EFFECTS OF TOPICAL APPLICATION OF *MARANTODES PUMILUM* GEL ON THE VAGINA OF OVARIECTOMIZED, SEX STEROID DEFICIENT RATS

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DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF MEDICAL SCIENCE

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ABSTRACT

Kacip Fatimah or *Marantodes pumilum* (MP) is known for its potential use as a herb to boost the women general well-being. Despite its widespread use, there is no scientific evidence which showed its beneficial effects in alleviating vaginal atrophy in the post-menopausal period. This study, therefore, intends to investigate the effects of MP on the vagina of post-menopausal rat model and possible mechanisms involved. **Methods:** Thirty-six female Sprague-Dawley (SD) rats were divided into six (6) groups (n=6) as follows: sham (ovariectomized), ovariectomized control (receiving plain vaginal gel treatment), ovariectomized, receiving MP at different concentrations (100 μg/mL, 250 μg/mL, and 500 μg/mL) and 0.005% estriol (E) topically for seven consecutive days. The rats were euthanized on the eighth day and vagina were then harvested for histological study and protein expression and distribution analysis by Western blotting and immunostaining, respectively. Vaginal ultrastructural changes were observed under transmission electron microscope (TEM) study. **Results:** Histological findings reveal dose-dependent increased in vaginal epithelial thickness following administration of MP. E treatment group had the thickest epithelia. An increase in the distribution and expression of cell proliferative proteins (PCNA and VEGF), tight junctional proteins (Occludin), water channel proteins (AQP-1 and AQP-2) and proton extruder proteins (V-ATPase A1) were observed in the vagina of MP and E-treated ovariectomized rats. TEM shows desmosome formation and approximation in intercellular space following treatment with 250 and 500 μg/mL MP and E. **Conclusions:** MP helps to alleviate vaginal atrophy in the sex steroid deficient state likely via inducing vaginal cell proliferation, promoting vaginal lubrication, improving vaginal acidity, and providing better intercellular integrity.

ABSTRAK

Kacip Fatimah atau *Marantodes pumilum* (MP) terkenal akan potensi kegunaannya sebagai herba untuk meningkatkan kesihatan umum wanita. Walaupun kegunaannya amat meluas, tiada kajian saintifik menunjukkan kesan baik dalam menangani atrofi faraj di kalangan wanita selepas menopaus. Kajian ini bertujuan untuk mengkaji kesan-kesan penggunaan MP secara topikal pada faraj dan mekanisma yang mungkin terlibat. **Kaedah:** 36 ekor tikus SD betina, dibuang ovari dan kekurangan hormon steroid seks dibahagikan kepada enam kumpulan (n=6), masing-masing menerima rawatan gel faraj berbeza : sham (ovariektomi), kawalan (ovariektomi, menerima rawatan gel kosong pada faraj), gel MP pada kepekatan berbeza (100 μg/mL, 250 μg/mL, dan 500 μg/mL) dan estriol (E) 0.005% selama tujuh hari berturutan. Tikus dibunuh pada hari kelapan dan tisu faraj diambil untuk kajian histologi, kajian ekspresi protein melalui *'immunostaining'* dan '*Western blotting'*, dan kajian transmisi mikroskop elektron (TEM). **Keputusan:** Kajian histologi menunjukkan peningkatan ketebalan epitelium faraj selari dengan peningkatan dos, dengan kumpulan rawatan E mempunyai epitelia paling tebal. Peningkatan ekspresi pada protein proliferasi (PCNA dan VEGF), protein persimpangan ketat (Occludin), protein laluan air (AQP-1 dan AQP-2), protein membuang proton (V-ATPase A1) telah didapati di dalam '*immunohistochemistry'* dan '*Western blotting'*. TEM menunjukkan pembentukan desmosom dan penghampiran ruang antara sel selepas rawatan dengan 250 dan 500 μg/mL MP dan E. **Kesimpulan:** MP boleh membantu mengurangkan atrofi faraj di dalam keadaan kekurangan hormon steroid seks dengan meningkatkan proliferasi sel, menggalakkan lubrikasi faraj, menambah keasidan faraj, dan memberi integriti lebih baik antara sel.

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Deepest gratitude to my parents and spouse for being supportive and helpful along this journey. To my beloved daughters, thanks for cheering me every day in your own way.

May God bless all of you.

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LIST OF SYMBOLS AND ABBREVIATIONS

APS	:	Ammonium persulphate
AQP	:	Aquaporin
BCA	:	Bicinchoninic acid
BSA	:	Bovine serum albumin
D.P.X	:	Distyrene, plasticizer, xylene
DAB	:	3,3'-diaminobenzidine
E	:	Estrogen/estriol
GADPH	:	Glyceraldehyde 3-phosphate dehydrogenase
H & E	:	Haematoxylin and eosin
H_2O_2	:	Hydrogen peroxide
HCl	:	Hydrochloric acid
HRP	:	Horseradish peroxidase
HRT	:	Hormone Replacement Therapy
IACUC	:	Institutional Animal Care and Use Committee
IHC	:	Immunohistochemistry
kDa	:	kilodaltons
KH_2PO_4	:	Potassium Dihydrogen Phosphate Anhydrous
LDL	:	Low-density lipoprotein
MP	:	Marantodes pumilum
Na_2HPO_4	:	di-Sodium Hydrogen Phosphate Anhydrous
OVX	:	Ovariectomized
PBS	:	Phosphate buffer saline
PBST	:	Phosphate buffer saline containing Tween-20
PCNA	:	Proliferating Cell Nuclear Antigen

PCOS	:	Polycystic ovarian syndrome
PVDF	:	Polyvinylidene difluoride
REVIVE	:	REal Women's VIews of Treatment Options for Menopausal Vaginal ChangEs)
SD	:	Sprague-Dawley
SDS	:	Sodium dodecyl sulphate
SDS-PAGE	:	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	:	Standard error mean
TEM	:	Transmission Electron Microscopy
TEMED	:	Tetramethylenediamine
UM	:	University of Malaya
UVP	:	Ultraviolet photometer
V-ATPase	:	Vacuolar-Adenosine TriPhosphatase
VEGF	:	Vascular Endothelial Growth Factor
VEGFR	:	Vascular Endothelial Growth Factor Receptor
w/w	:	Weight per weight (percentage solution)
WB	:	Western blot

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Marantodes pumilum (MP), or known as Kacip Fatimah is a small herbal plant which grows mainly in the tropical region of South East Asia. In Malaysia, there are three main varieties which are MP var. *alata*, MP var. *pumila* and MP var. *lanceolata.* The first two varieties are usually consumed via decoction drink and are the most studied (Aladdin *et al.*, 2016). As the queen of herbs as it is claimed, MP is believed to provide health benefits to female reproductive system at different reproductive stages. Consumers with menstrual problem claimed that MP usage alleviates their menstrual pain and regulates their monthly cycle (Nadia *et al.*, 2012). This herb was also claimed to improve the libido and sexual function (George *et al.*, 2014). Perinatal usage of MP has been a tradition by the Malay community especially during the postpartum period (Ezumi *et al.*, 2007).

Post-menopausal women consume MP to lessen their menopausal symptoms such as hot flushes, vaginal dryness, decreased libido etc. The resulting hypoestrogenemia in women after menopause greatly affected their sexual life which was due to diminished estrogen effects. Most post-menopausal women suffered from vaginal dryness and dyspareunia (painful intercourse) as reported by REVIVE (Real Women's Views of Treatment Options for Menopausal Vaginal Changes) survey (Wysocki *et al.*, 2014). Currently, women opt to use lubricants, vaginal moisturizers, and vaginal estrogen to avoid the systemic side effects of hormone replacement therapy (HRT) (Lobo *et al.*, 2012). There is a preference to using herbs as an alternative to HRT.

Large clinical trials on HRT had shown serious side effects on women health that raised worldwide concern regarding its safety (Marjoribanks *et al.*, 2017; Rossouw *et al.*, 2002). The risks of breast cancer (Jones *et al.*, 2016), venous thromboembolism in the

form of stroke event and pulmonary embolism (Binkowska, 2014), cardiovascular diseases (Whayne & Mukherjee, 2015), ovarian cancer (Collaborative Group on Epidemiological Studies of Ovarian, 2015) and endometrial cancer (Brinton & Felix, 2014) had been shown with longer duration of HRT treatment and adversely affect women within the late post-menopausal stage. Local vaginal estrogen therapy has recently emerged as more effective ways to treat menopausal vagina symptoms, however the risk of endometrial hyperplasia and breast cancer with systemic absorption has suggested its usage with lowest dose as possible (Krause *et al.*, 2010). The clinicians have made recommendations to reduce the risks of unwanted side effects following the treatment with various types of HRT (Cobin & Goodman, 2017).

Up-to-date, application of topical vaginal herbs for the treatment of vaginal atrophy after menopause are very minimal. It was hypothesized that MP could help to relief vaginal atrophy via increasing vaginal epithelial thickness and inducing vaginal epithelial proliferation as well as increasing vaginal lubrication via regulation of the tight junction and expression of aquaporin channels in the vagina. It was also hypothesized that MP could restore vaginal acidity via upregulating expression of proton extruders which was decreased in the sex steroids deficient state. The aims of this study were to investigate effects of MP on parameters that were changed in vaginal atrophy such as vaginal thickness, proliferation, secretion, acidity, and vaginal ultrastructure. Elucidating its effects could help to justify the beneficial effect of this herb in relieving vaginal atrophy in the post-menopausal conditions. This study is important as it provides evidence for the use of MP as an agent to relieve vaginal menopausal symptoms, therefore supporting its use as an alternative treatment for vaginal atrophy for the post-menopausal women.

This study hypothesized that:

1. MP increases vagina epithelial thickness and induces vaginal proliferation.
2. MP increases vaginal lubrication via up-regulating tight junction and expression of aquaporin channels.
3. MP enhances vaginal acidity via upregulating expression of proton pump in the vagina.

1.1 OBJECTIVES

1.1.1 General objective 1

To investigate the effects of MP on vaginal epithelial in sex steroid deficient rat model (post-menopausal model).

Specific objectives

1. To determine the thickness of vaginal epithelium in rats treated with topical MP via histology.
2. To determine vaginal cell proliferation via investigating the changes in proliferative proteins including PCNA and VEGF.

1.1.2 General objective 2

To investigate the mechanisms underlying effects of MP in alleviating vaginal dryness in sex steroid deficient rat model (post-menopausal model).

Specific objectives

1. To determine the changes in expression and morphology of the tight junction protein (e.g. Occludin) in rats treated with MP.
2. To investigate the changes in expression of water channel proteins such as AQP-1 and AQP-2.

1.1.3 General objective 3

To investigate the effects of MP on changes in vaginal acidity in sex steroid deficient rat model (post-menopausal model).

Specific objectives

1. To determine changes in expression of proton pump proteins such as V-ATPase A1.

1.2 SIGNIFICANCE OF STUDY

Despite its use mainly via oral consumption to alleviate the post-menopausal symptoms including vaginal atrophy, its direct application to the vaginal tissue that could help to enhance its effect in alleviating vaginal atrophy are not known. This study could help to justify the use of MP in relieving vaginal atrophy symptoms and pave the way for its use to treat this vaginal condition.

CHAPTER 2: LITERATURE REVIEW

2.1 *Marantodes pumilum* (MP)

Figure 2.1: *Marantodes Pumilum* plant.

Figure 2.1 shows the *Marantodes pumilum* plant in the forest. *Marantodes pumilum* (MP) or known as Kacip Fatimah in Malaysia is a small plant with height of about 40 cm and grows in hill forests and lowlands. Its leaves are approximately 30 cm in length and 13 cm in width, with dark green coloration on the upper side and light green to reddish purple on the underside. Its white to pink flowers are clustered and it can produce rounded bright red to purple fruits of half centimetre when ripe (Foster, 2010).

Out of the three main varieties, MP var. *alata* is the most commonly consumed by the local population in Malaysia (Jamal *et al.*, 2017). Traditionally, Malay women used this

herb in the form of decoction drink. It is believed to benefit to women of reproductive age to overcome various health condition. It is also used as abortifacient and to facilitate delivery. Postnatal women used MP to facilitate uterine involution, toning of abdominal muscles, contracting birth canal and recovering the general health (Burkill, 1966; Ezumi *et al.*, 2007). Besides being used to overcome the menstrual irregularities, it is also used to treat dysentery, joint pain and muscle pain, inflammation, gonorrhoea, and flatulence (Burkill, 1966; Ezumi *et al.*, 2007). It is also claim help to alleviate post-menopausal symptoms (Abdul Kadir *et al.*, 2012).

2.1.1 Pharmacological properties of MP

Public interest for MP usage despite its scientifically unproven effects is increasing. Researchers studied the pharmacological properties of MP related to phytoestrogen. Additionally, MP was also found to have antibacterial (Karimi *et al.*, 2013), antifungal (Karimi *et al.*, 2013), antioxidant (Akowuah, 2011; Karimi *et al.*, 2011a; Nadia *et al.*, 2012), anti-inflammatory (Nadia *et al.*, 2012; Sanusi *et al.*, 2013), anti-photoaging (Choi *et al.*, 2010), antihistaminergic, anticholinergic and mast cell stabilizing effects (Akowuah, 2011). Phytoestrogenic properties of MP are summarized in the following table.

Table 2.1: Phytoestrogenic properties of MP

Organs/ Systems	Reported effects	References
Cardiovascular system	Maintains elastic lamina structure in ovariectomized rats	Al-Wahaibi *et al.* (2008)
Lipid metabolism	Reduces LDL in post-menopausal women	Abdul Kadir *et al.* (2012)
	Reduces cholesterol and triglycerides in polycystic ovarian syndrome (PCOS) rats	Manneras *et al.* (2010)
Uterus	Uterotrophic effects in ovariectomized and PCOS rats.	Fazliana *et al.* (2009) Manneras *et al.* (2010)
Bone	Protects gene changes of the bone of post-menopausal rat models.	Fathilah *et al.* (2013)
	Prevents osteoporotic changes in rat post-menopausal rat models.	Fathilah *et al.* (2012)
	Increases bone strength	Fathilah *et al.* (2012)

2.1.2 Phytochemicals in MP

The discovery of the beneficial effects leads the researchers identifying its phytochemical compounds. Several phytochemicals have been reported to be present in different MP varieties and in different parts of the plant itself. Total phenolic content was found highest in the leaves of MP var. *alata* while leaves of MP var. *pumila* has highest total flavonoid content. Gallic acid and pyrogallol were reported to be the main phenolic compounds in MP var. *alata* leaves while kaempferol, naringin and quercetin were the main flavonoid compounds detected in MP var. *pumila* leaves (Karimi & Jaafar, 2011).

Gallic acid is known to exert antimicrobial, anticarcinogenic, antimutagenic, anti-inflammatory and antiangiogenic activity (Choubey *et al.*, 2015).

Phenolic compounds found in MP include gallic acid, caffeic acid and pyrogallol (Karimi & Jaafar, 2011; Karimi *et al.*, 2011b). These phenolics have been shown to exert antioxidant activities by free radical scavenging and ferric reducing antioxidant potential activities (Karimi & Jaafar, 2011). Other types of phenolic acids were also demonstrated such as salicylic acid, syringic acid, vanillic acid, coumaric acid, protocatechin acid, and chlorogenic acid (Chua *et al.*, 2011). Flavanoids compound found in MP include apigenin, kaempferol, myricetin, naringin, quercetin, rutin, catechin, epigallocatechin, anthocyanins, daidzein and genestein (Chua *et al.*, 2011; Karimi & Jaafar, 2011; Karimi *et al.*, 2011b; Norhaiza *et al.*, 2009). These flavonoids are responsible for antioxidant activity, antibacterial, anti-inflammatory, anti-cancer and anti-viral activity (Karimi *et al.*, 2013; Kumar & Pandey, 2013).

Recent study also demonstrated saponins content in the MP (Avula *et al.*, 2011). It includes ardisiacrispin A, ardisicrenoside B, ardisimamilloside H and 3-O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-α-L-arabinopynanosyl cyclamiretin A (Avula *et al.*, 2011). Saponins are known to have antifungal activity (Hu *et al.*, 2018), antiviral (Xiao *et al.*, 2018), anti-cancer (Man *et al.*, 2010), anti-inflammatory (Wu *et al.*, 2017) and antibacterial (Asati & Yadava, 2018). No studies have been done yet on the biological activities of saponins in MP.

2.1.3 Safety of MP

No serious adverse effects have been shown in MP treated groups in the human studies at dose ranging from 200 to 560 mg/day (Abdul Kadir *et al.*, 2012; George *et al.*, 2014;

Nik *et al.*, 2008). In animal studies, maximum dose with no observe adverse effects including reproductive toxicity is 800 mg/kg/day (Ezumi *et al.*, 2007). Meanwhile, the highest dose with no teratogenicity is 1000 mg/kg/day (Mohd Fuad *et al.*, 2005), and no genotoxicity effect is 2000 mg/kg/day (Zaizuhana *et al.*, 2006).

2.2 The vagina

2.2.1 Anatomy and physiology

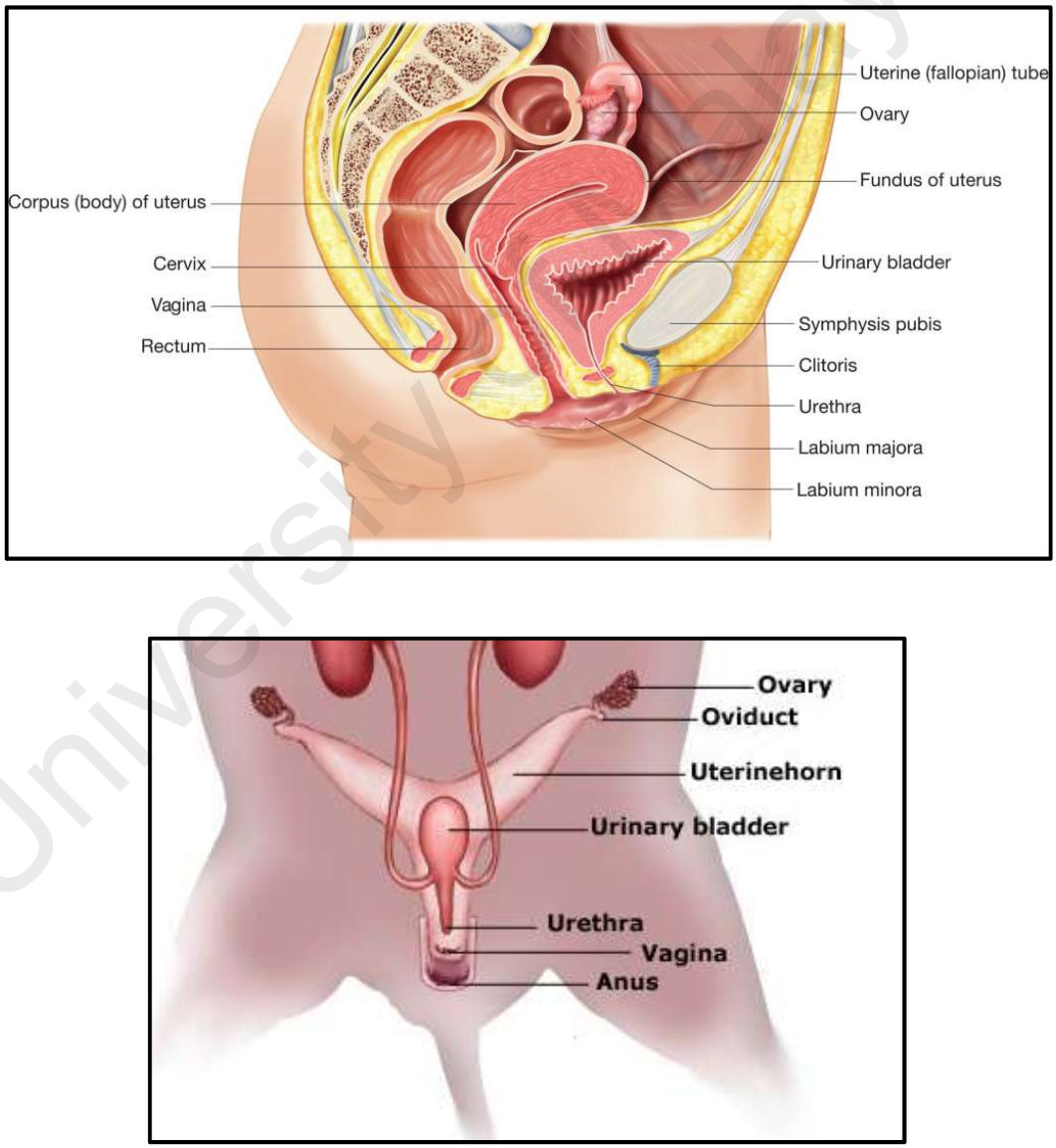

Figure 2.2: Anatomy of the human vagina (above) and rat vagina (below).

Figure 2.2 shows the overview of vaginal anatomy and its surrounding structures. Vagina is a fibromuscular passage lined by non-cornified, stratified squamous epithelium. Anterior to it lies the base of the urinary bladder and the running down urethra while posteriorly, it is related to pouch of Douglas, the rectum and perineal body. It functions as a passageway for menstrual blood outflow, being part of the birth canal and received seminal fluid during sexual intercourse (Monga & Dobbs, 2011).

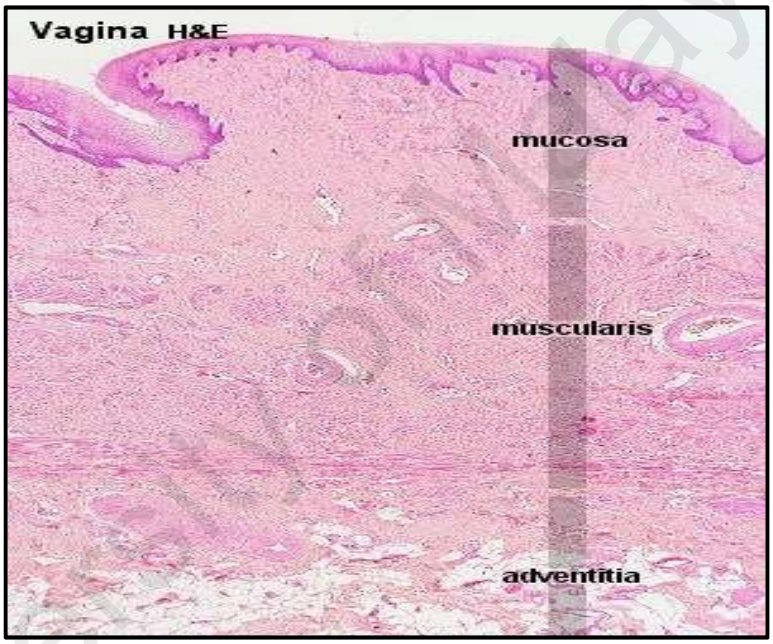

Figure 2.3: Histology of the vagina.

Figure 2.3 shows the vaginal histology stained with hematoxylin and eosin. Vaginal epithelium consists of three layers; inner mucosal layer, intermediate muscular layer, and outer adventitial layer. Vagina is well developed at birth due to estrogen influence from the mother. The maternal estrogen effects ceased after several weeks causing a rise in vaginal pH to 7 and epithelial atrophy. This condition is reversed during puberty under

the influence of estrogen. However, menopausal condition causes atrophies again the vagina epithelium and causes shrinkage of the vagina (Monga & Dobbs, 2011).

Thick vaginal epithelium is rich in glycogen particularly after ovulation and the reverse occur before puberty and after menopause due to the lack of estrogen. Glycogens are broken down by normal vaginal commensals which include Doderlein's lactobacillus, that form lactic acid. This results in acidic environment of around pH 4.5. Vaginal acidity is protective against pathogenic bacterial growth (Aldunate *et al.*, 2013). Variation in vaginal pH occurs throughout the menstrual cycle. A pH of 6.6 to 4.2 which are acidic and toxic to the sperm occurs before ovulation (Wagner & Ottesen, 1982). Vaginal pH is elevated to neutral pH (7.0) during sexual intercourse within seconds, due to sexual excitement and transudation produced by the vagina epithelium which provide temporary lubrication of the vagina. In spite of the alkaline pH of semen, decreased in vagina acidity could be preserved up to two hours following ejaculation (Fox *et al.*, 1973) in order protect spermatozoa viability.

Vagina epithelium does not contain glands; thus, uterine and cervical secretions moisten the vagina. Cervical columnar cells secrete mucus which contribute partially to the vagina fluid content. Secretions from the sebaceous and sweat glands in the vulva and mucus secretion comes from Bartholin's glands also may contribute to vaginal moisture. Microorganisms living in the vagina can produce substances which partly formed the vaginal fluid content (O'Hanlon *et al.*, 2013; Ocana & Elena Nader-Macias, 2004). Besides, vagina epithelium also maintained its moist luminal environment by allowing transudation from blood vessels through its epithelial walls (Nakano *et al.*, 2015).

2.2.2 Vaginal changes after menopause

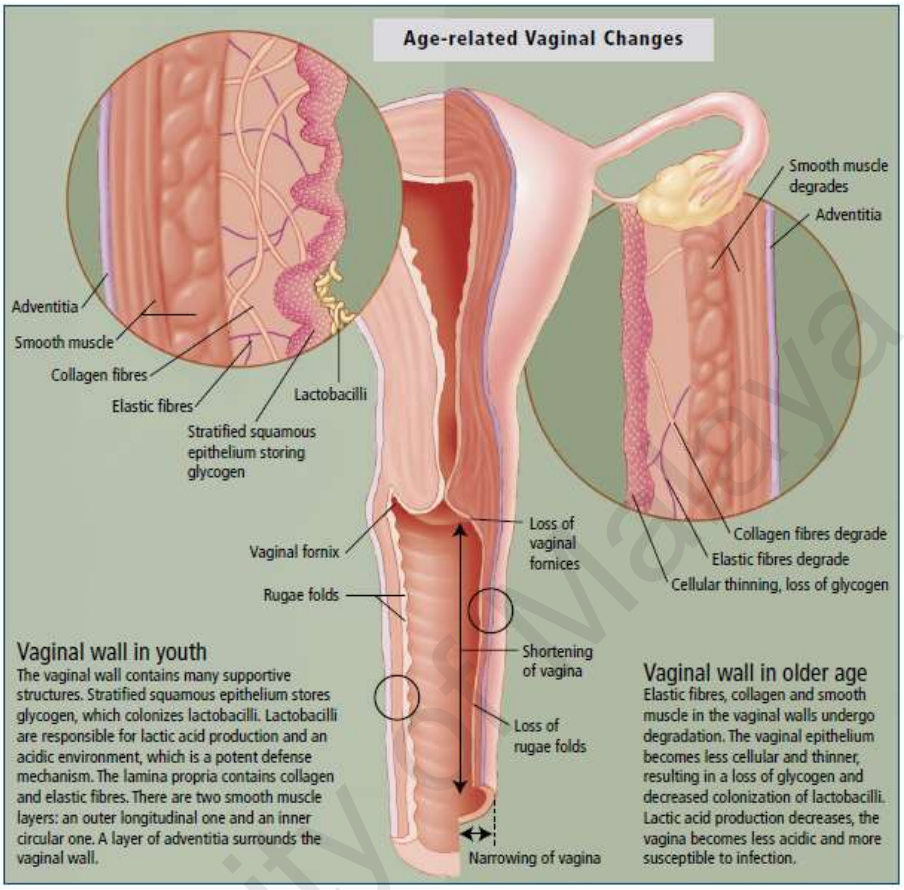

Figure 2.4: Vaginal changes after menopause (Johnston, 2002).

Figure 2.4 illustrates age-related structural vaginal changes following menopause event. Natural menopause is defined as permanent cessation of menstruation where the women having amenorrhea for 12 months without any obvious physiological such as pregnancy, or pathological causes for instance polycystic ovarian syndrome (PCOS) (Casper, 2014). Menopause reflects the ovarian follicular reserve, as they may be completely or almost completely depleted (Sherman, 2005). These result in hypoestrogenemia and high levels of follicular stimulating hormone (FSH) as a result of diminished estrogen negative feedback activity to the pituitary glands (Casper, 2014).

In menopause, aging of tissues inevitably take place. The thick vaginal epithelium reduced in its cellular number and thinning, with progressive reduction of rugal folds and loss of the vaginal fornices (Johnston, 2002). Elastic fibers lose their integrity as they become fragmented. Collagen fibers becomes hyalinize and smooth muscles of the vaginal walls degenerate (Sumino *et al.*, 2004). The vagina becomes less elastic and less stretchable, with shortening and narrowing of the vagina organ, making it more prone to trauma. As the vaginal blood flow ceases, vaginal fluid transudation also reduces during sexual arousal and coitus, contributing to dyspareunia (Edwards & Panay, 2016). Thinning of vaginal wall is associated with the loss of glycogen. Subsequently, lactobacilli colonization and production of lactic acid by the microorganisms decrease (Brotman *et al.*, 2014). The defensive mechanism by the normal vagina acidity is lost. As a result, enteric microbiota dominates the population growth in the vaginal flora and increased vulnerability to infection in the vagina (Brotman *et al.*, 2014).

2.2.3 Aquaporins (AQP)

Water contributes the main component that forms the cells and tissues including the vagina. Prior to the discovery of water channels proteins, aquaporins, the transport of water was assumed to be mediated passively via simple diffusion across the biological, lipid bilayer membrane (Agre & Kozono, 2003). Aquaporins family consists of small molecular weight 28 to 30 kDa and it is an integral membrane protein that mainly mediate the transport of water, with some other solutes such as urea, glycerol, etc. (Zhu *et al.*, 2015). Osmotic gradients drive the water movement across the plasma membrane. Thirteen AQP (AQP-0 to 12) isoforms has been found and twelve of them, excluding AQP-10 have been demonstrated in the female reproductive tract organ (Zhu *et al.*, 2015).

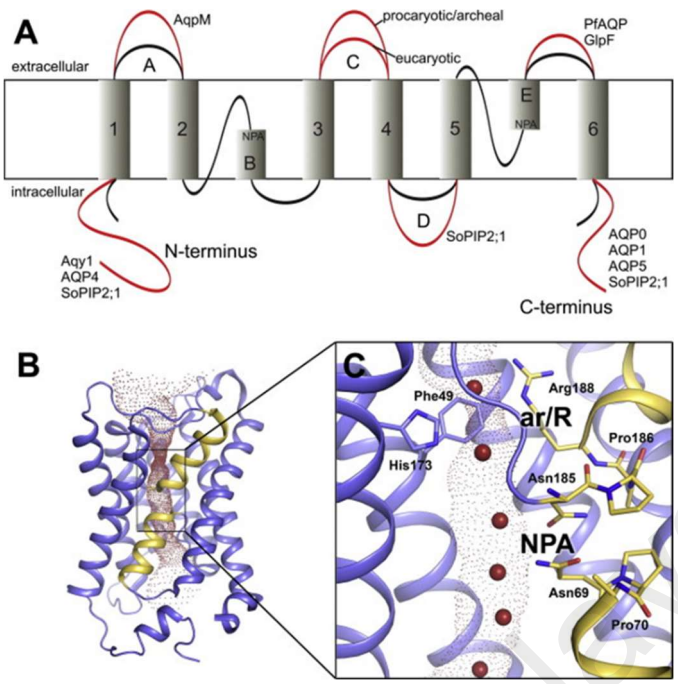

Figure 2.5: Overview on aquaporin water channel protein structure (Tornroth-Horsefield *et al.*, 2010).

Figure 2.5 shows an overview of aquaporins structure. Figure 2.5A demonstrated the topology map of aquaporin showing the basic aquaporin fold and areas that appear to be unusually long were remarked by red color. Figure 2.5B shows the general structure of aquaporin family members, where the six trans-membrane folds are blue-colored and the two yellow-colored half-helices entering in the transmembrane structure from loops B and E, creating a 7th transmembrane pseudohelix. Figure 2.5C shows more detailed structure of the NPA (asparagine, proline, alanine) and aromatic/arginine (ar/R) motifs of the aquaporin family (Tornroth-Horsefield *et al.*, 2010). Water molecules are shown in red. Figure 2.5B and 2.5C are illustrated from human AQP-5 X-ray structure (Horsefield *et al.*, 2008).

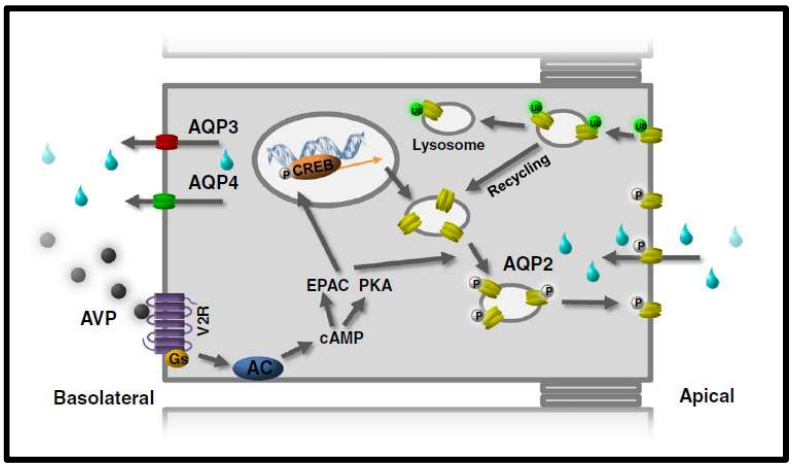

Figure 2.6: Regulation of AQP2 expression in renal collecting ducts (Kortenoeven & Fenton, 2014).

This water channel protein does not exclusively involve in water transport in cell volume regulation, fluid secretion and absorption, as they are also involved in other cell physiological process unrelated to water transport such as cell adhesion, cell proliferation, cell migration and differentiation (Galan-Cobo *et al.*, 2016). Figure 2.6 shows the regulation of water absorption by the aquaporins in the principle cells of renal collecting ducts. At the basolateral membrane, arginine vasopressin (AVP) or more commonly known as antidiuretic hormone (ADH) binds to the vasopressin type-2 receptor (V2R) on the principle cells of collecting ducts and connecting tubule cells (Kortenoeven & Fenton, 2014). Adenylate cyclase (AC) becomes activated following a signaling cascade from Gs protein, resulting in increased in intracellular cAMP, protein kinase A (PKA) activation and probably the Exchange factor directly activated by cAMP (EPAC), and AQP2 phosphorylation (Kortenoeven & Fenton, 2014).

Phosphorylation of AQP2 resulted in translocation of intracellular AQP2 vesicles to the apical membrane (Kortenoeven & Fenton, 2014). Water will move into the principle cells through AQP2 by osmotic gradient, and further driven into the blood circulation through AQP3 and AQP4 in the basolateral membrane, leaving the collecting ducts with

concentrated urine. Long term vasopressin action on its receptor will phosphorylate cAMP responsive element binding protein (CREB) and subsequently promotes transcription from AQP2 promoter (Kortenoeven & Fenton, 2014). These resulted in increased AQP2 expression. As water balance is reestablished, levels of AVP will reduced and internalization of AQP2 occurs by ubiquitination. These AQP2 can either be recycled or degraded by lysosomes (Kortenoeven & Fenton, 2014).

Several studies in humans, rodents and guinea pigs reported the tissue distribution and protein expression of AQP-1 to 6 in the vagina of mammals (Kim *et al.*, 2009; Kim *et al.*, 2011; Park *et al.*, 2008; Pei *et al.*, 2013; Yin *et al.*, 2006). Common expression and tissue distribution of AQP-1 to 3 were observed in the vagina of healthy female SD rats (Park *et al.*, 2008), diabetic rats (Pei *et al.*, 2013), and premenopausal human subjects (Kim *et al.*, 2011). Studies in rats had demonstrated localization of AQP-1 predominantly in the venules and capillaries of the vagina (Kim *et al.*, 2011). Other study demonstrated AQP-1 concentration within the lamina propria capillaries (Kim *et al.*, 2009) and also its distribution in the visceral smooth muscle cells of the oviducts and vagina of rats (Gannon *et al.*, 2000).

AQP-2 was found to occupy the cytoplasm of the vaginal epithelium while AQP-3 was mainly localized to the plasma membrane of vaginal epithelium (Park *et al.*, 2008; Pei *et al.*, 2013). A study by Yin *et al.* (2006) also found AQP-3 expression in the plasma membrane of intermediate cells layer of the vaginal epithelium in mouse and AQP-4 is distributed in the basolateral membrane of the superficial cells layer of the vaginal epithelium in similar study. In addition, AQP-5 and AQP-6 are found to present mainly in the cytoplasm of the vaginal epithelium (Kim *et al.*, 2011).

AQP are very important in female mammals' vagina during sexual arousal as it is involved in the vaginal lubrication, where rapid movement of water occur with increased

in vaginal blood flow resulting in enhanced vaginal lubrication and subsequently g. Although the exact mechanisms underlying the physiology of vaginal lubrication are not fully understood, several studies in women and rats have proposed the potential roles of AQP in vaginal lubrication (Kim *et al.*, 2011; Park *et al.*, 2008).

Expression of AQP in the female reproductive system can be modulated by steroid hormones such as estrogen (Li *et al.*, 1997; Oliveira *et al.*, 2005). Estrogen replacement in ovariectomized mice was found to induce shift in AQP-1 expression from the myometrium to the vasculature of the uterine stroma (Richard *et al.*, 2003). Another study with ovariectomized mouse has also shown the up-regulation of AQP-2 and AQP-3 expression in the myometrium and epithelial cells of the mouse uterus, suggesting the important role of AQP in uterine water imbibition during the peri-implantation period (Jablonski *et al.*, 2003).

In another study by He *et al.* (2006), AQP-2 expression level in the endometrium were positively correlated with 17β-estradiol concentrations in the serum. This resulted in increased water permeability of the endometrium epithelial cells, allowing more fluid to pass through the epithelial cells into the lumen, decreasing the luminal fluid viscosity and prepares the uterine for blastocyst implantation. Other studies had demonstrated the regulation of AQP expression by sex steroid hormones in the uteri of ovariectomized rats (Chinigarzadeh *et al.*, 2016).

AQPs were also expressed in other female reproductive organ such as cervix, ovaries, oviduct, and placenta. Cervix, for example, undergoes important changes during pregnancy and parturition which are modulated by AQP. During these period, cervix becomes soften as a result of the reduction in the cross-linkage in the collagen fibers network and increased in water content, which is termed cervical remodeling (Timmons *et al.*, 2010). Cervical remodeling is important for cervical dilatation to allow the passage

of fetus through the birth canal. AQP helps in water transport in the cervix and their increasing expression during labor has been demonstrated in preterm and term labor mice model (Anderson *et al.*, 2006).

2.2.4 V-ATPases

The role of Vacuolar-ATPase as an active proton pump is crucial to allow normal physiological activity in certain organs, such as kidney, epididymis, ear, bone, and nose (Beyenbach & Wieczorek, 2006; Forgac, 2007; Harvey & Xiang, 2012; Kartner & Manolson, 2012; Koch *et al.*, 2010; Marshansky & Futai, 2008; Saroussi & Nelson, 2009). However, less is known regarding its function in the female reproductive system. The V-ATPase is an enzyme complex, consisting of several subunits forming two domains which are transmembrane V0 domain and cytosolic V1 domain (Cipriano *et al.*, 2008; Forgac, 2007; Marshansky & Futai, 2008; Smith *et al.*, 2003; Wagner *et al.*, 2004). This proton pump is a rotatory pump and energized by the hydrolysis of ATP.

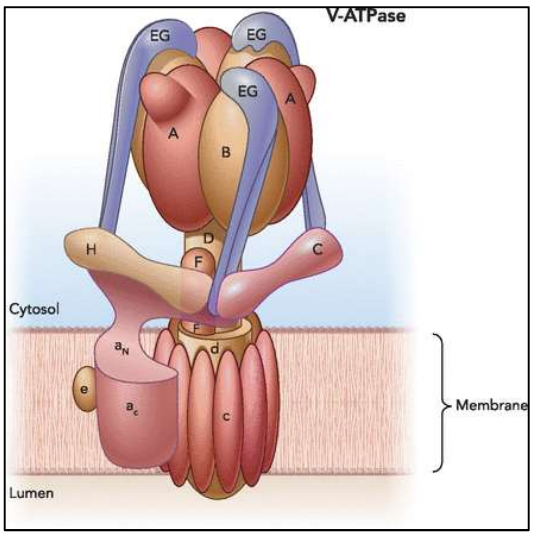

Figure 2.7: Structure and organization of V-ATPase subunit (Breton & Brown, 2013)

Figure 2.7 shows V1 cytosolic domain of V-ATPase which is formed by subunits A, B, C, D, E, F, G, and H, while V0 transmembrane domain is formed by subunits a, c, d, and e. The catalytic sites for ATP are situated at the interface between B/A subunit. Transport of protons takes place between the proteolipid c-ring and subunit a (Breton & Brown, 2013). The rotor function of V-ATPase is performed by subunits D, F, d, and the c-ring. The V-ATPase pump has three EG complexes joined with the subunits C and H to make a stator. This stator is linked to the transmembrane subunit a and AB hexamer. Meanwhile, role of subunit e is still unknown (Breton & Brown, 2013).

Gorodeski *et al.* (2005) had proposed in their study that the pH of the vaginal lumen is controlled by the net proton secretion from the apical ion transporter in the vaginal epithelial cell membrane. Using *in vitro* cell culture study, the result support their hypothesis with evidence of estrogen influence in the proton secretory mechanism of the human vaginal/ectocervical epithelial cells. In another study using vaginal/ectocervical cell culture from premenopausal and post-menopausal women surgical specimens, absence of estrogen attenuated the active H^+ secretion in the apical membrane of the vagina with the effects were reversed by 17β-estradiol treatment (Gorodeski, 2007b).

Besides the vagina, V-ATPase has also been found to play an important role in regulating the uterine fluid pH under the influence of progesterone, which is important for successful embryo implantation (Karim *et al.*, 2016). During the menstrual cycle, uterine fluid pH increased under estrogen influence in the proliferative phase. During the luteal phase which is under the influence of progesterone, the uterine fluid pH decreased (Gholami *et al.*, 2013). Studies have shown the important roles of V-ATPase during embryo implantation by providing acidic environment (Xiao *et al.*, 2017). Besides, V-ATPase was also found to play a role in inflammatory reactions during embryo implantation. The presence of seminal fluid in the uterus could trigger V-ATPase

expression, resulting in increased in macrophage influx and initiating an inflammatory environment together with stimulating effect of cleaved V-ATPase domain from the capacitated sperm on genes activation such as leukemia inhibitory factors (LIF) and interleukins (IL1b) (Jaiswal *et al.*, 2012). V-ATPase A2 has been demonstrated to be partly responsible to the setting of inflammatory milieu in the uterus by coordination of macrophage recruitment through the cytokine and chemokine networks regulation to allow growth and development of the placenta (Jaiswal *et al.*, 2012).

2.2.5 PCNA

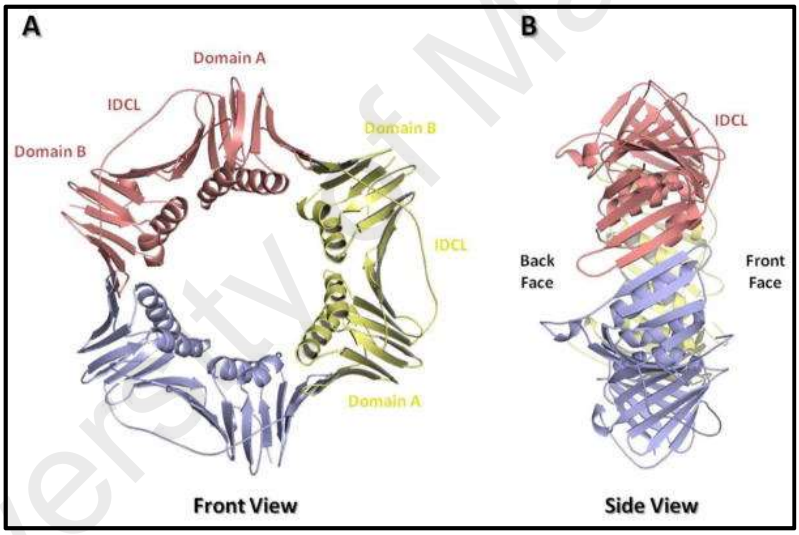

Figure 2.8: Structure of PCNA (Dieckman *et al.*, 2012).

Figure 2.8 shows the ribbon diagram of the PCNA trimer (PDB ID: 1PLQ) from the front view (A) and the lateral side (B). Each PCNA subunits are colored yellow, red, and blue linked together by the inter-domain connector loop (IDCL). The width of the PCNA ring reached 30 Å wide, having both front and back interfaces. Inter-domain connector loop (IDCL) is located at the front face of PCNA, involving in many protein-protein communications. In protein replication, DNA polymerases and DNA ligases bring out

their actions on the DNA at the PCNA ring's front face (Dieckman *et al.*, 2012). Currently, the role of the back face of PCNA is still unclear. The posterior face is emerging as a post-translational modification of PCNA, possibly playing a role in recruitment of protein factors to replication forks and keeping them in reserve until required on the anterior face of PCNA (Dieckman *et al.*, 2012).

Proliferating Cell Nuclear Antigen (PCNA) has been established as an important marker for cellular proliferation (Strzalka & Ziemienowicz, 2011). PCNA is a non-histone, nuclear protein that is important in synthesis of DNA and serve as an accessory protein for DNA polymerase delta, an enzyme that is elevated during G1/S phase of the cell cycle (Strzalka & Ziemienowicz, 2011). A very low level of PCNA mRNA can be detected in quiescent and senescent cells (Barani *et al.*, 2003). Moreover, this protein plays critical role in the metabolism of nucleic acid by being a part in the mechanism of DNA replication and repair (de Oliveira *et al.*, 2008).

The female reproductive organs undergo substantial changes of development since birth to maturity, including proliferation, differentiation, and degeneration. Studies have demonstrated PCNA expression all over the female reproductive tract, including the ovaries, uterus, cervix, and the vagina. In the ovaries, scientist has opted to utilize PCNA as a marker to identify and quantify non-growing follicles (NGF) in the ovary to estimate the total human ovarian reserve (Kelsey *et al.*, 2010; Muskhelishvili *et al.*, 2005; Phoophitphong *et al.*, 2012).

PCNA distribution has been observed in the endometrium lining of the uterus (Lai *et al.*, 2000) and in pregnancy state (Oner *et al.*, 2010; Sequeira *et al.*, 2016). In the ovaries, PCNA has been found to regulate the assembly of primordial follicle by promoting apoptosis of oocytes in the neonate mouse ovary (Xu *et al.*, 2011). Estrogen and phytoestrogen have been found to regulate PCNA expression in the vagina (Carbonel *et*

al., 2011; Xu *et al.*, 2015; Zujewski, 2002) with various levels of expression throughout the estrous cycle (Madhumati *et al.*, 2012; Santos *et al.*, 2017). PCNA expression was also associated with some malignancy such as cervical cancer (Madhumati *et al.*, 2012; Santos *et al.*, 2017) and uterine cancer (Yoshida *et al.*, 2012).

2.2.6 VEGF

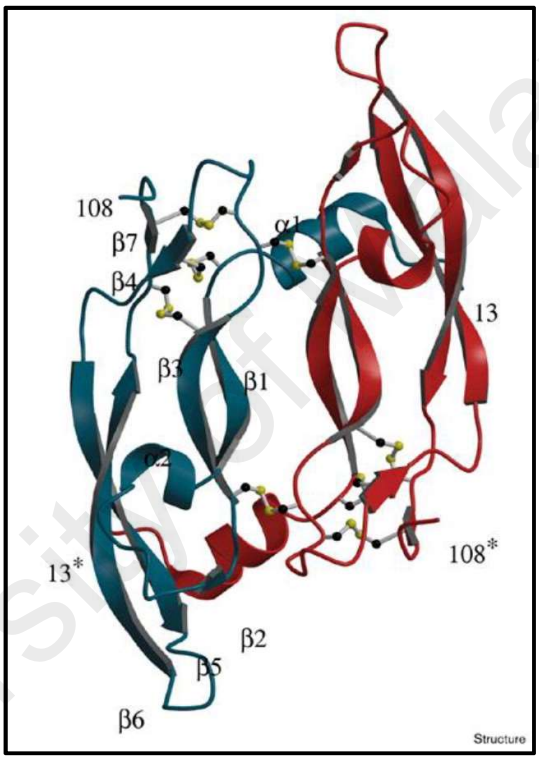

Figure 2.9: VEGF molecular structure.

Figure 2.9 shows a ribbon diagram of VEGF structure. VEGF dimer, colored red and blue, are interconnected to each other by many disulfide bonds (white) and sulfur atoms (yellow). The secondary structure elements of each monomer are shown: α1 (residues 16–24), β1 (27–34), α2 (35–39), β2 (46–48), β3 (51–58), β4 (67–69), β5 (73–83), β6 (89–99) and β7 (103–105). The asterisks symbols indicate the terminal end of each monomers.

Vascular Endothelial Growth Factor (VEGF) or previously known as Vascular Permeability Factor (VPF) is responsible for angiogenesis and vasculogenesis. The VEGF family in mammals consists of five members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PGF) (Shibuya, 2011). This homodimer glycoprotein expression is regulated by various factors such as hypoxia, cytokines, hormones, oncogenes, and nitric oxide (Berra *et al.*, 2000; Traver & Zon, 2002; Zhang & Zhu, 2002).

Two receptors are mainly mediating the VEGF actions: the Fms-like tyrosine kinase 1 (Flt-1) and fetal liver kinase 1/ kinase insert domain-containing receptor (Flk-1/KDR) (Shibuya, 2011). Flk-1/KDR has been shown to modulate most of the VEGF action such as induction of endothelial cells proliferation and migration, while Flt-1 was found to control the interaction among the endothelial cells and between endothelial cells and basal lamina, although the underlying mechanism is still unclear (Stuttfeld & Ballmer-Hofer, 2009).

These two receptors formed the basis of angiogenic action of VEGF. Studies by van Bruggen *et al.* (1999) showed the strong action of VEGF in increasing vascular permeability, being 50,000 times more potent than histamine. It is well established that VEGF plays an important role in tissue edema in brain, nasal, and uterine tissues (Azizzadeh Delshad *et al.*, 2016; Infanger *et al.*, 2004; Kazi & Koos, 2007; Liu *et al.*, 2016).

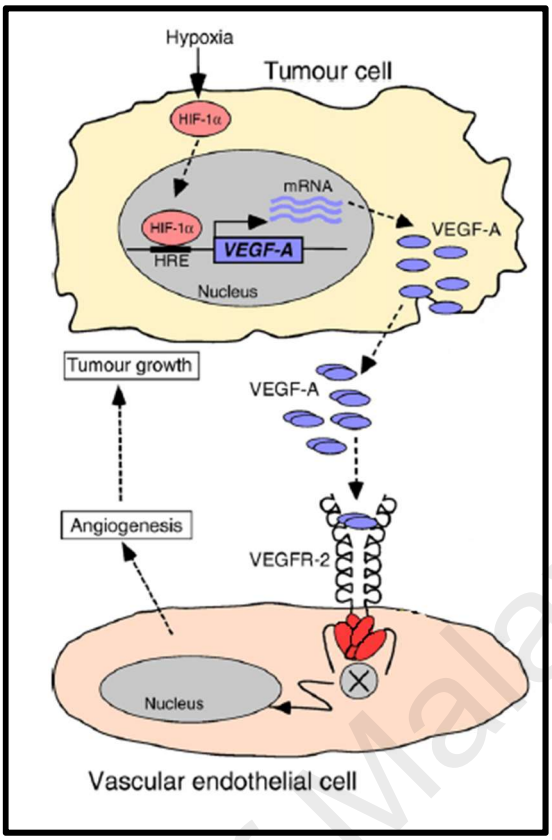

Figure 2.10: Mechanisms of VEGF synthesis and mode of action (Holmes *et al.*, 2007).

Figure 2.10 shows VEGF expression in the tumor cells. In tumor, VEGF expression can be stimulated by hypoxia or genetic mutations (Goel & Mercurio, 2013). VEGF synthesized from tumor cells will become available in cellular cytoplasm and reach the interstitial fluid to bind to its receptor in the endothelial cells (Holmes *et al.*, 2007). Several therapeutic approaches such as anti-VEGF antibody and VEGF receptor blocker had been implemented to suppress the tumor growth (Holmes *et al.*, 2007).

Studies have demonstrated VEGF expression in female reproductive organs, including uterus, ovary, oviduct, and the vagina. It involved in various female reproductive events such as ovulation, embryo implantation, and development of placenta (Jaffe, 2000). For example, in the proliferative phase of the female menstrual cycle, gonadotropin-induced folliculogenesis and increased blood vessels formation were accompanied by the

increased in VEGF expression in the thecal cell layer (Mattioli *et al.*, 2001). VEGF also increased vascular permeability (Bates, 2010) in the thecal cells, allowing the growing ovarian follicles to accumulate follicular fluid from plasma extravasation (Monteleone *et al.*, 2008). The increased in vascular permeability allowed lipid precursors of androgen to be delivered in the thecal cells and being aromatized to form estrogen (Cui *et al.*, 2013). Higher estrogen concentration was found in follicles with higher VEGF expression (Danforth *et al.*, 2003; Mattioli *et al.*, 2001), and anti-VEGF antibody suppresses both folliculogenesis and steroidogenesis (Zimmermann *et al.*, 2001), thus supporting the evidence of VEGF function in both events.

High levels of VEGF were also detected in the endometrium during the mid-proliferative phase and secretory phases of the menstrual cycle (Nayak & Brenner, 2002; Sugino *et al.*, 2002), which responsible for the increased in proliferation of endothelial cells in proliferative phase and increased vascular permeability during secretory phase (Demir *et al.*, 2010; Nardo, 2005). Studies had shown the sex steroid hormones influence on the endometrial VEGF expression, suggesting estrogen and progesterone role in increasing VEGF levels during the mid-proliferative phase and mid-secretory phase, respectively (Demir *et al.*, 2010).

Nevertheless, strong VEGF expression was also observed in human endometrium during menstruation, when both sex steroid hormones levels were low, thus explaining the role of hypoxia in modulating this protein expression (Sharkey *et al.*, 2000). Tissue ischemia during menstruation lead to endometrial shedding and upregulation of VEGF expression (Sharkey *et al.*, 2000). Instead, upon successful fertilization and implantation of the embryo, estrogen and progesterone levels will increase with marked VEGF expression in the endothelial and decidual cells (Sugino *et al.*, 2002). Anti-VEGF antibody administration in pregnant rats has resulted in failure of rat embryo implantation,

thus remarking the importance of VEGF for successful implantation (Rabbani & Rogers, 2001).

VEGF expression was also found in the cervix and vagina. In the cervix, exogenous VEGF induces cervical epithelium growth, edema, increases paracellular epithelial space, and immune cells recruitment into the cervical lumen in pregnant and non-pregnant mice (Donnelly *et al.*, 2013). This explained the role of VEGF in cervical remodeling during pregnancy. Meanwhile, VEGF and VEGF receptor-1 (VEGFR-1) expression were demonstrated and their levels increased in the vagina upon treatment with phytoestrogen in ovariectomized rats compared to ovariectomized control rats (Yin *et al.*, 2013). As ovariectomy induced a sex steroid deficient state up-regulated VEGF protein levels could help to alleviate vaginal atrophy in ovariectomized rats.

2.2.7 Occludin

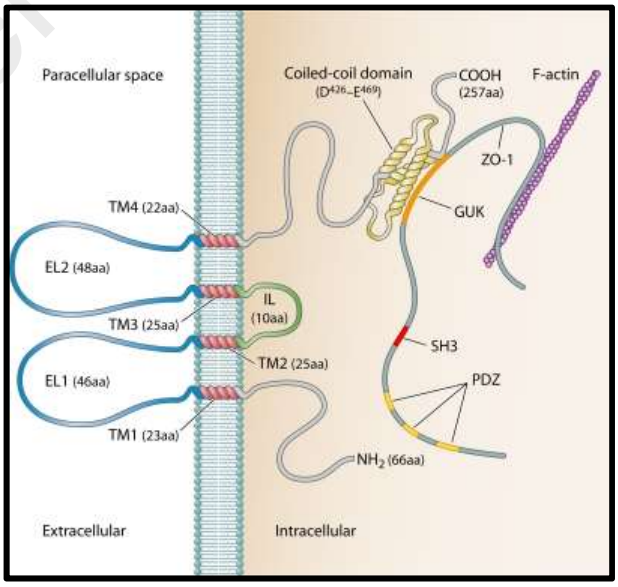

Figure 2.11: Structure of occludin within the plasma membrane (Cummins, 2012).

Figure 2.11 shows the structure of occludin in the plasma membrane. Occludin is a tight junction protein, encoded by OCLN gene (Meloni *et al.*, 2010). It is a 65-kDa protein situated within the plasma membrane (Furuse, 2010). Both occludin and claudin form the main building blocks of the tight junction structure. Occludin acquired a multidomain, tetra-span protein structure where each domains showing important function and regulatory features (Feldman *et al.*, 2005). Fig. 2.3 shows the C-terminus which is extended into the intracellular cytoplasm and has an interaction with ZO-1, thus mediating the tight junction trafficking within the cell. This terminus also played important roles in cellular signaling and mediated dimerization of occludin (Li *et al.*, 2005; Walter *et al.*, 2009a; Walter *et al.*, 2009b).

Several studies have shown occludin expression in the female reproductive tract. In uterine epithelium, the distribution of tight junction proteins including occludin were altered during the estrous cycle in rats (Mendoza-Rodriguez *et al.*, 2005). Occludin was detected in the tight junction structure during the proestrus day when mating occurrs, and not detected during metestrus, estrus, and diestrus days. These suggested that changes in tight junction proteins throughout the estrous cycle may contribute towards successful fertilization and implantation by providing optimized uterine environment (Mendoza-Rodriguez *et al.*, 2005).

On the other hand, studies have also demonstrated occludin expression in the cervix, which was associated with increased expression of tight junction proteins upon induction by prostaglandin during the first trimester of pregnancy (Ghule *et al.*, 2012). Occludin underwent rapid turnover during pregnancy, thus contributed to alteration in trans-epithelial resistance and permeability, although the exact mechanisms were unclear (Ghule *et al.*, 2012).

Estrogen has been found to regulate occludin expression in the vagina. Occludin expression was downregulated following bilateral ovariectomy and the condition was reversed following 17β-estradiol treatment (Oh *et al.*, 2016). Tight junctions resistance was also decreased following estrogen treatment, which was demonstrated by matrix metalloproteinase-7 (MMP-7) modulation on occludin (Gorodeski, 2007a; Zeng *et al.*, 2004). This might explain the mechanisms underlying vaginal atrophy condition in post-menopause following estrogen cessation.

2.2.8 Epithelial intercellular junction

In the female reproductive tract, epithelial cells served as a physical barrier to guard against environmental pathogen. The epithelial intercellular junction of the upper female reproductive tract (FRT) is lined with the columnar cells, which exhibit numerous tight junction structure to preserve the single layer mucosal integrity in the endocervix, uterus, and Fallopian tubes. In contrast, lower FRT including the vagina and ectocervix were lined by stratified squamous epithelium, which are loosely connected (Ochiel *et al.*, 2008).

Intercellular junctions are defined as connections between two neighboring cells, controlling the epithelial permeability via paracellular route. These junctions prevent the apical compartment from being mixed with the basolateral compartment (Godfrey, 1997; Simons & Wandinger-Ness, 1990). Tight junction consists of peripheral membrane proteins and transmembrane proteins which made a complex network adhering to each other (Matter *et al.*, 2005; Shin *et al.*, 2006). To date, three main types of intercellular junctions were identified: tight junctions, adherens junctions, and desmosome (Langbein, 2002; Marchiando *et al.*, 2010).

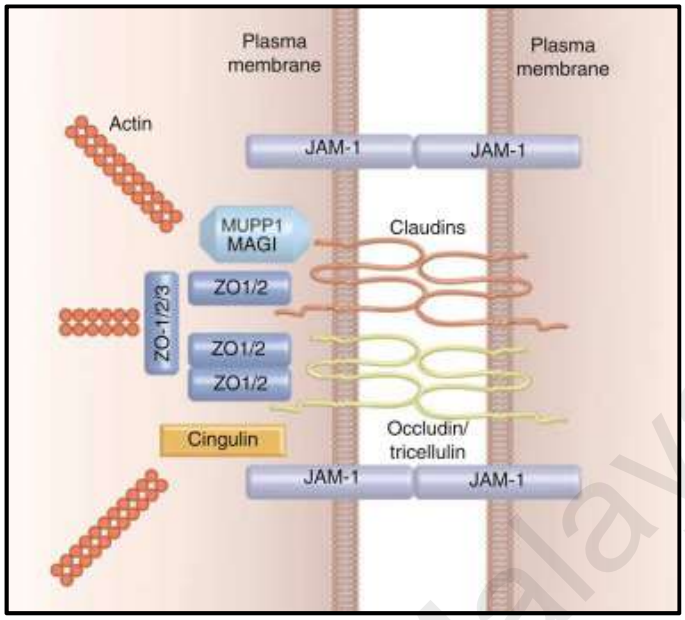

Figure 2.12: Schematic diagram of tight junction structure (Niessen, 2007).

Figure 2.12 shows a schematic diagram of tight junction structure (Niessen, 2007). Tight junctions or zonula occludens consist of networks of strands, sealing the two adjacent cell membranes together. Each strand is made of transmembrane proteins in both cellular plasma membranes. Its extracellular domains were linked directly to each other. Claudins and occludins contributed to the major proteins that form this junction. These transmembrane protein strands were associated with various membrane proteins, for instance the zonula occludens-1 (ZO-1) situated on the intracellular side of the cell membrane. ZO-1 further anchored the tight junction proteins to the actin component of the cytoskeleton, thus connecting the cytoskeletons of adjoining cells (Anderson & Van Itallie, 2009).

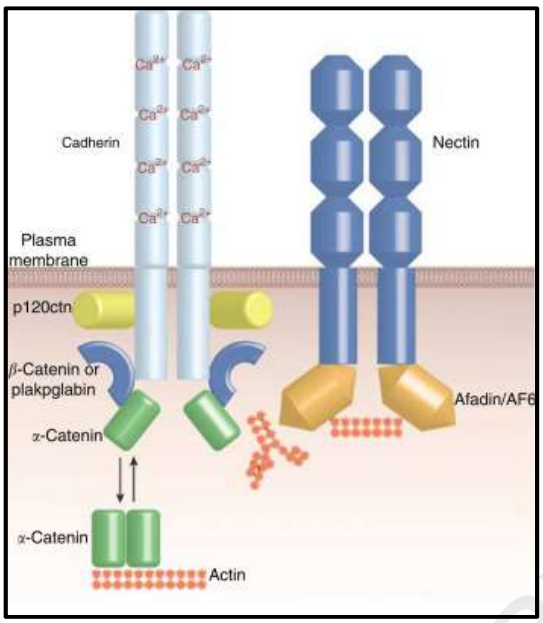

Figure 2.13: Schematic diagram of adherens junction structure (Niessen, 2007).

Figure 2.13 shows a schematic diagram of zonula occludens structure (Niessen, 2007). Adherens junction or zonula adherens is a cellular junction having the cytoplasmic side connected to actin cytoskeleton. This type of junction can exist in the form of bands surrounding the cell (zonula adherens) or as adhesion plaques, attaching the cell to the extracellular matrix (Meng & Takeichi, 2009).. Zonula adherens is mainly composed of cadherins, delta-catenin, gamma-catenin, and alpha-catenin. This rod-shaped type of junction bridged a pair of cell membrane, leaving a 10-20 nm distance in between them (Hirokawa & Heuser, 1981; Miyaguchi, 2000). The cytoplasmic side of this junction is related to the condensed actin filaments. Adherens junction usually situated below the tight junctions (Marchiando *et al.*, 2010; Meng & Takeichi, 2009).

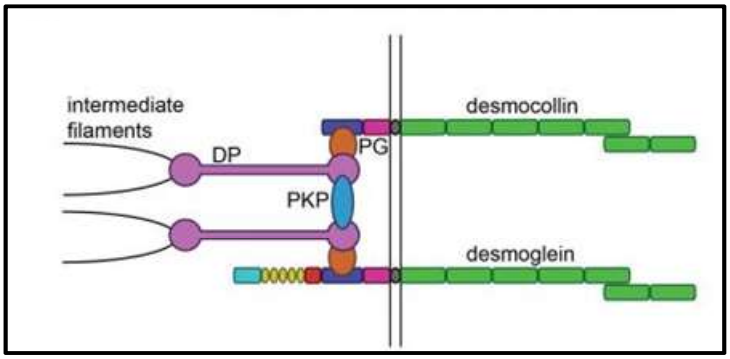

Figure 2.14: Desmosomes architecture (Kowalczyk & Green, 2013).

Figure 2.14 shows desmosomes architecture having desmocolin, desmoglein, and interconnected proteins linked them to the intermediate filaments intracellularly (Kowalczyk & Green, 2013). Desmosomes (macula adherens) is a type of junctional complex, which exist as spot-like adhesions on the lateral sides of the cell membranes. It connects keratin intermediate filaments from cell to another, forming a structural framework of great tensile strength (Gorodeski, 2007a; Miyaguchi, 2000). It has a wide intercellular space of about 30 nm. Desmosomes facilitated the epithelium to resist shearing forces and can be found in simple and stratified squamous epithelia such as vagina. The desmosomal adhesion proteins JAM3 (also known as [a.k.a.] JAM-C), desmoglein, and desmocollin are anchored to intermediate filaments via a scaffold network of plakin and armadillo proteins (Green & Simpson, 2007).

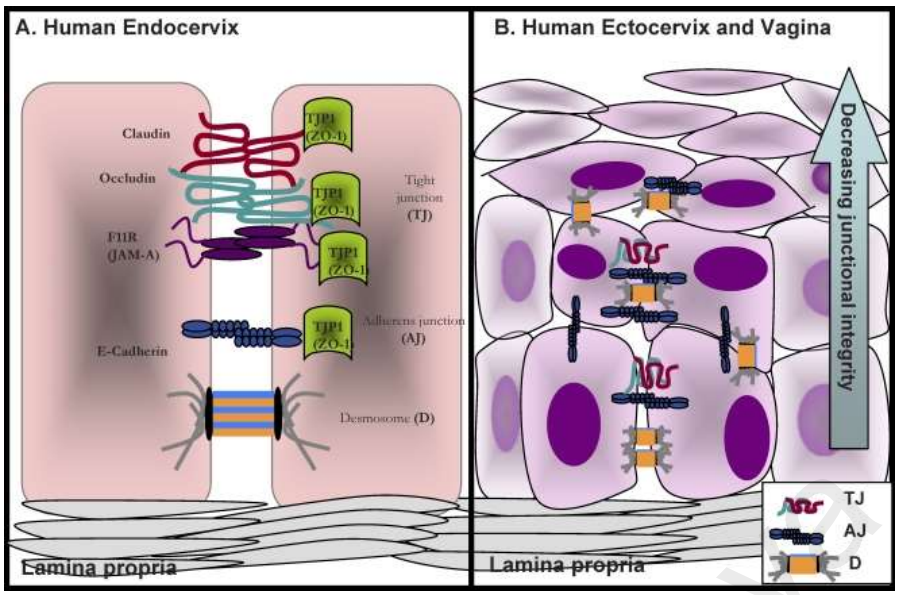

Figure 2.15: Comparison on different localization of intercellular junctions molecules between the upper FRT and lower FRT (Blaskewicz *et al.*, 2011).

Fig. 2.15 demonstrated the classic tripartite intercellular junctions in the endocervical epithelium (Blaskewicz *et al.*, 2011). Towards the apical surface of the cells, tight junctions seals the epithelium, and preserves cellular polarity. Immediately below it, adherens junctions are localized, being in charge for cell to cell adhesion. E-cadherin was found to be the most common transcellular component in the epithelial adherens junctions, and is attached to the actin cytoskeleton via vinculin and alpha and beta catenins (Blaskewicz *et al.*, 2011). The most basal structure of the paracellular adhesion molecules was `the desmosomes, providing the tissue with mechanical resistance and strength. Desmoglein and desmocollin formed most desmosomes components, connected to intermediate filaments of neighboring cells via an intracellular scaffolding network (Blaskewicz *et al.*, 2011).

The lower female reproductive tract, a different pattern of intercellular junction distribution. In stratified squamous epithelium of the ectocervix and vagina, parabasal epithelium showed the most abundant cellular junctions, immediately above the basal cell

layer which being in contact with the basement membrane (Blaskewicz *et al.*, 2011). Among the three main intercellular junctions, adherens junctions and desmosomes were predominantly abundant (Blaskewicz *et al.*, 2011). The junctional integrity was gradually diminished as epithelial cells were forced toward the apical side. The building stratified epithelium became cornified, leaving behind all cellular contacts, and were sloughed into the luminal area (Blaskewicz *et al.*, 2011).

CHAPTER 3: MATERIALS AND METHODOLOGY

3.1 Materials

3.1.1 Animals

Rat species was chosen as research model in this study for its convenience, well adaptation to new environment, close similarity to human genetic, behavioral, and biological characteristics thus making it a suitable model to replicate the post-menopausal condition in human. Thirty adult female Sprague-Dawley (SD) rats aged 10 to 12 weeks, weight 200 to 300 g were attained from the Animal Supplier, Selangor. They were divided into six groups and housed in polycarbonate cages with stainless steel cover, each cage having six rats. Food pellets and tap water ad libitum were freely accessed with daily maintenance of housing hygiene. External environment is maintained constantly with temperature of 24-26˚C, 30% to 50% humidity with 12 hours' light/dark cycle. All experimental procedures were accepted by the Institutional Animal Care and Use Committee (IACUC), University of Malaya (UM), ethics reference no. 2017-180105/PHYSIO/R/NS.

3.1.2 *Marantodes Pumilum* (MP)

MP var. *alata* fresh leaves of 2.0 kg were bought from a trader in Tapah, Perak, Malaysia, and leaves sample were sent to Herbarium, Rimba Ilmu, UM, Kuala Lumpur for further identification (Identification No. KLU49047).

3.1.3 Chemicals

All the chemicals used in this study are listed in the following table.

Table 3.1: List of chemicals consumed

No.	Chemicals/ Consumables	Company
1.	Polysine slides	Thermo Scientific, USA
2.	D.P.X mountant	R&M Chemicals, Malaysia
3.	Paraplast (paraffin wax)	Leica Biosystems, Wetzlar, Germany
4.	Haematoxylin	Sigma-Aldrich, USA
5.	Eosin	R&M Chemicals, Malaysia
6.	Aluminium Potassium Sulphate Dodecahydrate	Sigma-Aldrich, USA
7.	Ethanol	John Kollin Chemicals, UK
8.	Methanol	John Kollin Chemicals, UK
9.	Xylene	Friendemann Schmidt, Australia
10.	Bovine Serum Albumin (BSA)	Sigma-Aldrich, USA
11.	Trisodium citrate dihydrate	Friendemann Schmidt, Australia
12.	Tween-20	Croda International Plc, UK
13.	Antibodies (primary and secondary)	Santa Cruz Biotechnology, USA
14.	Phosphate Buffer Saline (PBS)	HiMedia Laboratories Pvt. Ltd., India
15.	30% Hydrogen peroxide	
16.	Peroxidase Stain Diaminobenzidine (DAB) Kit	Nacalai Tesque, Kyoto, Japan
17.	Cover slip	Marienfeld Superior, Germany
18.	PRO-PREP lysis buffer	iNtRON Biotechnology, Korea
19.	Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific, USA
20.	Greiner UV Transparent 96 Well Plate	Thermo Scientific, USA
21.	Sodium dodecyl sulphate (SDS)	Vivantis Inc. USA
22.	Ammonium persulphate (APS)	HiMedia Laboratories Pvt. Ltd., India
23.	Tetramethylenediamine (TEMED)	Sigma-Aldrich, USA
24.	Polyvinylidene difluoride (PVDF) membrane	Bio-Rad Laboratories Inc., USA

Table 3.1 continued

25.	BLUeye Prestained Protein Ladder	GeneDirex, Bio-Helix Co., Taiwan
26.	Acrylamide	Vivantis Inc. USA
27.	Bis-acrylamide	Vivantis Inc. USA
28.	Tris base	Promega Corporation, USA
29.	Glycerol	Sigma-Aldrich, USA
30.	2-mercaptoethanol	Sigma-Aldrich, USA
31.	Bromophenol blue	Sigma-Aldrich, USA
32.	Pierce 1-Step Transfer buffer	Thermo Fisher Scientific, USA
33.	Glycine	HiMedia Laboratories Pvt. Ltd., India
34.	Sodium chloride	Vivantis Inc. USA
35.	Potassium chloride	Friendemann Schmidt, Australia
36.	di-Sodium Hydrogen Phosphate Anhydrous (Na_2HPO_4)	Friendemann Schmidt, Australia
37.	Potassium Dihydrogen Phosphate Anhydrous (KH_2PO_4)	Friendemann Schmidt, Australia

3.2 Methods

The herbs leave aqueous extract were initially obtained and followed by preparation of the animals for ovariectomy procedure. These sex steroid deficient rats were left for 21 days for surgical recovery and the treatment phase with topical vaginal gel of MP were initiated. After completing the treatment phase, vaginal tissues were harvested for protein expression study and transmission electron microscopy study. Figure 3.1 shows the summary of methodical stages done in this study.

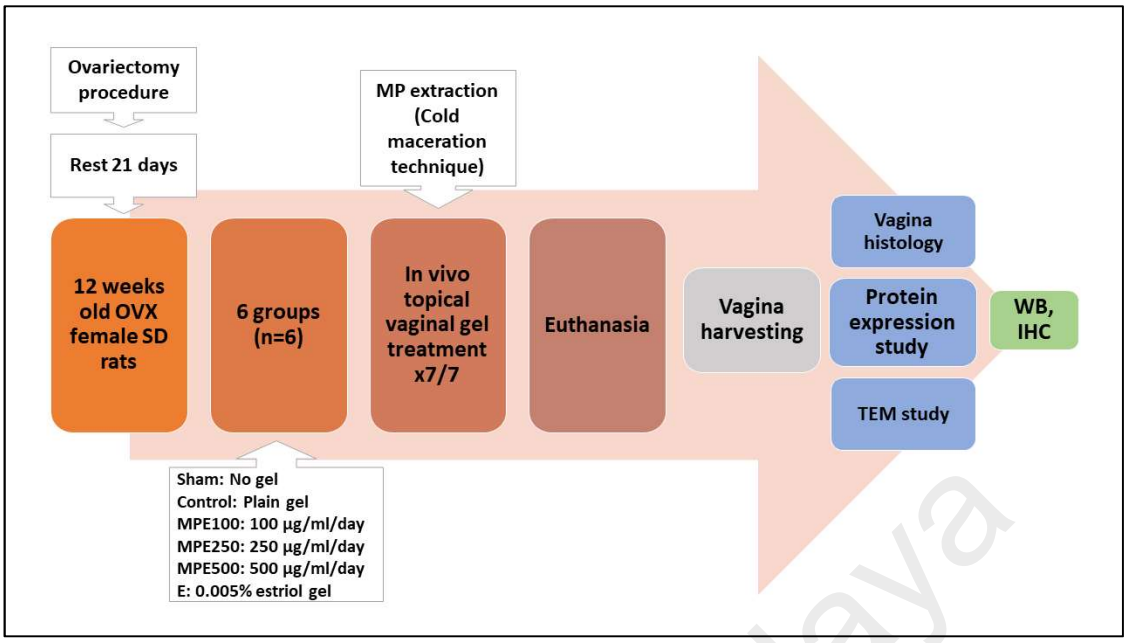

Figure 3.1: Study design and method overview of this study

3.2.1 MP var. *alata* leaves extraction

MP var. *alata* was chosen to be used in this study for its prevalence in Malaysia which contribute to its widespread usage among the locals (Jamal *et al.*, 2017). In comparison with the root and the stem, MP leaves have been shown to have highest flavonoids and phenolic contents. MP var. *alata* leaves have higher total phenolic content although the total flavonoids content was similar to the MP var. *pumila* leaves (Karimi *et al.*, 2011a). Recent study has shown that MP contains higher total phenolic contents compared to total flavonoid content in any method of extractions (Chua *et al.*, 2011).

MP var. *alata* leaves were extracted by aqueous extraction using cold maceration technique. Besides MP was traditionally consumed in decoction drink preparation, water extraction was chosen to discard any potential effect of toxicity from other solvents such as ethyl acetate to the experimental animals. A study by Azrie *et al.* (2014) has shown a higher yield of extraction when using water as a solvent. Upon water extraction, a higher content of gallic acid was presented compared to other solvents which corresponds to one

type of phenolics found in MP (Azrie *et al.*, 2014). Previous study also had used water extraction of MP to assess its toxicity level (Mohd Fuad *et al.*, 2005; Singh *et al.*, 2009; Zaizuhana *et al.*, 2006).

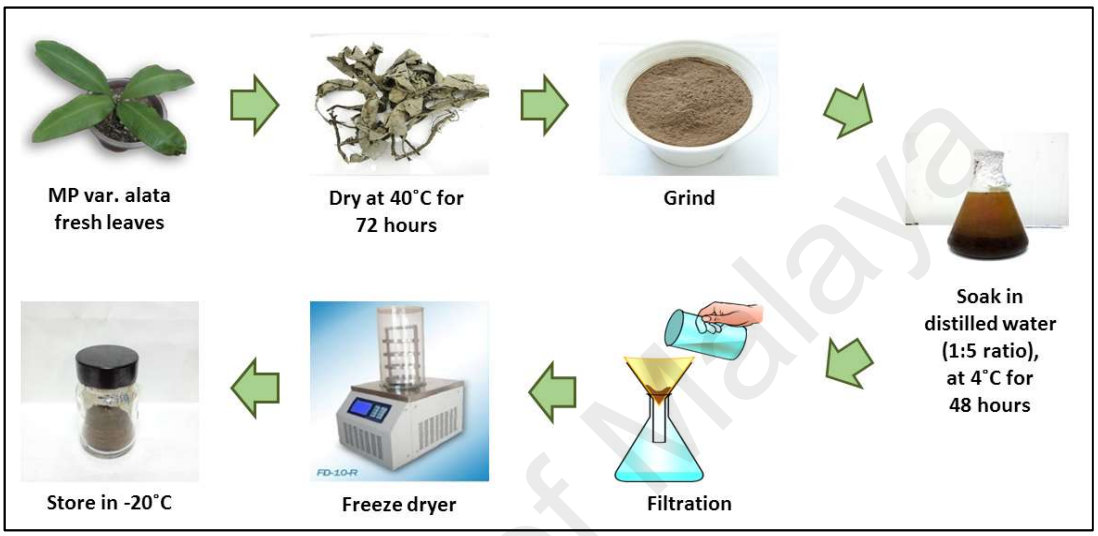

Figure 3.2: Aqueous extraction of MP var. *alata* leaves (cold maceration technique)

Figure 3.2 shows the aqueous extraction process in stepwise manner of MP var. *alata* leaves using cold maceration technique. After obtaining the fresh leaves, they were dried for 72 hours at temperature of 40˚C which lessens the disruption of active compound structure despite the drying time is lengthy. The dried leaves then ground finely to obtain powder form. The leaves powder then macerated at 4˚C by soaking 400g of MP leaves powder in two litres of distilled water with a ratio of 1:5 and constantly stirred for 48 hours. The macerated leaves solution was then filtered to obtain the filtrate with Whatmann® filter paper No. 1. (Sigma-Aldrich, USA). To collect the final extract, the filtrate had to be freeze-dried. The extract then stored at -20˚C for further usage. The yield of extraction obtained relative to the fresh leaves was 5.25% w/w following the below calculation method.

$$Yield\ of\ extraction\ percentage = \frac{Dry\ weight\ of\ extract\ (g) \times 100\%}{Dry\ weight\ of\ M.pumilum\ leaves\ (g)}$$

$$= \frac{21\ g \times 100\%}{400\ g}$$

$$= 5.25\%\ w/w$$

3.2.2 Ovariectomy procedure

3.2.2.1 Pre-operative management

Animals will be left to adapt for one week upon received. They were housed in pathogen-free accommodations in the Satellite Unit of Department of Physiology, under a 12 hours dark and light cycle, with free access to water and pelleted diet. Rats succeeding health examination before the operative procedure were included for the study (Sophocleous & Idris, 2014).

3.2.2.2 Ovariectomy

Ovariectomy was done to remove the effects of endogenous sex steroids hormone. Based on double dorsolateral approach as described by Park *et al.* (2010), induction of anesthesia was done with 5% isoflurane and sustained throughout the procedure with 2.5% isoflurane. Rat was set in prone position on the operating table with oxygen supply using a coaxial nose cone.

The fur was initially shaved around the target incision site. Skin incision site was made on the medial part of the most bulging area of the flank. The external oblique muscle is then exposed and dissected to allow access into the peritoneal space and exposure of the ovary. The surrounding adipose tissue of the ovary were drawn gently to prevent any

detachment of minute pieces of the ovary. Ovary is then identified, and ligation was made at distal uterine horn before removal of the ovary totally. Muscle layers and skin were then closed. The procedure is repeated on the other side.

3.2.2.3 Post-operative care

After administration of analgesic, animals will be left uninterrupted in a clean recovery box at 27–30 °C until they were completely awake. For rats with lengthened recovery, injection of warm sterile saline subcutaneously or intraperitoneally will be done for fluid replacement to avoid dehydration. Upon regaining full consciousness and being active, they will be moved to a clean polycarbonate cage with new bedding. Over the 72 hours postoperative, they were observed for any signs of distress or pain daily. Removal of metal clips and non-absorbable sutures were performed in the second week after surgical procedure (Sophocleous & Idris, 2014). Rats were left to heal and rest for 21 days before initiation of MP topical gel treatment.

3.2.3 MP gel preparation and treatment

3.2.3.1 Gel preparation

Plain gel was initially prepared using the following ingredients proportion.

- Sodium carboxymethyl cellulose (gelling agent) …… 1.0 g
- Propylene glycol (Humectant)…… 12 mL
- Methyl hydroxybenzoate (Preservative)……..... 0.05 g
- Purified water up to …… 50 mL

The above ingredients amount was multiplied by ten times in estimation of preparing 500 mL plain gel. First, methyl hydroxybenzoate (0.5 g) was dissolved in 120 mL of propylene glycol. Sodium carboxymethyl cellulose (10 g) was then added to the previous mixture. On the other side, 500 mL of water was heated separately at 65˚C. The previous propylene glycol mixture was then added to the water and stir cool using a glass rod. Precaution was made during stirring as rapid stirring may cause air bubbles entrapment into the gel.

Five sterile urine containers were used to store 100mL of plain gel each, labelled with normal control group, MP100, MP250, MP500 and estrogen group. Normal control group contained only plain gel inside the container. The other four containers were added with MP extract or estriol powder immediately and stirred while the gel is still warm. This aimed to allow adequate dissolution of the extract with the gel. The amount of MP extracts was calculated following the concentration desired (MP 100 µg/mL, MP 250 µg/mL and MP 500 µg/mL).

$$MP\ 100\ \mu g/ml = \frac{(x)\ \ MP\ extract}{100\ mL\ plain\ gel}$$

$$(x)\ \ MP\ extract = 100\ \mu g/mL \times 100\ ml$$

$$= 10000\ \mu g\ MP\ extract$$

$$= 10\ mg\ MP\ extract$$

MP extract of 10 mg, 25 mg, and 50 mg were then added to respective container containing plain gel. This vaginal gel was formulated based on previous preparation by Gupta *et al.* (2016) which used µg/mL as concentration unit. Doses of 100, 250 and 500 µg/mL/day of MP was selected with slight modification from a study conducted by Dianita *et al.* (2015) which utilized doses of 100, 200 and 400 mg/kg/day oral MP treatment for 28 successive days. Doses selection for our study were increased slightly as the treatment period is short (seven days) and still much lower than the toxicity level (800 to 2000 mg/kg/day) detected by previous animal toxicological studies (Ezumi *et al.*, 2007; Mohd Fuad *et al.*, 2005; Zaizuhana *et al.*, 2006).

Meanwhile, estrogen containing gel was prepared based on preparation done by Lopez-Belmonte *et al.* (2012) which used 0.005% estriol gel for rats in which 5.8 mg gel will contain 0.29µg estriol. Recent studies have preferred the usage of a very low concentration of estriol in vaginal gel preparation (0.005% estriol) and showed its efficacy and therapeutic effects in overcoming vaginal atrophy symptoms (Cano *et al.*, 2012; Caruso *et al.*, 2016). Prepared gels were kept in 4˚C refrigerator. For a roughly 100 mg plain gel, the amount of estriol added was calculated as follows:

$$Estriol = \frac{100\ mg}{5.8\ mg} \times 0.29\mu g$$

$$= 5\ \mu g$$

$$= 0.005\ mg$$

3.2.3.2 Insertion of gel into the rat vagina

Thirty-six ovariectomized rats were then divided into six groups, n=6 per groups:

A- ovariectomy, sham control, no treatment

B- ovariectomy, normal control rats receiving plain gel intravaginally

C- ovariectomy, treated with MP 100 µg/mL gel intravaginally

D- ovariectomy, treated with MP 250 µg/mL gel intravaginally

E- ovariectomy, treated with MP 500 µg/mL gel intravaginally

F- ovariectomy, treated with 0.005% estriol gel intravaginally

Gel was applied intravaginally using Eppendorf Combitip positive displacement pipette (Liu *et al.*, 2015; Lopez-Belmonte *et al.*, 2012) and left *in situ* for seven consecutive days. On the eighth day, animals were anaesthetized by using intraperitoneal injection of ketamine (50 mg/kg) and xylazine (5mg/kg) mixture to draw blood through cardiac puncture prior to euthanasia by pentobarbital overdose (250 mg/kg). Vagina tissues were then harvested for further histological, protein expression study and transmission electron microscopy.

3.2.4 Histological study

3.2.4.1 Slides preparation

Following euthanasia, vagina tissues were dissected and fixed immediately in 10% formalin in pre-labelled sterile urine container. Tissues were left for more than 24 hours at room temperature to allow complete penetration and fixation by the formalin. Prior to

tissue processing procedure, vagina tissues with 4 mm thickness were fixed in 50% ethanol for few hours to allow gradual dehydration. Tissue processing was then performed in Department of Anatomy, Faculty of Medicine, University Malaya using a rotatory tissue processor and left overnight. Steps of tissue processing are shown in the following table 3.2.

Table 3.2: Tissue processing procedures

Process Bath	12 Hour Cycle (Time in Hours)	Temperature
70% ethanol	1	37˚C
80% ethanol	1	37˚C
95% ethanol	1	37˚C
Absolute alcohol	1	37˚C
Absolute alcohol	1	37˚C
Absolute alcohol	1	37˚C
Xylene	1	37˚C
Xylene	1	37˚C
Xylene	1	37˚C
Paraffin	1	63˚C
Paraffin	1	63˚C
Paraffin	1	63˚C

Tissues were immersed in increasing concentration of ethanol series to allow dehydration to occur gradually. Xylene was used following ethanol immersion as clearing agent to displace ethanol, clearing any fat remnants and act as an intermediate solution before tissues being infiltrated by paraffin wax. On the following day, vagina tissues were embedded in paraffin blocks to support the tissue structure and kept at -20˚C before being sectioned. Paraffin blocks of vagina specimens were cut into 5 µm thin cross section using a microtome and mounted on polysine slides. All slides were incubated at 37˚C for at least 3 days to permit proper adherence of specimens onto the slides before being used for any study.

3.2.4.2 Hematoxylin and eosin staining

To study vaginal morphology, staining by hematoxylin and eosin (H&E) was done to visualize the vaginal epithelial thickness and layers. The prepared polysine slides containing vagina sections from each experimental group were selected and dewaxed by three changes of xylene. Sections were then undergoing hydration process by serial changes of ethanol in decreasing concentration manner before being immersed in distilled water and stain with filtered hematoxylin. Sections were washed with tap water until no more excess hematoxylin available and further differentiate by acid alcohol for few seconds to remove excess background staining.

Bluing of sections were done using tap water to change nuclear coloration from reddish purple to crisp blue/purple before being stain with eosin. Eosin will stain the cytoplasm and connective tissues while hematoxylin will stain the nucleus. Sections were dehydrated again using increasing concentration of ethanol and further cleared from any water content by xylene. Slides were left to dry before being mounted and covered with mounting media and cover slip. Table 3.3 shows the overall procedure of H&E staining and time allocated for each step.

Table 3.3: Hematoxylin and eosin staining procedure

PROCEDURE	TIME CONSUMED
DEWAXING	
Xylene I	3 minutes
Xylene II	3 minutes
Xylene III	3 minutes
HYDRATION	
Absolute alcohol I	2 minutes
Absolute alcohol II	2 minutes
95% alcohol I	2 minutes
95% alcohol II	2 minutes
80% alcohol	2 minutes
70% alcohol	2 minutes
50% alcohol	2 minutes
Distilled water	3 minutes
Filtered hematoxylin	20 minutes
Tap water	Until no more excess hematoxylin
DIFFERENTIATION	
Acid alcohol	2-10 seconds
BLUING	
Running tap water	5 minutes
Eosin	5 minutes
DEHYDRATION	
95% alcohol I	2 minutes
95% alcohol II	2 minutes
Absolute alcohol I	2 minutes
Absolute alcohol II	2 minutes
Absolute alcohol III	2 minutes
CLEARING	
Xylene I	3 minutes
Xylene II	3 minutes
Xylene III	3 minutes

3.2.5 Protein distribution study by immunohistochemistry

Immunohistochemistry involved a binding reaction between antigen and antibody. This method can provide visualization of distribution and location of target antigen in tissue sections. This method comprises mainly three components: (1) binding of primary antibody to specific antigen; (2) incubation with secondary antibody (conjugated with enzyme) will formed antigen-antibody complex; (3) enzyme is then catalyzed to produce

colored deposits at antigen-antibody binding sites using certain substrates and chromogen (Ramos-Vara, 2011).

Immunohistochemistry is done as described by Karim *et al.* (2016) with some modifications. Slides were dewaxed by three changes of xylene for 5 minutes each and further washed with absolute ethanol twice for 10 minutes each. Vagina sections were further washed with 95% ethanol twice for 10 minutes, phosphate buffer saline (PBS) twice for 5 minutes each and distilled water once for 2 minutes. Sections were incubated in 0.01 M citrate buffer, pH 6.0 for antigen retrieval. They were boiled twice at 50˚C for 10 minutes and twice at 90˚C for 10 minutes. Partial cooling was allowed in between each boiling interval for 1 minutes. Sections together with the citrate buffer were then left for 20 to 30 minutes for cooling before being washed with distilled water for 2 minutes.

Slides were carefully cleaned before incubation of tissue sections with 3% hydrogen peroxide (H_2O_2) in PBS for 30 minutes, aiming for neutralization of endogenous peroxidase. Proper washing with PBS thrice for 5 minutes each were done later and sections were further incubated in 5% bovine serum albumin (BSA) (Sigma-Aldrich, USA) for 1 hour which will block non-specific antigens. Targeted primary antibody (Santa Cruz Biotechnology, USA) at a dilution of 1:100 was then prepared in 5% BSA to incubate the sections for overnight at 4˚C. Vagina sections were washed with PBS thrice for 5 minutes on the next day prior to incubation with secondary antibody at a dilution of 1:200 in 5% BSA for 1.5 hour. Primary and secondary antibodies used are given in Table 3.5.

Washing with PBS thrice was repeated and tissue sections were subjected to peroxidase stain with diaminobenzidine (DAB) kit (Nacalai Tesque, Kyoto, Japan) until suitable dark-brown precipitation developed about 5 to 10 minutes. The reaction was terminated by washing with distilled water and nucleus was further counterstained with

filtered hematoxylin for 40 seconds to 1 minute. Slides were then washed with distilled water and dehydrated using increased concentration of ethanol series (95% ethanol twice and absolute ethanol twice, each for 20 seconds) before being cleared by xylene wash thrice for the same period (20 seconds). Slides were left to dry and tissue sections were secured with mounting media and cover slip. Sections were then viewed at 40x magnification under phase contrast microscope (Nikon H600L, Tokyo, Japan).

3.2.6 Protein expression study by Western blotting

Western blotting has the advantage of quantifying the desired proteins compared to immunohistochemistry procedure. This technique allowed identification of specific proteins extracted from the vagina cells. Generally, this technique comprises of three main elements: (1) protein separation according to its molecular weight, (2) protein transfer to a membrane for a solid support, and (3) targeted protein marking using appropriate primary and secondary antibody to allow further visualization (Mahmood & Yang, 2012). Proteins were initially extracted and quantify before initiation of Western blotting procedure.

3.2.6.1 Protein extraction

Proteins were extracted following the protocol described by the manufacturer. Following vagina harvesting during animal euthanasia, vagina tissues were frozen immediately in liquid nitrogen and stored in -80˚C before being used for extraction. Vagina tissue sample of 30-40 mg were obtained and immersed in liquid nitrogen, being crushed into powder form using a clean mortar and pestle. The powder form was transferred in 2.0 mL Eppendorf tube and mixed with 1000 µL of PRO-PREP solution

(Intron, Korea). The mixture was homogenized for 30 seconds before being incubated for 30 minutes at -20˚C to allow tissue lysis. The suspension was centrifuged at 24562 $\times g$ centrifugal force for 15 minutes at 4˚C. The supernatant was collected and transferred to a new 2.0 µL Eppendorf tube before being stored at -20˚C.

3.2.6.2 Protein quantification

Protein quantification was done using the following procedures: initially, preparation of diluted albumin (BSA) standards was done according to the protocol describe by the manufacturer to achieve the desired final BSA concentration. Nine vials of standard solution having different final BSA concentration were labelled as letter A to I. Phosphate buffer solution (PBS) was used as diluent in preparing standard dilution and sample dilution. Then, 10µL from each group of vagina protein samples were also diluted in 190 µL PBS. Next, preparation of Micro BCA Working Reagent was done. 25 parts of reagent MA were mixed with 24 parts of reagent MP and 1 part of reagent MC (25:24:1, Reagent MA: MB: MC). The total volume needed for Working Reagent is determined by calculating total standard and sample prepared and multiplying them by 3 replicates.

Each standard solution and diluted sample were then pipetted into the microplate well by three replicates each. Each well will contain 50 µL of standard or sample solution, added with 50 µL of Micro BCA Working Reagent. Microplate were then wrapped tightly with aluminum foil and incubated at 37˚C for 2 hours. Plate was cooled at room temperature and absorbance was measured 562 nm using a plate reader. A standard curved was then plotted using the measurement from the standard protein samples. A value of R^2>0.95 is sufficient and consider accurate to be utilized to quantify samples' protein concentration.

3.2.6.3 Western blotting technique

(a) ***SDS-PAGE gel preparation***

The preparation of SDS-PAGE was performed on the experiment day. A resolving gel of 8 to 12% were determined to be prepared, depending on target protein molecular weight. Resolving gel was prepared in specific glass plate and left for about 10 minutes to allow solidification. 4% stacking gel was then added on top of it with immediate insertion of 1.0 mm well comb to provide wells for loading the proteins. Gel formulation for two gels were shown in the following table.

Table 3.4: SDS-PAGE gel formulation.

	Resolving gel 10%	**Resolving gel 12%**	**4% Stacking gel**
Distilled water	3800µL	3200 µL	1500 µL
30% Acrylamide	3400 µL	4150 µL	335 µL
1.5M Tris (pH8.8)	2600 µL	2600 µL	-
1.5M Tris (pH6.8)	-	-	625 µL
10% SDS	100 µL	100 µL	25 µL
10% APS	100 µL	100 µL	25 µL
TEMED	10 µL	10 µL	5 µL

(b) ***Sample preparation***

20µg of protein sample from each group were added with loading dye in a ratio of 4:1. Solutions were mixed equally with vortex and further boiled for 5 minutes at 95˚C. Samples were then vortex again and ready to be loaded into the SDS-PAGE gel.

(c) ***Running SDS-PAGE gel***

The glass slabs containing the gel were fixed into the cassettes. Running buffer was poured into the inner chamber and 1.0 mm well comb was removed. All samples and 3-5 µL BLUeye Prestained Protein Ladder (GeneDirex, Bio-Helix Co., Taiwan) were loaded

into the wells. Running buffer was then poured into the outer chamber. Gel electrophoresis was run at 100V for around 2 to 2.5 hours until the blue loading dye migration reached the end of the glass plates but does not migrate off the gel.

(d) ***Transferring protein to membrane***

Protein transfer was done using Thermo Scientific Pierce G2 Fast Blotter as described by manufacturer. Each gel required four sheets of Western blotting filter paper (~0.83mm thick) and one sheet of PVDF membrane cut equally. Filter papers were equilibrated in Thermo Scientific™ Pierce™ 1-Step Transfer Buffer for around 5 minutes. PVDF membrane was initially activated in methanol before being equilibrated in Pierce™ 1-Step Transfer Buffer.

Following gel electrophoresis, gels were removed from the cassettes and briefly immersed in deionized water or transfer buffer to allow even wetting, help proper gel placement, and provide good contact between the gel and the membrane. Blotting sandwiches were prepared in the following arrangement; 2 sheets of filter paper at the bottom followed by PVDF membrane, gel, and another 2 sheets of filter paper covering the sandwich top. The sandwich was then placed on the bottom of the blotter cassette (anode) and a blot roller was used to discard air bubbles which may trapped in between. The top of the cassette (cathode) was lock into place and the whole cassette was slide into the control unit. Transfer process timing was selected in accordance to targeted protein molecular weight and in accordance to pre-programmed methods. Membrane was then removed and soaked in deionized water before being blocked in blocking serum.

(e) ***Blocking and incubation with antibodies***

Membrane was then incubated in 5% BSA for at least one hour in room temperature. Blocking with BSA aimed to prevent binding of non-specific antibodies that can lead to high background signaling during detection process. Membrane was then cut into two strips to be immunoblotted for the target protein and the housekeeping protein e.g. β-Actin, GADPH (Santa Cruz Biotechnology, UK). Site of cutting depend on the molecular weight of target protein. This step is important to certify a similar amount of protein samples were used.

After blocking with 5% BSA, the respective membrane strips were then incubated in target protein primary antibody and housekeeping protein primary antibody diluted in PBST buffer in 1:1000 ratio for overnight at 4˚C on a shaker. On the following day, membrane was washed thrice with PBST for 5 minutes each before being incubated with secondary antibody (Santa Cruz Biotechnology, UK) diluted in PBST (1:5000 ratio) for at least one hour at room temperature. All primary and secondary antibodies used in this study were listed in the following Table 3.5.

Table 3.5: Primary and secondary antibodies used

No.	Target protein	Primary antibody	Secondary antibody
1.	PCNA	Goat polyclonal: sc-9857	Donkey anti-goat IgG-HRP: sc-2020
2.	VEGF	Rabbit polyclonal: sc-507	Goat anti-rabbit IgG-HRP: sc-2030
3.	Occludin	Goat polyclonal: sc-8145	Donkey anti-goat IgG-HRP: sc-2020
4.	AQP-1	Rabbit polyclonal: sc-20810	Goat anti-rabbit IgG-HRP: sc-2004
5.	AQP-2	Rabbit polyclonal: sc-28629	Goat anti-rabbit IgG-HRP: sc-2004
6.	V-ATPase A1	Rabbit polyclonal: sc-28801	Goat anti-rabbit IgG-HRP: sc-2004
8.	β-Actin	Mouse monoclonal: sc-47778	Goat anti-mouse IgG-HRP: sc-2005

(f) ***Detection by colorimetric technique***

After incubation with secondary antibody, membranes were washed with PBST for three times and ready for detection. Peroxidase Stain DAB Kit (Nacalai Tesque, Japan) was prepared in metal enhancer for DAB stain solution (Nacalai Tesque, Japan). Membranes were exposed to DAB solution until suitable staining developed. Membranes were washed with distilled water to stop the reaction and allowed to dry before images of bands developed being captured using UVP BioSpectrum Imaging System (UVP, Canada).

(g) ***Statistical analysis***

The protein bands density was measured using ImageJ 1.49v software and statistical one-way analysis of variance was done using GraphPad Prism 5 software. Tukey's multiple comparison test was done if statistically significance were found. A value of $P<0.05$ was taken as statistically significance. The mean ± SEM of target protein/housekeeping protein ratio were used to quantify the protein expression.

3.2.7 Transmission Electron Microscopy

Vagina tissues were initially fixed in 4% glutaraldehyde for more than 4 hours to allow proper fixation. Vagina tissues were further trimmed to 1 to 2 mm and washed two to three times with cacodylate buffer. Samples were incubated at 4˚C in a mixture of osmium tetroxide with cacodylate buffer (1:1 ratio) for 2 hours. Samples were then washed again with cacodylate buffer thrice and left in the buffer for overnight at 4˚C.

On the following day, vagina samples were washed with distilled water thrice. Samples were then spin down in 35% alcohol, 50% alcohol, and 70% alcohol for 10 minutes each and further 15 minutes each in 95% alcohol and three changes of 100% alcohol solution.

Alcohol solution was then discarded and changed to propylene oxide. Samples were soaked in 2 changes of propylene oxide for 15 minutes each. Then, samples were spin down in the mixture of propylene oxide with Epon (1:1 ratio) for one hour and further slowly centrifuged in propylene oxide: Epon mixture (1:3 ratio) for another 2 hours. The end of sample processing was completed by leaving the samples immerse and slowly spin in Epon solution for overnight.

Samples were then embedded in Epon at 37˚C for 5 hours and at 60˚C for overnight. Epon blocks containing vagina samples were then trimmed into trapezius shape to allow adequate visualization of samples from any desired angles. The Epon blocks were then sectioned into 0.5µm thick sections and transferred to a drop of water on the polysine slides. The water will flatten the sections and avoid wrinkles formation. Slides were then dried on slide warmer and stained with toluidine blue for 2 minutes. Sections were then observed under microscope to locate precise site to be cut for ultrathin sections. Sections were then sectioned to 60-90 nm thick and placed on grids, leaving them for overnight before being stained with uranyl acetate and lead citrate. Sections were then observed under transmission electron microscope. All procedures were done in Electron Microscopy Unit, Faculty of Medicine, University of Malaya.

CHAPTER 4: RESULTS

This chapter will present all results conducted in this study including histological changes in the vagina, immunohistochemistry findings of targeted protein expression, Western blotting data and images from transmission electron microscope viewing on vagina ultrastructure.

4.1 Vagina histological changes.

Rats treated with MP 500 µg/mL/day gel had the thickest vaginal epithelium among MP-treated group approaching that of estrogen-treated rats (Fig. 4.1). Meanwhile, no difference in vaginal epithelial thickness was observed between sham and control rats. The thickness of the vaginal epithelium in MP100 vaginal gel-treated group was significantly higher when compared to the control and sham groups (Table 4.1). There was a dose-dependent increase in the thickness of the vaginal epithelial with increasing dose of MP. MP500 showed 7-folds epithelial thickness while MP250-treated group had 4-folds epithelial thickness when compared to control. Group receiving estrogen gel had the highest epithelial thickness with 9-folds epithelial thickness from the control vaginal epithelium.

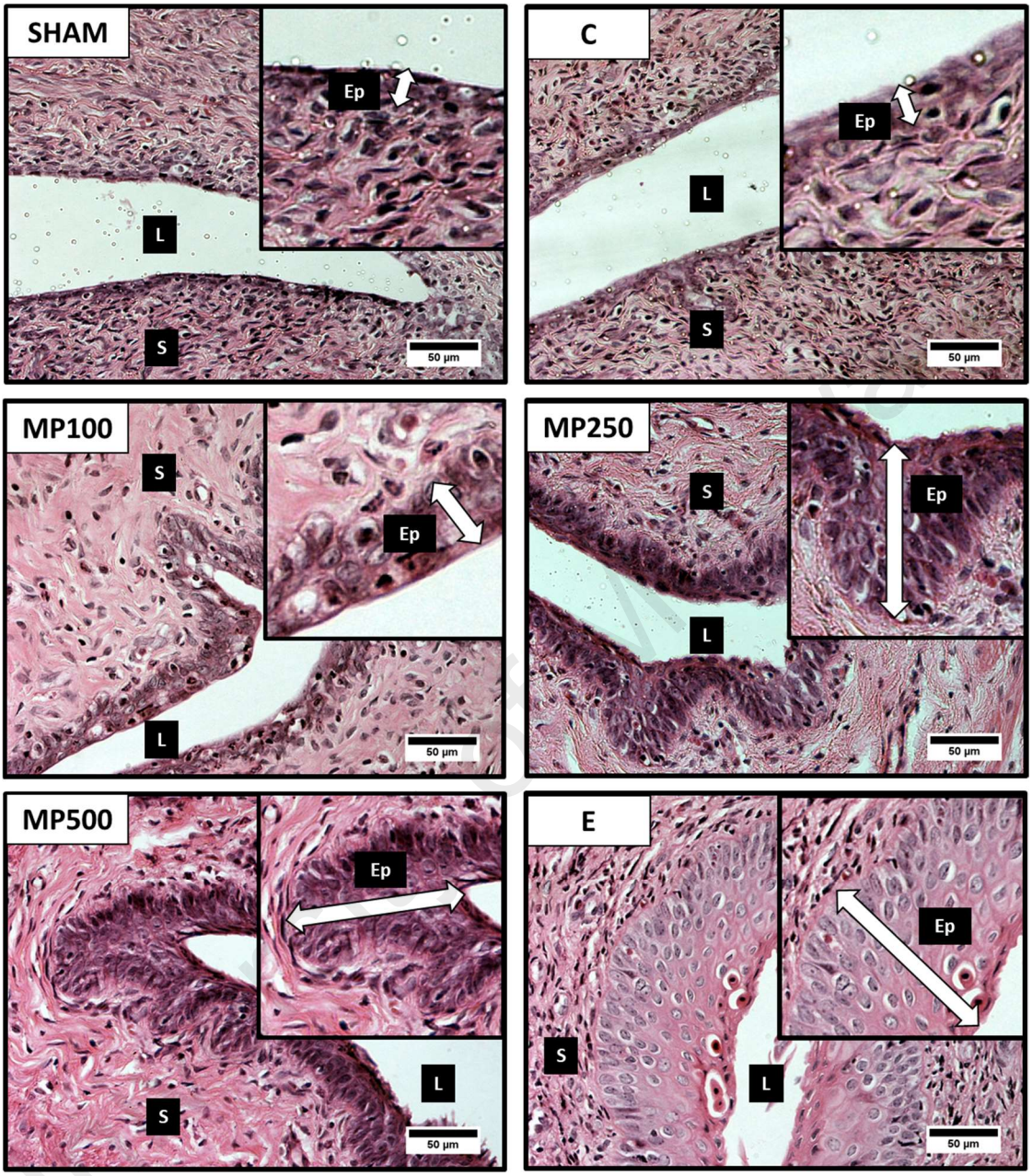

Figure 4.1: Images of the vagina epithelium stained with hematoxylin and eosin (H&E) in sex steroid deficient rats treated with topical MP vaginal gel.

Sham (no gel), C = Control (plain gel), MP100 = MP 100 µg/mL/day gel, MP250 = MP 250 µg/mL/day gel, MP500 = MP 500 µg/mL/day gel, E = 0.005% estriol gel, Ep = Epithelium, S = Stroma, L = Lumen. White arrow = vaginal epithelium span. All images were taken in 400 times total magnification. Scale bar = 50µm.

Table 4.1: Quantitative measurement of vaginal epithelial thickness.

Groups (n = 6)	Epithelial thickness (µm)
Sham	10.45 ± 2.62
Control	10.63 ± 1.21
MP100	31.84 ± 5.85*
MP250	46.38 ± 3.78*
MP500	72.49 ± 16.16*
E	97.85 ± 7.35*

Values represent means ± S.E.M for 6 rats per group. *P<0.05 compared to sham and control. No significant difference observed between sham and control groups.

4.2 Expression of proliferative proteins in the vagina of sex steroid deficient rats receiving MP gel treatment.

Proliferative proteins that are demonstrated in this section includes PCNA and VEGF proteins. Results are presented as immunohistochemistry images findings to visualize expressed protein location and Western blotting data as a complementary data for protein quantification.

4.2.1 Proliferating Cell Nuclear Antigen (PCNA)

No significance difference in the PCNA expression level was seen between sham and control groups (Fig. 4.2). However, significant increase in PCNA expression level was observed in the vagina of sex steroid deficient rats following treatment with MP 500 µg/mL/day vaginal gel as compared to control. The levels of PCNA expression were also higher following treatment with MP 100 and MP 250 vaginal gel when compared to control ($p<0.05$). No significant different in PCNA expression level was observed between MP500 and estrogen-treated rats, which were higher than control.

Immunohistochemistry images (Fig. 4.3) showed PCNA was distributed mainly in the nuclei of the basal cells of the vagina epithelium. PCNA distribution could be found in the vagina of all groups with high distribution was observed in MP 500 µg/mL/day and estrogen treated groups.

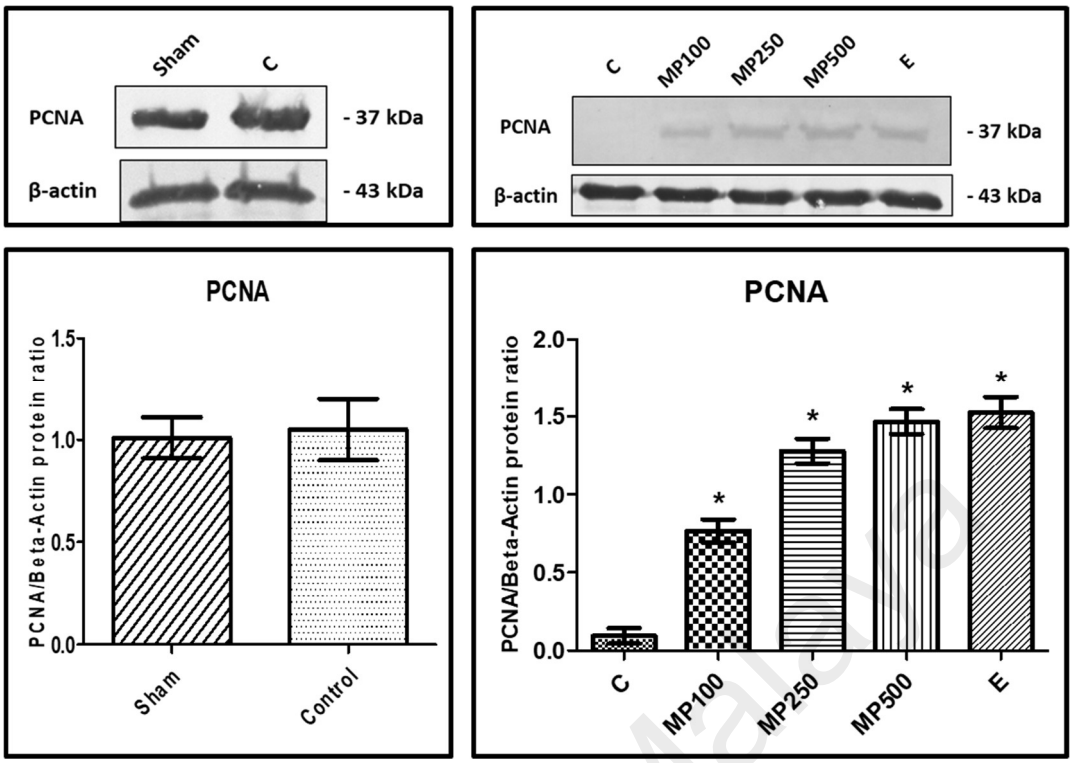

Figure 4.2: Representative Western blot images of PCNA and analysis of PCNA/β-actin protein band density ratio in the vagina of sex steroid deficient rats treated with MP vaginal gel.

Higher concentrations of proteins were used to compare between sham and control groups. In bar graph, *$p<0.05$ as compared to C. Sham (no gel), C = Control (plain gel), MP100 = MP 100 µg/mL/day gel, MP250 = MP 250 µg/mL/day gel, MP500 = MP 500 µg/mL/day gel.

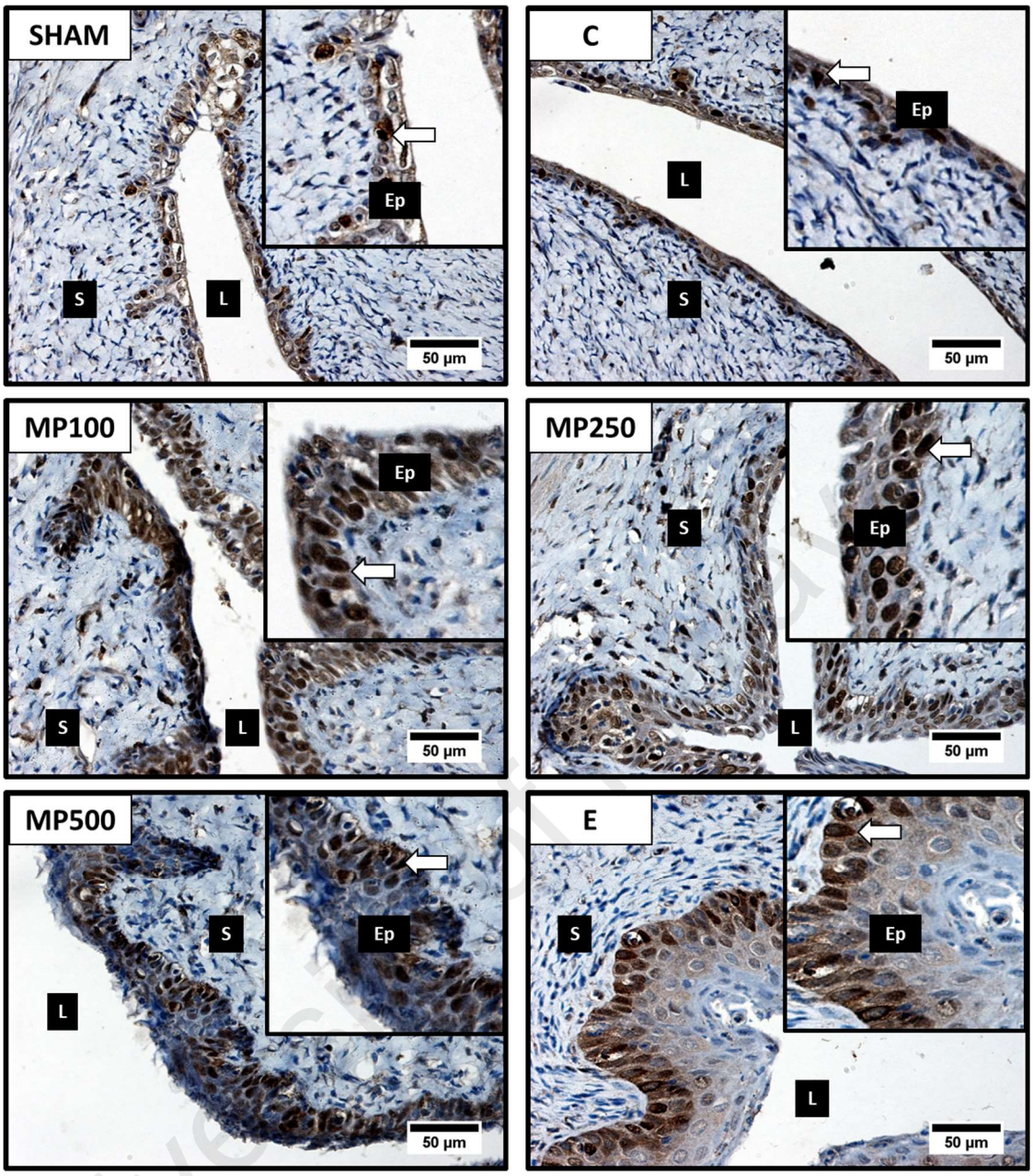

Figure 4.3: Immunohistochemistry images showed PCNA distribution in the vaginal epithelium of sex steroid deficient rats treated with MP vaginal gel.

Sham (no gel), C = Control (plain gel), MP100 = MP 100 µg/mL/day gel, MP250 = MP 250 µg/mL/day gel, MP500 = MP 500 µg/mL/day gel, E = 0.005% estriol gel, Ep = Epithelium, S = Stroma, L = Lumen. White arrow = PCNA expression on the basal cells nuclei of vagina epithelium. All images were taken in 400 times total magnification. Scale bar = 50µm.

4.2.2 Vascular Endothelial Growth Factor (VEGF)

Significant increase in VEGF expression was observed in the vagina of sex steroid deficient rats treated with 0.005% estriol vaginal gel when compared to control (Fig. 4.4). There was no significance difference in VEGF protein expression between sham and control groups. There was a slight but significant increase in the VEGF protein expression level following 250 and 500 MP gel treatment as compared to control.

Immunohistochemistry images (Fig. 4.5) showed VEGF was distributed mainly in the cytoplasm of the vaginal epithelial cells. The distribution could be seen throughout the epithelial layer. All treated groups showed expression of VEGF with highest density following MP 500 μg/mL/day and estrogen treatments.

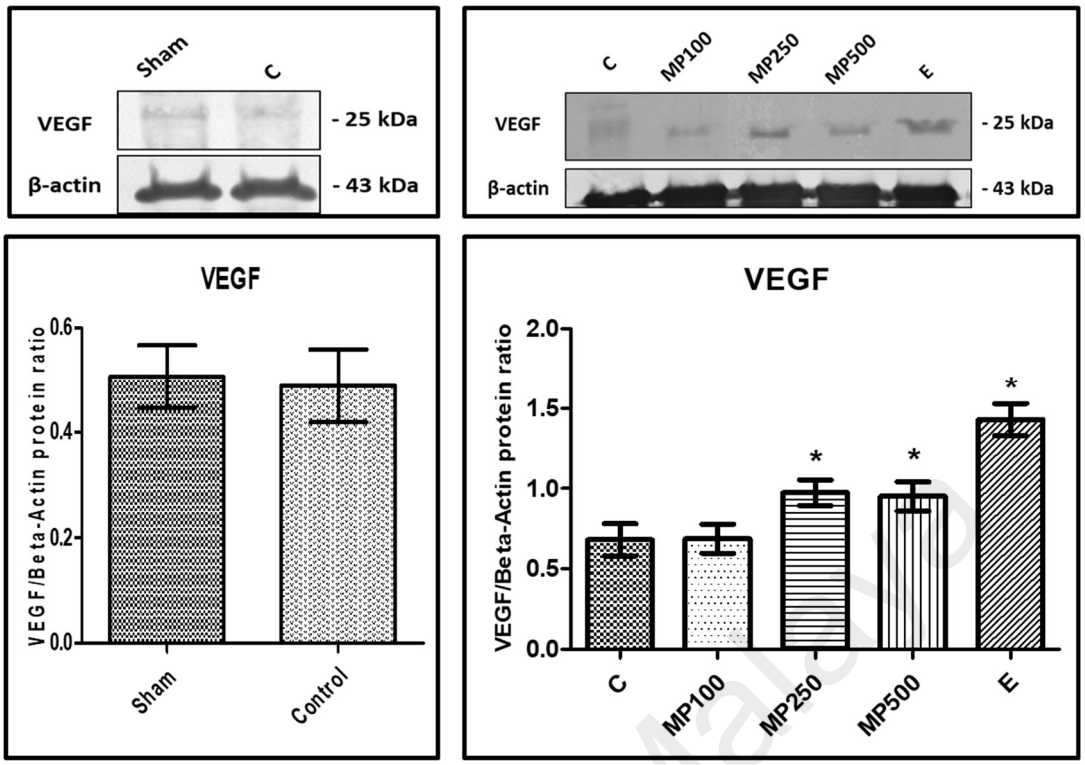

Figure 4.4: Representative Western blot images of VEGF and analysis of VEGF/β-actin protein band density ratio in the vagina of sex steroid deficient rats treated with MP vaginal gel.

Higher concentrations of proteins were used to compare between sham and control groups. In bar graph, *p<0.05 as compared to C. Sham (no gel), C = Control (plain gel), MP100 = MP 100 µg/mL/day gel, MP250 = MP 250 µg/mL/day gel, MP500 = MP 500 µg/mL/day gel.

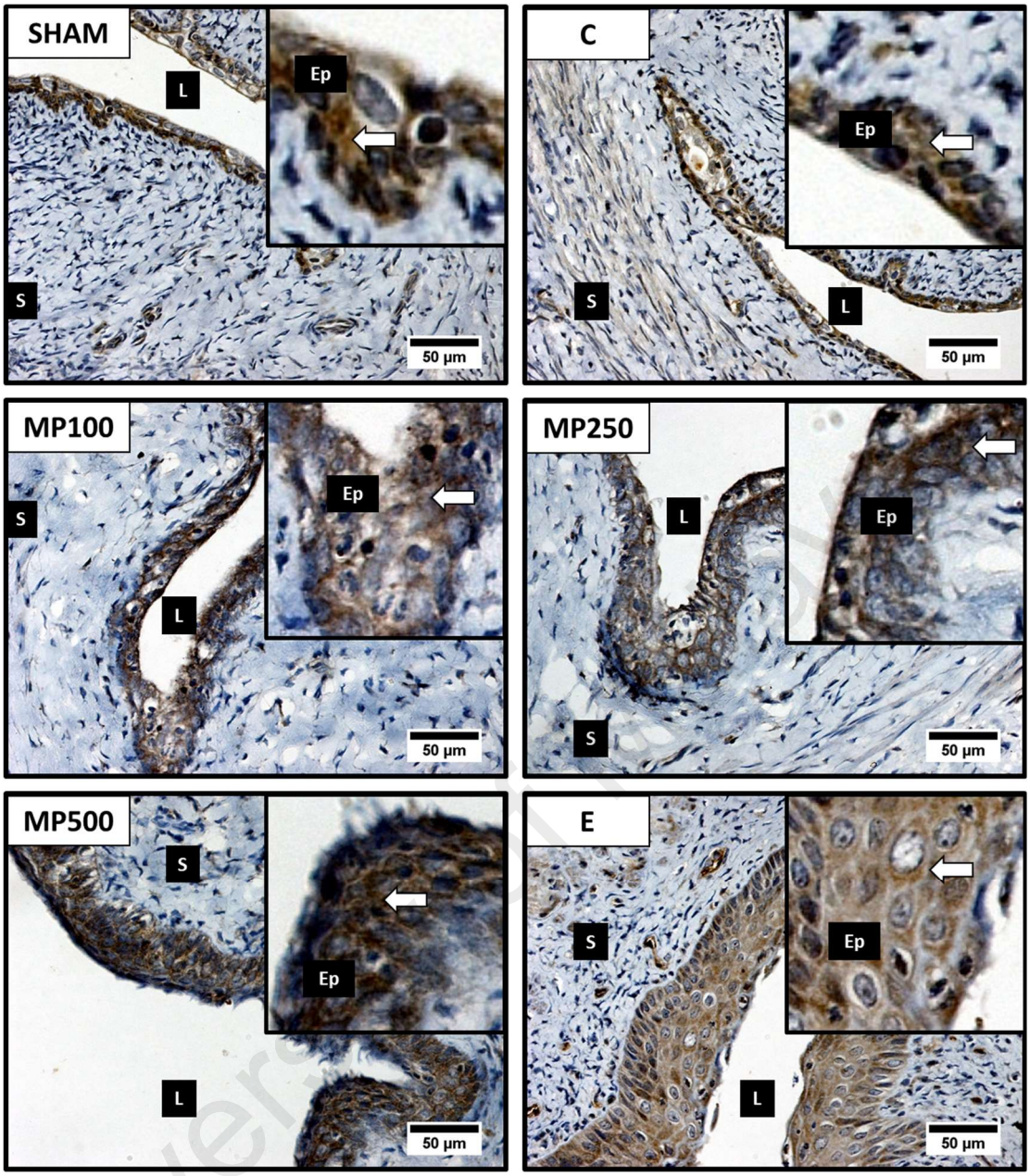

Figure 4.5: Immunohistochemistry images showing VEGF protein distribution in the vagina epithelium of sex steroid deficient rats treated with MP vaginal gel.

Sham (no gel), C = Control (plain gel), MP100 = MP 100 µg/mL/day gel, MP250 = MP 250 µg/mL/day gel, MP500 = MP 500 µg/mL/day gel, E = 0.005% estriol gel, Ep = Epithelium, S = Stroma, L = Lumen. White arrow = VEGF expression in the cytoplasm of vagina epithelium. All images were taken in 400 times total magnification. Scale bar = 50µm.

4.3 Expression of tight junction protein in the vagina of sex steroid deficient rats treated with MP and vaginal ultra-morphology.

Tight junction protein that is demonstrated in this section includes Occludin protein. Results are presented as Western blotting data for protein quantification. Details of intercellular junction information are demonstrated in transmission electron microscope images.

4.3.1 Occludin

Significant increase in occludin protein expression level was observed in MP100, MP250, MP500 and E treated groups when compared to control group (Fig. 4.6). The highest expression of occludin was observed in estrogen treated group followed by MP 500 µg/mL/day and MP 250 µg/mL/day MP gel-treated groups. There is no significance difference in protein expression between sham and control groups.

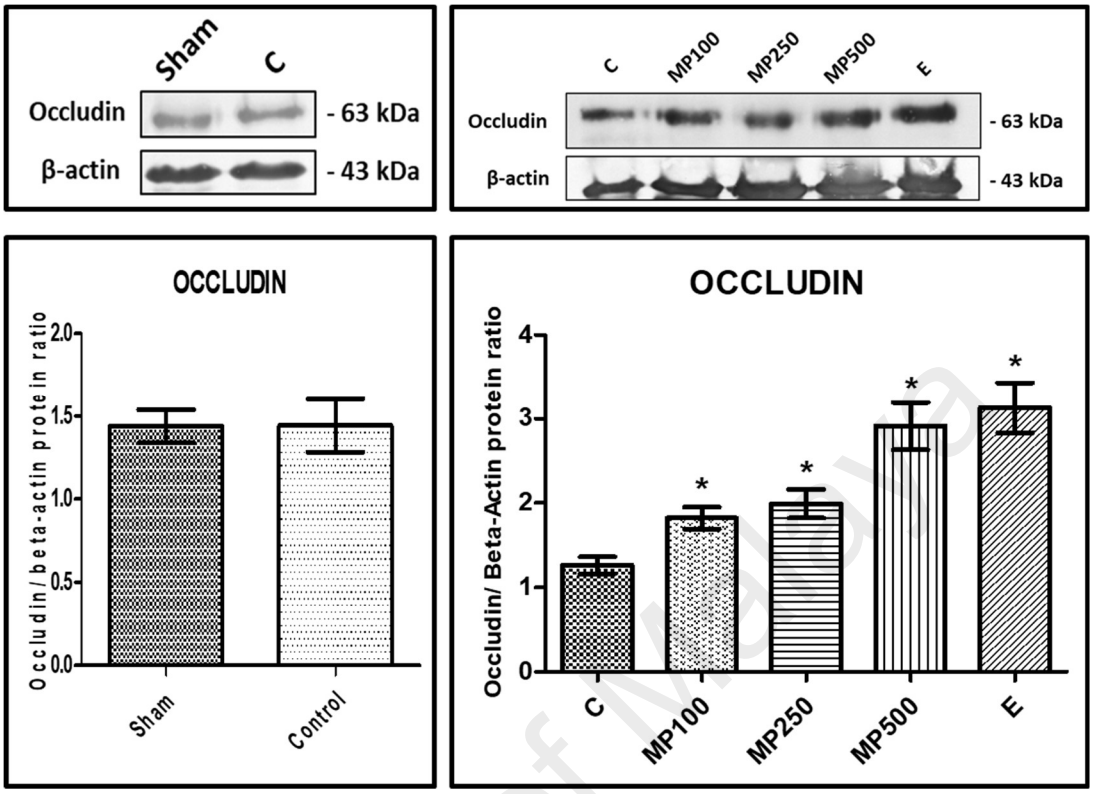

Figure 4.6: Representative Western blot images showing density of Occludin protein and analysis of Occludin/β-actin protein band ratio in the vagina of sex steroid deficient rats treated with MP vaginal gel.

Higher concentrations of proteins were used to compare between sham and control groups. In bar graph, *p<0.05 as compared to C. Sham (no gel), C = Control (plain gel), MP100 = MP 100 µg/mL/day gel, MP250 = MP 250 µg/mL/day gel, MP500 = MP 500 µg/mL/day gel, E = 0.005% estriol gel.

4.3.2 Vaginal tight junction morphology under transmission electron microscopy (TEM)

The ultramorphology of the vagina epithelium of sex steroid deficient rats treated with MP topical vaginal gel was observed under transmission electron microscope (TEM), showed intercellular spaces became more approximate following treatment with MP250, MP500 and E (Fig. 4.7). Desmosome started to be formed in MP250 group and its formation seems to be complete in MP500 and E treated groups. However, formation of zonula occludens and zonula adherens were minimally seen. In sham group, no desmosomes could be seen in control and sham groups.

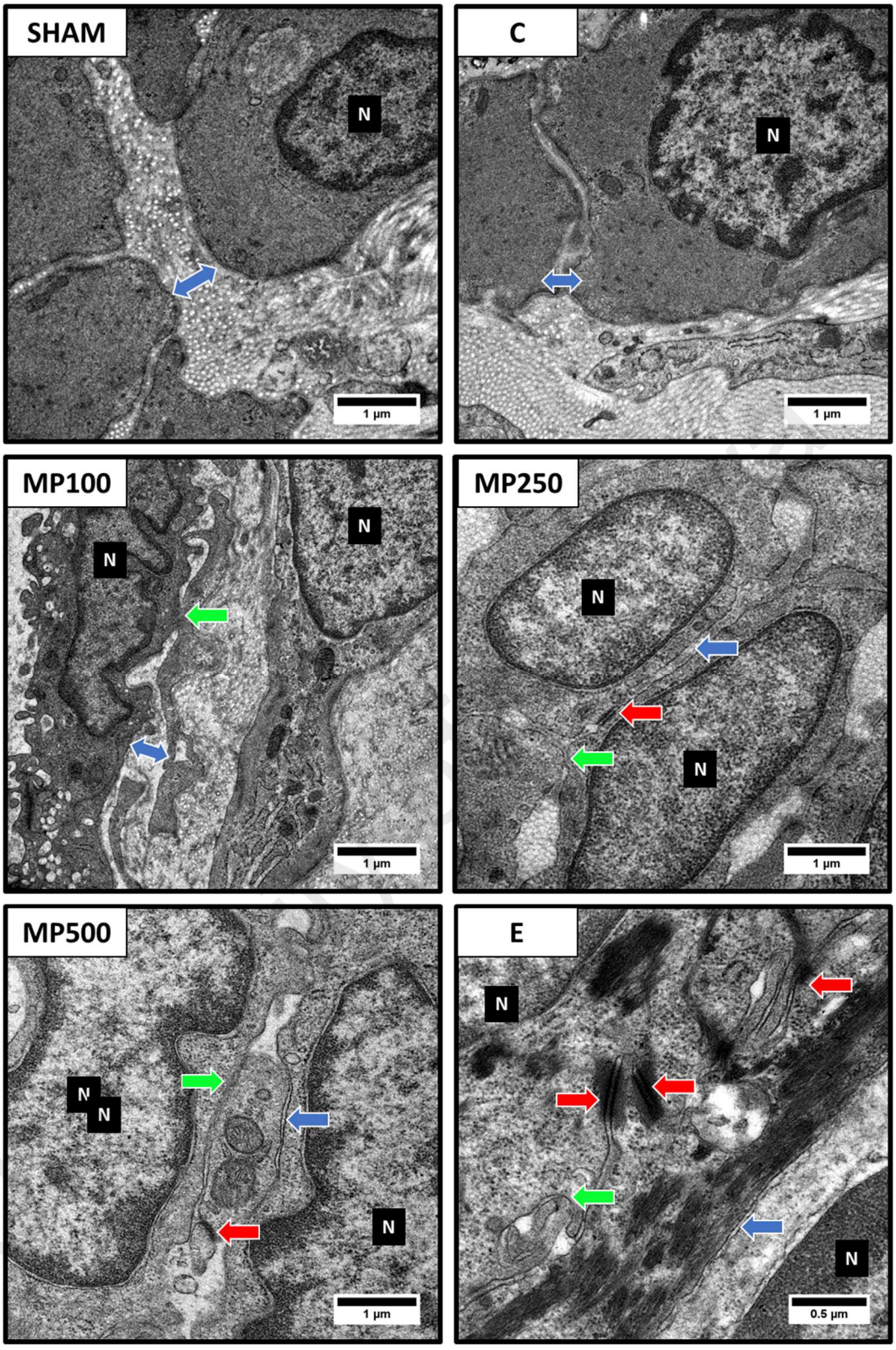

Figure 4.7: Images of vagina epithelium under transmission electron microscopy (TEM) showing the junctional area between cells.

Blue arrows = spaces between cells. Red arrows = Desmosomes. Green arrows = Zonula occludens. Sham (no gel), C = Control (plain gel), MP100 = MP 100 µg/mL/day gel, MP250 = MP 250 µg/mL/day gel, MP500 = MP 500 µg/mL/day gel, E = 0.005% estriol gel. All images were taken in 20,000 (Sham, C, MP100, MP250, MP500) and 40,000 times total magnification (E). Scale bar = 1 µm (Sham, C, MP100, MP250, MP500) and 0.5 µm (E).

4.4 Expression of aquaporin proteins in the vagina of sex steroid deficient rats treated with MP

A significant increase in AQP-1 protein expression was observed with increasing dose of MP gel. Expression levels in MP treated groups were significantly higher than control (Fig. 4.8). The highest expression was seen in E treated group followed by MP250 and MP500 groups. There is no significant difference in AQP-1 protein expression level between sham and control groups. Immunohistochemistry images of AQP-1 (Fig. 4.9) showed its distribution mainly in the blood vessels.

Meanwhile, AQP-2 protein expression level demonstrated significant increased in all MP groups and estrogen treated groups as compared to control group (Fig. 4.10). MP250 showed slightly higher expression of protein compared to MP500 while E-treated group showed highest level of protein expression above all. No significant difference can be seen in the protein expression level between sham and control groups. Immunohistochemistry findings of AQP-2 (Fig. 4.11) showed its distribution in the cytoplasm of vagina epithelium. The levels of distribution of AQP-2 was relatively higher following MP250, MP500, and estrogen treatments.

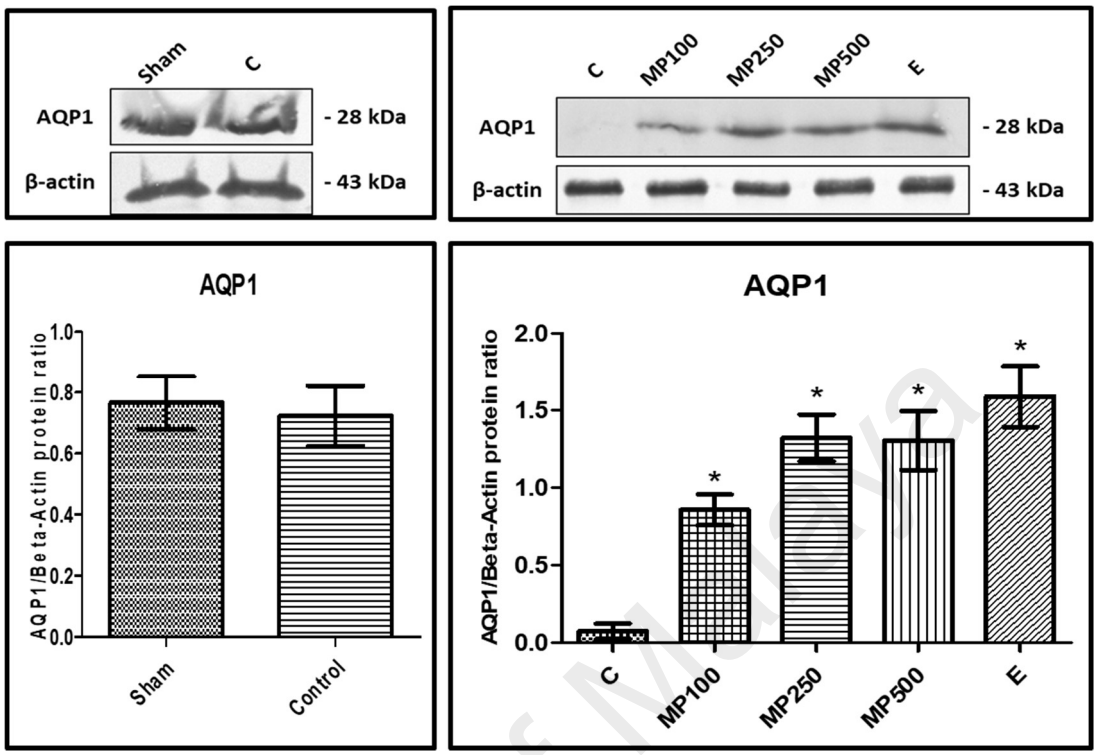

Figure 4.8: Representative Western blot images of AQP-1 and analysis of AQP-1/β-actin protein band density ratio in the vagina of sex steroid deficient rats treated with MP vaginal gel.

Higher concentrations of proteins were used to compare between sham and control groups. In bar graph, *$p<0.05$ as compared to C. Sham (no gel), C = Control (plain gel), MP100 = MP 100 µg/mL/day gel, MP250 = MP 250 µg/mL/day gel, MP500 = MP 500 µg/mL/day gel.

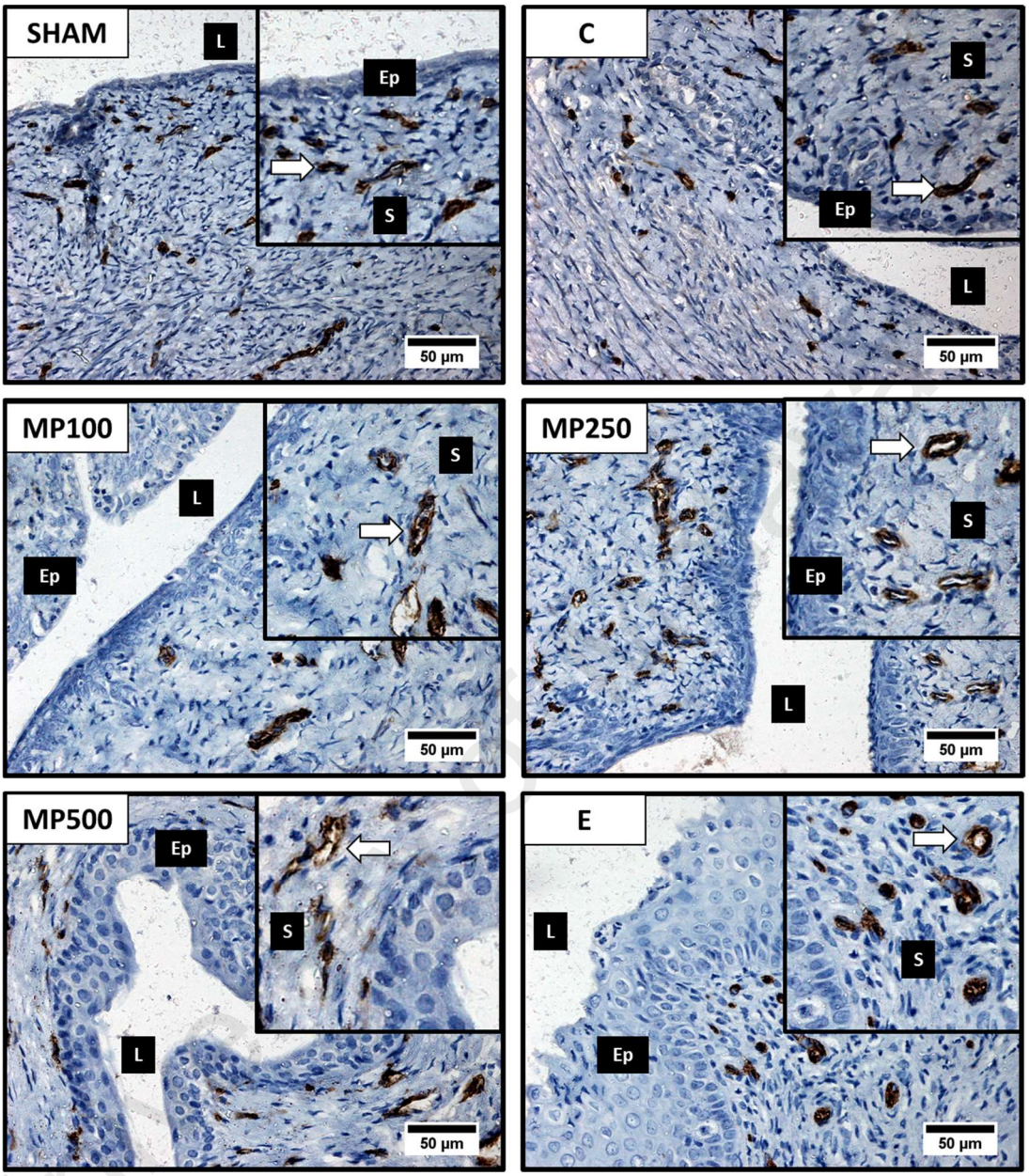

Figure 4.9: Immunohistochemistry images showing AQP-1 protein distribution in the vagina epithelium of sex steroid deficient rats treated with MP vaginal gel.

Sham (no gel), C = Control (plain gel), MP100 = MP 100 µg/mL/day gel, MP250 = MP 250 µg/mL/day gel, MP500 = MP 500 µg/mL/day gel, E = 0.005% estriol gel, Ep = Epithelium, S = Stroma, L = Lumen. White arrow = AQP-1 expression in the capillaries and blood vessels of vagina epithelium. All images were taken in 400 times total magnification. Scale bar = 50µm.

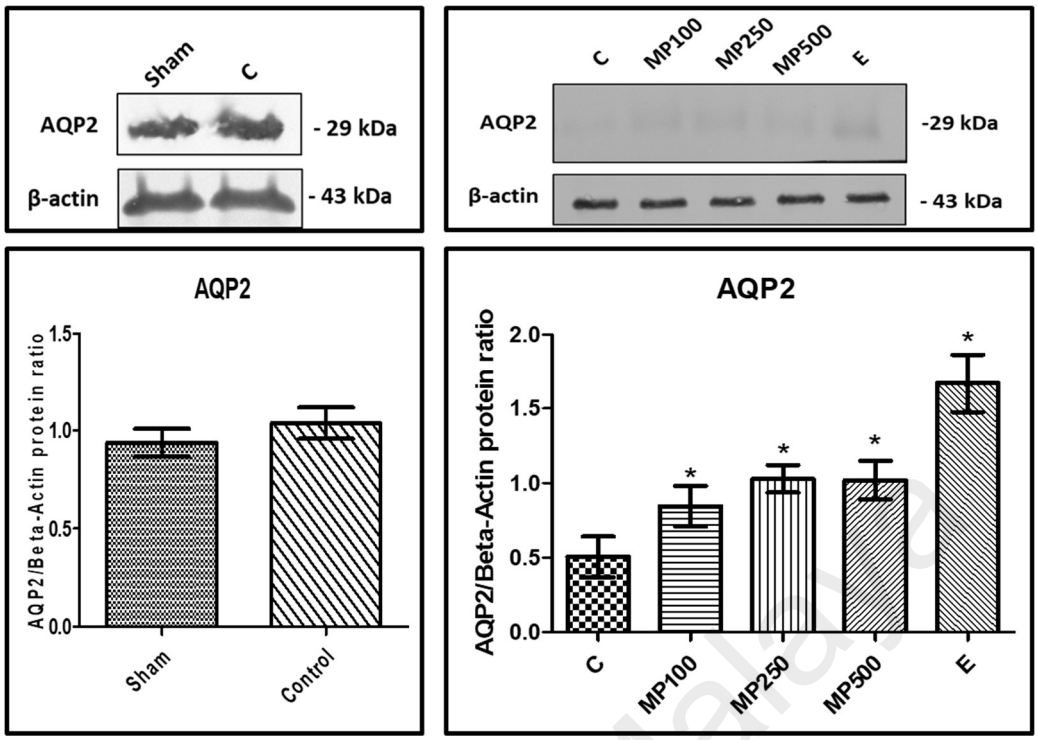

Figure 4.10: Representative Western blot images of AQP-2 with analysis of AQP-2/β-actin ratio in the vagina of sex steroid deficient rats treated with MP vaginal gel.

Higher concentrations of proteins were used to compare between sham and control groups. In bar graph, *p<0.05 as compared to C. Sham (no gel), C = Control (plain gel), MP100 = MP 100 µg/mL/day gel, MP250 = MP 250 µg/mL/day gel, MP500 = MP 500 µg/mL/day gel, E = 0.005% estriol gel.

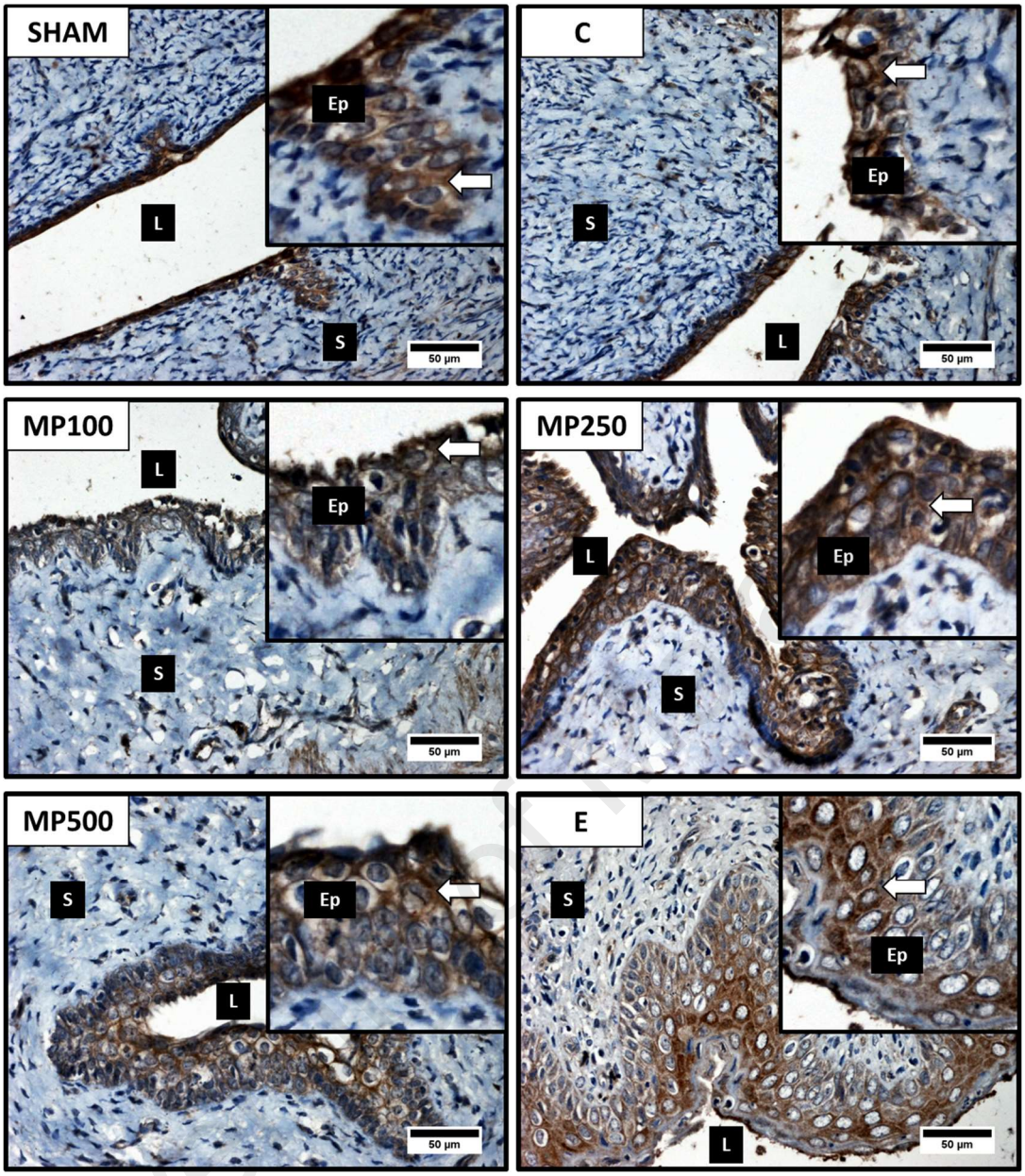

Figure 4.11: Immunohistochemistry images of AQP-2 distribution in the vagina epithelium of sex steroid deficient rats treated with MP vaginal gel were also shown.

Sham (no gel), C = Control (plain gel), MP100 = MP 100 µg/mL/day gel, MP250 = MP 250 µg/mL/day gel, MP500 = MP 500 µg/mL/day gel, E = 0.005% estriol gel, Ep = Epithelium, S = Stroma, L = Lumen. White arrow = AQP-2 expression in the cytoplasm of vagina epithelium. All images were taken in 400 times total magnification. Scale bar = 50µm.

4.5 Expression of V-ATPase A1 proteins in the vagina of sex steroid deficient rats treated with MP.

A significant increase in V-ATPase A1 protein expression level was observed following MP 500 and estrogen treatments which was significantly higher as compared to control. Meanwhile, there was no different in the protein expression level between sham and control groups (Fig. 4.12).

Immunohistochemistry images (Fig. 4.13) showed V-ATPase A1 was distributed exclusively in the vaginal epithelial cells. Distribution levels were relatively higher with increasing doses of MP vaginal gel treatment. High distribution was also observed in estrogen treated rats.

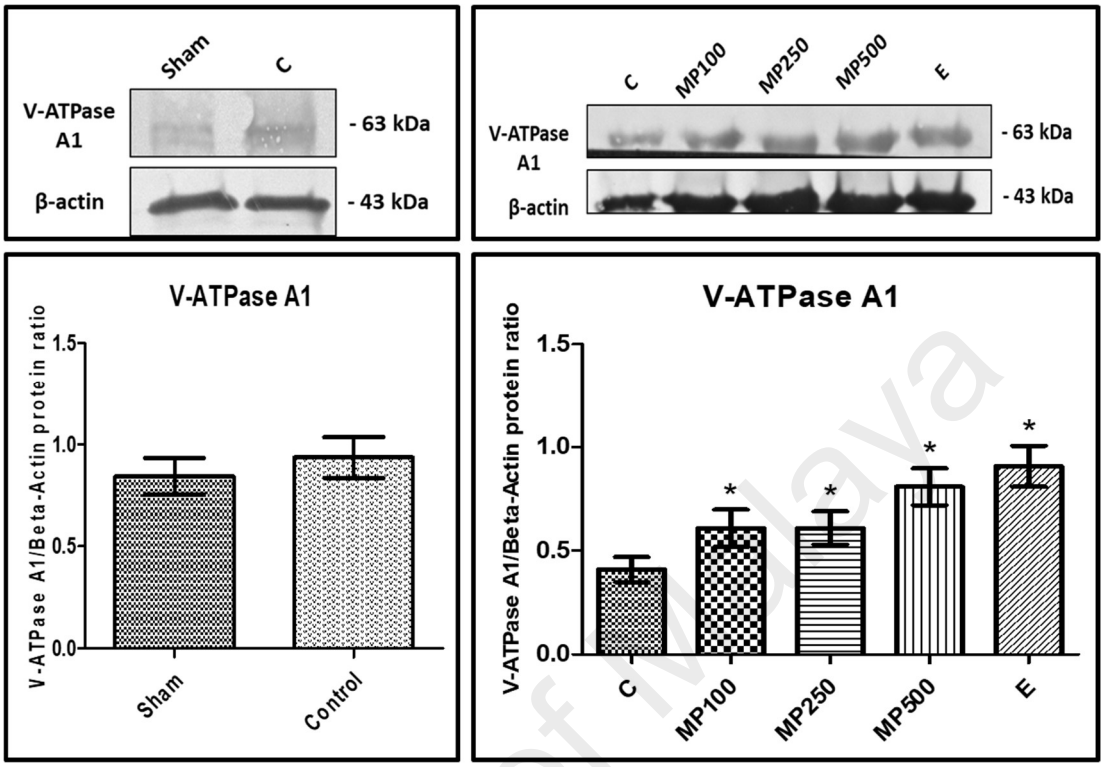

Figure 4.12: Representative Western blot images showing V-ATPase A1 protein and analysis of V-ATPase A1/β-actin protein density ratio in the vagina of sex steroid deficient rats treated with MP vaginal gel.

Higher concentrations of proteins were used to compare between sham and control groups. In bar graph, *$p<0.05$ as compared to C. Sham (no gel), C = Control (plain gel), MP100 = MP 100 µg/mL/day gel, MP250 = MP 250 µg/mL/day gel, MP500 = MP 500 µg/mL/day gel, E = 0.005% estriol gel.

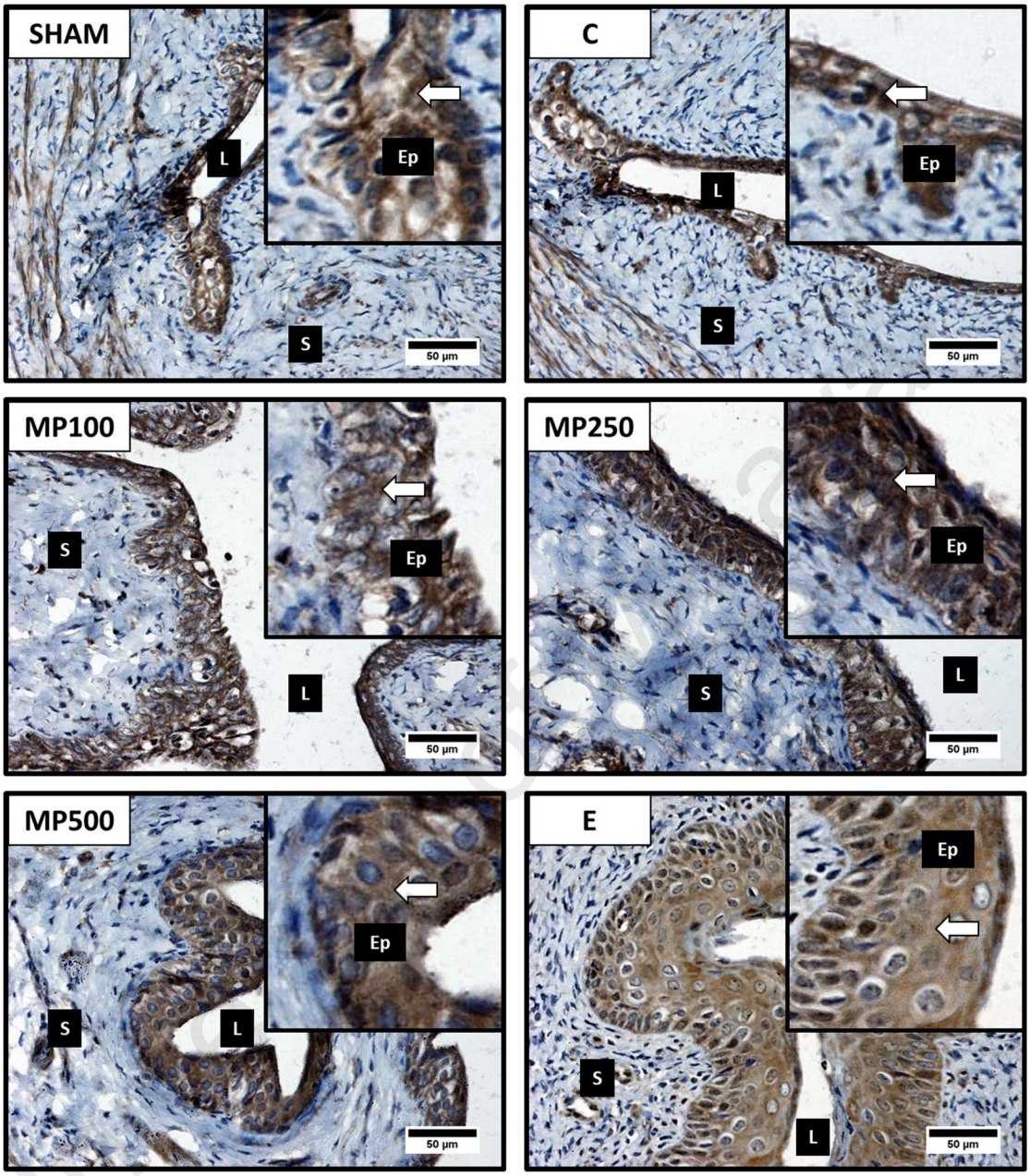

Figure 4.13: Immunohistochemistry images of V-ATPase A1 protein distribution in the vagina epithelium of sex steroid deficient rats treated with MP vaginal gel were also shown.

Sham (no gel), C = Control (plain gel), MP100 = MP 100 µg/mL/day gel, MP250 = MP 250 µg/mL/day gel, MP500 = MP 500 µg/mL/day gel, E = 0.005% estriol gel, Ep = Epithelium, S = Stroma, L = Lumen. White arrow = V-ATPase A1 expression in the cytoplasm of vagina epithelium. All images were taken in 400 times total magnification. Scale bar = 50µm.

CHAPTER 5: DISCUSSION

In this study, ovariectomy was performed to eliminate the endogenous sex steroids in order to create a condition resembling post-menopause that is usually associated with vaginal atrophy. This study has shown that different doses of topical vaginal gel containing MP herbs were found to possess phytoestrogenic effects on the vagina, as their effects resemble that of estradiol. Phytoestrogens are known to exert their actions via binding to the estrogen receptors, similar to the action of estradiol (Poluzzi *et al.*, 2014). In this study, administration of plain gel was used as a negative control while low dose estriol gel was used as a positive control. The latter was used to compare the efficacy of MP herbs gel on the atrophic vagina.

Sham treatment was given which was intended to eliminate the possibility that any changes were caused by the gel as it is made of chemicals that might induce some vaginal changes. We have shown that no differences in the morphology and protein expression levels were noted in the vagina between sham and control groups indicating that the plain gel has no stimulatory effect on vaginal proliferation and growth.

5.1 Effects of MP on vagina epithelia in sex steroid deficient rat model (post-menopausal model)

Estrogen is important for the vaginal epithelium to maintain its physiological functions (Miyagawa & Iguchi, 2015). Studies have shown that estrogen replacement in the post-menopausal women caused increased cellular proliferation of the vaginal epithelium (Pickar *et al.*, 2016). In menopause, the cessation of ovarian function could result in hypoestrogenemic state which is responsible for vaginal atrophy and thinning

of vaginal epithelium (Casper, 2014). This study has shown the increase in thickness of vagina epithelium occur following MP gel administration even with the lowest dose of MP topical vaginal gel (100μg/mL). Without treatment with either MP or estrogen, atrophy of the epithelial layer with only one layer of basal cells was seen in the vagina. These findings indicated that MP possesses strong estrogen-like effects which were consistent with the reports by other studies (Abdul Kadir *et al.*, 2012; Al-Wahaibi *et al.*, 2008; Fathilah *et al.*, 2012; Manneras *et al.*, 2010).

Besides that, there were evidence that MP induces proliferation of the vaginal epithelium. This was indicated by increased expression of the proliferative protein, PCNA in the vagina. MP exerts strong proliferative effects at increasing doses, reaching that of estrogen. PCNA is an important protein involved in repair and replication of cellular DNA. Its expression is used as proliferative cell marker as it remains longer in G1/S phase when the cell proliferates (Bologna-Molina *et al.*, 2013). We found that PCNA was mainly expressed in the nuclei of vagina basal cells, which is known to be mitotically active and responsible to replenish epithelial loss by proliferation (Bragulla & Homberger, 2009). Our findings were supported by recent studies which showed the phytoestrogens' ability to increase PCNA expression in the vagina (Rimoldi *et al.*, 2007; Xu *et al.*, 2015). An increase in proliferation would explain the histological finding whereby vaginal epithelium thickness increased following treatment with MP.

In addition, there were evidence that MP may increase vascular proliferation in the vagina as indicated by increased expression of VEGF in the vaginal tissue. The effects of MP were as strong as estrogen. A recent study has also demonstrated an increase in VEGF expression in the vagina of ovariectomized rats following phytoestrogen treatment (Yin *et al.*, 2013). VEGF is known to be an important factor in angiogenesis which is the formation of new blood vessels from pre-existing vessels (Hoeben *et al.*, 2004). A rise in

vagina epithelial VEGF distribution as observed in this study may explain the reversal of atrophic effect in ovariectomized rats' vagina due to adequate vascularization to the vaginal epithelium.

5.2 Effects of MP in alleviating vaginal dryness in a sex steroid deficient rats model (post-menopausal model).

As vagina has no glands and receives secretions from the cervix and uterus, transudation of fluid from the vaginal walls is important for vaginal lubrication. This could be achieved through the regulation of intercellular connection and intracellular route. Occludin is one of the main protein together with claudin to form the tight junctions. Estrogen regulation on the occludin had demonstrated consistent results. In humans, expression of occludin was found to decrease in vaginal-ectocervical epithelial cells in post-menopausal women and was upregulated following estrogen treatment (Gorodeski, 2007b), suggesting that estrogen is required to increase the transepithelial permeability thus allowing more plasma transudation to pass through the vagina epithelial wall and provides vaginal lubrication.

Similar findings were reported in rats where Oh *et al.* (2016) showed marked reduction in occludin expression in ovariectomized rats and occludin up-regulation upon estrogen replacement. These could explain vaginal dryness and atrophy in menopause and therapeutic approach for such symptoms. Our data has shown a significant increase in occludin expression in the MP500-treated group as well as estrogen replacement group which were consistent with the findings by Oh *et al.* (2016) and others.

Visualization under transmission electron microscope (TEM) had shown an approximation in the intercellular space when MP250 treatment was given with more desmosomes formed. There were also reduced intercellular spaces in MP500 and

estrogen-treated rats' vagina. The increased expression of occludin and desmosomes could contribute towards the paracellular formation in the vagina epithelium following MP and estrogen treatments. Oh *et al.* (2016) pointed the possibility of structural changes in the tight junction proteins despite increased in tight junction expression which could explain the restoration of vaginal lubrication in estrogen replacement in post-menopausal condition.

In addition, this study suggests the possible role of MP in improving vagina epithelial cellular integrity following estrogen deprivation by its ability to increase the tight junction protein expression, reducing intercellular space with more intercellular connections formation. A reduction in intercellular space will prevent intercellular fluid loss, preserving cellular hydration, and thus restoring the vagina lubrication as in healthy vagina epithelial cells.

Besides ultrastructural changes in the vagina, the intracellular mechanism for vaginal lubrication could be contributed by changes in the expression of the water channels. A significant increase in AQP-1 expression was found in MP250 treated groups and estrogen-treated group. The previous study had demonstrated the distribution of AQP-1 in vagina being concentrated in the capillaries and small veins (Kim *et al.*, 2009; Kim *et al.*, 2011; Park *et al.*, 2008). We have shown that AQP-1 was expressed in the blood vessels with a relatively higher distribution following MP and estrogen treatments.

AQP-2 expression was also increased in MP250 and estrogen-treated groups. The expression of AQP-2 was localized to the cytoplasm of vaginal epithelial cells, consistent with previous studies (Kim *et al.*, 2009; Kim *et al.*, 2011). AQPs expression in the vagina has been associated with increased in vaginal secretion (Kim *et al.*, 2009). Thus, there is a possibility that AQPs playing a role in vaginal lubrication via increasing water transport

across the vaginal epithelial cells. Different types of AQPs may have a different role in water transport in the vagina according to their distribution.

5.3 Effects of MP in alleviating changes in vaginal pH in sex steroid deficient rat model (post-menopausal model).

The menopausal condition has been associated with pH elevation in the vagina due to estrogen deficiency (Panda *et al.*, 2014). This condition will increase the susceptibility for pathogen invasion and sexually transmitted diseases as normal vaginal acidity are protective to the vaginal epithelium. Lactic acid production by lactobacilli has been established as the main contributor in vaginal acidity through lactobacilli action on glycogen (Boskey *et al.*, 1999). In addition, recent studies had demonstrated a possible role for proton extruder proteins in regulating vaginal acidity (Gorodeski *et al.*, 2005).

Our data had shown a significant increase in V-ATPase A1 expression in MP250 and MP500 treated groups compared to control group. V-ATPase A1 is mainly distributed in the cytoplasm of the vaginal epithelium. Gorodeski *et al.* (2005) had demonstrated that the luminal acidification in the vagina contributed by apical V-ATPase H^+ secretion and regulated by estrogen. Upon estrogen deprivation, the luminal pH was increased. This condition was reversed upon administration of 17β-estradiol. Bafilomycin A_1, which is a very specific V-ATPase blocker had been shown to alter luminal acidity by increasing the luminal pH thus indicating the role of V-ATPase in the regulation of vaginal acidity (Gorodeski *et al.*, 2005). However, in this study, we were unable to measure the rats' vaginal pH as the vaginal fluid of the animal is very scanty. Thus, the impression for vaginal acidity is made through the expression of V-ATPase proton pump channels, which is known to involve actively in H^+ transport.

CHAPTER 6: CONCLUSION

6.1 Conclusion

In conclusion, our observations indicated that topical MP can be used to reduce vaginal atrophy in the sex steroid deficient state, resembling the effect of estrogen therapy albeit lesser. MP actions are possibly mediated, at least partially through activation of estrogen receptor pathways, though this has not been elucidated in this study. This study has shown the potential role of MP when applied topically as a vaginal gel in alleviating vaginal atrophy in post-menopausal condition via inducing vagina epithelium cellular proliferation, improving vaginal lubrication, and restoring vaginal acidity. These data may provide a better approach in future studies in discovering the potential health benefits of MP herbs as an agent to alleviate vaginal atrophy symptoms, providing an alternative therapy for post-menopausal women, particularly when applied topically as a gel.

6.2 Limitations of the study

In this study, rat models were chosen to imitate post-menopausal condition in women. Although this study provides promising finding, there is lack of certainty of the results to exert the same effect as in the human study. Rat's vagina is very small compared to the human vagina, thus MP effect might be different in various parts of the human vagina.

6.3 Future studies recommendation

As there are no current studies regarding the toxicity levels of local MP intravaginal treatment, future studies could be suggested to investigate this concern. This study had used a very low dose of topical MP gel formulation yet providing promising results in treating vaginal atrophy in the post-menopausal condition in rats. A combination of active compounds activity in MP has been shown to increase vaginal epithelial proliferation and might provide a safer choice than hormonal preparation, although in this study MP has not reach that of low dose estriol formulation effects. Following the safety profile study, MP gel formulation could be refined to suit for human study. Further gel stability and analysis could be done to provide a better formulation. This gel may serve as an excellent, natural alternative for menopausal women in order to avoid the side effects of hormonal gel preparation and hormone replacement therapy (HRT).

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