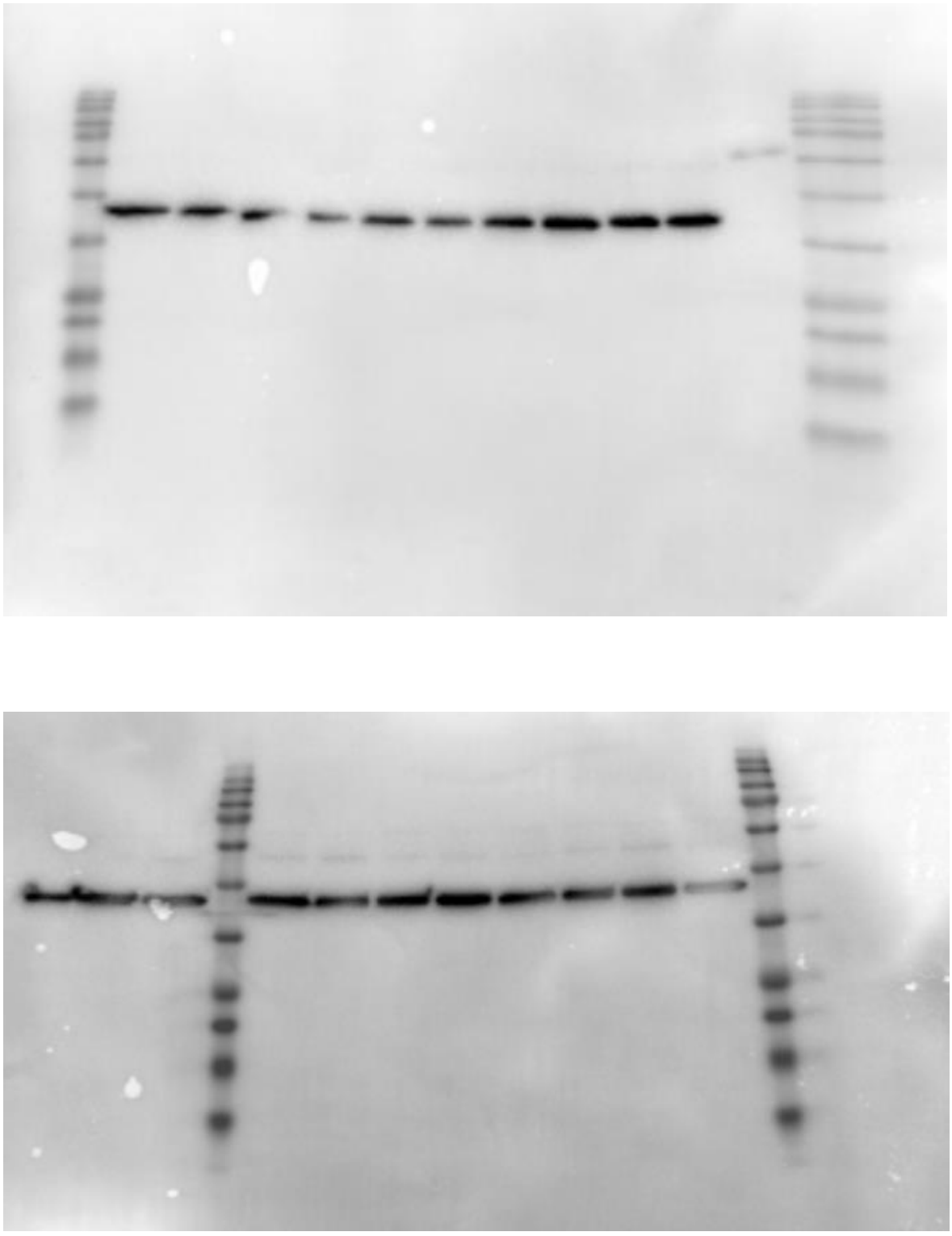

Figure 8.18 ERβ 48-2 ERβ

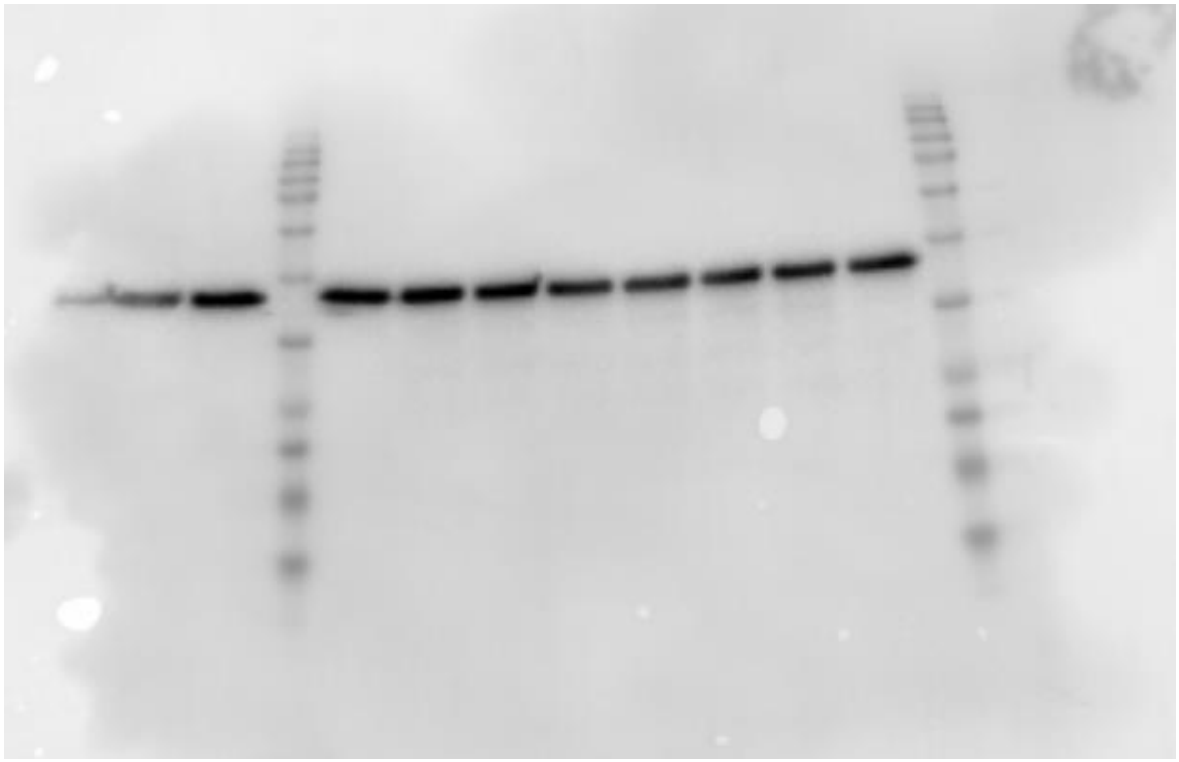

Figure 8.19 ERβ 48-2 BA

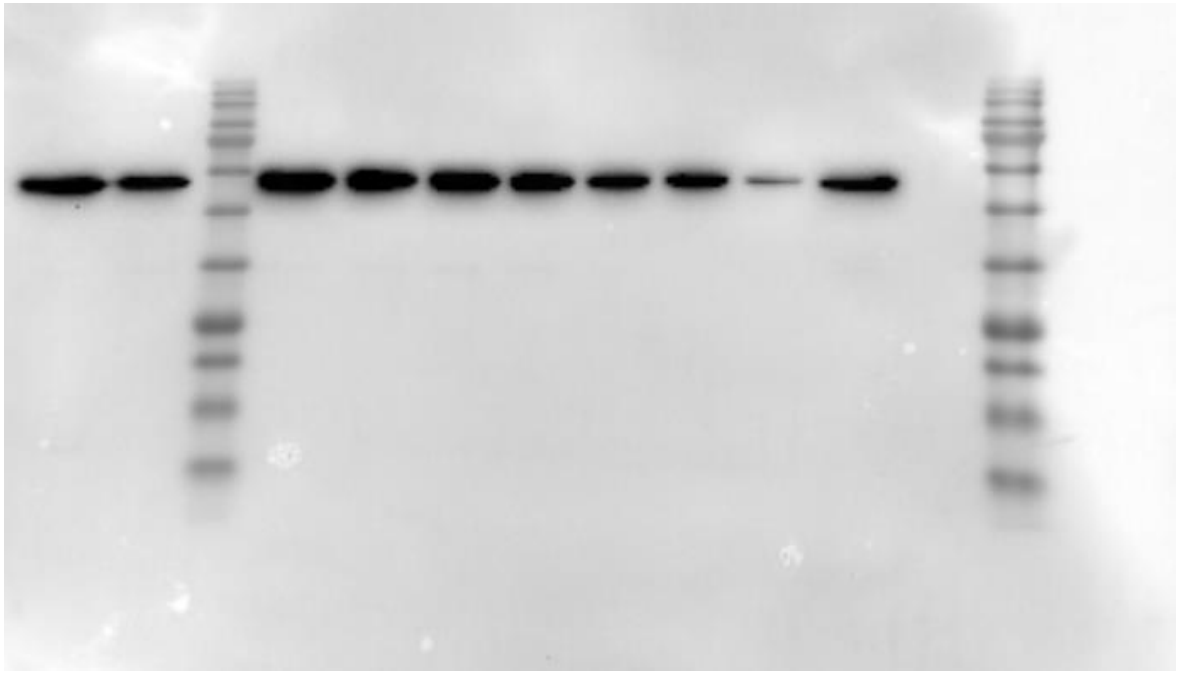

Figure 8.20 ERβ 48-3 ERβ

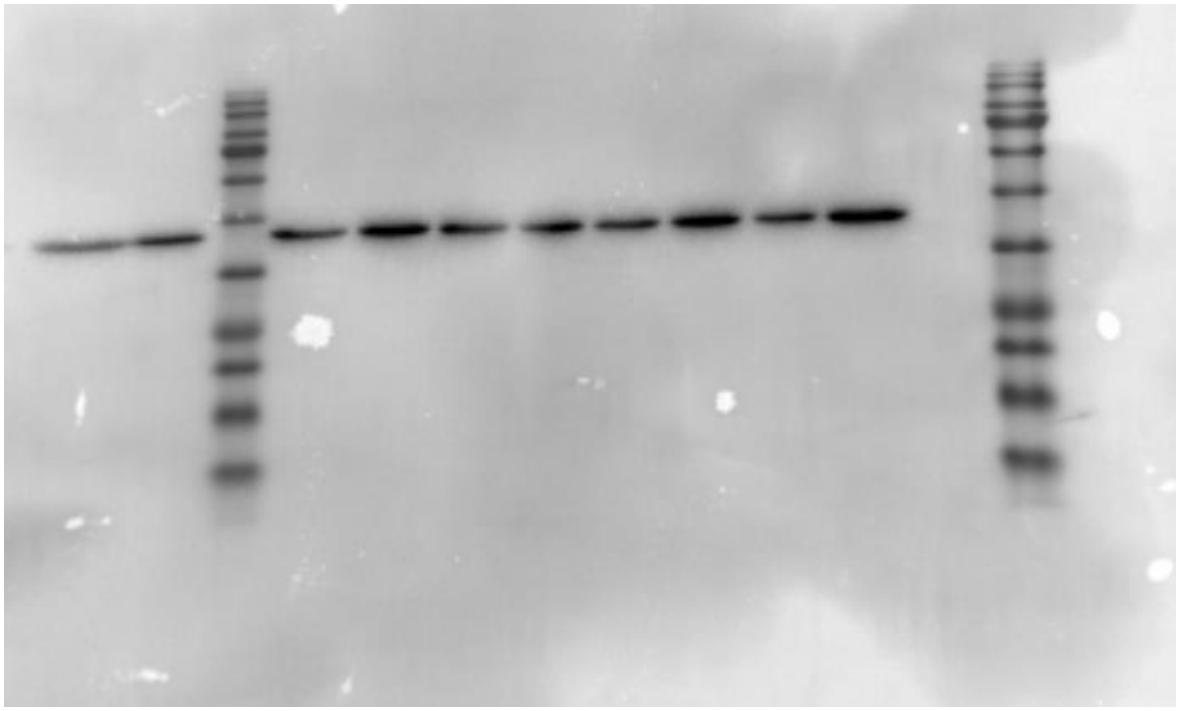

Figure 8.21 ERβ 48-3 BA

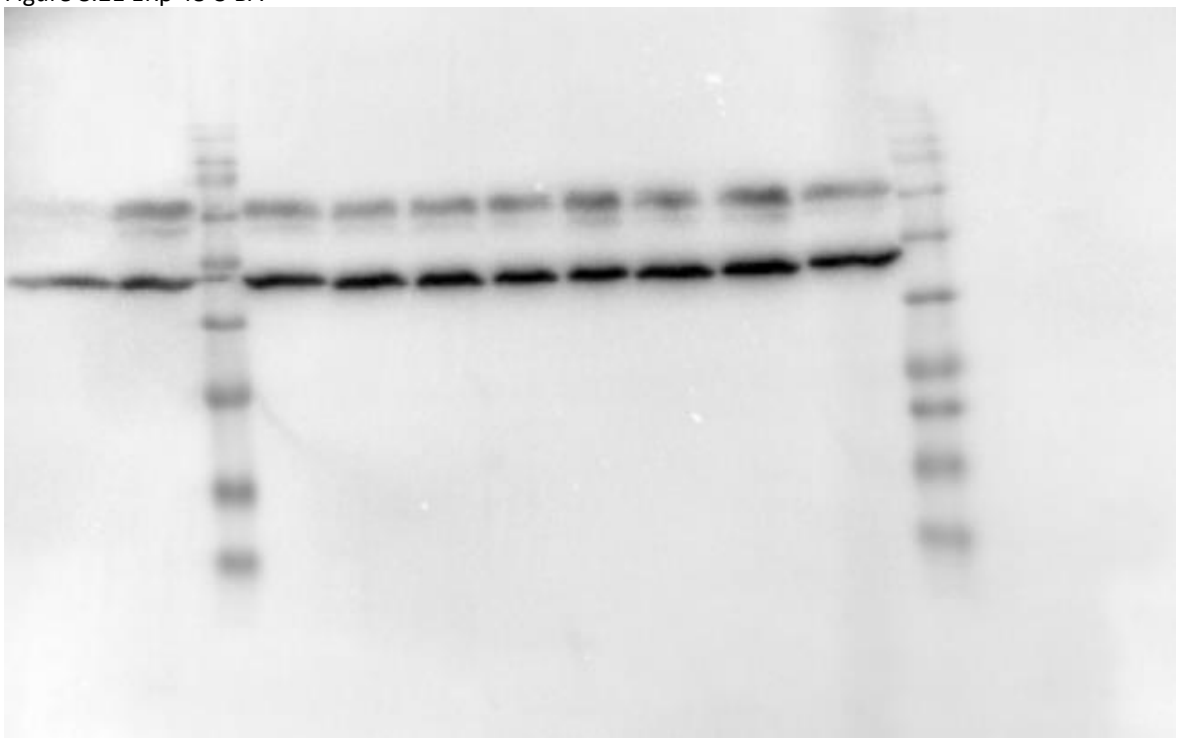

Figure 8.22 PRβ 24-1 PRβ +BA

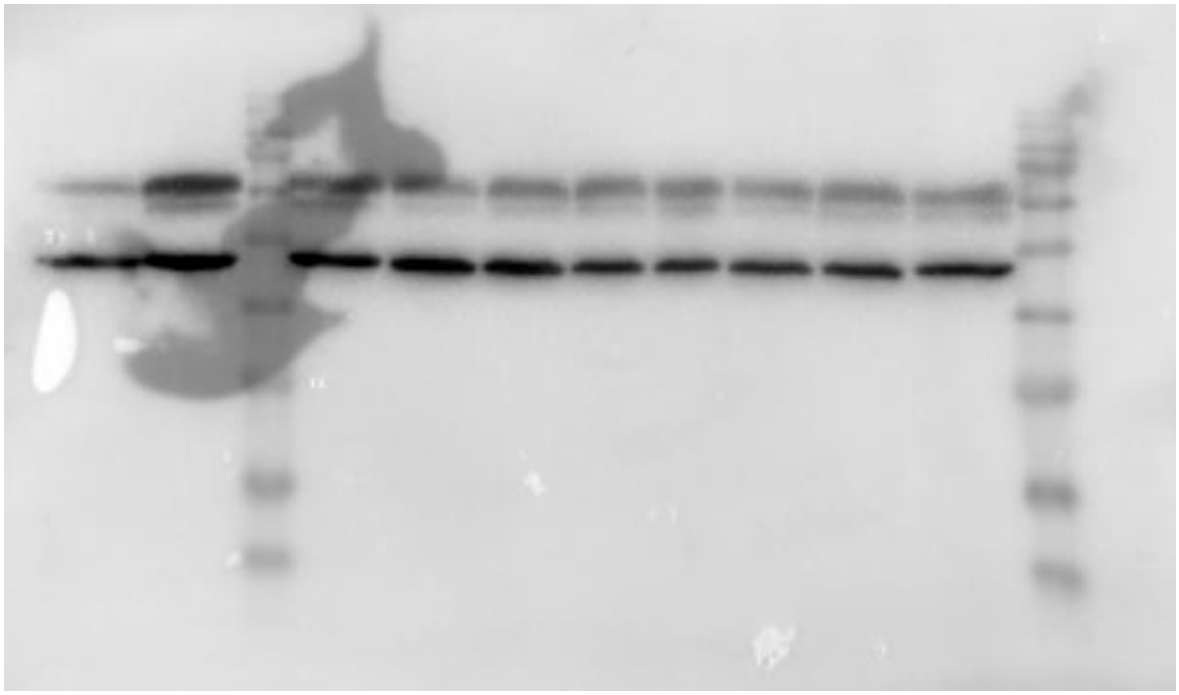

Figure 8.23 PRβ 24-2 PRβ +BA

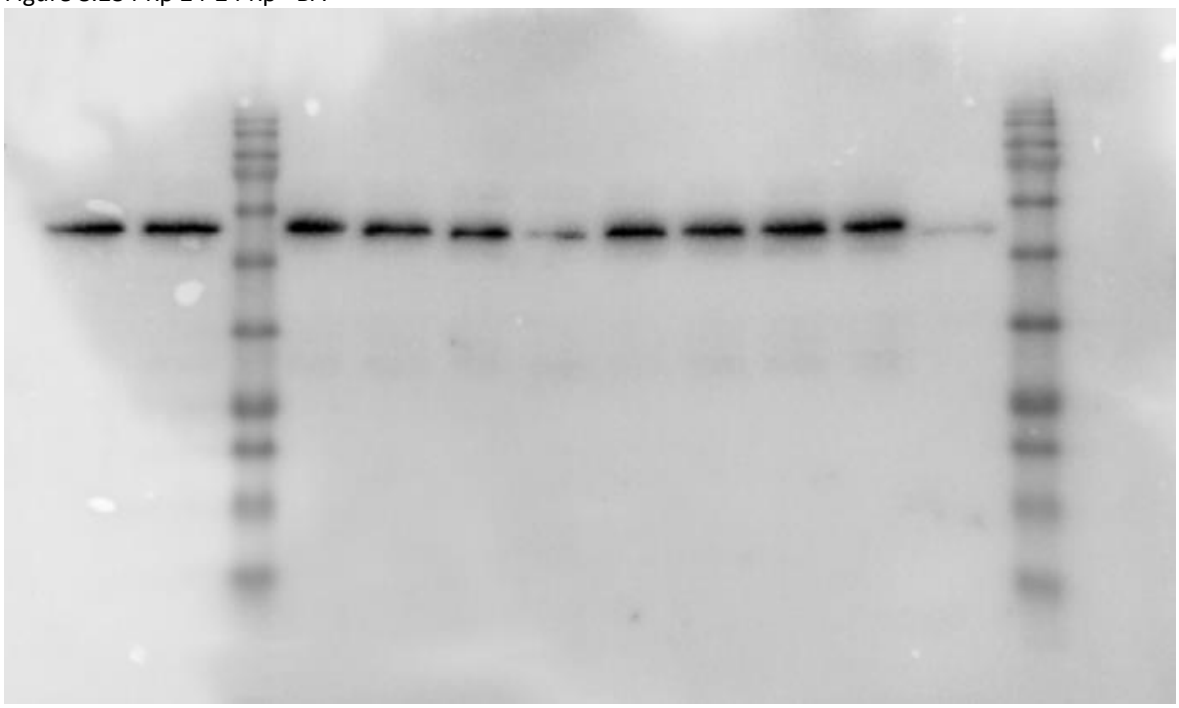

Figure 8.24 PRβ 24-3 PRβ

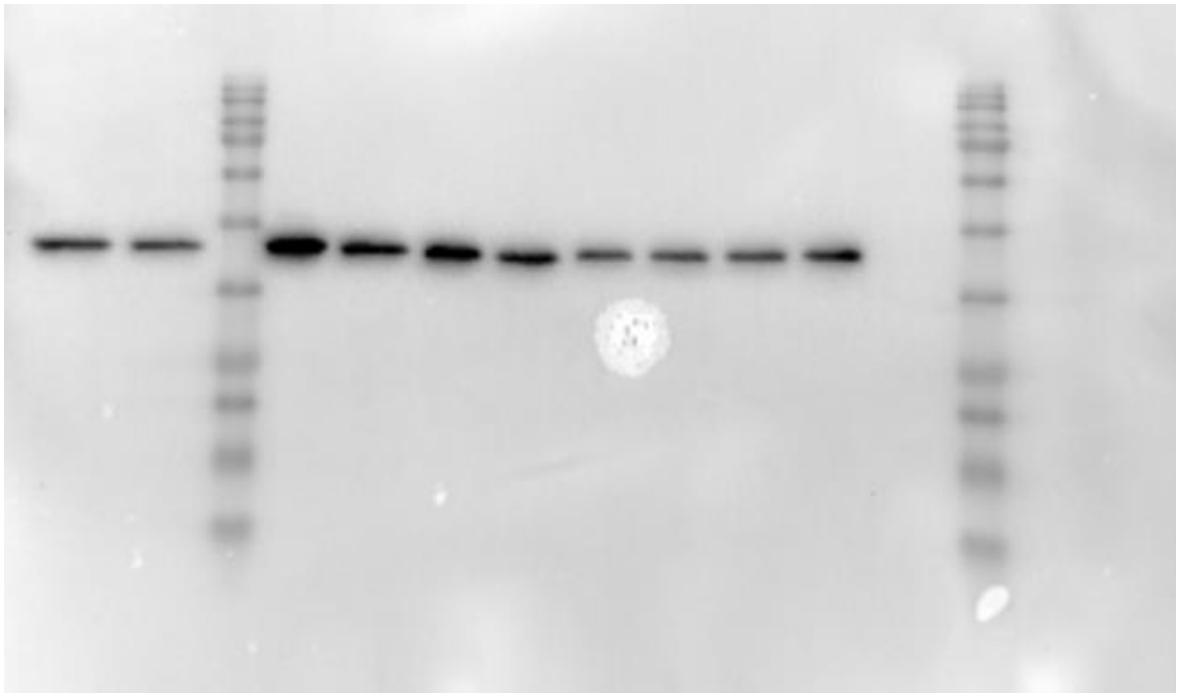

Figure 8.25 PRβ 24-3 BA

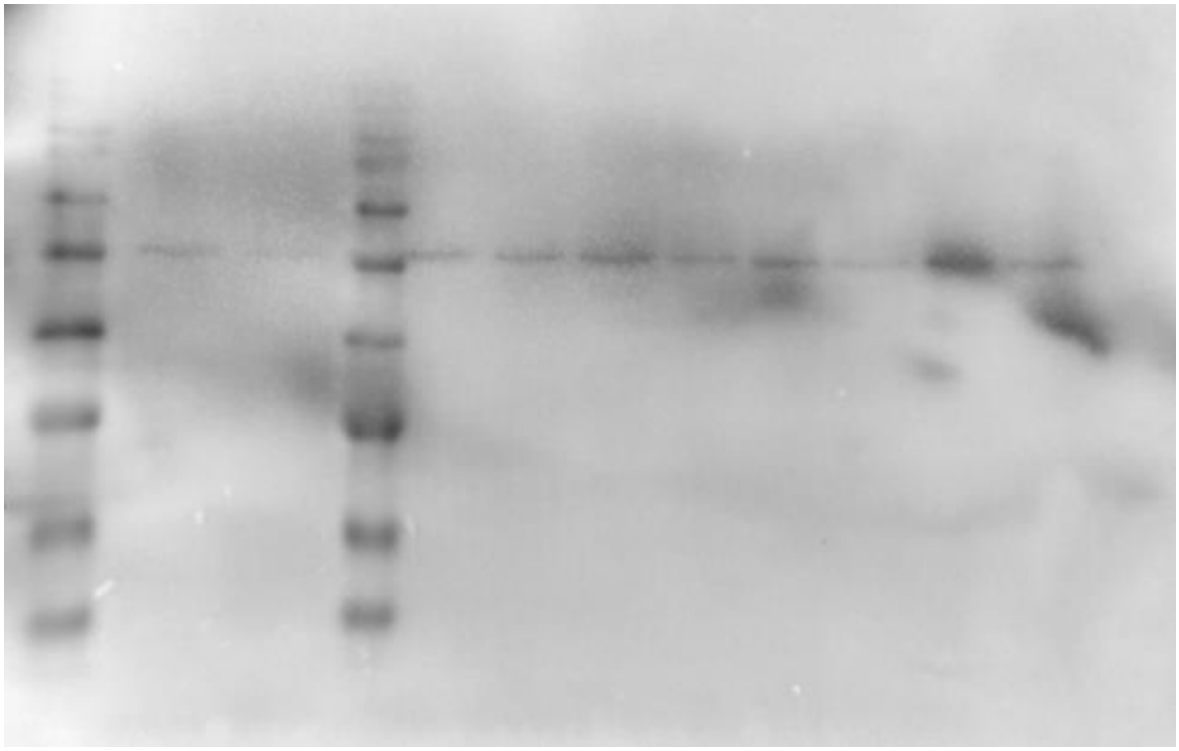

Figure 8.26 PRβ 48-1 PRβ

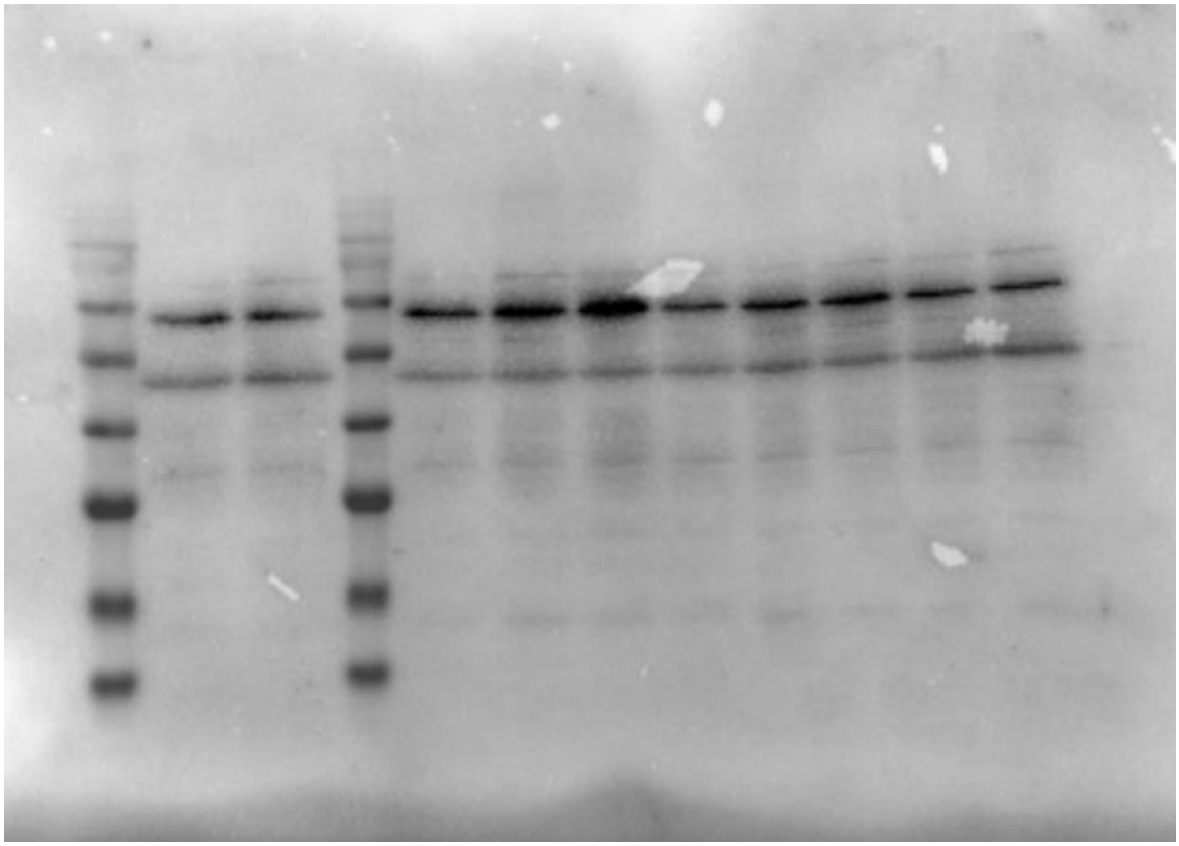

Figure 8.27 PRβ 48-1 BA

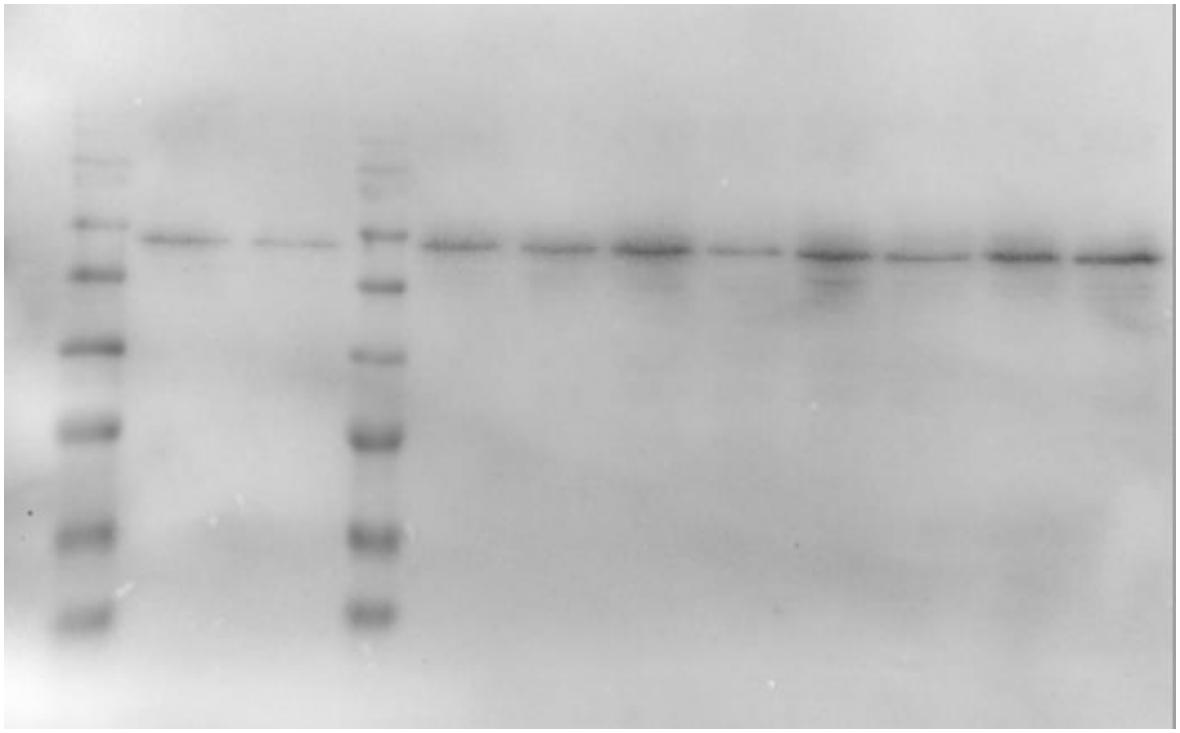

Figure 8.28 PRβ 48-2 PRβ

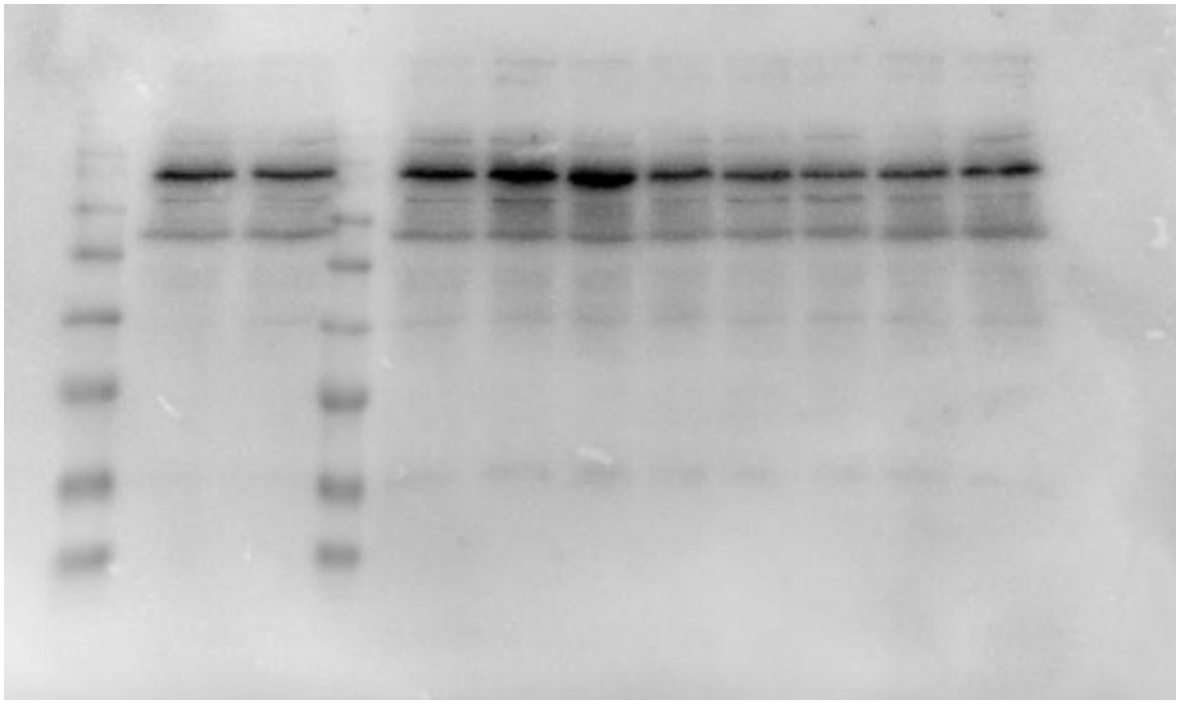

Figure 8.29 PRβ 48-2 BA

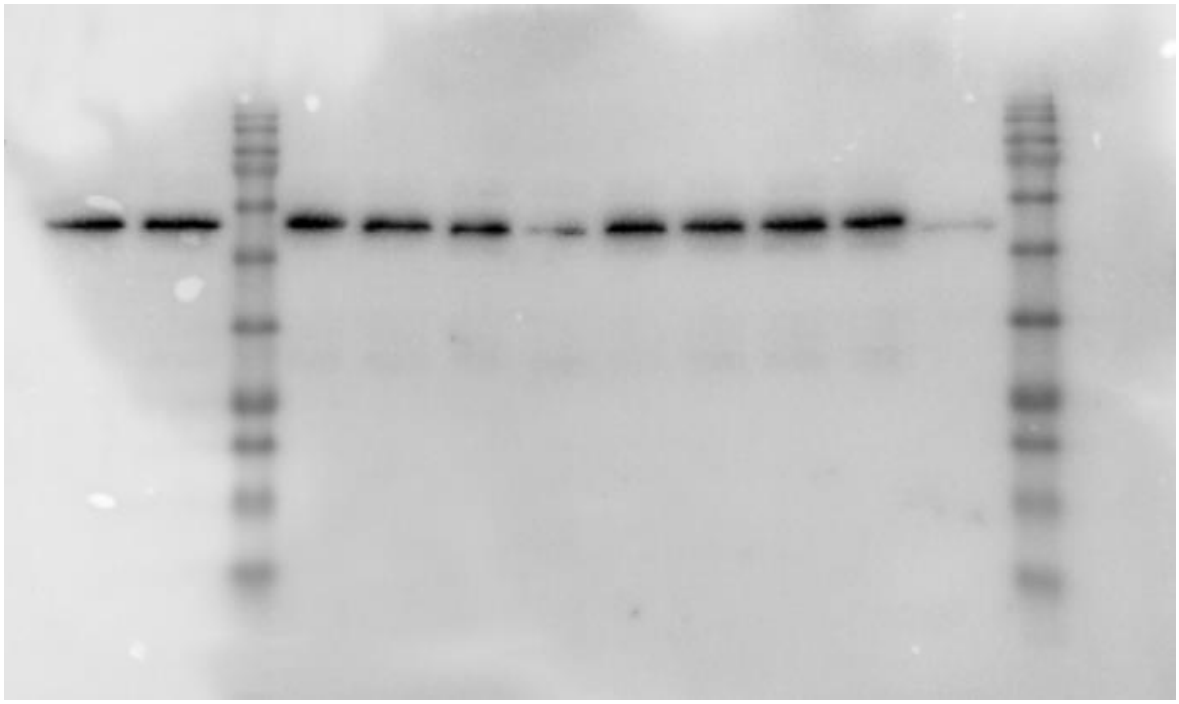

Figure 8.30 PRβ 48-3 PRβ

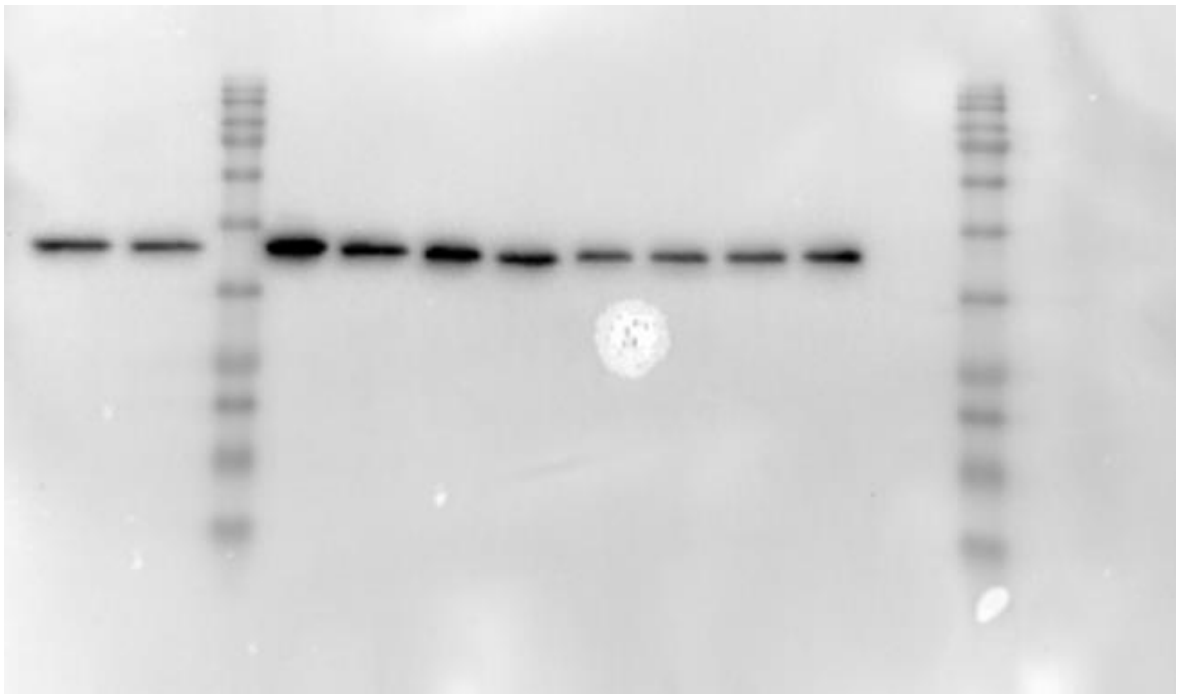

Figure 8.31 PRβ 48-3 BA

XV. UPR 16

FORM UPR16

Research Ethics Review Checklist

Please include this completed form as an appendix to your thesis (see the Postgraduate Research Student Handbook for more information

University of Portsmouth

Postgraduate Research Student (PGRS) Information			Student ID:	UP300489		
PGRS Name:	Rebecca O'Cleirigh					
Department:	Pharmacy and Biomedical Sciences	First Supervisor:	Asmita Sautreau			
Start Date: (or progression date for Prof Doc students)		September 2011				
Study Mode and Route:	Part-time	☒	MPhil	☐	MD	☐
	Full-time	☐	PhD	☒	Professional Doctorate	☐

Title of Thesis:	Exploring the haematological effects of a Chinese herbal formula on endometrial receptivity
Thesis Word Count: (excluding ancillary data)	43652

If you are unsure about any of the following, please contact the local representative on your Faculty Ethics Committee for advice. Please note that it is your responsibility to follow the University's Ethics Policy and any relevant University, academic or professional guidelines in the conduct of your study

Although the Ethics Committee may have given your study a favourable opinion, the final responsibility for the ethical conduct of this work lies with the researcher(s).

UKRIO Finished Research Checklist:
(If you would like to know more about the checklist, please see your Faculty or Departmental Ethics Committee rep or see the online version of the full checklist at: http://www.ukrio.org/what-we-do/code-of-practice-for-research/)

a) Have all of your research and findings been reported accurately, honestly and within a reasonable time frame?	YES	☒
	NO	☐
b) Have all contributions to knowledge been acknowledged?	YES	☒
	NO	☐
c) Have you complied with all agreements relating to intellectual property, publication and authorship?	YES	☒
	NO	☐
d) Has your research data been retained in a secure and accessible form and will it remain so for the required duration?	YES	☒
	NO	☐
e) Does your research comply with all legal, ethical, and contractual requirements?	YES	☒
	NO	☐

Candidate Statement:	
I have considered the ethical dimensions of the above named research project, and have successfully obtained the necessary ethical approval(s)	
Ethical review number(s) from Faculty Ethics Committee (or from NRES/SCREC):	SFEC 2017-037
If you have *not* submitted your work for ethical review, and/or you have answered 'No' to one or more of questions a) to e), please explain below why this is so:	

JPR16 – August 2015

Signed *(PGRS)*:		Date: 01/08/2017