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Studies on *Labisia pumila* var. *alata* extract with phytoestrogenic effects:

impact on biological activities and gene expression

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To my family with LOVE

ABSTRACT

In Malaysia, *Labisia pumila* var. *alata* (LPva) has been used by women for generations. Traditionally, the plant is boiled, either alone or in decoction with other herbs. It is claimed to have health benefits such as to contract the uterus after childbirth, allay painful menstruation and irregular periods and to generally alleviate fatigue. Therefore, we aimed to investigate the scientific basis of LPva phytoestrogenic activities in different animal models and cell lines.

In **Paper I,** we investigated the effects of a standardized water extract of LPva (10, 20 and 50 mg/kg body weight/day) and compared to estrogen replacement (ERT), on body weight gain, uterus weight, adipose tissue mRNA and protein levels of adipokines in ovariectomized (OVX) female Sprague-Dawley rats. After a month of oral treatment, ERT- and LPva-treated OVX rats showed significantly less weight gain compared to untreated OVX rats. Ovariectomy caused plasma leptin levels to decrease significantly but when treated with LPva or ERT, plasma leptin and mRNA levels increased to levels higher or comparable to that seen in the sham operated control rats (SHAM). In contrast, the elevated plasma resistin concentrations in OVX rats were significantly reduced in rats given ERT and LPva extracts. The uterus to body weight ratio of untreated OVX rats was significantly low compared to SHAM, but showed dose-dependent increase upon treatment with LPva. The study provides evidence that LPva exerts uterotrophic effect and regulates body weight gain.

In **Paper II,** we evaluated the effects of LPva on 11beta-hydroxysteroid dehydrogenase type 1 (*Hsd11b1)* and corticosterone (CORT) expressions in OVX rats. *Hsd11b1* was chosen because it was highly expressed in a microarray analysis of OVX rat liver compared to SHAM. Using samples from **Paper I**, *Hsd11b1* expressions were measured and found that mRNA levels in liver of OVX rats were significantly increased when compared to SHAM and restored to normal level after treatment with LPva or ERT. In adipose tissues, the *Hsd11b1* mRNA level of OVX group was increased by 55 % in comparison to SHAM, normalized in LPva. Protein levels of 11β-HSD1 were down-regulated in both liver and adipose tissue of LPva- and ERT-treated rats, in comparison to OVX rats. CORT levels in OVX group increased significantly compared to SHAM. The results showed that the treatment with LPva normalized *Hsd11b1* mRNA expression and 11β-HSD1 levels in OVX rats, in parallel with decreased CORT levels. Thus, LPva is useful for postmenopausal treatment based upon its regulation at body weight partially via inhibition of *Hsd11b1* expression in adipose tissue and liver.

In **Paper III,** we investigated the effect of LPva on body composition and metabolic features in a rat model of polycystic ovary syndrome (PCOS). LPva (50 mg/kg body weight daily) increased uterine weight (27%) and insulin sensitivity (36%) measured by euglycemic hyperinsulinemic clamp compared to control PCOS rats. Lipid profile was improved in LPva rats and plasma resistin levels were increased. In adipose tissue, LPva decreased leptin mRNA expression but did not affect expression of resistin and adiponectin. No effects on body composition, adipocyte size or plasma leptin levels were observed. Therefore, in this study, LPva increases uterine weight, indicating estrogenic effects, and improves insulin sensitivity and lipid profile in PCOS rats without affecting body composition.

In **Paper IV**, the effects of LPva treatment on urinary tract infection (UTI) were investigated in an infection model using uropathogenic *Escherichia coli* (UPEC) strain CFT073 and the bladder epithelial cell line, T24. Our results demonstrate that LPva treatment induced apoptosis and significantly reduced the number of intracellular *E.coli* in bladder epithelial cells. LPva-induced apoptosis was coupled with up-regulated expression of pro-apoptotic caveolin-1. LPva treatment down-regulated the expression of β1 integrin as indicated by reduced levels of gene specific mRNA. However, LPva did not exhibit direct antimicrobial properties and did not influence antimicrobial peptide levels in cells. These findings suggest that LPva facilitates the exfoliation of infected bladder cells and may thereby mediate beneficial effects during UTI.

Key words: *Labisia pumila*, ovariectomy, PCOS, adipokines, apoptosis

LIST OF PUBLICATIONS

I. **Fazliana Mansor**, Wan Nazaimoon Wan Mohamud, Harvest F. Gu, Claes-Göran Östenson. *Labisia pumila* extract regulates body weight and adipokines in ovariectomized rats.
Maturitas 2009; 62(1): 91-7.

II. **Fazliana Mansor**, Wan Nazaimoon Wan Mohamud, Harvest F. Gu, Claes-Göran Östenson. *Labisia pumila*, a Malaysian plant, down-regulates 11beta-hydroxysteroid dehydrogenase type 1 (*Hsd11b1*) and corticosterone levels in ovariectomized rats.
Manuscript

III. Louise Mannerås *, **Fazliana Mansor***, Wan Nazaimoon Wan Mohamud, Malin Lönn, Harvest F. Gu, Claes-Göran Östenson, Elisabet Stener-Victorin. Beneficial metabolic effects of the Malaysian herb *Labisia pumila* var. *alata* in a rat model of polycystic ovary syndrome.
Journal of Ethnopharmacology 2010; 127(2): 346-51.

IV. **Fazliana Mansor***, Nubia Ramos*, Petra Luthje, Musa Sekikubo, Åsa Holm, Wan Nazaimoon Wan Mohamud, Annelie Brauner. *Labisia pumila* var *alata* reduces bacterial load by inducing uroepithelial cell apoptosis.
Submitted manuscript

* Authors contributed equally to the work

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LIST OF ABBREVIATIONS

11β-HSD1	11beta-hydroxysteroid dehydrogenase type 1 (enzyme)
ANOVA	Analysis of variance
BEC	Bladder epithelial cells
CORT	Corticosterone
DEXA	dual-emission X-ray absorptiometry
DHT	dihydrotestosterone
ELISA	Enzyme-linked immunosorbent assay
ERT	Estrogen replacement
Hsd11b1	11beta-hydroxysteroid dehydrogenase type 1 (gene)
LPva	*Labisia pumila* var. *alata*
OVX	Ovariectomized
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
RIA	Radioimmunoassay
RT-PCR	Reverse Transcription PCR
SHAM	Sham-operated
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary tract infection

Abbreviations used only once are described where they appear in the text

1. BACKGROUND

1.1 Introduction to Malaysian traditional medicine

In contrast to the two well-developed systems of traditional medicine found in Indian Ayurvedic and Chinese traditional medicine, which have been extensively documented over centuries (*Newman 2000*), there is very little documentation for Malaysian traditional medicine. Most literature on medicinal plant use in Malaysia has emerged only in the last century. Books on Malay medicine featuring herbal ingredients were written in the 1880s in traditional Jawi script, thus were available only to educated Malays of the time (*Foster 2009*). An 1886 script by Munshi Ismail was translated by John Dermont Gimlette, an English physician, and IH Burkill, then director of the Singapore Botanic Gardens, as The Medical Book of Malayan Medicine (*Gimlette and Burkill 1930*). Later, A Dictionary of Economic Products of the Malay Peninsula was written by Burkill (1935), a reference that still serves as the most comprehensive work on the Malay herbal, economic and medicinal plants (*Foster 2009*).

1.2 *Labisia pumila* var. *alata* (LPva)

Figure 1. ***Labisia pumila*** (a synonym latin name also known as *L.pothoina*). From Malaysian Herbal Monograph (Volume 1) (1999). Zhari Ismail, Noorhayati Ismail, Jaafar Lassa (contributors). Malaysian Monograph Committee. Kuala Lumpur

Labisia pumila or locally known as Kacip Fatimah in Malaysia, is a very popular herb amongst the Malaysia womenfolk. It is believed to be used for centuries but only documented in the early 20th century. From the family of Myrinaceae, it is a subherbaceous plant and exist in three varieties, namely, *L. pumila* var. *alata* (LPva), *L. pumila* var. *pumila* and *L. pumila* var. *lanceolata* (*Stone 1988*). Each variety commands different uses and thus, it is important to ensure that the right variety is used in each case. Water extract of the plant LPva is traditionally consumed by Malay women as a herbal medicine to treat menstrual irregularities, painful menstruations and to help contracting the birth channel after delivery and also for "sickness in the bones" (*Burkill 1935, Zakaria and Mohd 1994*). Used regularly, it is believed to help promote overall well-being, sexual health and vitality (*Bodeker 2009*). Today, the herb, or formulations with other herbs, is readily available in powders or extracts in pills, capsules, mixtures with coffee or tea, and as a canned beverage (*Foster 2009*). In addition, the family of Myrinaceae has a notable use for the treatment of uterine disorders. It was reported that another Malaysian plant from the same genus, *Ardisia lanceolata*, is being used for postpartum protective remedy (*Wiart 2006*). This possibly explains that similar compounds which exert these effects could be found in LPva too. The LPva extract is used widely as a traditional medicine and warrants further investigation on scientific basis. Although this plant has not been subjected to a detailed pharmacological investigation, some biological activities have recently been documented. The water extract of LPva has been found to inhibit estradiol binding to antibodies raised against estradiol, suggesting the presence of estrogen-like compounds in the extract (*Husniza et al. 2000*). It has been found that LPva increases thickness of endometrial wall, numbers and perimeter of endometrial glands in rats (*Effendy et al. 2004*). *Ayida Al-Wahaibi et al.* (*2008*) reported that treatment with water extract of LPva maintained the elastic lamellae architecture of the ovariectomized (OVX) rat aortae in a manner comparable to that of the normal rats. This implied a possible role for LPva in modulating postmenopausal cardiovascular risks. There is also a possible mechanism for LPva in modulating postmenopause adiposity in a manner similar to that reported for estrogen through the initiation of lipolysis in adipose tissue and with a possible effect on weight management (*Ayida Al-Wahaibi et al. 2007*). In another study, an aqueous extract of the plant was shown to decrease corticosterone levels with no effect on the immune status of pregnant rats (*Pandey et al. 2008*).

1.3 Components in LPva

In leaves, anti-oxidant activities are found more in LPva than in the other variety, *L. pumila* var. *pumila.* Some components discovered in water extracts are flavanoids, ascorbic acid, beta-carotene, anthocyanin and phenolic content (*Norhaiza et al. 2009*). Chemical and spectroscopic methods reveal a multi-class of natural products belonging to phenolic compounds containing long chains, glycerogalactolipid, cerebrosides, alpha-tocopherol, sterols and lipids in the methanolic extract of LPva (*Ali et al. 2009*). LPva also contains triterpene saponins, including the compound ardisiacrispin A, which is reported in *Ardisia crispa* for its utero contracting activities (*Jansakul et al. 1987, Avula et al. 2010*). This possibly explains the phytoestrogenic activity of LPva. A further analysis by Direct Analysis in Real Time (DART™) showed that LPva contains gallic acid, which is a hypoglaecemic agent (*Wan Nazaimoon WM, personal communication, July 2010*). Interestingly, with advanced research, the potential uses are explored even more. LPva markedly inhibited TNF-alpha production and cyclooxygenase-2 expression. Decreased collagen synthesis of human fibroblasts by ultra-violet B was restored back to normal level after treatment with LPva extract since decreased collagen synthesis is one of the major causes of wrinkle formation in the skin (*Fisher et al. 1997*). These results collectively suggest LPva extract has tremendous potential as an anti-photoaging cosmetic ingredient (*Choi et al. 2010*). In a different study, it has been shown that ethyl acetate fraction of LPva has T helper1 upregulating activity and suggests its possible usefulness as a therapeutic agent in immune compromised patients (*Pandey et al. 2010*).

1.4 Dosage and safety

In our studies (**Papers I, II and III**), the rationale for the LPva doses used was based on an earlier study which showed that when given at a dose of 17.5 mg/kg/day, LPva resulted in body weight loss and lipolysis of adipose tissue in OVX Sprague-Dawley rats (*Ayida Al-Wahaibi et al. 2007*). We therefore chose the three concentrations, 10, 20 and 50 mg/kg/body weight, to further evaluate the effects. The concentrations used were also proven to be safe, since 50 mg/kg had no adverse effect (*Singh et al. 2009*). In higher concentrations as used in another study, rats given 100, 700 or 2000 mg/kg rat body weight did not suffer genotoxic effect (*Zaizuhana et al. 2006*). Findings in a study suggested that aqueous extracts

of LPva up to 800 mg/kg/day did not show any significant reproductive toxicity or complication in pregnancy and delivery in rats (*Wan Ezumi et al. 2007*). LPva is generally considered safe for human consumption when used in the form of a traditional decoction. A pilot study on post-menopausal women indicated dosages of up to 560 mg/day as safe for consumption (*Hazlina et al. 2009*). On the other hand, a study by Effendy et al. (*2006*) found that LPva had toxic effects on kidney and liver of rats. However, this study used high doses injected subcutaneously over a short period of time in a petroleum ether extraction, which is a very different preparation and therefore would yield a set of different compounds than those found in traditional and conventional products using water as the extraction solvent. These purely experimental results therefore have no relevance to real-world usage, where solely water decoctions are used in human. However, further investigations on pharmacokinetics and side effects of LPva consumption should be carried out.

1.5 Phytoestrogens

Phytoestrogens belong to a large family of plant derived molecules possessing various degrees of estrogen like activity. The majority of phytoestrogens are introduced into the diet as inactive compounds. After the consumption of plant lignans and isoflavones, a complex enzymatic conversion occurs in the gastrointestinal tract resulting in the formation of compounds with a steroidal structure similar to estrogens (*Albertazzi and Purdie 2002*). Dietary phytoestrogens are being promoted as alternatives to synthetic estrogens for hormone therapy, particularly after a few trials revealing that hormone therapy may induce increased risk of breast cancer (*Beral et al. 2002, Chlebowski et al. 2003*). Phytoestrogens have been associated with a decreased risk of breast cancer (*Adlercreutz 1990, Messina et al. 1994*) and in some studies have been shown to reduce the elevated gonadotropin levels associated with menopause in women (*Wilcox et al. 1990, Cassidy et al. 1994*). Despite their widespread use and increasing acceptance, the data on the estrogenic actions of phytoestrogens in women are confounded by many factors including the different doses used, the presence of endogenous estrogens, ages of the subjects, and the treatment regimes used (*Ososki and Kennelly 2003*).

1.6 Animal models

1.6.2 Menopause and ovariectomized rat model (Papers I and II)

Menopause occurs naturally when the ovary ceases folliculogenesis or artificially by surgical and/or medical ablation of the ovarian function (*Wang et al. 2007*). It is a hypo-estrogenic state, which may adversely affect estrogen target tissues (*Harman 2006*). Menopause, which is characterized by a reduction of estrogen production, is followed by a rise in the incidence of hypertension, a shift in body-fat distribution toward a more central or male-type distribution and an aggravation of the lipoprotein profile, leading to metabolic syndrome and subsequently a rise in the incidence in cardiovascular disease (*Tremollieres et al. 1999, Tchernof and Poehlman 2000, Seed and Knopp 2004, Carr 2003, Kannel and Wilson 1995*). This has led to the assumption that endogenous estrogens may have a protective role in the development of central obesity and the metabolic syndrome. This is supported by the fact that postmenopausal hormone replacement therapy is capable of reducing the effects of menopause on fat redistribution as well as on some of the major cardiovascular risk factors, including the worsening of the lipoprotein profile and blood pressure and a reduction of the incidence of type 2 diabetes in women with known ischemic heart disease (*Kannel and Wilson 1995, Tchernof et al. 2000, Seed and Knopp 2004, Harrison-Bernard and Raij 2000, Kanaya et al. 2003*).

OVX rodents are traditionally used as a model for menopausal women as they mimic estrogen insufficiency and show a progressive increase in body weight (*Chen and Heiman 2001, Clegg et al. 2006, Kalra et al. 1996*). Withdrawal of estrogen with OVX increases food intake and decreases motor activity, a phenomenon which occurs in rodents (*Lindén et al. 1990, Shimomura et al. 2002*) and humans (*Kalra et al. 1996, Wade and Schneider 1992*). Furthermore, in OVX rats, uterine weight is decreased and this effect is attenuated by estrogen administration (*Rachoń et al. 2007*). Therefore, OVX rats are often used to study the pathogenesis of increasing adiposity in human after menopause and have been used as human menopause clinical model to test the function of hormones and various drugs.

In a different aspect, ovariectomy-induced osteopenia in the rat produces skeletal responses similar to that in a post-menopausal woman. In the OVX rat, high bone turnover and subsequent bone loss, like in the human post-menopausal condition, can be prevented by estrogen replacement. Because of the striking resemblance of skeletal responses in humans and rats in the state of estrogen deficiency, the OVX rat is considered to be a gold standard model for evaluating drugs for prevention and reversal of osteoporosis (*Kharode et al. 2008*).

Since the OVX model effectively demonstrated the response of known agents such as conjugated equine estrogen in a fashion similar to that in postmenopausal women (*Engler-Chiurazzi et al. 2009*), the OVX rat model has been an animal model of choice for evaluation of LPva in our study.

1.6.2 Polycystic Ovary Syndrome rat model (Papers III)

Polycystic ovary syndrome (PCOS) is one of the most common endocrine and metabolic disorders, affecting approximately 5–10% of women in reproductive age. It is a complex endocrine and metabolic disorder associated with ovulatory dysfunction, hyperandrogenism, polycystic ovaries, insulin resistance, abdominal fat and obesity (*Ehrmann 2005, Balen et al. 1995, Azziz 2004, Barber 2006, Hirchberg, 2009*).

The etiology of PCOS is unclear. One hypothesis is that PCOS is a genetically determined ovarian disorder in which excessive androgen production early in life may provide a hormonal insult that leads to PCOS in adulthood (*Apter 1998, Crosignani and Nicolosi 2001, Franks et al 2006*). Its management is often unsatisfactory or requires a diversified approach. The heterogeneity of PCOS is reflected by the existence of several animal models. A rat model has been developed that exhibits both ovarian and metabolic disturbances similar to human PCOS in which continuous exposure to nonaromatizable dihydrotestosterone (DHT), starting before puberty, results in polycystic ovarian morphology, adiposity and enlarged adipocytes, as well as insulin resistance (*Mannerås et al. 2007*). Female rats received the nonaromatizable DHT by continuous administration, beginning before puberty, to activate androgen receptors. Adult DHT rats had irregular cycles, polycystic ovaries characterized by cysts formed from atretic follicles and a diminished granulosa layer. They also displayed metabolic features,

including increased body weight, increased body fat and enlarged mesenteric adipocytes, as well as elevated leptin levels and insulin resistance.

In women with PCOS, insulin sensitivity is decreased by 35–40%, independently of obesity, although obesity further exacerbates insulin resistance (*Dunaif and Thomas 2001*), and an association between androgen and insulin levels has been suggested (*Morin-Papunen et al. 2000*). DHT induced insulin resistance and increased abdominal fat mass and adipocyte size, as in women with PCOS and hyperandrogenemia (*Dunaif and Thomas 2001*); both are independently associated with insulin resistance and prediction of type 2 diabetes (*Barber et al. 2006*). In female rats (*Holmäng et al. 1990*), testosterone administration induced insulin resistance, attributed to effects on glucose transport (*Rincon 1996, Holmäng et al. 1992*). Another finding was reduced capillary density in the muscle, despite increased muscle weight (*Rincon et al. 1996*). Because DHT rats became insulin resistant, with increased body and muscle weight, they also might have had reduced capillary density in muscle. In line with *Mannerås et al.* (2007) findings in DHT-exposed rats, elevated androgen levels are partly responsible for insulin resistance in muscle in PCOS women (*Holte 1996*).

As LPva has shown promising results on body weight development, adipokine concentrations, and uterine weight in OVX rats (**Paper I),** the effects of LPva on metabolic features and body composition were studied in the DHT-induced rat model of PCOS (**Paper III**).

1.7 11beta-hydroxysteroid dehydrogenase type 1 (Paper II)

Our focus on the enzyme 11beta-hydroxysteroid dehydrogenase type-1 (11β-HSD1) and *Hsd11b1* gene was based on results of microarray gene expression analysis of liver where the expression was 10-fold increased in OVX as compared to SHAM (**Paper II**). We have chosen liver for microarray analysis because it is one of the most important organs in the body and serves a variety of important functions including metabolic, vascular, immunological, secretory and excretory functions. It plays a key role in the carbohydrate, protein and fat metabolism in the human body (*Mitra and Metcalf 2009*). Meanwhile, for further real-time RT-PCR confirmation, we also used adipose tissue because it is now recognized not only as a storage depot for lipid but also as a regulator of metabolism, through hormones known as adipokines (*Santos and Fonseca 2009*). 11β-HSD1 enzyme is an NADPH-dependent enzyme mainly expressed in key metabolic tissues including liver and adipose tissue (*Lakshmi and Monder 1988, Bujalska et al. 1997*).

In man, 11β-HSD1 reduces inactive cortisone to cortisol that binds to glucocorticoid receptors (*Staab and Maser 2010*) (Figure 2). In rats, dehydrocorticosterone is reduced to the active corticosterone (CORT) (*Jamieson et al. 1995*). Activation of 11β-HSD1 results in the production of excess tissue glucocorticoids. This in turn may affect glucose homeostasis, insulin action and adiposity, all of which are associated with visceral obesity and development of type 2 diabetes (*Masuzaki et al. 2001, Whorwood et al. 2002, Johansson et al. 2001*). In rodents, the role of *Hsd11b1* in the development of obesity and features of the metabolic syndrome is well established (*Masuzaki et al. 2001, Masuzaki and Flier 2003, Kotelevtsev et al. 1997, Morton et al. 2001, Morton and Seckl 2008*). In humans, glucocorticoid excess leads to the development of visceral obesity and other features of the metabolic syndrome (*Friedman et al. 1996, Arnaldi et al. 2003*). In contrast, reduction of *Hsd11b1* expression, by either pharmacological inhibition or targeted gene disruption, prevents regeneration of active glucocorticoids from the inactive 11-keto forms, attenuates intrahepatic glucocorticoid action and increases insulin sensitivity (*Walker and Best 1995, Alberts et al. 2002, Kotelevtsev et al. 1997, Morton 2010*). In **Paper II**, we analyzed the plasma CORT levels and *Hsd11b1* expressions in liver and adipose tissues.

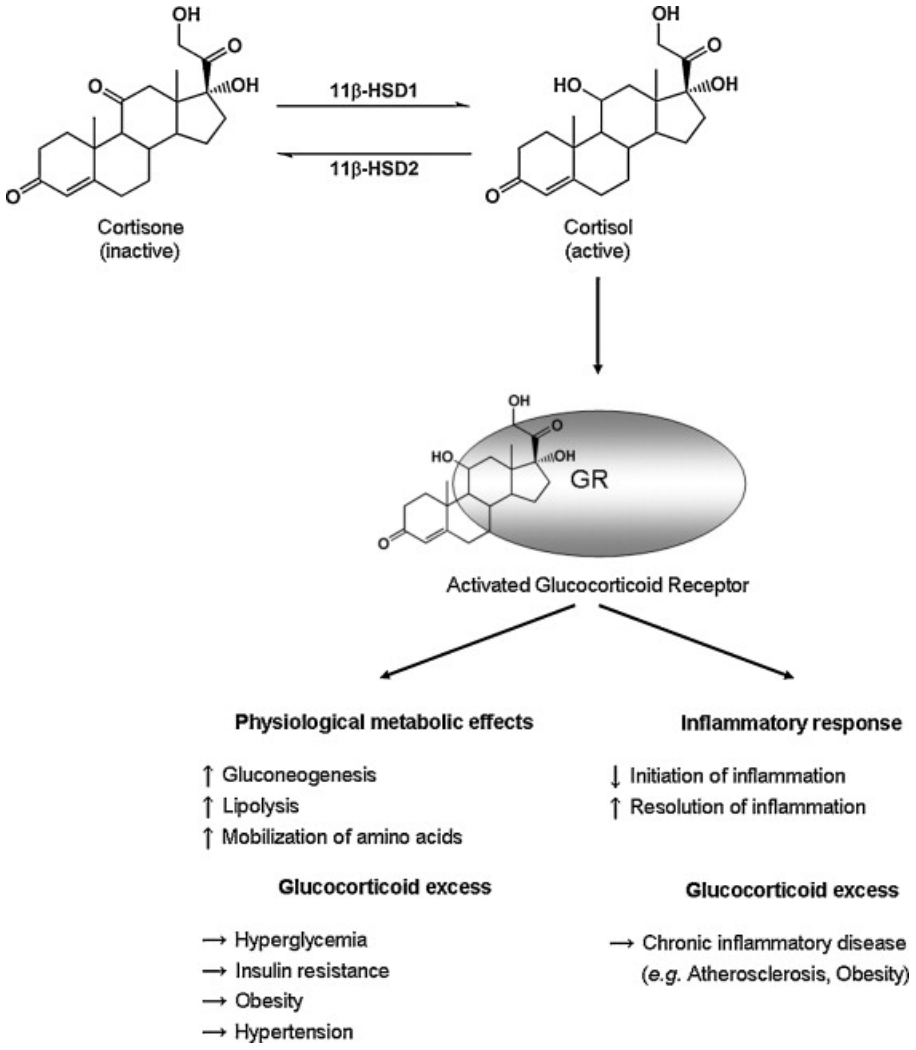

Figure 2. **Interconversion of cortisone and cortisol by the 11β-HSD system and glucocorticoid responses.**

From: Staab CA, Maser E. 11beta-Hydroxysteroid dehydrogenase type 1 is an important regulator at the interface of obesity and inflammation. *J Steroid Biochem Mol Biol. 2010;119(1-2):56-72. Review.*

1.8 Urinary tract infection and LPva

Urinary tract infection (UTI) is a highly prevalent bacterial infection in women (*Foxman 2003*). Up to 50% of women will suffer at least one episode of UTI in their lifetime (*Hooton et al 1996*) with 25% likelihood to suffer recurrence (*Brown 1999, Kunin 1994, Stamm and Hooton 1993, Stapleton 1999*).

Under healthy conditions the urinary tract, except for the external part of urethra, is maintained free from microbial colonization. Yet, the perineum is a region heavily colonized by bacteria originating from the gastrointestinal tract. These bacteria can invade the urinary tract but are mostly cleared from the bladder, mainly by the urine flow, antimicrobial peptides and other factors effective against uropathogenic bacteria (*Chromek et al. 2006*). When bacteria colonize the bladder epithelial cells without strong immunological response, it is described as asymptomatic bacteriuria (*Hooton et al. 2000*). When, on the other hand, bacteria acquire different virulence properties which increase their ability to adapt to new niches they may also cause infections. Although some properties are in common, uropathogenic bacteria causing UTI do not have a single profile (*Snyder et al. 2004, Tullus et al. 1991*).

Uropathogenic *Escherichia coli* (UPEC) is the most common cause of uncomplicated UTI (*Gupta et al. 1999*). To cause UTI, the bacteria use several steps; adhesion and colonization, invasion and survival and host damage. The virulence properties of relevance for the current study are properties enabling adhesion. *E. coli* have several distinct adhesive properties, so called fimbriae or pili (*Mulvey et al. 2002*), which are expressed one at a time, when needed (*Snyder et al. 2005*). In the early stage of UTI, type 1 fimbriae are of importance.

With increasing age, UTI becomes more common. Among postmenopausal women, high frequency of UTI (*Hu et al. 2004*) has been associated with reduced estrogen levels (*Raz & Stamm 1993*). The high incidence of UTI has led to investigation of plant products, such as cranberry, which may help to prevent infection (*Guay 2009*). In light of our previous findings on the phytoestrogenic activity of LPva (*Fazliana et al. 2009*), in **Paper IV** we investigated effects of LPva on the uroepithelium and its interaction with UPEC infection.

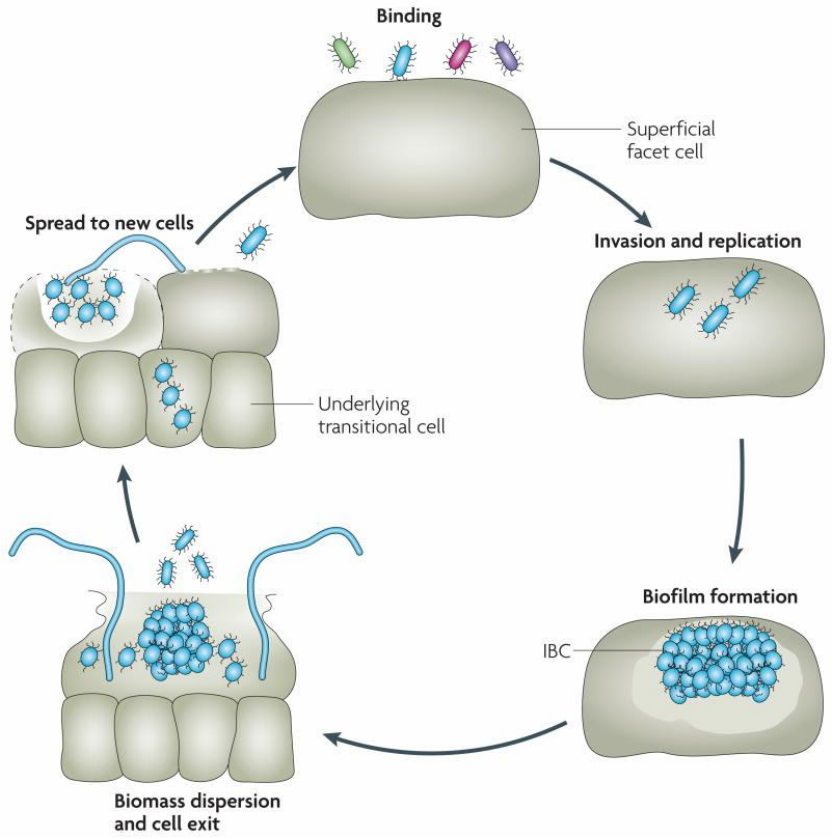

Figure 3: **Multi-step pathogenic cascade of uropathogenic *Escherichia coli* (UPEC)**
UPEC coordinate highly organized temporal and spatial events to colonize the urinary tract. UPEC bind to and invade the superficial facet cells that line the bladder lumen, where they rapidly replicate to form a biofilm-like intracellular bacterial community (IBC). In the IBC, bacteria find safe haven, are resistant to antibiotics and subvert clearance by host innate immune responses. UPEC can persist for months in a quiescent bladder reservoir following acute infection and challenge current antimicrobial therapies. Quiescent bacteria can re-emerge from their protected intracellular niche and be a source of recurrent urinary-tract infections. Insight into the processes that accompany IBC formation and biofilm dispersal, as well as the factors that drive bacteria into the reservoir, may aid the design of preventive or therapeutic strategies for recurrent infections.
From: *Cegelski et al.* The biology and future prospects of antivirulence therapies. Nat Rev Microbiol. 2008;6(1):17-27. Review.

2. AIMS OF THE THESIS

The general aim of the work in this thesis was to identify phytoestrogenic/biological activities by *Labisia pumila* var *alata* (LPva). The specific aims in the individual papers were:

1. To investigate the effects of LPva extracts in relation to estrogen replacement (ERT) on body weight gain, uterine weight, plasma and mRNA expression adipokines such as leptin, adiponectin and resistin, using ovariectomized (OVX) rats as a model.

2. To elucidate the mechanism of action of LPva by evaluating its effects on 11beta-hydroxysteroid dehydrogenase type 1 (*Hsd11b1*) expression with the possible regulatory effects on body weight in the OVX rats.

3. To investigate the effect of LPva on metabolic features and body composition in the dehydrotestosterone (DHT)-induced rat model of polycystic ovary syndrome (PCOS).

4. To determine if LPva mediates protective functions against urinary tract infection (UTI), and investigate the mechanisms of action of LPva in a cell culture model of *E. coli* UTI.

3. MATERIAL AND METHODS

This chapter describes the principles of methods that are central to the work presented in this thesis. For more information about these and the other methods used, please refer to the specific paper.

3.1. Plant material and extraction procedure

Preparation of the standardized extract of LPva was carried out at a Good Manufacturing Practice facility in Malaysia. Briefly, extraction involved simmering the plant material in water at 80°C for 3 hours followed by spray-drying to obtain the final LPva extract. A patent has been applied for the process for preparation of LPva extract to obtain an optimized and reproducible chemical profile by high performance liquid chromatography (USPTO Patent Application - 20070082069).

3.2. Animals and treatment

3.2.1 Papers I and II

We used six-weeks old female Sprague-Dawley rats in studies of **Papers I and II** (Figure 4). The rats were OVX and randomly assigned to different treatments (ERT- estrogen replacement and LPva10, 20 and 50-*Labisia pumila* 10, 20 and 50mg/kg respectively). A control group was sham-operated (SHAM). At the time of surgery, all rats had similar body weight. Treatments were started 2 weeks after OVX. The study protocol was approved by the Animal Care and Use Committee (ACUC), Ministry of Health Malaysia and the Stockholm North Animal Ethics Committee. The ERT dose used in this study was an extrapolation of the human dosage of 0.625 mg/day.

At the end of the experiments, all animals were anaesthesized with pentobarbital and blood was collected, centrifuged, and plasma was stored at -20°C. Liver and adipose tissue were removed and stored in RNA later (detail information in 'tissue collection' section).

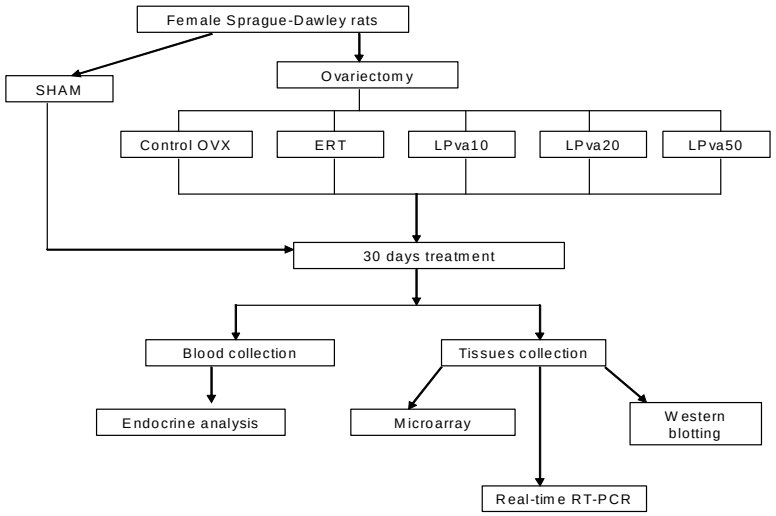

Figure 4. **The schedule of treatment.**

3.2.2 Paper III

At the age of 21 days, the female Wistar rats were implanted subcutaneously in the neck with a 90-d continuous-release pellet (Innovative Research of America, Sarasota, FL) containing 7.5 mg of DHT (daily dose, 83 μg) (Figure 5). A microchip (AVID, Norco, CA) with an identification number was inserted along with the pellets. After 7 wk of DHT exposure, rats were randomized into two groups, PCOS LPva and PCOS control. The PCOS LPva group received extracted LPva orally (50 mg/kg of body weight dissolved in 1 ml of distilled water) daily during 4-5 weeks; the PCOS control group received 1 ml of distilled water. The rats were weighed weekly from the day of pellet implantation to the end of the study. The study was concluded when the rats were 14–15 wk of age (i.e., after 10–11 wk of DHT administration). Animals were cared for in accordance with the principles of the Guide to the Care and Use of Experimental Animals and the study was approved by the Animal Ethics Committee of the University of Gothenburg.

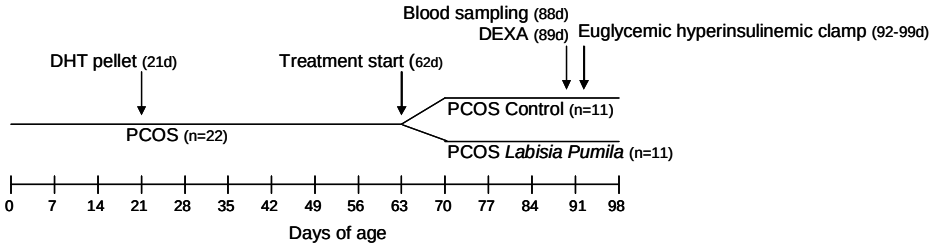

Figure 5. **Schematic diagram of LPva treatment on DHT-induced PCOS rats.**

3.3 Blood sampling, tissues collection and endocrine analysis (Papers I-III)

For **Papers I** and **II**, at the end of animal experiments, the rats were killed and liver and adipose tissues were put into 5 volumes of RNA*later* stabilization solution (Ambion, Austin, TX) for 24 h at 4°C and then stored at –80° C for mRNA analyses. Uterus were dissected and weighed. Blood was collected for plasma analysis of leptin (RIA kit, LINCO Research, Inc., St. Charles, MO, USA), resistin (ELISA kit, *Diagnostic Systems* Laboratories, Webster, TX), adiponectin (ELISA, GenWay Biotech Inc., San Diego, USA) and corticosterone (ELISA kit, *Diagnostic Systems* Laboratories, Webster, TX).

For **Paper III**, tail blood was taken after an overnight fast for analyses of lipid profile (total cholesterol [TC], low-density lipoprotein cholesterol [LDL-C], high-density lipoprotein cholesterol [HDL-C], and triglycerides [TG]), leptin, adiponectin and resistin. Plasma concentrations of TC, LDL-C, HDL-C, and TG were determined enzymatically with a Konelab autoanalyzer 2.0 (Thermo Clinical Labsystems, Espoo, Finland). Enzyme immunoassays were used to determine the levels of leptin (rat leptin ELISA kit, EZRL-83K; Lincon Research, St. Charles, MO), adiponectin (rat adiponectin ELISA kit, CYT288; Chemicon International, Billerica, MA, and resistin (rat resistin ELISA, RD391016200R; BioVendor, Heidelberg, Germany). Human insulin, given during the clamp, was measured with an enzyme immunoassay (insulin ELISA, 10-1113-01; Mercodia, Uppsala, Sweden).

3.4 Body composition (Paper III)

Body composition was analyzed at 14 wk of age by whole-body dual-emission X-ray absorptiometry (DEXA) with a whole-body densitometer (QDR-100/W, Hologic, Waltham, MA). DEXA was originally intended for bone density analysis. However, it has become a widely used non-invasive technique for the investigation of body composition in humans and animals. The technique is based on the physics principle that X-rays of different energies are attenuated differently when passing through the body. The differential attenuation of the two energies is used to estimate the bone mineral content and the soft tissue composition.

3.5 Euglycemic hyperinsulinemic clamp (Paper III)

At 14–15 wk of age (i.e., after 4–5 wk of treatment, 10–11 wk after pellet implantation), rats were subjected to an euglycemic-hyperinsulinemic clamp. It is regarded as a gold standard to quantify insulin sensitivity *in vivo*. This technique measures the steady state amount of glucose metabolized per unit body weight during a whole-body exposure to a pre-determined amount of insulin, while maintaining the amount of plasma glucose within the euglycemic range (*DeFronzo et al. 1979*). The clamp technique is the most widely used method in the research setting and is highly reproducible (*Soop et al 2000*). Plasma insulin concentration is acutely increased and maintained by a continuous infusion of insulin. Blood glucose levels are "clamped" at basal levels (euglycemic levels) by a simultaneous variable glucose infusion. Because endogenous hepatic glucose production is inhibited at high insulin concentrations, the amount of glucose infused per unit of time to maintain euglycemia reflects the amount of glucose metabolized in the peripheral tissues and is therefore an indirect index of the sensitivity of tissue to exogenous insulin. The insulin sensitivity index obtained by the clamp technique is basically the mean glucose infusion rate at steady state and termed glucose infusion rate (GIR) or glucose disposal rate (GDR or M), which is normalized to body weight (mg/kg x min). The degree of insulin resistance is inversely proportional to the glucose uptake by tissues during the procedure. Accordingly, an insulin-resistant subject requires less glucose to maintain euglycemia and has therefore a low GIR/GDR value.

The rats were anesthetized with thiobutabarbital sodium (130 mg/kg i.p.; Inactin, Sigma, St. Louis, MO). Catheters were inserted into the left carotid artery and the right jugular vein. A tracheotomy was performed to facilitate respiration. Body temperature was maintained at 37°C with a heating pad. Insulin (100 IU/ml; Actrapid, Novo Nordisk, Bagsvaerd, Denmark) together with 0.2 ml of albumin and 10 ml of physiological saline was infused at 24, 16, and 12 mU/min/kg for 1, 2, and 3 min respectively, followed by 8 mU/min/kg for the rest of the clamp. Simultaneously, a 20% glucose solution in physiological saline was administered to maintain blood glucose levels at a euglycemic level (6.0 mM). The glucose infusion rate was guided by measuring glucose concentration every 5 min with a B-glucose analyser (Hemocue, Dronfield, Derbyshire, UK). The mean glucose infusion rate, normalized to body weight, was calculated at steady state (after

approximately 50–70 min) as an index of insulin sensitivity. Blood samples were taken at the end of the clamp to determine insulin concentrations.

3.6 RNA extraction and Taqman real-time RT-PCR (Papers I-IV)

One way of analyzing the expression of peptides or proteins is to study the levels of their messenger RNAs (mRNA). Real-time reverse transcriptase (RT)-PCR is thought to be the most sensitive method for the detection and quantification of specific mRNA.

This method is based on the general principle of PCR, by which the target is amplified during the process. However, real-time RT-PCR is a "two-step" method for quantifying mRNA. Firstly, total RNA which is unstable is isolated and reversely transcribed into complementary DNA (cDNA), which is very stable and may be stored for years in the freezer. In the second step, PCR amplifies the cDNA using primers and a probe complementary to the target sequence.

The Taqman probes have a reporter fluorophore in its 5' end and quencher in its 3' end. This assay is based on the fluorescence resonance energy transfer (FRET) which means that when the probe is intact the fluorescence emission of the reporter absorbed by the quencher. As soon as the probe has annealed to a target sequence and *Taq* DNA polymerase cleaves the probe, in the extension phase of the PCR reaction, the reporter and quencher will be separated from each other and fluorescence can be detected and measured (*Arya et al 2005*). This technique continuously monitors the cycle-by cycle accumulation of the fluorescently labeled PCR produced (*Walker 2002*).

Quantification can either be relative to an external standard curve or to one or more co-amplified internal mRNAs. Quantification relative to internal control mRNAs compares the Ct (threshold cycle) values from target RNAs to those of one or more internal reference genes (housekeeping genes) and results are expressed as ratios of the target-specific signal to the internal reference (**Papers I-III**). In **Paper IV**, relative quantification was done according to the comparative Ct ($2^{-\Delta\Delta Ct}$) method. 18S ribosomal RNA (18S rRNA) was used as endogenous control.

Total RNA isolations were performed using TRI reagent (**Paper I**), RNeasy Lipid Tissue Mini Kit (Qiagen) (**Paper II**) and RNeasy Mini Kit (Qiagen) (**Papers II and IV**) and RiboPure Kit (Ambion) (**Paper III**). The tissues were disrupted using mini beadbeater (BiospecProducts, Bartlesville, OK, USA) with 0.5 ml of 2-mm diameter Zirconia beads or (1-mm glass beads) and further processed with the manufacturers' protocol. Total RNA was transcribed into cDNA using Omniscript Reverse Transcriptase (Qiagen) in **Paper I**, Reverse Transcription Reagents (Applied Biosystem, Foster City, CA, USA) in **Papers II and III**, and DyNAmo cDNA Synthesis Kit (Finnzymes, Espoo, Finland) in **Paper IV** with random hexamer primers. Reaction volume was 20µl in all studies. Pre-developed Taqman Gene Expression Assay (Applied Biosystems) containing probe and primers for genes were evaluated in all papers.

3.7 Western blotting (Papers II and IV)

Western blot is a technique to identify and locate proteins with help of specific antibodies. The proteins are analyzed from liver and adipose tissues (**Paper II**) and T24 cells (**Paper IV**) that have been lysed in lysis buffers with protease inhibitors. The proteins in the lysates are separated by molecular mass, on 10 % and 12% Tris-HCl gels using electrophoresis. After that the proteins are blotted on to 0.45 µm PVDF membrane (Invitrolon, PVDF/Filter Paper Sandwich, LC2007, Invitrogen) using an electrical field. The transferred proteins are bound to the surface of the membrane providing access for the reaction with antibodies. The membrane is first blocked with 5% non-fat milk to prevent unspecific antigen interactions. Then the antibody-antigen complexes are identified with horseradish peroxidase (HRP) enzyme conjugated to the secondary anti-IgG antibody (species specific). The HRP-conjugated antibody can easily be detected by a phosphor imager instrument.

Antibodies used were rabbit anti-11β-HSD1 (1:300; Abcam, Cambridge, MA, USA) and HRP-conjugated anti-rabbit antibody (1:50,000; Abcam) for **Paper II**. For **Paper IV**, antibodies used were goat anti-caveolin-1 (1:500; Abd Serotec, Oxford, UK) and HRP-conjugated anti-goat antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

For a loading control, we used rabbit anti-β-tubulin conjugated with HRP at a 1:200 dilution of 200 mg/ml (Abcam) and rabbit anti-GAPDH antibodies (1:1000; Cell

Signaling Technology, Danvers, MA, USA) for **Papers II** and **IV** respectively. Proteins were visualized using a chemiluminescence detection kit, Super Signal West Pico kit (Pierce, Thermo Scientific, Waltham, MA, USA) and imaged using a FujiLas CCD camera. Band intensity was quantified with ImageJ (NIH) and normalized to β-tubulin (**Paper II)** and GAPDH (**Paper IV**) in the same sample.

3.8 Microarray (Paper II)

The target preparation starts with isolating total RNA. Total RNA was prepared from frozen tissue using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) and purified using RNeasy Mini Kits (Qiagen, Valencia, CA, USA). The RNA was reversed transcribed into cDNA by the use of primer that includes a poly(T) tail and a T7 polymerase binding site. Both first strand and second strand cDNA synthesis were performed. The double stranded cDNA was used in *in vitro* transcription (IVT) where biotinylated nucleotides were incorporated into the target, resulting in a biotin-labeld complementary RNA (cRNA) sample. The synthesized labeled cRNA was then hybridized onto Affymetrix Rat genome 230 2.0 array, which were washed and scanned. Output files from microarray analyses were analyzed using Affymetrix GeneChip Operating Software. Scanned data files were analyzed using Partek software (Partek Inc., St Louis, MO, USA). Probe set data were summarized, background adjusted, and quantile normalized using robust multichip average methodology. A false discovery rate correction was applied to post hoc pairwise group comparisons of Pearson's correlation coefficients obtained by ANOVA. Heat map and clustering of the gene expression data were also generated using Partek. $P < 0.05$ was considered significant, with an arbitrary threshold of 3-fold difference between the six groups analyzed simultaneously. After retrieving a list of statistically significant genes, one has to verify the findings with some other methods. In our studies, real-time RT-PCR and Western blotting were used to confirm the results.

3.9 Cell culture, LPva treatment and bacteria (Paper IV)

The T24 human bladder epithelial cell (BEC) line (ATCC HTB-4), obtained from the American Type Culture Collection (Rockville, MD, USA), was used to model the human uroepithelium. T24 cells were maintained in McCoy’s 5A medium with L-glutamine (Gibco, Carlsbad, CA, USA) and supplemented with 10% fetal bovine serum (Gibco). Cells were grown in 6- or 24-well tissue culture plates (Sarstedt Inc,

Newton, NC, USA and Costar, Corning, NY, USA, respectively) and maintained before and during infections at 37°C in a humidified incubator with 5% CO_2. Cells were treated with 100 or 1000 µg/ml LPva, beginning on the day after cells seeding. The LPva concentration used was determined by extrapolation of doses used in previous animal experiments (*Fazliana et al. 2009*). Medium containing LPva was renewed daily and experiments were performed after 72 h of treatment. In all experiments, untreated (control) cells were grown and handled in parallel with LPva-treated cells.

Viability and proliferation of cells treated with LPva was confirmed by XTT assay. Well characterized UPEC strain *E.coli* CFT073 was used. Prior to experiments, UPEC strain CFT073 was cultured in Luria-Bertani (LB) broth with agitation (37°C) to log phase. The culture was centrifuged (2500 x *g*, 10 min) and the pellet resuspended in PBS. The concentration of the suspension was determined by spectrophotometer (OD 600 nm). A final concentration of approximately 1.0×10^7 CFU/ml was used for infection.

3.10 Apoptosis assays (Paper IV)

T24 cells grown on coverslips overnight in 24-well plates were treated with 100 µg/ml or 1000 µg/ml LPva for 6, 24 or 72 h. Staining of cells was performed using the Annexin-V-FLUOS staining kit and the *in situ* cell death detection kit (TUNEL assay) (Roche Applied Science), according to manufacturer instructions. Annexin-V-FLUOS labels phosphatidylserine which is produced in the early stages of apoptosis while the TUNEL assay labels DNA strand breaks generated during apoptosis. Coverslips were mounted onto glass slides using ProLong Gold Antifade Reagent (Invitrogen). Cells were examined with a Leica TCS SP5 confocal microscope at 20x objective and appropriate filter settings.

3.11 Infection assays (Paper IV)

The effect of LPva treatment on UTI was assessed by employing two assays to measure total number of cell-associated bacteria (attachment assay, modified from *Kai-Larsen et al. 2009*) and the number of intracellular bacteria only (invasion assay, modified from *Guignot et al. 2009*). Cells grown in 24-well plates were infected with *E.coli* CFT073 for 30 minutes, then washed three times with PBS to

remove non-adherent bacteria. To collect the total number of bacteria, cells were lysed with 1% Triton X-100 in PBS. The lysate was serially diluted and plated on blood agar plates. Bacterial counts were determined following overnight incubation at 37°C.

To determine the number of intracellular bacteria, fresh medium was added and cells were incubated for a further 90 min to allow bacterial entry into BEC. Cells were washed once with PBS and incubated for 1 h with fresh medium containing 100 µg/ml gentamicin (Sigma-Aldrich) to kill extracellular bacteria. Thereafter, cells were washed with PBS, lysed and treated as above. In treated cells, medium supplemented with LPva was used throughout the entire experiment.

3.12 Statistical analysis

All values reported are expressed as mean±SEM. Differences between means were analyzed for significance by one-way ANOVA, followed by Tukey's post hoc test (**Papers I** and **II**) and Mann-Whitney U test (**Paper III and IV**). $p < 0.05$ was considered significant.

4. Results

4.1 Uterotrophic effects of LPva

Administration of LPva to ovariectomized rats dose-dependently increased uterus-to-body weight ratio, indicating that LPva contains estrogen-like compounds (**Paper I**) (Figure 6). In **Paper III**, the uterus was 27% heavier in the PCOS LPva group than in PCOS controls (Figure 7).

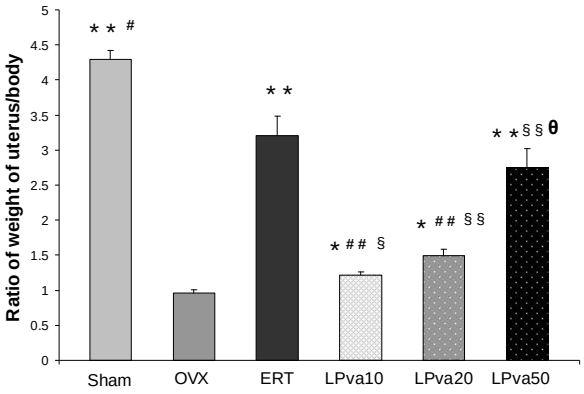

Figure 6. **Uterus to body weight ratio.**
Data are means ± SEM. *p<0.05 vs OVX control, **p<0.01 vs OVX control, # p<0.05 vs ERT, # # p<0.01 vs ERT, § p<0.05 vs Sham, §§ p<0.01 vs Sham and θ p<0.05 vs LPva10.

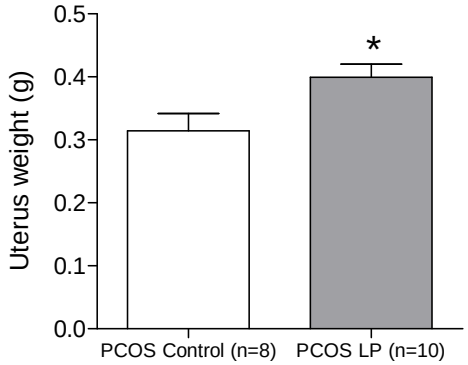

Figure 7. **Uterus weight in PCOS control rats and PCOS-LPva rats.**
Values are mean ± SEM. * $P < 0.05$

4.2 Body weight gain.

OVX rat model (**Paper I**) showed a dose-dependent trend with increasing concentrations of LPva towards a decrease in body weight gain that was significant ($P < 0.05$) at a dose of 50 mg/kg/day (Figure 8). However, LPva had no effect on body weight in the PCOS model (**Paper III**).

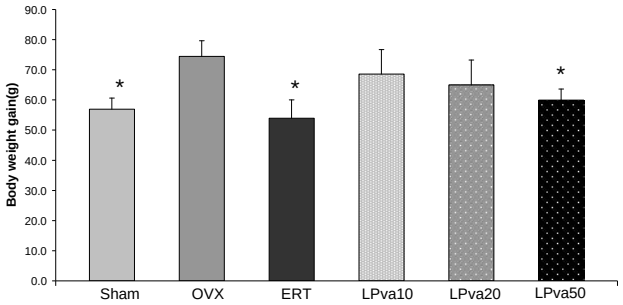

Figure 8. **Body weight gain.** *p<0.05 vs OVX control.

4.3 Food intake

Compared to the SHAM group, OVX rats showed highest daily food consumption at the end of the study (Figure 9). At week-6, mean food intake was lowest amongst the ERT rats (p<0.05). Although LPva50 showed significant difference compared to OVX, there was no significant difference in food intake amongst the LPva-treated groups.

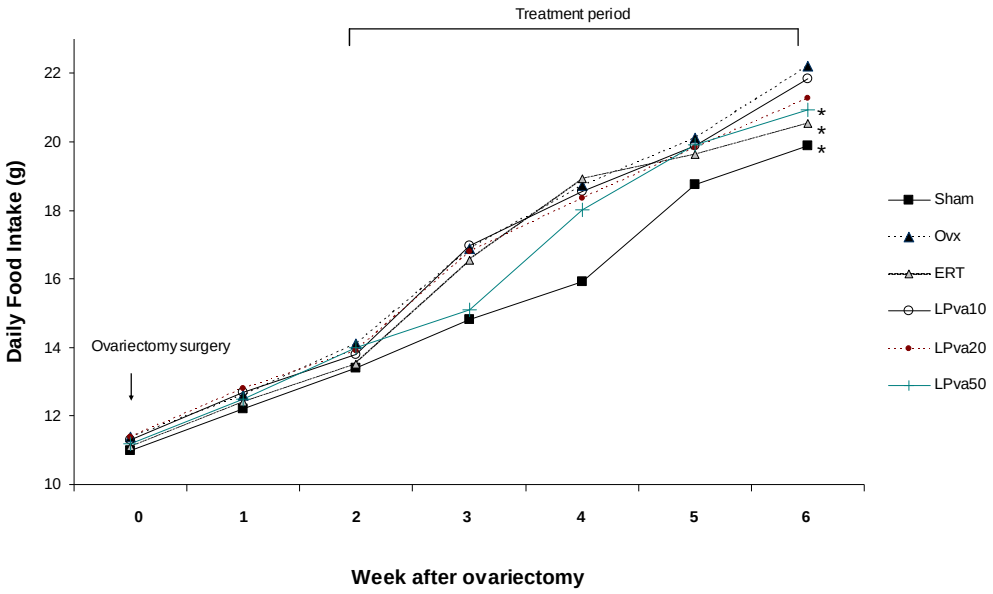

Figure 9. **Daily food intake.** *p<0.05 vs OVX control

4.4 Glucose and insulin in plasma and insulin sensitivity

In **Paper I**, fasting plasma insulin levels were significantly higher in the ERT-treated and LPva50 rats compared to the OVX control group. However, there was no significant change in the levels of plasma glucose. Interestingly, in the PCOS model (**Paper III)**, the glucose infusion rate, determined by a euglycemic-hyperinsulinemic clamp, was 35% higher in LPva-treated rats than in controls, indicating improved insulin sensitivity (Figure 10).

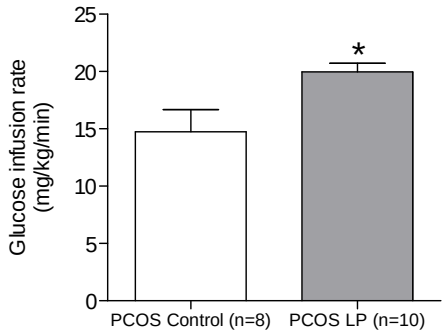

Figure 10. **Glucose infusion rate,** at steady state, required to maintain euglycemia during euglycemic-hyperinsulinemic clamp in PCOS control rats and PCOS LPva rats. Values are mean ± SEM. * $P < 0.05$

4.5 Adipokines expressions

Ovariectomy decreased leptin mRNA levels, and LPva treatment but not ERT significantly increased the levels, the highest being in the LPva10 group ($p < 0.01$ vs OVX, SHAM, ERT). Similar trend was also observed for plasma leptin. The low leptin levels in OVX control were restored when OVX rats received ERT or LPva extracts ($p < 0.05$) (Figure 11).

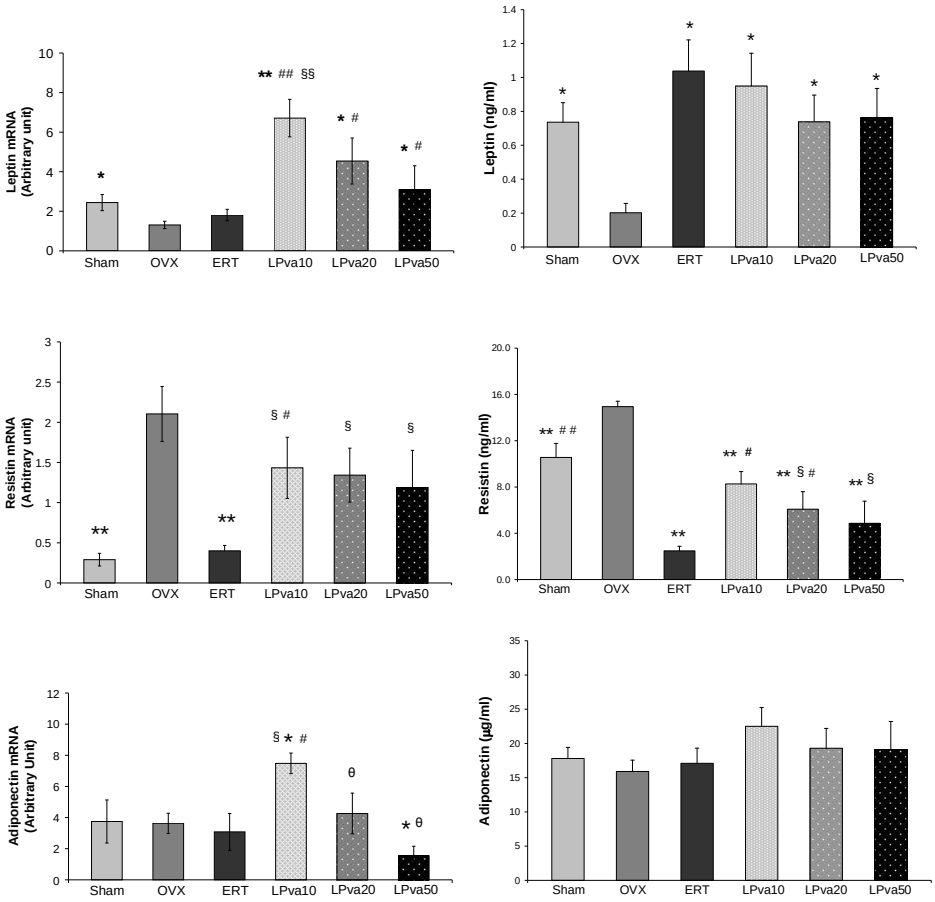

Figure 11. **Expressions of leptin, resistin and adiponectin at mRNA (left column) and protein levels (right column).** * p<0.05 vs OVX control, ** p<0.01 vs OVX control, # p<0.05 vs ERT, ## p<0.01 vs ERT, § p<0.05 vs Sham, §§ p<0.01 vs Sham and θ p<0.05 vs LP10.

The plasma and mRNA levels of resistin were increased after ovariectomy and decreased remarkably when rats were treated with ERT ($p < 0.01$). Although not significant, reduction of mRNA levels in the adipose tissue was also observed in the OVX rats treated with LPva. Compared to the SHAM group, plasma resistin levels were significantly lower in LPva20, LPva50-treated and ERT-treated rats ($p < 0.05$ for all).

Ovariectomy had no significant effect on the protein and mRNA expression levels of adiponectin. Plasma adiponectin levels in SHAM, OVX control, ERT and LPva groups remained comparable. However, compared to the SHAM, OVX

control and ERT, mRNA expression levels of adiponectin were elevated in LPva10 group ($p < 0.05$).

In **Paper III**, resistin levels were higher in the LPva group than in PCOS-controls (Table 1). Circulating adiponectin levels trended higher after treatment with LPva, but the difference was not statistically significant. Circulating leptin levels were unaffected by treatment, despite decreased the expression of leptin mRNA in mesenteric adipose tissue (Figure 12).

Adipokine	**PCOS control (*n* = 10–11)**	**PCOS LPva (*n* = 11)**	**Mann–Whitney *P* value**
Resistin (ng/ml)	20.72 ± 1.54	25.32 ± 0.92	*0.023
Adiponectin (µg/ml)	18.26 ± 2.02	24.02 ± 2.00	0.071
Leptin (ng/ml)	6.05 ± 1.14	4.52 ± 0.75	0.360

Table 1: **Circulating levels of adipokines.** Values are mean ± SEM.

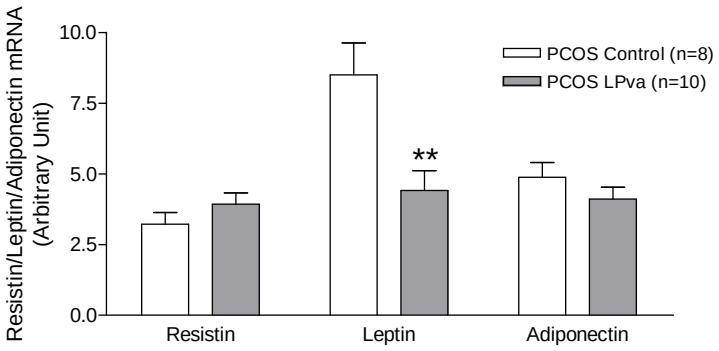

Figure 12. **Relative mRNA expression levels of resistin, adiponectin and leptin** in adipose tissue of PCOS control rats and PCOS LPva rats. Values are mean ± SEM. ** $P < 0.01$

4.6 Lipid profiles

After treatment with LPva, PCOS rats displayed an improved lipid profile, illustrated by decreased levels of TC and TG. HDL-C and LDL-C levels were similar in PCOS LPva rats and PCOS controls (Figure 13).

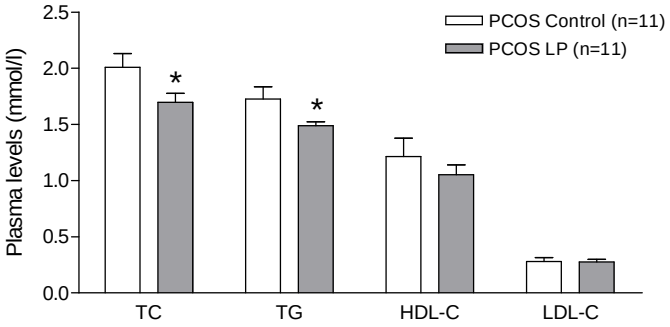

Figure 13. **Lipid profile in PCOS LPva rats and PCOS controls.**
Values are mean±SEM. *P < 0.05 vs. control (Mann–Whitney U test).

4.7 Hsd11b1

4.7.1 Real-time RT-PCR analysis

The microarray analysis of liver tissues revealed that *Hsd11b1* expression was 10-fold increased in OVX compared to SHAM. All three treatment doses of LPva reduced the *Hsd11b1* expression more than three times as compared to OVX, the expressions however being 2 to 3 fold increased as compared to SHAM (Table 2**).** These reductions were not as big as noted after treatment by ERT. To confirm the microarray data, we carried out real-time RT-PCR on mRNA from liver and adipose tissue. Even though the trends seemed similar in both tissues, the *Hsd11b1* mRNA expression in OVX increased more in the liver than in adipose tissue (Figure 14A and 14B**).** This increase was normalized in both liver and adipose tissue by treatment with all doses of LPva (LPva10, LPva20, LPva50), while ERT normalized the expression only in liver.

Groups	Fold-change vs SHAM
OVX	10.42
ERT	1.43
LPva10	2.98
LPva20	2.62
LPva50	2.39

Table 2. **Microarray results of *Hsd11b1* gene**

4.7.2 Protein levels by Western blot and corticosterone

In parallel, the 11β-HSD1 protein levels, as observed in Western blotting analysis, increased in both tissues in OVX rats, and were normalized after treatment with LPva or ERT (Figure 14C and 14D). OVX significantly increased plasma CORT levels compared to SHAM group. All doses of LPva and ERT reduced the plasma CORT levels (Table 3).

Groups	Corticosterone ng/ml
SHAM	53 ± 18
OVX	135 ± 11*$^{\#\,\S}$
ERT	88 ± 22
LPva 10	85 ± 27
LPva 20	95 ± 23
LPva 50	92 ± 16

Table 3. **Plasma corticosterone levels were measured by ELISA.** *$p < 0.05$ vs SHAM, #$p<0.05$ vs ERT, § $p < 0.05$ vs. all LPva10, LPva20 and LPva50.

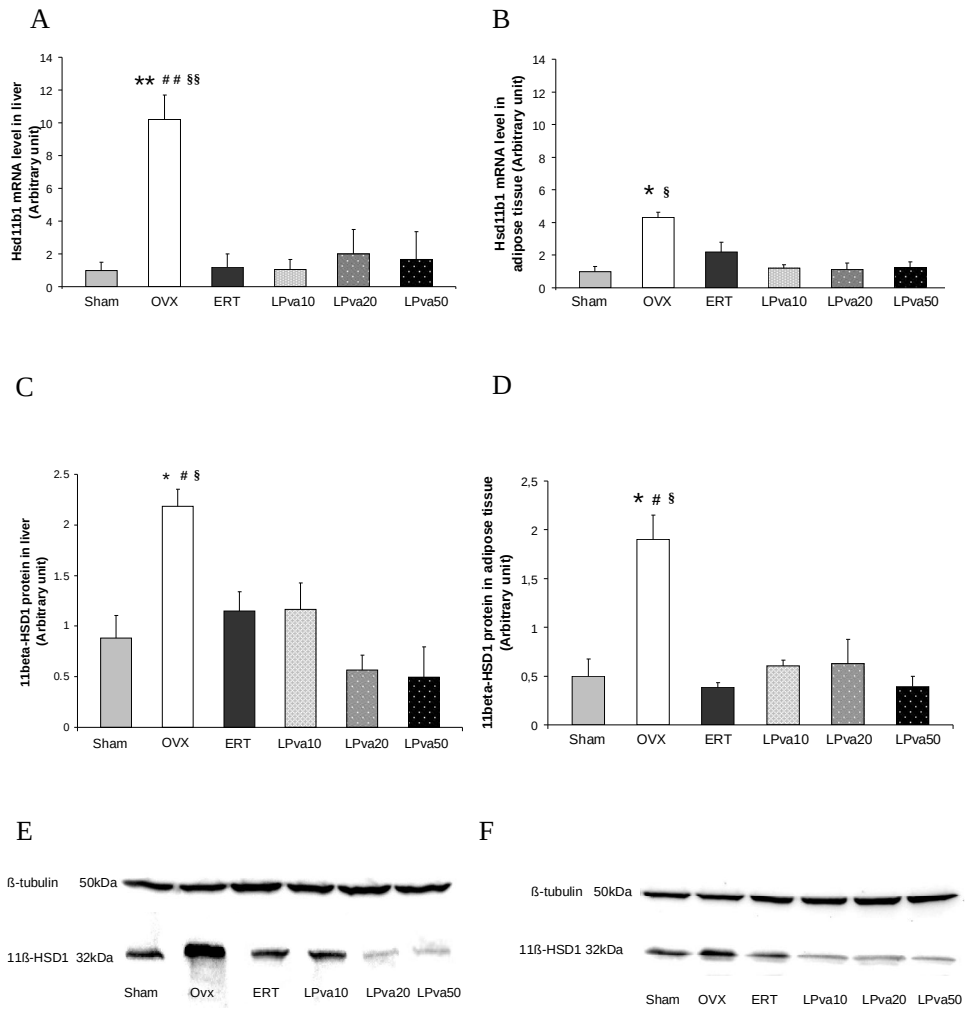

Figure 14. ***Hsd11b1*** **mRNA expression levels** in liver **(A)** and adipose tissue **(B). 11ß-HSD1 protein levels** in liver **(C)** and adipose tissues **(D)**. **E** and **F,** representative Western blots for the 11ß-HSD1 proteins for liver and adipose tissues, respectively.

4.8 UPEC and LPva

4.8.1 LPva induces apoptosis in bladder epithelial cells.

We investigated the effect of LPva on epithelial cells and demonstrate that LPva treatment induces programmed cell death of BEC. Apoptosis was seen at both 6 and 24 h using the Annexin-V-FLUOS assay, suggesting that LPva had the potential to induce cell death (Figure 15).

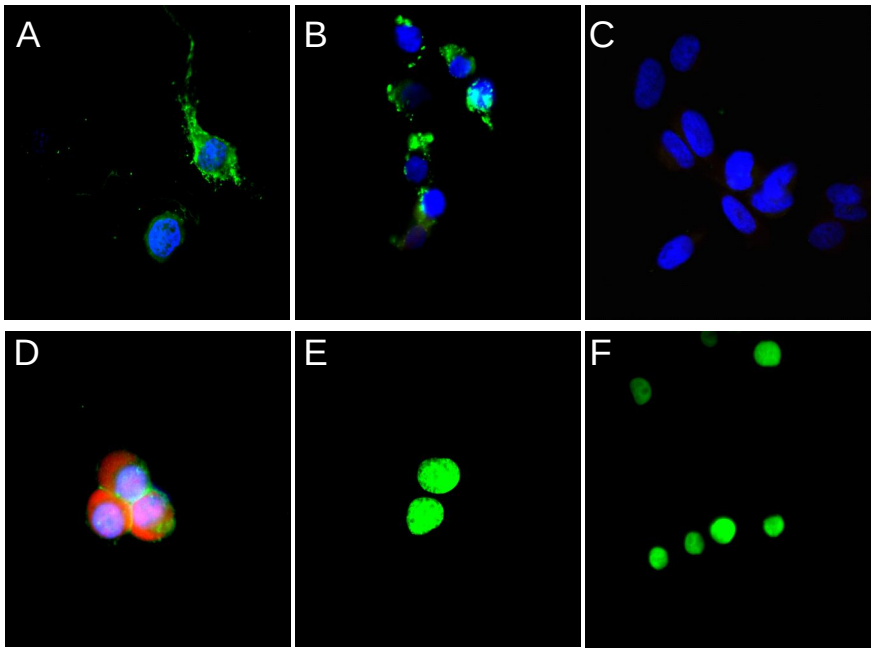

Figure 15. **Apoptosis induced by LPva treatment in T24 bladder epithelial cells.** The Annexin assay revealed apoptosis in 100 µg/ml and 1000 µg/ml LPva-treated cells. **(A)** Apoptosis (stained **green** with Annexin-V) was seen among non-infected 24 h 100 µg/ml LPva-treated cells and **(B)** following UPEC CFT073 infection. **(C)** No apoptosis was seen in untreated control cells however, **(D)** necrotic cells (stained **red** with propidium iodide) were detected following UPEC infection. All cells were stained with DAPI (**blue**) to detect the cell nucleus. Images shown are those following 24 h of treatment. Similar results were seen after 6 h. In the TUNEL assay, apoptosis (stained green with TUNEL reaction) was observed in all 6 h **(E)** and 24 h **(F)** 1000 µg/ml LPva-treated cells.

4.8.2 LPva treatment increases expression of caveolin-1.

Due to our finding that LPva treatment induces apoptosis in T24 cells, we next explored the expression of caveolin-1 which has been associated with this process (*Liu et al 2001, Gargalovic and Dory 2003*). A significant upregulation of caveolin-1 was found in LPva-treated cells compared to untreated cells, on both the mRNA and protein levels (Figure 16).

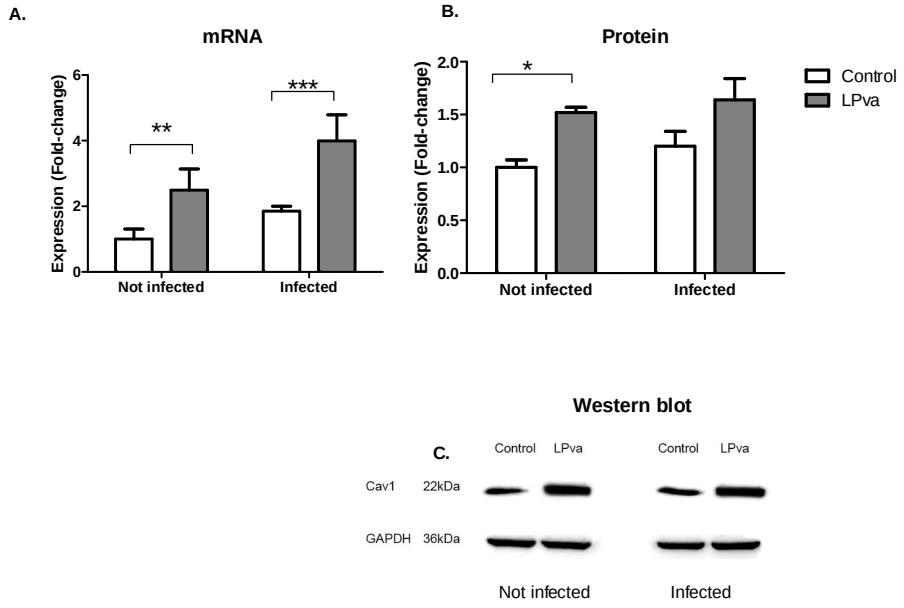

Figure 16. **The mRNA and protein levels of caveolin-1 in LPva-treated T24 bladder epithelial cells with and without *E. coli* CFT073 infection. (A)** Expression of caveolin-1 mRNA was significantly up-regulated in 6 h 100 µg/ml LPva-treated non-infected cells compared to untreated controls (***P* = 0.01). An upregulation was also observed among treated cells following *E. coli* CFT073 infection compared to untreated cells (4 independent assays with 3 replicates) (****P* = 0.001). **(B)** In uninfected 72 h LPva-treated cells, caveolin-1 protein was expressed significantly compared to untreated cells (3 independent experiments) (**P* < 0.05). Blots were stripped and reprobed with antibodies against GAPDH to correct for differences in protein loading. Quantification of Western blot analyses was performed using ImageJ Software. All values shown are means ± standard error of the mean. (**C)** Representative Western blots for the caveolin-1 protein expressed in untreated or LPva-treated cells with and without UPEC infection.

4.8.3 LPva does not exhibit antimicrobial activity.

To elucidate the possible antimicrobial effect of LPva, sensitivity assays were performed. No detectable zone of inhibition was induced by LPva, indicating no direct inhibitory activity. Furthermore, mRNA expressions of the antimicrobial peptides LL-37 and HBD-2 were not upregulated compared to untreated cells suggesting that LPva did not influence BEC antimicrobial responses.

4.8.4 LPva reduces the amount of intracellular UPEC.

The number of intracellular bacteria was significantly reduced in LPva-treated cells (P = 0.01) (Figure 17A). However, this was not due to interference of FimH on type 1 fimbriae nor to an overall decreased bacterial attachment (Figure 17B). Instead, we demonstrate that the expression of the invasion mediating protein, β-1 integrin decreased significantly.

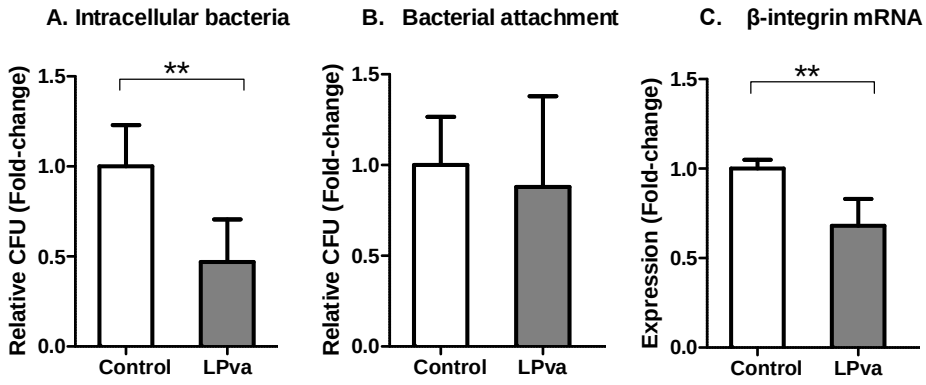

Figure 17. **Level of attachment and invasion by UPEC CFT073 in LPva-treated BECs.**
(A) Treatment was associated with a significant decrease in the level of intracellular bacteria (3 independent assays with 6 replicates) (**P = 0.01) **(B)** However, LPva treatment did not affect attachment. Values shown are means ± standard error of the mean. **(C)** β1 integrin mRNA expression was significantly down-regulated in LPva-treated cells compared to controls (4 independent assays with 3 replicates) (**P = 0.01). Values shown are means ± standard error.

5. Discussion

In our studies, we have found various effects by LPva in different animal models and cell lines. Even though LPva extract did not bind to estrogen receptor-alpha and -beta in the receptor binding assays (data not shown), it has been shown to mimic estrogenic effects to certain extent. These findings indicated that the components of LPva induced estrogenic effects through targets and signaling pathways other than those of estrogen; for example, by promoting adrenal gland secretion or local biosynthesis at peripheral tissues (*Ma et al. 2010*).

5.1 Uterotrophic effects and body weight changes by LPva

In OVX rats, the lack of estrogen causes atrophy of the uterus. Therefore, as found in our study, OVX resulted in a dramatic decrease in the uterine weight (*Rachon´ et al. 2007, García-Pérez et al. 2006*). LPva significantly recovered the weight of the uterus atrophied by OVX (Figure 6), indicating that LPva exerts estrogen-like effects.

As expected and as have been reported by others, ERT administration decreased body weight gain in the OVX rats (*Shinoda et al. 2002, Tanaka et al. 2001, Sivan et al. 1998, Alonso et al. 2007, Gorzek et al. 2007*). Interestingly, LPva exerted similar effect and at doses ranging between 10 to 50 mg/kg/day prevented further increase in body weight gain in the control OVX rats. However, no change in body weight by LPva treatment was observed in the PCOS rats model. Effects of LPva differ in some parts from findings in the **Papers I** and **III**. The main reason for this is most likely the use of two animal models displaying different endocrine and metabolic characteristics. Despite these differences regarding effects on body weight, LPva exerted an uterotrophic effect in both models.

5.2 Insulin sensitivity and adipokines

In the OVX study (**Paper I**), plasma glucose levels did not differ between the groups, but plasma insulin concentrations were elevated in the ERT and LPva50 groups, indicating decreased insulin sensitivity. Treatment of OVX rats with 17-β estradiol has been shown to decrease the amount of peripheral insulin receptors and lead to reduce insulin sensitivity (*Gonzales et al. 2002*). These effects were dose-dependent and a high dose of 17-β estradiol was related to the down-regulation of

insulin receptor substrate-1 (IRS-1) expression (*Gonzales et al. 2003*). These effects indicate the presence of phytoestrogen in the extract.

In concert with a previous study (*Yoneda et al 1998*), we found inverse correlation between adipose tissues leptin mRNA expression and body weight gain in the OVX rat model. In fact, as body weight increased following ovariectomy, leptin declined significantly to levels less than 50% of those seen in the SHAM rats. Our results are thus in agreement with several other studies where ovariectomy was shown to result in decreased plasma leptin levels in the experimental rats studies (*Chu et al. 1999, Yoneda et al. 1998, Brann et al. 1999*). Decrease in leptin has been suggested to result in reduced control of appetite and the lower amount of energy spent, and hence, increase in body weight in the OVX rats (*Kimura et al. 2002*). The upregulation of leptin mRNA expression in the adipocyte tissues and the higher plasma leptin levels seen in OVX rats treated with LPva is at least in part, due to the presence of phytoestrogens in the extract.

Leptin mRNA levels were restored in the estradiol-treated rats, comparable to that observed in the SHAM rats, suggesting the involvement of ovarian steroids in the regulation of leptin gene (*Brann et al. 1999*). Estrogen also caused increased leptin mRNA levels in cultured adipocytes *in vitro* (*Kristensen et al. 1999*) while in another study, OVX induced a 25% decrease in leptin gene mRNA expression in fat cells (*Machinal et al. 1999*). Compared to ERT, LPva induced significantly higher leptin mRNA levels in the adipose tissue of the treated rats.

Resistin is a signaling molecule that is induced during adipogenesis and secreted by the adipocytes (*Steppan et al. 2001*). The molecule is proposed to be involved in insulin resistance and type 2 diabetes. Our results indicate that resistin levels of mRNA and in plasma are increased in OVX rats and reduced when rats were given ERT and LPva. These results clearly showed that resistin protein and mRNA expression was suppressed by ERT and LPva. Huang et al. (2005) showed that mRNA levels were down-regulated by estrogen both *in vivo* and *in vitro*.

In **Paper III**, the plasma resistin levels were increased in the PCOS LPva group. Since LPva improved insulin sensitivity without influencing body weight or adipocity, the increase in plasma resistin levels appeared to lack clinical effect in

this context, or was counter-acted by other factors regulating insulin sensitivity. Furthermore, insulin resistance in PCOS appears independent of obesity, since even lean women with PCOS are more insulin resistant than weight-matched controls (*Dunaif et al. 1989*).

If LPva works through estrogenic pathways, this is consistent with *in vitro* studies of adipocytes showing that 17β-estradiol stimulates resistin gene expression (*Chen et al. 2006, Lee et al. 2008*). Despite increased plasma levels of resistin in LPva-treated rats in the present study, mRNA expression was not significantly different between the two groups. A possible explanation is that resistin expression is affected by LPva differentially in different adipose depots or other cell types (*Steppan and Lazar 2004*).

Adiponectin is an adipocyte-derived plasma protein with important insulin-sensitizing properties. In contrast to other adipokines e.g. leptin, reduced levels of adiponectin are associated with metabolic abnormalities such as insulin resistance and obesity (*Weyer et al. 2001*). In **Paper III,** there was a tendency toward higher circulating levels of adiponectin after treatment in LPva-treated rats than in controls, despite similar body weight and body composition. This finding supports the theory that LPva has positive metabolic effects in our PCOS rat model. However, adiponectin mRNA expression levels were similar in the two groups. In **Paper I**, ovariectomy has no effect on the plasma adiponectin levels and there was no significant difference between OVX control and other treated-groups (*Nishizawa et al. 2002*).

No difference was found in circulating leptin levels between PCOS LPva and PCOS control rats, probably because of the lack of change in body fat after LPva exposure. In contrast, leptin expression in mesenteric adipose tissue of LPva-treated group was decreased. One possible explanation for this discrepancy is that LPva directly affects leptin mRNA expression in mesenteric adipose tissue but not in other adipose depots.

5.3 *Hsd11b1* and corticosterone

Our focus on *Hsd11b1* is based upon microarray analysis of liver tissues where the expression of this gene was 10-fold augmented in OVX compared to SHAM. Up-regulation of *Hsd11b1* expression in OVX rats has been suggested to be related to overweight rather than lack of estrogen (*Paulsen et al. 2008*). However, we have demonstrated in **Paper I** that LPva and ERT exerted similar effects in reducing the body weight gain as seen in OVX rats (*Fazliana et al. 2009*). This may, at least in part, explain the effect of LPva and ERT on regulation of *Hsd11b1*.

In rats, 11β-HSD1 converts 11-dehydrocorticosterone to active CORT. Our finding that OVX induces a rise in CORT production is in agreement with other studies showing that ovariectomy induced rise in serum CORT production in rats (*Petersson et al. 2005, Cao et al. 2008, Kour et al. 2010, Pelleymounter et al. 1999*). The CORT levels were normalized when the OVX rats were treated with LPva and ERT, suggesting a reduction of 11β-HSD1 protein and *Hsd11b1* mRNA expression levels by a direct effect. We also observed positive relations between mRNA expression of *Hsd11b1* in liver and adipose tissues and circulating levels of CORT. Increased 11β-HSD1 activity in adipose tissues and liver might contribute to the several features of metabolic syndrome (*Anagnostis et al. 2009*). The mRNA expressions in both liver and adipose were increased in the OVX group but significantly reduced by LPva treatment. It is proven that 17-beta estradiol inhibits 11β-HSD1 activity in rodent adipocytes. (*Tagawa et al. 2009*). Our result is concordant with Jamieson et al (1999) which showed that estrogen suppresses hepatic *Hsd11b1* expression. In another study, *Blum et al.* (*2009*) explained that the inhibition of *Hsd11b1* counter-acted fat accumulation and related metabolic abnormalities in type 2 diabetes. The finding that ERT and LPva normalized the increased expressions of *Hsd11b1* in the liver and adipose of OVX rats might explain this phenomenon.

In this context, it is of interest that a study had demonstrated that insulin decreased *Hsd11b1* mRNA in cultured hepatocytes (*Jamieson et al. 1995*). In **Paper I** (*Fazliana et al. 2009*), insulin was increased in LPva treated rats, despite normal blood glucose levels. It is possible that increased plasma insulin levels, at least partly, can account for the reduced *Hsd11b1* expression following LPva treatment.

As this is the first attempt to explore differential display of *Hsd11b1* gene expression in OVX rat liver and adipose treated with LPva, this study partly explains the regulation of CORT and body weight change. Based on our results, we hypothesize that the anti-obesity effect of LPva is attributed, at least to some extent, to the inhibition of *Hsd11b1* expression in adipose tissue and liver. Therefore, the use of LPva can be explored to reduce glucocorticoid action as the basis for novel therapy of type 2 diabetes and obesity.

5.4 Apoptosis in uroepithelial cells

In this paper, we show that LPva treatment induced apoptosis in BECs, accompanied by elevated levels of caveolin-1. The plant antioxidant component, anthocyanin, also present in LPva (*Norhaiza et al. 2009*), was recently shown to induce apoptosis (*Zikri et al. 2009*). Furthermore, a reduction in intracellular bacteria was observed which may, in part, be due to reduced expression of β1 integrin. LPva did not directly exhibit or elicit antimicrobial responses and also did not affect binding of type 1 fimbriae. These findings collectively suggest that LPva treatment promotes exfoliation of superficial bladder cells and protects the uroepithelium from bacterial invasion.

Apoptosis is a beneficial host response which removes UPEC from the urinary tract due to exfoliation of apoptotic cells into the urine (*Mulvey et al. 1998*). We demonstrate that LPva induced apoptosis of BECs, with and without UPEC infection. This finding also indicates that LPva exerts cell protection under the condition of infection. Interestingly, Annexin-V staining of cell culture medium revealed detached apoptotic LPva-treated cells which mimics *in vivo* exfoliation of dead cells and removal in the urine. Elevated levels of caveolin-1 have been proposed as a marker of apoptosis in macrophages (*Gargalovic and Dory 2003*) and have furthermore been shown to 'sensitize' T24 BECs to other apoptosis-inducing stimuli (*Liu et al. 2001*). Type 1 fimbriae, one such kind of stimuli, induce apoptosis-like exfoliation upon attachment to the uroepithelium (*Mulvey et al. 1998*). In the present model, we suggest that UPEC infection further induced apoptosis in cells already sensitized to such stimulus by LPva treatment.

A significant reduction in intracellular bacteria in LPva-treated cells likely reflects LPva- and UPEC-induced apoptosis and exfoliation of dead infected BECs.

Nevertheless, decreased expression of invasion-mediating β1 integrin (*Eto et al. 2007*) may also play a role. A downregulation of expression of β1 integrin mRNA could not be confirmed on the protein level in the current study. However, this is likely due to the high stability of the protein (*Lindberg et al. 2010*). Thus, we cannot rule out that at later time points, reduced levels of β1 integrin protein would occur. Based on our findings, we suggest that following LPva-induced apoptosis and consequent exfoliation of dead cells, decreased expression of β1 integrin may thus protect deeper layers of the uroepithelium from UPEC invasion.

6. Concluding remarks and future perspectives

The major findings

Paper I – The study provides evidence that LPva exerts uterotrophic effect and regulates body weight gain at least partly by modulating secretion of leptin and resistin, and expression of the adipokines in adipose tissues.

Paper II - The study confirms that estrogen deficiency induced by ovariectomy in rats results in an excessive increase in *Hsd11b1* gene expression which can be reversed by LPva treatment. Although LPva plays a significant role in the regulations of *Hsd11b1*, it is unclear whether this effect is primary or secondary to changes in body composition.

Paper III - The study indicates that LPva increases uterine weight, indicating estrogenic effects, and improves insulin sensitivity and lipid profile in PCOS rats without affecting body composition.

Paper IV – We demonstrate that LPva has potential therapeutic applications against UTI. Our finding of reduced UPEC invasion of bladder epithelial cells following LPva treatment is likely due to LPva-induced apoptosis and possible down regulation of β1 integrin. This study provides new insight into the mechanisms of action of LPva which may help to reduce the burden of UTI faced by women.

Future perspectives

The results published in this thesis are useful to improve our understanding of LPva actions. In spite of the limitations for each study design, we were reasonably able in showing LPva biological activities through different mechanisms via modulation of gene expression levels. However, they inevitably lead to further questions and debate.

We have shown that LPva exerts its effects to some extent mimicking estrogen mode of actions. In some aspects, LPva and ERT did not show similar effects, mainly due to a collective of compounds in the extract. Therefore it is necessary to evaluate its refined active compounds. In this area, work will be done closely with phytochemistry research group. Also, little is known about its side effects and pharmacokinetics. Therefore, identifying bioactive compounds in LPva could be necessary to further investigate toxicology effects, especially at molecular levels.

Furthermore, it would be valuable to further investigate the microarray analysis to obtain a comprehensive analysis of genetic profiles. Additional information may be mined from this to provide clues to many areas including the metabolic pathways involved. It is also important to study the metabolism of LPva in liver and its effect on kidney in future studies. It would be a great interest to carry out clinical trials among women in Malaysia and perform the endocrinological analyses and molecular studies.

Nevertheless, current and future investigations will contribute to bridge the traditional knowledge of LPva with scientific evidence.

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