POTENTIAL PROTECTIVE EFFECTS OF TUALANG HONEY AND *FICUS DELTOIDEA* JACK VAR. DELTOIDEA AGAINST BISPHENOL A INDUCED TOXICITY IN THE REPRODUCTIVE SYSTEM OF PRE-PUBERTAL FEMALE RATS

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2016

UNIVERSITY OF MALAYA

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ABSTRACT

Bisphenol A (BPA) is one of the most ubiquitous environmental endocrine disrupting chemicals that can disrupt the normal development and functions of the female reproductive system. In the last few decades, considerable amount of evidence has shown that young women are put at high risk of reproductive infertility from their routine exposure to numerous BPA-products since BPA induces increased production of reactive oxygen species (ROS), which is responsible for reducing the levels of endogenous antioxidant enzymes and increasing the levels of lipid peroxides. Thus, natural products containing high antioxidant properties such as tualang honey (Malaysian wild local honey) and *Ficus deltoidea* (Malaysian local herbal plant), were selected to study their possible potentials to counter the detrimental effects of BPA on the female reproductive system. The objective of the present study was to investigate the potential protective effects of Tualang honey and *Ficus deltoidea* against BPA-induced toxicity in the female reproductive system of prepubertal female Sprague Dawley rats. Animals were divided into six groups (n=8 in each group) that consist of (i) control group (received corn oil), (ii) BPA-exposed group (received BPA), (iii) TH+BPA group (received Tualang honey before receiving BPA), (iv) TH control group (received Tualang honey alone), (v) FD+BPA group (received *Ficus deltoidea* before receiving BPA) and (vi) FD control group (received *Ficus deltoidea* alone). The administration of the various treatment agents was performed once daily by oral gavage for six consecutive weeks. Uterine and ovarian toxicity of BPA-exposed rats were evident from the changes in the estrous cycle, disruption in the gonadotropins hormone levels (FSH and LH), follicular development and secretion of sexual steroid hormones (17β-estradiol and progesterone) by the ovary. BPA toxicity also results in disruptive effects on the uterus by inducing morphological abnormalities, increasing oxidative stress and dysregulating the expression and

distribution of the estrogen sensitive genes, ERα, ERβ and C3. Pretreatment with Tualang honey and *Ficus deltoidea* in the BPA-exposed rats showed significant protection on the reproductive system as shown by the increase in the percentage of rats with normal estrous cycle, increase in the level of gonadotropins hormone (FSH), reduction in the formation of the atretic follicles and normalization of the progesterone secretion by the ovary. In addition, there was lesser degree of abnormalities in the uterine and ovarian morphology and reduced disruptions at the transcriptional and translational levels of ERα, ERβ and C3, as well as reducing lipid peroxidation and subsequently the level of oxidative stress within the uterus. More importantly, there were no obvious estrous cycle, morphological, hormonal, as well as expression and distribution of ERα, ERβ and C3 changes observed in rats treated with Tualang honey and *Ficus deltoidea* alone. In conclusion, we suggest that Tualang honey and *Ficus deltoidea* have the potential protective role to counter the toxicity effects of BPA on the female reproductive system, possibly by their phytochemical properties, and further future studies can be conducted to determine the mechanisms involved in such activities.

ABSTRAK

Bisphenol A (BPA) adalah salah satu daripada bahan kimia pengganggu endokrin yang tertinggi terdapat di alam sekitar yang boleh mengganggu perkembangan normal dan fungsi sistem reproduktif betina. Dalam beberapa dekad kebelakangan ini, terdapat banyak bukti yang menunjukkan bahawa wanita muda berisiko tinggi mengalami masalah ketidaksuburan akibat rutin harian mereka kerap terdedah kepada produk-produk yang mengandungi BPA memandangkan BPA sebagai penyebab kepada pertambahan penghasilan Spesis Reaktif Oksigen (ROS) yang mana penyebab kepada pengurangan paras enzim antioksidan dalaman dan meningkatkan paras peroksida lipid. Dengan ini, produk-produk semulajadi yang mengandungi bahan-bahan antioksidan yang tinggi seperti madu Tualang (madu liar tempatan Malaysia) dan *Ficus deltoidea* (pokok herba tempatan Malaysia) telah dipilih untuk dikaji kemungkinan potensi mereka bagi mengatasi kesan-kesan kerosakan yang disebabkan oleh BPA ke atas sistem reproduktif betina. Objektif kajian ini adalah untuk mengkaji potensi kebolehan perlindungan Madu Tualang dan *Ficus deltoidea* ke atas kesan ketoksikan BPA pada sistem reproduksi tikus pra-baligh jenis *Sprague Dawley*. Haiwan kajian telah dibahagikan kepada enam kumpulan (n=8 dalam setiap kumpulan) iaitu terdiri daripada (i) kumpulan kawalan (diberi minyak jagung), (ii) kumpulan terdedah-BPA (diberi BPA), (iii) kumpulan TH+BPA (diberi Madu Tualang terlebih dahulu sebelum BPA), (iv) kumpulan kawalan TH (hanya diberi Madu Tualang), (v) kumpulan FD+BPA (diberi terlebih dahulu *Ficus deltoidea* sebelum BPA) dan (vi) kumpulan kawalan FD (hanya diberi *Ficus deltoidea*). Rawatan pelbagai bahan kajian dilakukan sekali sehari secara gavaj oral untuk tempoh enam minggu. Ketoksikan pada uterus dan ovari kumpulan tikus terdedah-BPA terbukti daripada perubahan kitar estrus, gangguan paras hormon gonadotropin (FSH dan LH), perkembangan folikel dan rembesan hormone seks steroid (17β-estradiol dan progesteron) oleh ovari. Ketoksikan BPA juga menghasilkan kesan-kesan gangguan ke

atas uterus iaitu menyebabkan keabnormalan morfologi, meningkatkan tekanan oksidasi dan gangguan pengawalaturan ekspresi dan taburan gen-gen sensitif estrogen, ERβ, ERα dan C3. Pra-rawatan dengan Madu Tualang dan *Ficus deltoidea* kepada tikus-tikus terdedah BPA telah menunjukkan kesan perlindungan terhadap sistem reproduksi dengan meningkatkan peratusan tikus-tikus dengan kitar estrus yang normal, meningkatkan paras hormon gonadotropin (FSH), mengurangkan penghasilan folikel atretik dan penormalan rembesan progesteron oleh ovari. Selain itu, hanya sedikit kadar ketidaknormalan morfologi dalam uterus dan ovari dan berkurangnya gangguan terhadap paras transkripsi dan translasi bagi ERβ, ERα dan C3, serta menurunkan peroksidasi lipid dan seterusnya paras tekanan oksidasi di dalam uterus. Apa yang lebih penting, tiada perubahan yang dikesan bagi kitar estrus, morfologi, hormon-hormon serta ekspresi dan taburan ERβ, ERα dan C3 pada tikus-tikus yang diberi rawatan Madu Tualang dan *Ficus deltoidea* sahaja. Sebagai kesimpulan, kami mencadangkan bahawa Madu Tualang and *Ficus deltoidea* mempunyai potensi sebagai pelindung kepada sistem reproduktif haiwan betina daripada kesan ketoksikan BPA, yang mana berkemungkinan disebabkan oleh kandungan fitokimia terkandung di dalam Madu Tualang dan *Ficus deltoidea*. Walau bagaimanapun, kajian lanjut adalah perlu bagi menentukan mekanisma yang terlibat.

ACKNOWLEDGEMENTS

Alhamdullilah, all praise to Allah SWT. First and foremost, I would like to express my deepest appreciation and gratidute to my supervisors, Prof. Dr. Normadiah M Kassim and Dr Shatrah Othman, as they have been tremendous mentors to me. I really appreciate their contribution in terms of times and ideas, as well as their continuous support, supervision and guidance that have finally made this research complete. Their valuable advice on both my research as well as my career have been priceless.

I gratefully acknowledge the funding sources that has made my PhD work achievable: University of Malaya for providing the Postgraduate Research Grant (PG087-2012B), and the Ministry of Higher Education and University of Putra Malaysia for awarding me the "Skim Latihan Akademik IPTA" (SLAI) fellowship for tutorship allowances.

I wish to thank all academic and non-academic staff of the Department of Anatomy and Department of Molecular Medicine, University of Malaya. Special thanks are due to Dr Intan and Dr Giri for being my unofficial mentors, who gave tireless advice in the molecular and immunohistochemistry staining works, respectively. To all my fellow friends: Helmi, Lina, Siti Rosmani, Asma, Huma and Dennis – thank you very much for your friendships as well as your good advice and collaboration throughout the study. In immunohistochemistry study, Helmi and Lina are the fellow lab mates who gave their tireless advice and assistance. In regards to the gene expression study, I am particularly indebt to Dennis, who gave his wonderful advice and specific guidance for all methods of purification, reverse transcription and real-time PCR.

Last but not least, to my beloved husband, Dr Faiz, I am truly grateful for your continuous support, understanding, patience and encouragement that inspire me to pursue and to complete my PhD study. To my two beloved sons, Hakeem and Harith, thank you for being such good boys that cheered me away from the worries.

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BPA	= Bisphenol A
EDCs	= Endocrine disrupting chemicals
PCOS	= Polycystic ovaries syndrome
LH	= Luteinizing hormone
FSH	= Follicle stimulating hormone
UVB	= Ultraviolet B
ELISA	= Enzyme-linked immunosorbent assay
TBARS	= Thiobarbituric acid reactive substances
PCR	= Polymerase chain reaction
MDA	= Malondialdehyde
ERα	= Estrogen receptor alpha
ERβ	= Estrogen receptor beta
C3	= Complement component 3
E_2	= Estrogen
SERM	= Selective estrogen receptor modulators
ERE	= Estrogen responsive element
DNA	= Deoxyribonucleic acid
RNA	= Ribonucleic acid
mRNA	= Messenger RNA
cDNA	= Complementary DNA

CHAPTER 1: INTRODUCTION

1.1 Introduction

In recent years, exposure to environmental toxicants has become a serious health concern. Anxiety over exposure to endocrine disrupting chemicals (EDCs) in human and wildlife has escalated since they have detrimental effects on the reproduction development and functions (Ibtihaq, Anisa, Eman, & Amany, 2011; Wetherill et al., 2007). One of the EDCs is the bisphenol A (BPA) that is widely used in the industries as plasticizer for the production of polycarbonate plastics and epoxy resins (Von Goetz, Wormuth, Scheringer, & Hungerbuhler, 2010). Exposure to BPA in humans received dramatic attention when it was detected in serum, follicular and amniotic fluids (Kuo & Ding, 2004), fetal serum (Sun et al., 2000), milk of nursing mothers (Brede, Fjeldal, Skjevrak, & Herikstad, 2003) and in the urine (Gould et al., 1998; Maffini, Rubin, Sonnenschein, & Soto, 2006). These findings have generated both scientific and public interests in assessing BPA as one of the potential EDCs to health risk.

Numerous studies have reported that BPA could induce alterations in both morphology and functions of the female reproductive system (Kuiper et al., 1998). Exposure to BPA cause disruptions of the uterine morphology, reducing the weight of uterus, dysregulating the expression of estrogen-sensitive genes ERα and ERβ as well as reducing the immunity via dysregulation of complement C3 expression (Schonfelder, Friedrich, Paul, & Chahoud, 2004; Seidlova-Wuttke, Jarry, & Wuttke, 2004). In the ovary, BPA exposure has been reported to have negative effects on the granulosa cell steroidogenesis (Biles, McNeal, & Begley, 1997; Markey, Wadia, Rubin, Sonnenschein, & Soto, 2005), reduces the pool of primordial follicles (Takeuchi & Tsutsumi, 2002), increases antral follicles while reducing the percentage of corpora lutea (Takeuchi,

Tsutsumi, Ikezuki, Takai, & Taketani, 2004). BPA also increases the risk for the development of polycystic ovaries syndrome (PCOS) (Xu et al., 2002) and decreases the levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels (Lee et al., 2003). As a consequence, these may predispose the tissue to earlier onset of diseases, reduced fertility and even cancer.

BPA exposure has been claimed to promote oxidative stress (OS) and inflammation of reproductive system in women (Kurosawa et al., 2002). Several compounds with antioxidant properties have been extensively studied to counter disease-associated OS (Kabuto, Hasuike, Minagawa, & Shishibori, 2003). Thus, with these concerns in mind, we propose to use natural products with high antioxidant activities, namely Tualang honey (Agromas, Malaysia) and *Ficus deltoidea* as potential therapeutics to counter the deleterious effects of BPA on the reproductive system. The beneficial effects of Tualang honey and *Ficus deltoidea* were claimed to originate mainly from their antioxidant properties (Al-Mamary, Al-Meeri, & Al-Habori, 2002; Aljadi & Kamaruddin, 2004; Khalil, Alam, Moniruzzaman, Sulaiman, & Gan, 2011; Mahaneem, Sirajudeen, Swamy, Nik, & Siti, 2010) .

Regarding to the reproductive system, the capabilities of Tualang honey shown to prevent uterine and vaginal atrophy (Zaid, Sulaiman, Sirajudeen, & Othman, 2010) as well as osteoporotic bone (Zaid et al., 2012) in postmenopausal animal model, protects rat testis against damage and oxidative stress induced by cigarette smoke (Mahaneem, Siti, Hasnan, & Kuttulebbai, 2011) has been scientifically proven. In streptozotoxin-induced diabetic rats, Tualang honey has shown to reduce the OS levels in the renal and pancreas (Erejuwa, Sulaiman, Wahab, Sirajudeen, et al., 2010). It also has the capability to induce antiproliferative effects on oral squamous cell carcinomas (OSCC) (Ghashm, Othman, Khattak, Ismail, & Saini, 2010), human osteosarcoma (Abdulmlik, Nor, Mohammed, Noorliza., & Rajan, 2010) and keloid fibroblasts (Mohamad, Ahmad, Siew,

& Shaharum, 2011).

Medicinal plants have been traditionally used in various parts of the world as traditional treatment for health maintenance. A medicinal plant named *Labisia pumila* ("Kacip Fatimah") has been reported to protect osteoporotic bone in estrogen-deficient rat model (Fathilah, Nazrun Shuid, Mohamed, Muhammad, & Nirwana Soelaiman, 2012) and protects skin cells from photo aging caused by UVB radiation (Choi et al., 2010). The other medicinal plant that is well known for its efficacy on erectile function improvement is the *Eurycoma longifolia* ("Tongkat Ali") (Kotirum, Ismail, & Chaiyakunapruk, 2015) while *Andrographis paniculata* ("Hempedu bumi") was reported to have anticancer and anti-malarial activities (Dua et al., 2004; R. A. Kumar, Sridevi, Kumar, Nanduri, & Rajagopal, 2004).

Extensive pharmacological studies have validated the traditional use of *Ficus deltoidea* ("mas cotek"), particularly for maintenance and fertility of the female reproductive system (Salleh & Ahmad, 2013). This medicinal plant has also been reported to have antidiabetic (Kalman, Schwartz, Feldman, & Krieger, 2013), anti-inflammatory and antinociceptive (Sulaiman et al., 2008), antimelanogenic and antiphotoaging (Oh et al., 2010), antibacterial (Samah, Zaidi, & Sule, 2012), wound healing (Abdalla, Ahmed, Abu-Luhoom, & Muhanid, 2010), anticancer and cytotoxicity activities (Farsi et al., 2013).

In this study, systematic analysis on investigating the effect of BPA on the female reproductive system was conducted. Prepubertal female rats were exposed to BPA by oral gavage over a six weeks period. Using an image analyzer, we investigated the morphological changes in the uterus to verify whether exposure to BPA induced abnormalities in the cellular level. The morphology of the ovary was also investigated to verify whether BPA induced disruption to the follicular development. Disruptive effects of BPA on the reproductive system would also include changes in the estrous cycle,

reproductive hormones and lipid peroxidation. Patterns of estrous cycle were detected by daily evaluation of vaginal smears. All serum blood hormone levels of 17β-estradiol, FSH, LH and progesterone were measured using ELISA method.

In uterus, Thiobarbituric Acid Reactive Substances (TBARS) assay was used to measure lipid peroxidation level. Subsequently, using the real-time PCR approach, we investigated the differences in the mRNA expression of closely-related estrogen-sensitive genes, ERα, ERβ and C3 to examine the possible disruptive effect of BPA at the gene transcription level that may affect the uterus. The protein expressions of these genes in the uterus were also analysed to verify the consequences at the protein level.

1.2 Objectives of the study

1.2.1 General objectives

Many reports have been demonstrated that early exposure of life to numerous products of BPA might led to high risk of permanent reproductive infertility (B. Yi et al., 2011). In many reports, Tualang honey and *Ficus deltoidea* have been scientifically proven to have protective effects on the female reproductive system. However, thus far, no scientific studies have been conducted to investigate the potential protective effects of these natural products in preventing the disruptive effects of BPA on the reproductive system. To address the lack of information, our present study was designed to elucidate comprehensively and systematically the potential protective effects of Tualang honey and *Ficus deltoidea* against BPA induced toxicity of the reproductive system of prepubertal female rats. It is hoped that the findings from our study will provide new important scientific information to support the rational intake of natural products in daily life to prevent further reproductive toxicity due to BPA exposure.

1.2.2 Specific objectives

1. To investigate the ability of Tualang honey and *Ficus deltoidea* in reducing the disruptive effects of BPA on estrous cycles, follicular development of the ovary as well as morphology and morphometry of the uterus.

2. To investigate the ability of Tualang honey and *Ficus deltoidea* in normalizing the disruptive effects of BPA on the gonadotropins (FSH and LH) and steroid hormones (17β-estradiol and progesterone).

3. To investigate the ability of Tualang honey and *Ficus deltoidea* in reducing the disruptive effects of BPA on lipid peroxidation (MDA level) as well as expression of estrogen-sensitive genes and proteins of ERα, ERβ and C3 in the uterus.

1.3 Hypothesis of the study

1.3.1 General hypothesis

The general hypothesis is that Tualang honey and *Ficus deltoidea* have potential protective effects to reduce toxicity induced by BPA in the female reproductive system.

1.3.2 Specific hypothesis

1. BPA disrupts the normal estrous cyclicity and concurrent treatment with either Tualang honey or *Ficus deltoidea* can prevent these changes.

2. BPA causes regression in the morphology of the reproductive organs (uterus and ovary) and disrupts the normal ovarian follicular development. Concurrent treatment with either Tualang honey or *Ficus deltoidea* may prevent these changes.

3. BPA disrupts the levels of gonadotropins and steroid hormones (FSH, LH, 17-β estradiol and progesterone). Concurrent treatment with either Tualang honey or *Ficus deltoidea* can improve the levels of these hormones.

4. BPA increases the level of lipid peroxidation (MDA). Concurrent treatment with either Tualang honey or *Ficus deltoidea* can reduce the MDA levels.

5. BPA disrupts the expressions of estrogen-sensitive genes and proteins (ERα, ERβ and C3). Concurrent treatment with either Tualang honey or *Ficus deltoidea* can prevents these disruptive changes.

1.4 Significance of the study

Tualang honey and *Ficus deltoidea* are natural products that have been extensively evaluated for its nutritional and medicinal properties. Thus, this study provides scientific information regarding the potential protective effects of Tualang honey and *Ficus deltoidea* in reducing the disruptive effects of BPA on the reproductive system.

CHAPTER 2: LITERATURE REVIEW

2.1 Bisphenol A

2.1.1 Historical background of Bisphenol A

Bisphenol A (BPA) was discovered in 1891 by a Russian chemist, Aleksandr Dianin. In 1905, a German chemist by the name of Theodor Zincke found a formula to synthesize BPA by a condensation reaction of phenol with acetone (2 to 1 ratio, respectively) in the presence of strong acid catalyst (hydrochloric acid) (Huang et al., 2012) (Figure 2.1).

In the middle of the 20th century, the usefulness of BPA became evident when a Bayer chemist, Dr. Hermann Schnell discovered an efficient formula to synthesize polycarbonates by reacting BPA with phosgene. In October 1953, a new material was patented under the name Makrolon and since 1960, the production of polycarbonate has rapidly increased to industrial levels (Allard & Colaiacovo, 2011). From that moment until now, BPA has been widely used in the plastic industry. Currently, world leading BPA manufactures include BASF, Bayer Material Science, Dow Chemicals, Hexion Specialty Chemicals, SABIC Innovative Plastics, Shell and Sunoco chemicals.

BPA is an organic compound that consists of two phenolic rings connected by a single carbon carrying two methyl groups (Figure 2.2). It is a monomer that is used extensively in the manufacturing of polycarbonate, epoxy resins and as a non-polymer additive in plastics such as polyvinyl chloride (PVC) (Welshons, Nagel, & vom Saal, 2006). It is a convenient compound for the manufacturing industries because BPA-based plastics are lightweight, clear and highly durable.

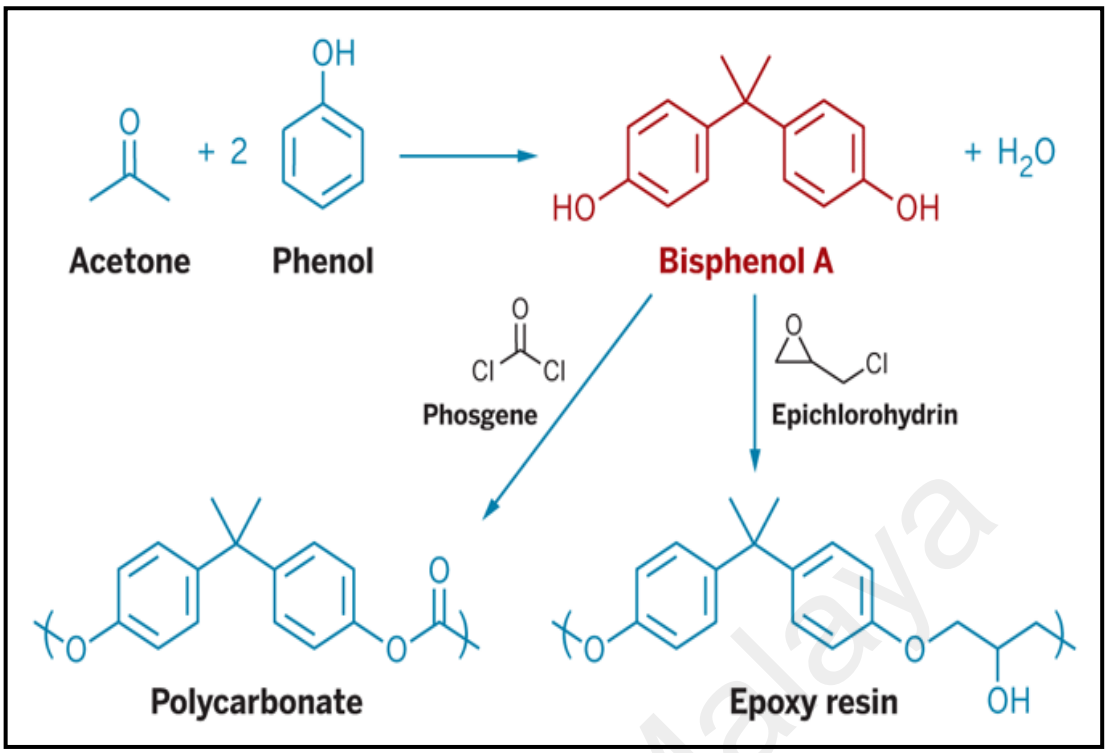

Figure 2.1: Synthesis of Bisphenol A for the production of polycarbonate and epoxy resin. (Adapted from: http://www.pubs.acs.org)

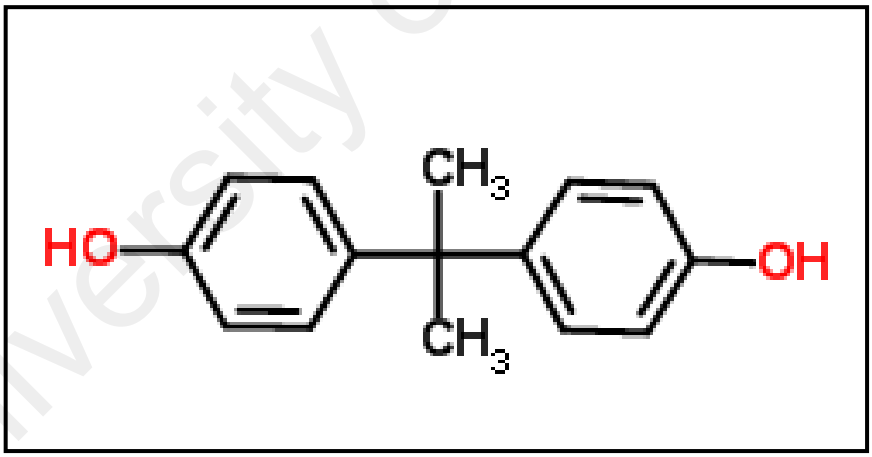

Figure 2.2: Chemical structure of Bisphenol A. www.chemspider.com)

Epoxy resins which are commonly formed using BPA, are widely used as the inner coating of food and beverage cans. Furthermore, BPA is widely used in numerous products such as digital media (CDs and DVDs), electronic equipment, automobiles, construction glazing, sports safety equipment, medical devices, tableware, reusable bottles (e.g., baby milk bottles), eyeglass lenses, toys, water supply pipes, some flame

retardants and food containers (Huang et al., 2012; Jain, Mahendra Kumar, Umesh, & Pramod, 2011; Santhi, Sakai, Ahmad, & Mustafa, 2012) (Figure 2.3). Thus, BPA is one of the highest volume compound produced worldwide with the global demand observing dramatic increase from 3.9 million tons in 2006 to about 5 million tons in 2010 with over 100 tons released into the atmosphere by yearly production (Alonso-Magdalena et al., 2012; Santhi et al., 2012).

2.1.2 Sources of Bisphenol A

BPA is highly produced in the environment particularly from anthropogenic activities. Unfortunately, BPA is directly released into the water bodies and atmosphere during its manufacturing process. In addition, unreacted or uncured BPA is indirectly released to the air during processing and handling of various commercial products (polycarbonate and epoxy resins). Other potential exposure to BPA sources are via oral intake, contaminated water, soil, sediment and landfill leakages.

Oral intake was suggested to be the primary source of human exposure to BPA (Huang et al., 2012). It is expected that the most significant migration of BPA by oral intake comes from canned food lined with epoxy resins, drinking water in polycarbonate bottles and saliva from dental sealants (De Coensel, David, & Sandra, 2009; Grumetto, Montesano, Seccia, Albrizio, & Barbato, 2008; Le, Carlson, Chua, & Belcher, 2008; Maia et al., 2010) (Figure 2.3). Numerous studies have found BPA leaching from epoxy resins lining in canned pet foods (Kang & Kondo, 2002), vegetables (Brotons, Olea-Serrano, Villalobos, Pedraza, & Olea, 1995; Yoshida, Horie, Hoshino, & Nakazawa, 2001), fish (Munguia-Lopez, Gerardo-Lugo, Peralta, Bolumen, & Soto-Valdez, 2005) and infant formula (Biles et al., 1997; Kuo & Ding, 2004) (Figure 2.4).

The main roles of epoxy resin as inner coating of metallic food cans are to protect from rusting and corrosion. This resin is synthesized by the condensation of BPA with epichlorohydrin to create a compound called BADGE (bisphenol A diglycidyl ether). However, incomplete polymerization during the processing of plastic container may lead to BPA contamination in the stored food. In 2002, Takao et al reported on the influence of high temperature on the release of BPA from epoxy resin lining that lines metal cans. He found that BPA concentration was increased by an average of 18 times higher when the epoxy resins lining was heated at 100˚C.

A plastic container with a level of 30 µg/g BPA has a potential to dispense 6.5 µg of BPA to food (Nerin, Fernandez, Domeno, & Salafranca, 2003). According to the food packaging investigation report, polyvinyl chloride stretch films contained BPA at measurable levels that ranged from 43 to 483 mg/kg film (Lopez-Cervantes & Paseiro-Losada, 2003). These reports have illustrated the risks of BPA contamination of consumer food products. Other chemical analysis has found lower range of BPA (0.19 to 26 mg/kg) in food packed with recycled paper products (Brede et al., 2003; Lopez-Espinosa et al., 2007; Mountfort, Kelly, Jickells, & Castle, 1997; Sajiki & Yonekubo, 2004; Sun et al., 2000).

Since 1960's, BPA diglycidyl methacrylate has been used widely in the manufacture of dental products. About 60% to 80% of this monomer is polymerized *in situ* but the unpolymerized may leach into saliva and absorbed by the body. The risk of BPA exposure from dental sealants was evident from a study by Olea et al (1996) that found that the levels of BPA in saliva of 18 adult patients was between 3.3 to 30.0 µg/ml following one hour application of 50 mg of dental sealant to 12 molars.

Figure 2.3: Examples of baby milk bottles which are made from polycarbonate plastics. (www.baumhedlundlaw.com)

Figure 2.4: Examples of epoxy resin used as inner coating of metalic food cans. (www.cleveland.com)

Contamination of water source by BPA could induce wide range of health risk in human. In a study by Santhi et al (2012), the author indicated that 93% of Langat River are contaminated with BPA. More seriously, this study also found out that the BPA levels in the water samples obtained near the industrials and sewage treatment plants outlets were six fold-higher than in the Malaysian rivers. In addition, BPA levels that were detected in tap water ranged from 3.5 to 59.8 ng/L with the highest level detected in samples collected from PVC pipes and water filter devices. Typically, BPA concentrations are much higher in cities which are located in highly developed industrial and commercial regions (Huang et al., 2012). Additionally, BPA pollution has also been detected in underground waters (Figure 2.5). Several studies have shown that BPA can be detected in landfill leachates. In Japan, a study by Kawagoshi et al. (2003) has identified the leaching of BPA from landfills as the main contributor for the EDCs pollution into the groundwater source (740 ng/ml) (Kawagoshi, Fujita, Kishi, & Fukunaga, 2003). This study claimed that the high level of BPA in leachates was contributed by the large volume of plastic wastes in the landfill.

Air is another potential source for BPA exposure to human. In an urban setting, concentrations of BPA in Osaka (2001), Sapporo (2009), Chennai (2007), Mumbai (2008) and Auckland (2004) were in the range of 10 to 1920 $\mu g/m^3$, 70 to 930 $\mu g/m^3$, 200 to 17400 $\mu g/m^3$, 100 to 9820 $\mu g/m^3$ and 4 to 1340 $\mu g/m^3$, respectively (Huang et al., 2012). In fact, concentrations of BPA in indoor air are found to be significantly higher than those in the outdoors due to the presence of household goods at home and furnishing materials in the office (Wilson, Chuang, & Lyu, 2001; Yasuhara et al., 1997). In one survey, BPA has been found to be present in 86% of house dust samples at concentrations ranging from 0.2 to 17.6 µg/g (Rudel, Camann, Spengler, Korn, & Brody, 2003).

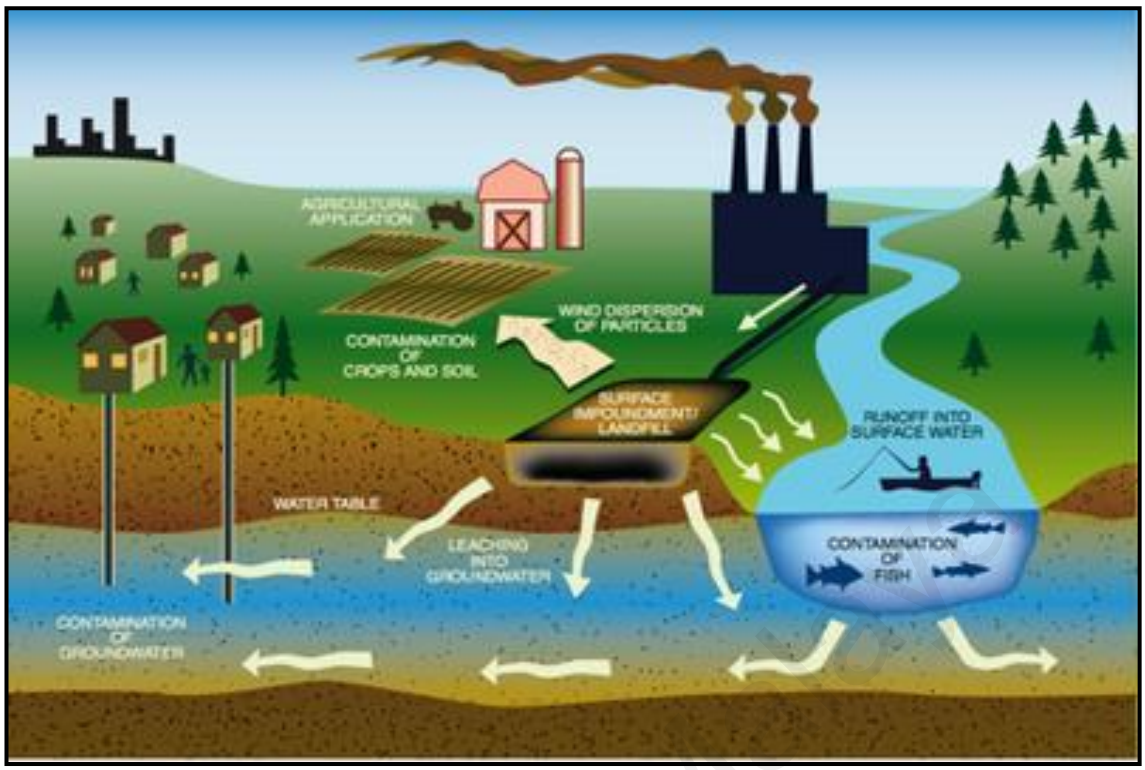

Figure 2.5: Leaching of landfill leachates into the groundwater sources. (www.geltechsolutions.com)

2.1.3 Detection of Bisphenol A levels in human

In the previous decades, numerous studies on BPA levels in human fluids and tissues have been conducted. Daily human exposure to BPA was estimated to be 0.48 to 4.8 µg/kg body weight/day (Kang, Kondo, & Katayama, 2006). Starting from 1999, several analytical techniques such as gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC), derivation with different chemical agents followed by gas chromatography (GC) and enzyme-linked immunosorbent assay(ELISA) have been used to determine BPA levels in human serum (Sajiki, Takahashi, & Yonekubo, 1999).

Several studies on pregnancy-associated fluids have detected the BPA levels in serum of pregnant women, umbilical cord blood and fetal plasma (Ikezuki, Tsutsumi, Takai, Kamei, & Taketani, 2002; Schonfelder et al., 2002; Yamada et al., 2002) (Table

2.1). In 2002, Ikezuki et al. has shown that the levels of BPA in human maternal sera during early pregnancy, late pregnancy as well as in umbilical cord serum and follicular fluid were 1.5 ng/mL, 2.2 ng/mL and 2.4 ng/mL respectively. However, the level of BPA in fetal amniotic fluid dropped dramatically to 1.1 ng/mL during late gestation period. Meanwhile, another study has found that the BPA levels in the umbilical cord and placenta sera were at 2.9 ng/mL and 11.2 ng/g, respectively (Schonfelder et al., 2002).

Results from these studies on BPA levels in pregnancy-associated fluids also indicate that BPA could traverse the maternal-fetal placental barrier. More seriously, BPA is a lipophilic compound that can dissolve into fat and breast milk (Vandenberg, Hauser, Marcus, Olea, & Welshons, 2007), thus, developing neonate has a risk of BPA exposure from the breast feeding mother. In addition, a study by Kuruto et al (2007) has demonstrated the concentration of BPA was at a range of 1 to 7 ng/mL in 101 human colostrum samples. Colostrum is the first lacteal secretion produced by the mammary gland prior to the production of milk. It is produced in a small quantity and it has high levels of antibodies, carbohydrates, protein and low level of fat. However, lower level of BPA was found at a range of 0.28 to 0.97 ng/mL in breast milk (Sun et al., 2004).

Limited numbers of studies were conducted to examine BPA levels in other bodily fluids, namely the follicular fluid and semen. In one study, 2.0 ng/mL of BPA was detected in follicular fluid from women undergoing *in vitro* fertilization (IVF) procedures (Ikezuki et al., 2002) while in semen, BPA was measured at 2.0 ng/mL by ELISA detection system and 0.5 ng/ml by HPLC-MS method (Inoue et al., 2002). Additionally, urinary BPA concentrations have also been measured in human urine worldwide.

In one study, BPA glucuronide was detected in urine samples of 48 female Japanese college students at concentrations ranging from 0.2 ng/mL to 19.1 ng/mL (Ouchi & Watanabe, 2002). BPA levels were measured in the morning spot urine samples from two

different years of Japanese university students. Overall, the urinary BPA levels in the students in 1992 were significantly higher than those in 1999. This study assumed that canned beverages (coffee and tea) may be a contributory factor for BPA exposure in the 1992 cohort but not in the 1999 cohort. They also suggested that the decreasing levels of BPA in 1999 could also be due to the recent changes in the canning process.

Table 2.1: BPA levels in human serum and tissues (Vanderberg et al., 2007)

Human serum/tissue	Detected levels [ng/mL (ppb), mean±S.E.M)
Healthy human serum	0-1.6
Female non-pregnant serum	2.0±0.146
Early pregnancy serum	1.5±0.197
Late pregnancy serum	1.4±0.148
Fetal (cord) serum	2.2±0.318
Amniotic fluid (15-18 weeks)	8.13±1.573
Late amniotic fluid	1.1±0.162
Follicular fluid	2.4±0.133
PCOS female serum	1.04±0.1
Normal female serum	0.64±0.1
Serum-non-obese PCOS	1.05±0.10
Serum-non-obese normal	0.71±0.09
Serum-obese-PCOS	1.17±0.16
Serum-obese normal	1.04±0.09
Abnormal fetal karyotype maternal serum	2.97 (median)
Normal maternal serum	2.24 (median)
Serum women with recurrent miscarriage	2.59±0.780
Serum control healthy women	0.77±0.067
Human colostrum	3.41±0.013
Breast milk	0.61±0.042
Saliva immediately after Delton sealant application	42.8±10.22
Saliva prior to dental sealant application	0.30±0.043
Serum from women with simple endometrial hyperplasia	2.9±0.632
Serum from healthy control women, normal endometrium	2.5±0.452

2.1.4 Pharmacokinetics of Bisphenol A

In human, BPA (unconjugated form) that is orally administered will rapidly be absorbed from the gastrointestinal tracts (Figure 2.6). Then, this biologically active form of BPA (unconjugated) will rapidly metabolize in the liver by glucuronic acid (first-pass-metabolism process) to the glucuronide which is a main conjugated form of BPA (biologically inactive metabolite) (Volkel, Colnot, Csanady, Filser, & Dekant, 2002). Finally, this conjugated BPA is rapidly cleared (within 24 hours) from blood via excretion through the urine, feces and sweat (Genuis, Beesoon, Birkholz, & Lobo, 2012). In fact, the bioavailability and biotransformation of BPA in the body system are significantly dependent on the route of exposures whether by oral, intraperitoneal or subcutaneous (Volkel, Colnot, Csanady, Filser, & Dekant, 2002). Oral exposure has lower bioavailability of BPA compared to the other routes due to intensive biotransformation of BPA in the liver (Matthews, Twomey, & Zacharewski, 2001; Pottenger et al., 2000).

Compared to the human, BPA in rats is metabolized by enterohepatic circulation process (Pottenger et al., 2000) (Figure 2.7). The enterohepatic circulation starts from absorption of BPA by the small intestines, then passes to the liver via the hepatic portal vein for metabolization process and its metabolites travel back into the small intestine and liver before being excreted in the feces (Pottenger et al., 2000). In fact, this enterohepatic circulation of BPA glucuronide does not occur in human due to higher threshold of biliary elimination of BPA in human compared to the rats (route for the elimination of drugs or other substances) (Dobrinska, 1989).

In fact, the disruptive effects of BPA in the body still occur even after its rapid elimination. In many tissues, β-glucuronidase enzyme is able to reverse conjugation of BPA (conjugated) to its unconjugated form (biologically active form) (Ginsberg & Rice, 2009). Thus, some portions of BPA still accumulate and are biologically active in many

tissues to exert a variety of disruptive effects. In addition, 3D models study has identified that 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP) is a biologically active metabolite of BPA with its transcriptional activity to ERs being 1000-fold higher than BPA (Baker & Chandsawangbhuwana, 2012). According to this study, first phenolic ring of BPA is able to mimic the binding activity of A ring of E_2 to the ERs. However, the second ring of BPA has a shorter distance or lacks of some key contacts that exist between E_2 and ERs (Figure 2.8). Therefore, this could be an explanation to the lower estrogenicity of BPA compared to the E_2. However, metabolism of BPA to its metabolite, MBP increases the spacing between the two phenolic rings that mimic the contact between E_2 with the ERs and finally interfere with the normal functions of E_2.

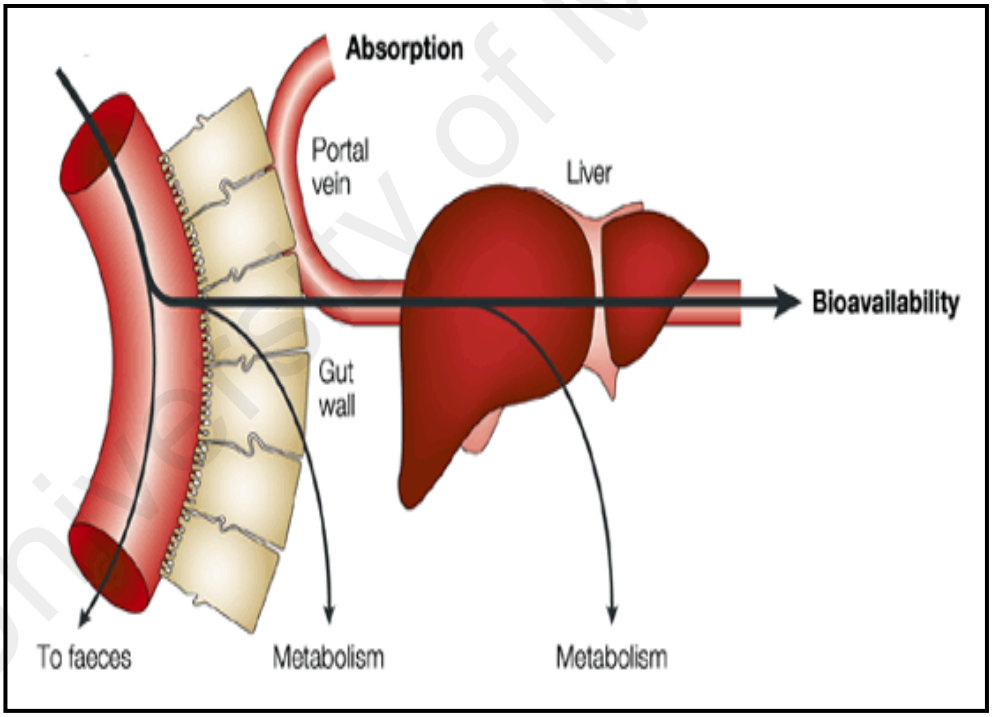

Figure 2.6: Process of the first-pass-metabolism of BPA in the human liver (https://canna-pet.com).

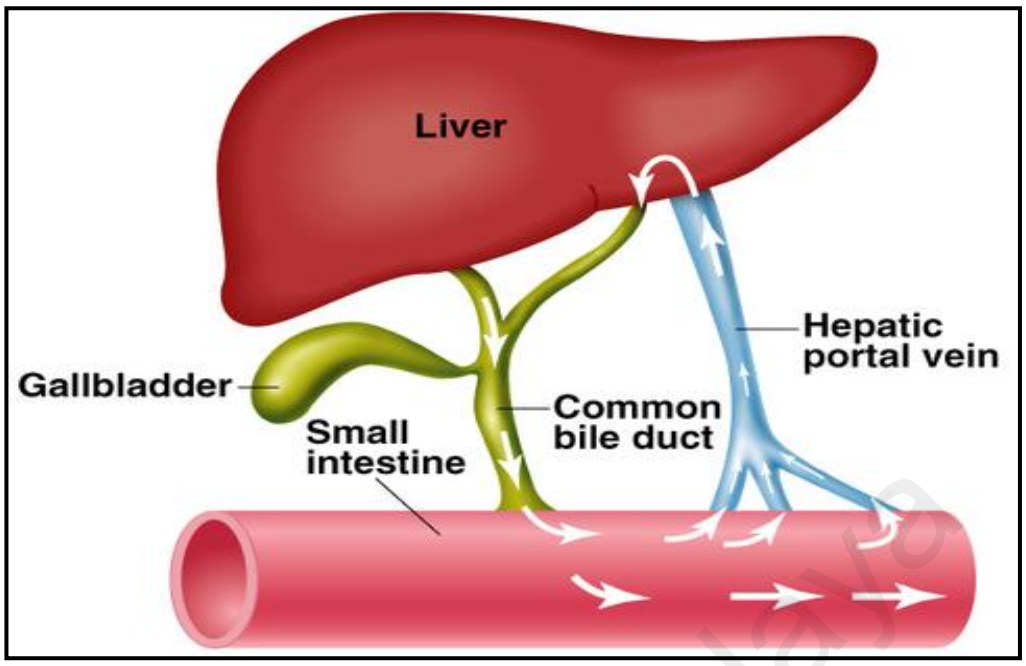

Figure 2.7: Process of the enterohepatic circulation of BPA in rats (www.quizlet.com).

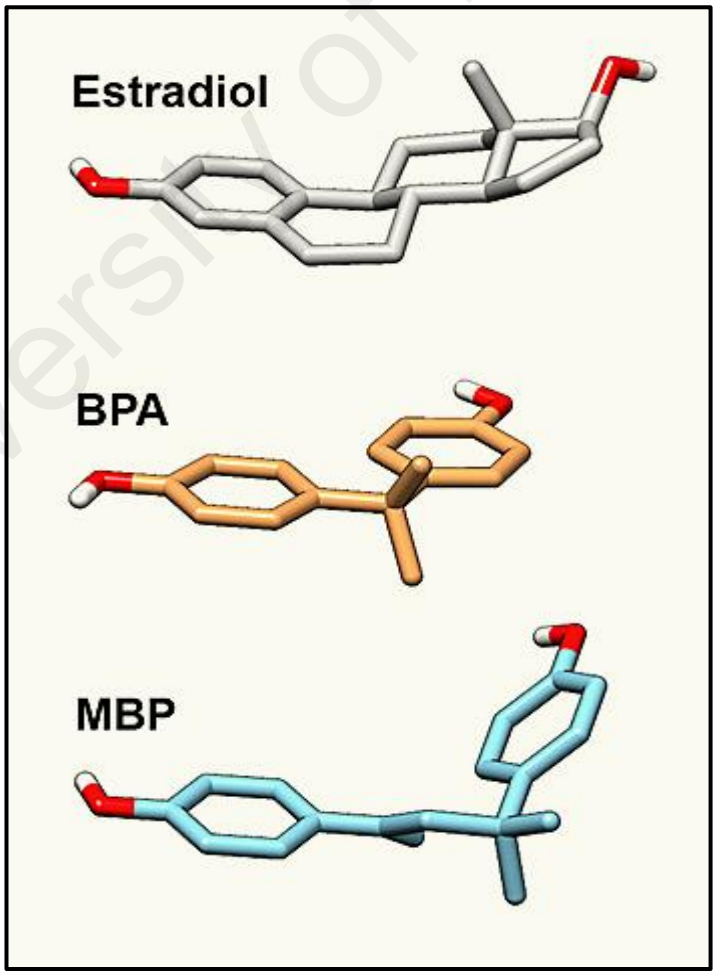

Figure 2.8: 3D models structures of E2, BPA and MBP (ucsdnews.ucsd.edu).

2.1.5 Mechanisms of action and effects of Bisphenol A

The endocrine system is a communication system in the body that maintains normal physiological balance in various organ systems by regulating the activity of body system in reaction to variations in body temperature, activity level, stress and circulating levels of nutrients and hormones required for growth, reproduction and metabolism (Kolle et al., 2012) (Figure 2.9). According to Jintelman et al (2003), the primary function of an endocrine system is to transform exogenous stimuli into chemical messengers and hormones which result in the expression of genes and synthesis of proteins and/or activation of already existing tissue-specific enzymes.

According to the World Health Organization (WHO) in 2002, an endocrine disruptor is defined as "an exogenous substance or mixture that alters normal functions of an endocrine system and consequently causes adverse effects in an intact organism, or its progeny". Similarly, The U.S.- Environmental Protection Agency (EPA) has defined an endocrine disruptor as "an exogenous agent that interferes with the synthesis, secretion, transportation, binding, action and elimination of natural hormones in the body, which are responsible for the maintenance of homeostasis, reproduction, development and behaviour" (Wetherill et al., 2007).

The most prominent classes of chemicals containing EDCs are synthetic hormones, pesticides, BPA, phthalates, parabens, organic solvents, organohalogens (Gultekin & Ince, 2007), some metals as well as plant constituents (phytoestrogens) (Harvey & Darbre, 2004; Lottrup et al., 2006; Luderer et al., 2004; Maffini et al., 2006; North & Golding, 2000; Queiroz & Waissmann, 2006). There are three major endocrine disruption endpoints including estrogenic (mimics or block natural estrogens activities), androgenic (mimic or block natural testosterone activities) and thyroidal (directly or indirectly impacts on the thyroid functions) (Synder, Westerhoff, Yoon, & Sedlak, 2003).

Furthermore, endocrine disrupting chemicals also can disrupt the synthesis, metabolism and clearance of endogenous hormones and thereby influence hormone bioavailability (Brouwers et al., 2007). EDCs are associated with a wide variety of adverse health effects in organism and/or their progeny including disorders of the reproductive system and reduction in reproductive fitness, hormone-dependent cancers (Alum, Yoon, Westerhoff, & Abbaszadegan, 2004; Synder et al., 2003), urogenital birth defects (Brouwers et al., 2007) and sometimes unpredictable consequences (Figure 2.10).

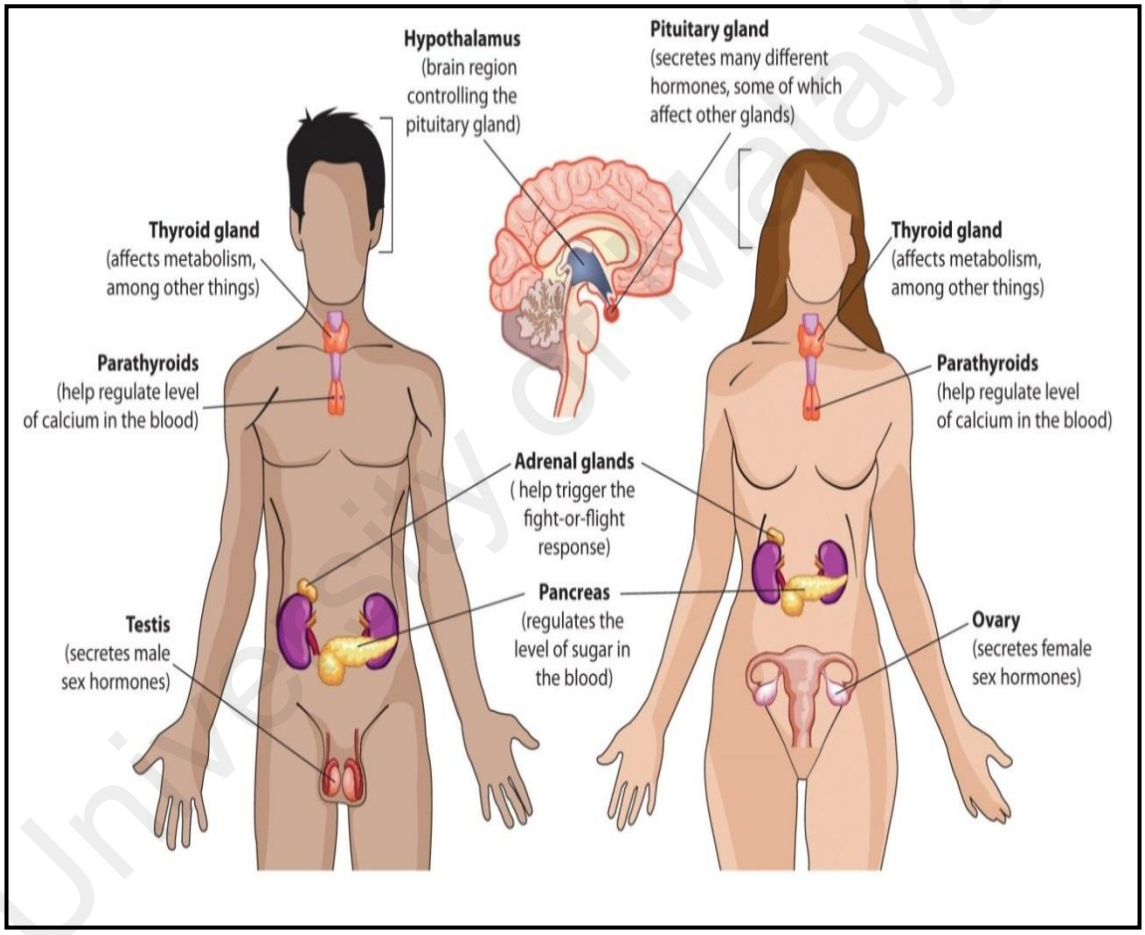

Figure 2.9: Major endocrine systems in the human body (www.premedhq.com).

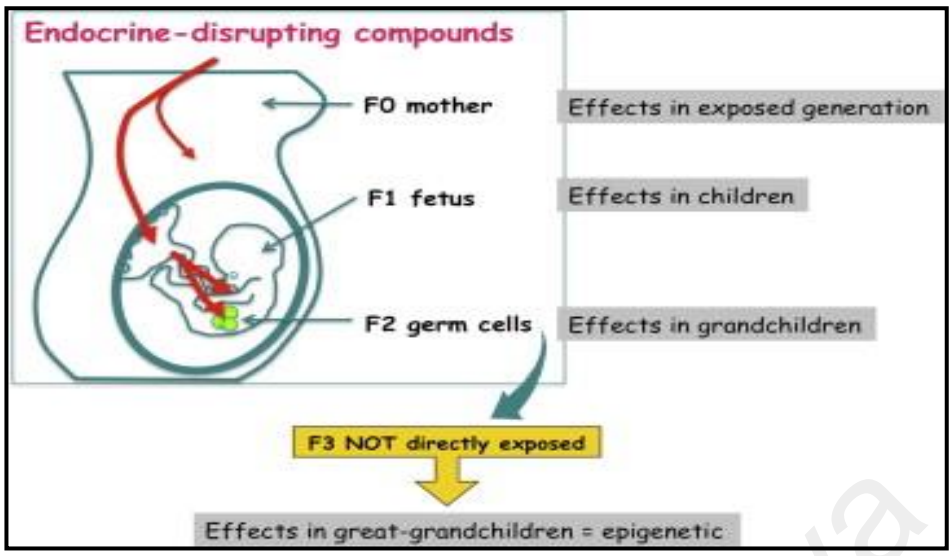

Figure 2.10: Transgenerational effects of BPA. FO mother who is directly exposed to BPA, that will also directly expose her F1 fetus to BPA disturbance. The F2 is also affected to the BPA disturbance via F1 germ cells. Postnatal F3 generation may show BPA disruption without direct exposure of BPA in the F1 and F2 generations (Fowler et al., 2012).

BPA is one of the ubiquitous xenoestrogens, which has been classified as an EDC. Throughout the years, BPA has been narrowly defined as environmental estrogen or selective estrogen receptor modulator (SERM). Such definitions were adopted due to its pleiotropic mechanisms of actions that bind to nuclear estrogen receptors either by agonist and/or antagonist actions. More seriously, BPA was incorrectly pointed that it can mimic all actions of natural estrogens (Welshons et al., 2006). However, the use of these self-limiting definitions is indeed inaccurate. A study by Seidlova-Wuttke et al (2004) has confirmed that BPA is not purely estrogenic in the reproductive tissues and bone. BPA has been found to have no significant effect on uterus weight but reduces the thickness of endometrial and myometrial layers and bone density. Quantitative RT-PCR analysis also revealed the dissimilarities effects of BPA with estradiol, particularly in ERα, ERβ and complement component 3 (C3) mRNA expressions. Hence, BPA cannot be defined to have 'classical estrogenic effects'. In addition, BPA is not a 'selective' estrogen receptor because it also binds to other nuclear receptor such as androgen and thyroid receptors (Lee, Chattopadhyay, Gong, Ahn, & Lee, 2003).

Estrogen receptor (ER) is a member of steroid receptor superfamily, a ligand-activated enhancer protein that is activated by the hormone estrogen (17β-estradiol) and is able to regulate gene transcription via estrogen responsive element (Klinge, 2001). Unfortunately, it can also be activated by other compounds including endocrine disrupting chemical such as BPA (Hiroi et al., 1999). ER is encoded by two subtype genes, namely the ERα and ERβ, with their functions as signal transducers and transcription factors in modulating the expression of target genes (Couse & Korach, 1999). The endogenous estrogen (17β-estradiol) has a lower binding affinity to ERβ than ERα (Kuiper et al., 1997) but both receptors share similarity in terms of transactivation via estrogen responsive element (ERE) (Pace, Taylor, Suntharalingam, Coombes, & Ali, 1997). In contrast, they possess dissimilar functions with regards of their roles in transcription activation, which depend very much on the ligands and their responsive elements (Peach et al., 1997). Complement C3 is involved in innate immunity. The crucial role of C3 is to regulate any activation of host cell damage by promoting phagocytosis, initiating local inflammatory responses against pathogens and to instruct adaptive immune response to select appropriate antigens for a humoral response (Sahu & Lambris, 2001).

Natural estrogens have been defined as a group of steroid hormones secreted primarily from the ovaries of premenopausal women and they are also produced by other organs or tissues including adrenal gland, placenta, testes, adipose tissues and brain (Okoh, Deoraj, & Roy, 2010). 17β-estradiol (E_2) is a predominant circulating endogenous estrogen and is the most biologically active ovarian hormone (Alonso-Magdalena et al., 2012). It has a half-life of about three hours and is consequently subjected to a very rapid and irreversible oxidation into the estrogen metabolites estrone (E_1) and estriol (E_3) (Okoh, Deoraj, & Roy, 2010). Estrogen plays a crucial role in sexual determination, promote the growth and maintenance of female reproductive system and in controlling

the menstrual cycle and pregnancy (Ferreira, Westers, Albergaria, Seruca, & Hofstra, 2009). Besides these crucial functions, estrogens also play important roles in bone strengthening, cholesterol metabolism, influencing the central nervous system and gastrointestinal physiology (Nilsson & Gustafsson, 2000; Roy & Liehr, 2000).

It has been long acknowledged that BPA is a weak estrogen since its binding affinity to the ERα and ERβ is approximately 1000 to 10000-fold less than the natural hormone estradiol (Matthews et al., 2001; Routledge, White, Parker, & Sumpter, 2000) (Figure 2.11). BPA may bind to estrogen receptors but it has a relatively higher binding affinity by 6.6-fold with ERβ compared to ERα (Alonso-Magdalena et al., 2012; Kuiper et al., 1997; Matthews et al., 2001). In a particular cell or tissue type, BPA exhibits estradiol-like agonist activity via ERβ but a mixed agonist and/or antagonist activity via ERα (Kurosawa et al., 2002). Numerous studies have shown that BPA interrupts the normal activity of endogenous estrogens (17β-estradiol) by disrupting the proper activities of estrogen nuclear hormone receptors in various tissues (Ackermann, Brombacher, & Fent, 2002; Adachi et al., 2005; Gould et al., 1998; Hall & Korach, 2002; Kuiper et al., 1997; Kurosawa et al., 2002; Matthews et al., 2001; Mueller et al., 2003; Nagel et al., 1997; Olsen, Meussen-Elholm, Samuelsen, Holme, & Hongslo, 2003; Pennie, Aldridge, & Brooks, 1998; Recchia et al., 2004; Routledge et al., 2000; Satoh, Ohyama, Aoki, Iida, & Nagai, 2004; Seidlova-Wuttke et al., 2004; Vivacqua et al., 2003; Watson, Campbell, & Gametchu, 1999).

In 1938, Dodds and Lawson have successfully proven that BPA has estrogenic properties by injecting 100 mg/kg of BPA in ovariectomized rats (Allard & Colaiacovo, 2011). This injection has successfully induced an estrus phase in the rats. Five decades later in 1993, a group of scientists inadvertently rediscovered the estrogenic activity of BPA when it leached from polycarbonate flasks during autoclaving into cell culture media. Following that, further experiments were conducted to confirm the findings. From

the experiments, they have found that BPA could elicit an estrogenic response in a well-established estrogen responsive cell line (MCF-7) that showed competitive binding of BPA to estrogen receptor and induction of progesterone receptors with the lowest effective dose of 10 to 20 nM (Krishnan, Stathis, Permuth, Tokes, & Freldman, 1993). In fact, two years earlier in 1991 the estrogenic properties of nonyl-phenol released from polystyrene were discovered by Soto and colleagues. These experiments findings have led to further research on the effects of BPA exposure in animal models and humans.

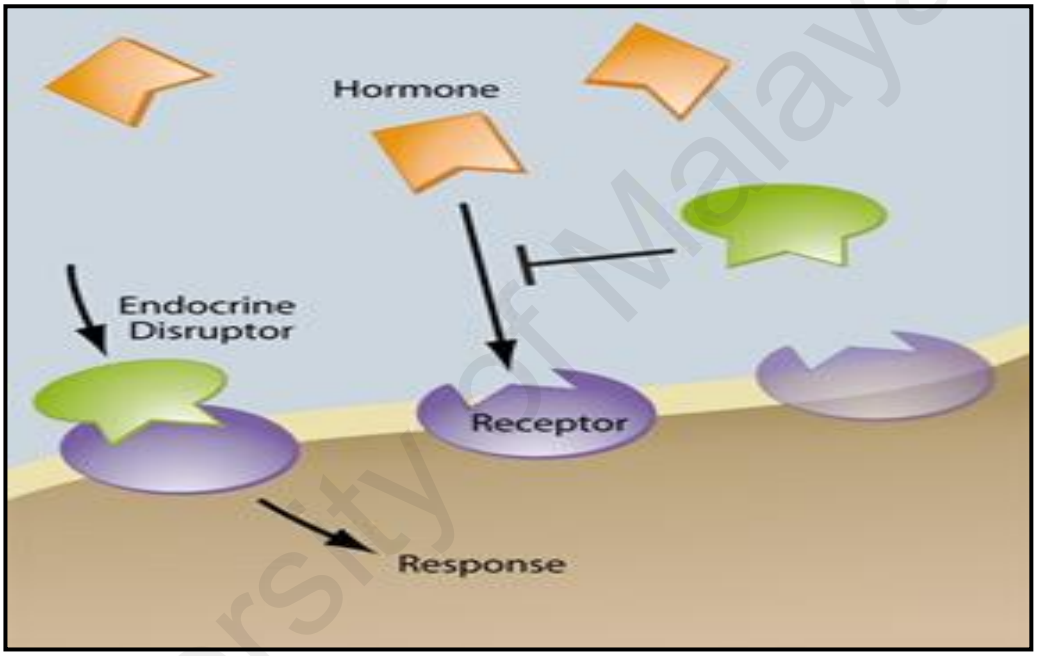

Figure 2.11: BPA interrupts the normal activities of endogeneous estrogen by disrupting the proper activities of estrogen nuclear hormone receptor in various tissues (www.pkdiet.com).

Concerned with the high potential risk of BPA, in the 1980s, the U.S.- EPA declared 50 mg/kg/day as the lowest observable adverse effect level (LOAEL) for BPA (http://www.epa.gov/iris/subst/0356.htm). The declaration was made based on Reference Dose for Chronic Oral Exposure (RfD) in rat that estimates a daily exposure to the human population without an appreciable risk of deleterious effects during a life time. Generally, the 'low dose effects' of environmental EDC refer to effects being reported at doses lower

than those used in traditional toxicological studies for risk assessment purposes (Richter et al., 2007).

However, a mountain of evidences reveals that a wide variety of biological pathways such as non-classical estrogen pathways may be induced by BPA at very low concentrations at similar or even at a higher efficiency than estrogen (Nadal, Diaz, & Valverde, 2001; Quesada et al., 2002; Watson, Bulayeva, Wozniak, & Finnerty, 2005) (Figure 2.12). In 2007, a group of scientists provided a comprehensive review related to these findings (Richter et al., 2007). According to this review, more than 40 *in vivo* laboratory rodent studies have revealed the effects of BPA at/or below the calculated safe dose particularly after fetal, neonatal or perinatal exposure and after adult exposure in a broad spectrum of tissues and cell types (Table 2.2).

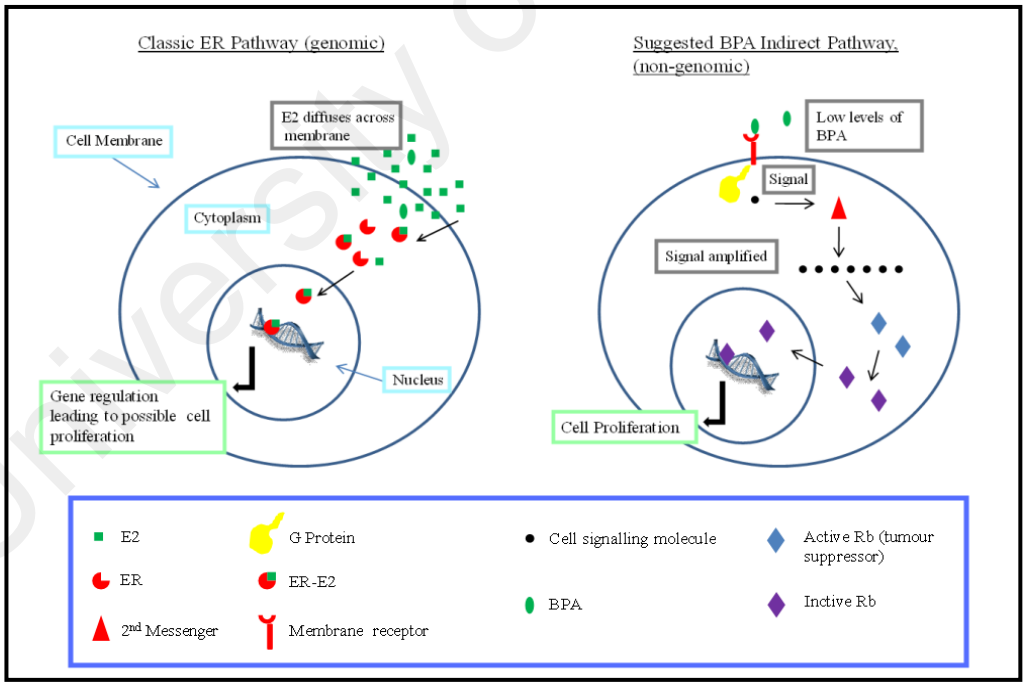

Figure 2.12: Non-classical estrogen pathways induced by BPA.

These pronounced effects included genital malformations, earlier onset of estrus cycle and puberty, protein induction in the uterus, mammary gland disorganization and cancer, prostate reduction weight and cancer, disorganization of sexually dimorphic circuits in

the hypothalamus, body weight disruption and others (Table 2.3). One study revealed that there were alterations in fetal mouse genital tract after exposure to low doses of BPA (0.025 and 0.25 µg/kg/day) *in utero* (Markey et al., 2005) while a study on male mice has shown that a mere dose of 5 µg/kg/day may decrease the weights of testes and seminal vesicles (Al-Hiyasat, Darmani, & Elbetieha, 2002). *In vitro* study, the thyroid hormone action was disrupted by BPA via recruiting transcriptional co-repressors to the thyroid hormone receptor (Moriyama et al., 2002).

Table 2.2: Summary of published papers on the physiological effects of BPA in animal model (Allard & Colaiacovo, 2011).

Topic	Reference	Species/strain	Route of exposure	Time of exposure	Endpoints
Female Reproduction	(Rubin, Murray, Damassa, King, & Soto, 2001)	Rat/Sprague Dawley	Oral	E 6 – PND 21	Reduced the LH level.
	(Fernandez, Bianchi, Lux-Lantos, & Libertun, 2009)	Rat/Sprague Dawley	Subcutaneous	PND 1-10	-Reduced the LH released from GnRH. -Increased the GnRH pulsatility.
	(Adewale, Jefferson, Newbold, & Patisaul, 2009)	Rat/Long Evans	Subcutaneous	PND 1-4	-Early onset of puberty. -Abnormal estrous cycle between PND 50-105. -Early onset of puberty.
	(Howdeshell, Hotchkiss, Thayer, Vandenbergh, & vom Saal, 1999)	Mouse/CF-1	Oral	E 11-17	-Ovarian cysts. -Cancerous lesions in ovary, oviduct and uterus.
Male Reproduction	(Newbold, Jefferson, & Padilla-Banks, 2007)	Mouse/CD-1	Subcutaneous	PND 1-5	Decreased the testosterone level.
	(Akingbemi, Sottas, Koulova, Klinefelter, & Hardy, 2004)	Rat/Long Evans	Oral	PND 21-35	-Reduced the weight of testis and epididymis.
	(Chitra, Latchoumycandane, & Mathur, 2003)	Rat/Wistar	Oral	PND 45-90	-Increased the ventral prostate.

Table 2.2: Summary of published papers on the physiological effects of BPA in animal model (Continued).

Topic	Reference	Species/strain	Route of exposure	Time of exposure	Endpoints
Mammary gland	(Durando et al., 2007)	Rat/Wistar	Osmotic pump	E 8 – E 23	-Increased the duct hyperplasia at PND 110. -Increased the tumor multiplicity at PND 50.
	(Munoz-de-Toro et al., 2005)	Mouse/CD-1	Osmotic pump	E 9-PND 4	Increased the terminal end buds at PND 30.
Brain and behavior	(Kubo et al., 2003)	Rat/Wistar	Oral	E 1 – PND 21	-Eliminaton of sex differences in open-field test at 6 weeks. -Elimination of sexual dimorphism of locus coeruleus at 14 weeks.
	(Della Seta, Minder, Dessi-Fulgheri, & Farabollini, 2005)	Rat/Sprague Dawley	Oral	E 1 – PND 21	-Decreased maternal behavior. -Increased body weight.
Adipogenesis	(Rubin et al., 2001)	Rat/Sprague Dawley	Oral	E 6 – PND 21	Accelerated differentiation into adipocytes, accumulation of triglycerides.

E: embryonic day (plug day = day 1); PND: postnatal day (birth = day 1)

Table 2.3: Broad spectrum effects of BPA in human body (Vandenberg et al, 2007).

Effects of BPA in human body
1. Impared female reproductive development
2. Miscarriage
3. Genitalia deformity
4. Early onset of puberty
5. Defects and lower sperm count
6. Breast and prostate cancer
7. Diabetis
8. Obesity
9. Hyperactive
10. Impaired learning and memory
11. Impaired, altered and compromised immune system and functions
12. Adverse effects on thyroid function

Numerous studies have also reported that BPA could induce morphological and functional alterations in uterus (Markey et al., 2005; Suzuki et al., 2002). The main function of uterus is regulated by the cyclic changes in sexual steroid hormones and therefore it has been widely used as an excellent classical target organ to detect estrogenic effects of BPA (Diel et al., 2000). A study in 2004 clearly revealed that BPA promotes uterine disruption by influencing expression and distribution of ERα and ERβ (Schonfelder, Friedrich, Paul, & Chahoud, 2004). In addition, BPA also has been reported to reduce uterine immunity by reducing the complement C3 gene expression thereby contributing to ascending infections (Seidlova-Wuttke et al., 2004). Early exposure to BPA in pregnant rats (gestation day 4.5) adversely affects the transport of embryo, preimplantation, embryo development and uterine receptivity that could lead to failure of

embryo implantation or induced infertility in the female (Xiao, Diao, Smith, Song, & Ye, 2011).

BPA has been reported to induce morphological and functional alterations of the ovaries, even at low, presumably environmentally relevant doses (Adewale et al., 2009) (Figure 2.13). *In vitro* studies have shown that BPA could negatively affect granulosa cells steroidogenesis by regulating the steroidogenic enzymes and cause uncontrolled neovascularization by the induced stimulatory effects on vascular endothelial growth factor (VEGF) (Grasselli et al., 2009; Zhou, Liu, Liao, & Han, 2008). *In vivo* studies also claimed that BPA could induce disruption in follicular development of rodent animals (Markey, Coombs, Sonnenschein, & Soto, 2003; Rodriguez, Santambrosio, Santamaria, Munoz-de-Toro, & Luque, 2010). Other *in vivo* study by Hunt et al (2005) reported that low-dose exposure to BPA causes meiotic aneuploidy in mouse oocytes. Thus, such findings indicate that BPA could induce disruption in female meiosis which is one of the leading causes of miscarriage in humans (Hassold & Hunt, 2001). Furthermore, BPA is also implicated in the development of polycystic ovaries (PCOS) (Kato, Ota, Furuhashi, Ohta, & Iguchi, 2003). These findings have provided substantial evidence that reproductive tissues, ovary and uterus are highly sensitive to BPA.

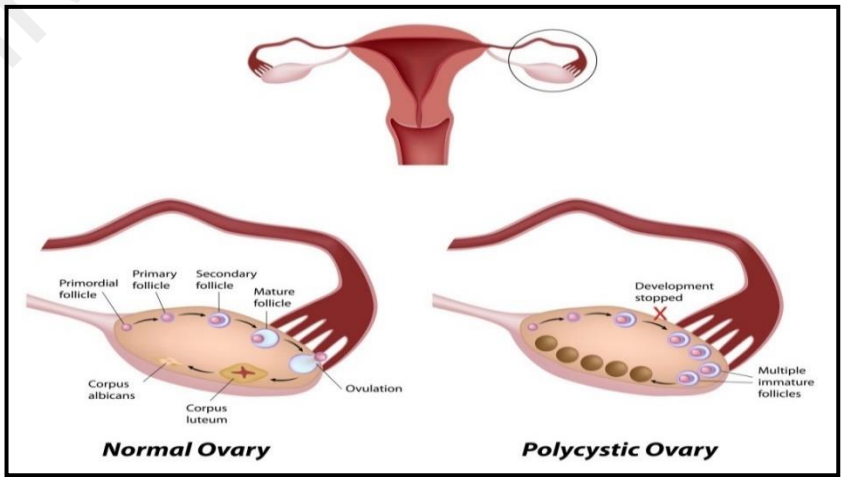

Figure 2.13: Development of polycystic ovary induced by BPA (www.clearpassage.com).

2.2 Female Reproductive System

The female reproductive system is a set of organs of ovary, uterus, vagina and mammary gland that is located inside the pelvis of the female body (Figure 2.19). The ovary has crucial functions for the reproductive functions, including production of ovum and reproductive hormones (E_2 and progesterone) (Y. Li et al., 2013). Uterus is an important part of the female reproductive system where the fertilized ovum is implanted, provides nutrients to the growing fetus throughout the gestation period, undergoes muscular contractions during parturition process and excretes the blood and mucosal tissue from its inner lining as a part of the menstrual cycle (Diel et al., 2000). The functions of vagina are to receive the male penis during coitus, passage of a full-term offspring during parturition process and channels the menstrual blood out from uterus (Li & Davis, 2007). The mammary gland is located in the breasts of the female that is responsible for the production and secretion of milk for a newborn offspring (Allard & Colaiacovo, 2011).

The reproductive organs are highly sensitive to the estrogenic effects of EDCs that induce alterations in the normal functions of reproductive system via a variety of pathways (Alum et al., 2004; Synder et al., 2003). The present study used uterus and ovary as target organs to determine the effects of BPA on the reproductive system where their significant roles in the reproductive functions are regulated by cyclical changes of the reproductive hormones.

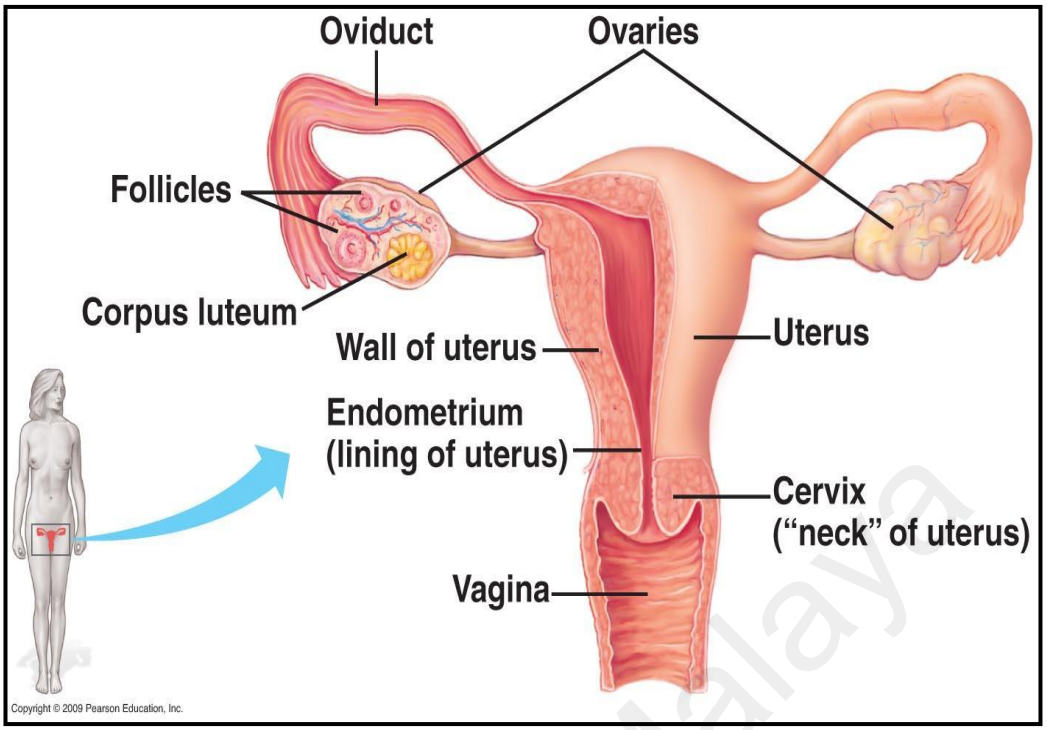

Figure 2.14: The human female reproductive system is located inside the pelvis of the body (www.studyblue.com).

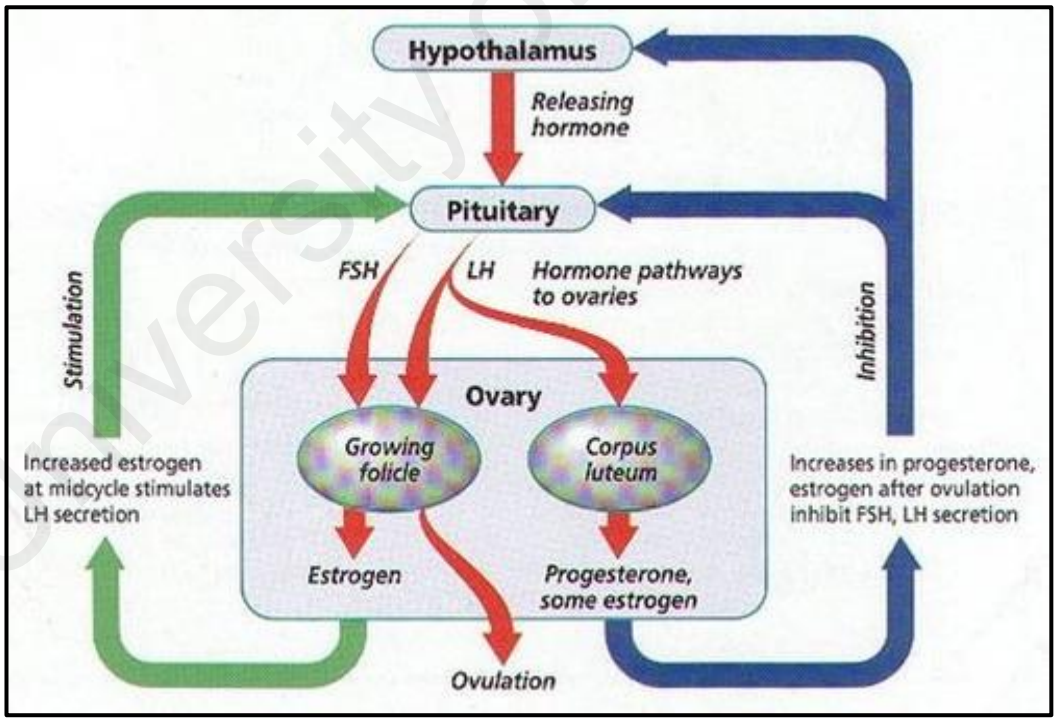

Figure 2.15: The neurophysiological pathway that involves the hypothalamus, the pituitary gland and the gonad (https//:quizlet.com).

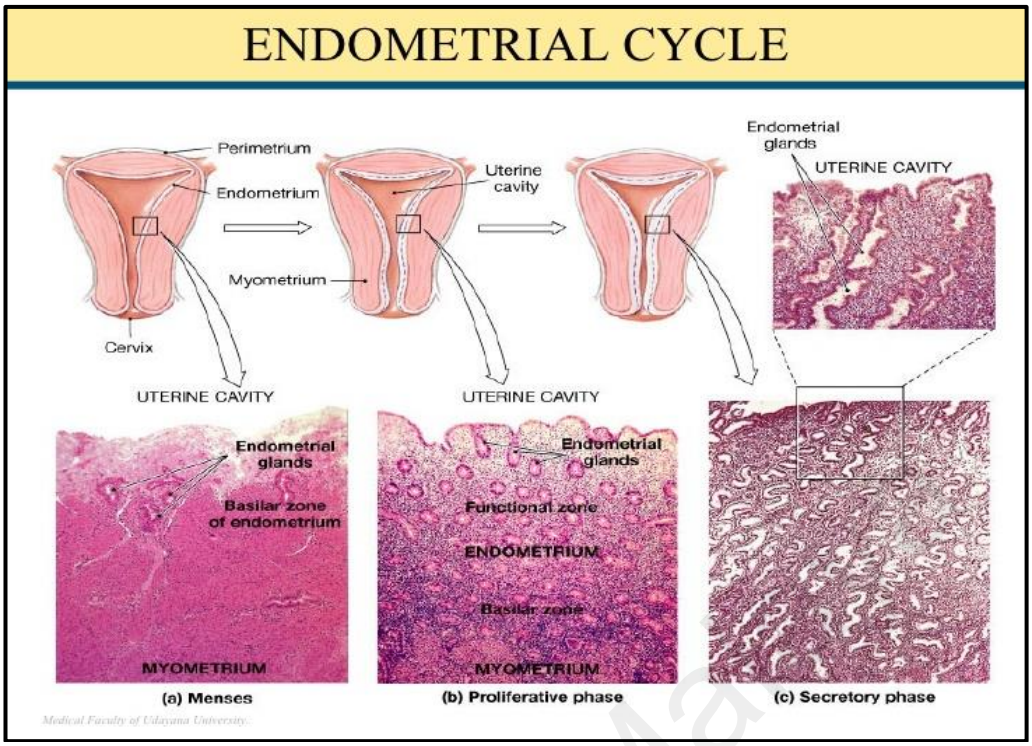

Figure 2.16: Histological changes of human endometrium during normal reproductive menstrual cycle (www.slideshare.net)

Evaluation of the reproductive cycles is used for the detection of alterations in the normal female reproductive system. In human, during the reproductive years, the menstrual cycle is controlled by a sequence of the hormonal system which includes follicle stimulating hormone (FSH), luteinizing hormone (LH), estrogen and progesterone (Stoppard, 1994; Young, Lowe, Stevens, & W.Heath, 2006). These hormones are produced and regulated by three regulatory systems, namely the hypothalamus, the anterior pituitary gland and the ovaries with its growing follicles (Figure 2.15). The relationship among these hormones is regulated by a negative feedback mechanism, whereby when the levels of one hormone rises, the levels of other hormones decrease. The standard menstrual cycle in human spans is 28 days which is counted from the first day of menstruation and is divided into two phases, namely the follicular phase and the secretory phase. There are considerable variations in the duration of menstrual cycle among normal individuals.

At the beginning of the follicular phase, menses occurs between two to seven days (Stoppard, 1994). Following that, the hypothalamus in the brain releases a substance called gonadotropins releasing hormone (GnRH) which passes down to the pituitary gland. The pituitary gland responds to the GnRH by secreting rising amounts of FSH and a lesser amount of LH. These two hormones are known as gonadotropins. Both of these gonadotropins are secreted from the gonadotrophs cells in the anterior pituitary and act synergistically (Li & Davis, 2007). Under the influence of a rising amount in FSH, five to seven tertiary-stage ovarian egg follicles are recruited for entry into the next menstrual cycle.

These egg follicles, which are growing by a process known as folliculogenesis, will develop and compete with each other for dominance. However, only one egg follicle becomes dominant and will continue to maturity. Meanwhile, all these follicles secrete increasing amounts of estradiol, which is an estrogen. Estrogen initiates the formation of a new layer of endometrium in the uterus, which is histologically identified as the proliferative endometrium (2.17). Subsequently, the rising level of estrogen exerts a negative feedback on the hypothalamus and the pituitary gland by producing a smaller amount of FSH. When the level of estrogen peaks near the midcycle, the hypothalamus and the pituitary gland respond again with a large secretion of LH at around day 12 of the cycle and it may last for 48 hours. The surge of LH matures the dominant egg follicle and weakens the wall of this egg follicle in the ovary. This process leads to an event called ovulation whereby mature ovum is released into the fallopian tube at around day 14 of the cycle.

After the follicular phase, the secretory phase will ensue. Following ovulation at day 14 of the cycle, the residual follicle transforms into a structure called corpus luteum which produces progesterone and estrogen for approximately two weeks. Progesterone plays a vital role in converting the proliferative endometrium into a secretory lining that is

receptive for implantation of an embryo and early pregnancy support. During this phase, if implantation does not occur, the corpus luteum will die, causing a sharp drop in levels of both progesterone and estrogen. This causes the uterus to shed its lining at approximately day 28 of the cycle and results in a process termed menstruation. This will set the stage for the next cycle by stimulation of gonadotrophin hormones to begin the whole cycle again.

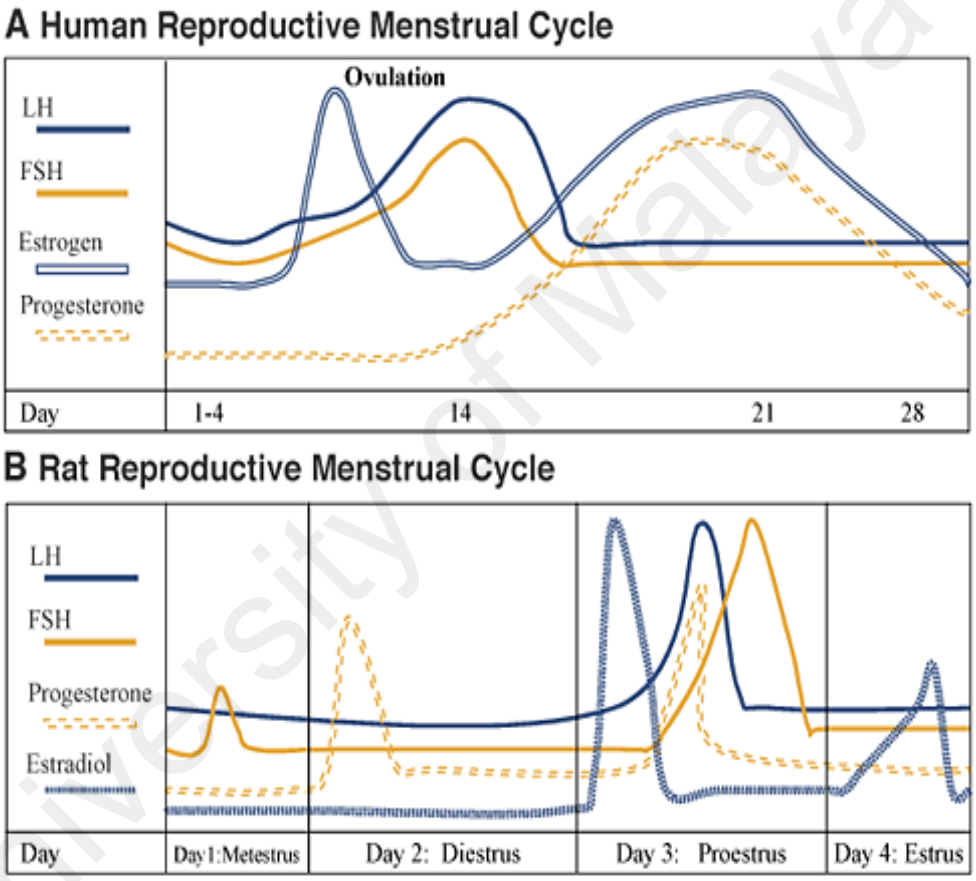

Figure 2.17: The progression of the reproductive cycles in human and rat (pubs.niaaa.nih.gov)

Human and rat are mammals that have similarities in their hypothalamic-pituitary-gonadal axis functions which is crucial for the development and regulatory of the reproductive system (Figure 2.17) (Sengupta, 2013). However, both species are significantly different from each other in the detailed functions. In rat, the estrous cycle is a short period of 4 to 5 days with the endometrium reabsorbed if the conception does

not occur and only sexually active during the estrus phase (termed as 'in heat') (Li & Davis, 2007). The short estrous period and not easily disrupted by routine stress in the animal facility have made rats to be an ideal animal model for investigation of changes in the reproductive cycle (Caligioni, 2009). In addition to that, rat has been scientifically proven for their utility as an animal model in terms of further understanding the effects and mechanisms of EDCs in the reproductive system (Li & Davis, 2007). In toxicity studies, the reliable and standard endpoint for the onset of puberty is manifested by the development of the external orifice opening of the vaginal canal (Li & Davis, 2007).

2.3 Natural products with potential therapeutic effect

In almost all parts of world, natural products and medicinal plants have been widely used in the maintenance of health and treatments of various ailments (Table 2.4).

Natural products from the beehive such as propolis, honey and royal jelly have ameliorative effects on the peripheral blood leucocytes and lung inflammation in the asthma mouse model (El-Aidy et al., 2015). A medicinal plant called *Labisia pumila* ("Kacip Fatimah") has been scientifically proven to protect osteoporotic bone in estrogen-deficient rat model (Fathilah et al., 2012). Other scientific findings has proven the beneficial effects of medicinal plants known as *Eurycoma longifolia* ("Tongkat Ali") used to enhance erectile functions (Kotirum et al., 2015) while *Andrographis paniculata* ("Hempedu Bumi") has been claimed to have anticancer and anti-malarial activities (Dua et al., 2004; Kumar et al., 2004).

Regarding to the protective effects of natural products or medicinal plants on the disruptive effects of BPA, *Triticum aestivum* (Wheat Sprout) juice has been suggested as a potential detoxification agent against BPA-toxicity in young women (Yi et al., 2011).

A Chinese herbal medicine, *Lycium barbarum* (Goji berry) has also been reported to have protective effects on testis spermatogenic injury induced by BPA in rats (Zhang et al., 2013) (Figure 2.18).

Table 2.4: Natural products as medicine

Natural products	Medicinal uses
1. Beehive product -Honey, propolis, royal jelly.	-Reproductive system maintenance, wound healing, anticancer, anti-inflammatory, probiotics, immunomodulatory.
2. Plant -*Labisia pumila* ("Kacip Fatimah"), *Ficus deltoidea* ("Mas Cotek")	-Female reproductive system maintenance
-*Eurycoma longifolia* ("Tongkat Ali")	-Male sex performance
-*Andrographis paniculata* ("Hempedu Bumi")	-Anti-cancer and anti-malarial
-*Amaranthus spinosus* (spinach), *Arundina graminifolia* (orchid), *Carica papaya* (papaya), *Citrus grandis* (pomelo), *Garcinia atroviridis* (perennial plant), *Callicarpa arborea* (purple beauty berry), *Zingiber officinale* (ginger)	-Anti-gastric
-*Coleus amboinicus* (perennial plant)	-Constipation
-*Curcuma domestica* (turmeric)	-Bloating
-*Psidium guajava* (guava)	-Diarrhea and stomach ache
3. Fungi -Penicilin -Betulinic acid -Doxorubicin	-Antibiotic -Weak inhibitor of HIV replication -Leukaemia, soft tissue and bone sarcomas, lung cancer, thyroid cancer.
4. Algae	Antibacterial and antifungal.

Till now, no scientific study has been conducted to investigate the use of local Malaysian wild honey, Tualang honey and Malaysian medicinal plant, *Ficus deltoidea* (Mas Cotek) as natural therapeutic agents against the disruptive effects of BPA on the female reproductive system. Thus, in the present study we propose to use these two natural products as potential therapeutics to prevent the disruptive effects of BPA on the female reproductive system.

A) Royal jelly (keepingbee.org)

B) *Labisia pumila* (vagifirm.com)

C) *Eurycoma longifolia* (tongkataliextra.com)

D) *Andrographis paniculata* (ms.wikipedia.org)

E) *Triticum aestivum* (ww.123rf.com)

F) *Lycium barbarum* (earthfoods.com)

Figure 2.18: Natural products that are useful for health maintenance

Tualang honey and *Ficus deltoidea* (Mas Cotek) have been scientifically proven for their abilities in maintaining the health and reproductive functions. Tualang honey has the capability to reduce oxidative stress levels in the renal and pancreas (Omotayo et al., 2010). With regards to the reproductive health, Tualang honey has been reported to prevent atrophy state in the uterus and vagina (Zaid et al., 2010) and protects rat testis from oxidative stress induced by cigarette smoke (Mohamed, Sulaiman, Jaafar, & Sirajudeen, 2011). Meanwhile, *Ficus deltoidea* (Mas Cotek) was claimed to have stimulatory effects on the uterus and vaginal muscles that facilitates the process of birth (Salleh & Ahmad, 2013) and as an aphrodisiac tonic to enhance sexual desire (Bunawan, Amin, Bunawan, Baharum, & Mohd Noor, 2014).

2.4 Honey

2.4.1 Variations of honey

Honey is a sweet natural substance that contains a highly concentrated solution of a complex mixture of sugars produced by honey bees in almost every country of the world and extracted generally from nectars (Blasa et al., 2006; Gomez-Caravaca, Gomez-Romero, Arraez-Roman, Segura-Carretero, & Fernandez-Gutierrez, 2006). Honey has been used extensively since ancient times and has been appreciated as the only concentrated form of sugar available worldwide (Nagai, Sakai, Inoue, Inoue, & Suzuki, 2001).

Previous works have indicated that the composition of honey varies widely according to the floral source and geographical origin, climate, environmental condition and post-harvest processing condition such as processing, handling and storage (Al-Mamary, Ali Al-Meeri, & Molham Al-Habori, 2002; Beretta, Granata, Ferrero, Orioli, & Facino, 2005;

Chen, Mehta, Berenbaum, Zangerl, & Engeseth, 2000; Cherchi, Spanedda, Tuberoso, & Cabras, 1994; Frankel, Robinson, & Berenbaum, 1998; Gheldof & Engeseth, 2002; Gheldof, Wang, & Engeseth, 2002; Kucuk et al., 2007; Turkmen, Sari, Poyrazoglu, & Velioglu, 2006; Yao et al., 2004). A study has shown that the Italian honey collected from the nectar of wild blossoms on hill zones and mountain zones contains higher organoleptic characteristics and nourishing values, low moisture content, high density and has a stability which guarantees long shelf live without the risk of fermentation or changes in its properties (Blasa et al., 2006).

Multifloral honey differs from unifloral honey in that it is darker in color with solid crystallization and is thick while unifloral honey has light color, transparent appearance and is thin (Blasa et al., 2006). The color of honey is affected by the content of minerals, pollens and phenolics (Baltrusaityte, Venskutonis, & Ceksteryte, 2007; Lazaridou, Biliaderis, Bacandritsos, & Sabatini, 2004). Darkening of honey during storage may occur due to Maillard reactions, fructose caramelization and reactions of polyphenols as well as temperature and/or time of storage (Bertoncelj, Dobersek, Jamnik, & Golob, 2007). These compositional differences can influence the value of a specific honey for medicinal or health-promoting purposes (Gomez-Caravaca et al., 2006). However, the main constituents of all honey from all countries of the world are almost similar.

2.4.2 Biochemical content of honey

Honey, a supersaturated aqueous solution of inverted sugars, mainly contains fructose (38%) and glucose (31%) as well as a small amount of complex mixture of other saccharides such as disaccharides, trisaccharides and oligosaccharides. It also contains small amounts of enzymes, amino and organic acids, phytochemicals, carotenoid-like substances, Maillard reaction products, vitamins and minerals (Antony, Han, Rieck, &

Dawson, 2000; Bertoncelj et al., 2007; Blasa et al., 2006; Cherchi et al., 1994; Gomez-Caravaca et al., 2006; Vit, Soler, & Tomas-Barberan, 1997). The average composition of honey is given in Table 2.5.

Table 2.5: Nutrient values of honey adopted from National Honey Board, 2002.

Nutrient	Average amount per 1 Tbsp serving (21g)	Average amount per 100g
Water	3.62g	17.10g
Calories	64	304
Total Carbohydrate	17.46g	82.40g
Fructose	8.16g	38.50g
Glucose	6.57g	31.00g
Maltose	1.53g	7.20g
Sucrose	0.32g	1.50g
Other carbohydrates	0.85g	4.00g
Dietary fiber	0.04g	0.20g
Total fat	0	0
Cholesterol	0	0
Total protein	0.06g	0.30g
Ash	0.04g	0.20g
Vitamins		
(data not available for biotin and vitamin B-12)		
Riboflavin	0.01mg	0.04mg
Niacin	0.03mg	0.12mg
Pantothenic acid	0.01mg	0.07mg
Vitamin B-6	0.01mg	0.02mg
Vitamin B-12	0	0
Folate	0.42mcg	2.00mcg
Vitamin C	0.11mg	0.50mg
Minerals		
Calcium	1.27g	6.00mg
Phosphorus	0.85g	4.00mg
Sodium	0.85mg	4.00mg
Potassium	11.02mg	52.00mg
Iron	0.09mg	0.42mg
Zinc	0.05mg	0.22m
Magnesium	0.42mg	2.00mg
Selenium	0.17mg	0.80mg
Copper	0.01mg	0.04mg
Manganese	0.02mg	0.08mg

Honey is considered to be a suitable supplement for humans with its high nutritional value (304 kcal/100 g honey) and fast absorption of its carbohydrates. It has a density of about 1.36 kg/liter (40% denser than water), with acidic environment ranging from 3.2 to 4.5 pH and high osmotic pressure and low water activity (Jeffrey & Echazareta, 1996). Raw honeys are usually produced by small farms and left in their natural state without undergoing process such as thermal or pasteurization to alter their natural composition. However, the extraneous matter that is contained in raw honey is removed in order to make it marketable on a large scale (Blasa et al., 2006).

The main enzymes found in honey which are derived from the hypopharyngeal glands of worker honey bees are invertase, diastase (amylase), catalase, glucose oxidase and acid phosphatase (Jeffrey & Echazareta, 1996). Honey contains a number of amino acids such as proline, lysine, phenylalanine, tyrosine, glutamic and aspartic acids which are contributed by the pollens, nectar or by honey bees themselves. The predominant organic acid in honey is gluconic acid while the others are butyric, acetic, formic, lactic, succinic, malic, citric, maleic, oxalic and pyroglutamic. Gluconic acid in honey originates largely from the activity of enzymatic glucose oxidase reaction (Jeffrey & Echazareta, 1996). Indeed, it contributes a slight tartness to the flavor of honey and also adds antimicrobial properties as well as increase in calcium absorption**.**

In recent years, many studies have been conducted to identify the phytochemicals present in honey. Phytochemicals are non-nutrient compounds commonly found in plants such as fruits and vegetables (Kuhnle, Dell'Aquila, Runswick, & Bingham, 2009) and have health-promoting activities by reducing the risk of oxidative tissue (Al-Mamary et al., 2002; Aljadi & Kamaruddin, 2004). Honey is well known to be rich in both enzymatic and non-enzymatic antioxidants, including catalase, ascorbic acid, flavonoids, alkaloids**,** glucose oxidase, phenolics acid, carotenoid derivatives, Maillard reaction products, amino acid and proteins (Al-Mamary et al., 2002; Aljadi & Kamaruddin, 2004; Gheldof

& Engeseth, 2002; Gheldof et al., 2002; Gheldof, Wang, & Engeseth, 2003; Schramm et al., 2003). Pinocembrin, a unique flavonoid is highly present in propolis and honey, while the others such as quercetin, chrysin, galangin, luteolin and kaempferol were also reported to be found in honey (Baltrusaityte et al., 2007).

Previous works have indicated that the antioxidant activity of honey varies widely depending on the floral sources (Al-Mamary et al., 2002; Bertoncelj et al., 2007; Chen et al., 2000; Frankel et al., 1998; Gheldof & Engeseth, 2002; Nagai et al., 2001; Yao et al., 2004). Several studies have shown that a strong correlation between honey colour and antioxidant power, with darker and more crystallized honey having higher total phenolic content and hence a stronger antioxidant capacity than lighter and transparent honey (Bertoncelj et al., 2007; Blasa et al., 2006; Nagai et al., 2001) as well as honey with higher content of water (Aljadi & Kamaruddin, 2004; Frankel et al., 1998). Moreover, the color of honey depends on the potential alkalinity and ash content, as well as on the antioxidatively active pigments, including carotenoids and flavonoids (Frankel et al., 1998).

2.4.3 Therapeutic uses of honey

Nowadays, ancient remedies still survive and in use together with modern medicine. They are termed ‘modern folk medicine’ since their effectiveness has not been scientifically proven through clinical trials. The use of honey as modern folk medicine is common for coughs, sore throats and eye diseases (using lotus honey) in India, infected leg ulcers in Ghana, earaches in Nigeria, topical treatment for measles and eye measles to prevent corneal scarring, as well as for the treatment of gastric ulcers and constipation (Ankra-Badu, 1992; Imperato & Traore, 1969; Kandil, El-Banby, Abdel-Wahed, Abdel-Gawwad, & Fayez, 1987; Obi, Ugoji, Edun, Lawal, & Anyiwo, 1994; Zumla & Lulat,

1989). In Germany, honey with cod liver oil was used for treatment of ulcerations, burns, fistulas and boils. During the World War I, Russian soldiers used honey to prevent infections in wounds as well as to accelerate healing (Bergman, Yanai, Weiss, Bell, & David, 1983).

The mechanism of the effects of honey on human health has gradually become apparent in a number of scientific studies. The scientific support has been well documented with a proliferation in publications on the successful therapeutic uses of honey in several general medical and surgical conditions (Adesunkanmi & Oyelami, 1994; Efem, 1988; Efem, Udoh, & Iwara, 1992; Grange, 1990; Hejase, Simonin, Bihrle, & Coogan, 1996; Ndayisaba, Bazira, & Habonimana, 1992; Ndayisaba, Bazira, Habonimana, & Muteganya, 1993; Phuapradit & Saropala, 1992; Subrahmanyam, 1991, 1993, 1994, 1996, 1998; Wood, Rademaker, & Molan, 1997).

The most active medical effect of honey is its antimicrobial property. The mechanism of this function is due to its high osmolarity (Willix, Molan, & Harfoot, 1992), acidity and content of inhibines, such as peroxide (Bang, Buntting, & Molan, 2003; Burdon, 1995; Cooper, Molan, & Harding, 1999; Mani, 2006; Molan, 2001, 2002; Patton, Barrett, Brennan, & Moran, 2006; Postmes, van den Bogaard, & Hazen, 1993), flavonoids and the phenolic acids (caffeic and ferulic acid) (Baltrusaityte et al., 2007; G. Cao, Sofic, & Prior, 1997; Frankel et al., 1998; Wahdan, 1997).

Honey has been proven to be effective in treating infected surgical wounds, burns and decubitus ulcer. A research found that local application of honey in the postoperative management of patients who had undergone radical vulvectomy for vulva carcinoma lead to succesful healing (Cavanagh, Beazley, & Ostapowicz, 1970). The rate of wound healing was accelerated and less bacterial colonization was observed. This observation has been proven in an animal model when pure commercially available honey applied on

12 mice significantly healed wounds faster than those of the control group (Bergman et al., 1983). Subrahmanyam (1998) reported his findings in a randomized controlled trial study in which honey was found to be a more effective dressing for burns as compared to silver sulfadiazine. Treatment with silver sulfadiazine, the most widely used agent to prevent or clear infection in burns, resulted in 7% of the patients controlled from wound infection within seven days while treatment with honey resulted in 91% of the wounds to be sterile within the same period.

Honey has also been used for a long time in the treatment of various gastrointestinal diseases. The effect was proven when an oral rehydration therapy (ORT) solution containing 5% honey was given to infants and children suffering from bacterial gastroenteritis (*Salmonella, Shigella* and *E.coli)* (Haffejee & Moosa, 1985; Jeddar et al., 1985). The convincing results showed that recovery time from bacterial diarrhea was significantly faster in patients given ORT solution containing equal concentration of glucose and concluded that honey can safely be used as a substitute for glucose in solution with electrolytes and in promoting sodium and water from the gut.

In recent years, attention has highly focused on natural food as nutraceuticals for the treatment of illnesses and disease. The mechanism of the effect of honey on human health has gradually become apparent in a number of studies. Many researchers demonstrated that honey contains a rich source of natural antioxidants and phytochemicals, which are effective in reducing the risk of heart disease, cancer, immune-system decline, cataracts, different inflammatory processes, as anti-allergic, anti-thrombotic, vasodilatory actions as well as its impact on gastrointestinal health and energy metabolism (Bertoncelj et al., 2007).

2.4.4 Tualang honey (Agromas, Malaysia)

Tualang honey is produced by bees (*Apis Dorsata*) in which their hives are built hanging under the branches of Tualang tree (*Kompassia excels*). These trees are found in the rainforest of Malaysia, including the ones at Sik, Kuala Nerang and Baling forest in Kedah, Malaysia (Figure 2.19). Tualang honey is collected from forests where the levels of dangerous chemicals in the atmosphere are insignificant and the environmental pollution is minimal. Furthermore, it is kept by the *Apis Dorsata* in fixed comb log and hives wall without the use of artificial comb foundations, sugar feeding and antibiotics in such hives. Therefore, Tualang honey is considered as Malaysian pure wild honey with dark brown color.

Figure 2.19: Tualang honey (Agromas, Malaysia)

The phenolic content, antioxidant capacity, chemical compositions, volatile compositions and hydroxymethylfurfural were extensively studied in Tualang honey. A good correlation of total phenolics and flavonoids contents with the antioxidant capacity was reported in Tualang honey (Khalil et al., 2011). Interestingly, Tualang honey has also been claimed to have better antioxidant effects against a variety of ROS compared to other local Malaysian honey, Gelam honey and commercial honey, Indian forest and

Pineapple honey (Kishore, Halim, Syazana, & Sirajudeen, 2011). It has been claimed to share some similarity in terms of properties and biochemical characteristics with the well-researched Manuka honey (New Zealand monofloral honey) (Ahmed & Othman, 2013). However, the phenolic and flavonoid content is higher in Tualang honey compared to the Manuka honey.

The chemical compositions include furfural such as hydroxymethylfurfural, furfural, 2-furylmethyletone, 5-methyl furfural and fatty acids such as palmitic acid, ethyl linoleate and ethyl oleate (Man, Mahaneem, & Siti Amrah, 2010). A total of 35 volatile compounds were detected such as hydrocarbons, acids, aldehydes, alcohols, ketones, terpenes and furans were diluted in honey (Nurul Syazana, Gan, & Halim, 2010).

A lot of scientific evidences has revealed the beneficial effects of Tualang honey including ameliorating the oxidative stress effects in renal and pancreas of streptozotocin-induced diabetic rat (Erejuwa et al., 2012; Erejuwa, Sulaiman, Wahab, Salam, et al., 2010), preventing uterine and vaginal atrophy as well as osteoporotic bone in postmenopausal animal model (Zaid et al., 2012; Zaid et al., 2010), and protecting rat testis against damage and oxidative stress induced by cigarette smoke (Mahaneem et al., 2011). Tualang honey also has been reported to have beneficial effects on oral squamous cell carcinomas (OSCC), human osteosarcoma (HOS) and keloid fibroblast (Mohamad et al., 2011). A cancer research group claimed that Tualang honey has a potential to induce anticancer activity in breast cancer cell lines via the upregulation of double strand DNA repair enzymes thereby preserving the cellular DNA integrity (Yaacob & Ismail, 2014) or by promoting apoptotic cell death (Yaacob, Nengsih, & Norazmi, 2013). In UVB radiation study by the same research group, they also found that treatment with Tualang honey to PAM212 cells resulted in the induction of the number of cyclobutane pyrimidine dimers and 8-oxo-dG-positive cells (biomarkers of oxidative damage) due to the improvement of DNA repair (Ahmad, Jimenez, Yaacob, & Yusuf, 2012).

It was also found that daily intake of Tualang honey at 20 mg/day for 4 months has similar effect on bone densitometry when compared with hormone replacement therapy (Lily Husniata et al., 2010). Besides that, it was reported that Tualang honey has antimicrobial properties on full thickness burn wound in rats (Halima, Kirnpal-Kaur, Doraia, Azmana, & Khooa, 2010). Combination of Tualang honey supplementation and jumping exercise may also elicit beneficial effects on tibial bone mineral density (Kiew et al., 2010). It is also potentially used as therapeutic agent for diabetic foot wounds (Nawfar, Han, Paiman, & Iskandar, 2010). Honey is an effective agent against yeast infections (Tuan Noorkorina & Mazatul Haizam, 2010) and can reduce apoptosis in leukemia cells (Rosline et al., 2010). Last but not least, it was found that Tualang honey was significantly effective as a prophylactic measure in reducing acute respiratory symptoms among Hajj pilgrims (Siti Amrah et al., 2010).

2.5 *Ficus deltoidea* Jack (Mas Cotek)

2.5.1 Botanical history of *Ficus deltoidea* (Mas Cotek)

The *Ficus* plant originates from Southeast Asia (Malaysia, Thailand and Indonesia) and can be found widely in other parts of the world (Sulaiman et al., 2008). According to the botanical scientific classification, *Ficus* genus plant is an 'angiosperms' (flowering plant), which is under the order of 'rosales' and belongs to the family of 'moraceae' (known as mulberry or fig family) (Weiblen, 2000). Throughout worldwide, *Ficus* plant has more than 800 species, which are recognized by its morphological differences (Figure 2.20). *Ficus deltoidea* is one of the most well-known and available *Ficus* species in Malaysia, which is traditionally used by Malay community for the health maintenance purposes particularly in the maintenance of reproductive system (Bunawan et al., 2014) (Figure 2.21).

The botanical name for *Ficus deltoidea* is *Ficus deltoidea* Jack while its local names are given according to the different parts of the world. In Malaysia, *Ficus deltoidea* is locally known as "Mas Cotek", "Serapat Angin", "Sempit-sempit" and "Telinga Beruk". For Indonesian, this species is called "Tabat Barito", "Ara Jelatih", "Ara Tunggal", "Api-api Gajah" or "Api-api Telinga Kera". In Africa and Philippines, is known as "Kangkalibang" and "Angulora", respectively. Several subspecies of *Ficus deltoidea* are as follows: *Ficus deltoidea* var *deltoidea*, *Ficus deltoidea* var *augustifolia*, *Ficus deltoidea* var *angustissima*, *Ficus deltoidea* var *arenaria*, *Ficus deltoidea* var *bilobota*, *Ficus deltoidea* var *borneensis*, *Ficus deltoidea* var *subhirsuta*, *Ficus deltoidea* var *kunstleri*, *Ficus deltoidea* var *lutescens*, *Ficus deltoidea* var *longipedunculata*, *Ficus deltoidea* var *subsessilis*, *Ficus deltoidea* var *peltata*, *Ficus deltoidea* var *recurvate*, *Ficus deltoidea* var *trengganuensis* and *Ficus deltoidea* var *oligoneura* (Bunawan et al., 2014).

A) *Ficus carica* (www.dreams.time) B) *Ficus palmate* (www.arkive.org)

C) *Ficus tinctoria* (www.wikiwand.com) D) *Ficus nervosa* (www.wm-sec.com)

Figure 2.20: Different species of *Ficus* plant.

Figure 2.21: Female (A) and male (B) plant of *Ficus deltoidea*. The female plant has a big round long leaves while male plant has more elongated and small leaves.

Ficus deltoidea is an evergreen shrubby plant with whitish grey stem and their leaves are arranged in spiral and ascending twigs. The shape of the leaves are broad spoon-shaped to obovate, 4 cm to 8 cm of length, bright green color on the surface and rust-red to olive-brown on the underneath side. The shape of the figs (fruit) of this plant is spherical to round-shaped, width of 1.0 cm to1.5 cm and orange-red of ripen-color. In Malaysia, the name Mas Cotek was given due to tiny golden spots on the surface of the leaves on this plant. *Ficus deltoidea* can be divided into female and male plants, which is identified by the differences in the leaf structure. The size of female leaves is bigger, thicker and has more clear lines compare to the male leaves. The structure of male leaves is small, long and thin. Both of these plant sexes have some spots at the back of leaves, where red spots in the female and black spots in the male.

In fact, the leaves of *Ficus deltoidea* have its own interesting morphology, considering that the shape resembles that of the female reproductive structure (labia majora) and that it has been specifically used to treat female and male infertility (Hakiman & Mahmood, 2009). In Malaysia, *Ficus deltoidea* var *deltoidea* is mostly used by the

Malay community. In the traditional Malay community, the leaves of *Ficus deltoidea* are not only boiled as decoction for various ailments but it can also be obtained commercially in capsules, tea bags or as tonic tea as well as massage oil.

2.5.2 Biochemical content of *Ficus deltoidea*

A comprehensive study on the volatile compounds produced by the fruit of *Ficus deltoidea* began in 2002 (Grison-Pige, Hossaert-McKey, Greeff, & Bessiere, 2002). In this study, the isolated volatile compounds were terpenoids and aliphatic groups which are also present as floral fragrances in plants. In 2011, an antibacterial compound known as lupeol was isolated from the leaves of *Ficus deltoidea* (Suryati, Nurdin, Dachriyanus, & Lajis, 2011). Subsequently, two other bioactive compounds in the leaves of *Ficus deltoidea*, vitexin and isovitexin were isolated that useful for the treatment of diabetic (Choo, Sulong, Man, & Wong, 2012).

Phytochemical studies have revealed that the antioxidant activity of *Ficus deltoidea* is attributed to the presence of flavonoids, phenolics, vitamin C, terpenoids, alkaloids, saponin and tannins (Omar, Mullen, & Crozier, 2011; Zunoliza et al., 2009). The leaves of *Ficus deltoidea* has also been shown to contain enzymatic antioxidants including ascorbate oxidase, peroxidase, catalase, and ascorbate peroxidase (Hakiman & Mahmood, 2009). According to the cell culture studies, some of the flavonoid compounds such as rutin, quercetin and naringen in the *Ficus deltoidea* are influenced by different carbon sources (Ong, Ling, Poospooragi, & Moosa, 2011).

In antioxidant studies, three major groups of the phytochemicals that are present in the *Ficus deltoidea* are phenolic compounds, tannins and phenylisopropanoid (Abdullah et al., 2009). Phenolic compounds accumulate in different parts of the plant tissues and

cells during ontogenesis (development) that serve important roles for the biological activities in the plant. Localization of phenolic compounds in the cell walls, vacuoles and cell nuclei contribute to the anti-inflammatory and anti-septic properties in the plants (Yi, Fischer, Krewer, & Akoh, 2005).

The chemical constituents under phenolic compounds are catechin, flavones, naringin, vitexin, isovitexin, anthocyanins and proanthocyanins. The beneficial role of naringin is to reduce the cholesterol level in the body by lowering plasma lipid concentrations (Choi et al., 2001). Strong antioxidants properties of vitexin and isovitexin were also reported (Zunoliza et al., 2009). Supplementation of tannins reduces the plasma glucose and lipid profile levels as well as attenuating oxidative stress in hypercholesterolemia associated diabetes model rats (Velayutham, Sankaradoss, & Ahamed, 2012).

2.5.3 Ethnomedicinal uses and pharmacological activities of *Ficus deltoidea* Jack

Traditionally, different parts of this plant are used to treat various kinds of ailments. The powdered form of the root and leaves are applied externally to wounds, sore and rheumatism joints, while the fruits are chewed to relieve toothache, headache and cold. Decoctions of boiled leaves are taken during confinement period to constrict the womb and strengthen vaginal muscles as well as to treat menstrual cycle problems. The entire plant, on the other hand is used as an aphrodisiac tonic to enhance sexual desire (Bunawan et al., 2014).

Important findings were reported from a study on *Ficus deltoidea* that showed its potential as an antidiabetic agent (Adam, Khamis, Ismail, & Hamid, 2012). The mechanisms of antihyperglycemic activity of *Ficus deltoidea* are mediated through stimulation of insulin secretion from pancreatic β cells, increasing the glucose uptake by

adipocytes cells and augmentation of the adiponectin secretion from adipocytes cells. The rational use of *Ficus deltoidea* for the treatment of microbial infections was supported by scientific *in vitro* study, which found antimicrobial activity of the chloroform, methanol and aqueous extracts of this medicinal plant on the fungus, Gram-positive and Gram-negative bacteria strains (Abdsamah, Zaidi, & Sule, 2012).

Ficus deltoidea was reported to have antimelanogenic activity by suppressing tyrosinasegene expression in B16F1 melanoma cells (Oh et al., 2010). The finding from this study has been suggested that *Ficus deltoidea* extract has potential to be used as a novel depigmenting agent for the cosmetics. Regarding to the reproductive system, *Ficus deltoidea* has potential to develop as a natural uterotonic agent to facilitate delivery process and also treatment for post-partum haemorrhage (Umi Romaizatul Amiera, Nihayah, Farah Wahida, & Rajab, 2014). The mechanism related to the uterotonic effects of *Ficus deltoidea* is via induction of contraction that mediated by multiple uterotonin receptors (muscarinic, oxytocin and prostaglandin) (Salleh & Ahmad, 2013).

Many other extensive pharmacological studies on the traditional uses of *Ficus deltoidea* including as an anti-inflammatory and antinociceptive (Abdullah, Hussain, Zhari, & Rasadah, 2009; Sulaiman et al., 2008), antiphotoaging (Hasham, Choi, Sarmidi, & Park, 2013; Oh et al., 2010) and antiulcerogenic (Zahra, Mahmood, Hapipah, Suzita, & Salmah, 2009). In addition, *Ficus deltoidea* has also been shown to have antibacterial (Abdsamah et al., 2012), wound healing activity (Abdulla, Abdul-Aziz Ahmed, Abu-Lahoom, & Muhanid, 2010), anticancer (Akhir, Chua, Majid, & Sarmidi, 2011) and cytotoxicity activity (Farsi et al., 2013).

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Animal

Healthy female Sprague Dawley rats at 21-day of age (80 g of body weight) were obtained from the Animal Husbandry Unit, Faculty of Medicine, University of Malaya. All the experimental design and procedures were conducted according to the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) that has been approved by the Animal Care and Use Committee (ACUC) of University of Malaya [ANA/27/07/2014/SSMZ (R)]. Throughout the experimental period, the rats were maintained under the standard laboratory conditions (temperature 25 ± 2°C, 50 ± 15% relative humidity and normal photoperiod of 12 hours dark and 12 hours light) and supplied *ad libitum* with tap water and commercial pellet diet (Gold Coin Feedmills Pte. Ltd, Malaysia). The rats were placed in stainless steel cages with wood bedding and tap water was supplied in glass bottles to minimize additional exposures to endocrine disruptors. They were acclimatized to the laboratory environment for seven days before the commencement of the experiments. At 28-day of age, the rats were divided into six groups (n=8).

The sample size of rats was determined based on a previous study on female reproductive system (Phrakonkham et al., 2007) using PS Power and Sample Size Calculation software version 3.0.4.3. The power of the study was set at 80% with 95% confidence interval. The standard deviation (σ) was set at 2.1 for uterine relative weight. The differences in population mean (δ) was set at 4.0 of uterine relative weight. Finally, the number of sample sample size was eight rats per group.

3.1.2 Tualang honey (Agromas, Malaysia)

Tualang honey was purchased from the Federal Agricultural Marketing Authority (FAMA), under the Ministry of Agriculture and Agro-Based Industry, Malaysia (Figure 3.1). Tualang honey is a wild multifloral honey collected from *Apis dorsata's* beehive that is built on a giant tree *Koompassia excels* (locally known as Tualang tree) in the rain forest of Kedah, Malaysia. At the Honey Processing Centre in Kuala Nerang, Kedah, the Tualang honey was processed through several stages (quality inspection, dehydration, packaging and labeling). Tualang honey was filtered to remove solid particles and concentrated in an oven at 40°C. Tualang honey was subjected to commercialized sterilization procedure using γ irradiation (25 kGy) at Sterilgamma (M) Sdn. Bhd. (Selangor, Malaysia). The water concentration of the Tualang honey was standardized by FAMA at 18%.

3.1.3 Aqueous extract of *Ficus deltoidea* (Mas Cotek)

Aqueous extract of *Ficus deltoidea* was purchased from Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia (UTM), Johor, Malaysia (Figure 3.2). Herba Bagus Sdn. Bhd is the main supplier for the *Ficus deltoidea* leaves. Following taxonomical authentication, the leaves (voucher specimen: MFD 6) were deposited at the Herbarium of the Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia (UTM), Johor. Briefly, 10 kg of the fresh leaves were boiled with 80 L of distilled water for two hours. Then, the extract was filtered and freeze-dried at 180ºC (inlet temperature) and 108ºC (outlet temperature). The freeze dried extracted material was kept in an air-tight container and stored in the refrigerator at 4ºC until use.

Figure 3.1: Tualang honey at the Honey processing Centre in Kuala Nerang, Kedah. (penpasksgb.blogspot.com)

Figure 3.2: *Ficus deltoidea* at the Institute of Bioproducts Development (IBD), Universiti Teknologi Malaysia (UTM), Johor, Malaysia. (www.ibd.utm.my)

3.1.4 Materials

Table 3.1: Materials use in the experiments

Chemical	1) Bisphenol A (Sigma-Aldrich, USA) 2) Corn oil (Sigma-Aldrich, USA) 3) Ketamine (Troy Laboratories, Australia) 4) Xylazil (Troy laboratories, Australia) 5) RNAlater (Ambion, USA) 6) Potassium chloride (NaCl) (Sigma-Aldrich, USA) 7) Kalium Chloride (KCl) (Sigma-Aldrich, USA) 8) Na_2HPO_4 (Sigma-Aldrich, USA) 9) KH_2PO_4 (Sigma-Aldrich, USA) 10) Absolute ethanol 99.8% (BDH, USA) 11) Ethanol 95% (BDH, USA) 12) Xylene (BDH, USA) 13) Formaldehyde (BDH, USA) 14) Paraplast (BDH, USA) 15) Hematoxylin (Sigma-Aldrich, USA) 16) Eosin-Y (Sigma-Aldrich, USA) 17) Acid hydrochloric (Sigma-Aldrich, USA) 18) Canada balsam (Sigma-Aldrich, USA) 19) Phosphate Buffered Saline (PBS) (Santa Cruz, USA)
Kit/antibody	1) ImmunoCruz Rabbit ABC staining system (sc-2018, Santa Cruz, USA) 2) Primary antibody ER-α MC-20 (sc-542, Santa Cruz, USA) 3) ImmunoCruz Rabbit ABC staining system (sc-2023, Santa Cruz, USA) 4) Primary antibody C3 V-20 (sc-14612, Santa Cruz, USA) 5) Rabbit Specific HRP/DAB (ABC) detection IHC (Abcam, USA) 6) ER-β antibody ab3576 (Abcam, USA) 7) FSH (Cusabio, Delaware, USA)

	8) LH (Cusabio, Delaware, USA) 9) 17-β estradiol (Cusabio, Delaware, USA) 10) Progesterone (Cusabio, Delaware, USA) 11) Thiobarbituric Acid Reactive Substances (TBARS) assay (OxiSelect TBARS assay kit, Cell Biolabs, USA) 12) RNeasy Protect Mini Kit (Qiagen, USA) 13) High Capacity RNA-to-cDNA kit (Applied Biosystem, USA) 14) TaqMan Gene Expression Assays (Applied Biosystem, USA)
Equipment	1) Electronic analytical balance (Denver Instrument Company AA-160) 2) Centrifuge (Christ Heraeus, Germany) 3) Fridge (Pensonic, Thailand) 4) Frozen -80ºC (Pensonic, Thailand) 5) Image analyzer attach to the light microscope (NIS-Elements Advanced Research, Nikon, Japan) 7) Tissue processor (Citadel 2000, Thermo Scientific, USA) 8) Microwave (Panasonic, Thailand) 9) Oven 37ºC (Echo Thermo, USA) 10) Microplate reader (BioTek, USA) 11) NanoDrop (BioTek, USA) 12) Thermal cycler (Thermo Scientific, USA). 13) StepOne Plus Real-Time PCR System (Applied Biosystem, USA).

3.2 Methodology

3.2 Study design

3.2.1 Administration of animal

After acclimatization for one week, 48 postweaning female Sprague Dawley rats at 28-day of age (P28) were divided into the following six groups (n=8) (Figure 3.3 and Figure 3.4). They were administered with BPA (10 mg/kg) alone or concurrently with Tualang honey (200 mg/kg) or *Ficus Deltoidea* (100 mg/kg) by oral gavage as follows:

1) NC (Negative Control group): Treated with vehicle corn oil.
2) PC (Positive Control group): Treated with BPA at 10 mg/kg.
3) TH (Tualang honey group): Treated with Tualang honey at 200 mg/kg and BPA at 10 mg/kg.
4) THC (Tualang honey Control group): Treated with Tualang honey at 200 mg/kg and vehicle corn oil.
5) F (*Ficus deltoidea* group): Treated with *Ficus deltoidea* at 100 mg/kg and BPA 10 mg/kg.
6) FC (*Ficus deltoidea* Control group): Treated with *Ficus deltoidea* at 100 mg/kg and vehicle corn oil.

NC group (Negative Control) was treated with vehicle (0.2 ml of corn oil). PC group (Positive Control) was treated with BPA suspended in vehicle at 10 mg/kg body weight. TH group (Tualang honey group) was administered with 200 mg/kg body weight of Tualang honey 30 min before they were administered with BPA (10 mg/kg body weight). THC group (Tualang honey Control) was administered with 200 mg/kg body weight of Tualang honey 30 min before administration of corn oil. F (*Ficus deltoidea* group) group was administered with 100 mg/kg body weight of *Ficus deltoidea* 30 min before they

were administered with BPA (10 mg/kg body weight). FC (*Ficus deltoidea* Control group) group was administered with 100 mg/kg body weight of *Ficus deltoidea* 30 min before administration of corn oil. BPA was solute with the corn oil to make it easily administered and prepared in absorbable form in rats.

The administration was performed once daily in the morning (between 09:00 and 10:00 AM) by oral gavage for six consecutive weeks. The oral route was chosen to mimic the most likely route of human exposure to BPA. Throughout the administration period, daily body weight of individual rats was recorded. Vaginal smear was evaluated daily to determine the changes in estrous phase. After the last dose of treatment, all the rats were only sacrificed at the onset of diestrous phase. Standardization of diestrous phase during sacrifice is important because histological features and physiology of female reproduction varies greatly, corresponding to the different phases of estrous cycle. Thus, standardization of estrous phase during sacrifice will minimizing these variability which consequently provides more reliable and consistent interpretations of results. Blood samples were collected from the abdominal aorta under general anaesthesia and the extracted blood serum samples were stored at −20°C until further analysis.

The wet weight of the whole uterus was measured. The left horn of the uterus was immediately fixed in 10% buffered formalin for histopathological analysis. One half the right horn of uterus was kept in phosphate buffers for malondialdehyde (MDA) determination while the other half was kept in RNAlater® (Ambion, USA) for mRNA extraction. Subsequently, both of these right horns were stored at -80ºC until analysis. As for the ovaries, the weights of the ovaries were immediately measured; then the ovaries were fixed in 10% buffered formalin for histopathological analysis.

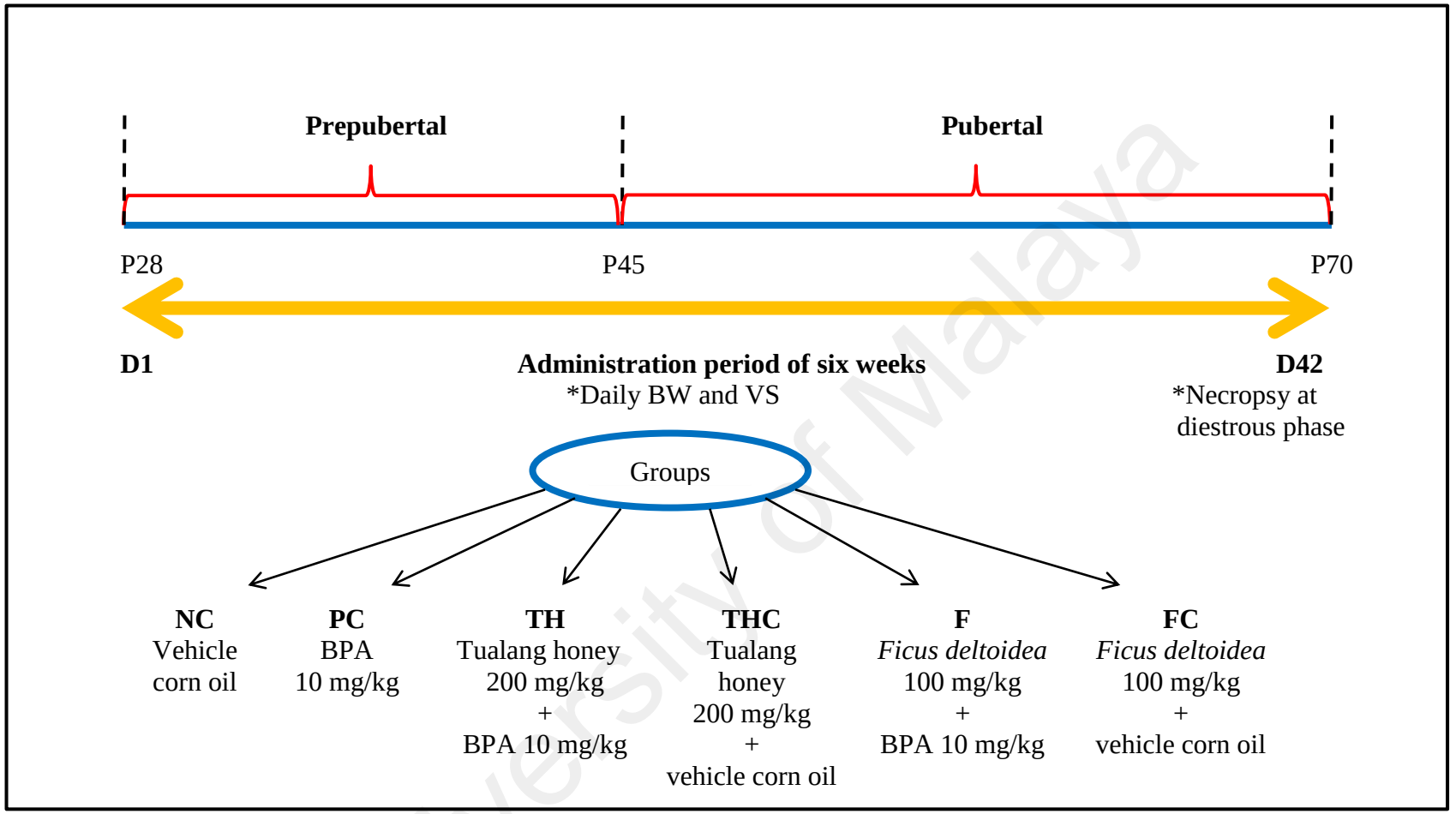

Figure 3.3: Schematic representation of the study design used to analyse the disruptive effects of prepubertal exposure to BPA and protective effects by concurrent treatment with Tualang honey and *Ficus deltoidea*. P: postnatal day; D1: Beginning or day 1 of administration; D42: Last dose or day 42 of administration; BW: body weight; VS: vaginal smears; NC: Negative Control group; PC: Positive Control group; TH: Tualang honey group; THC: Tualang honey Control group; F: *Ficus deltoidea* group; FC: *Ficus deltoidea* Control group.

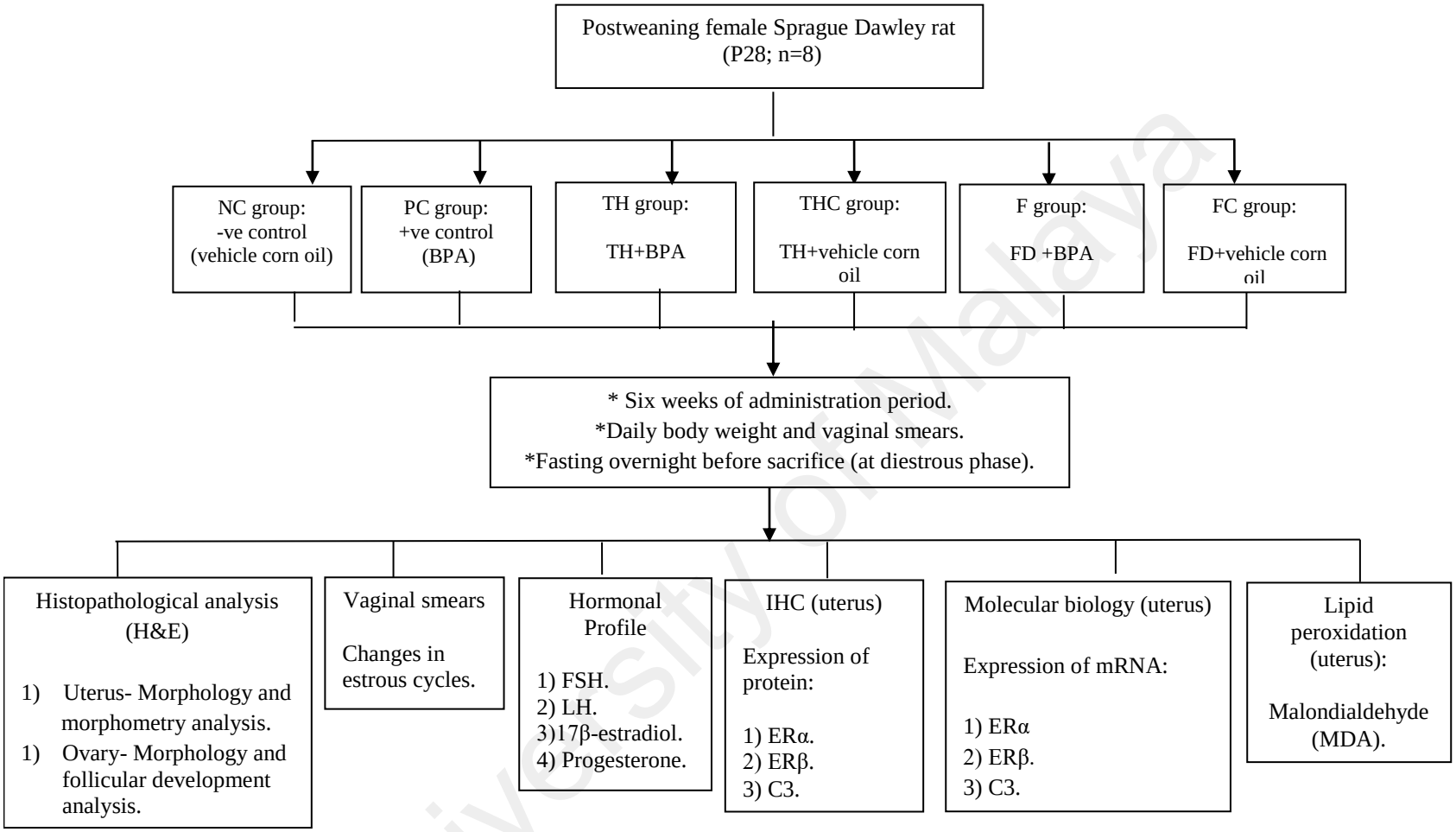

Figure 3.4: Flow chart of study design

BPA= Bisphenol A 10 mg/kg bw; TH= Tualang honey 200 mg/kg bw; FD= *Ficus Deltoidea* 100 mg/kg bw. NC: Negative control; PC: Positive control; TH: Tualang honey; THC: Tualang honey control; F: *Ficus deltoidea*; FC: *Ficus deltoidea* control; IHC: Immunohistochemistry.

3.2.2 Justification of dose for BPA

BPA is present in the numerous daily-used products, particularly in the packed food and drinks. Therefore, oral is the most relevant route of human exposure to BPA. Consequently, oral route exposure in the laboratory animal studies could be as relevant references for the safety assessments of human exposure to no-effect or lowest effects levels.

It is very important to note that the selected dose of BPA at 10 mg/kg is lower than the lowest observable adverse effect level (LOAEL) for BPA (50 mg/kg) (http://www.epa.gov/iris/subst/0356.htm) that is declared by U.S.-EPA (United States Environmental Protection Energy). The declaration was made based on the Reference Dose for Chronic Oral Exposure (RfD) in rat that estimates a daily exposure to the human population without an appreciable risk of deleterious effects during a life time. Indeed, toxicity study has identified the dose below than the 50 mg/kg of BPA as no adverse effect dose level (NOAEL) on the reproductive and developments in the offspring (Tyl et al., 2002).

Nonetheless, previous studies have found that at a low dose of 10 mg/kg BPA is still able to induce disruptive effects on morphological and biochemical parameters in the reproductive system (Anjum et al., 2011; Li et al., 2013; Okuda, Takiguchi, & Yoshihara, 2010; Suzuki et al., 2002). Therefore, the dose selection in the present study (10 mg/kg of BPA) was based on these studies and to verify whether low dose exposure to BPA could induce disruptive effects on the female reproductive organ in the present study.

3.2.3 Justification of doses for Tualang honey and *Ficus deltoidea*

The dose of Tualang honey at 200 mg/kg was based on a previous study that showed positive biological effects on female reproductive organs and the dose used was equivalant to one table spoon that is routinely taken by an adult human (Zaid et al., 2010). Among the many variety of Malaysian honey, the wild local Tualang honey was chosen for this study because of their purity and quality that are guaranteed and fully monitored by the Malaysian Agricultural Research and Development Institute (MARDI), Malaysia and its also has been supported by scientific studies (Ahmed & Othman, 2013; Krishna Kishore, Halim, Syazana, & Sirajudeen, 2011; Mahaneem et al., 2010; Mahaneem et al., 2011).

Ficus deltoidea var *deltoidea* (FDD) and *Ficus deltoidea* var augustifolia (FDA) were the most popular and commonly used by Malay community in Malaysia. In the present study, FDD was selected because it contained more or different bioactive compounds compared to FDA, which is involved in the uterotonic effect (Umi Romaizatul Amiera et al., 2014). In addition, FDD also contained higher phenolic acid, polyphenol and flavonoids compared to the FDA (Hakiman & Mahmood, 2009). The selection dose of 100 mg/kg body weight of aqueous extract of *Ficus deltoidea* was based on a previous subchronic toxicity study where no signs of toxicity effects were observed in hematological and biochemical parameters in rats (Fazliana, Muhajir, Hazilawati, Shafii, & Mazleha, 2008). The aqueous extract of leaves of *Ficus deltoidea* showed positive effects on the reproductive system which showed uterotonic effects (Amiera, Nihayah, Wahida, & Rajab, 2014) and this form of extraction is the most common preparation, where it is routinely prepared as tea that is derived from the infusion of the leaves in hot water. The female leaves of *Ficus deltoidea* was selected due to its higher antioxidants levels than the male leaves, according to most non enzymatic and enzymatic

antioxidant assays (Hakiman & Mahmood, 2009) and that the extract of the leaves has been shown to be more potent in cytotoxicity activity than the fruit extract (Norrizah, Norizan, Sharipah Ruzaina, Dzulsuhaimi, & Nurul Hidayah, 2012).

The quality of aqueous extract of *Ficus deltoidea* has been guaranteed by Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia (UTM), Malaysia since the production of extract is according to the ISO Certified Laboratory, which is the purity and quality are follows the guidelines of international standard.

In order to preserve the freshness and to prevent any loss of antioxidant content, the working dose of Tualang honey and *Ficus deltoidea* were freshly prepared every morning by dissolution in deionized water.

3.2.4 Determination of estrous cycle

Vaginal opening in the rats is a reliable and standard endpoint as an external index for the determination of puberty onset in the toxicity studies and its occurs simultaneously with the first ovulation (Li & Davis, 2007). Vaginal opening can be detected by a simple visual examination of the vulva, which is identified by the formation of external orifice of the vaginal canal during their age at 5 to 8 weeks (Figure 3.5). In the present study, monitoring for the onset of vaginal opening in the rats was started one week earlier where their age was 4 weeks old.

In the present study, the first day of daily vaginal smear was commenced once vaginal opening was detected in the rats (between 09:00 and 10:00 AM daily). For the collections of vaginal secretions, the micropipette tips filled with approximately 0.2 ml of normal saline (NaCl 0.9%) was inserted into the vagina to a depth of 2 to 5 mm (Figure 3.6). Normal saline was used as an isotonic solution with osmolarity closed to the blood that provide longer period for the cells from lysis. Then, normal saline was flushed and

drew back into the micropipette tips for three times before the sample was collected. A drop of the cell suspension was smeared onto a labeled glass slide and immediately viewed under a light microscope, using 10x (for determination of proportion the three cells types) and 40x objective lenses (for characterization of dominant cells).

Cytological appearance of estrous phase was determined as follows (Figure 3.7):

1) The proestrous phase (twelve to fourteen hours) was defined by the predominance of nucleated epithelial cells.

2) An estrous phase (twenty-five to twenty-seven hours) primarily consisted of anucleated cornified cells.

2) The metestrous phase (six to eight hours) indicated by equal proportions of leucocytes, cornified and nucleated epithelial cells.

3) The diestrous phase (fifty-five to fifty-seven hours) primarily consisted of the predominance of leucocyctes.

The estrous cycle patterns are described as follows:

1) Normal cycle (NC): A 4 to 5-day of estrous cycle in which the estrous phase was observed at least twice during the sampling period.

2) Persistent diestrous: Known as prolonged diestrous that lasts 4 or more days of diestrous phase during most of the cycle.

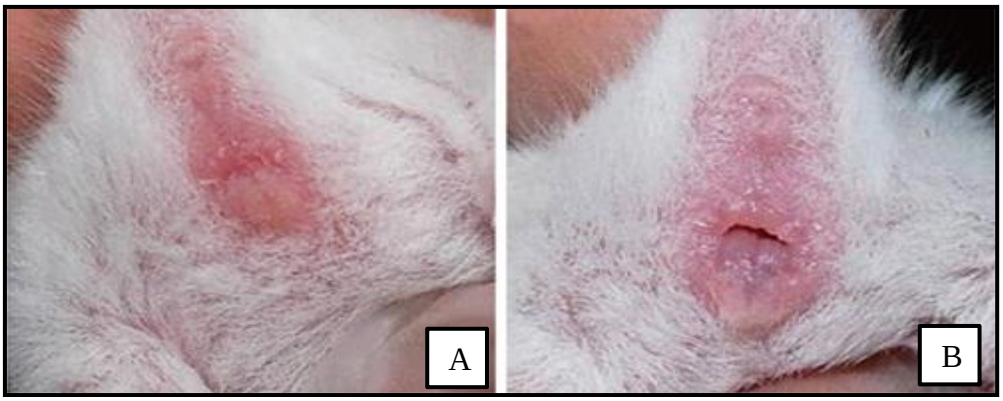

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Figure 3.5: Photographs of vaginal opening. The photo (A) showed the vulva of the rat before the onset of vaginal opening. The formation of vaginal opening was identified by the formation of external orifice of the vulva (B). (http://ecerm.org/)

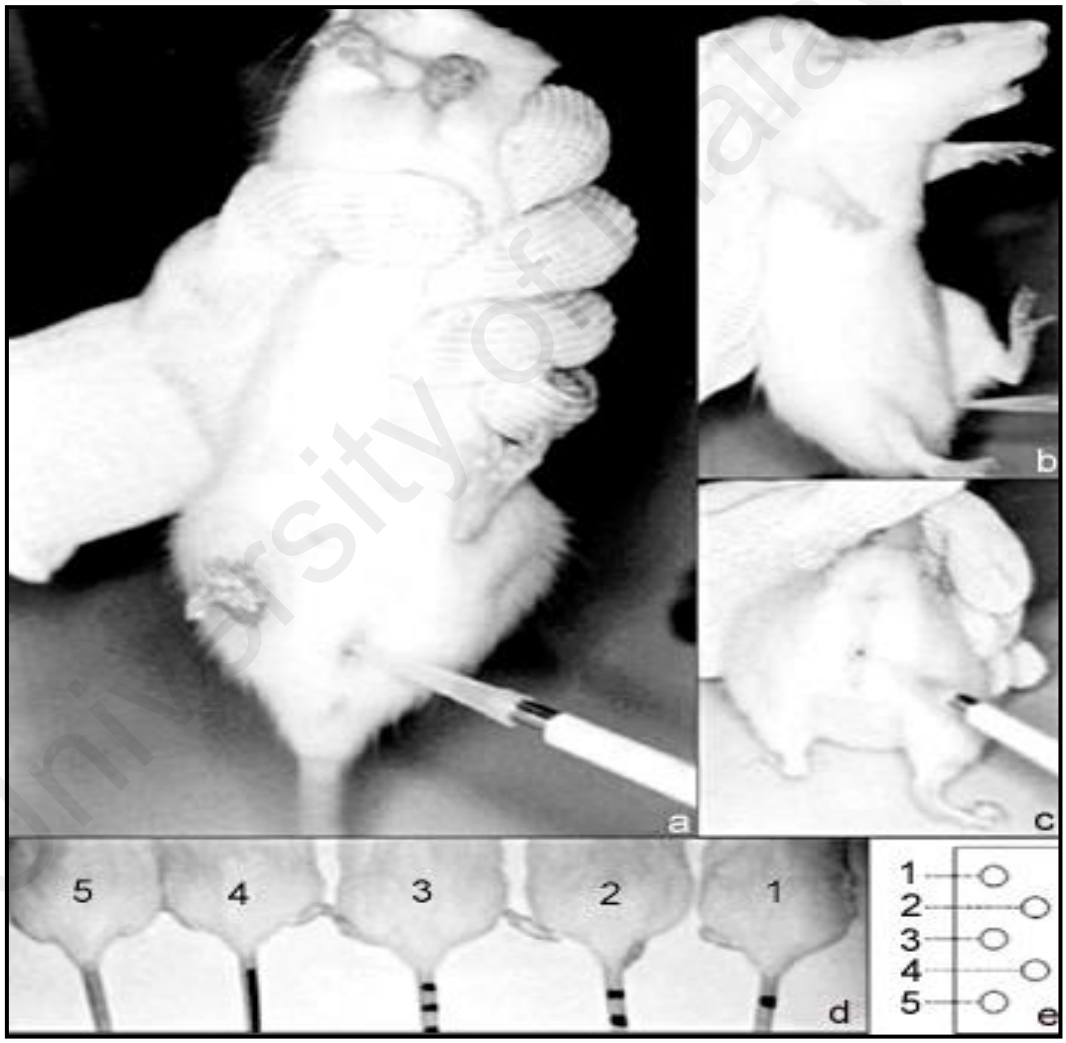


Figure 3.6: Method of vaginal secretion collections from the rats.
(Marcondes, Bianchi, & Tanno, 2002)

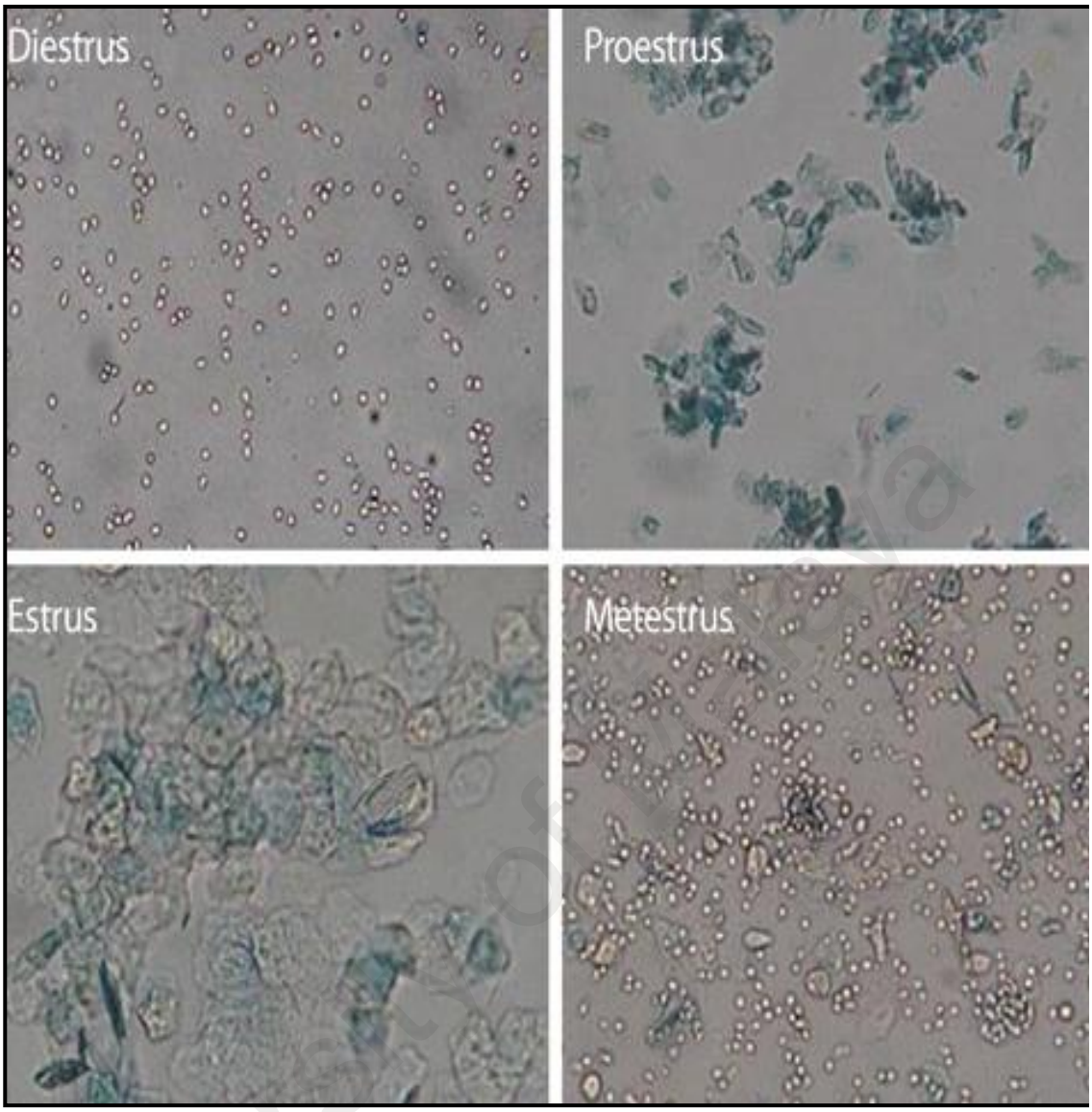


Figure 3.7: Photomicrographs indicating the cytological appearances of different phases of the estrous cycle from the vaginal secretions of rats. Diestrous phase primarily consisted of leucocytes cells. Proestrous phase is defined by the predominance of mucleated epithelial cells. Estrous phase is dominated by anucleated cornified cells. Metestrous phase is indicated by equal proportions of leucocytes, anucleated cornified cells and nucleated epithelial cells (400X). (Nah, Park, & Gye, 2012)

3.2.5 Blood samples collection

Laparatomy was performed while the rats were under general anaesthesia. Blood samples were carefully taken from abdominal aorta using a syringe (5 ml) with needle (size 21 G). The blood samples were collected into plain tubes and left aside for one hour to ensure complete clotting. Subsequently, blood samples were centrifuged (Christ Heraeus, Germany) at 2500 rpm for 15 minutes at 4˚C to extract the serum, and the aliquots of the serum were kept frozen (Pensonic, Thailand) at -20˚C until further analysis of the hormonal profile for 17-ß estradiol, progesterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH).

3.2.6 Collection of the ovary and uterus

The abdominal cavity was opened with a longitudinal cut. The urethra and rectum were carefully dissected from the anterior and posterior walls of the vagina. Then, the vagina with the two horns of uterus and ovaries (left and right) were dissected cut from the surrounding fat. Figure 3.8 shows the ovary and uterus of a rat.

Separation of uterus from vagina was done by cutting the uppermost point of the cervix. Measurements of weights of selected tissues were done using electronic analytical balance (Denver Instrument Company AA- 160). The right ovary was immediately weighed and fixed in 10% buffered formalin for histopathological analysis. The wet weight of the whole uterus was measured for uterotrophic response evaluation. The left horn of uterus was immediately fixed in 10% buffered formalin for histopathological analysis. The proximal half of the right horn of uterus was kept in phosphate buffer for malondialdehyde (MDA) determination while the distal half was kept in RNAlater® (Ambion, USA) for mRNA extraction. Subsequently, both of these right horns were stored at -80ºC until analysis.

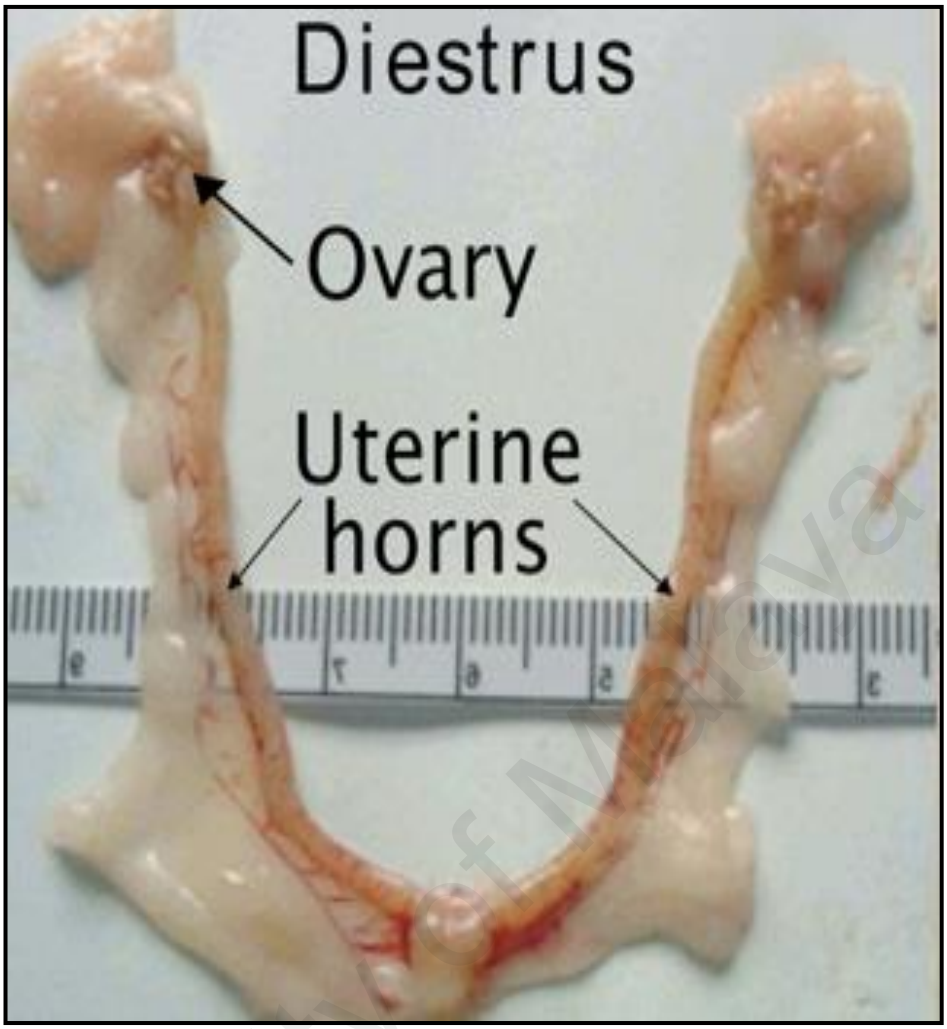


Figure 3.8: Ovary and uterus from rats during diestrous phase. (www.mdpi.com)

3.2.7 Histopathological analysis

The formalin-fixed tissues (ovary and left horn of uterus) were processed by dehydration in a graded series of ethanol, cleared by xylene and infiltrated in paraffin using an automatic tissue processor (Citadel 2000, ThermoScientific, USA). Subsequently, these processed tissues were embedded in paraffin. The uterus paraffin block was sectioned at 5 μm thickness while the ovary paraffin block was serially sectioned at 5 μm thickness. The sections were mounted onto labeled glass slides. Subsequently, the sections were deparaffinized in xylene, stained with hematoxylin and

eosin (Sigma Aldrich, USA), dehydrated in a graded series of ethanol, cleared in xylene and mounted with Canada Balsam (Sigma-Aldrich).

Morphological changes and morphometric analysis were conducted under a light microscope (Olympus CH-B145-2) attached to an image analyzer (NIS-Elements Advanced Research, Nikon, Japan). Classification and quantification of ovarian follicles from eight rats of each group were performed on 62 total field areas measured with grid lines using NIS-Elements software (Figure 3.9).

3.2.7.1 Histomorphometry of uterus

The pathologist who evaluated the histological changes of uterine sections was blinded to the treatments that the rats received. For histomorphometric analysis, the slides were reviewed and the clearest section on each slide was photographed at 20X and 40X magnifications.

The mean values of the endometrial and myometrial thickness were measured on six randomly chosen areas of the sections, and the height of the uterine luminal epithelial cells were also measured (Figure 3.10). All measurements (in μm) were manually determined by tracing on-screen images with a cursor using a computer-image analyzing program (NIS-Elements Advanced Research, Nikon, Japan).

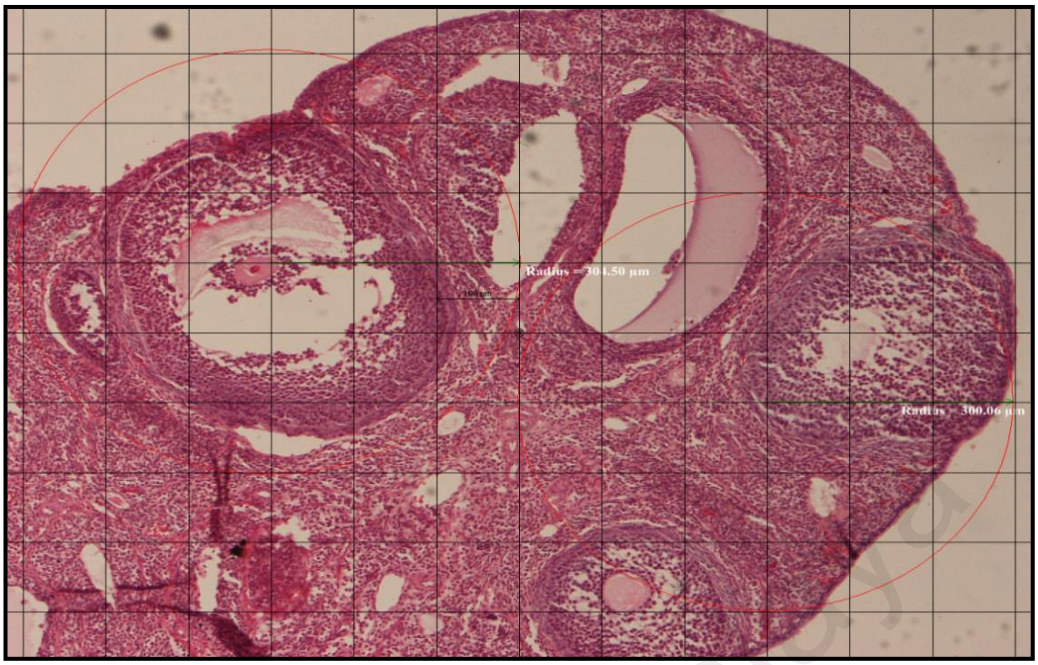


Figure 3.9: Grid lines used for the classification and quantification of ovarian follicles using NIS Elements software (10X, H&E)

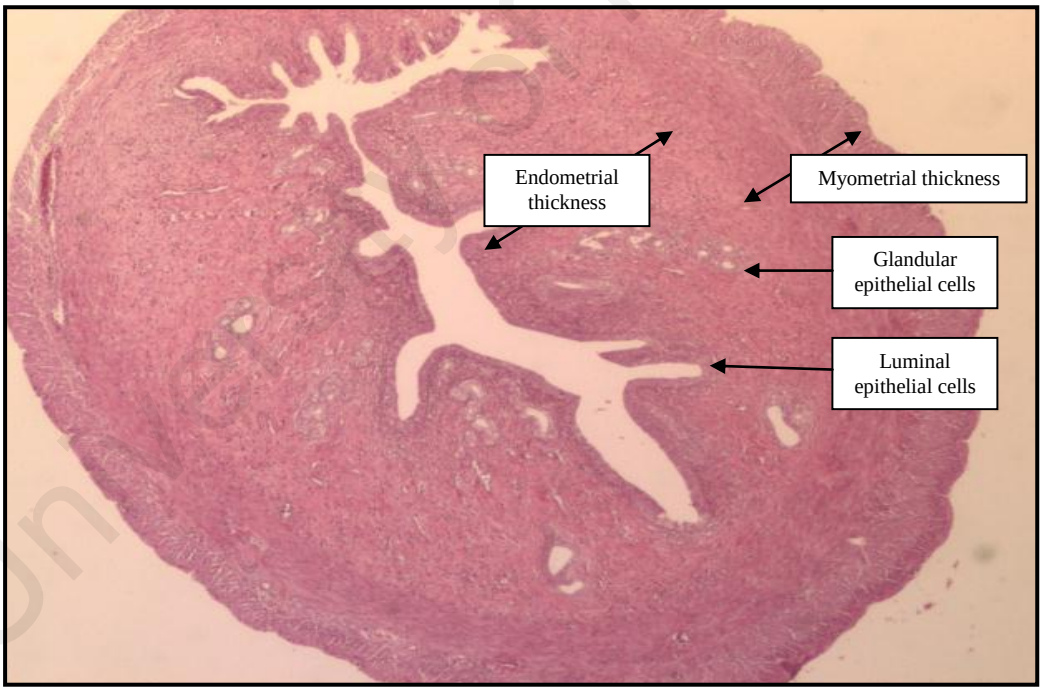
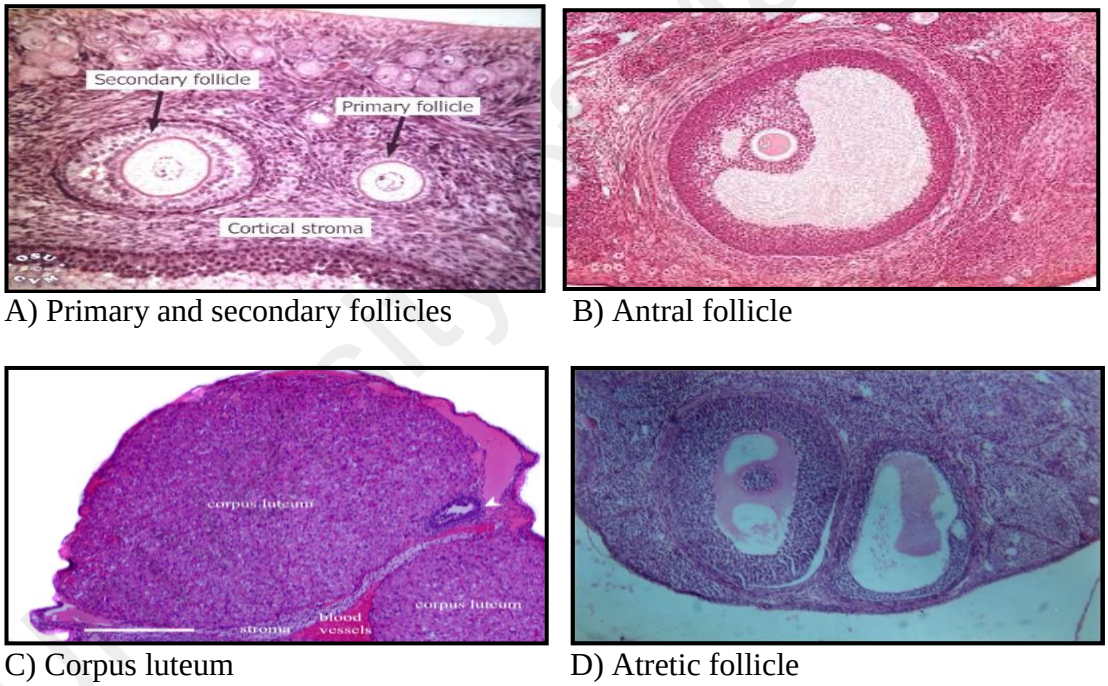


Figure 3.10: Histomorphometric measurements of the uterus (4X, H&E).

3.2.7.2 Classification and quantification of ovarian follicles

All ovarian sections were observed under a light microscope (Olympus CH-B145-2) attached to an image analyzer (NIS-Elements Advanced Research, Nikon, Japan). Classification and quantification of ovarian follicles were conducted according to the criteria described in a previous study (Zhuang et al., 2010) (Figure 3.11). One section was chosen out of every 20 sections with five sections collected from each ovary. Each of selected ovarian sections were traced around the tissue boundaries with the computarised software and a sampling grid is superimposed over the section. The standardized size of counting fields were manually traced on the gridlines.



A) Primary and secondary follicles B) Antral follicle

C) Corpus luteum D) Atretic follicle

Figure 3.11: Classification of ovarian follicles. A) Primary follicles comprise of an oocyte surrounded by a single layer of cuboidal granulosa cells while secondary follicle is consists of an oocyte surrounded by more than one layer of cuboidal granulosa cells and no visible antrum. B) Antral follicle has clearly defined antral space and cumulus granulosa cell layer. C) Corpus luteum is comprised of lutein cells and formed only after ovulation. D) Atretic follicle is degenerated follicle with inspissated follicular fluid, degenerated oocyte, disorganized and thickened granulosa layers or filled with organizing fibrinous material in the antrum. (www.flickr.com).

3.2.8 Assay of serum 17β-estradiol, FSH, LH and progesterone

Measurement of these hormone levels were performed using ELISA based kits (Cusabio, USA) following similar principal and protocols. Each hormone was analysed using different kits according to the specific Antibody and Horseradish Peroxidase. The Catalog Numbers for each kit are as follows:

Table 3.2: Catalog Number of kits for each hormone.

Hormone	Catalog Number
17β-estradiol	CSB-E07279r
FSH	CSB-E06869r
LH	CSB-E12654r
Progesterone	CSB-E07282r

In brief, 50 µl duplicates of Standards, Samples and Blank were added to the representative wells coated with E_2. Subsequently, 50 µl of specific HRP conjugate were added to each well (except to Blank wells). Then, 50 µl of specific antibody for each hormone was added to each well. The solutions in the wells were mixed and incubated for two hours in a 37˚C incubator (Echo Thermo, USA). After the incubation period, these mixed solutions were carefully aspirated from each well and the wells were rinsed for three times using Wash Buffer solutions. Subsequently, 50 µl of the Substrate A and Substrate B were added to each well and incubated again for another 15 minutes at 37˚C.

Finally, the reactions were terminated using 50 µl of Stop Solution. A microplate reader (BioTek, USA) was used to measure the OD at wavelength of 450 nm. A standard curve was constructed by plotting a graph of the absorbance of each reference standard against its corresponding levels and used to determine each hormone level.

3.2.9 Malondialdehyde (MDA) determination

MDA levels of uterus were measured by the double heating method (Draper & Hadley, 1990) using Thiobarbituric Acid Reactive Substances (TBARS) assay (OxiSelect TBARS assay kit, Cell Biolabs, USA).

The uterus of each rat was homogenized in phosphate buffered saline (PBS) containing butylated hydroxytoluene (BHT). Subsequently, the uterine homogenate was centrifuged at 10,000 g for five minutes to collect the supernatant for the TBARS assay. 100 µl of supernatant samples and standards were mixed with 100 µl of SDS lysis solution in appropriate tubes and incubated for five minutes at room temperature. Then, 250 µl of TBA reagent was added to each tube followed by 60 minutes incubation at 95ºC.

The tubes were cooled at room temperature in ice bath for five minutes. All tubes were centrifuged at 3000 rpm for 15 minutes and 200 µl of the supernatant was transferred to a 96 well-microplate for measurement of absorbance using a spectrophotometer at 532 nm wavelength. The concentration of MDA was calculated using the absorbance coefficient of the MDA-TBA complex and expressed as micromoles per micro gram protein (µM/µg protein).

3.2.10 Immunohistochemistry

3.2.10.1 ERα and C3

The distributions of ERα and C3 in uterus of rats were evaluated using immunoCruz Rabbit ABC staining system sc-2018 kit (Santa Cruz, USA) and immunoCruzGoat ABC staining system sc-2023 kit (Santa Cruz, USA), respectively. Immunohistochemistry for ERα and C3 used similar principal and protocol, but incubated with their correspond antibodies.

Uterine sections were deparaffinized, hydrated, boiled in 0.1 M sodium citrate buffer (pH 6.0) for 15 minutes and incubated with 0.1% - 1% of hydrogen peroxide for 10 minutes to quench the endogenous peroxidase activity. Non-specific binding was blocked by incubation with 1.5% blocking serum. Subsequently, the sections were incubated with primary antibody at 1:100 of ER-α (MC-20:sc-542, Santa Cruz Biotechnology, USA) or 1:100 of C3 (V-20:sc-14612, Santa Cruz Biotechnology, USA) for overnight at 4ºC.

On the following day, the sections were incubated with biotinylated secondary antibody for one hour. Subsequently, they were incubated with AB enzyme reagent for 30 minutes. The color developed after incubation with peroxidase abstract for 10 minutes and counterstained with hematoxylin. The sections were dehydrated through 2x 95% of ethanol, 2x 100% and 3x xylenes for 10 seconds each. Finally, the sections were mounted with Canada Balsam (Sigma-Aldrich) and covered with glass coverslip before viewing under a light microscopy. The negative control tissue (not incubated with primary antibody) was included to ensure no false positive staining and accurate interpretation of the staining results.

3.2.10.2 ERβ

The distribution of estrogen receptor-β in rat's uterus was evaluated using Rabbit Specific HRP/DAB (ABC) Detection IHC kit ab64261 (Abcam, USA). Initially, the sections were deparaffinized, hydrated, boiled in 0.1 M sodium citrate buffer (pH 6.0) for 15 minutes and incubated with hydrogen peroxide for 20 minutes to quench the endogenous peroxidase activity. Non-specific binding was blocked by incubation with protein block. Subsequently, the sections were incubated with primary antibody at 1:200 (ER-β antibody ab3576, Abcam, USA) for overnight at 4ºC.

The next day, the sections were incubated with biotinylated secondary goat anti-polyvalent antibody for 30 minutes at room temperature. Color was developed after incubation with DAB chromogen for 10 minutes, followed by counterstained with haematoxylin. The sections were dehydrated through 2x 95% of ethanol, 2x 100% and 3x xylenes for 10 seconds each. Finally, the sections were mounted with Canada Balsam (Sigma-Aldrich) and covered with glass coverslip for viewing under a light microscopy. The negative control tissue (not incubated with primary antibody) was included to ensure no false positive staining and accurate interpretation of the staining results.

3.2.11 mRNA expression of ERα, ERβ and C3

3.2.11.1 Purification of total RNA

Purification of total RNA of uterus was done using RNeasy Protect Mini Kit (Qiagen, USA). Thirty mg of uterus was homogenized in Buffer RLT. The lysate was centrifuged at 12000 rpm. The supernatant sample was transferred into a new 1.5 ml tube and one volume of 70% of ethanol was added. This sample was then transferred into an RNeasy spin column, centrifuged and the flow-through was discarded. Subsequently, buffer RPE was added to the RNeasy spin column, centrifuged for one minute at 10000 rpm and the flow-through was again discarded. This step was repeated but centrifuged for two minutes.

Finally, the spin column was placed in a new 1.5 ml tube, added with 30 µl of RNase-free water and centrifuged at 10000 rpm for one minute to elute the RNA. Measurements of the concentration and purity of RNA were obtained by using a NanoDrop (BioTek, USA) at A_{260}:A_{280} ratio of absorbance. The values for absorbance ratios, purity and concentrations were averages of two readings. The high quality and purity of RNA were

within the range of 1.8 to 2.0 of absorbance ratio. Total RNA yields for all samples were calculated manually from the concentration and volume of RNA obtained. The RNA was kept at -80ºC until further analysis.

3.2.11.2 Reverse transcription of RNA to cDNA

Reverse transcription (RT) of RNA to single-stranded cDNA was executed using the Applied Biosystem kit, USA. Equal amounts of RNA (300 ng) obtained from each uterus sample were reverse-transcribed into cDNA. Each RT reaction mixture consisted of 2X RT buffer (10 µl), 1 µl of reverse transcriptase, nuclease-free water and RNA sample made up to a total volume of 20 µl. The reaction mixtures were briefly centrifuged to spin down the contents and to eliminate air bubbles. Finally, the tubes were placed in a programmed thermal cycler (Thermo Scientific) for 60 minutes at 37ºC followed by five minutes at 95ºC to terminate the reaction. The cDNA obtained was subsequently used for real-time PCR or stored at -20ºC until further analysis.

3.2.11.3 Quantitative Real-time PCR of selected genes

Quantitative Real-time PCR of selected genes was performed using Applied Biosystem Kit, USA. The PCR reaction mixture (reaction size of 20 µl) was prepared in triplicates. Each PCR reaction mix consists of 20x TaqMan Gene Expression Assay (1 µl), 2x TaqMan Gene Expression Master Mix (10 µl), cDNA template (4 µl) and RNase-free water (5 µl). The endogenous control gene used in this study was β-actin mRNA. In order to warrant specific amplification, three types of controls (water only, reaction without primers and templates derived without reverse transcriptase) were included in the PCR reaction. The prepared PCR reaction mixture was gently inverted to mix the reaction components, followed by a brief centrifugation to spin it down. Twenty µl of PCR

reaction mix was transfer to each appropriate 96-well reaction plate and briefly centrifuged.

Finally, the plate was placed in the StepOne Plus Real-Time PCR system (Applied Biosystems, USA). The point at which exponential amplification of the PCR products begins (values for cycle threshold=C_T) was determined using the Applied Biosystems software (Sequence Detection System). The experiment was repeated three times to ensure the validity of the results. The relative mRNA expression levels for each selected genes were calculated in relation to the β-actin internal control. The sequences of primer used for the amplification of gene are shown in the Table 3.

Relative abundances of the target mRNA were calculated using the $2^{-\Delta\Delta CT}$ method, where 2 was the fold change between C_T values and $\Delta\Delta C_T$ was the normalized cycle change between normal and treated samples (Livak & Schmittgen, 2001).

Table 3.3: Sequences of primers and references for Taqman-PCR .

Gene	Forward primer	Reverse primer	Amplicon size (bp)	Accession number and references
ER α	5'-AAGCTGGCCTGACTCTGCAG-3'	5'-GCAGGTCATAGAGAGGCACGA-3'	144	X61098 (Spreafico, Bettini, Pollio, & Maggi, 1992)
ER β	5'-CTCTGTGTGAAGGCCATGAT-3'	5'-GGAGATACCACTCTTCGCAATC-3'	159	U57439 (G.G Kuiper, Enmark, Pelto-Huikko, Nilsson, & Gustafsson, 1996)
C3	5'-CTGTACGGCATAGGGATATCACG-3'	5'-ATGCTGGCCTGACCTTCAAGA-3'	199	X52477 (Misumi, Sohda, & Ikehara, 1990)

3.2.12 Statistical analysis

All statistical evaluations were performed with Statistical Package for Social Sciences (SPSS Inc. Chicago, Illinois, USA version 18.0 for windows). Firstly, Shapiro-Wilk W test was performed to test whether all results followed a normal distribution (normally distributed if P value was greater than 0.05). Parametric variables were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni test for multiple comparisons to identify significant difference between groups. Values are reported as mean ± S.E.M. $P < 0.05$ is considered as significant.

CHAPTER 4: RESULTS

All results in this study have been analyzed and divided into two sets of studies as follows:

1) Protective effects of Tualang honey against Bisphenol A induced toxicity in the reproductive system.

2) Protective effects of *Ficus deltoidea* against Bisphenol A induced toxicity in the reproductive system.

In the Tualang honey study, all results from BPA-exposed rats (PC group-BPA 10 mg/kg) were analyzed and compared with control rats, (NC group-vehicle corn oil). Consequently, in order to evaluate the preventive effects of Tualang honey against toxicity effects of BPA, all results from BPA-exposed rats treated with Tualang honey (TH group-Tualang honey 200 mg/kg + BPA 10 mg/kg) were compared with BPA-exposed rats alone (PC group- BPA 10 mg/kg). Finally, the degree of the preventive effects of Tualang honey against the toxicity effects of BPA was compared with the control rats (NC group-vehicle corn oil), to evaluate whether Tualang honey is able to completely prevent those effects to the normal level. Evaluation to determine whether Tualang honey itself has effects on the reproductive functions was made by comparing all the results from the rats treated with Tualang honey alone (THC group-Tualang honey 200 mg/kg) with control rats (NC group-vehicle corn oil). Similar approach of results analysis was conducted for *Ficus deltoidea* study, accordingly.

Results from the two sets of studies were analyzed and presented separately to evaluate the protective effects of each natural product on the disruptive effects of the reproductive system induced by BPA.

4.1 Protective effects of Tualang honey against Bisphenol A induced toxicity in the reproductive system

4.1.1 Body and organ weights

Treatment with BPA and Tualang honey on female Sprague Dawley rats was commenced at prepubertal age (P28), according to their respective groups. Throughout the treatment period, daily body weights were recorded. After six weeks of treatment (P71), the rats were sacrificed during diestrous phase and weights of the uterus and ovaries were measured for evaluation of any changes.

The changes in the body weight and organ weights of the uterus and ovary of the rats in all experimental groups are shown in Table 4.1 and Figure 4.1, Figure 4.2 and Figure 4.3, respectively. The mean body weight gain in each group was obtained from the difference in values between the final body weight (at P71) and the initial body weight (at P28). This body weight gain was divided by the final body weight (at P71) to obtain the percentage of body weight gain.

Six weeks of chronic exposure to BPA in the PC group caused a slight increment (17.30%) in the body weight gain compared to the control rats (NC group). Comparable increment (16.50%) in body weight gain was also noted in rats concurrently treated with Tualang honey (TH group), indicating that TH does not have any effect on the changes caused by BPA. The changes in body weight gain for Tualang honey treated alone (THC group) was comparable to the control rats (NC group), reflecting that Tualang honey itself has no effect on the body weight. These changes in the body weight of rats in the various groups are not statistically significant.

The change in the uterine weight was recorded at the end of the administration period as an indicator for the changes of its normal functions. After six weeks of BPA exposure in the rats of PC group, there was a significant decline of 25.39% in relative uterine weight

compared to the control rats (NC group). However, concurrent treatment of rat with Tualang honey (TH group) significantly prevented this effect. The relative uterine weight in rats treated with Tualang honey alone (THC group) was comparable to the control rat (NC group), reflecting that Tualang honey has no effect on the weight of the uterus.

A different trend of changes was observed for ovarian weight compared to the uterine weight. The relative weight of the ovary in the PC group was significantly higher than the NC group by 20.83%. Interestingly, treatment with Tualang honey in BPA-exposed rat (TH group) showed significant reduction in the relative ovarian weight compared to the PC group and this value was comparable to the control rat (NC group). The relative ovary weight in rats treated with Tualang honey alone (THC group) was comparable to the control rats (NC group).

Table 4.1: Effect of BPA and Tualang honey on body weight and weights of uterus and ovary of rats (n=8).

Group	Body weight gain (g)	Changes in body weight gain (%)	Uterine wet weight (mg)	Relative weight of uterus (wet weight/ body weight)	Ovarian wet weight (mg)	Relative weight of ovary (wet weight/ body weight)
NC	78.88±14.61	48.15±6.60	296.25±26.58	1.89 ± 0.12^{bbb}	36.88±1.88	0.24 ± 0.01^{b}
PC	99.25±9.90	56.52±3.49	246.25±13.08	$1.41\pm0.04^{aaa,c,dd}$	49.38±1.13	$0.29\pm0.01^{a,c,d}$
TH	92.50±4.62	56.12±1.94	285.00±5.34	1.73 ± 0.04^{b}	40.00±1.64	0.24 ± 0.02^{b}
THC	89.50±10.64	52.39±3.47	315.71±29.75	1.83 ± 0.07^{bb}	38.75±0.82	0.23 ± 0.01^{b}

Data are expressed as Mean ±SEM.

1) $^{a}P<0.05$ and $^{aaa}P<0.001$ vs. NC
2) $^{b}P<0.05$, $^{bb}P<0.01$and $^{bbb}P<0.001$ vs. PC
3) $^{c}P<0.05$ vs. TH
4) $^{d}P<0.05$ and $^{dd}P<0.01$ vs.THC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

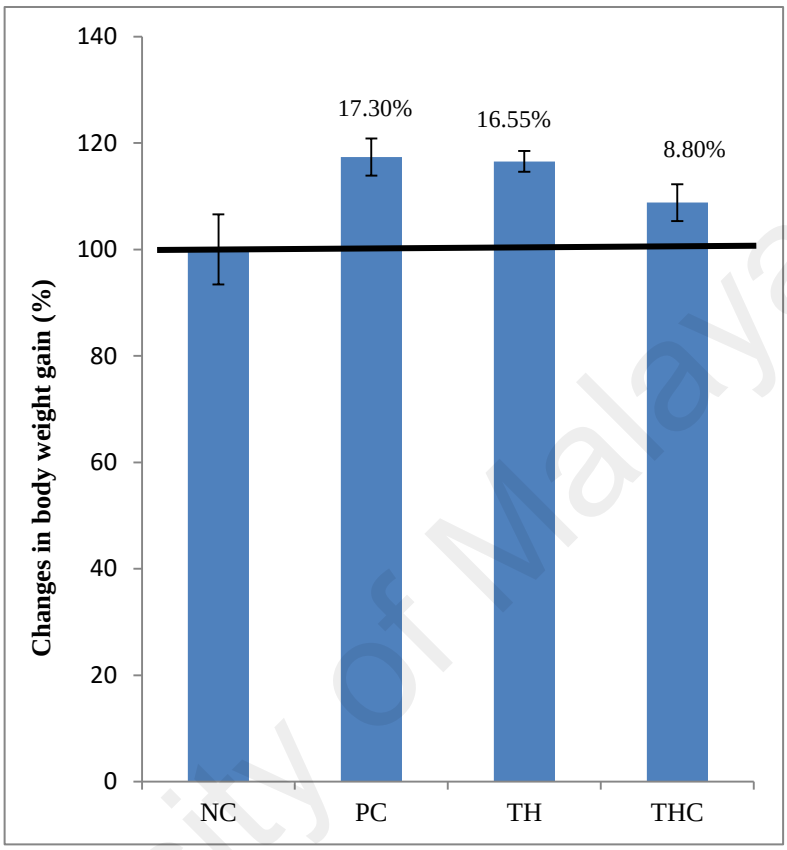


Figure 4.1: Effect of BPA and Tualang honey on the body weight of rats.

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

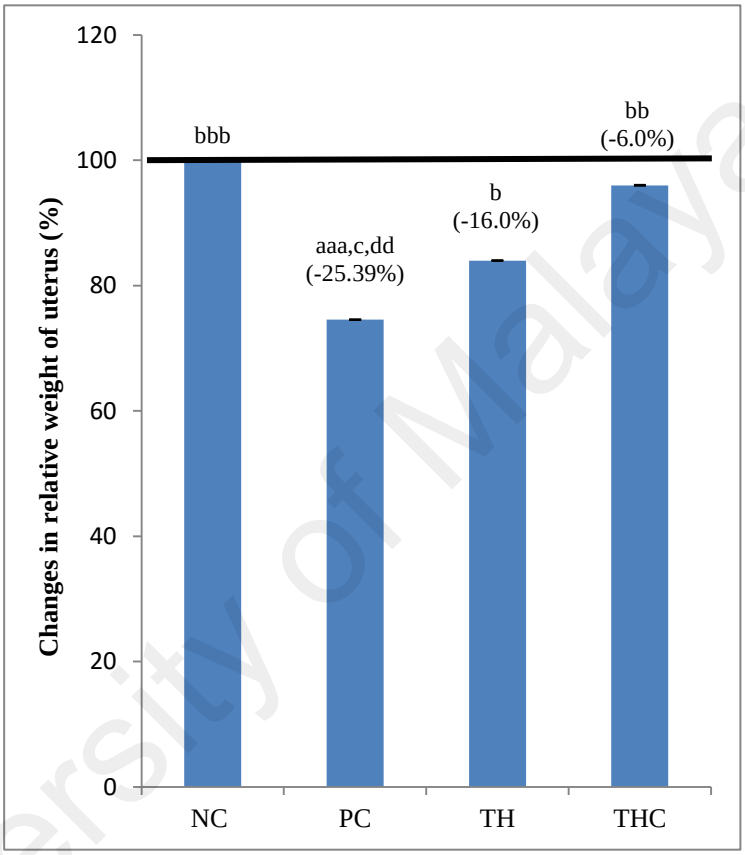


Figure 4.2: Effect of Tualang honey and BPA on the relative weights of uterus of rats.

1) $^{aaa}P<0.001$ vs. NC
2) $^{b}P<0.05$, $^{bb}P<0.01$ and $^{bbb}P<0.001$ vs. PC
3) $^{c}P<0.05$ vs. TH
4) $^{dd}P<0.01$ vs. THC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

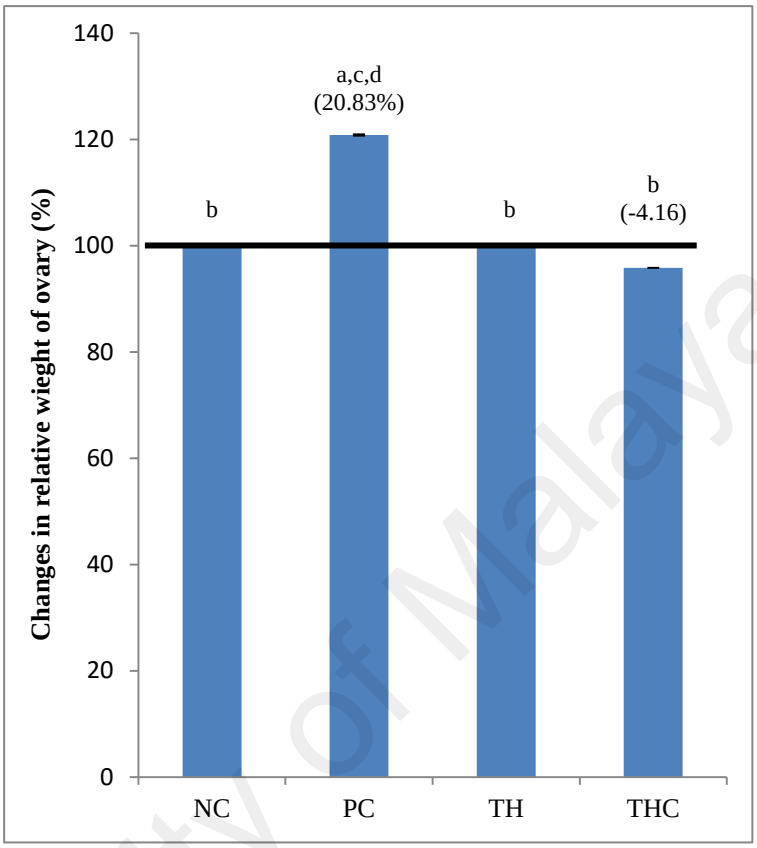


Figure 4.3: Effect of Tualang honey and BPA on the relative weight of the ovary in rats.

1) [a]P<0.05 vs. NC
2) [b]P<0.05 vs. PC
3) [c]P<0.05 vs. TH
4) [d]P<0.05 vs. THC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

4.1.2 Effects of BPA and Tualang honey on estrous cycle

To evaluate the effect of BPA and Tualang honey on the estrous cycle, vaginal smears were performed daily on the rats beginning from P41 until P71. The onset of the first estrous cycle in each rat may vary, anytime between at 5th to 8th weeks of age. Thus, evaluation of the estrous cycle has been standardized at P41 where all the rats had experienced their first estrous cycle. In NC group and THC groups, all rats maintained in their normal estrous cycle (100%). In PC group, only 12.5% of the rats presented normal estrous cycle with the rest showing persistent diestrous phase. These estrous cycles pattern were significantly different from both NC group and THC groups. Treatment with Tualang honey in BPA-exposed rats showed significantly higher percentage in normal estrous cycle (62.5%) with lesser number of rats in persistent diestrous phase compared to the PC group.

Table 4.2: Effect of BPA and Tualang honey on the estrous cycle (n=8).

Group	Rats with normal estrous cycle , % (n)
NC	100% (8/8)[b]
PC	12.5% (1/8)[a,c,d]
TH	62.5% (5/8)[b]
THC	100% (8/8)[b]

1) [a]P<0.05 vs. NC
2) [b]P<0.05 vs. PC
3) [c]P<0.05 vs. TH
4) [d]P<0.05 vs. THC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

4.1.3 Hormonal profile

The gonadotropins hormones (FSH and LH) as well as 17β-estradiol and progesterone are major reproductive hormones that regulate the functions of reproductive system in the mammals.

The function of FSH is to stimulate the growth and recruitment of immature ovarian follicles in the ovary while ovulation of matured ovarian follicles is triggered by LH (Li & Davis, 2007). 17β-estradiol is the most biologically active ovarian hormone that plays an important role for the growth and maintenance of the endometrium and myometrium lining of uterus (Ferreira et al., 2009). Progesterone is produced by residual ovulated follicle named corpus luteum that is crucial for converting the proliferative state of endometrial lining into a secretory lining that is receptive for implantation of an embryo (Li & Davis, 2007). Therefore, profiling of these reproductive hormones has been performed to determine the effects of BPA and Tualang honey in the treated rats.

4.1.3.1 Follicle stimulating hormone (FSH) and luteinizing hormone (LH)

Table 4.3, Figure 4.4 and Figure 4.5 show the changes in the FSH and LH levels in all experimental groups. The serum levels of FSH and LH were significantly reduced in PC group by 60.19% and 80.58%, respectively compared to the control (NC group). It was noted that concurrent treatment with the Tualang honey in BPA-exposed rats has significantly prevented the reduction of FSH level. However, the reduction in LH level was not prevented by concurrent treatment with Tualang honey in BPA-exposed rats. The levels of FSH and LH in rats treated with Tualang honey alone (THC group) was not significantly different compared to the NC group.

4.1.3.2 17β-Estradiol (E_2) and progesterone (P_4)

The E_2 levels were significantly higher in BPA-exposed rats (PC group) compared to the control rats (NC group) by 28.46% (Table 4.3 and Figure 4.6). Concurrent treatment with Tualang honey in BPA-exposed rats (TH group) also showed a higher level of E_2 compared to the NC group but in lesser magnitude compared to the PC group. Treatment with Tualang honey alone (THC group) did not significantly increase the E_2 level in the rats compared to the NC group.

All treated groups, PC and TH demonstrated lower progesterone levels compared to the NC group (Table 4.3 and Figure 4.7). The level of progesterone in rats in the PC group is significantly lower that the control group, NC. Concurrent treatment with Tualang honey in BPA-exposed rats (TH group) has significantly prevented this reduction. Treatment with Tualang honey alone (THC group) resulted comparable progesterone level with the NC group.

Tab**le 4.3:** Level of reproductive hormones in all experimental groups (n=8).

Group	Follicle Stimulating Hormone (FSH)	Luteinizing Hormone (LH)	17β-Estradiol	Progesterone
NC	$78.50\pm4.96^{bbb,ccc}$	$10.25\pm2.71^{bb,cc}$	19.04±3.99	64.65±6.28
PC	$31.25\pm5.96^{aaa,ddd}$	$1.99\pm0.32^{aa,dd}$	24.46±2.22	34.95±4.56
TH	$52.87\pm2.34^{aaa,b,ddd}$	$1.91\pm0.12^{aa,dd}$	22.42±2.07	42.78±5.36
THC	$72.00\pm7.25^{bbb,ccc}$	$9.30\pm1.19^{bb,cc}$	21.42±1.62	52.16±4.57

Data are expressed as Mean ±SEM.

1) $^{aa}P<0.01$ and $^{aaa}P<0.001$ vs. NC
2) $^{b}P<0.05$, $^{bb}P<0.01$and $^{bbb}P<0.001$ vs. PC
3) $^{cc}P<0.01$ and $^{ccc}P<0.001$ vs. TH
4) $^{dd}P<0.01$ and $^{ddd}P<0.001$ vs. THC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

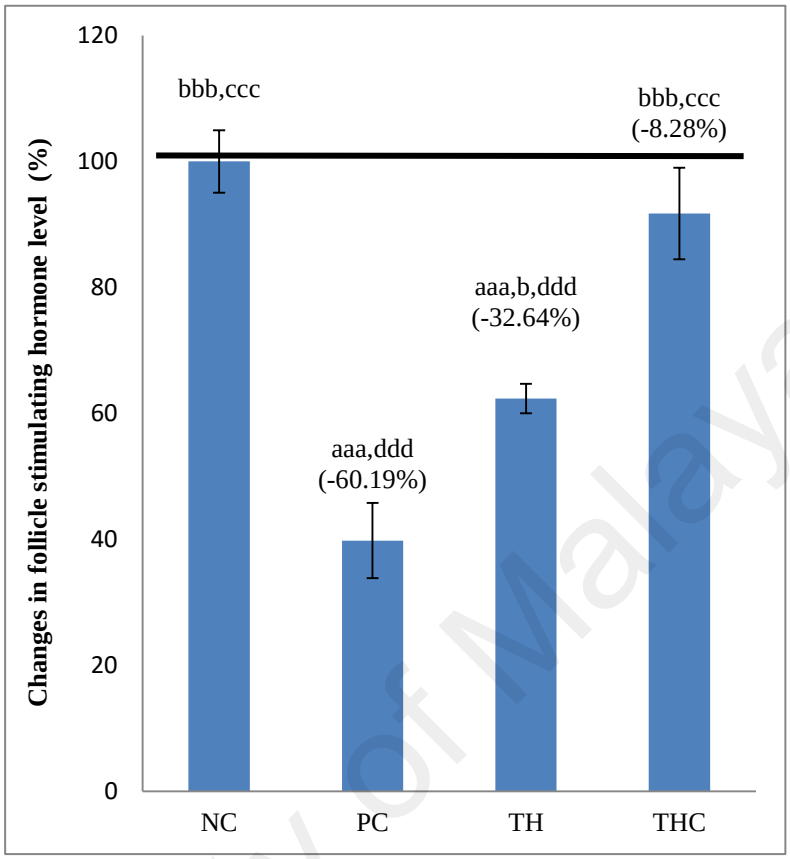


Figure 4.4: Effect of BPA and Tualang honey on the level of follicle stimulating hormone (FSH) in all experimental groups. After six weeks exposure to BPA (PC group), a significant reduction in FSH level was observed. Concurrent treatment with Tualang honey (TH group) in BPA-exposed rats significantly prevented this effect.

1) $^{aaa}P<0.001$ vs. NC
2) $^{b}P<0.05$, $^{bbb}P<0.001$ vs. PC
3) $^{ccc}P<0.001$ vs. TH
4) $^{ddd}P<0.001$ vs. THC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

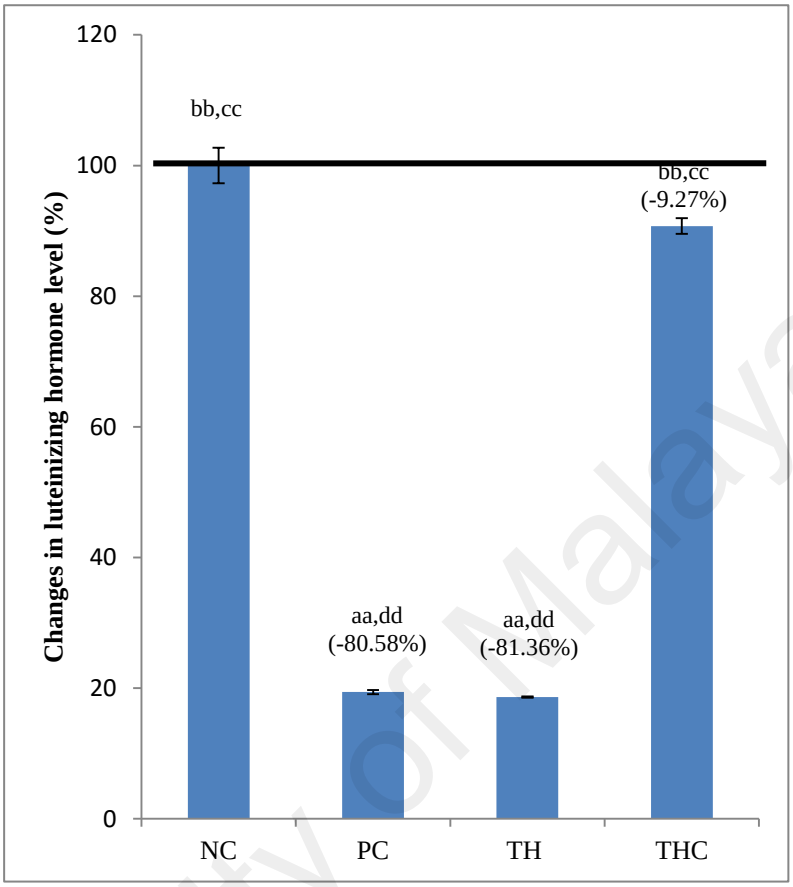


Figure 4.5: Effect of BPA and Tualang honey on the level of luteinizing hormone (LH) in all experimental groups. After six weeks exposure to BPA (PC group), a significant reduction in LH level was observed. Concurrent treatment with Tualang honey (TH group) in BPA-exposed rats did not significantly prevented this effect.

1) $^{aa}P<0.01$ vs. NC
2) $^{bb}P<0.01$ vs. PC
3) $^{cc}P<0.01$ vs. TH
4) $^{dd}P<0.01$ vs. THC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

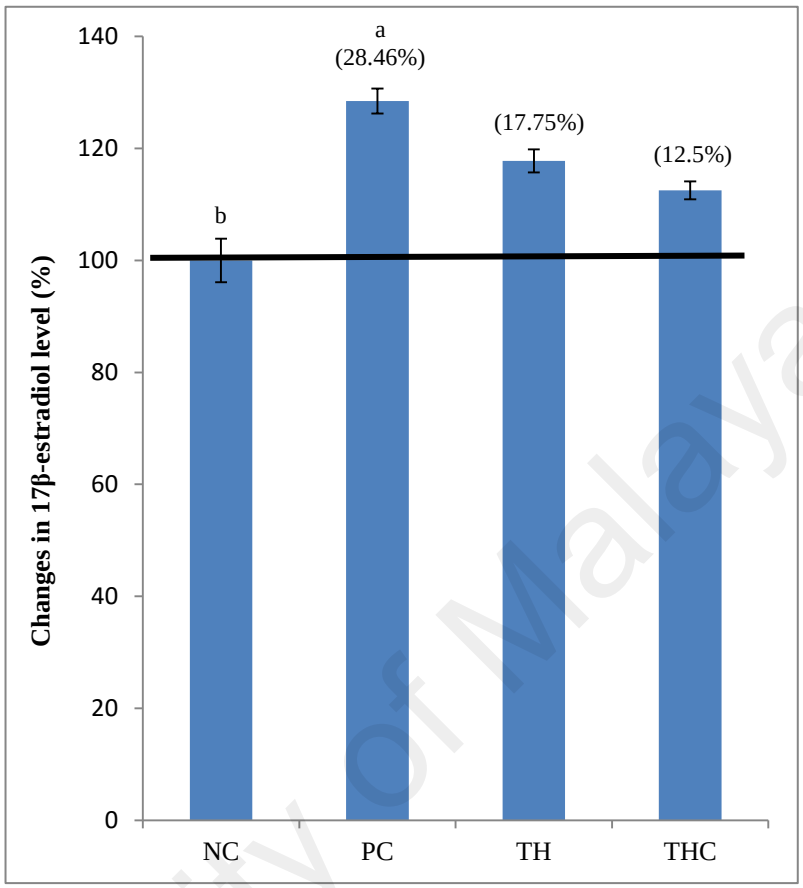


Figure 4.6: Effect of BPA and Tualang honey on the level of 17β-estradiol (E_2) in all experimental groups. After six weeks exposure to BPA (PC group), a significant increasing E_2 level was observed. Concurrent treatment with Tualang honey (TH group) in BPA-exposed rats was slightly prevented this effect.

1) $^{a}P<0.01$ vs. NC
2) $^{b}P<0.01$ vs. PC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

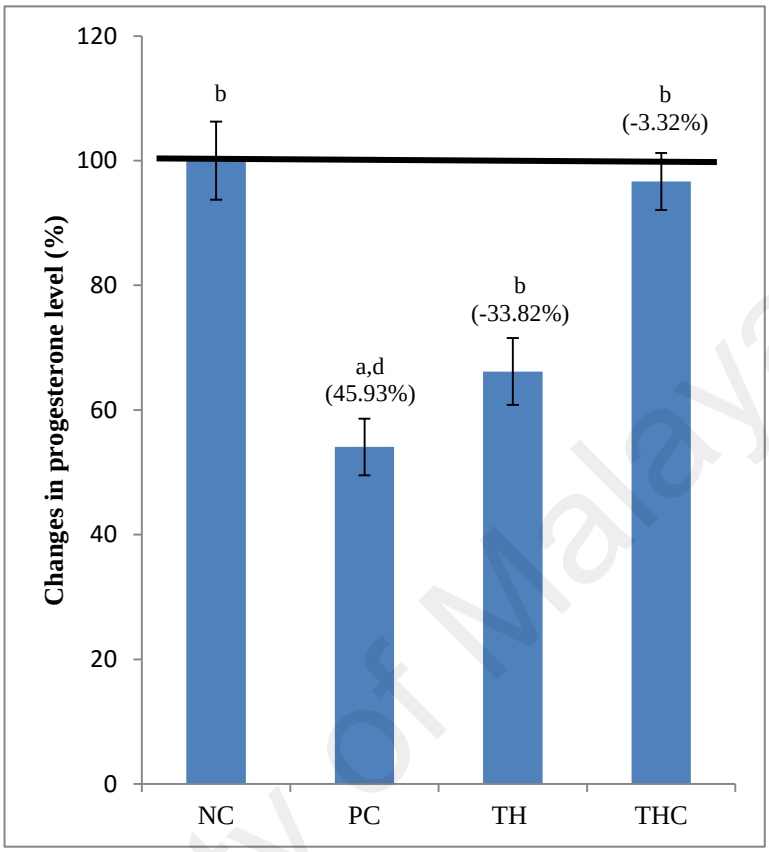


Figure 4.7: Effect of BPA on the level of progesterone in all experimental groups. After six weeks exposure to BPA (PC group), a reduction in progesterone level was observed. Concurrent treatment with Tualang honey (TH group) in BPA-exposed rats was significantly prevented this effect.

1) [a]P<0.05 vs. NC
2) [b]P<0.05 vs. PC
3) [d]P<0.05 vs. THC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

4.1.4 Malondialdehyde (MDA) level

The level of oxidative marker of MDA was measured to determine cellular damage of tissue that occurs via lipid peroxidation process by BPA.

As depicted in Table 4.4 and Figure 4.8, significant increase in the MDA levels by 100% was observed in rats exposed to BPA (PC group) compared to the control rats (NC group). However, concurrent treatment with Tualang honey led to significant reduction in the MDA levels (TH group). The MDA level was not affected by Tualang honey treatment alone (THC group) and the value is comparable to the normal rats (NC group).

Table 4.4: Effect of BPA and Tualang honey on the level of malondialdehyde (MDA) in all experimental groups (n=8).

Group	Malondialdehyde (MDA) (μM/μg protein)
NC	0.0037 ± 0.00026^{bbb}
PC	$0.0074\pm0.00053^{aaa,c,ddd}$
TH	0.0049 ± 0.00064^{bb}
THC	0.0036 ± 0.00089^{bbb}

Data are expressed as Mean ±SEM.

1) $^{aaa}P<0.001$ vs. NC
2) $^{bb}P<0.05$ and $^{bbb}P<0.001$ vs. PC
3) $^{c}P<0.05$ vs. TH
4) $^{ddd}P<0.001$ vs. THC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

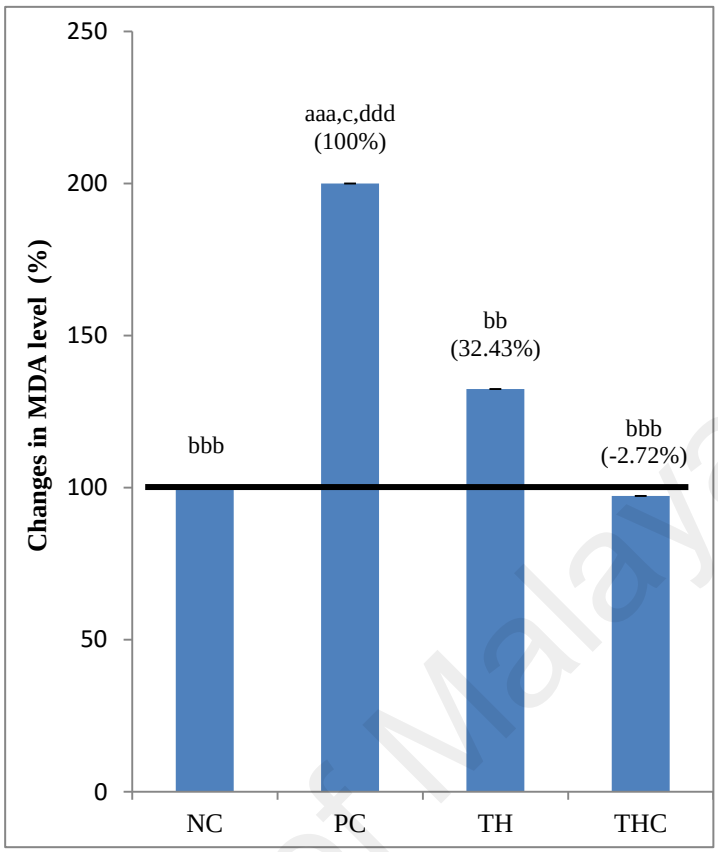


Figure 4.8: Effect of BPA and Tualang honey on the level of malondialdehyde (MDA) in all experimental groups. Malondialdehyde level was significantly increased in BPA-exposed rats (PC group). However, concurrent treatment with Tualang honey (TH group) has significantly prevented the increase.

Data are expressed as Mean ±SEM.

1) $^{aaa}P<0.001$ vs. NC
2) $^{bb}P<0.05$ and $^{bbb}P<0.001$ vs. PC
3) $^{c}P<0.05$ vs. TH
4) $^{ddd}P<0.001$ vs. THC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

4.1.5 Histopathology

4.1.5.1 Uterine morphology

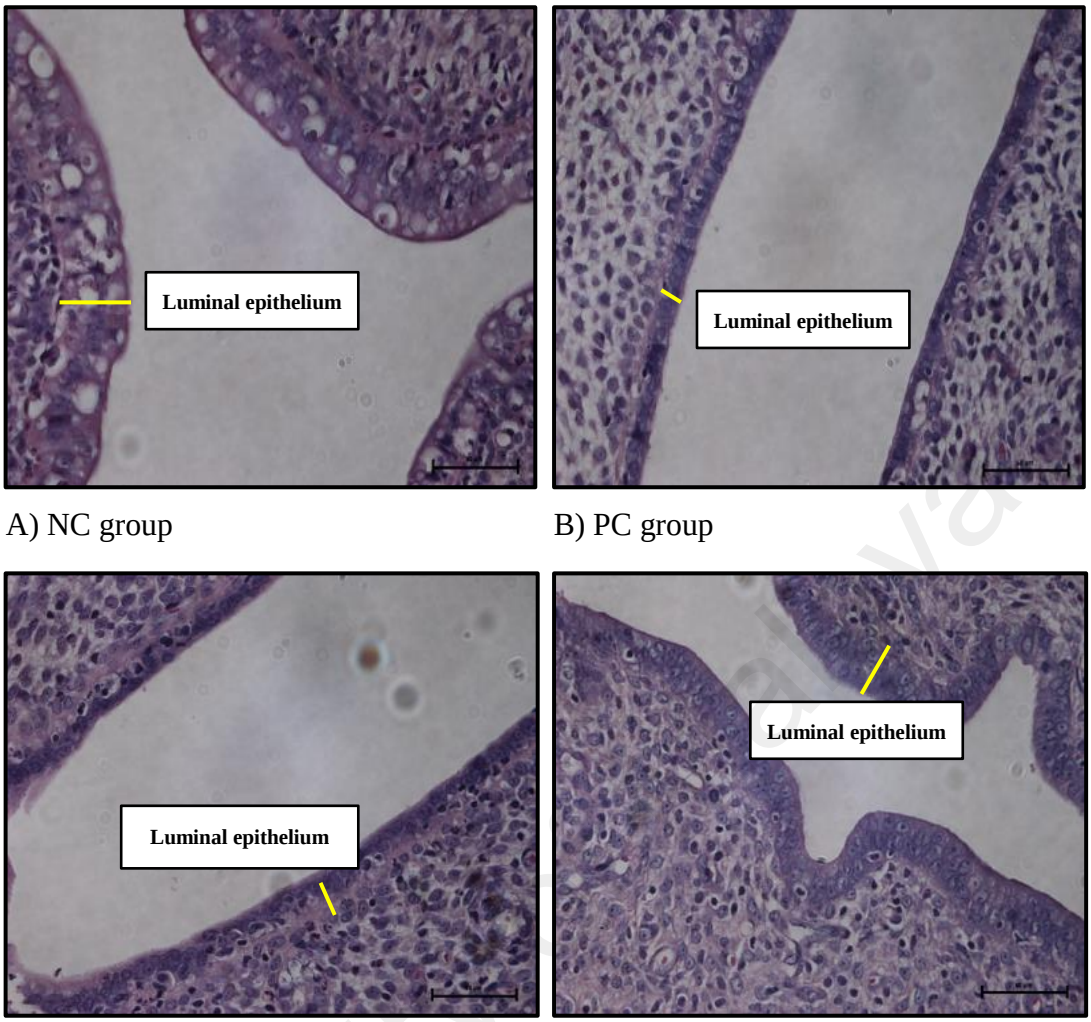
Histological examinations of uterine cross section from representative rats in all groups are shown in Figure 4.9, 4.10 and 4.11. The histological changes were consistent with the histomorphometric analysis (Section 4.1.5.2).

In the control rats (NC group), normal histological appearance was observed (Figure 4.9A, Figure 4.10A and Figure 4.11A). The luminal epithelial cells were tall, cylindrical with well-rounded nuclei localized on a prominent basement membrane and had tall pseudo-stratified columnar epithelium (Figure 4.9A). High cellular content and healthy endometrial glands were also noted in the stroma (Figure 4.10A). The myometrium was normal in appearance (Figure 4.11A). The histological findings of rats treated with Tualang honey alone (THC group) were comparable with the control rats (NC group) (Figure 4.9D, Figure 4.10D and Figure 4.11D).

As shown in Figure 4.9B, Figure 4.10B and Figure 4.11B, there were significant disruptive changes in the uterus of BPA-exposed rats (PC group) when compared to the control rats (NC group). The luminal epithelial cells were cuboidal in shape and less organized and distorted with irregular shaped nuclei (4.9B). The nuclei had more condensed chromatin and there were very little interstitial spaces between the stroma cells (4.10B). The endometrial glands appeared disrupted and smaller in size while the epithelial cells were distorted with irregular shaped nuclei and reduced in number. The smooth muscle bundles of the myometrium seemed smaller and shrunken while the inner circular and outer longitudinal smooth muscle fibers looked disintegrated (4.11B).

In comparison with BPA-exposed rats (PC group), reduced disruption of uterine morphology was noted in the group concurrently treated with Tualang honey (TH group)

(Figure 4.9C, Figure 4.10C and Figure 4.11C). There were more interstitial space between the stromal cells and the stromal cells appeared larger in size with the glandular epithelium close to the normal histology. Moreover, the myometrium appeared to the normal histology.

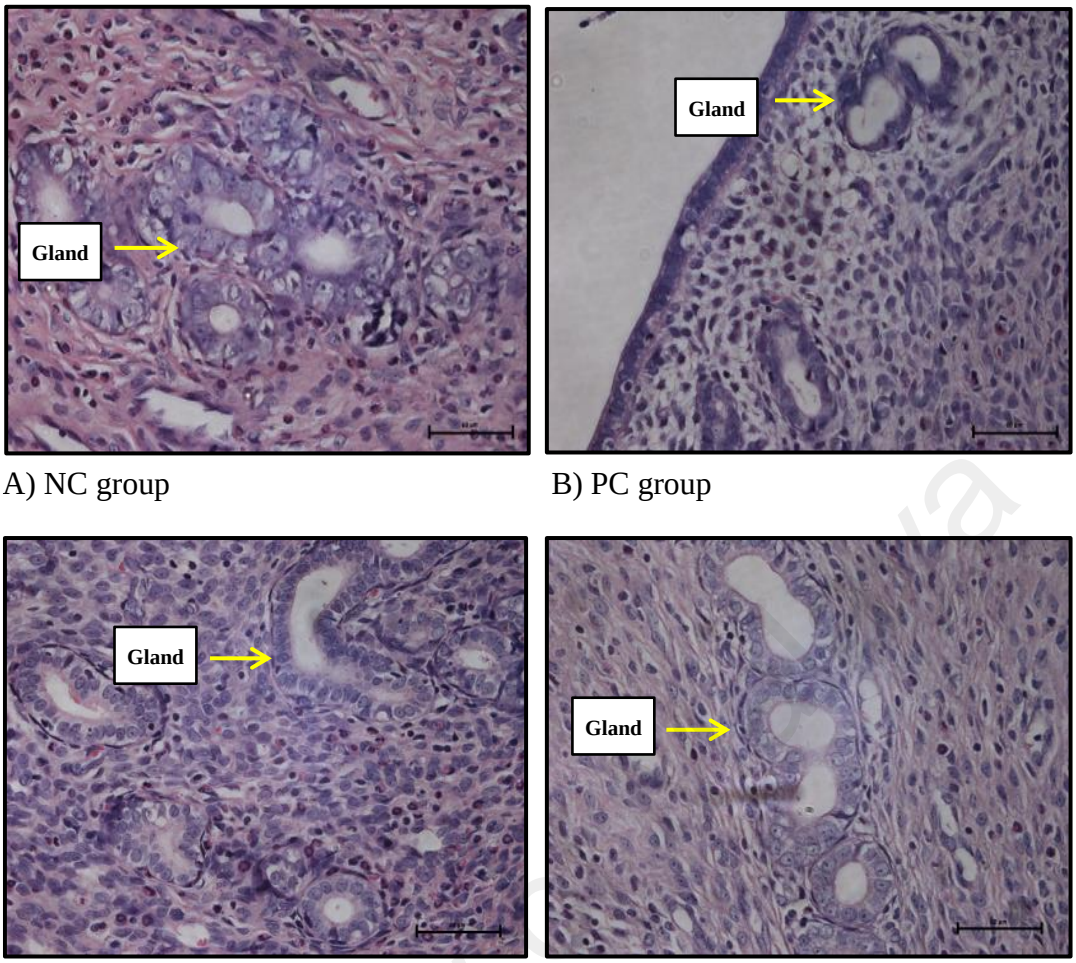


A) NC group B) PC group

C) TH group D) THC group

Figure 4.9: Photomicrographs of uterine luminal epithelium from all experimental groups. A) In NC group, normal histological appearance was observed with luminal epithelial cells were tall, cylindrical in shape with well-rounded nuclei and localized on a prominent basement membrane and had a tall pseudo-stratified columnar epithelium. B) In BPA exposed rats (PC group), the disruptive changes were more obvious where the cells less organized and distorded with irregular shaped nuclei in the luminal epithelial cells. C) However, the disruptive effects were reduced with concurrent treatment of Tualang honey (TH group). D) The histological findings in THC group were comparable to the NC group. (H&E, 400X)

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

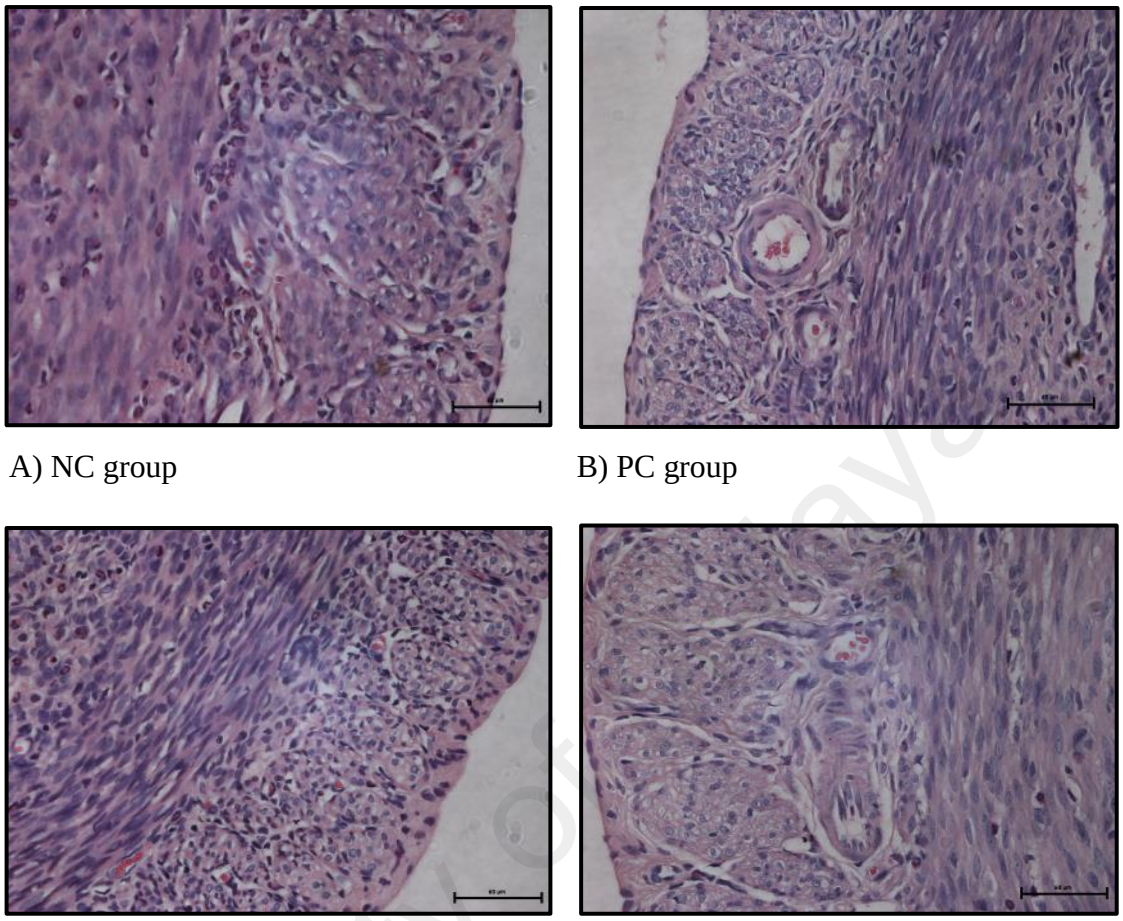


A) NC group B) PC group

C) TH group D) THC group

Figure 4.10: Photomicrographs of uterine gland and stroma from all experimental groups. A) In NC group, normal histological appearance were observed with high cellular content of stroma and healthy endometrial glands. B) Disruptive effects were observed in BPA-exposed rats (PC group) with very little interstitial space between the stroma cells and unhealthy endometrial cells were smaller in size. C) In TH group, some improvements were observed when BPA-exposed rats were concurrently treated with Tualang honey. The stromal cells appeared larger in size and more interstitial space between the cells as well as plumper glandular epithelium cells. D) The histological findings in THC group were comparable to the NC group. (H&E, 400X)

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)



A) NC group B) PC group

C) TH group D) THC group

Figure 4.11: Photomicrographs of uterine myometrium from all experimental groups. A) In NC group, normal histological appearance of myometrium was observed. B) In BPA exposed rats (PC group), disruptive changes were observed with smaller and shrunken smooth muscle bundles of the myometrium with the inner circular and outer longitudinal smooth muscle fibers looked disintergrated C) In TH group, myometrium was close to the normal histology. D) The histological findings in THC group were comparable to the NC group. (H&E, 400X)

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

4.1.5.2 Histomorphometry of the uterus

Histomophometry analysis of the uterus was performed to investigate and quantitatively validate the histological findings of the effects of BPA and Tualang honey on the uterus of rats. Uterine histomorphometry parameters measured were the height of the luminal epithelial cells and thickness of the endometrium and myometrium layers.

In general, histological findings and weight of the uterus rats from all experimental groups were consistent with their uterine histomorphometry results. As shown in Table 4.5 and Figure 4.12, Figure 4.13 and Figure 4.14, it was noted that the decline in all histomorphometry parameters (luminal epithelial cells, thickness of endometrium and myometrium layers) were consistent with the disruptive histological changes and weight reduction in the uterus of BPA-exposed rats (PC group). These histomorphometry changes in BPA-exposed rats were significant compared to the control rats (NC group). However, these changes were significantly prevented by concurrent treatment with Tualang honey (TH group).

In BPA-exposed rats (PC group), significant reduction was observed for the height of luminal epithelial cells compared to the NC group (38.11%) (Figure 4.12). This reduction was significantly prevented with concurrent treatment of Tualang honey (TH group). Meanwhile, although the value of this parameter is slightly lower in rats treated with Tualang honey alone (THC group) compared to the control rats (NC group), the value is not statistically significant.

As shown in Figure 4.13, a significant reduction was observed for endometrial thickness (14.88%) in BPA-exposed rats (PC group) compared to the NC group. However, this reduction was significantly prevented in BPA-exposed rats concurrently

treated with Tualang honey (TH group). The value of endometrial thickness in rats treated with Tualang honey alone (THC group) was similar to the NC group. While thickness of the myometrium was significantly reduced in BPA-exposed rats (23.66%) compared to the NC group (Figure 4.14). This reduction was significantly prevented when BPA-exposed rats concurrently treated with Tualang honey (TH group). The myometrial thickness in rat treated with Tualang honey treated alone (THC) was similar with the NC group.

Table 4.5: Histomorphometry analysis of the uterus in all experimental groups (n=8).

Group	Height of luminal epithelial cells (μm)	Thickness of endometrium (μm)	Thickness of myometrium (μm)
NC	$30.51 \pm 1.37^{bbb,c}$	571.87 ± 5.14^{bb}	299.18 ± 6.86^{bb}
PC	$18.88 \pm 0.81^{aaa,cc,ddd}$	$486.74 \pm 3.15^{aa,cc,ddd}$	$228.39 \pm 4.91^{aa,c,dd}$
TH	26.86 ± 0.68^{bbb}	546.30 ± 3.78^{b}	284.51 ± 6.63^{bb}
THC	$28.23 \pm 0.48^{a,bbb}$	574.25 ± 5.45^{bbb}	299.26 ± 4.92^{bb}

Data are expressed as Mean ±SEM.

1) $^{a}P<0.05$, $^{aa}P<0.01$ and $^{aaa}P<0.001$ vs. NC
2) $^{b}P<0.05$, $^{bb}P<0.01$and $^{bbb}P<0.001$ vs. PC
3) $^{c}P<0.05$ and $^{cc}P<0.01$ vs. TH
4) $^{dd}P<0.01$ and $^{ddd}P<0.001$ vs. THC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

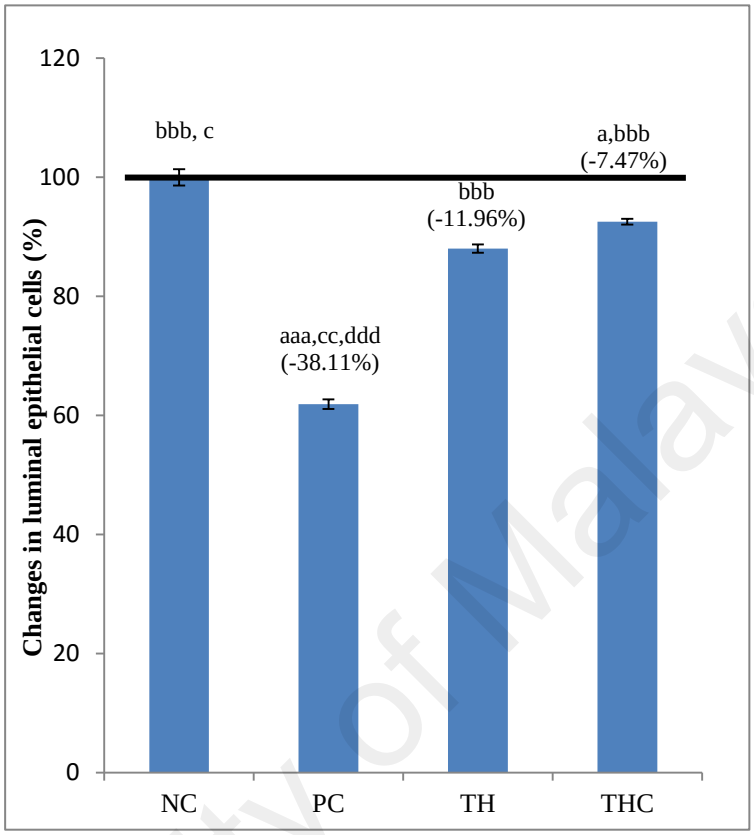


Figure 4.12: Effect of BPA and Tualang honey on the height of luminal epithelial cells of uterus in all experimental groups. In PC group, reduction in luminal epithelial cells was significantly prevented by concurrent treatment with Tualang honey (TH group).

1) $^{a}P<0.05$ and $^{aaa}P<0.001$ vs. NC
2) $^{bbb}P<0.001$ vs. PC
3) $^{c}P<0.05$ and $^{cc}P<0.01$ vs. TH
4) $^{ddd}P<0.001$ vs. THC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

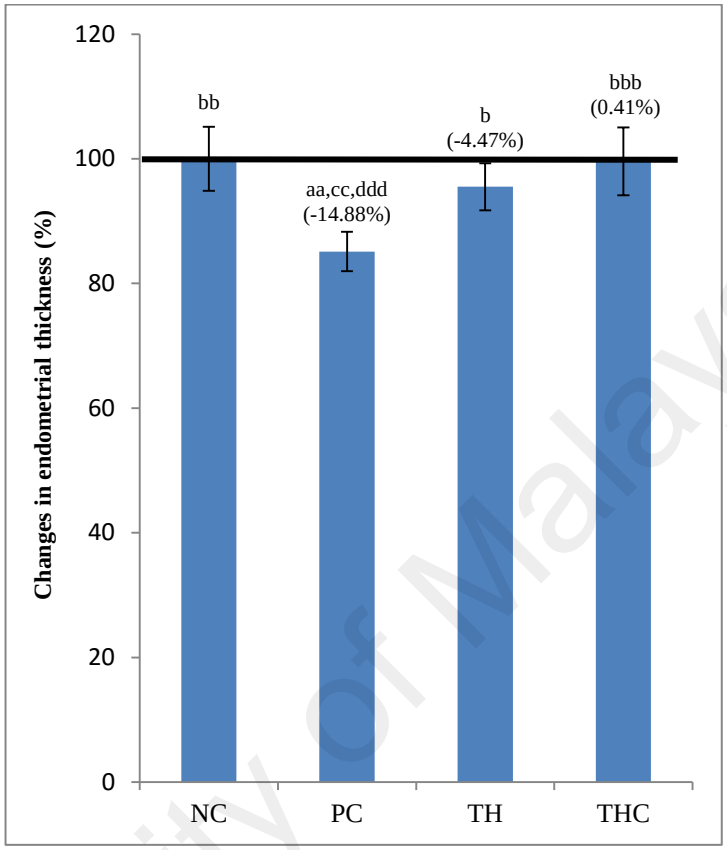


Figure 4.13: Effect of BPA and Tualang honey on the thickness of endometrium of uterus in all experimental groups. In PC group, reduction in endometrium layer was significantly prevented by concurrent treatment with Tualang honey (TH group).

1) $^{aa}P<0.01$ vs. NC
2) $^{b}P<0.05$, $^{bb}P<0.01$ and $^{bbb}P<0.001$ vs. PC
3) $^{cc}P<0.01$ vs. TH
4) $^{ddd}P<0.001$ vs. THC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

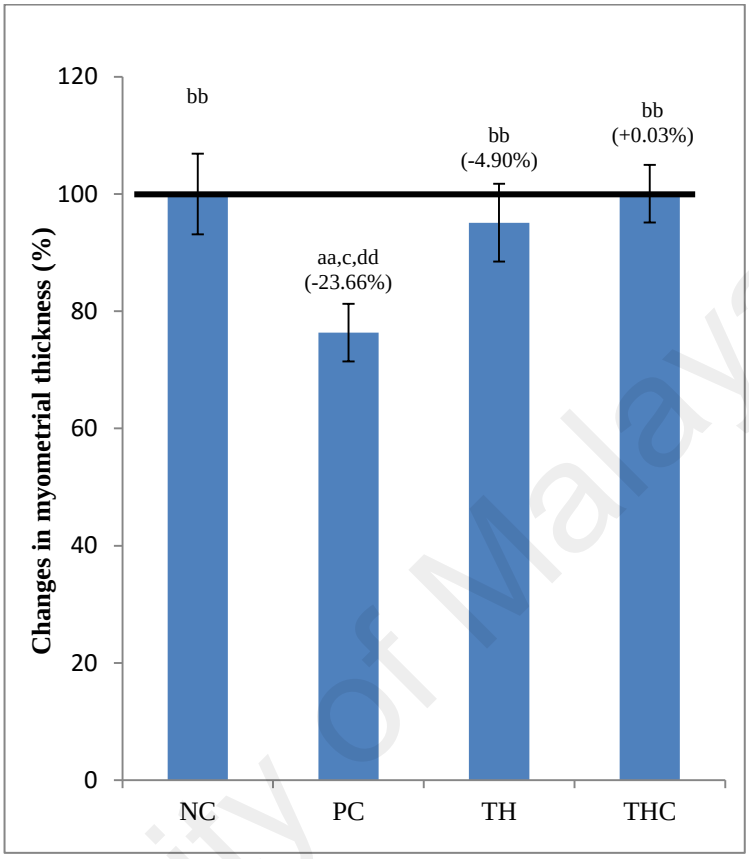


Figure 4.14: Effect of BPA and Tualang honey on the thickness of myometrium of uterus in all experimental groups. In PC group, reduction in myometrium layer was significantly prevented by concurrent treatment with Tualang honey (TH group).

1) $^{aa}P<0.01$ vs. NC
2) $^{bb}P<0.01$ vs. PC
3) $^{c}P<0.05$ vs. TH
4) $^{dd}P<0.01$ vs. THC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

4.1.5.3 Ovary

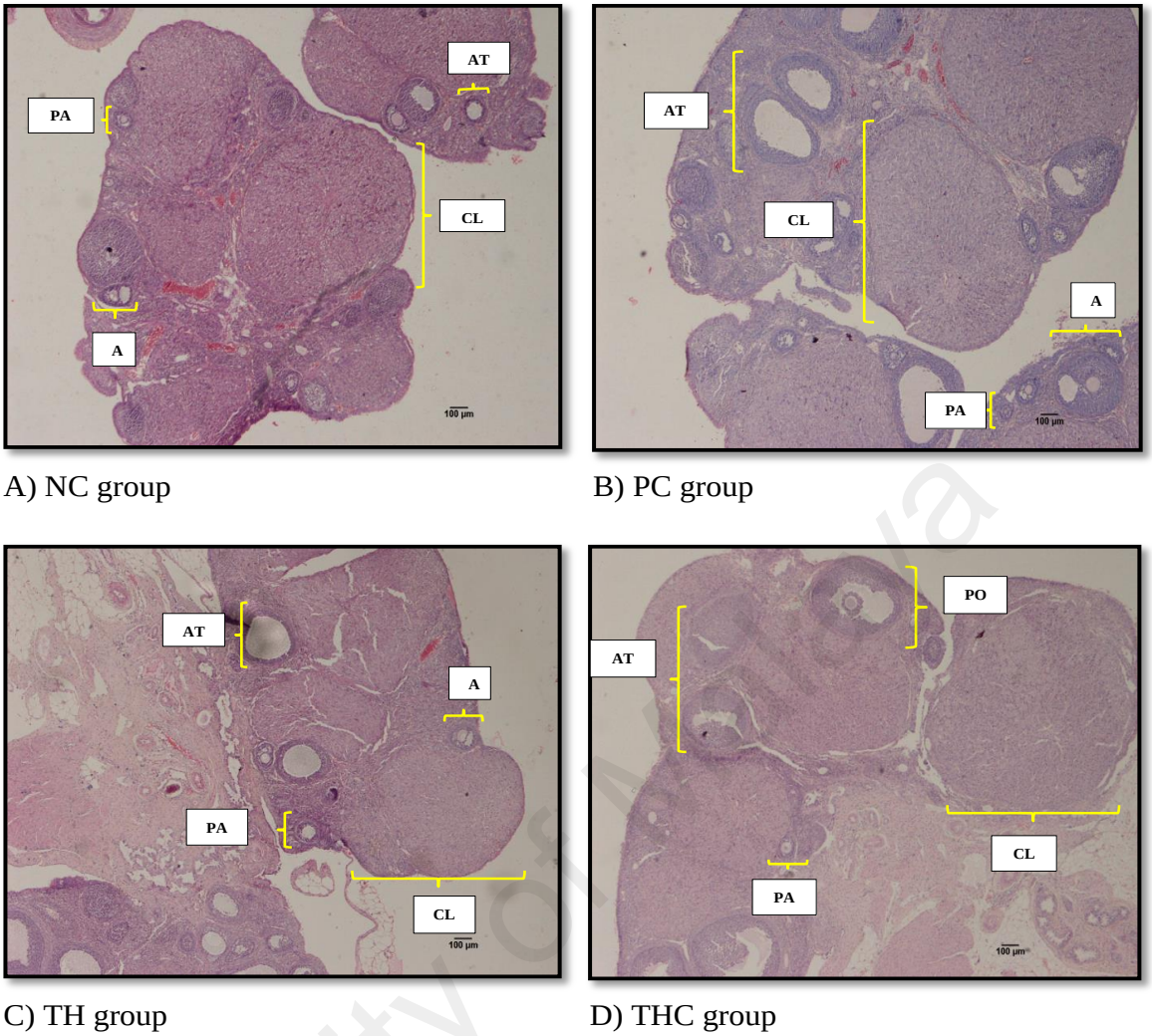
4.1.5.3.1 Ovarian morphology

Histological examinations of representative ovary from rats in all groups are shown in Figure 4.15. Generally, ovaries from all experimental groups were examined for all stages of follicular development that consists of preantral, antral, corpus luteum and atretic follicles.

Normal histological findings of the ovaries were observed in Control and Tualang honey treated alone rats (NC and THC groups, respectively). No large antral-like follicle and atretic cystic-like follicles were found in the ovaries from both groups.

In contrast, both of the PC and TH groups showed some histological abnormalities of the ovaries with large antral-like follicles that did not arrive at ovulation. The presences of atretic cystic-like follicles with less number of corpora lutea were observed in both groups. However, the degree of abnormalities was more apparent in the ovaries of BPA-exposed rats alone (PC group) compared to the BPA-exposed rats concurrently treated with Tualang honey (TH group).

In order to confirm these ovarian histological findings, further quantitative results on the follicular number in all ovaries from all experimental groups were shown in the Section 4.1.5.2.2.



A) NC group

B) PC group

C) TH group

D) THC group

Figure 4.15: Representative histological sections from the ovary of rat from all experimental groups (H&E, 40X).

A) Normal histological appearance was observed in control group (NC group) (B) The disruptive changes were observed in BPA-exposed rats (PC group) with the presence of atretic cystic-like follicles (C) However, less disruptive changes were observed when BPA-exposed rats concurrently treated with Tualang honey (TH group) (D) The histological findings in rats treated with Tualang honey alone (THC group) were comparable to the NC group.

PA: Preantral; **A**:Antral; **At**:Atretic; **CL**:Corpus Luteum; **PO**:Preovulatory.

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

4.1.5.3.2 Ovarian follicular counting

Follicular counting analysis of the ovary was performed to investigate and quantitatively validate the histological findings of the effects of BPA and Tualang honey on the ovaries of rats. Follicles counted were preantal, antral, corpus luteum and atretic follicles. In general, results of the ovarian histological changes and follicular counts from all experimental groups are in agreement (Figure 4.16). Overall, ovarian follicles of different stages were found in the ovaries of all experimental groups.

The numbers of all follicles (preantal, antral, corpus luteum and atretic follicles) in the control rats (NC group) were comparable to the rats treated with Tualang honey alone (THC group). These follicular counts results were in agreement with the histological findings, with normal histological appearance of the ovaries in the NC and THC groups (Figure 4.15).

Disruptive histological changes in the rats exposed to BPA (PC group) were consistent with the results of the follicular counting. The number of preantral follicles and corpus luteum in BPA-exposed rats (PC group) were reduced compared to the control rats (NC group). BPA-exposed rats concurrently treated with the Tualang honey (TH group) also showed similar number of these follicles compared to the BPA-exposed rats alone (PC group). Apart from that, the number of antral follicles was higher in the BPA-exposed rats alone (PC group) compared to the control rats (NC group). Concurrent treatment with Tualang honey in BPA-exposed rats (TH group) produced slightly less number of antral follicles than the BPA-exposed rats alone. However, the trend in these follicular numbers is not statistically significant between the comparative groups due to small sample size of rats.

The number of the atretic follicles were significantly higher in BPA-exposed rats (PC group) compared to the control rats (NC group). Nonetheless, concurrent treatment

with Tualang honey in BPA-exposed rats (TH group) slightly reduced the number of atretic follicles. This result could be related to the histological findings that found more apparent atretic cystic-like follicles in the BPA-exposed rats alone (PC group) compared to the BPA-exposed rats concurrently treated with Tualang honey (TH group).

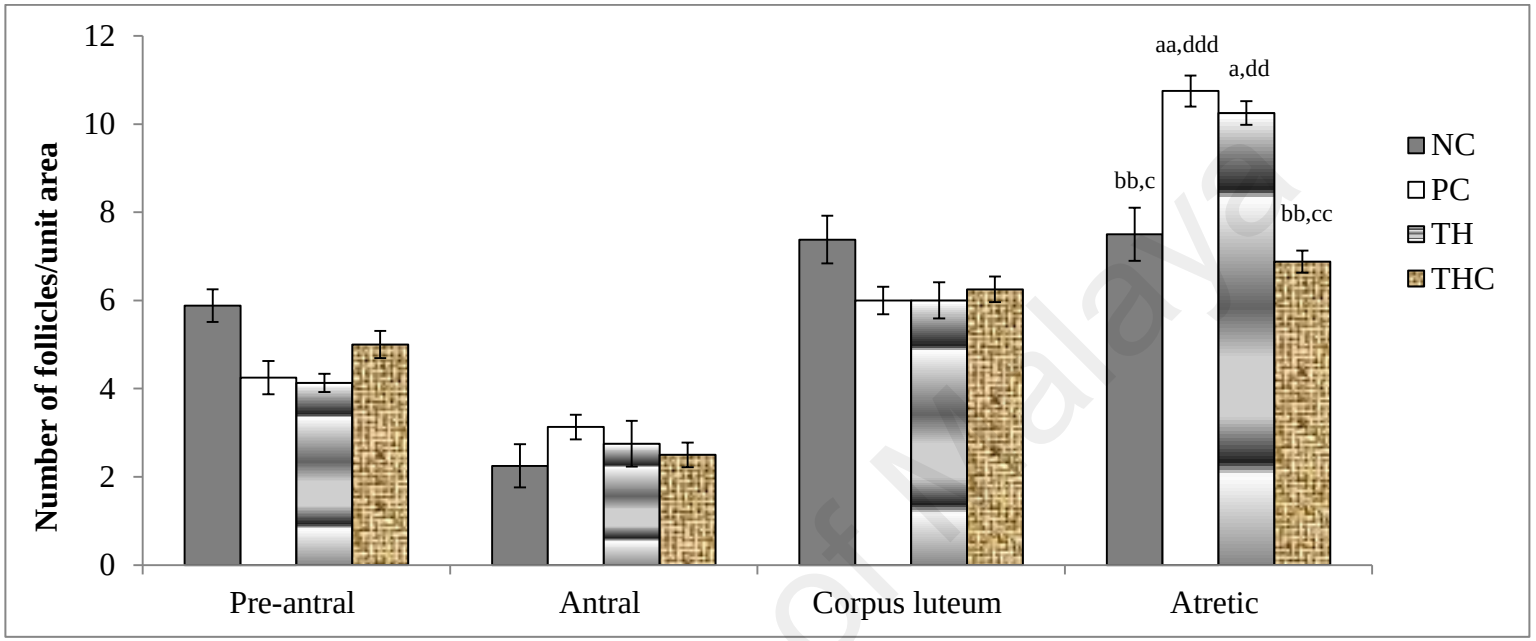


Figure 4.16: Number of different follicles in all experimental groups. The number of atretic follicles was significantly higher in BPA-exposed rats (PC group) compared to the Control rats (NC group). Concurrent treatment with Tualang honey in BPA- exposed rats (TH group) slightly reduced the number of atretic follicles compared to the BPA-exposed alone rats (PC group).

Data are expressed as Mean ±SEM. (n=8)

1) $^{a}P<0.05$ and $^{aa}P<0.01$ vs. NC
2) $^{bb}P<0.001$ vs. PC
3) $^{c}P<0.05$ and $^{cc}P<0.01$ vs. TH
4) $^{dd}P<0.01$ and $^{ddd}P<0.001$ vs. THC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

4.1.6 Immunohistochemistry (IHC)

IHC is a method to detect the location and expression of specific antigen (target protein) in the cells of a tissue section based on the principle of specific binding of antibody to its antigens in biologically tissues.

The basic protocol involves application of a primary antibody directed against a specific tissue antigen (target protein) (Figure 4.17). Then, amplification of a signal will be occurred when a secondary antibody is localized to the primary antibody. A horseradish peroxidase enzyme (HRPO) molecule will be conjugated to this secondary antibody (labeled secondary antibody). Finally, the brownish stains will be produced after diaminobenzidine (DAB) chromogen reacts with HRPO.

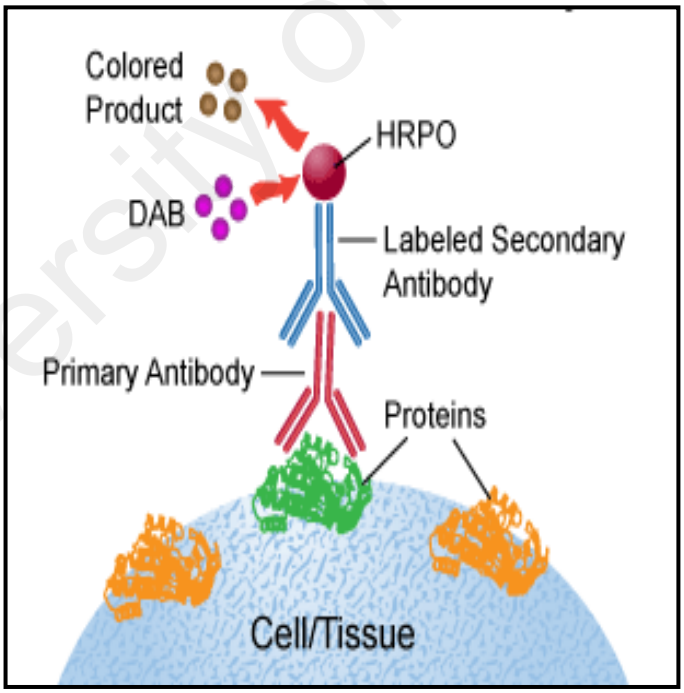


Figure 4.17: Illustration of immunohistochemistry method (www.leinco.com)

4.1.6.1 ERα, ERβ and C3 protein distribution

The representative uterine tissue sections were immunohistochemically stained to evaluate cell specific changes in the ERα, ERβ and C3 proteins. These proteins were localized to the nuclei of epithelial and stromal cell types in the uterus. In general, the staining intensity of all target proteins in all representative uterine sections were highest in the luminal and glandular epithelial cells while the endometrial stroma had lower staining intensity (about 50 to 80%).

As shown in the Figure 4.18, the most pronounced immunostaining intensities of ERα was observed in control rats (NC group) that were localized to the cells of luminal, stroma and glands (Figure 4.18 C, D). As expected, lower immunostaining intensities were observed in BPA-exposed rats (PC group) (Figure 4.18 E, F). Immunostaining intensities in BPA-exposed rats concurrently treated with Tualang honey (TH group) was similar to the rats treated alone with BPA (PC group) (Figure 4.18 G, H). Immunostaining intensities in the Tualang honey treated alone rats (THC group) was comparable to the NC group (Figure 4.18 I, J).

As depicted in Figure 4.19, the immunostaining patterns and intensities were different in ERβ as compared to the ERα. Highest immunostaining intensities of the luminal, stroma and glands cells were observed in the BPA-exposed rats (PC groups) compared to the other groups (Figure 4.19 E,F). In TH group, lower immunostaining intensities were detected in all cellular compartments (luminal, stroma and gland cells and) compared to the BPA-exposed rats (PC group). Comparable immunostaining intensities were observed in rats from NC (Figure 4.19 C, D) and THC (Figure 4.19 I, J). Overall, immunostaining intensities in all cellular compartments of uterus rats from the NC, THC and TH groups were comparable to each other and lower than the PC group.

For complement C3 protein expression, the highest immunostaining intensities were observed in the control rats (NC group), particularly in the luminal and glandular cells (Figure 4.20, C,D). Lowest immunostaining intensities were observed in these cellular compartments in rats treated with BPA alone (PC group) (Figure 4.20 E, F). However, higher intensities of immunostaining were observed in these cellular compartments in BPA-exposed rats concurrently treated with Tualang honey (TH group) (Figure 4.20 G, H). Tualang honey treated rats (THC group) has comparable immunostaining intensities with the rats from NC group (Figure 4.17 I, J).

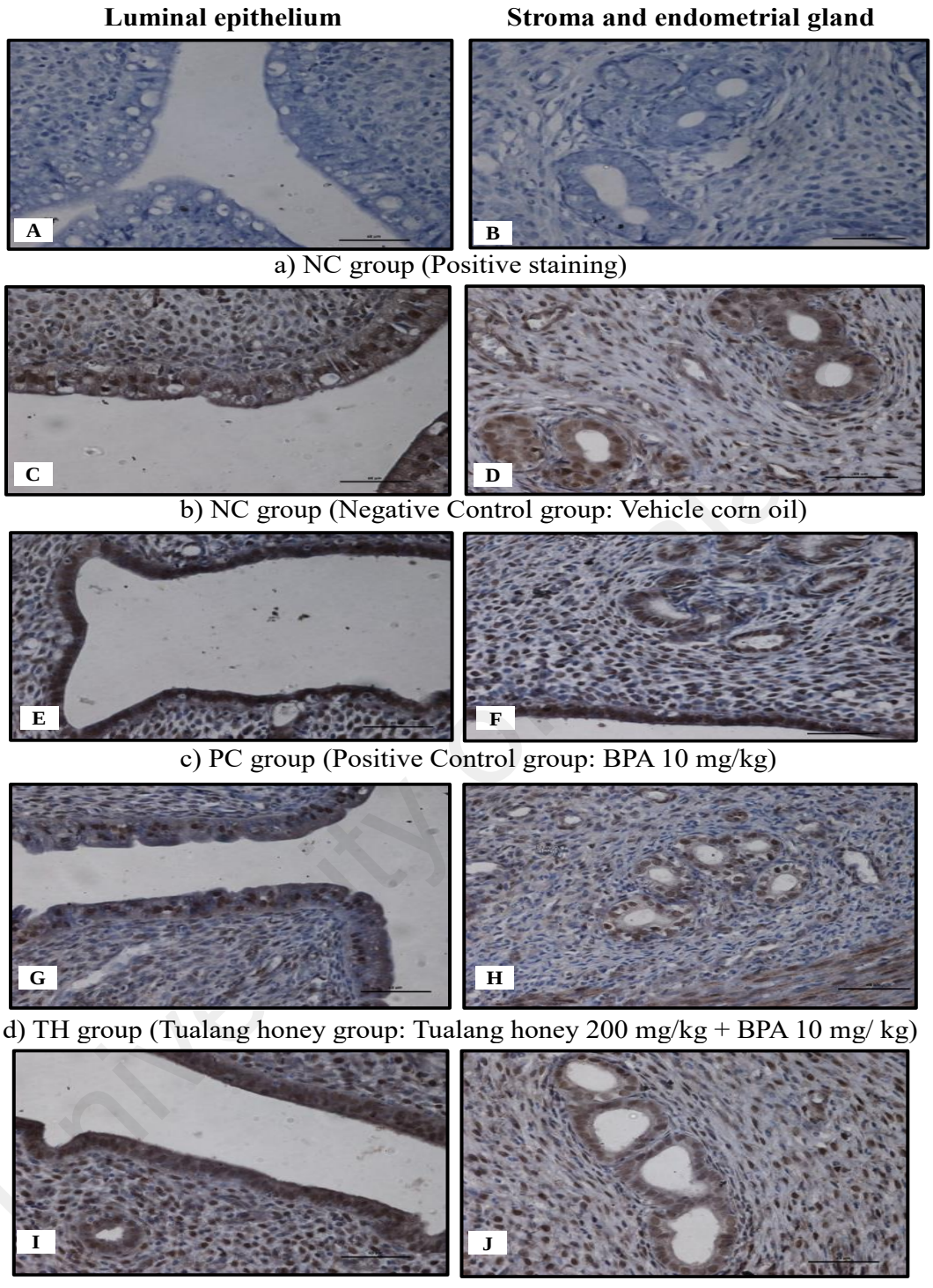


Figure 4.18: Immunohistological localization of ERα in uterine sections from all experimental groups. Lower immunostaining intensity were observed in PC (E,F) compared to the control rats (NC group). Comparable intensity of immunostaining were observed in both rats from the PC and F groups. (400X).

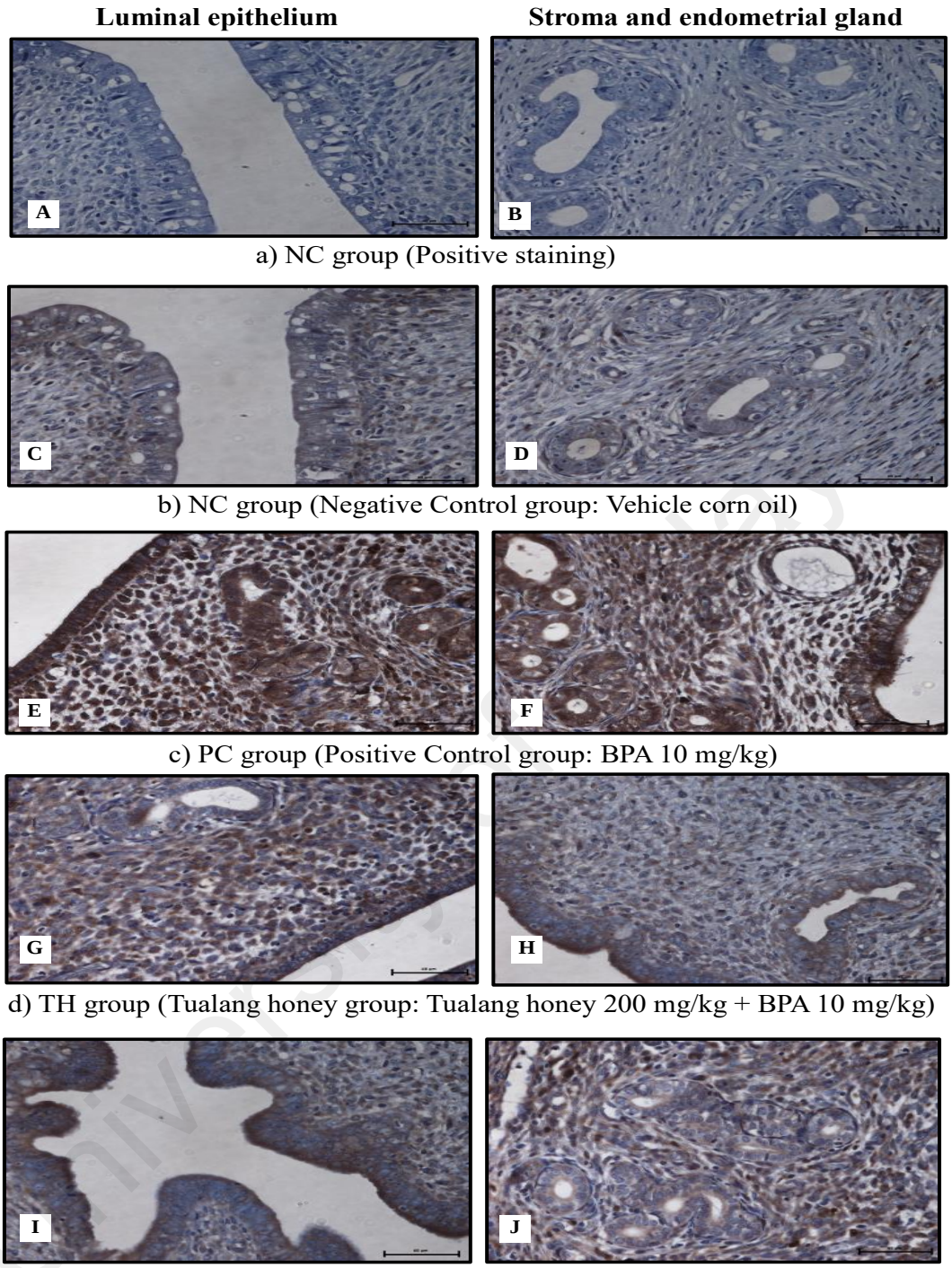


Figure 4.19: Immunohistological localization of ERβ in uterine sections from all experimental groups. The immunostaining intensity were higher in the rats from the PC group (E,F) compared to the control rats (NC group). Compare to the rats from the PC group, immunostaining intensity were lower in rats from the TH group (G, H). (400X).

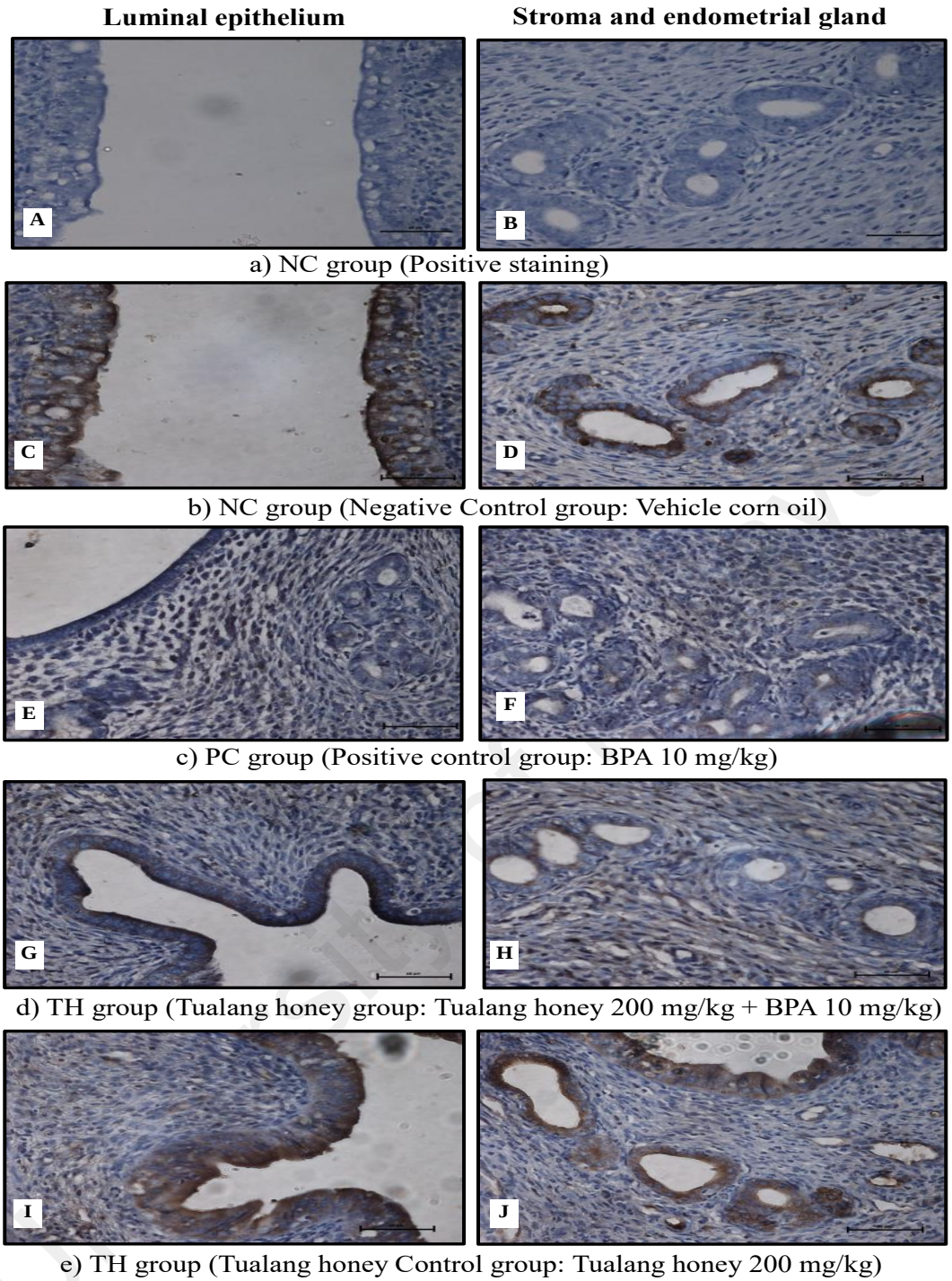


Figure 4.20: Immunohistological localization of C3 in uterine sections from all experimental groups. The lowest intensity of C3 immunostaining was observed in PC group (E, F) while higher intensity of immunostaining were observed in TH group (G, H). The highest immunostaining intensity were observed in NC group (C, D) and THC group (I, J) (400X).

4.1.6.2 ERα, ERβ and C3 mRNA expression

Realtime PCR was performed to quantitate mRNA expression levels of ERα, ERβ and C3 genes. These results are important to relate and support the findings of protein expression of ERα, ERβ and C3 derived from the immunohistochemistry analysis.

As shown in Figure 4.21, BPA exposure in PC group rats were able to significantly downregulate the ERα mRNA expression by approximately 4-fold compared to the control rats (NC group). However, the magnitude was reduced to about 3-fold by concurrent treatment with Tualang honey (TH group). In contrast, treatment with Tualang honey (THC group) showed a 1.5-fold induction in the ERα mRNA expression compared to the control rats (NC group).

Figure 4.22 shows a dissimilar pattern of ERβ mRNA expression compared to ERα mRNA expression. As compared to the control rats (NC group), the expression of ERβ was significantly upregulated by approximately 1.4-fold in BPA-exposed rats (PC group) and this was significantly reduced to 1.1-fold by concurrent treatment of Tualang honey (TH group). This indicates that concurrent treatment with Tualang honey was able to reduce ERβ mRNA expression in BPA-exposed rats by 80%. Meanwhile, Tualang honey alone (THC group) had no effect on the ERβ mRNA expression with the value of expression comparable to the control rats (NC group).

As shown in Figure 4.20, BPA exposure (PC group) caused a dramatic suppression of the complement C3 mRNA expression by almost 90% compared to the control rats (NC group). Surprisingly, this effect was completely reversed with concurrent treatment with Tualang honey (TH group). In fact, the C3 mRNA expression in the TH group was 1.8-fold higher than the control rats (NC group). Tualang honey alone had no effect on the C3 mRNA expression with the value of expression comparable to the control rats (NC group).

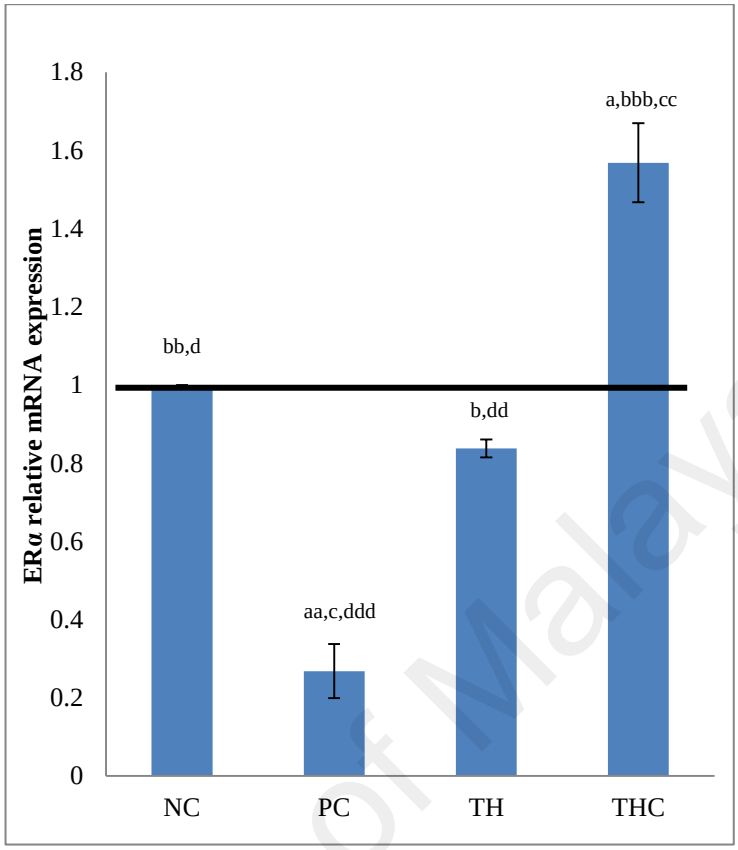


Figure 4.21: Relative quantitative expression of the ERα gene for all experimental groups. The profile of ERα gene for each experimental group is shown as fold change in the rats from PC, TH and THC groups compared to the control rats (NC group) which was assigned as 1.0, performed using StepOne Plus Real-time PCR system. Results shown are pooled from 3 independent experiments (n=8) and errors are expressed as Standard Error.

Data are expressed as Mean ±SEM.

1) $^{a}P<0.05$ and $^{aa}P<0.01$ vs. NC
2) $^{b}P<0.05$, $^{bb}P<0.01$ and $^{bbb}P<0.001$ vs. PC
3) $^{c}P<0.05$ and $^{cc}P<0.01$ vs. TH
4) $^{d}P<0.05$, $^{dd}P<0.01$ and $^{ddd}P<0.001$ vs. THC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

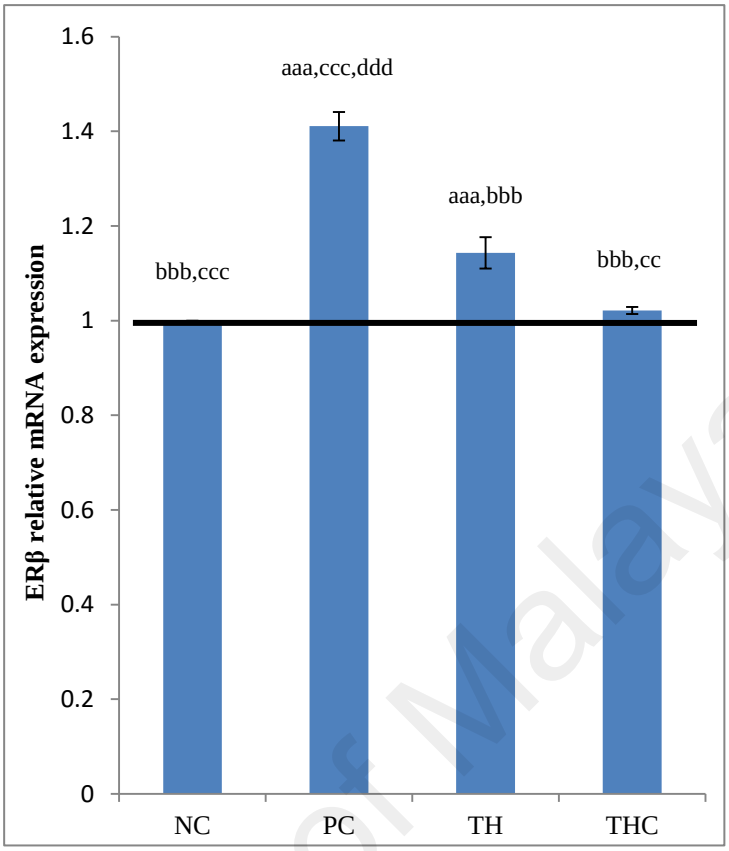


Figure 4.22: Relative quantitative expression of the ERβ gene for all experimental groups. The profile of ERβ gene for each experimental group is shown as fold change in the rats from PC, TH and THC groups compared to the control rats (NC group) which was assigned as 1.0, performed using StepOne Plus Real-time PCR system. Results shown are pooled from 3 independent experiments (n=8) and errors are expressed as Standard Error.

Data are expressed as Mean ±SEM.

1) $^{aaa}P<0.001$ vs. NC
2) $^{bbb}P<0.001$ vs. PC
3) $^{cc}P<0.01$ and $^{ccc}P<0.001$ vs. TH
4) $^{ddd}P<0.001$ vs. THC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

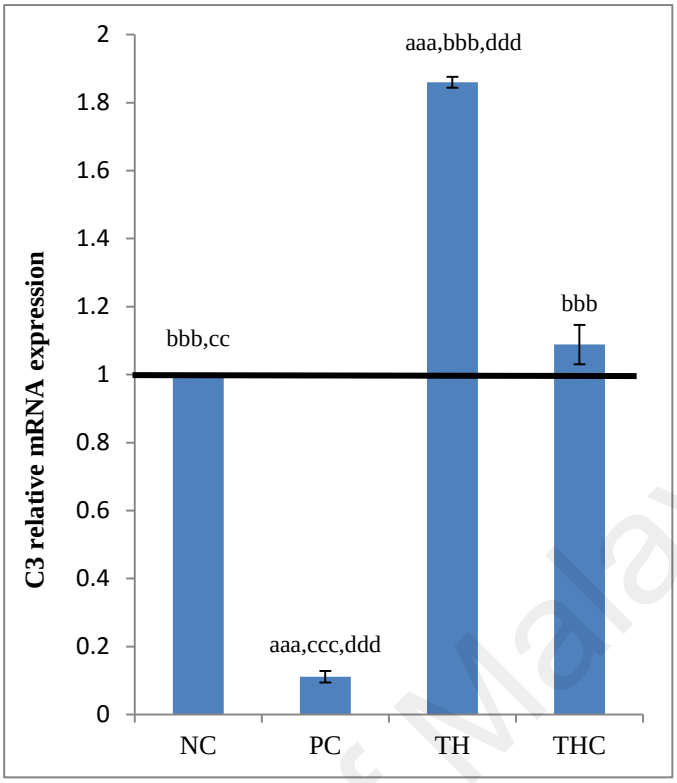


Figure 4.23: Relative quantitative expression of the C3 gene for all experimental groups. The profile of C3 gene for each experimental group is shown as fold change in the rats from PC, TH and THC groups compared to the control rats (NC group) which was assigned as 1.0, performed using StepOne Plus Real-time PCR system. Results shown are pooled from 3 independent experiments (n=8) and errors are expressed as Standard Error.

Data are expressed as Mean ±SEM.

1) $^{aaa}P<0.001$ vs. NC
2) $^{bbb}P<0.001$ vs. PC
3) $^{cc}P<0.01$ and $^{ccc}P<0.001$ vs. TH
4) $^{ddd}P<0.001$ vs. THC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)

4.2 Protective effects of *Ficus deltoidea* against Bisphenol A induced toxicity in the reproductive system

4.2.1 Body and organ weights

The changes in body, uterus and ovary weights of rats in all experimental groups are shown in Table 4.6 and Figure 4.24, Figure 4.25 and Figure 4.26, respectively.

As shown in Figure 4.24, 18.30% increment in the body weight gain was observed in rats exposed to BPA alone (PC group) compared to the control rats (NC group). The increment in the body weight gain was lower (8.7%) when BPA-exposed rats were concurrently treated with *Ficus deltoidea* (F group). Meanwhile, comparable changes in the body weight gain were observed in rats treated with *Ficus deltoidea* (FC group) and control rats (NC group).

As shown in the Figure 4.25, compared to the control rats (NC group) a decline in the relative uterine weight (25.39%) was observed in rats exposed to BPA alone (PC group). However, concurrent treatment with *Ficus deltoidea* in BPA-exposed rats (F group) significantly prevented the decline. Meanwhile, the relative uterine weight in rats treated with *Ficus deltoidea* alone (FC group) was comparable to the control rat (NC group).

As shows in Figure 4.26, rats exposed to BPA alone showed significant increment in the relative ovary weight (20.83%) compared to the control rats (NC group). This increment was significantly prevented by concurrent treatment with *Ficus deltoidea* (F group). The relative ovary weight in the rats treated with *Ficus deltoidea* alone (FC group) was slightly lower compared to the control rat (NC group), however their weights are still comparable.

Table 4.6: Effect of BPA and *Ficus deltoidea* on the body weight and weights of uterus and ovary of rats (n=8).

Group	Body weight gain (g)	Changes in body weight gain (%)	Uterus wet weight (mg)	Relative uterus weight (wet weight/ body weight)	Ovary wet weight (mg)	Relative ovary weight (wet weight/ body weight)
NC	78.88±14.61	48.15±6.60	296.25±26.58	1.89 ± 0.12^{bb}	36.88±1.88	0.24 ± 0.01^{b}
PC	99.25±9.90	56.52±3.49	246.25±13.08	$1.41\pm0.04^{aa,c,d}$	49.38±1.13	$0.29\pm0.01^{a,c,d}$
F	87.88±2.45	52.36±0.65	298.75±8.75	1.78 ± 0.05^{b}	35.00±1.34	0.21 ± 0.01^{bbb}
FC	89.12±5.45	53.33±2.29	305.71±17.30	1.84 ± 0.10^{b}	33.13±2.49	0.20 ± 0.02^{bbb}

Data are expressed as Mean ±SEM.

1) $^{a}P<0.05$ and $^{aa}P<0.01$ vs. NC (Negative control)
2) $^{b}P<0.05$, $^{bb}P<0.01$and $^{bbb}P<0.001$ vs. PC (BPA 10 mg/kg)
3) $^{c}P<0.05$ vs. F (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
4) $^{d}P<0.05$ vs. FC (*Ficus deltoidea* 100 mg/kg)

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

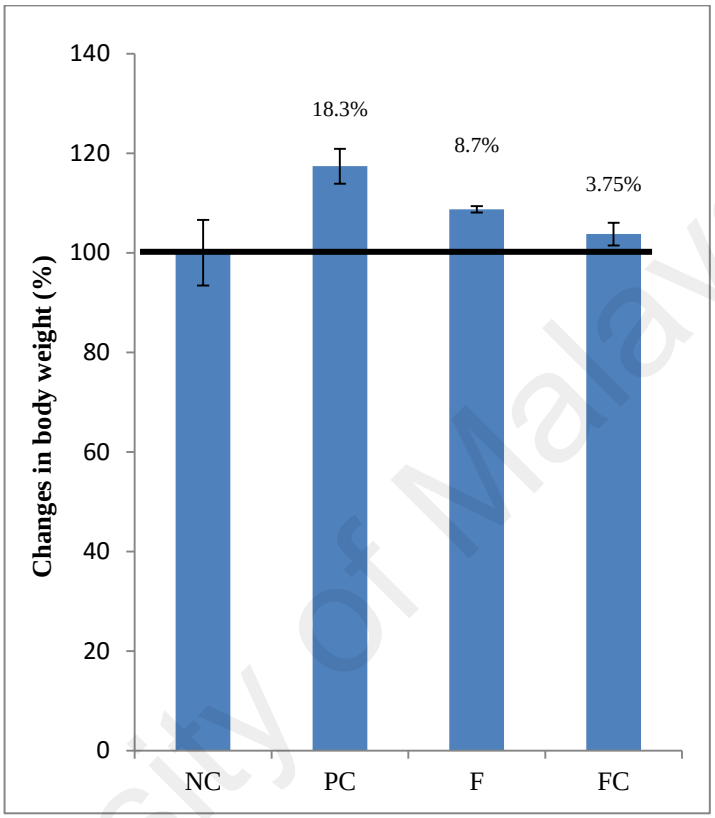


Figure 4.24: Effect of BPA and *Ficus deltoidea* on the body weight of rats.

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

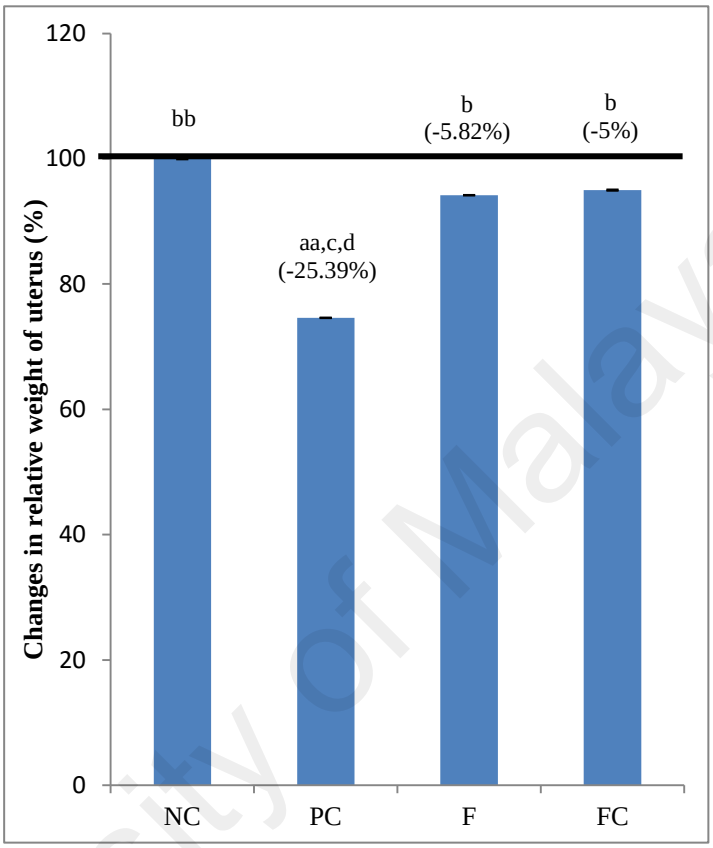


Figure 4.25: Effect of BPA and *Ficus deltoidea* on the **c**hanges in relative weight of the uterus in rats. A significant decline in relative uterine weight was observed following six weeks of BPA exposure (PC group). This effect was significantly prevented by concurrent treatment with *Ficus deltoidea* (F group).

1) $^{aa}P<0.01$ vs. NC
2) $^{b}P<0.05$ and $^{bb}P<0.01$ vs. PC
3) $^{c}P<0.05$ vs. F
4) $^{d}P<0.05$ vs. FC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

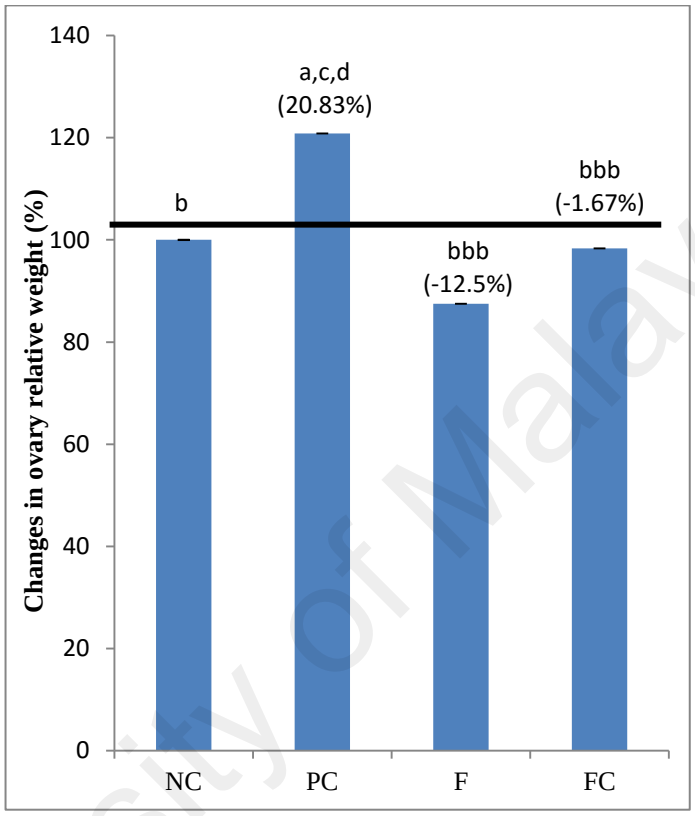


Figure 4.26: Effect of BPA and *Ficus deltoidea* on the relative weight of the ovary in rats. A significant increase in relative ovary weight was observed in BPA exposed rats (PC group). This effect was significantly prevented by concurrent treatment with *Ficus deltoidea* (F group).

1) $^{a}P<0.05$ vs. NC
2) $^{b}P<0.05$ and $^{bbb}P<0.001$ vs. PC
3) $^{c}P<0.05$ vs. F
4) $^{d}P<0.05$ vs. FC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

4.2.2 Effect of BPA and *Ficus deltoidea* on estrous cycle

As shown in Table 4.7, all rats in the NC and FC groups maintained their normal estrous cycles (100%). However, for BPA-exposed rats (PC group) only 12.5% were in normal estrous cycles while the rest were demostrating persistent diestrous phase and these estrous cycle patterns were significantly different compared to the NC and PC groups. Concurrent treatment with *Ficus deltoidea* in BPA-exposed rats showed improved percentage of rats in the normal estrous cycles (62.5%) with a reduction of rats in persistent diestrous phase compared to the BPA-exposed rats alone (PC group).

Table 4.7: Effect of BPA and *Ficus deltoidea* on the estrous cycle. (n=8)

Group	Rat with normal estrous cycle, % (n)
NC	100% (8/8)[b]
PC	12.5% (1/8)[a,c,d]
F	62.5% (5/8)[b]
FC	100% (8/8)[b]

1) [a]P<0.05 vs. NC
2) [b]P<0.05 vs. PC
3) [c]P<0.05 vs. F
3) [d]P<0.05 vs. FC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

4.2.3 Hormonal profile

4.2.3.1 Follicle stimulating hormone (FSH) and luteinizing hormone (LH)

As shown in Table 4.8, Figure 4.27 and Figure 4.28, serum FSH and LH levels were significantly reduced in BPA-exposed rats (PC groups) compared to the control rats (NC group) (60.19% and 80.58%, respectively). BPA-exposed rats that were concurrently treated with *Ficus deltoidea* had significantly higher FSH levels. However, reduction in LH level was not significantly prevented by *Ficus deltoidea* treatment in BPA-exposed rats. Both these hormonal levels were comparable in rats from FC and NC groups.

4.2.3.2 17β-Estradiol (E_2) and progesterone (P_4)

In BPA-exposed rats (PC group), the level of E_2 was higher (28.46%) than the control rats (PC group) (Table 4.8 and Figure 4.29). Comparable increment of E_2 level was observed in BPA-exposed rats concurrently treated with *Ficus deltoidea* (F group) but in at a lower magnitude compared to the rats exposed to BPA alone. E_2 level in rats treated with the *Ficus deltoidea* alone (FC group) was comparable to the control rats (NC rats).

As shown in Table 4.8 and Figure 4.30, the level of progesterone in BPA-exposed rats was significantly lower (45.93%) compared to the control rats (NC group). However, concurrent treatment with *Ficus deltoidea* in BPA-exposed rats has significantly prevented this reduction. Meanwhile, the level of progesterone in *Ficus deltoidea* treated rats was comparable to the control rats (NC group).

Table 4.8: Levels of reproductive hormones in all experimental groups. (n=8)

Group	Follicle Stimulating Hormone (FSH)	Luteinizing Hormone (LH)	17β-Estradiol	Progesterone
NC	$78.5\pm4.96^{bbb,ccc}$	$10.25\pm2.71^{bbb,cc}$	19.04±3.99	64.65 ± 6.2^{b}
PC	$31.25\pm5.96^{aaa,ddd}$	$1.99\pm0.32^{aaa,d}$	26.46±2.22	$34.95\pm8.56^{a,d}$
F	$53.18\pm1.17^{aaa,b,dd}$	3.18 ± 0.16^{aa}	23.69±2.03	45.80 ± 6.62^{b}
FC	$71.69\pm7.32^{bbb,cc}$	7.73 ± 0.34^{b}	21.89±1.59	63.06 ± 3.30^{b}

Data are expressed as Mean ±SEM.

1) $^{a}P<0.05$, $^{aa}P<0.01$ and $^{aaa}P<0.001$ vs. NC
2) $^{b}P<0.05$ and $^{bbb}P<0.001$ vs. PC
3) $^{cc}P<0.01$ and $^{ccc}P<0.001$ vs. F
4) $^{d}P<0.05$, $^{dd}P<0.01$ and $^{ddd}P<0.001$ vs. FC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

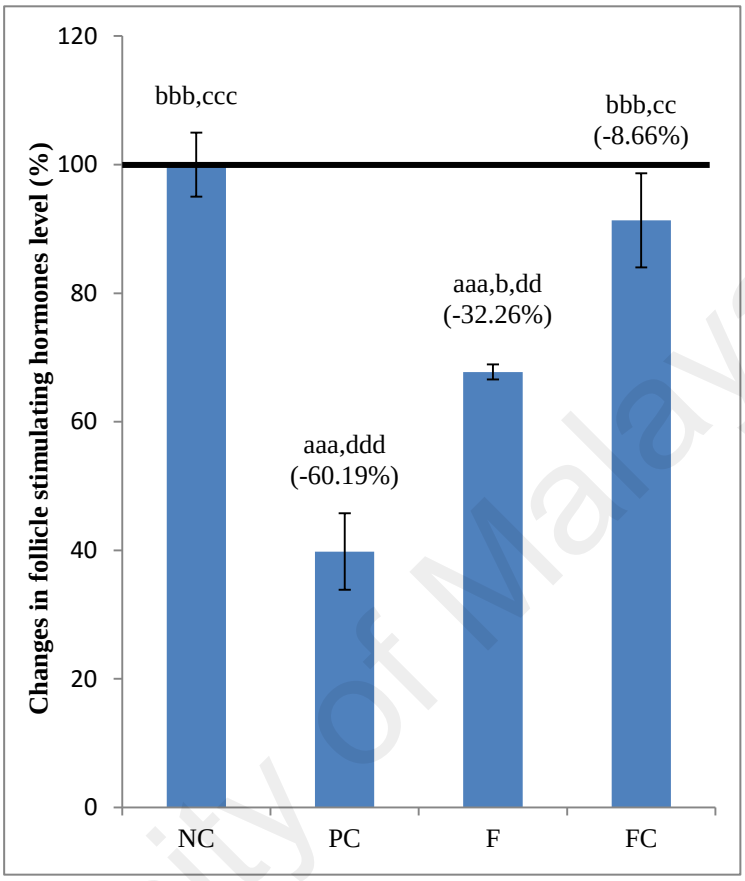


Figure 4.27: Effect of BPA and *Ficus deltoidea* on the level of follicle stimulating hormone (FSH) in all experimental groups. A significant reduction in FSH level was observed following six weeks exposure to BPA. Concurrent treatment with *Ficus deltoidea* in BPA exposed rats (F group) significantly prevented this effect.

1) $^{aaa}P<0.001$ vs. NC
2) $^{b}P<0.05$ and $^{bbb}P<0.001$ vs. PC
3) $^{cc}P<0.01$ and $^{ccc}P<0.001$ vs. F
4) $^{dd}P<0.01$ and $^{ddd}P<0.001$ vs. FC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

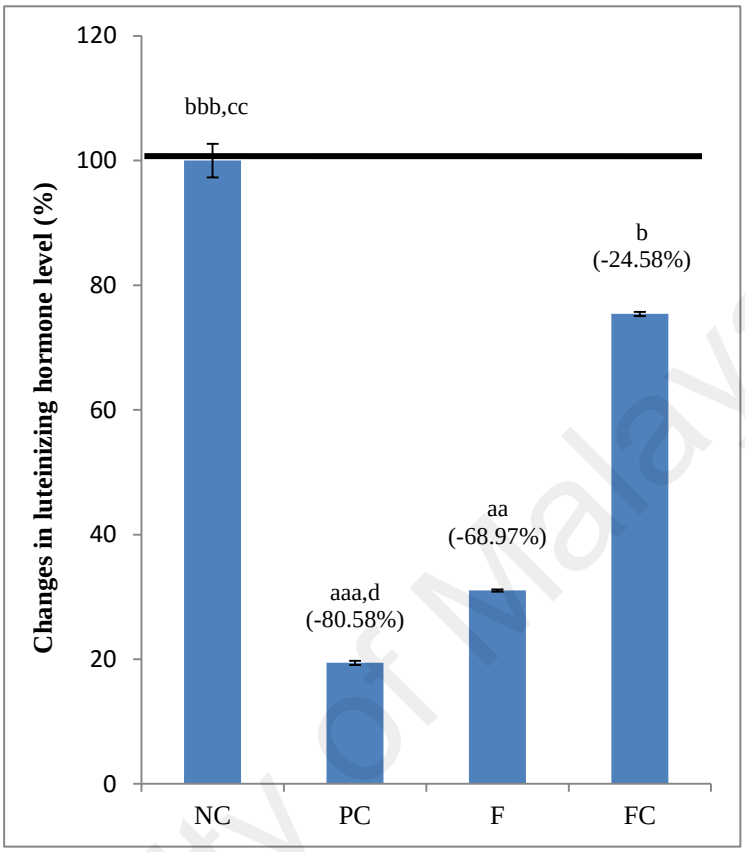


Figure 4.28: Effect of BPA and *Ficus deltoidea* on the level of luteinizing hormone (LH) in all experimental groups. A significant reduction in LH level was observed. Concurrent treatment with *Ficus deltoidea* (F group) in BPA-exposed rats did not significantly prevent this effect.

1) $^{aa}P<0.01$ and $^{aaa}P<0.001$ vs. NC
2) $^{b}P<0.05$ and $^{bbb}P<0.001$ vs. PC
3) $^{cc}P<0.01$ vs. F
4) $^{d}P<0.05$ vs. FC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

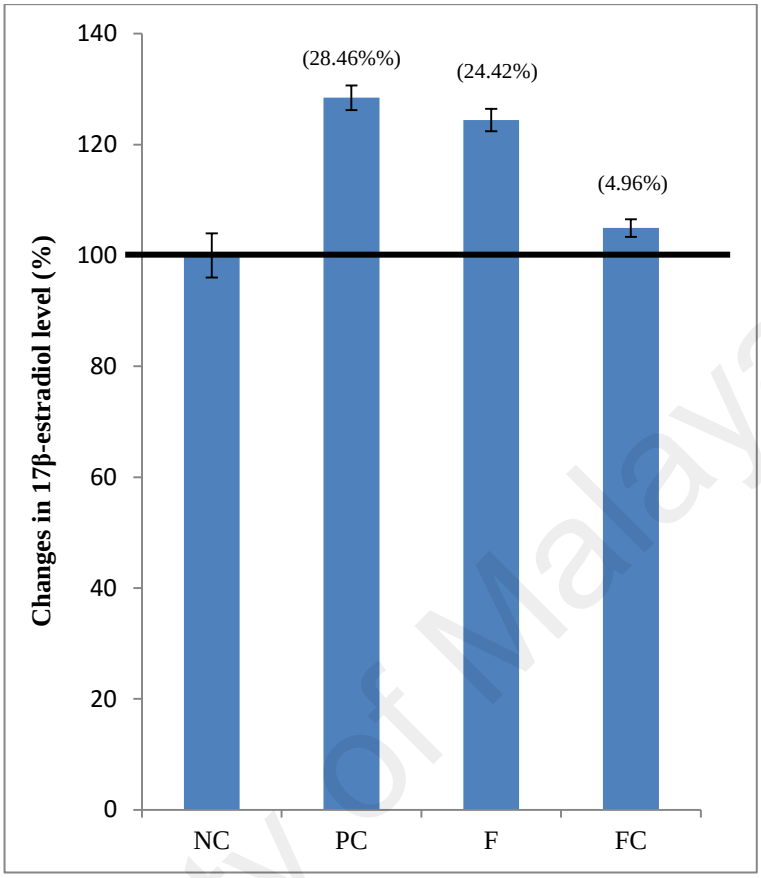


Figure 4.29: Effect of BPA and *Ficus deltoidea* on the level of 17β-estradiol in all experimental groups. In PC group, six weeks exposure to BPA cause slightly increases in the E_2 level. Concurrent treatment with *Ficus deltoidea* (F group) in BPA-exposed rats significantly prevented this effect.

1) $^{a}P<0.05$ vs. NC
2) $^{b}P<0.05$ vs. PC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

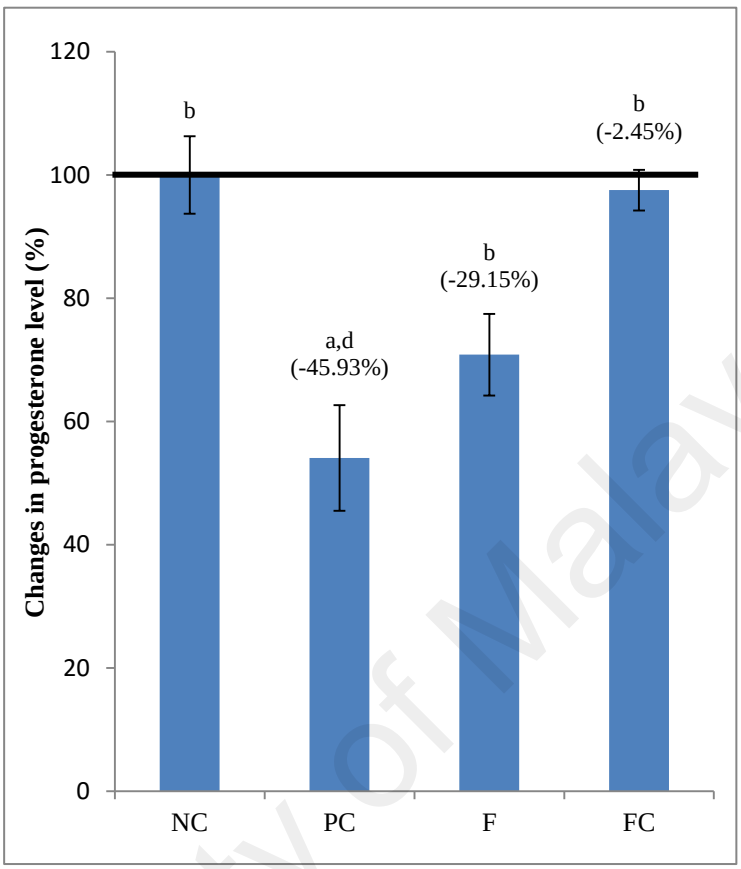


Figure 4.30: Effect of BPA and *Ficus deltoidea* on the level of progesterone in all experimental groups. A reduction in progesterone level was observed in BPA-exposed rats (PC group). Concurrent treatment with *Ficus deltoidea* (F group) in BPA-exposed rats significantly prevented this effect.

1) $^{a}P<0.05$ vs. NC
2) $^{b}P<0.05$ vs. PC
3) $^{d}P<0.05$ vs. FC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

4.2.4 Malondialdehyde (MDA) level

As shown in Table 4.9 and Figure 4.31, the MDA level was significantly increased by 100% in rats exposed to BPA compared to the control rats (NC group). However, this MDA level was significantly reduced in BPA-exposed rats concurrently treated with *Ficus deltoidea* (F group). The MDA level in rats treated with *Ficus deltoidea* alone (FC group) was comparable to the normal rats (NC group).

Table 4.9: Effect of BPA and *Ficus deltoidea* on the level of malondialdehyde (MDA) in all experimental groups. (n=8)

Group	Malondialdehyde (MDA) (μM/μ protein)
NC	0.0037 ± 0.00026^{bbb}
PC	$0.0074 \pm 0.00053^{aaa,ccc,ddd}$
F	0.0048 ± 0.00023^{bb}
FC	0.0036 ± 0.00024^{bbb}

Data are expressed as Mean ±SEM.

1) $^{aaa}P<0.001$ vs. NC
2) $^{bb}P<0.05$ and $^{bbb}P<0.001$ vs. PC
3) $^{ccc}P<0.001$ vs. F
4) $^{ddd}P<0.001$ vs. FC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

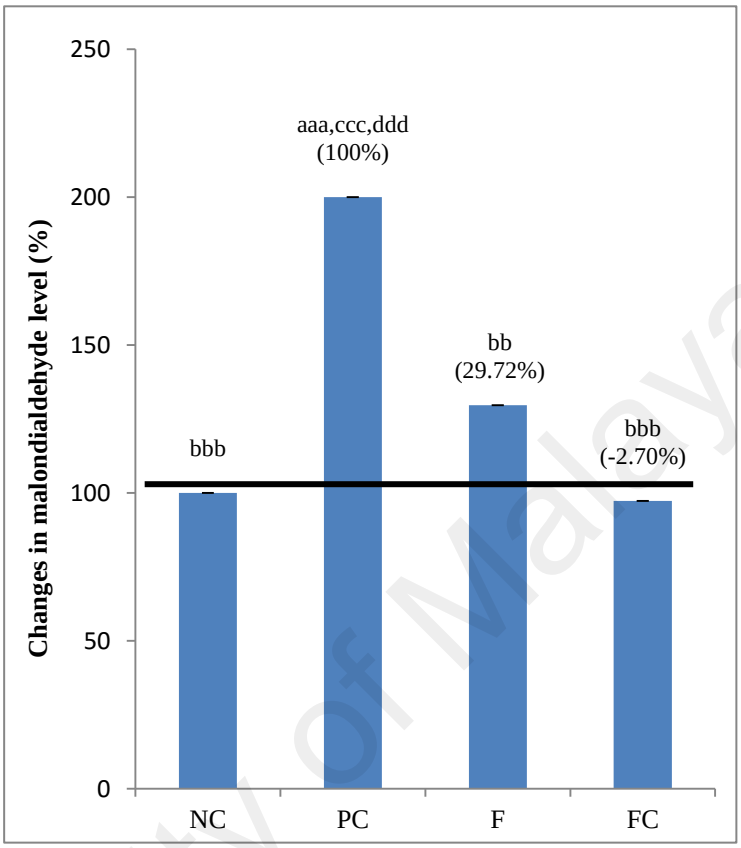


Figure 4.31: Effect of BPA and *Ficus deltoidea* on the level of malondialdehyde (MDA) in all experimental groups. In PC group, malondialdehyde level was significantly increased compared to the PC group. However, concurrent treatment with *Ficus deltoidea* (F group) has significantly reduced this increment.

Data are expressed as Mean ±SEM.

1) $^{aaa}P<0.001$ vs. NC
2) $^{bb}P<0.05$ and $^{bbb}P<0.001$ vs. PC
3) $^{ccc}P<0.001$ vs. F
4) $^{ddd}P<0.001$ vs. FC

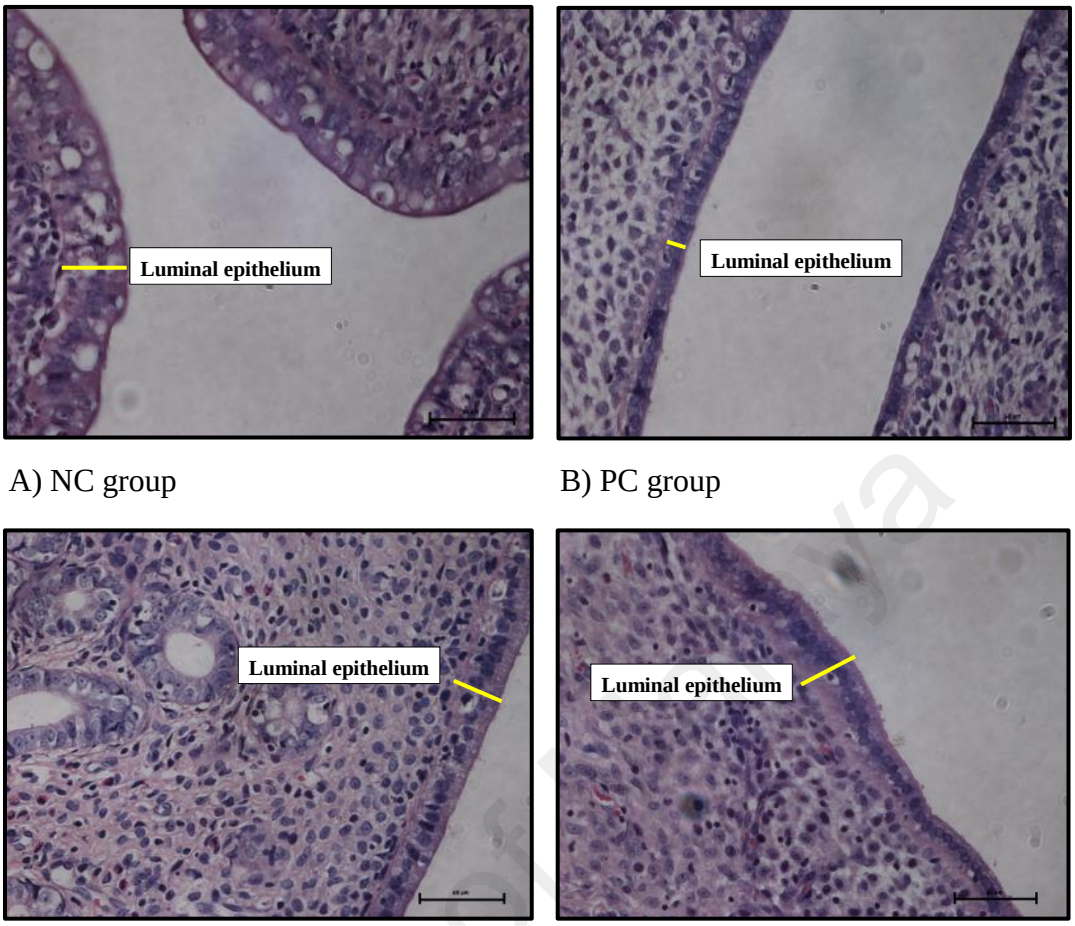
NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

4.2.5 Histopathology

4.2.5.1 Uterine morphology

The histological changes in the uterus of rats from all experimental groups were consistent with the histomorphometric analysis (Figure 4.32, Figure 4.33 and Figure 4.34). Figure 4.32A, Figure 4.33A and Figure 4.34A demonstrate normal histological appearance in the control rats (NC group) and these have been described in Section 4.1.5.1. The disruptive changes in the BPA-exposed rats are shown in Figure 4.32B, Figure 4.33B and Figure 4.34B and also previously in Section 4.1.5.1. The histological findings in rats treated with *Ficus deltoidea* alone (FC group) were comparable to the control rats (NC group) (Figure 4.32D, Figure 4.33D and Figure 4.34D).

The disruptive effects on uterine morphology were partially prevented in BPA-exposed rats concurrently treated with *Ficus deltoidea* (F group) (Figure 4.32C, Figure 4.33C and Figure 4.34C). There were more interstitial spaces between the stromal cells, the stromal cells appeared larger in size, the glandular epithelium was close to the normal histology and mitotic figures were also observed. The appearance of the myometrium was also closer to the normal histology.

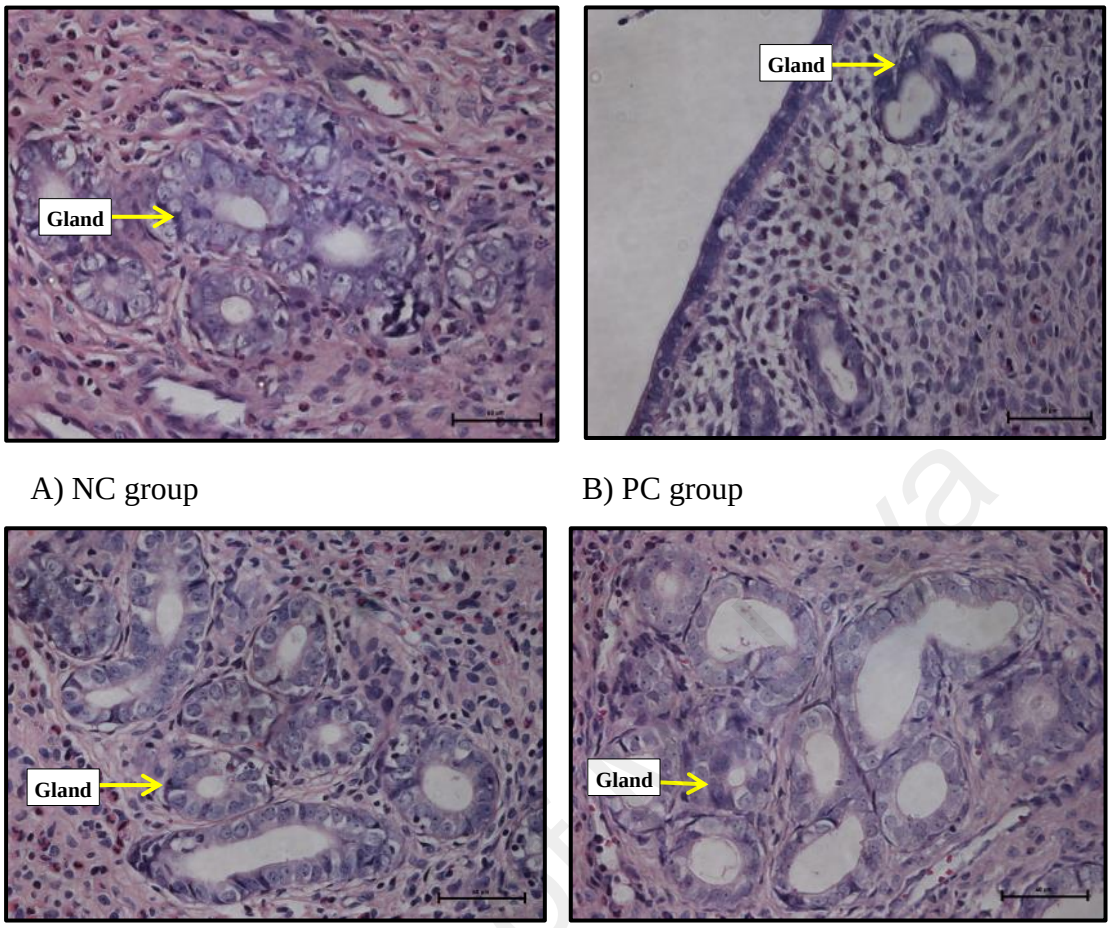


A) NC group B) PC group

C) F group D) FC group

Figure 4.32: Photomicrographs of uterine luminal epithelium from all experimental groups. A) Normal uterine histology was observed in rats from NC group. Their luminal epithelial cells were tall, cylindrical with well-rounded nuclei localized on a prominent basement membrane and had a tall pseudo-stratified columnar epithelium. B) In PC group, disruptive changes were observed in rats exposed to BPA. The luminal epithelial cells are less organized and distorted with irregular shaped nuclei. C) In F group, concurrent treatment with *Ficus deltoidea* in BPA-exposed rats has reduced these disruptive effects. D) The histological findings in FC group were comparable to the NC group. (H&E, 400X)

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

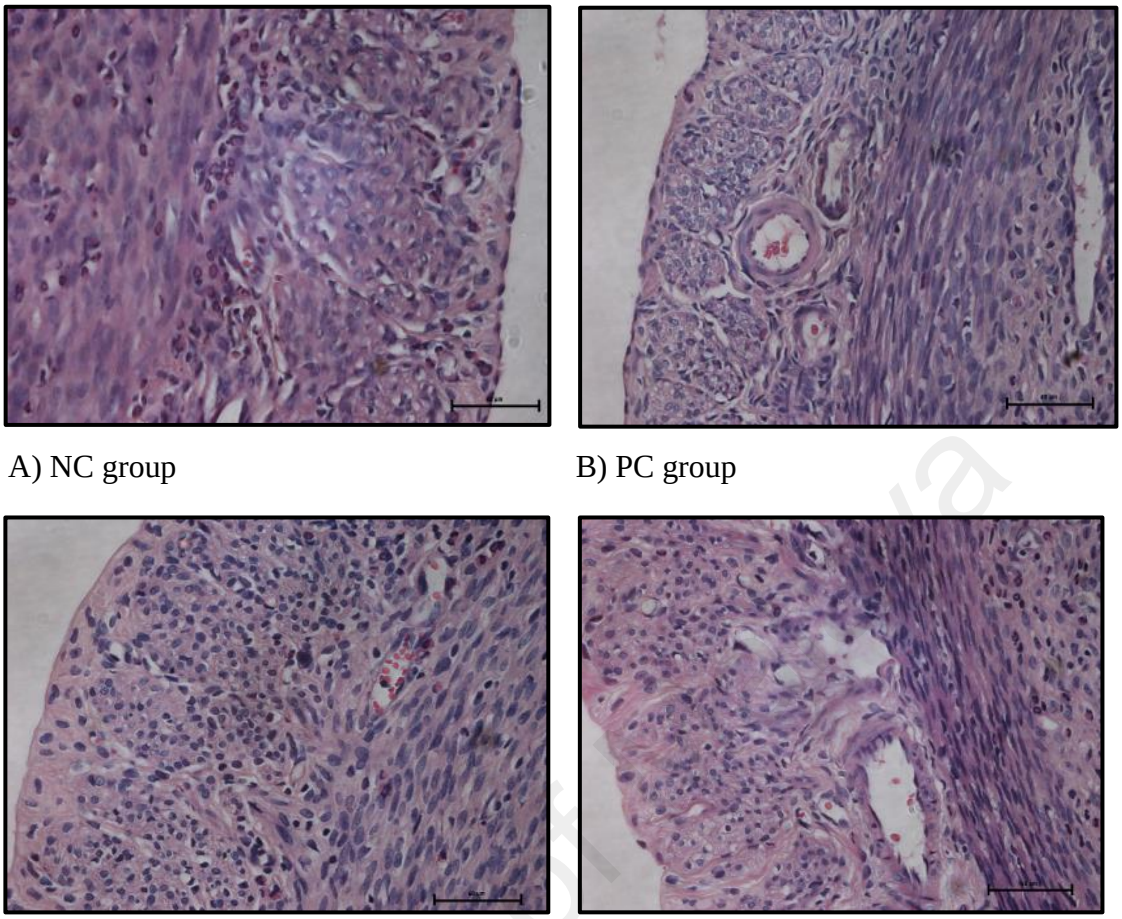


A) NC group B) PC group

C) F group D) FC group

Figure 4.33: Photomicrographs of uterine gland and stroma from all experimental groups. A) In control rats (NC group), normal histological appearance was observed. Healthy glands and high cellular content of stroma were present. B) In PC group, exposure to BPA has induced disruptive effects with unhealthy and smaller size of glandular cells while very little interstitial space were observed between the stroma cells. C) In FC group, concurrent treatment with *Ficus deltoidea* in BPA-exposed rats has improved the histological findings. The glandular cells become plumper while the sizes of the stromal cells were larger with more interstitial space between cells. D) The histological findings in FC group were comparable to the NC group. (H&E, 400X)

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
TH-Tualang honey group (Tualang honey 200 mg/kg + BPA 10 mg/kg)
THC- Tualang honey Control group (Tualang honey 200 mg/kg)



A) NC group B) PC group

C) F group D) FC group

Figure 4.34: Photomicrographs of uterine myometrium from all experimental groups. A) Normal histological appearance was observed in myometrium of control rats (NC group) B) Disruptive changes were observed in BPA-exposed rats (PC group) where smaller and shrunken smooth muscle bundles of the myometrium were observed. The inner circular and outer longitudinal smooth muscle fibers looked disintergrated C) In F group, myometrium was close to the normal histology. D) The histological findings in FC group were comparable to the NC group. (H&E, 400X)

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

4.2.5.2 Histomorphometry of uterus

Table 4.10, Figure 4.35, Figure 4.36 and Figure 4.37 demonstrate the results of uterine histomorphometry (luminal epithelial cells, thickness of endometrium and myometrium layers) in all experimental groups.

In general, these histomorphometry results were consistent with the results of the histological changes and weight of the uterus rats. In BPA-exposed rats (PC group), the decline in all histomorphometry parameters were consistent with the disruptive histological changes and weight reduction of the uterus. The histomorphometry changes in BPA-exposed rats (PC group) were significantly reduced compared to the control rats (NC group). Concurrent treatment with *Ficus deltoidea* in BPA-exposed rats (F group) has significantly prevented the reduction in histomorphometry parameters.

A significant reduction in the height of luminal epithelial cells was observed in BPA-exposed rats (PC group) compared to the NC group (38.11%) (Figure 4.35). However, the reduction was significantly prevented with concurrent treatment of *Ficus deltoidea* (F group). Luminal epithelial cells height in rats treated with *Ficus deltoidea* alone (FC group) was comparable to the control rats (NC group).

As shown in Figure 4.36, endometrial thickness was significantly reduced (14.88%) in BPA-exposed rats (PC group) compared to the NC group. However, the reduction was significantly prevented by concurrent treatment with *Ficus deltoidea* (F group). A slightly higher endometrial thickness was observed in rats treated with *Ficus deltoidea* alone compare to the NC group, but the value is not statistically significant.

In BPA-exposed rats, the thickness of the myometrium was significantly reduced (23.66%) compared to the NC group (Figure 4.37). Concurrent treatement with *Ficus*

deltoidea (F group) has significantly prevented this effect. Comparable values of myometrial thickness were detected in *Ficus deltoidea* treated alone (FC) and NC groups.

Table 4.10: Histomorphometry analysis of the uterus in all experimental groups. (n=8)

Group	Height of luminal epithelial cells (μm)	Thickness of endometrium (μm)	Thickness of myometrium (μm)
NC	30.51 ± 1.37^{bbb}	571.87 ± 6.14^{bbb}	299.18 ± 6.86^{bb}
PC	$18.88 \pm 0.81^{aaa,cc,ddd}$	$486.74 \pm 4.15^{aa,cc,ddd}$	$228.39 \pm 4.91^{aa,c,dd}$
F	26.06 ± 0.68^{bb}	555.9 ± 4.07^{bb}	286.79 ± 3.84^{b}
FC	28.58 ± 0.48^{bbb}	595.18 ± 4.32^{bbb}	296.04 ± 4.30^{bb}

Data are expressed as Mean ±SEM.

1) $^{aa}P<0.01$ and $^{aaa}P<0.001$ vs. NC
2) $^{b}P<0.05$, $^{bb}P<0.01$ and $^{bbb}P<0.001$ vs. PC
3) $^{c}P<0.05$ and $^{cc}P<0.01$ vs. F
4) $^{dd}P<0.01$ and $^{ddd}P<0.001$ vs. FC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

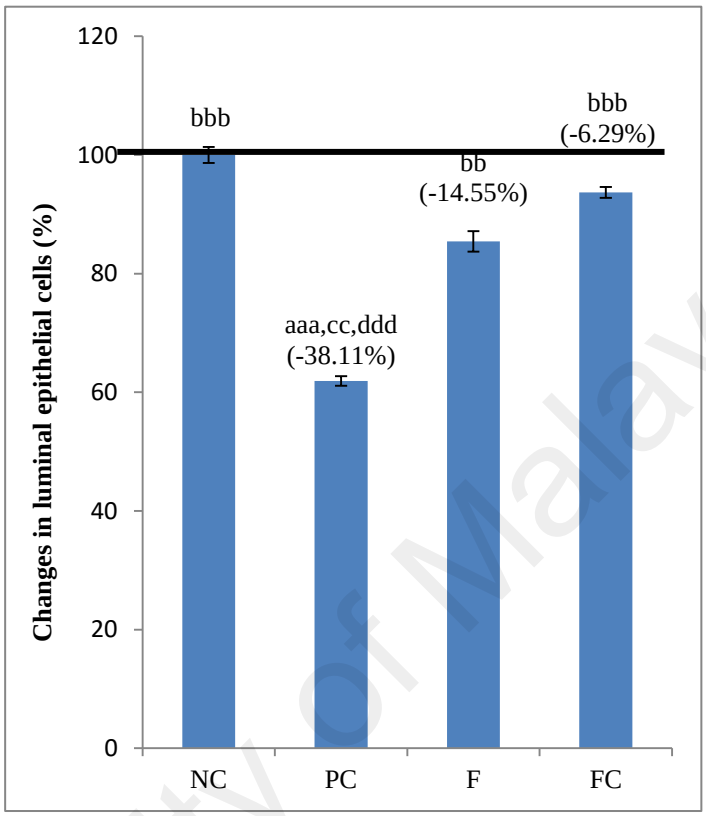


Figure 4.35: Effect of BPA and *Ficus deltoidea* on the height of luminal epithelial cells of uterus in all experimental groups. BPA-induced reduction in luminal epithelial cells (PC group) was significantly prevented by concurrent treatment with *Ficus deltoidea* (F group).

1) $^{aaa}P<0.001$ vs. NC
2) $^{bb}P<0.01$and $^{bbb}P<0.001$ vs. PC
3) $^{cc}P<0.01$ vs. F
4) $^{ddd}P<0.001$ vs. FC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

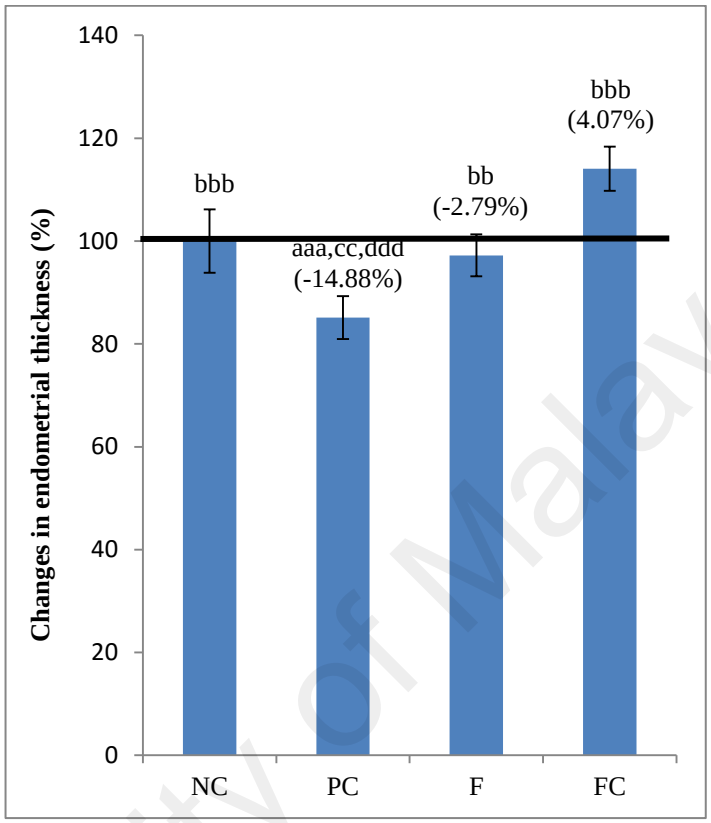


Figure 4.36: Effect of BPA and *Ficus deltoidea* on the thickness of endometrium of uterus in all experimental groups. BPA-induced reduction in endometrium layer (PC group) was significantly prevented by concurrent treatment with *Ficus deltoidea* (F group).

1) $^{aa}P<0.01$ vs. NC
2) $^{bb}P<0.01$ and $^{bbb}P<0.001$ vs. PC
3) $^{cc}P<0.01$ vs. F
4) $^{ddd}P<0.001$ vs. FC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

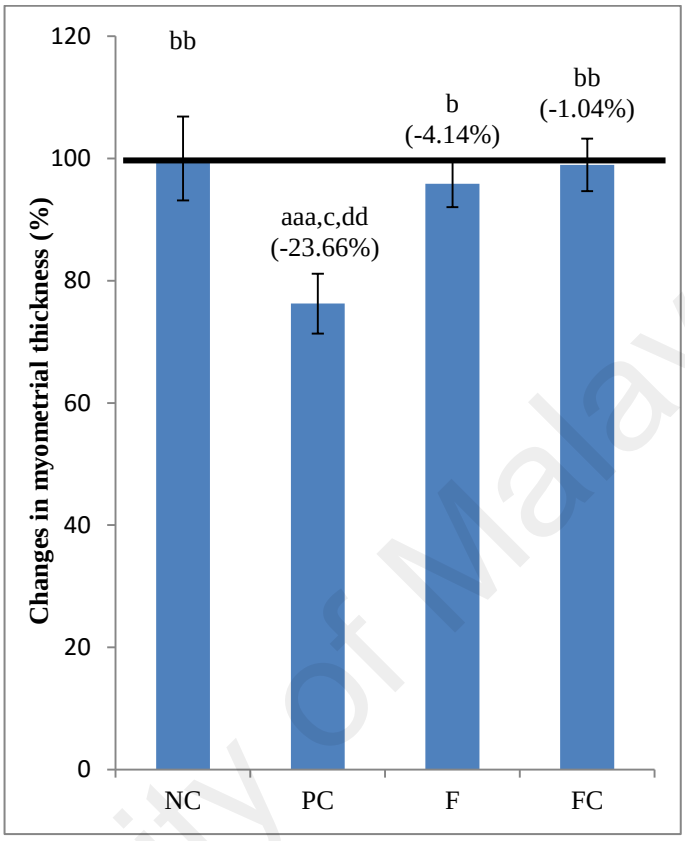


Figure 4.37: Effect of BPA and *Ficus deltoidea* on the thickness of myometrium of uterus in all experimental groups. BPA-induced reduction in myometrium layer (PC group) was significantly prevented by concurrent treatment with *Ficus deltoidea* (F group).

1) $^{aa}P<0.01$ vs. NC
2) $^{b}P<0.05$ and $^{bb}P<0.01$ vs. PC
3) $^{c}P<0.05$ vs. F
4) $^{dd}P<0.01$ vs. FC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

4.2.5.3 Ovary

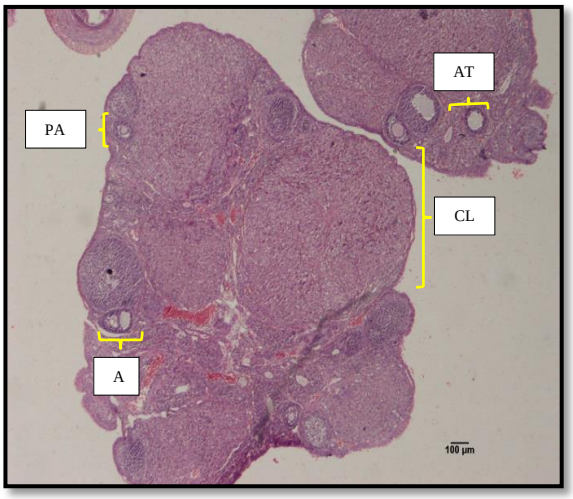
4.2.5.3.1 Ovarian morphology

Figure 4.38 shows histological examinations of representative ovaries from rats in all groups. In general, all stages of follicular development that consists of preantral, antral, corpus luteum and atretic follicles were present in the ovaries of rats from all experimental groups.

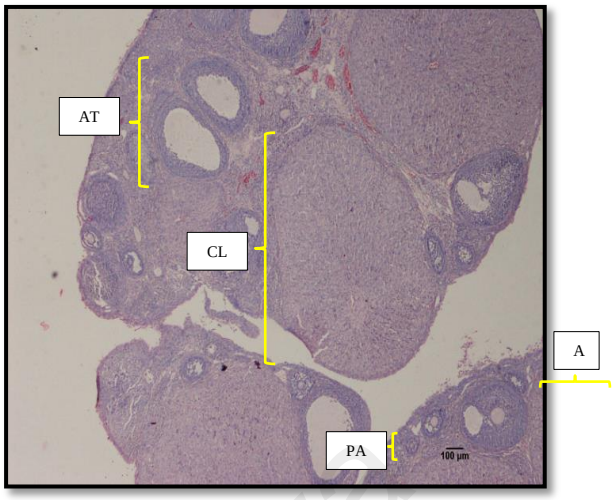
Control and *Ficus deltoidea* treated alone rats showed normal histological characteristics of the ovaries (NC and THC groups, respectively) where no large antral-like follicle and atretic cystic-like follicles were found.

Different histological findings were found in both PC and F groups, where abnormalities of the ovarian histology were observed with the existence of the large antral-like follicles that did not arrive at ovulation. In addition, atretic cystic-like follicles were also present. Less number of corpora lutea were found in both groups. The degree of abnormalities was more apparent in the ovaries of BPA-exposed rats alone (PC group) compared to the BPA-exposed rats concurrently treated with *Ficus deltoidea* (F group).

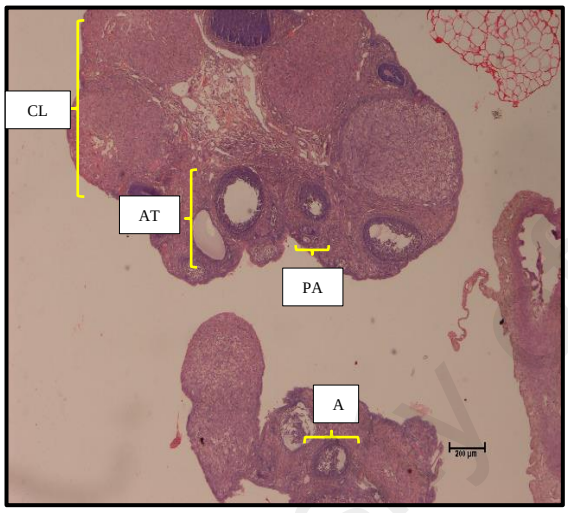
Further quantitative results on the follicular number in all ovaries from all experimental groups were done to detail out these ovarian histological findings (Section 4.2.5.2.2).



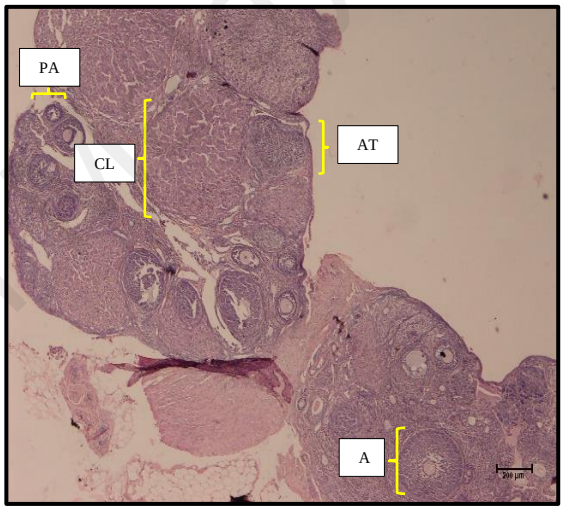
A) NC group



B) PC group



C) F group



D) FC group

Figure 4.38: Representative histological sections from ovary rat of all experimental groups (H&E, 40X).

A) In NC group, normal histological appearance was observed. B) More apparent atretic cystic-like follicles were observed in BPA-exposed rats (PC group). C) Less atretic follicles were observed in BPA-exposed rats concurrently treated with *Ficus deltoidea* (F group). (D) The histological appearance in rats treated with *Ficus deltoidea* (FC group) was comparable to the control rats (FC group).

PA: Preantral; **A**:Antral; **At**:Atretic; **CL**:Corpus Luteum.

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

4.2.5.3.2 Ovarian follicular counting

Histological findings of the effects of BPA and *Ficus deltoidea* on the ovary rats were quantitatively validated by follicular counting analysis on the preantal, antral, corpus luteum and atretic follicles. In general, consistent results of the ovarian histological changes and follicular counts were observed (Figure 4.39) and ovarian follicles of different stages were found in the ovaries of all experimental groups.

Both control groups, NC and FC groups have shown comparable numbers in all ovarian follicles (preantal, antral, corpus luteum and atretic follicles) that were in agreement with the histological findings, which found normal histological appearance of the ovaries in the NC and FC groups.

Rats exposed to BPA alone (PC group) have different histological and follicular counting results than the NC and FC groups. Disruptive histological changes in rats from PC group were consistent with the follicular counting results. In this group, the number of preantral follicles and corpus luteum were reduced compared to the control rats (NC group). When these rats were concurrently treated with the *Ficus deltoidea* (F group), the number of preantral follicles was higher compared to the BPA-exposed rats alone (PC group). However, the number of corpus lutem in rats from F group was similar to the PC group. For antral follicles, the number was higher in the BPA-exposed rats alone (PC group) compared to the control rats (NC group). Treatment with *Ficus deltoidea* in BPA-exposed rats (F group) showed slightly less number of antral follicles than the BPA-exposed rats alone. Although the trend could be observed in the follicular number, they were not statistically significant between the comparative groups due to small sample size of rats.

In BPA-exposed rats (PC group), the number of atretic follicles was significantly higher compared to the control rats (NC group). There was a slightly lower number of

atretic follicles observed following concurrent treatment of *Ficus deltoidea* in BPA-exposed rats (F group). The higher number of atretic follicles in BPA-exposed rats alone could be related to the histological findings were more apparent of the atretic cystic-like follicles was observed compared to the rats concurrently treated with *Ficus deltoidea* and BPA (F group).

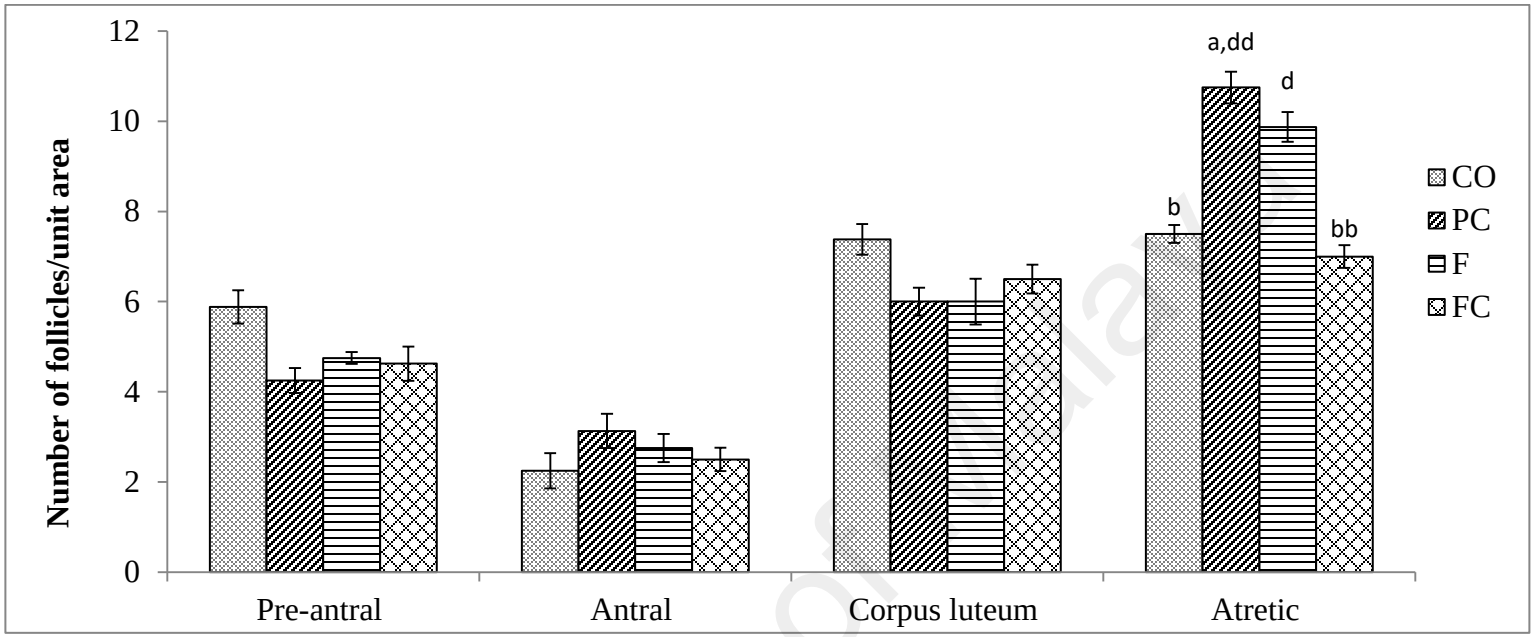


Figure 4.39: Number of follicles in all experimental groups. In BPA-exposed rats (PC group), the number of atretic follicles was significantly higher compared to the control rats (NC group). *Ficus deltoidea* was concurrently treated in the BPA-exposed rats (F group) and slight reduction was observed the number of atretic follicles compared to the BPA-exposed to *Ficus deltoidea* alone.

Data are expressed as Mean±SEM (n=8)

1) $^{a}P<0.05$ vs. NC
2) $^{b}P<0.05$ and $^{bb}P<0.01$ vs. PC
3) $^{d}P<0.05$ and $^{dd}P<0.01$ vs. FC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

4.2.6 Immunohistochemistry

4.2.6.1 ER*α*, ERβ and C3 protein distribution

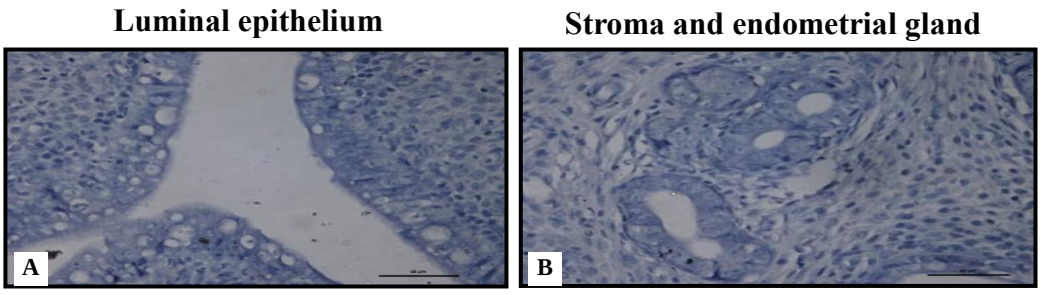
From the general observations, staining intensities of all target proteins in all representative uterine sections were highest in the luminal and glandular epithelial cells while the endometrial stroma had lower staining intensities that those in cells of endometrial stroma (about 50 to 80%).

Figure 4.40 shows localization of the ERα in the representative uterine tissues from all experimental groups. The most pronounced immunostaining intensities were observed in control rats (NC group) (Figure 4.40 C, D). The intensity of immunostaining in the BPA-exposed rats (PC group) was lower compare to the control rats (NC group) (Figure 4.40 E, F). However, immunostaining intensity was slightly higher in BPA-exposed rats concurrently treated with *Ficus deltoidea* (F group) compared to the BPA-exposed rats alone (PC group) ((Figure 4.40 G, H). Intensities of immunostaining in the *Ficus deltoidea* treated alone rats (FC group) were comparable with the control rats (NC group) (Figure 4.40 I, J).

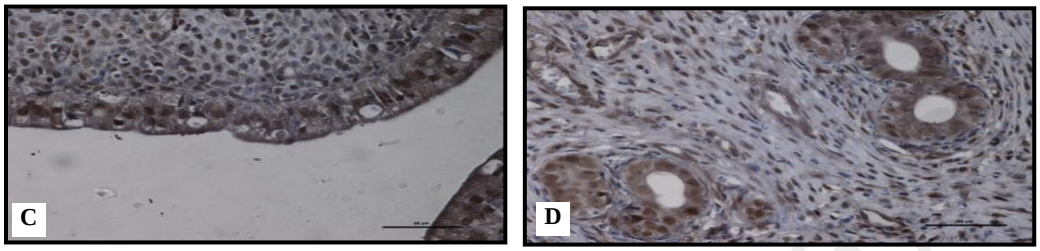
Figure 4.41 indicates the differences in the immunostaining patterns and intensities of ERβ compared to the ERα. Rats exposed to BPA (PC group) showed the most pronounced intensity of immunostaining (Figure 4.41 E, F). However, concurrent treatment with *Ficus deltoidea* in BPA-exposed rats (F group) showed slightly lower intensity of immunostaining compared to the BPA-exposed rats alone (PC group) (Figure 4.41 G, H). Meanwhile, comparable immunostaining intensities were observed in the rats from NC and FC groups (Figure 4.41 C, D and Figure 4.41 I, J, respectively) with lower

intensities in all cellular compartments (luminal and glandular epithelial cells and stroma) compared to the BPA-exposed rats (PC group).

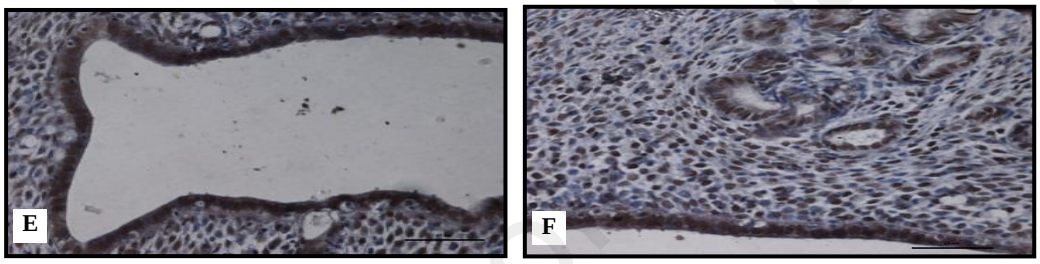
Figure 4.42 shows localization of the C3 in the representative uterine tissues from all experimental groups. Among all groups, the lowest immunostaining intensities of C3 were observed in BPA-exposed rats (PC group) (Figure 4.42 E, F). Concurrent treatment with *Ficus deltoidea* in BPA-exposed rats (F group) showed higher intensities of immunostaining (Figure 4.42 G, H). Compared to these groups, the highest and comparable immunostaining intensities were observed in both control (NC group) and *Ficus deltoidea* treated alone (FC group) rats (Figure 4.42 C, D and Figure 4.42 I, J, respectively).



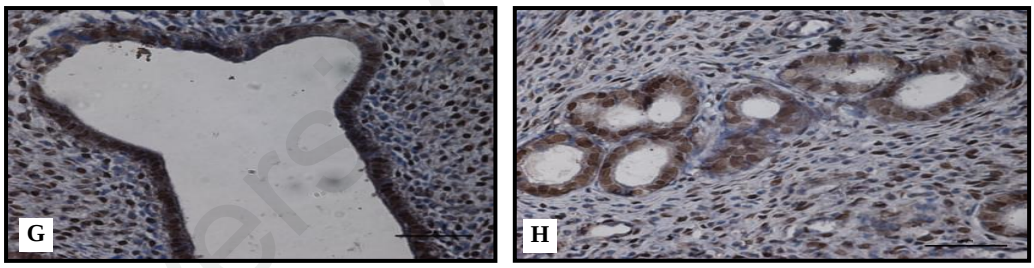
a) NC group (Positive staining)



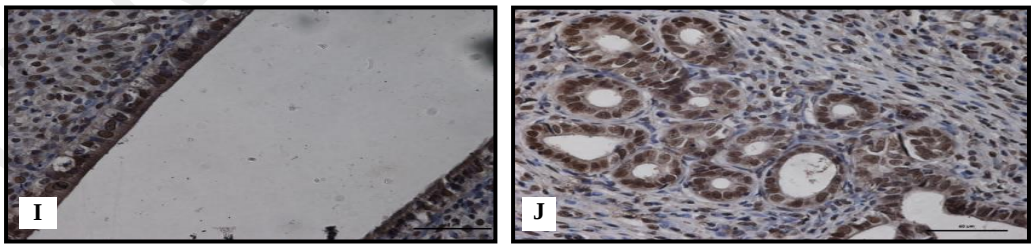
b) NC group (Negative Control group: Vehicle corn oil)



c) PC group (Positive Control group: BPA 10 mg/kg)



d) F group (*Ficus deltoidea* group: *Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)



e) FC group (*Ficus deltoidea* Control group: *Ficus deltoidea* 100 mg/kg)

Figure 4.40: Immunohistological localization of ERα in uterine sections from all experimental groups. The immunostaining intensity in BPA-exposed rats (E, F) (PC group) was lower than the control rats (NC group). Concurrent treatment with *Ficus deltoidea* in BPA-exposed rats (F group) (G, H) showed slightly higher immunostaining intensity compared to the BPA-exposed rats alone (PC group). (400X).

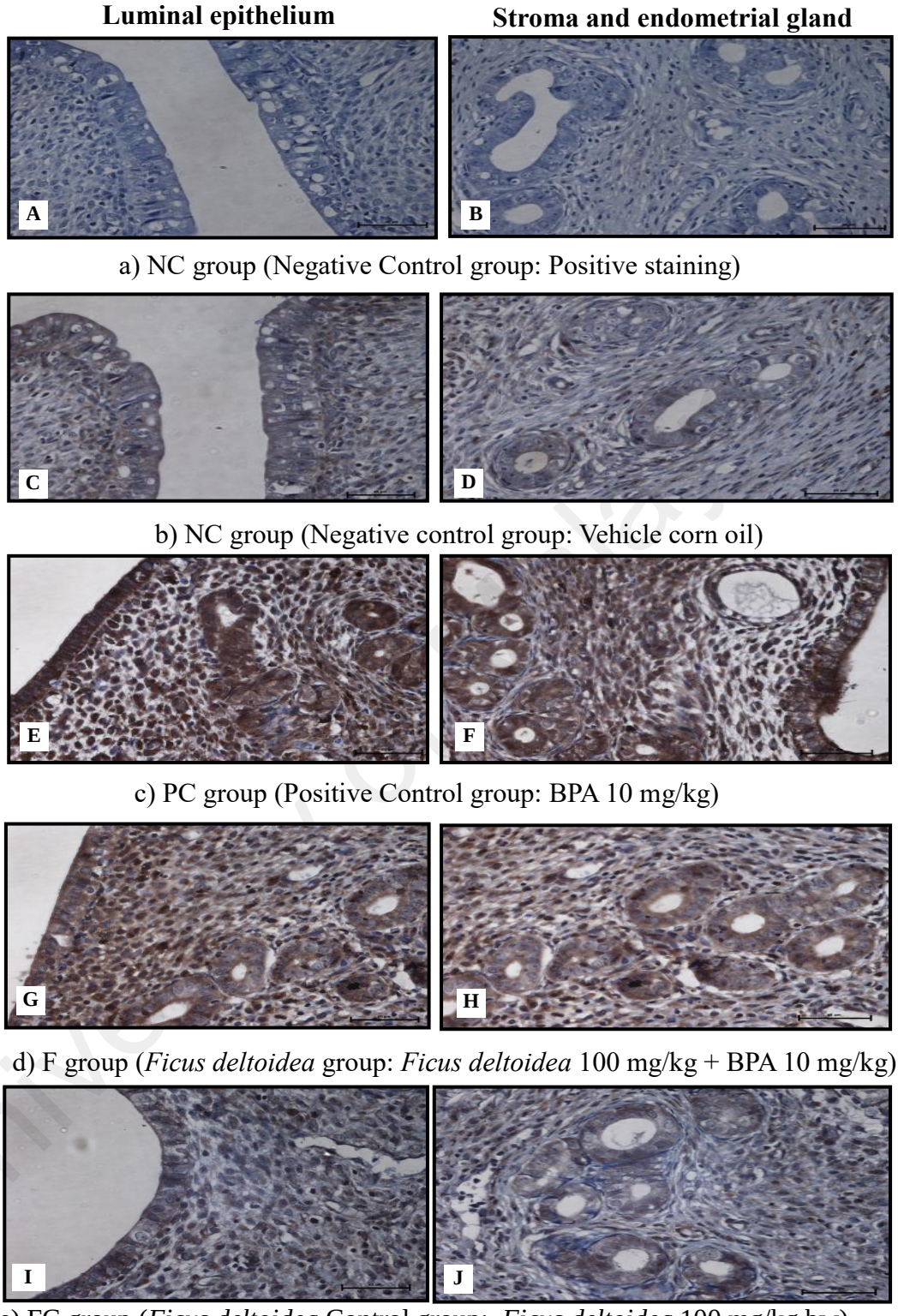


Figure 4.41: Immunohistological localization of ERβ in uterine sections from all experimental groups (x400). The immunostaining intensity in BPA-exposed rats (PC group) (E, F) was higher than the control rats (NC group). Lower immunostaining intensity was observed in BPA-exposed rat treated with *Ficus deltoidea* (F group) (G, H) compared to the BPA-exposed rats alone (PC group).

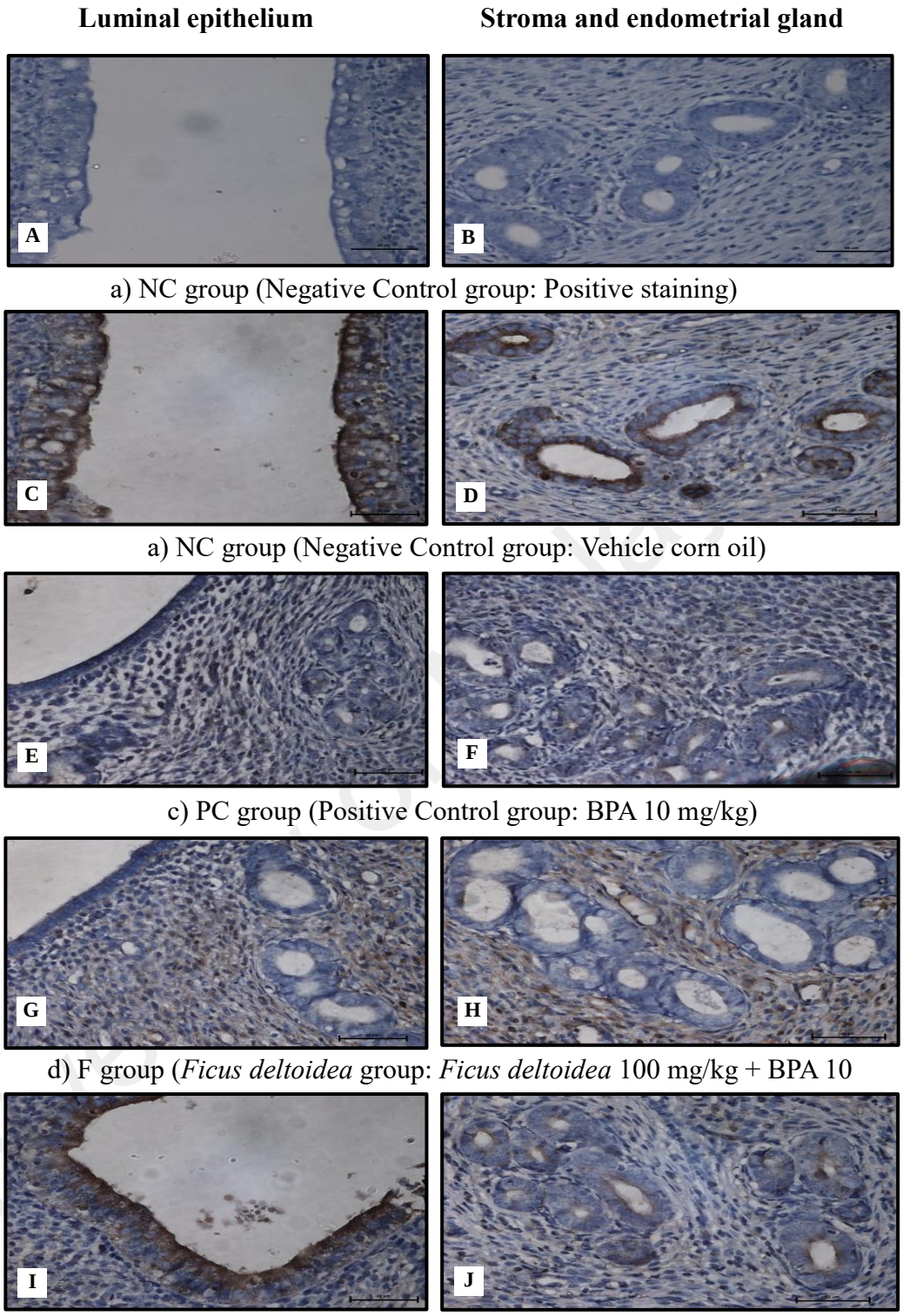


Figure 4.42: Immunohistological localization of C3 in uterine sections from all experimental groups. The immunostaining intensity in BPA-exposed rats (PC group) (E, F) was lower than the control rats (NC group). Higher immunostaining intensity was observed in BPA-exposed rat treated with *Ficus deltoidea* (F group) (G, H) compared to the BPA-exposed rats alone (PC group). (400X).

4.2.6.2 ERα, ERβ and C3 mRNA expression

The levels of mRNA expression of ERα, ERβ and C3 were quantitatively performed by realtime PCR to relate and support their protein expressions from the immunohistochemistry analysis.

Figure 4.43 shows the reduction in expression of mRNA ERα in BPA-exposed rats (PC group) by approximately 4-fold compared to the control rats (NC group). The suppression effect of BPA on ERα expression was significantly prevented by 3-fold in BPA-exposed rats concurrently treated with *Ficus deltoidea* (F group). Interestingly, mRNA ERα expression was highest in rats treated with *Ficus deltoidea* alone (FC group) which is 1.6-fold above the value of control rats (NC group).

Figure 4.44 indicates a different pattern of mRNA expression of ERβ compared to the ERα. In BPA-exposed rats (PC group), expression of ERβ was significantly upregulated by approximately 1.4-fold. However, this upregulation was reduced to 1.1-fold in BPA-exposed rats concurrently treated with *Ficus deltoidea* (F group). On the other hand, concurrent treatment with *Ficus deltoidea* in BPA-exposed rats was able to reduce the ERβ expression by 80%. Rats treated with *Ficus deltoidea* alone (FC group) showed comparable values of ERβ expression compared to the control rats (NC group).

Complement C3 mRNA expression is shown in Figure 4.45. In BPA-exposed rats (PC group), the expression of C3 was significantly downregulated to about 4-fold compared to the control rats (NC group). The effect was significantly inhibited to about 3-fold in BPA-exposed rats concurrently treated with *Ficus deltoidea* (F group). Rats treated with *Ficus deltoidea* alone had no effect on the C3 expression with the value of expression was comparable to the control rats (NC group).

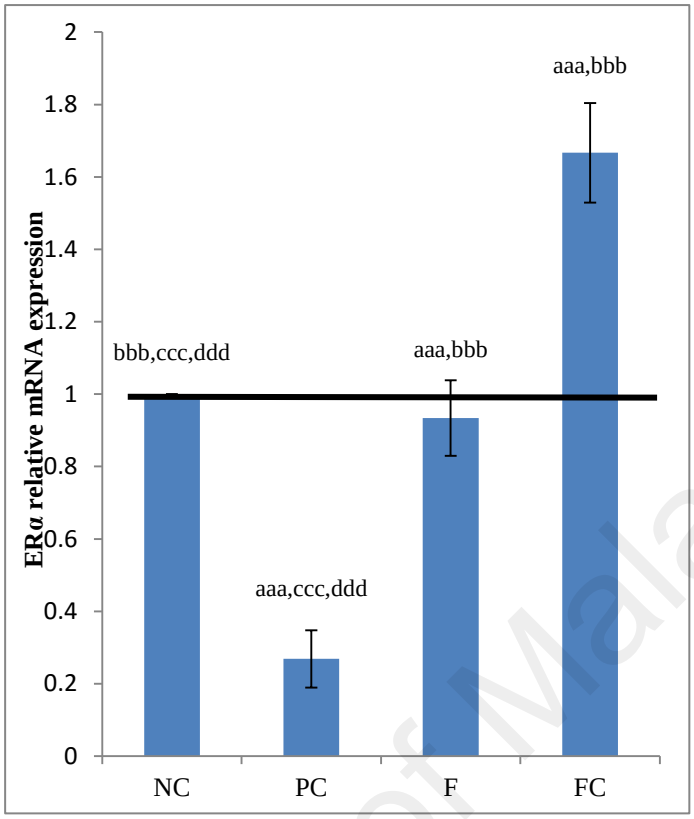


Figure 4.43: Relative quantitative expression of the ERα gene for all experimental groups. The profile of ERα gene for each experimental group is shown as fold change in the rats from PC, F and FC groups compared to the control rats (NC group) which was assigned as 1.0, performed using StepOne Plus Real-time PCR system. Results shown are pooled from 3 independent experiments (n=8) and errors are expressed as Standard Error.

Data are expressed as Mean ±SEM.

1) $^{aaa}P<0.001$ vs. NC
2) $^{bbb}P<0.001$ vs. PC
3) $^{ccc}P<0.001$ vs. F
4) $^{ddd}P<0.001$ vs. FC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

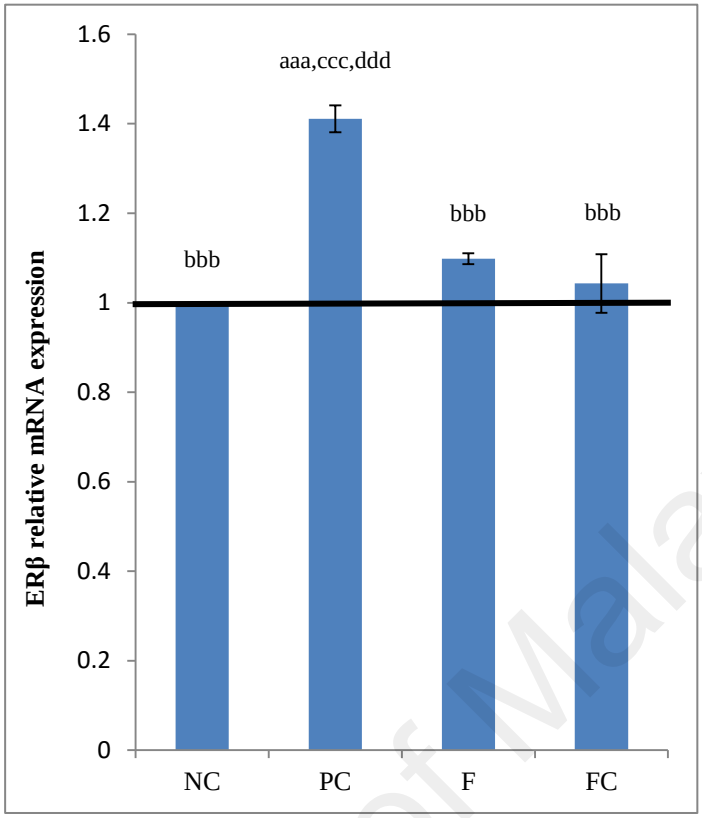


Figure 4.44: Relative quantitative expression of the ERβ gene for all experimental groups. The profile of ERβ gene for each experimental group is shown as fold change in the rats from PC, F and FC groups compared to the control rats (NC group) which was assigned as 1.0, performed using StepOne Plus Real-time PCR system. Results shown are pooled from 3 independent experiments (n=8) and errors are expressed as Standard Error.

Data are expressed as Mean ±SEM.

1) $^{aaa}P<0.001$ vs. NC
2) $^{bbb}P<0.001$ vs. PC
3) $^{ccc}P<0.001$ vs. F
4) $^{ddd}P<0.001$ vs. FC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

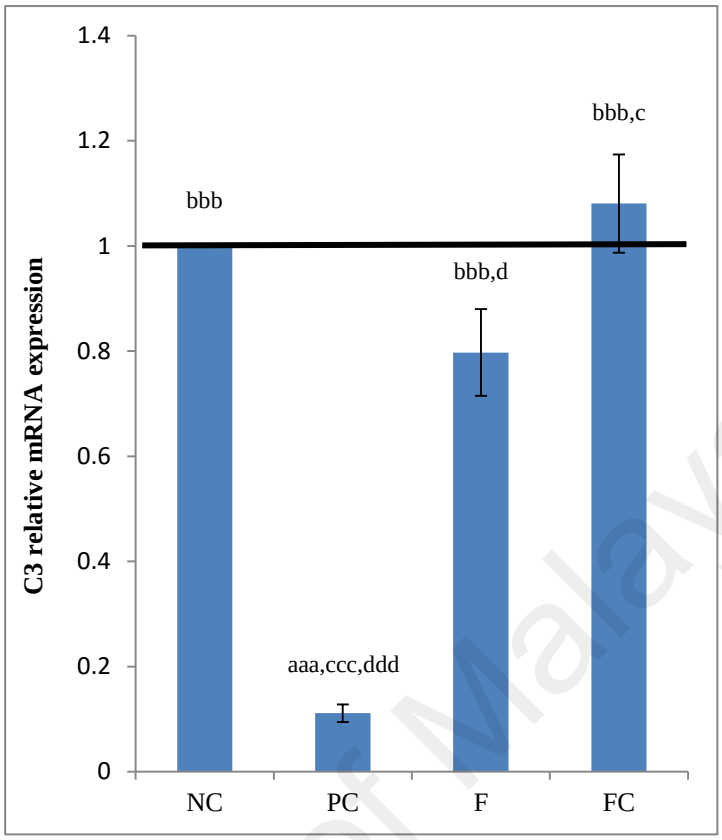


Figure 4.45: Relative quantitative expression of the C3 gene for all experimental groups. The profile of C3 gene for each experimental group is shown as fold change in the rats from PC, F and FC groups compared to the control rats (NC group) which was assigned as 1.0, performed using StepOne Plus Real-time PCR system. Results shown are pooled from 3 independent experiments (n=8) and errors are expressed as Standard Error.

Data are expressed as Mean ±SEM.

1) $^{aaa}P<0.001$ vs. NC
2) $^{bbb}P<0.001$ vs. PC
3) $^{c}P<0.05$ and $^{ccc}P<0.001$ vs. F
4) $^{ddd}P<0.001$ vs. FC

NC- Negative Control group (vehicle corn oil)
PC- Positive Control group (BPA 10 mg/kg)
F-*Ficus deltoidea* group (*Ficus deltoidea* 100 mg/kg + BPA 10 mg/kg)
FC- *Ficus deltoidea* Control group (*Ficus deltoidea* 100 mg/kg)

CHAPTER 5: DISCUSSION

5. 1 Reproductive toxicology

Reproductive toxicology can be defined as a study of the occurrence of biologically adverse effects on the reproductive systems, male or female, that result from exposure to environmental agents, particularly xenoestrogenic compounds (Hood, 2006). The toxicity effect may be expressed as an alteration in the organs and related endocrine system or pregnancy outcome.

In reproductive toxicology studies, ovary and uterus have been extensively used as study models for the reproductive function because of their crucial roles in the reproductive system. These organs are highly sensitive to the xenoestrogenic effects of the environmental compounds, particularly during the prepubertal period of life. The ovary was selected as a target organ due to the fact that it serves a number of important functions that are critical for the primary reproductive activities, including ovulation of the oocytes and production of the sexual steroid hormones, namely the estrogen and progesterone (Li et al., 2013). Meanwhile, the main function of the uterus is regulated by the cyclical changes of the sexual steroid hormones, and for that reason, uterus has been widely used as target organ to study the estrogenic effects of BPA (Diel et al., 2000). In toxicity studies, the pathological changes observed in the uterus are regarded as the consequence of the pharmacological activity of the estrogen in the ovary, which corresponds to the beginning of the changes in the hypothalamus-pituitary axis, as reflected in the reproductive cycle (Li & Davis, 2007).

In recent years, there is increasing concern about xenoestrogenic compounds, particularly the disruptive effects of BPA, ever since BPA was first detected in infant formula and known to leach from polycarbonate baby bottles during washing, boiling and brushing (Biles et al., 1997; Kuo & Ding, 2004). More seriously, considerable amount of

evidences have demonstrated that continuous exposure of children to numerous BPA-containing products until they grow into young adults may lead to long-lasting changes that cause reproductive infertility (Yi et al., 2011).

The prepubertal period is a critical time for child development where it refers to the period of accelerated growth of the gonad preceding maturity (Saunders, 2007). According to the schedule of comparative age categories based on reproductive development, the prepubertal period in rats generally begins at 21 to 45-days of age, which corresponds to 2 to 11 years of age in human (childhood period) while the pubertal period in rats begins at 45 to 90-day of age that is equivalent to 12 to 16 years old in human (adolescence period) (Hood, 2006).

During the prepubertal age, the neuroendocrine development of the hypothalamus-pituitary-gonadal axis is still immature and therefore, the levels of sex hormones in the body are still relatively low compared to the pubertal age (Sun et al., 2000). In the present study, the rationale for administration of BPA at prepubertal age (28-day) is to investigate the effects of BPA at this "sensitive" window of development where the reproductive system is highly susceptible to the estrogenic effects of EDCs compared to adults. Therefore, children are highly vulnerable to BPA exposure from contaminated foods and drinks as well as baby bottles.

In general, the onset of puberty is characterised by the activation of the hypothalamic-pituitary-gonadal axis, development of secondary sexual characteristics, capability in the sexual reproduction and a growth spurt (Kakarla & Bradshaw, 2003). In girls, the onset of puberty is initiated by changes in the expression of hypothalamic neurotransmitters that stimulate the functions of the ovary for the estrogen secretions (Fowler et al., 2012). Unfortunately, BPA, as one of the EDCs, can interfere with the

process of maturation of the hypothalamus-pituitary-gonadal axis through a variety of pathways.

With this concern in mind, our present study had investigated the toxicity effects of BPA on the female reproductive physiology of rats starting from prepubertal until pubertal period in rat with special reference to the estrous cycle, gonadotropins hormones (FSH and LH) level, follicular development and sexual steroid hormones secretion by the ovary (17β-estradiol and progesterone). Meanwhile, the toxicity effects of BPA in the uterus were evaluated on the morphology, MDA level (lipid peroxidation marker), and both proteins and mRNA expressions of estrogen-sensitive genes, namely the ERα, ERβ and C3. Consequently, the potential protective roles of Tualang honey and *Ficus deltoidea* as natural products in preventing the toxicity effects of BPA on the above-mentioned selected parameters were evaluated.

In the present study, the toxic effects of BPA was studied in the female reproductive system using rats as the animal model. The administration of BPA was from 28-day to 70-day of age, which corresponds to 2-year old (weaning age of children) to 15-year old (pubertal age of adolescent) in human. Exposure of BPA was given by oral route since the most primary source of BPA in daily human life was commonly derived from various packaging products of foods and drinks (Huang et al., 2012). Throughout the six weeks of treatment, Tualang honey or *Ficus deltoidea* was administrated first prior to BPA exposure in order to allow the body system of the rats to acquire the optimum protection from these natural products before being exposed to the toxic effects of BPA.

The findings of this study have been analyzed and divided into two sets of data: protective effects of Tualang honey and *Ficus deltoidea* against the disruptive effects of BPA on the reproductive system. Due to the resemblance in the protective effects observed between the two natural products, the discussion is presented as follows:

1) Toxicity of Bisphenol A in the reproductive system.

2) Protective effects of Tualang honey and *Ficus deltoidea* against Bisphenol A toxicity in the reproductive system.

5.2 Toxicity of Bisphenol A in the female reproductive system

Over the last few decades, the epidemic of obesity has risen dramatically worldwide. The typical causes for this epidemic occurrence were highly focused on the high calorie diet and sedentary life style of modern society. In fact, EDCs may also increase the risk of developing obesity-associated disorders, mainly by altering the metabolic functions of the body (Alonso-Magdalena et al., 2012).

BPA is one of the EDCs that have been reported to induce weight gain in rodent models (Howdeshell et al., 1999; Rubin et al., 2001). Several *in vitro* studies have been conducted to elucidate the actual mechanisms for the induction of body weight by BPA. In 2005, Masuno *et al* had shown that BPA exposure in mouse pre-adipocyte cell line led to high accumulation of triglycerides and lipoprotein lipase. These effects were through the activation of phosphatidylinositol 3-kinase (PI 3-kinase), which dramatically turned the fibroblastic cells (3T3-L1) into adipocytes. In another study, adiponectin, as an adipocyte-specific hormone that protects against metabolic syndrome, was found to be inhibited by BPA. However, the mechanisms involved remained unclear (Hugo et al., 2008).

In the present study, the mean body weight of BPA-exposed rats showed slight increment, but was not significantly different compared to the control rats. This suggests that BPA may not be directly correlated with weight gain and this is in agreement with previous reports (Kwon, Stedman, Elswick, Cattley, & Welsch, 2000; Tan & Ali Mohd, 2003; Tinwell, Haseman, Lefevre, Wallis, & Ashby, 2002). A study by Kwon *et al* (2000)

found that oral administration of BPA at high dose had no effect on the body weight of rats. Similar findings from another study also reported no difference in the body weight of offspring rats that were perinatally-exposed to low dose of BPA (Rubin et al., 2001). In fact, the discrepancy in the findings of body weight changes remains enigmatic and could be due to many factors such as differences in the sensitivity of the strain used, dose, route of exposure, window of exposure (age) and duration of exposure (Allard & Colaiacovo, 2011; Mendoza-Rodriguez et al., 2011; Steinmetz et al., 1998).

Nonetheless, in this study, there was a slight increment in the body weight in BPA-exposed rats compared to the control rats. This could be accounted by the action of BPA on adipocytes but other related mechanisms cannot be disregarded (Hugo et al., 2008; Masuno, Iwanami, Kidani, Sakayama, & Honda, 2005). Hence, it may be possible that significant differences in the body weight between BPA-exposed rats and control rats can be achieved if a longer period of treatment is adopted in future experimental design.

Many previous studies have reported on the disruptive effects of BPA on the estrous cycle of rats (Kato et al., 2003; Markey et al., 2003; Rubin et al., 2001; Schonfelder et al., 2002). In agreement with these, our present study showed that the duration of estrous cycle in BPA-exposed rats was prolonged (more than 4 or 5 days) compared to the control rats. This was likely due to the long diestrous phase as shown in our results. The disruption of estrous cycle in BPA-exposed rats could be due to the alteration in the normal functions of the hypothalamic-pituitary axis, which interfered with the normal production of gonadotropin releasing hormone (GnRH) and thereby decreasing the secretion of FSH and LH levels. The interference by BPA on the production of gonadotropin hormones (FSH and LH) could affect the gonadal function, which consequently disrupt the production of sexual steroid hormones, namely E_2 and progesterone from the ovary. Thus, disruptive effects of BPA on the hypothalamic-pituitary-gonadal axis could finally induce abnormalities in the uterine and ovarian

morphology and functions. Thus, disruptive effects of BPA on the hypothalamic-pituitary-gonadal axis could finally induce abnormalities in the uterine and ovarian morphology and functions. Our findings are in agreement with the previous report by Rubin *et al* (2001).

In the brain, the sexually dimorphic region, namely the hypothalamic rostral preoptic area, is said to play an important role for the maintenance of normal estrous cycle and estrogen positive feedback (Petersen & Barraclough, 1989). In this region, ERs are localized within GnRH neurons (Petersen, Ottem, & Carpenter, 2003; Simonian, Spratt, & Herbison, 1999). This important role of GnRH neurons is to provide the primary hypothalamus signal for the gonadotrophin synthesis and secretion, and therefore establishing the preovulatory LH surge that is required for ovulation (Maffini et al., 2006). During the critical period of the sexual differentiation in the female and male, ERs and aromatase in this region convert *in situ* testosterone to estrogen (McEwen & Alves, 1999). Thus, it is important to note that an exogenous xenoestrogen compound like BPA can also influence this region of the brain, especially during the critical period of sexual differentiation, by binding to ERs within the GnRH neurons (Popa et al., 2014). Exogenous xenoestrogen compound has the capability to cause hypothalamic stimulation and pituitary inhibition, which in turn accelerate GnRH pulse frequency and reduce serum LH level, respectively. For these reasons, such disruptions are strongly associated with reproductive infertility.

A recent study had reported that BPA induced persistent impairment in the female reproductive functions of primates (female rhesus monkey). This impairment was caused by direct influence of BPA of the hypothalamic neuroendocrine functions via suppression of the GnRH level (Kurian et al., 2015). In fact, many earlier studies had also described the persistent effects of BPA exposure on the hypothalamus-pituitary-gonadal axis functions and proposed the underlying mechanisms involved. One study reported that in

BPA-exposed male rats, the ERβ mRNA expression in preoptic area of the brain showed 4-fold increase compared to the control rats (Ramos et al., 2003). Meanwhile, another study had found that neonatal exposure to BPA in both sexes of rats induced disruptions on the ERα and ERβ expressions throughout the mediobasal hypothalamus and amygdala of the brain (Cao et al., 2013). Apart from the xenoestrogenic properties, the disruptive effects of BPA in the brain have also been shown to be mediated by ROS generation. It is believed that the generation of ROS by BPA leads to the increase in the level of lipid peroxidation product (MDA) while reducing the level of antioxidant glutathione (GSH) (Aydogan, Korkmaz, Barlas, & Kolankaya, 2008).

The ovary and uterus are the main target organs for xenoestrogenic compounds as proven in many scientific studies (Newbold et al., 2007; Rodriguez et al., 2010; Vigezzi et al., 2015; Xu et al., 2002; Zhou et al., 2008). Changes in the uterine weight have been used as a basis for comparing relative potency of estrogenic compounds in bioassay analysis. Toxicological data on organ weight showed that the effects of BPA appeared to be very specific on the ovary and uterus. In the present study, the weights of the ovary and uterus of BPA-exposed rats were increased by 20% and reduced by 25%, respectively, compared to the control rats. Based to these results, it appears that BPA affected the weight of both organs, without having significant effects on the body weight. These are also in agreement with a previous report by Ashby *et al.* (2003). In fact, any changes of 10% or greater in the organ weight is a strong indicator of toxic effects, particularly when it is associated with morphological changes. In this study, the significant changes in the organ weights and size of ovary and uterus of BPA-exposed rats were in fact coupled with overall disruptive alterations observed in their histological structures.

The ovary has several major crucial functions that are important for the reproductive activities, including the production of estrogen from the developing ovarian

follicles and progesterone from the corpora lutea (Li & Davis, 2007). Proper development of follicles ensures proper secretion of the respective hormones at the right time. Hence, these events have to take place systematically. In contrast to the normal histological findings in the ovary of control rats, the presence of large-antral and atretic-cystic like follicles in BPA-exposed rats were evident. These abnormal disrupted follicles reflected the pathological alteration in the secretion of gonadotropins hormones (decreasing levels of FSH and LH) and possibly account for the increase in the ovarian weight. Thus, the disruptive effect of BPA on folliculogenesis is evident. This subsequently results in disruption of follicular and ovarian functions, especially those related to hormone production.

Similar study by a recent research work had also revealed that prepubertal exposure to BPA at low doses may induce disruptions in the normal functions of the ovary, which affects early folliculogenesis process (Gamez et al., 2015). Continuous BPA exposure from prepubertal until pubertal periods may cause permanent effects on the process of ovulation and production of sexual steroid hormones (E_2 and progesterone) in the ovaries. Thus, disruption on these ovarian functions will consequently induce further disruptions in the uterine functions (Westwood, 2008).

In the present study, reduction in the LH level observed in the BPA-exposed rats may be the reason for the formation of cystic follicles (anovulation follicles) and consequently reduction in the formation of corpora lutea in the ovary (formed after ovulation). When the formation of corpora lutea is reduced, the secretion of progesterone is low. Cystic follicles are large antral-like follicles surrounded by thin layer of granulosa cells with non-detectable theca cell layers that do not support ovulation process in the ovary (Adewale et al., 2009; Fernandez et al., 2009). The observed increase in the number of cystic-antral like follicles with reduced corpora lutea in BPA-exposed rats in this study is in agreement with previous findings (Markey et al., 2003; Rodriguez et al., 2010).

Interference with the normal development of follicles in the ovary was evidenced by reduction in the number of preantral follicles and increase in the number of atretic-cystic like follicles in BPA-exposed rats. Our results confirmed the findings of previous study that showed positive correlation between the total number of antral-cystic like follicles in BPA-exposed rats and the ovarian weight (Murasawa et al., 2005). Interestingly, changes in the follicular populations and histology in the ovary of BPA-exposed rats were similar to those seen in aging rats (Nishijima, 2013).

As expected, alterations of the sexual steroid hormones (estrogen and progesterone) of BPA-exposed rats subsequently result in morphological changes in the uterus. The histological changes in the uterus of BPA-exposed rats identified in this study include reduction in the thickness of luminal and interstitial space between the stromal cells, presence of unhealthy glands and reduction in the thickness of the muscular layer. These findings are in agreement with the previous BPA studies (Yoshida, 2001; Markey, 2005). These histological abnormalities in the uterus of BPA-exposed rats were reported to closely resemble those seen in old rats, which is part of the normal female aging process (reproductive senescence) (Bosquiazzo, Vigezzi, Munoz-de-Toro, & Luque, 2013). Physiologically, this process is termed as persistent anestrus or persistent diestrous (Westwood, 2008). In old rats, the decline in reproductive function is indicated by the alteration in the expression of estrogen sensitive genes (Khalyfa et al., 2003). These previous findings were also seen in the current study, where occurrence of persistent diestrous phase and alteration in the expression of estrogen-sensitive genes were observed in the BPA-exposed rats.

The disruptive effects of BPA in the uterus and ovary of rats may be summarized as follows: BPA causes reduced secretion of the gonadotrophins hormones (FSH and LH) that in turn induce further disruptive effects on the ovarian follicular development and functions, particularly causing anovulation of the follicles and interruption of production

of the sexual steroid hormone (E_2 and progesterone) by the ovary. As a consequence of this, the development and functions of the uterus are also unquestionably affected.

To explain the mechanism involved in the disruptive effects of BPA in the uterus, we analysed the role of oxidative stress, as proposed in a previous report (Popa et al., 2014). BPA has been suggested as one of the EDCs that induce ROS generation, which play an important role in the pathology of female reproductive diseases (Yi et al., 2011). Lipid peroxidation (LPO) is a process of interaction between ROS and the cellular membrane that induces damage to the cellular macromolecules and DNA (Guney et al., 2007). MDA is a secondary product of LPO and is generally accepted as an endpoint marker for oxidative stress (Aslan, Sekeroglu, Tarakcioglu, & Koylu, 1997). In this study, the uterine MDA level in BPA-exposed rats was enormously higher compared to the control rats. This suggests that BPA has the capability to generate ROS that subsequently results in increased lipid peroxidation and MDA formation. Therefore, it is evident that the disruptive effects of BPA on the reproductive organs could be due to the generation of oxidative stress as a result of the xenoestrogenic activity of BPA.

The unifying relationship of ET-ROS-OS framework (electron transfer-reactive oxygen species-oxidative stress) provides a novel and reasonable hypothesis for the physiological effects of the BPA toxicity (Kovacic, 2010). Many experimental studies could be related to this theoretical framework, including lipoperoxidation, oxidation of metabolic products, generation of ROS and depletion of antioxidant as well as oxidation of DNA. As discussed earlier (Chapter 2: 2.1.4 Pharmacokinetics of BPA), the predominant metabolic pathway for BPA is by the conjugation of a phenolic hydroxyl group of BPA to glucuronide (Knaak & Sullivan, 1966). However, some of the BPA is converted to 5-hydroxy bisphenol by cytochrome P-450-dependent mixed-function oxidases in the liver before it is finally transformed to bisphenol-o-quinone (Atkinson & Roy, 1995).

The bisphenol-o-quinone metabolite has the ability to bind and consequently induce the formation of adducts with DNA, which has been regarded as a contributing factor for the toxicity in the reproductive organs, liver, kidney, as well as the central nervous system (Kovacic, 2010). In many scientific studies, the relationship between oxidative stress and infertility among women has been proven in several scientific studies (Jozwik, Wolczynski, & Szamatowicz, 1999; T. Suzuki et al., 1999). Epidemiological data showed that higher levels of ROS and lower levels of antioxidant were detected in infertile women compared fertile women (Polak, Koziol-Montewka, Gogacz, Blaszkowska, & Kotarski, 2001; Wang, Sharma, Falcone, Goldberg, & Agarwal, 1997).

Due to the xenoestrogenic nature of BPA, it is known that the effects of this compound are likely to be mediated through the ERs. However, it has been proposed that the effects of BPA on certain cells or tissues may be dissimilar due to the presence of 2 different ER subtypes. Therefore, based on this, BPA is not only an estrogenic compound that has the ability to induce a variety of disruptive molecular effects, but also may ultimately impact the physiological response at the cellular level, depending on the ER subtypes affected. Further experiments on estrogen sensitive genes in the uterus were also performed to support the findings on the morphological and oxidative stress in the uterus. Our current findings are also in line with previous studies that revealed the toxicity effects of BPA on the development, growth and functions of the uterus by interrupting the regulation of mRNA expression and protein distribution of ERα, ERβ and complement C3 (Schonfelder et al., 2004; Seidlova-Wuttke et al., 2004; Vigezzi et al., 2015).

ER, a member of steroid receptor superfamily, is a ligand-activated enhancer protein that is activated by estrogen (17β-estradiol) and has the ability to regulate gene transcriptions via estrogen responsive elements (Klinge, 2001). Unfortunately, it can also be activated by other compounds including EDCs such as BPA (Hiroi et al., 1999). ER is encoded by two subtype genes, namely ERα and ERβ, and their functions as signal

transducers and transcription factors in modulating the expression of target genes (Couse & Korach, 1999). The endogenous estrogen (17β-estradiol) has a lower binding affinity to ERβ than ERα (Kuiper et al., 1997) but both receptors share similarity in terms of transactivation via estrogen responsive element (ERE) (Pace et al., 1997). In contrast, they possess dissimilar functions with regards of their roles in transcription activation, which depend very much on the ligands and their responsive elements (Peach et al., 1997).

BPA is considered a weak environmental estrogen since its binding affinity to ERα and ERβ is estimated to be 10,000-fold lower than the endogenous estrogen; with a relative binding affinity of 6-fold higher in ERβ compared to ERα (Hiroi et al., 1999; Kuiper et al., 1997). On the other hand, in some cell types, BPA might exhibit estradiol-like agonist activity via ERβ and a mixed agonist/antagonist activity via ERα (Kurosawa et al., 2002). Both subtypes contain a ligand-dependent transactivation domain, named AF2 (Activation Function 2) for activation of transcription of target genes by the recruitment of transcriptional co-factors steroid receptor coactivator-1 (SRC-1) and transcriptional intermediary factor-2 (TIF-2) (Voegel, Heine, Zechel, Chambon, & Gronemeyer, 1996). The disruptive role of BPA in this pathway significantly increases the recruitment of these co-factors (Routledge et al., 2000). This could be a possible explanation for our present data that showed significant effect of BPA at both transcriptional and translational levels by upregulating the expressions of mRNA and protein localization of ERβ in the uterus of BPA-exposed rats compared to the control rats.

In our study, the upregulation of ERβ in BPA-exposed rats could also contribute to the disruptive effects seen in the uterus. A report by 2000 by Weihua et al. (2000) revealed that in the uterus, ERβ acts as a modulator of ERα-mediated gene transcription. The role of ERβ is as a trans-dominant repressor that inhibits the ERα transcriptional activity (Hall & McDonnell, 1999). This inhibitory effects is due to the ability of ERβ to

form heterodimers with ERα, which in turn regulate estrogen receptor functions (Pettersson, Grandien, Kuiper, & Gustafsson, 1997). Thus, unlike ERα, ERβ does not have classic uterotrophic effects but rather disruptive effects on the uterus. Therefore, the high expression of ERβ induced by BPA has caused disruptive effects on the morphology of the uterus of BPA-exposed rats compared to the control rats.

Other studies via crystallography structure analysis and computer modeling studies have found that BPA also has higher binding affinity to ERRγ than the ERα (Matsushima et al., 2007; Nose & Shimohigashi, 2008). ERRγ is an orphan nuclear receptor that belongs to the ERR family (estrogen receptor-related) (Giguere, 2002). Although both ERα and ERRγ receptors share similar sequence homology, 17β-estradiol does not associate to ERRγ (Takayanagi et al., 2006). The BPA-ERRγ interaction was suggested to trigger heterodimerization between ERα and ERRγ which results in suppression of the transcriptional activity of the ERα (Matsushima et al., 2007). Similarly, other report has also found that the modulation of BPA as an inhibitor on ERα signaling is also via ERRγ (Huppunen & Aarnisalo, 2004). Such interaction could be the explanation as to why our present data showed significant down regulation of ERα mRNA expression in the uterus of BPA-exposed rats compared to the control rats. However, the protein localization of ERα in BPA-exposed rats did not differ much from the normal control rats. This indicates that the effect of BPA is less at protein translation level compared to the mRNA transcriptional level.

Previously, Seidlova-Wuttke *et al.* (2004) has used quantitative RT-PCR analysis to investigate the effects of BPA on C3 mRNA transcription in the uterus of rats. According to the authors, treatment with estradiol significantly upregulated the C3 mRNA expression. Interestingly, BPA exposure had caused down regulation of C3 mRNA expression. In fact, those effects also have been observed in our present study, where there was down regulations of C3 expressions at the transcriptional and

translational levels in the uterine of BPA-exposed rats compared to the control rats. These results suggest that BPA induces suppression in the immune system in the uterus.

5.3 Protective effects of Tualang honey and *Ficus deltoidea* against Bisphenol A induced toxicity in the reproductive system

In the present study, we investigated the effects of Tualang honey and *Ficus deltoidea* on the body weight of experimental rats both exposed and non-exposed to BPA. The slight increment of body weight in BPA-exposed rats was not prevented by treatment with Tualang honey and *Ficus deltoidea.* However, it is possible that prevention of the body weight could be seen if the rats were treated with the two natural products for a longer period of time. Meanwhile, the body weight of rats treated with Tualang honey and *Ficus deltoidea* alone remained unchanged as observed in normal control rats.

To determine the whether Tualang honey and *Ficus deltoidea* have protective effects on the reproductive cycle of BPA-exposed rats, changes in the estrous cycle of rats was monitored and analyzed. Results demonstrated that treatments with Tualang honey and *Ficus deltoidea* in BPA-exposed rats significantly increased percentage of rats with normal estrous cycle up to 62.5%. However, Tualang honey and *Ficus deltoidea* were not able to completely increase the percentage of rats with normal estrous cycle as seen in the control rats (100%). Treatment with Tualang honey and *Ficus deltoidea* alone did not cause any disruptive effects on the estrous cycle indicating that on their own, they do not have influence on the hypothalamic-pituitary functions.

Theoretically, the increase in the percentage of normal estrous cycle in BPA-exposed rat should essentially relate to the hypothalamic-pituitary axis, where normalization of gonadotropins hormone levels ought to be apparent. Improvement in the

percentage of normal estrous cycle could be associated with reduced harmful effects of BPA on the ovarian follicular development in BPA-exposed rats. However, here, the increase in the percentage of normal estrous cycle in BPA-exposed rats is only accompanied by an increase in the level of FSH but not LH, and the reasons for these remain enigmatic. It may be possible that both natural products may not be affecting the pituitary, but acting primarily the ovaries instead. They could be displaying their effects through estrogenic mechanisms via ERs at the ovaries, hence protecting the follicular development and ovulation, even though the LH level is not altered. Even at low LH, we have shown that the ovulation still occured as shown by the restoration of progesterone secreted by the corpus luteum in both in Tualang honey and *Ficus deltoidea* treated rats. Therefore, further investigation is warranted to define the mechanism that may be responsible to restore the normal estrous cycle in BPA-exposed rats treated with Tualang honey and *Ficus deltoidea* without significantly affecting the hypothalamic-pituitary functions.

Following this, we then investigated the protective effects of Tualang honey and *Ficus deltoidea* on the ovary and uterus. Changes in the weight of the ovary and uterus in BPA-exposed rats were prevented by Tualang honey and *Ficus deltoidea* treatments. The increased and decreased of ovarian and uterine weights, respectively, were normalized to the control levels. These suggest that these natural products have protective properties that prevent any changes in the weight of the reproductive organs. Subsequently, further investigation showed that normalization of these organ weights by the two natural products were correlated with the normalization of their morphology. In the ovary, the follicles were seen healthier. Meanwhile, protective effects of these natural products in the uterus could be observed through improvement in cells of the stroma, luminal epithelium, endometrial glands and muscles. These suggest the protective effects of both Tualang honey and *Ficus deltoidea* against the disruptive effects of BPA on the cellular

components of the reproductive organs. Comparable histological findings in these organs were observed between Tualang honey and *Ficus deltoidea* treated alone compared to the control rats, indicating that they have no pathological effects on these cellular organs. Although the morphological improvements in ovary and uterus were seen to be significant in BPA-exposed rats treated with Tualang honey and *Ficus deltoidea*, the protection exerted by these natural products seemed to be only partial compared to the control rats. This could be due to the incomplete elimination of ROS, thus enabling continual disruption on the cellular components and follicles. Moreover, even after elimination of ROS and BPA, the proteins/enzymes involved in the intracellular protective machinery may require a longer period of time to recover from the oxidative damage that has occured (Chevallet et al., 2003).

Further analysis on lipid peroxidation via quantitation of the MDA level (oxidative stress marker) was performed to relate with the oxidative stress that results in the morphological changes. Treatment with Tualang honey and *Ficus deltoidea* have significantly prevented the increased in MDA level, comparable to the normal levels as shown in the control rats. This shows that both natural products are able to prevent oxidative stress and lipid peroxidation caused by BPA. Meanwhile, the MDA levels in rats treated with Tualang honey *and Ficus deltoidea* alone were similar to the control rats, suggesting that on their own, these natural products do not influence lipid peroxidation.

Molecular analysis on the estrogen sensitive genes such as the ERα, ERβ and C3 in uterus of rats using realtime-PCR and immunohistochemistry were subsequently performed. We have demonstrated that the disruptions of the ERα, ERβ and C3 genes by BPA occur at both transcriptional and translational levels. These disruptions were significantly reduced when BPA-exposed rats were treated with Tualang honey and *Ficus deltoidea*. Interestingly, rats treated with Tualang honey and *Ficu sdeltoidea* alone

showed higher expression of ERα compared to the other groups. This again proves that these natural products have estrogenic properties that affect certain estrogen sensitive genes, yet the level of estrogen hormone itself is not affected. The mechanism for this is yet to be defined.

The positive effects of Tualang honey and *Ficus deltoidea* on estrous cycle, ovarian follicular development as well as the morphology, lipid peroxidation and transcriptional and translational of estrogen sensitive genes in the uterus of BPA-exposed rats are most likely associated with the variety of phytochemicals in these natural products. In addition, treatment with Tualang honey and *Ficus deltoidea* also managed to reduce the level of oxidative stress in BPA-exposed rats, possibly due to their antioxidant properties. This is in agreement with many previous studies that reported the beneficial effects of Tualang honey as natural antioxidants for the protection of human health and nutrition (Erejuwa, Gurtu, et al., 2010; Erejuwa et al., 2012; Erejuwa, Sulaiman, Wahab, Salam, et al., 2010; Omotayo et al., 2010; Yaacob & Ismail, 2014; Yaacob et al., 2013; Zaid, Othman, & Kassim, 2014; Zaid et al., 2012; Zaid et al., 2010; Zaid, Othman, & M Kassim, 2014).

In one clinical study that spanned 16 weeks on daily oral supplementation of 20g of Tualang honey (equal to one table spoon) in postmenopausal women, there was reduction in the levels of blood oxidative stress markers, which was comparable to those who received standard estrogen progestin therapy (EPT) (Shafin, Othman, Zakaria, & Hussain, 2014). In an animal study, Tualang honey was shown to significantly reduce lipid peroxidation, increase total antioxidant status, as well as normalizes the activities of antioxidant enzymes (GPx, SOD and CAT) (Mohamed et al., 2011).

A study on herbal plant by Omar *et al* (2011) has found that 85% of the total antioxidant activity of the aqueous extract of *Ficus deltoidea* is attributed to its 25 flavonoids (9 flavan-3-ols and 16 flavones) with the main constituents being flavan-3-ol monomers, proanthocyanidins and C-linked flavone glycosides. Proanthocyanidins are

also widely contained in other plants including pine bark, berries, grapes and cocoa products. Therefore, many previous studies have been conducted to reveal the beneficial effects of these bioactive compounds with radical-scavenging, anti-inflammatory and anti-carcinogenic properties (Goncalves, Dinis, & Batista, 2005; Mantena & Katiyar, 2006; Nandakumar, Singh, & Katiyar, 2008). In addition, in 2011, a study by the Malaysian Agricultural Research and Development Institute (MARDI) has also found that the scavenging activity of free radicals by *Ficus deltoidea* is related to its high total phenolic and flavonoid contents (Norra, 2011).

Antioxidant properties of certain bioactive compounds in Tualang honey and *Ficus deltoidea*, namely the quercetin and kaempferol, also contributed to the protective effects against BPA. Using a fluorescent probe, 2',7'-dichlorofluorescien diacetate (DCF-DA) that detected the generation of ROS, the study by Wattel *et al* (2003) demonstrated that quercetin and kaempferol were capable of scavenging and reducing ROS in the cells. Noteworthy, the antioxidant properties of quercetin are more potent than the kaempferol, and this may be due to the presence of additional free hydroxyl at C3' that dictates its antioxidant potency in neutralizing free radicals.

We have also demonstrated the cytoprotective and antigenotoxicity effects of Tualang honey and *Ficus deltoidea* against the toxicity caused by BPA at cellular and DNA levels. Cellular and DNA damage by BPA have been extensively conducted in many *in vitro* and *in vivo* studies (Iso, Watanabe, Iwamoto, Shimamoto, & Furuichi, 2006; Tiwari et al., 2012; Wu et al., 2012). The cytoprotective effects of Tualang honey and *Ficus deltoidea* were especially evident from the improvements in the histological morphology observed in the ovary and uterus of our experimental rats. The antioxidant activities of phytochemicals present in Tualang honey has the ability to protect cells from oxidative stress damage. The endogenous antioxidants present in the body combined with

exogenous antioxidants from Tualang honey may reduce the oxidative stress caused by BPA by scavenging ROS from the body.

An *in vitro* study by Yaacob *et al.* (2014) reported that the combination of Tualang honey and a chemotherapeutic agent called 4-hydroxytamoxifen (OHT), showed enhanced cytotoxicity and reduced proliferation of a cancerous cell line (MCF-7) by a mechanism associated with increasing DNA repair proteins. More interestingly, similar study also noted that combination of OHT-Tualang honey treatment had significantly reduced the side effects of OHT, most probably by reducing DNA damage on the non-cancerous cells (MCF-10A). Therefore, other than reducing the proliferation of cancerous cells, Tualang honey also has the capability to repair and preserve genomic stability in the normal cells. It has been reported that polyphenols in honey, namely caffeic acid phenyl ester (CAPE), pinocembrin, chrysin, galangin, quercetin, kaempferol, acacetin, apigenin and pinobanksin have both cytotoxic and genotoxic effects on a wide variety of cancer cells (Jaganathan & Mandal, 2009). Similar findings on the protective mechanisms against DNA damage were reported in studies using Buckwheat honey and honey from arid regions, which is in agreement with the current findings on Tualang honey (Habib, Al Meqbali, Kamal, Souka, & Ibrahim, 2013; J. Zhou et al., 2012).

Some herbal plants have been found to provide cytoprotection and gene protection by preventing excessive lipid peroxidation (Vidyashankar, Thiyagarajan, Sandeep, Sharath, & Babu, 2014). *Ficus deltoidea*, as a herbal plant, has also been characterized to contain a wide range of phytochemicals, including apigenin, quercetin, isovitexin, naringen, proanthocyanidins, luteolin, cathecin, terpenoids and alkaloids (Bunawan et al., 2014; Sirisha, Sreenivasulu, Sangeeta, & Madhusudhana, 2010). Thus, it is not surprising that this natural product also has the potential to provide cytoprotection, most likely by preventing excessive lipid peroxidation. This postulation is based on a study by Lin *et al*

(2002), who demonstrated that bioactive compounds such as apigenin, quercetin, isovitexin and naringenin are potent inhibitors for xanthine oxidase activity in the metabolism of DNA. In addition, naringenin was noted to stimulate DNA repair in prostate cancer cells (Gao et al., 2006). Proanthocyanidins that contain dimers, primers and other oligomers of catechin and epicatechin and gallic acid esters (Omar et al., 2011) were shown to improve lead-induced cognitive impairments in rats by inhibiting oxidative stress and inflammatory response (Liu et al., 2014).

Besides that, Tualang honey and *Ficus deltoidea* also contains non-enzymatic antioxidants such as vitamin E and C that act as lipophilic and hydrophilic free-radical scavengers, respectively (Ahmed & Othman, 2013; Hakiman & Mahmood, 2009). In the natural state, cells in our body are capable of maintaining the normal antioxidant-oxidative stress balance by neutralizing or directly minimizing the oxidative damage by means of enzymatic and non-enzymatic antioxidants (Oral et al., 2006). Hence, this could be another explanation for the protective effects of Tualang honey and *Ficus deltoidea* on the reproductive organs in BPA-exposed rats. Vitamin E is found to reside mainly on cell membrane, thus plays an important role in the maintenance of cell membrane (Baker, Brindle, Irvine, & Aitken, 1996). On the other hand, vitamin C is the major scavenger of free-radicals in the extracellular fluid, neutralizing radicals in the aqueous phase, protecting biomembranes against impairment by peroxidase and regenerating tocopherol (Vitamin E) from tocopheroxyl radicals in the membrane (Kumar, Rani, Dixit, Pratap, & Bhatnagar, 2009). Several studies have shown that both antioxidants can mitigate adverse pathological impacts by minimizing the genotoxicity and cytotoxicity effects in the cells (El-Neweshy & El-Sayed, 2011; Harabaway & Mosleh, 2014; Hounkpatin et al., 2012).

Other than antigenotoxicity effects, the protective effects of Tualang honey and *Ficus deltoidea* on C3, a gene related to the immune system, are also possibly due to the immunoprotective activities of certain bioactive compounds. In this study, Tualang honey

and *Ficus deltoidea* appeared to significantly normalize the suppression of the expression of this immunomodulatory gene. This is explained by the presence of polysaccharides in Tualang honey and *Ficus deltoidea* that may enhance various immune responses including stimulation on macrophages, activation of complement and proliferation of lymphocytes (Savant, Joshi, Reddy, Mannasahed, & Joshi, 2014). Furthermore, flavonoid, as an antioxidant, also serves as an immune stimulator that is crucial for the growth, development and immunity (Mahiunddin, 2010). Remarkably, Tualang honey was able to induce the expression of C3, not only back to normal, but also elicit a higher level of expression (almost double than normal). This suggests that there may be certain bioactive compounds in Tualang honey that is able to stimulate stronger immune response when challenged with toxicity caused by BPA. Several reports have described the presence of phytosterols in Tualang honey, namely tannins and saponins, that contribute to the immunostimulating activity that protect the cell-mediated immune response (Dey, Dutta, Pattanaik, & Sharma, 2014; Heroor, Beknal, & Mahurkar, 2011). This may be the reason behind the findings on C3 as demonstrated in this study.

Besides that, other bioactive compounds found in honey were also reported for their immunoprotective activities. For example, endotoxin, a lipopolysaccharidefound in an outer membrane of Gram-negative bacteria in honey can be a potent immunomodulator that elicit strong immune responses (Timm, Bartelt, & Hansen, 2008). Interestingly, cells of Mono Mac assay responded to honey by releasing a significant amount of interleukin-6 (IL-6), that stimulate the immune response in the body. In addition, the present study also found that honey induced ROS release from the HL-60 assay, an assay sensitive to the immunomodulating substances such as Gram-negative bacteria. In other *in vitro* studies, the immunomodulatory and immunoprotectiveactivies of honey were demonstrated via stimulation of B and T lymphocyctes, neutrophils, monocytes, eosinophils and natural killer cells (NK cells) during primary and secondary immune

responses (Abuharfeil, Al-Oran, & Abo-Shehada, 1999; Al-Waili, 2003; Al-Waili & Haq, 2004; Attia, Gabry, El-Shaikh, & Othman, 2008).

Honey was shown to provoke antitumor effect in macrophages (Attia et al., 2008). The mechanism proposed was that honey, as an immune booster whether indirectly or directly, contains fermented sugar with short chain fatty acid (SCFA) that may stimulate the immune system (Sanz et al., 2005; Schley & Field, 2002). In addition, nigerooligosaccharides (NOS) in honey has also been accounted for their immunopotential activity (Murosaki, Muroyama, Yamamoto, Liu, & Yoshikai, 2002).

In summary, Tualang honey and *Ficus deltoidea* are able to prevent the toxicity effects of BPA in the prepubertal female reproductive system in rats. The mechanisms implicated in these protective effects from the toxicity caused by BPA may likely be due to the antioxidant and estrogenic properties of Tualang honey and *Ficus deltoidea* as the main contributors. The current findings strongly support the traditional belief that Tualang honey and *Ficus deltoidea* has the potential to promote health, fertility and immunity, hence should be recommended as a dietary supplement.

CHAPTER 6: CONCLUSION

6.1 Conclusion

This study has shown that BPA interfered with the reproductive system of prepubertal rat, which was an evident from the changes in the estrous cycle, disruption in the gonadotropins hormone levels (FSH and LH), follicular development and sexual steroid hormones secretion by the ovary (17β-estradiol and progesterone). BPA toxicity also results in disruptive effects on the uterus by inducing morphological abnormalities, increasing oxidative stress and perturbing the expression of estrogen sensitive genes, including ERα, ERβ and C3, at both transcriptional and translational levels.

Subsequently, the study demonstrated significant protective effects of Tualang honey and *Ficus deltoidea* against the toxicity caused by BPA in the reproductive system. These natural products have the capabilities to prevent toxicity effects of BPA as shown by the increase in the percentage of rats with normal estrous cycle, increase in the level of gonadotropins hormone (FSH) and sexual steroid hormone (progesterone), reduction in the formation of the atretic follicles and normalization of the progesterone secretion by the ovary. Consequently, Tualang honey and *Ficus deltoidea* were also shown to improve the disruptive state of the uterus by reducing the abnormalities of the cells, reducing the disruptions at the transcriptional and translational levels of estrogen sensitive genes, ERα, ERβ and C3 as well as reducing lipid peroxidation and subsequently the level of oxidative stress within the uterus.

All these results concur with the hypotheses that were postulated in the present study. The current findings strongly support the traditional belief that honey and herbal plants are important as daily supplements in the diet for the promotion of long term health, fertility and immunity. In conclusion, Tualang honey and *Ficus deltoidea* are able to

prevent the toxicity effects of BPA on the prepubertal female reproductive system, possibly due to their variety of phytochemical properties. Hence, they may be particularly beneficial as health supplements to help in reducing the risk of permanent female reproductive infertility caused by prolonged BPA exposure, especially during prepubertal period of life.

6.2 Limitation

In the present study, time and/or cost are major contributors for the limitations. The limitations in the present study are as follows:

1) Small sample size of rats per group (8 rats per group) possibly contributes to the less or no significant value in the statistical analysis of some important parameters.
2) Short administration period (six weeks) may not be enough for recovery period from the toxicity effects of BPA.

6.3 Future studies

Subsequent the current study that has shown the protective effects of Tualang honey and *Ficus deltoidea* on BPA-exposed rats, it would be rational to study the therapeutic effects of the two natural products using similar research protocols. In addition, this study can also be expanded to the profiling of genes and proteins affected by treatment with Tualang honey and *Ficus deltoidea* on BPA-exposed rats using proteomic analysis and microarray, respectively. This will provide informative data on the overall effects of these natural products that can be used to analyse the metabolic pathways involved.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publications:

1) Title: Tualang honey protects against BPA-induced morphological abnormalities and disruption of ERα, ERβ and C3 mRNA and protein expressions in the uterus of rats.

Journal: Evidence-Based Complementary and Alternative Medicine.

Published: November 2015.

Authors: Siti Sarah Mohamad Zaid, Shatrah Othman and Normadiah M Kassim.

2) Title: Potential protective effect of Tualang honey on BPA-induced ovarian toxicity in prepubertal rat.

Journal: BMC Complementary and Alternative Medicine

Published: Nov 2014

Authors: Siti Sarah Mohamad Zaid, Normadiah M Kassim and Shatrah Othman.

Presentations:

1) Oral presentation.

Title: Potential Protective Effects of Tualang Honey Against BPA-induced Toxicity in the Reproductive System.

Conference: The 3rd Asia-Pacific Conference on Life Science and Engineering (Chiang Mai, Thailand).

Date: 18 November 2015.

2) Oral presentation.

Title: Potential Role if *Ficus deltoidea* in Reducing BPA-induced Ovarian Toxicity.

Conference: IICBBE International conference (Phuket, Thailand).

Date: 7 April 2015.

3) Oral presentation.

Title: Potential Role of Tualang Honey in Reducing BPA-induced Ovarian Toxicity.

Conference: International Conference on Biological Engineering and Natural Sciences (Singapore).

Date: 19 Januari 2015.

4) Poster presentation.

Title: Potential Protective Effects of Tualang honey on Bisphenol A Induced the Morphology of Prepubertal Rat Uterus

Conference: International Conference on Medical and Health Sciences, USM (Malaysia).

Date: 22 May 2013.

APPENDICES