THE EFFECT OF *Cinnamomum verum* AND *Clinacanthus nutans* AQUEOUS EXTRACT ON DEHYDROEPIANDROSTERON INDUCED POLYCYSTIC OVARIAN SYNDROME (PCOS) IN RAT

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by

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

%	Percent
°C	Degree Celcius
ARASC	Animal Research and Service Centre
ASRM	American Society of Reproductive Medicine
ASHRE	Europen Society for Human Reproductive and Embryology
cc	Clomiphene citrate
CN	*Clinacanthus nutan*
cm	Centimeter
CV	Cinnamomum verum
CVD	Cardiovascular disease
DHT	Non-aromatisable androgen dihydrotestosterone (DHT)
DHEA	Dehydroepiandrosterone
ESHRE	Rotterdam European Society for Human Reproduction
FSH	Follicle Stimulating Hormone
g	Gram
Hcl	Hydrochloric acid
H&E	Haematocylin and Eosin
IVF	In-vivo Fertilisation
LH	Luteinising Hormone
m	Meter
mm	Milimeter
ml	Millimeter
mg/kg	milligram per kilogram

m^2	meter square
NICHD	National institute for Child Health and Human diseases
NIH	National Institutes of Health
ng/ml	nanograms per milliter
OC	Oral contraceptive
P	p- value
PCOS	Polycystic Ovarian Syndrome
SD	Sprague Dawley
USM	Universiti Sains Malaysia
USA	United States of America
WHO	World Health Organisation

LIST OF APPENDICES

KESAN EKSTRAK AKUEUS *Cinnamomun verum* DAN *Clinacanthus nutans* TERHADAP MODEL TIKUS SINDROM OVARI POLISISTIK (PCOS) ARUHAN DEHYDROEPIANDROSTERON

ABSTRAK

Sindrom ovarium polikistik adalah gangguan utama yang dicirikan oleh peningkatan tahap androgen, jerawat dan hirsutisme yang tinggi. Rawatan semasa merangkumi metformin yang hanya berkesan terhadap gejala PCOS tanpa merawat penyebabnya. Di samping itu, metformin menyebabkan kesan sampingan yang ketara seperti gejala gastrousus, penambahan berat badan dan peningkatan daya tahan insulin. Objektif kajian ini adalah untuk menilai kesan ekstrak akueus *Cinnamomum verum* (CV) dan *Clinacanthus nutans* (CN) pada tikus yang disebabkan oleh PCOS. Kajian awal untuk induksi PCOS menggunakan dos 6mg/100g/0.2ml dehydroepiandrosterone (DHEA) dalam tempoh 21 hari telah dijalankan dalam tiga kumpulan tikus ini: (a) kumpulan kawalan, (b) kumpulan vehikel sahaja, dan (c) kumpulan DHEA + vehikel; untuk memastikan pembentukan ciri PCOS dalam model tikus yang digunakan sebelum meneruskan ke kajian utama. Tiga puluh ekor tikus SD betina dibahagikan kepada enam kumpulan lima haiwan masing-masing iaitu kumpulan kawalan, kumpulan DHEA, kumpulan CV (500mg / kg), kumpulan CV (1000mg / kg), kumpulan CN (500mg / kg) dan kumpulan CN (1000mg / kg). Rawatan ekstrak berair CV dan CN diteruskan selama 28 hari selepas induksi. Selepas rawatan selesai, semua tikus dikorbankan. Analisis hormon, berat badan, histopatologi dan kajian toksikologi ovari, hati, rahim dan buah pinggang dijalankan. Dalam kumpulan DHEA, paras

testosteron meningkat dengan ketara berbanding dengan tikus normal yang tidak diinduksi ($p = 0.1780$) ($p < 0.05$) yang berkurangan kepada paras ketara apabila kedua-dua ekstrak diberikan. Induksi PCOS pada tikus membawa kepada peningkatan ketara dalam sista folikular ($p < 0.0001$) dan penurunan dalam corpus luteum berbanding dengan kumpulan kawalan ($p < 0.0001$). Apabila rawatan diberikan kepada kumpulan PCOS, terdapat peningkatan ketara dalam jumlah corpus luteum pada kedua-dua ekstrak CV ($p = 0.0002$, $p = 0.0070$). Menariknya, bilangan sista folikular juga menurun dengan ketara dalam semua kumpulan rawatan CV dan CN ($p=<0.001$, $p=<0.001$, $p=<0.001$, $p=<0.001$). Tikus yang diberi rawatan CN menunjukkan struktur organ morfologi normal yang menunjukkan kesan tidak toksik bagi kedua-dua tumbuhan. Walau bagaimanapun, ekstrak CV menyebabkan perubahan degeneratif ringan pada hati dan buah pinggang pada 500mg/kg dan 1000mg/kg. Kesimpulannya CV dan CN mampu mengurangkan ciri PCOS pada model tikus yang diinduksi. Kajian ini bertindak sebagai asas untuk kajian lebih lanjut mengenai mekanisme kedua-dua tumbuhan dalam rawatan PCOS.

THE EFFECT OF *Cinnamomum verum* AND *Clinacanthus nutans* AQUEOUS EXTRACT ON DEHYDROEPIANDROSTERONE INDUCED POLYCYSTIC OVARIAN SYNDROME (PCOS) IN RAT

ABSTRACT

Polycystic ovarian syndrome is a major disorder characterised by elevated levels of androgens, acne and hirsutism. Current treatment includes metformin which is only effective against PCOS symptoms without treating the underlying cause. In addition, metformin causes significant side effects such as gastrointestinal symptoms, weight gain and increased insulin resistance. The objective of this study was to evaluate the effect of *Cinnamomum verum* (CV) and *Clinacanthus nutans* (CN) aqueous extract in PCOS induced rats. Preliminary study for PCOS induction using dosage 6mg/100g/0.2ml of dehydroepiandrosterone (DHEA) in 21 day duration was conducted in these three groups of rats: (a) control group, (b) vehicle only group, and (c) DHEA + vehicle group; to ensure the establishment of PCOS characteristics in the rat model used before proceed to the main study. Thirty female SD rats were divided into six groups of five animals each namely control group, DHEA group, CV group (500mg/kg), CV group (1000mg/kg), CN group (500mg/kg) and CN group (1000mg/kg). CV and CN aqueous extract treatment were continued for 28 days post-induction after induction of PCOS. After treatment completion, all rats were sacrificed. Analysis of hormones, body weight, histopathology and toxicology study of the ovary, liver, uterus and kidney were carried out. In DHEA group, testosterone levels increased significantly compared to non-induced normal rats ($p=0.0402$) ($p<0.05$) which reduced to significant level when both extracts were given. Induction of PCOS in rats lead to significant increase in follicular cyst ($p<0.0001$) and decrease in corpus

luteum compared to the control group ($p<0.0001$). When treatments were given to the PCOS group, there was a significant increase in the number of corpus luteum in both CV extracts ($p=0.0002$, $p=0.0070$). Interestingly, the number of follicular cysts also decreased significantly in all CV and CN group ($p=<0.001$, $p=<0.001$, $p=<0.001$, $p=<0.001$). CN treated rats showed normal morphological organ structure showing non-toxic effect of both plants. However, CV extracts induced mild degenerative changes in liver and kidney at 500mg/kg and 1000mg/kg. In conclusion CV and CN is able to reduce PCOS charasteristics in induced rats model. This study act as a basic for further study on the mechanism of both plants in the treatment of PCOS.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Polycystic ovarian syndrome (PCOS) is a heterogenous endocrine and reproductive disorder characterised by polycystic ovaries, chronic anovulation and hyperandrogenism leading to symptoms of irregular menstrual cycles, hirsutism, acne and infertility that affect about 5 to 10% of the female population worlwide (Heibashy, 2013; Arentz et al., 2014). According to the World Health Organisation (WHO), 3.4% (116 million) women worldwide were affected in 2012.

In Malaysia the prevalence of PCOS was 22.9% (Ishak et al., 2012). The ovaries are the primary sites of abnormality in PCOS and enlarged polycystic ovary in PCOS was due to excessive number of follicles and cysts compared to normal ovaries (Jonard et al., 2003; Webber et al., 2003).

The use of the insulin-sensitising drugs is an effective therapeutic approach to improve insulin resistance in the pathogenesis of PCOS. Metformin, clomiphene citrate (CC), aromatase inhibitors, tamoxifen and glucocorticoids are the main drugs used for the treatment of PCOS. However, the used of these drugs especially metformin led to various side effects such as hot flushes, arthritis and joint or muscle pain. Psychological side effects include irritability, mood swings, depression and bloating (Jadhav and Bhutani, 2005; Sasikala and Shamila, 2009). In addition, these drugs only alleviate the symptoms of PCOS without actually treating the underlying conditions. Due to these factors, alternative medicines are gaining importance.

Drug discovery which is largely based on terrestrial plants and plant-derived drugs play a dominant therapeutic role in the treatment of human diseases. Plant-derive products such as aloe-vera, cinnamon, chaste berry, kacip fatimah, spearmint tea, dandelion root and ginseng saponin are traditionally used to treat various symptoms of PCOS with no or lesser side effects compared to clinical drugs. However, many of these claims were not supported by scientific evidence. *Cinnamon Verum* (CV) is a popular spice around the world and one of the oldest medicinal herbs. In Malaysia, this spice is used in cooking thus it is easily obtained. *Cinnamomum verum* was shown to possess significant activities as hypoglycaemic and cholesterol lowering agent (Khan et al., 2003). In addition, polyphenolic polymer isolated from CV was shown to have insulin-like activity and antioxidant activity (Anderson et al., 2003). Due to this property, cinnamon extract was used in this study.

Clinacanthus nutans (CN) commonly known as Sabah snake grass or *Belalai Gajah* in Malaysia, is a well-known medicinal herb, especially in Southeast Asia. This plant has been traditionally used for the treatment of hepatitis, skin rashes, snake venom poisoning, as well as cancer (Mustapa *et al.*, 2015). In Malaysia, the fresh leaves are usually boiled with water and consumed as herbal tea (Alam *et al.*, 2016). CN is reported to possess various medicinal properties including blood glucose lowering effect, alpha-glucosidase inhibition activity, antioxidant activities, anti-cancer properties and anti-inflammatory effects (Zulkipli *et al.*, 2017; Khoo *et al.*, 2018). The effect of CN in treating PCOS is not well known. However, CN was reported to play a role in insulin resistance signalling (Sarega *et al.*, 2016) which may have the potential in the treatment of PCOS.

Since CV and CN have the properties required to improve and treat PCOS, both plants were used in this study. There is a need to scientifically validate the traditional used of these plants in PCOS. The effectiveness of CV and CN in PCOS will be compared.

1.2 Problem Statement

Currently the standard care of treatment for woman with PCOS is insulin sensitiser since the central core of PCOS etiology is through insulin resistance (Amoura et al., 2015). Such treatment is associated with substantial cost which is unaffordable and burdensome to some people. Currently, in the government hospitals, metformin is highly subsidised by the Ministry of Health Malaysia with the total cost of subsidy amounting to RM3.2 billion per year (Ministry of Health, 2020). Besides, various side effects resulting from the use of metformin have been reported such as gastrointestinal symptoms, weight gain and increased insulin resistance after prolonged usage (Koppala, 2017; Nowak et al., 2007). Studies have shown that natural products could offer similar or even better end-result with reduced or no side effects (Ismail & Imam, 2014). Both CV and CN has phytoestrogens such as flavonoids and phenols which are increasingly being used by women as an alternative to oestrogen (Romualdi et al. 2008). Thus, CV and CN extract will be thoroughly explored in this present study as an alternative treatment for PCOS.

1.3 Significance of Study

Concerning issues regarding PCOS include the huge economic burden carried by the world countries. For example, around 4 billion dollars are spent annually in the United States to screen for this disease and treat its various morbidities, including hirsutism, infertility, and diabetes mellitus (Azziz *et al.*, 2005). Australian Health System spends more than 800 million dollars every year to account for this disease (Azziz *et al.*, 2005). There are no published data on the economic burden of PCOS among Malaysian population. However, infertility treatment due to PCOS will cost around RM18000 for one *in-vitro* fertilisation (IVF) procedure (Infertility Aide, 2020). Moreover, patients with PCOS are twice more likely to be admitted to hospital compared to patients without PCOS because women with PCOS have a higher propensity to develop metabolic and cardiovascular disorders (Hart and Doherty, 2015). Therefore, early prevention and effective treatment of PCOS is necessary to reduce the economic burden, as well as to prevent future health comorbidities.

This research aim is to study the therapeutic effects of CV and CN on PCOS rat model, which may contribute to making CV and CN a promising alternative therapy for treating clinical and pathological abnormalities in PCOS. Through this research, the effectiveness of CV and CN in improving PCOS compared to the drug metformin will be investigated. The usage of natural products in medicine can also boost individuals and countries economy through increase demand and supplies of these products.

1.4 Objective

1.4.1 General Objective

To study the effects of *Cinnamomum verum* (CV) and *Clinacanthus nutan* (CN) extract on dehydroepiandrosterone (DHEA) induced PCOS rats.

1.4.2 Specific Objectives

1. To determine the percentage induction of PCOS characteristics on rats using DHEA as chemical inducer based on the morphological changes of the ovary
2. To assess the therapeutic effects of CV and CN aqueous extracts on the hormonal profile and the morphological changes of the ovary
3. To determine the effects of CV and CN aqueous extracts treatment on PCOS induced rats' oestrus cyclicity pattern
4. To evaluate the toxicity of CV and CN aqueous extract treatment on PCOS-induced rats' kidney, liver and uterus using haematoxylin and eosin stained tissue section.

1.5 Research Hypothesis

1. PCOS characteristics 100% established on rats using DHEA

2. CV and CN aqueous extract improved hormonal profile and significantly increased the number of follicle in ovary

3. CV and CN improves oestrus cyclicity pattern

4. CV and CN aqueous extract not toxic to kidney, liver and uterus

CHAPTER 2

LITERATURE REVIEW

2.1 Polycystic ovarian syndrome (PCOS)

Polycystic Ovarian syndrome is a common heterogeneous endocrine disorder of unknown aetiology commonly effect women of reproductive age, between puberty and menopause. In 1935, Stein and Levanthal first defined PCOS as endometrial hyperplasia, multiple follicle cysts with granulosa cell lining, and a noticeable absence of ovarian corpus luteum (Stein and Leventhal, 1935). PCOS is also known as Stein-Leventhal syndrome, hyperandrogenic anovulation syndrome, and polycystic ovarian disease. PCOS is often referred to as a 'syndrome,' since a patient with PCOS encounters several symptoms concurrently.

PCOS is the most common endocrine dysfunction in women with infertility (March et al., 2010). Women with PCOS have symptoms correlated with menstrual dysfunction and androgen toxicity which have a major effect on their quality of life (El-Hayek *et al.*, 2016). They may be at elevated risk of various morbidities, including obesity, insulin resistance, type 2 diabetes mellitus, cardiovascular diseases (CVD), infertility, cancer and psychological disorders (El-Hayek *et al.*, 2016).

After Stein and Leventhal's classical discovery in 1935, interest in PCOS has grown from 'gynaecological curiosity to endocrinopathy with multisystems' (Homburg, 1996). While Stein and Leventhal first described this syndrome in modern medicine, there was an earlier definition dated back to 1721, which reads: "Young married peasant women, moderately obese and infertile, with two ovaries larger than average, bumpy, shiny and whitish, just like pigeon eggs" (Farquhar, 2007).

There was more recognition in the nineteenth century when sclerocystic changes were first identified in the ovary, but it was not until Stein and Leventhal first presented their paper at the Central Association of Obstetricians and Gynaecologists in 1935 that this syndrome was identified in more detail (Farquhar, 2007). They first reported seven women with amenorrhoea, hirsutism and ovaries, several small cyst and thickened tunica (Stein and Leventhal, 1935). Figure 2.1 shows an ovarian section of normal women vs PCOS patient. There was a clear addition of cysts in PCOS patients compared to the age-matched non-PCOS women.

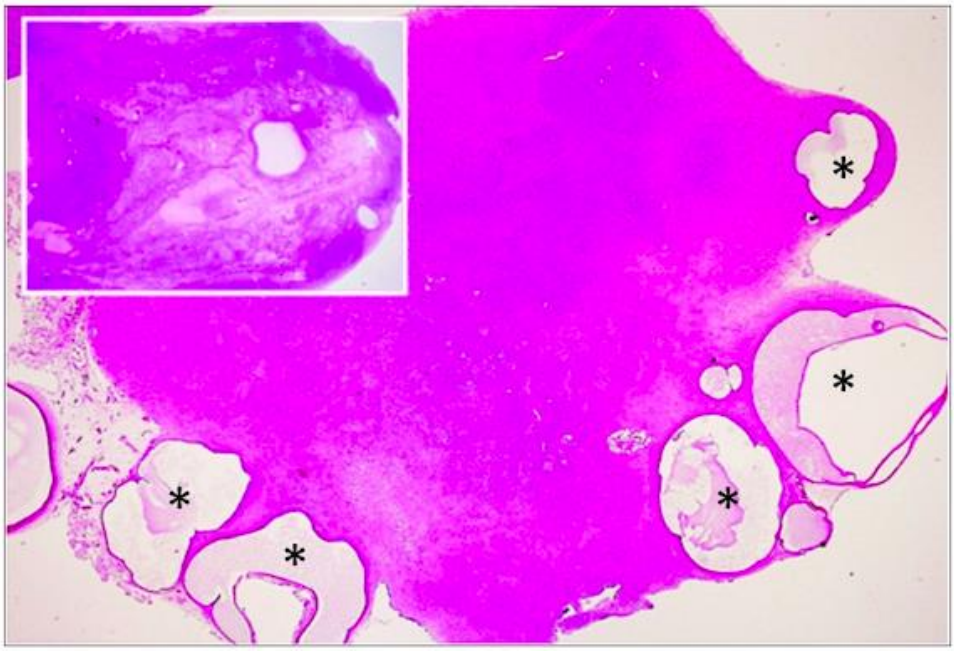

Figure 2.1 Haematoxylin and Eosin stained human polycystic ovary. An ovarian section of 41- year-old PCOS patient shows multiple cysts (star) in the periphery of the ovary. Insert, age-matching non-PCOS ovary. Figures at 100× magnification. Adapted from Oakley *et al.* (2011).

2.1.1 Clinical features of PCOS

Clinical features of PCOS are important in diagnosing PCOS. PCOS women can be sub-grouped based on the basis of possible clinical features of endocrinological disorders and can be examined appropriately for the collection of suitable treatment modalities (Ramanand *et al.*, 2013). Polycystic ovaries are defined as part of the clinical requirements for the diagnosis of PCOS by transabdominal or transvaginal ultrasound (Michelmore *et al.*, 2001). It is necessary to differentiate a follicle from an ovarian cyst that may resemble a follicle's appearance. The morphology of polycystic ovaries can be identified by an ultrasound scan with the presence of 10 or more follicles measuring 2 to 8 mm in diameter, arranged peripherally around a dense stroma center or scattered

over an increased amount of stroma (Dumensic et al., 2015) as shown in Figure 2.2. Nonetheless, it is also common for women without PCOS to have a number of cysts in their ovaries. In addition, multi-follicular ovaries may be a natural stage of development in puberty and early adulthood. Therefore, expanded ovarian size (more than 10mL) may be a clearer predictor of adult PCOS than follicular numbers in adolescent girls with hyperandrogen and oligomenorrhoea for at least 2 years postmenarche (Dumensic et al., 2015).

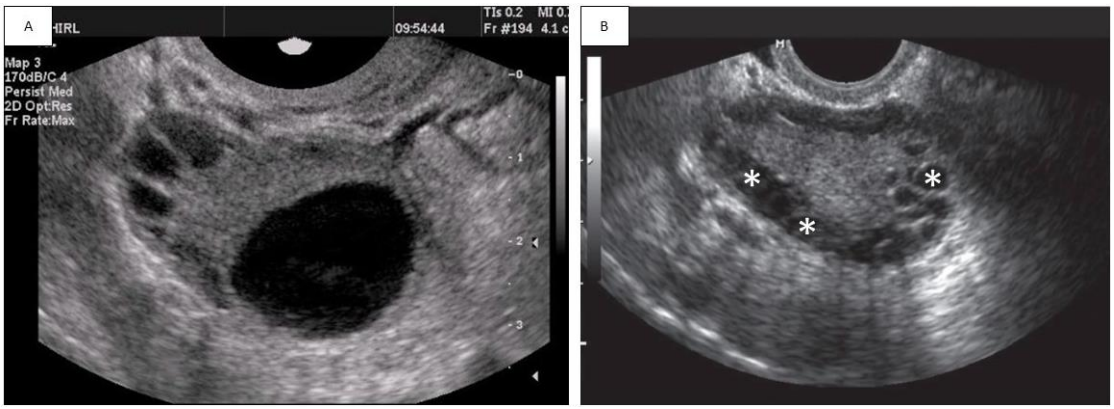

Figure 2.2 Ultrasound image of A) normal ovary and (B) polycystic ovary. Ovarian cysts are shown in the periphery of the ovary as dark circles (star). Adapted from Oakley et al. (2011) and Lamb (2016).

Hyperandrogenism, also known as excess androgen, is an endocrinological condition, characterised or caused by excessive androgen production and/or secretion in the female body. Hyperandrogenism is a significant pathophysiological characteristic with 60 to 80 percent prevalence (Markopoulos *et al.*, 2011). Some experts found hyperandrogenism to be the key characteristic of PCOS, and believe that PCOS is associated with hyperandrogenism and ovulatory dysfunction (Baptiste and Battista, 2013).

The foundation units of female reproductive biology are ovarian follicles which are regularly initiated for growth and development, resulting in ovulation or releasing an oocyte (Rodgers & Irving-Rodgers, 2010). Ovarian follicles consist of granulosa cells and follicular theca cells. Folliculogenesis describes the development of a number of small primordial follicles into large pre-ovulatory follicles during the menstrual cycle. From conception, human ovaries produce a number of primordial, immature follicles. These follicles each contain a primary oocyte of similar immature origin. At puberty, the primordial follicles undergo a series of essential changes in character, both histologically and hormonally. They first transform into primary follicles, and then into secondary follicles. The follicles then become tertiary, or antral follicles. They become dependent on hormones at this stage of development, especially FSH which causes their growth rate to rise substantially.

Histologically, primordial follicles comprise immature oocytes surrounded by smooth, squamous granulosa cells (supporting cells), which are isolated by the basal lamina from the oocyte setting. Primordia follicles display little or no biological activity. These follicles can remain dormant in humans until puberty. At this time, the granulosa cells of the primordial follicles shift from a flat to a cuboid shape during ovarian follicle activation, which marks the beginning of the primary follicle. Both the oocyte and follicle grow rapidly to approximately 0.1 mm in diameter. Stroma-like theca cells are recruited by signals secreted by the oocytes which penetrate the basal lamina and undergo differentiation to become theca externa and theca interna. A complex network of capillary vessels forms between the theca layers which bathe the follicles leading to follicular maturation.

By the end of the thirteenth day follicular phase of the menstrual cycle, the cumulus oophorus layer of the preovulatory follicle may create an opening, or stigma, and excrete the oocyte in a process called ovulation. Ovulation can occur in follicles which are at least 14 mm in natural cycles. The ruptured follicle will be transformed into corpus luteum, a steroidiogenic cluster of cells that maintain the endometrial layer of the uterus through the secretion of progesterone. Figure 2.7 shows histological maturation of a follicle starting from the primordial follicle.

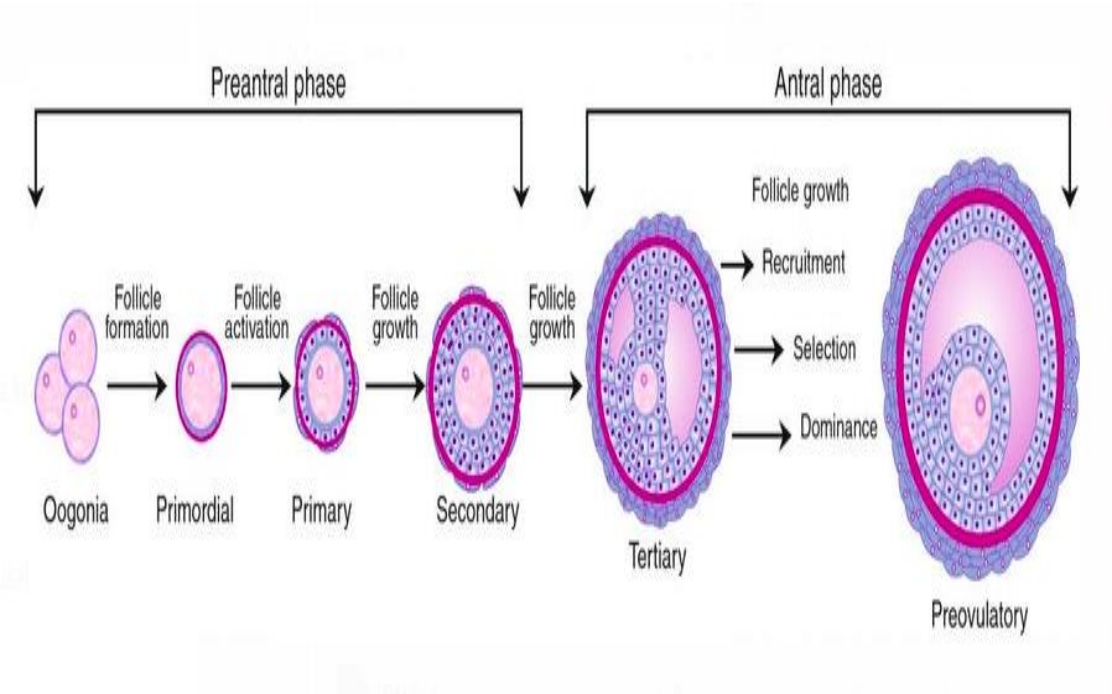

Figure 2.3 Histological maturation of a follicle starting from the primordial follicle. FSH stimulates the growth of a tertiary follicle and LH stimulates the production of oestrogen by the granulosa and theca cells. Once the follicle mature, it ruptures and releases the oocyte. Cells remaining in the follicle develop into corpus luteum. (Araujo et al., 2014)

Menstrual irregularity is a defective ovulation that involves oligo / amenorrhoea or chronic anovulation. As many as 85 percent of women with PCOS had clinical evidence of menstrual irregularities, according to the Androgen Excess / PCOS Society Task Force Report (Dumesic *et al.*, 2015). Menstrual cycle follows a specific pattern based on the hormones secreted by the pituitary gland in the brain and the ovaries. Follicle-stimulating hormone (FSH) and leutinising hormone (LH) are produced by the pituitary glands while the ovaries produce progesterone and oestrogen. Small increases in FSH stimulate the maturation of follicles. The follicles secreted oestrogen which thicken the uterine wall. LH level will then increase causing ovulation. Figure 2.3 shows the menstrual cycle phases and the hormones responsible. If fertilisation occurs, the fertilised ovum will move to the uterus via the Fallopian tube to be implanted. In PCOS sufferer, hormone testosterone and estradiol were high while LH and FSH were low. Many tiny follicles (tiny cysts 4 to 9 mm in diameter) develop in the ovary of females with PCOS, thus the name polycystic ovaries. None of these tiny follicles will expand to a mature size. As a result, the testosterone, progesterone, LH and FSH levels became imbalanced.

Androgens including testosterone, androstenedione, dehydroepiandrosterone (DHEA), and DHEA sulfate (DHEAS) are normally developed by PCOS patients' ovaries and adrenal glands. Androgens rise in women with PCOS due to the high level of LH but can also rise due to the high levels of insulin typically seen in women with PCOS. Access to androgens will therefore be given to the animal model in this study to stimulate PCOS by using DHEA.

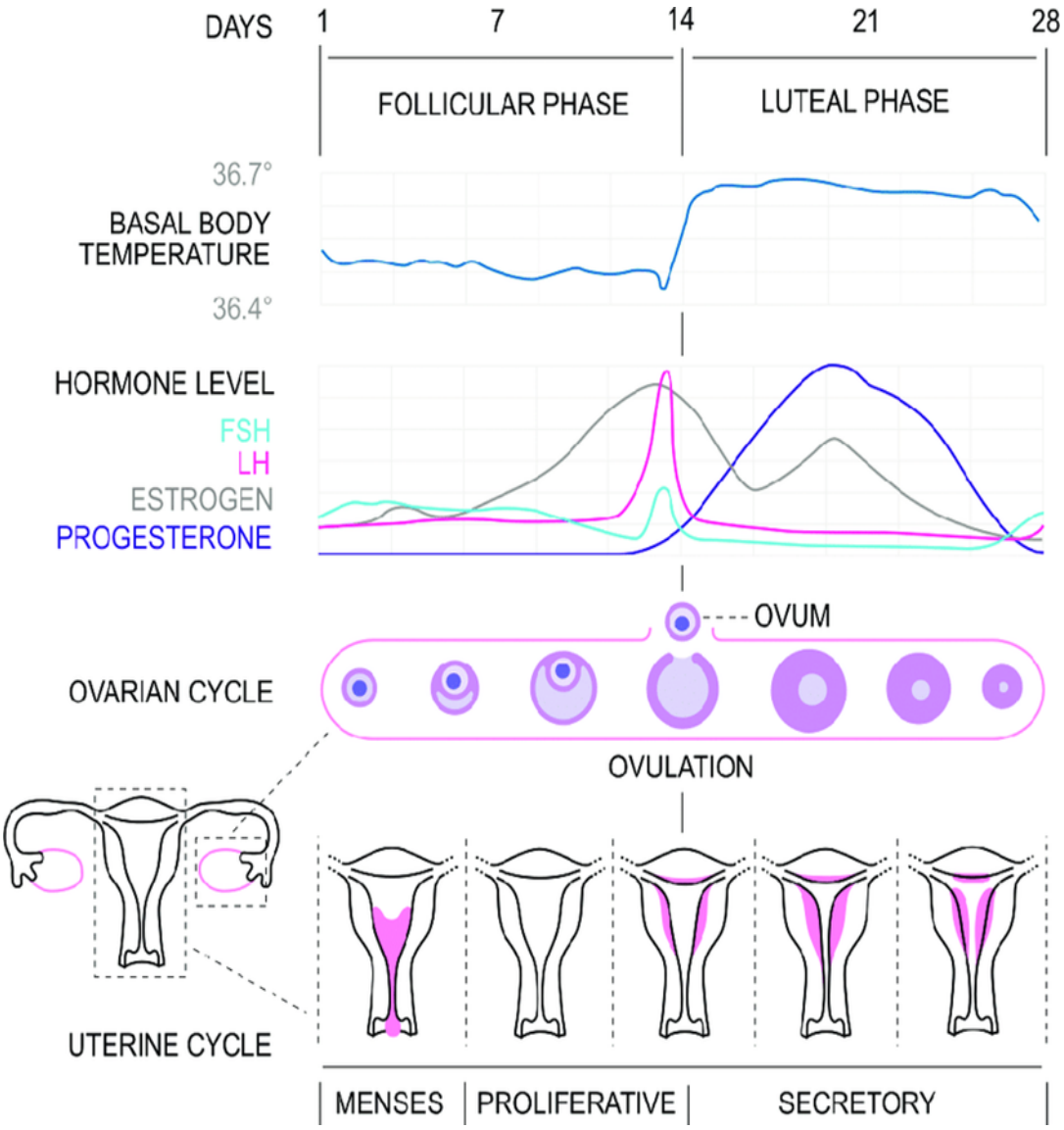

Figure 2.4 Hormone involved in the menstrual cycle phase. The dynamic changes of hormonal level in the body will stimulate different phases of the menstrual cycle (Pepin et al., 2017).

Another symptom of PCOS is oligomenorrhea and amenorrhoea. Oligomenorrhoea is diagnosed when the menstrual cycle lasts longer than 35 days or occurs fewer than 8 times a year, whereas amenorrhoea is characterised as menstruation cycle over 90 days. Throughout the presumed mid-luteal phase of the cycle, a serum progesterone level is calculated. The cycle is considered as oligo/anovulatory if the serum progesterone level is below 3 to 4 ng/mL (Dumesic *et al.*, 2015). Clinicians must be able to distinguish normal anovulation associated with puberty from pathological anovulation through close examination of the menstrual cycle patterns (Franks, 2002; Wiksten-Almströmer, Hirschberg and Hagenfeldt, 2008).

Hirsutism refers to the development of course, dark hair in places where women normally grow fine hair or have no hair at all that is above the lip and on chin, arms, abdomen and back. The elevated level of male hormones (androgens) is responsible for this excess hair development. While all women produce androgen, higher androgen levels may cause hirsutism (Sachdeva, 2010). The Ferriman-Gallwey hirsutism ranking as shown in Figure 2.4 can be used for diagnosis of hirsutism. This ranking system is developed by the Endocrine Society Guidelines published in 2008 (Martin *et al.*, 2008). A score of 6 to 8 is mild, 8 to 15 is extreme and over 15 is graded as an overt hirsutism (Bernier, 2012).

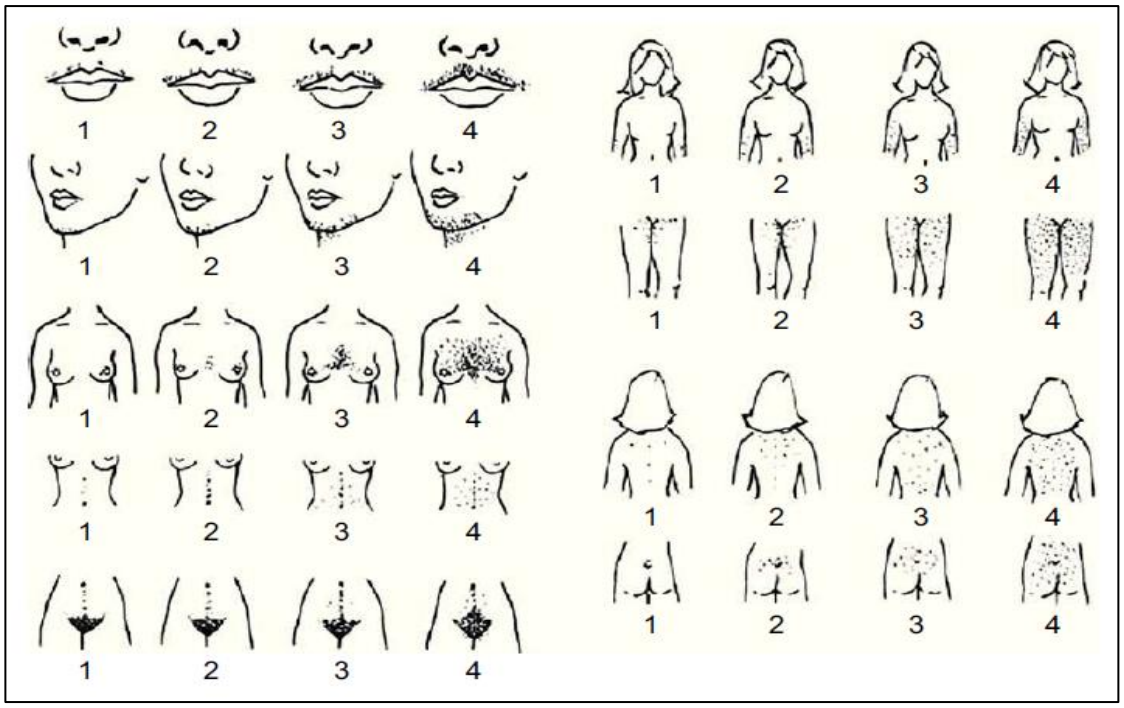

Figure 2.5 Ferriman-Gallwey hirsutism scoring system. Each of the nine body areas most sensitive to androgen is assigned a score from 0 (absence of terminal hairs) to 4 (extensive terminal hair growth), and these separate scores are summed to provide a hormonal hirsutism score. Adapted from Oliveira et al. (2010) .

Obesity is common in women suffering from PCOS. According to the study setting and the ethnic background of the subject, between 40 and 80% of women with PCOS are estimated to be overweight, obese or centrally obese (Cupisti et al., 2008; Vrbikova & Hainer, 2009). Obesity has a worse additive effect on PCOS characteristics such as resistance to insulin, hyperandrogenism, infertility, hirsutism and complications of pregnancy (Qin et al., 2013). The relationship between PCOS and obesity is complex, and interaction of genetic and environmental factors is most likely (Lim et al., 2013). It is demonstrated that central obesity is associated with increased risk of diabetes, hyperlipidemia, hypertension, atherosclerosis and resistance to insulin (Tehrani et al., 2010) Localised fat in the upper body is correlated with a significantly reduced overall insulin clearance, which has contributed to a significantly reduced overall insulin

clearance that contributes to hyperinsulinemia (Peiris et al., 1986). These are the metabolic abnormalities associated with PCOS sufferers.

2.1.2 Diagnostic criteria of PCOS

There is currently no standard diagnostic test for PCOS. However, three groups have proposed the diagnostic criteria for PCOS: 1) the National Institutes of Health / National Institute for Child Health and Human Diseases (NIH/NICHD), 2) the European Society for Human Reproduction and Embryology / American Society for Reproductive Medicine (ESHRE/ASRM), and 3) the Androgen Excess and PCOS Society (Sirmans & Pate, 2013). Table 2.1 summarised the criteria for the diagnosis of PCOS based on these three groups.

Table 2.1 Criteria for the diagnosis of polycystic ovarian syndrome. Adapted from Sirmans and Pate (2013).

NIH/NICHD[a] 1992	**ESHRE/ASRM[b] (Rotterdam criteria) 2004**	**Androgen Excess Society 2006**
Exclusion of other androgen excess or related disorders Includes ALL of the following: ▪ Clinical and/or biochemical hyperandrogenism ▪ Menstrual dysfunction	Exclusion of other androgen excess or related disorders Include**s** TWO of the following: ▪ Clinical and/or biochemical hyperandrogenism ▪ Oligo/anovulation ▪ Polycystic ovaries	Exclusion of other androgen excess or related disorders Include**s** ALL of the followin ▪ Clinical and/or biochemical hyperandrogenism ▪ Ovarian dysfunction and/or polycystic ovaries

[a] NIH/NICHD, National Institutes of Health/National Institute of Child Health and Human Disease.

[b] ESHRE/ASRM, European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine.

From 1935 until 1990, there was no formal tool for diagnosing PCOS (Bernier, 2012). In 1990, a group of researchers attending an NI /NICHD sponsored conference agreed that PCOS would be defined as menstrual irregularity (oligo/anovulation) and/or biochemical hyperandrogenism excluding other endocrinopathies, such as androgen-secreting tumours or Cushing 's syndrome (Dumesic *et al.*, 2015). Those parameters remained the principal diagnostic method for PCOS until the conference in Rotterdam was held in 2003.

ESHRE/ASRM held the conference in Rotterdam, The Netherlands. In this meeting, the diagnostic criteria for PCOS were revised, hence this diagnostic method is also known as Rotterdam criteria. It was determined in this context that the polycystic ovaries found by ultrasound should be treated as a diagnostic criterion (Bernier, 2012). Thus, the diagnostic criteria have been expanded to include at least two of the following characteristics: 1) clinical and/or biochemical hyperandrogenism excluding other endocrinopathies, 2) chronic oligo/anovulation, and 3) the presence of polycystic ovaries by ultrasound scans. A group of experts from the 2012 NIH Evidence-based PCOS technique workshop suggested that clinicians use the more current Rotterdam diagnostic criteria (Dumesic *et al.*, 2015) and these criteria have remained as the main diagnostic tool for PCOS until 2006.

In 2006, the Androgen Excess Society had recommended tightening up PCOS diagnosis requirements. Consequently, under the wider Rotterdam definition, the prevalence of PCOS has increased, including women without documentary ovulatory dysfunction or hyperandrogenism, and women with polycystic ovaries (Dumesic *et al.*, 2015). The diagnostic criteria have therefore been updated to include hyperandrogenism

as a required condition, excluding other endocrinopathies (Bernier, 2012). Therefore, a formal diagnosis required either oligo/anovulation or polycystic ovaries (Bernier, 2012). Currently, all three criteria concurrently play a major role as key diagnostic criteria of PCOS.

Blood glucose and cholesterol tests are normally conducted when PCOS is established to gauge the metabolic abnormalities in PCOS patients. Blood testing for testosterone and dehydroepiandrosterone sulfate (DHEAS) may be conducted in women with mild to extreme hirsutism. All women diagnosed with PCOS should be monitored by a health care provider. PCOS signs can seem mild and unpleasant and treatment can seem unnecessary. However, untreated PCOS may increase the likelihood of other health issues in a woman over time.

2.1.3 Risk Factors of PCOS

Risk factors is related to genetic and health behaviors. Research evidences reveal many gene loci have been linked with development of PCOS. Familial history of PCOS and health impacts are known. Further research on genome, transcriptome, proteome, and metabolome should be investigated in order to explore and suggest specific preventive strategies (Hiam et al., 2019). Unhealthy behaviors resulting in overweight, obesity, insulin resistance, hyperinsulinemia, and hyperandrogenism are also known to be risk factors for development of PCOS among women. Over consumption of diets and drinks containing high rich in sugar, fructose, transfat, animal fat, and processed foods are considered its leading causes. Modification of healthy dietary patterns containing natural nutrients and low glycemic index food items are

recommended to promote metabolic health and prevention of obesity, insulin resistance and PCOS pathogenesis. Doing regular exercise would be additional effective prevention approach to promote insulin sensitivity and improve PCOS manifestations (Ibáñez et al., 2017; Lagana et al., 2018). Lastly, metabolic health literacy and proactive health education should be advised and implemented among women and their families in order to raise their awareness and motivation to engage in preventive action and early detection of PCOS symptoms.

2.1.4 Treatment of PCOS

PCOS is a multifaceted syndrome that affects multiple metabolic and reproductive systems. Treatment objectives commonly focus on treating infertility, managing endometrial menstrual defence and controlling hyperandrogenic characteristics like hirsutism and acne.

Metformin is an insulin sensitiser which has been formulated as a type 2 diabetes treatment but may be recommended in limited circumstances for women with PCOS (Wang et al., 2017). While metformin is not a weight-loss drug, studies have shown that when metformin is prescribed in combination with low-calorie diet, women with PCOS lose slightly more fat compared to placebo (Lashen, 2010). Metformin is not usually prescribed for PCOS patient who have trouble getting pregnant, because it is not as successful as another ovulation induction. For these women, letrozole and clomiphene therapies were suggested in place of metformin.

Oral contraceptive (OC) with combined estrogen and progesterone is also prescribed for PCOS sufferer. OC is effective in treating hirsutism. An OC allows regular menstruation. However, that does not mean that PCOS is healed. Most common, irregular menstrual cycles resume when the OC is stopped.

Changes in lifestyle are seen as a cost-effective first-line approach that should be given to women with PCOS, since there is no evidence to indicate that medical care is any more effective (Harrison et al., 2011). It focuses not only on the obese patients but also on the non-obese PCOS patients. In women with PCOS, excess weight is associated with detrimental metabolic and reproductive health outcomes (El-Hayek et al., 2016). Weight reduction is one of the most successful methods to treat hormonal disturbances, menstrual disorders and other PCOS symptoms. Many overweight women with PCOS who lose as little as 5 percent of their body weight find that their cycles are becoming regulated (Barbieri & Ehrmann, 2020) thus alleviating PCOS symptoms. For morbidly obese women with PCOS, weight loss surgery may be an option.

2.2 Induction of PCOS in rats

Currently, rat model has been widely used for scientific study of PCOS, due to the small body size, short lifespan, high reproduction index and variety of genetic strains (Singh, 2005). PCOS studies using experimental rat models have focused on neonatal androgenisation and injection of steroidal analogs and endocrine-disrupting chemicals such as aromatase inhibitor (Walters et al., 2012). DHEA, testosterone, letrozole estradiol valerate and non-aromatisable androgen dihydrotestosterone (DHT) were

chemicals used to induce PCOS characteristics in animal models (Luchetti et al., 2004; Manneras et al., 2007). DHEA and its sulfaester (DHEA-S) are the most abundant circulating sex steroid hormones in women, mainly secreted by the adrenal glands, theca cells of ovarian follicle, and central nervous system (Yamada et al., 2010). DHEA has also been widely used in USA as an anti-aging supplement and is available without prescription. However, uncontrolled dosages of DHEA can lead to infertility in women (Legrain & Girard, 2003). Daily dosage of DHEA introduce into the body could lead to a high circulating levels of testosterone and androstenedione. High concentration of these metabolites were commonly detected in follicular cyst. In fact, DHEA-induced PCOS rat models present many features of human PCOS. (Lee et al., 1991; Anderson et al., 1992) and is ideal for the study of aromatase deficiency-induced classic PCOS. Hence, DHEA was used in this study as chemical inducer of PCOS in rat.

2.3 Oestrus cycle

Oestrus cycle is the reproductive cycle of female rats. Rat's oestrous cycle usually lasted for 4 to 5 days. This criteria make them an ideal model for investigation of reproductive cycle. Oestrus cycle is divided into pro-oestrus, oestrus, met-oestrus and di-oestrus, which are determined based on the cell type in the vaginal smear (Marcondes et al., 2002). Ovulation period can occur from the beginning of pro-oestrus to the end of oestrus (Young *et al.,* 1941; Schwartz, 1964). The level of prolactin, LH and FSH are low during oestrus phase and increase in the pro-oestrus phase. At met-oestrus phase, estradiols increases which peak at the pro-oestrus phase and returning to baseline level at the oestrus. Changes occurring in the mouse oestrous cycle are evident in the animal's physiology and anatomy. These changes can be detected using a variety of methods to determine the stage of oestrous which include evaluating vaginal

cytology, measuring electrical impedance, biochemical analysis of urine and visual observation of the external genital. Of these, evaluating vaginal cells is accepted as the most accurate method for identifying all stages of oestrous cycle although it is relatively labour intensive requiring vaginal cells to be collected, transferred to a glass slide, air dried, stained and viewed. The vaginal cytology method is best used when all 4 stages of the oestrous cycles need to be identified (Caligioni, 2009; Ramos et al., 2001; Achiraman et al., 2011). The characterisation of each phase is based on the percentage of cells observed in the vaginal smear; the epithelial cells, cornified cells and leukocytes as shown in Figure 2.8.

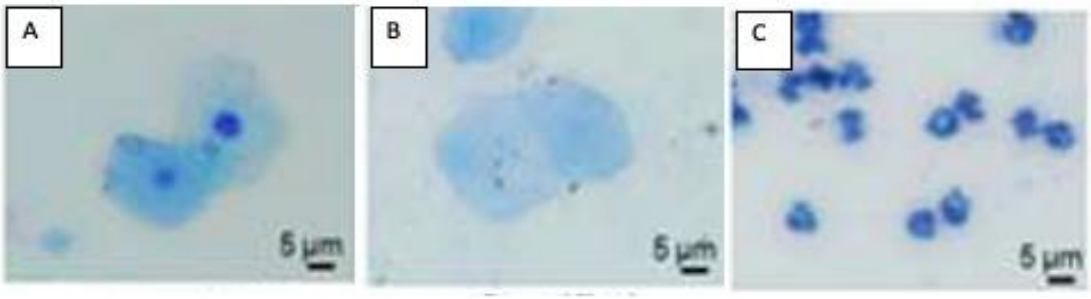

Figure 2.6 Characterisation of cells observed in vaginal smears A) epithelial cells B) cornified cells C) leukocytes cells

2.4 Medicinal plants

Natural products have been utilised as traditional medicine in many countries for various diseases. 50% of clinically approved drugs were based on natural products because they are not only effective but have less side effects compared to the synthetic drugs (Newman and Cragg, 2016). The World Health Organisation (WHO) has taken a big step by listing 21000 plants used for medicinal purposes around the world (Umashanker and Shruti, 2011). Active ingredients in medicinal plants such as flavonoid, phenolic acid and phenolic diterpenes (Pietta et al., 1998) have been shown to be more effective in most chronic diseases including cancer, cardiovascular disease and diabetes mellitus.

Some medicinal plants have multi-potential beneficial effects in PCOS, insulin resistance, hyperandrogenism, oligo/amenorrhea, and obesity. However, more pre-clinical and clinical studies are required to explore the effectiveness of herbal medicines in PCOS. Some plants such as *Asparagus racemosus, Grifola frondosa, Lepidium meyenii* and *Tinospora Cordifolia* have been highly regarded as sources that have the benefit of treating PCOS. The medicinal plants traditionally used in the treatment of PCOS that were scientifically studied are listed in Table 2.2.