Final Report

Impact of deoxymiroestrol and miroestrol, potent phytoestrogens from *Pueraria mirifica* tuberous roots on anti-inflammatory effects in RAW 264.7 mouse macrophages and mice

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Abstract

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Abstract: *Pueraria mirifica* is a leguminous plant has long been used as medicinal plant. The use of *P. mirifica* was first mentioned in a pamphlet as a source of rejuvenation and good health. There are at least 17 compounds were found in its tuberous roots such as puerarin, daidzein, tuberosin, mirificin, and kwakhurin. Among them, deoxymiroestol and miroestrol show the most potent estrogenic like-effect. Recently, there are studies shows *P. mirifica* and its active compounds possess various biological activities, including estrogenic activity, relief climacteric related symptoms, antioxidant and antitumor effect. However, the mechanism involved in inflammation and pain of deoxymiroestrol and miroestrol is still unclear. Therefore, this study aimed to determine the anti-inflammatory activities of miroestrol and deoxymiroestrol in both of *in vitro* and *in vivo*. The production of interleukin-6 and tumor necrosis factor-alpha expression at both of transcriptional and post-transcriptional levels were investigated in LPS-activated RAW 264.7 macrophages and investigated in mice by using mice paw edema model. The results showed that miroestrol and deoxymiroestrol possessed anti-inflammatory effects in both of *in vitro* and *in vivo*. The effect of miroestrol and deoxymiroestrol in LPS-induced expression leads to reduced inflammatory cytokine expression, specifically inducible interleukin-6 and tumor necrosis factor-alpha. Furthermore, miroestrol and deoxymiroestrol significantly decreased inflammation by 24.40 ± 3.13 % with 200 mg/kg miroestol and 38.2 ± 3.84 % with 200 mg/kg deoxymiroestrol, respectively ($p<0.05$). The results from

this study was useful as supportive studies and information about anti-inflammation property of deoxymiroestrol and miroestrol. This study will be provided risk-benefit assessment of using *P. mirifica* products and its related compound-deoxymiroestrol and miroestrol as alternative medication or health supplements due to their anti-inflammatory effects.

Keywords: Deoxymiroestrol, Miroestrol, *Pueraria mirifica*, anti-inflammatory effect

บทคัดย่อ

เลขที่สัญญาทุน: TRG5780229

ชื่อโครงการวิจัย : การศึกษาฤทธิ์ต้านอักเสบของสารไมโรเอสทรอลและดีออกซี่ไมโรเอสทรอล จากหัวกวาวเครือขาวในเซลล์ RAW 264.7 แมคโครฟาจและในหนูทดลอง

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บทคัดย่อ: กวาวเครือขาวเป็นพืชตระกูลถั่วที่ถูกนำมาใช้เป็นยาสมุนไพรเป็นเวลานาน ถือเป็นพืชสมุนไพรตัวแรกๆที่ใช้ได้ผลดีในทางอายุรเวช สาระสำคัญที่พบในหัวกวาวเครือขาวประกอบด้วยสารออกฤทธิ์อย่างน้อย 17 ชนิด เช่น puerarin daidzein tuberosin mirificin และ kwakhurin โดยพบว่าสาร deoxymiroestol และ miroestrol มีฤทธิ์คล้ายฮอร์โมนเอสโตรเจนมากที่สุด จากการศึกษาในปัจจุบันพบว่ามีการศึกษาฤทธิ์ทางชีวภาพของสารสำคัญที่สกัดได้จากกวาวเครือขาว ได้แก่ ฤทธิ์คล้ายฮอร์โมนเอสโตรเจน ฤทธิ์บรรเทาการวัยหมดประจำเดือน ฤทธิ์ต้านอนุมูลอิสระ และฤทธิ์ต้านมะเร็ง แต่อย่างไรก็ตามยังไม่มีการศึกษาฤทธิ์ต้านอักเสบของสาร deoxymiroestol และ miroestrol ที่ชัดเจน ดังนั้นการศึกษาวิจัยในครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาฤทธิ์ต้านอักเสบของสาร miroestrol และ deoxymiroestrol ทั้งในหลอดทดลองและสัตว์ทดลอง อีกทั้งยังศึกษาระดับและการแสดงออกของ interleukin-6 และ tumor necrosis factor-alpha ทั้งในระดับ both of transcription และ post-transcription โดยการผลิต interleukin-6 และ tumor necrosis factor-alpha ในเซลล์ที่ได้รับการกระตุ้นให้เกิดการอักเสบด้วย LPS ในเซลล์ RAW 264.7 macrophages และทดลองในหนูทดลองด้วยเทคนิค mice paw edema model ผลการศึกษาพบว่าสาร miroestrol และ deoxymiroestrol มีฤทธิ์ต้านอักเสบทั้งในหลอดทดลองและในสัตว์ทดลอง กล่าวคือสาร miroestrol และ deoxymiroestrol สามารถลดการสร้าง cytokine ที่ก่อให้เกิดการอักเสบ คือ interleukin-6 และ tumor necrosis factor-alpha ใน LPS-induced RAW 264.7 cell นอกจากฤทธิ์ในหลอดทดลองแล้วยังพบว่าสาร miroestrol และ deoxymiroestrol ที่ให้ในขนาด 200 มิลลิกรัมต่อกิโลกรัมน้ำหนักตัว สามารถลดการอักเสบได้ โดย miroestrol สามารถลดการบวมของเท้าหนูได้ ร้อยละ 24.40 ± 3.13 % ($p<0.05$) และ deoxymiroestrol สามารถลดการอักเสบได้ร้อยละ 38.2 ± 3.84 ($p<0.05$) ซึ่งจากผลการทดลอง

ดังกล่าวแสดงให้เห็นถึงฤทธิ์การต้านอักเสบของสาร deoxymiroestrol และ miroestrol ทำให้การศึกษาดังกล่าวเป็นข้อมูลสนับสนับสนุนการใช้กวาวเครือขาว และสารสำคัญคือสาร deoxymiroestrol และ miroestrol เป็นพืชสมุนไพรทางเลือกในการต้านอักเสบได้อีกทางหนึ่งด้วย

Keywords: ดีออกซี่ไมโรเอสทรอล ไมโรเอสทรอล กวาวเครือขาว ฤทธิ์ต้านอักเสบ

Chapter 1 Executive summary

1.1 Introduction to research

Inflammation is one of complex biological response of vascular tissues against harmful xenobiotics such as pathogens, irritants, or damage cells. It involves in a casade of biochemical events comprising the local vascular system, the immune system, and the different cell types found in the injured tissue (de Silveira et al., 2013). Various factors such as microbial infections, chemical and immunologic reactions can cause inflammation and caused many diseases, including rheumatoid arthritis, obesity, cardiovascular diseases, neurodegenerative diseases, diabetes, and cancer (Wang et al., 2013). Usually, Inflammation is initiated through the specific cytokines or chemokines characterized by recruitment of leukocytes to the damage site. During inflammation, excess production of pro-inflammatory molecules such as tumor necrosis factor-alpha (TNF-alpha), Interleukin-6 (IL-6), prostaglandin E_2 (PGE_2), and nitric oxide (NO) are response for modulating inflammation besides their critical role in immune-inflammatory response (Ravipati et al., 2012). Macrophages pathogenic substances through pattern-recognition receptors and subsequently initiate and regulate inflammatory response (Medzhitov and Janeway, 1997). Lipopolysaccharide (LPS) is one of the most powerful activators of macrophages. Macrophages and monocytes that have been activated by LPS are known to produce inflammatory mediators (Yang et al., 2012) such as NO and other cytokines. For in vivo tests, inflammation can be induced in animals by many substances. Mice paw edema is the most commonly used model for acute inflammation while subcutaneous implantation of biomaterial is usually used for inflammatory model (Wen et al., 2011) Therefore, inhibiting the production of TNF-alpha, IL-6, PGE2, and NO is an important therapeutic target in the development of anti-inflammatory agents.

Pharmaceutical development has led to a great number of medicines, however many of them have limited efficacy; patients can encounter escalating health-care costs; and adverse drug reactions are reported in some patients. As a result, there is an increased tendency to use traditional medicine, with an extent not less than 80% worldwide (Desalegn et al., 2019). Plants have been used since ancient times to treat

diseases and infections. Medicinal plants are cheap, easily available and affordable. Besides traditional use, scientific study of medicinal plants has found herbal medicines to be a medicinal resource for drug discovery (Bertin et al., 2016). A variety of medicinal plants have been used to treat pain and inflammation. These plants include *Malva verticillata*, *Otostegia integrifolia*, *Ocimum suave*, *Cucumis ficifolius*, *Arisaema schimperianum*, *Euclea racemosa*, *Malva verticillata*, *Impatiens tinctoria*, *Ehretia cymose* and a host of others (Masresha et al., 2012; Falls et al., 2013).

Pueraria mirifica (synonym: *Pueraria candollei* Wall. Ex Benth var. *mirifica*) Kwao Kruea Khao (in Thai), is a traditional herb for rejuvenation. The tuberous roots of P. mirifica have been used as sources of Thai traditional medicine (Cain, 1960). The phytoestrogens from tuberous roots of *P. mirifica* include chromenes (such as miroestrol and deoxymiroestrol) and isoflavonoids (such as puerarin, daidzin, daidzein, genistin, and genistein) (Chansakaow et al. 2000a, b). Due to the phytoestrogenic constituents, this plant has been postulated to exert estrogenic-like effects on reproductive organs in many species, such as ovariectomized rats (Malaivijitnond et al. 2004), cynomolgus monkeys (Trisomboon et al. 2005), and mice (Udomsuk et al. 2010). Safety of using this plant and its active compounds is also important, since phytoestrogens are natural compounds affecting sexual behaviors as well as morphology and function of male and female reproductive organs (Opalka et al., 2006). Chansakaow et al. (2000) identified deoxymiroestro and miroestrol as the most active phytoestrogens that found in *P. mirifica* tuber, a traditional Thai medicine. Interestingly, deoxymiroestrol and miroestrol exhibited stronger growth-promoting activity than estradiol in human breast cancer cells, MCF-7. We previously reported that root cultures of *P. mirifica* increased CYP2B9 mRNA expression and the activity of a marker enzyme, benzyloxyresorufin-O-dealkylase (Udomsuk et al., 2010). Moreover, *P. mirifica* and miroestrol showed antioxidant activity in ovariectomized mice (Chatuphonprasert et al., 2013). From these various effects of deoxymiroestrol and miroestrol, the anti-inflammation, which is one of the most important claims for herbal medicine against many of maladies, of these two compounds are not clear. Therefore, there are interesting points to further investigate its biopharmacological effects regarding anti-inflammatory activity.

1.2 Literature review

1.2.1 Botanical characteristic of *P. mirifica*

P. mirifica belongs to the family Leguminosae, subfamily Papilionoideae, tribe Phaseoleae. *P. mirifica* was used as a source of Kwao Kruea Khaw by Thai herbalists. Other names of Kwao Kruea Khaw are Kwao Kruea, Kruea Khao Pu, and Talan Kruea (Smitinand, 2001). *P. mirifica* can be found in Thailand and Burma. Botanical characters of both varieties were almost the same, but pods were different. The plant is a long-living twinning wood. The leaves are pinnately trifoliate terminal leaflet. The tuberous roots are varied in sizes and shapes depending on the environment in which they exist. The flowers are bluish-purple legume shaped. The length of the inflorescences of certain flowers is approximately 20-30 cm. The flower contains five sepals and the petals are one standard with two keels. The pods are slender typically short or elongate, with hairs or no hair, including 1-10 single seeds when fully mature and dry which turn into brown color (Yodpetch, 2007) as showed in the figure below (Figure 1).

Figure 1 Botanical characteristics of *Pueraria mirifica*

Moreover, *P. mirifica* were classified by ISSR-Touchdown PCR technique (Bunmanop et al., 2011). The elucidation of the genetic of *P. mirifica* by ISSR PCR is more effectively than botanical characterization. They also found that even *P. mirifica* taken from the same area, botanical characteristics were different. Because of being a leguminous plant like bean, *P. mirifica*, is capable to having cross pollination, which was likely to be a cause of high genetic variation among these plants.

1.2.2 Chemical constituents of *P. mirifica*

Major compounds of *P. mirifica* are isoflavonoids. The isoflavonoids, classified to 5 main groups (Table 1): chromenes, isoflavones, isoflavones glycosides, coumestans and pterocarpans (Chansakaow et al., 2000a) as show in Table 1. The other compounds that have been found in this plant are beta-sitosterol, stigmasterol, alkane alcohol, fat, and sugar (De-eknamkul and Tongruang, 2001).

Table 1 Chemical constituents of *Pueraria mirifica*

Group	Compounds	References
Chromenes	Miroestrol, Deoxymiroestrol, Isomiroestrol	Cain, 1960; Chansakaow et al., 2000a
Isoflavones	Daidzein, Genistein, Kwakhurin, Kwakhurin hydrate	Ingram et al., 1986; Tahara et al.; 1987 Ingram et al., 1989
Isoflavone glycosides	Daidzin, Genistin, Mirificin, Puerarin, Puerarin-6´-monoacetate	Ingram et al., 1986; Ingram et al., 1989
Coumestans	Coumestrol	Ingram et al., 1989
Coumestans	Mirificoumestan, Mirificoumestan glycol, Mirificoumestan hydrate	Tahara et al., 1987
Pterocarpans	Tuberosin, Puemiricarpene	Chansakaow et al., 2000a

1.2.3 Pharmacological activities of *P. mirifica* and its active compounds

Because of *P. mirifica* is a phytoestrogen, the estrogenic activity of this plant has been investigated. Their estrogenic activity and the other biological activities have been demonstrated for crude extract and certain purified components in mice, female mice, rats, monkeys, and in clinical trial in human (Cherdshewasart and Sriwatcharakul, 2008).

Sukavattana (1940) firstly reported that the alcoholic extract of *P. mirifica* stimulated the proliferation of vaginal and uterus epithelium in female rats and women.

Treatment with genistein at 0.7 mg/day prevented trabecular bone loss in ovariectomized (OVX) mice without hypertrophic effects on the uterus, while administration of 5 mg/day of genistein induced uterine hypertrophy. There is a marked difference between genistein dosages that protect against bone loss and those that induce uterine hypertrophy (Ishimi et al., 1999).

Muangman and Cherdshewasart (2001) showed that intake of crude *P. mirifica* powder relieved climacteric symptoms, such as hot flushes, frustration, sleep disorders and skin dryness in post-menopausal woman.

Evaluation of powder and extract from *P. mirifica* crude extract had shown that it had no toxic effects on the eyes and skin of rabbits and mice (Cherdshewasart et al, 2003; Udomsuk et al., 2010).

P. mirifica feeding prolonged the menstrual cycle length in adult cyclic cynomolgus monkeys (Trisomboon et al., 2004).

P. mirifica influenced the reproductive functions in both sexes of rats, but the response in females is greater than in males (Malaivijitnond et al., 2004).

Crude extract of P. mirifica stimulated reddening of sex skin in aged menopausal monkeys (Trisomboon et al., 2006).

P. mirifica exhibited prevention of 7, 12-DMBA-induced rat mammary tumors, with a mechanism of strong competitive binding of its phytoestrogen to ER-alpha and synthesis suppressor of ER-alpha (Cherdshewasart et al., 2007).

Rachon et al. (2007) speculated that due to the lack of luteinizing hormone suppressing effects in OVX rats, it is unlikely for daidzein and puerarin to alleviate vasomotor symptoms in postmenopausal women. In contrast, due to their uterotropic effects, high dose consumption of commercially available preparations containing daidzein or puerarin may expose women with an intact uterus to the risk of endometrial hyperplasia.

Urasopon et al. (2007) found that *P. mirifica* treatment may be useful to prevent an osteoporosis in elderly hypogonadism subjects without influences on reproductive organs.

The antioxidant activity was evaluated and found that the activity is correlated with puerarin (Cherdshewasart and Sriwatcharakul, 2008).

Udomsuk et al. (2010) reported that root cultures of *P. mirifica* significantly enlarged the uterus length in female mice comparable to those did by estradiol benzoate, while the weight of uterus was not markedly changed by either the root culture of *P. mirifica* or estradiol benzoate.

Root cultures of *P. mirifica* had anti-lipid peroxidation activity in mouse brain by thiobarbituric acid assay (Udomsuk et al., 2010).

Virojchaiwong and his colleague studied effects of *P. mirifica* in postmenopausal women, the results showed that both dosages (25 and 50 mg for 1 month) of *P. mirifica* were similar effective and safe in the treatment of menopausal symptoms in woman who had undergone a hysterectomy (Virojchaiwong et al., 2011).

The extract of *P. mirifica* at concentrations of 10 and 50 ug/ml exhibited considerable antioxidant activity with significant neuroprotection, based on the microscopic observations of cell morphology and the determination of cell viability and cell number (Sucontphunt et al., 2011).

Deoxymiroestrol modified the expression of several metabolism-related genes suggest the need for caution when using health supplements having phytoestrogenic activity (Udomsuk et al., 2011).

Miroestrol and deoxymiroestrol significantly up-regulated CYP2B9 while suppressing CYP1A2 at both transcriptional and enzymatic levels (Udomsuk et al., 2012).

Crude extract of *P. mirifica* and miroestrol significantly decreased total GSH content and they are promising alternative medicine with their ability to improve GSH level and activities of antioxidative enzymes, especially in OVX mice (Chatuphonprasert et al., 2013; Jearapong et al., 2013)

P. mirifica inhibits 17 beta-estradiol induced cell proliferation of human endometrial mesenchyma stem cells (Lin et al., 2017)

1.2.4 Inflammation

Inflammation is a part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells or irritants. It is characterized by redness, swollen joint that is warm to touch, joint pain, its stiffness and loss of joint

function. Inflammation is either acute or chronic. Under specific circumstance, it could turn into a chronic state and subsequently become a causative factor in the pathogenesis. Inflammation is a self-defense reaction in its first phase, hence regarded as the main therapeutic target and often, the best choice to treat the disease and alleviate the symptoms.

1.2.4.1 Acute inflammation

Acute inflammation may be an initial response of the body to harmful stimuli. An increased movement of plasma and leukocytes, especially granulocytes from the blood into the injured tissues is observed. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system and various cells within the injured tissue. Mast cells in the tissues, the key players of inflammation, are loaded with mediators of inflammatory response. When their toll-like receptors interact with pathogen associated molecular patterns these cells discharge the chemical mediators recruiting white blood cells to the site of inflammation. These include neutrophils, monocytes (that become macrophages when they leave the blood and enter the tissue), antigen-presenting dendritic cells, lymphocytes (B cells and T cells leading to an adaptive immune response) and natural killer cells.

The Inflammatory response stimulates release of TNF-alpha from stimulated mast cells. Other cells involved in inflammation have receptors for TNF-alpha. They are activated by the binding of TNF-alpha. Activation of these recruited cells produces their own mediators of inflammation. This positive feedback quickly amplifies the response. Phagocytes (macrophages and neutrophils) produce reactive oxygen species (ROS). Macrophages and activated platelets release interleukin (IL)-1, a cytokine. IL-1 causes fever by stimulating the release of prostaglandins (PGs), which act on the temperature control center of the hypothalamus. IL-1 is synthesized from a larger precursor that is cleaved by a caspase-1. Caspase-1 is part of two (or more) multiprotein complexes in the cytosol of macrophages and neutrophils that are called inflammasomes. Inflammasomes are activated by several different products produced by invading bacteria that interact with toll-like receptors (TLRs) thus providing a link

between the innate immune system and inflammation. Chemical mediators such as histamine and bradykinin induce the production of PGs and leukotrienes with a role to potentiate the plasma exudation. These potent mediators of inflammation are derivatives of arachidonic acid (AA), a 20-carbon unsaturated fatty acid produced from membrane phospholipids.

1.2.4.2 Chronic Inflammation

In chronic inflammation, the inflammatory response is out of proportion resulting in damage to the body. The different types of allergies and many autoimmune diseases including, asthma, rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus are a few examples.

1.2.5 Anti-inflammatory activities of medicinal plants

Inflammation is a part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells or irritants. It is characterized by redness, swollen joint that is warm to touch, joint pain, its stiffness and loss of joint function. Inflammation is either acute or chronic. Under specific circumstance, it could turn into a chronic state and subsequently become a causative factor in the pathogenesis. Inflammation is a self-defense reaction in its first phase, hence regarded as the main therapeutic (Shailasree et al., 2012). Inflammation results in the liberation of endogenous mediators like histamine, serotonin, bradykinin, prostaglandins etc. Prostaglandins are ubiquitous substances that indicate and modulate cell and tissue responses involved in inflammation.

These mediators even in small quantities can elicit pain response. Pain results in dropped muscular activities. In order to comprehend the inflammatory process, antagonists of mediators are generally employed in both Ayurveda and Allopathy treatment. Most of the anti-inflammatory drugs now available are potential inhibitors of cyclooxygenase (COX) pathway of arachidonic acid metabolism which produces prostaglandins. Prostaglandins are hyperalgesic, potent vasodialators and also contribute to erythema, edema and pain. Hence for treating inflammatory diseases analgesic and anti-inflammatory agents are required. These points to the utilization of

plants possessing anti-inflammatory and analgesic properties.target and often, the best choice to treat the disease and alleviate the symptoms (Anilkumar, 2010).

Phytomedicine could be in the form of crude preparations (extracts, tinctures, essential oils) containing a wide variety of compounds or could be pure molecules with a strong and specific activity. Identification of chemical compounds and the molecular targets of these compounds help validate the use of these medicines. Plant extracts or their constituents are responsible for the protective effect with powerful antioxidant capacity and protective properties. Chemical compounds from plants have been screened for their capacity to modulate the expression of pro-infammatory signals thereby assessing their capacity as anti-inflammatory agents. Polyphenols, flavonoids, terpenes, quinines, catechins, alkaloids, and antioxidants are phytochemical compounds targeted for anti-inflammatory activity. Potent anti-inflammatory plant compounds include guggulsterone [4,17(20)-pregnadiene-3,16-dione], a plant sterol from Commiphora mukul, boswellic acid, a pentacyclic triterpenic acid and its derivatives including, acetyl-b-boswellic acid, 11-keto-b-boswellic acid and acetyl-11-keto-b-boswellic acid, curcumin from turmeric, resveratrol from red grape seeds, genistein from Soy, quercetin (onions), silymarin (artichoke), withanolides (Ashwagandha), tea polyphenols, cranberries and peanuts. The mechanism of anti-inflammatory activity for these bioactives was identifed by inhibition of NF-kB activation and down-regulating the expression of inflammatory marker enzymes (Shailasree et al., 2012).

1.2.6 Plant-derived bioactives

Abrus precatorius **L:** Abruquinone A is a naturally occurring isoflavoquinone. It was originally isolated from the roots of *A. precatorius* (family Leguminosae). The roots of *A. precatorius* have been used as a folk medicine for dieresis, treatment of fever, sore throat, bronchitis and hepatitis. The anti-inflammatory effect of Abruquinone A was found to be partly via prevention of vascular permeability and inhibition of platelet aggregation. It could influence the release of chemical mediators from mast cells *in vitro* and to suppress plasma extravasation caused by these chemical mediators *in vivo.*

***Acacia catechu* L**: A. catechu (known as grar) is used as a cure for rabies in traditional medicine in Asia. This activity was attributed to catechin, a natural flavonoid isolated from *A. catechu*. It was tested for COX-2 and 5-LOX inhibition via enzyme, cellular, and in vivo models. Catechin inhibited both ovine COX-1 and COX-2 at IC50 of 15mg/mL. In *in vivo* studies, human osteosarcoma cells expressing COX2 showed decreased production of PGE_2. It could also inhibit leukotriene production in human cell lines including, immortalized THP-1 monocyte and HT-29 colorectal adenocarcinoma. *A. catechu* flavans (epicatechin, quercetin, catechin) with reported anti-inflammatory activity had dual specificity for inhibiting COX-2 and 5-LOX experimented in air pouch model created on the back of Balb/C mice.

***Alstonia scholaris* (L.)**: Three main alkaloids, picrinine, vallesamine and scholaricine from A. scholaris leaf produced anti-inflammatory and analgesic effect. In *in vitro* tests, alkaloids inhibited inflammatory mediators including, COX-1, COX-2 and 5-LOX. Further indole alkaloids, 16-formyl5a-methoxystrictamine, picralinal, and tubotaiwine isolated from this plant exhibited COX-2/5-LOX dual inhibition. They reduced inflammatory symptoms in xylene-induced ear edema and carrageenan-induced air pouch inflammatory model in mice.

***Andrographis paniculata* Wall**: *A. paniculata* was reported to exhibit analgesic, anti-pyretic and anti-inflammatory effect. Eight pure compounds including andrographolide, were analyzed for anti-inflammatory activity in in vitro studies using RAW 264.7 (Mouse leukaemic monocyte macrophage cell line) stimulated for inflammatory response by LPS/interferon (IFN)-g. A significant decrease in the levels of NFkB mRNA by compounds decreased levels of tumor necrosis factor (TNF)-a, IL-6, MIP-2 and nitric oxide (NO) by all the compounds was recorded.

Boswellia serrata Roxb: Frankincense, the gum resin of B. serrata and B. carterii has been used for the treatment of inflammatory diseases in the traditional medicine in many countries. Boswellic acid (BA), which belong to the ursane type pentacyclic triterpene saponines was identified as the active principle. It could inhibit leukotriene biosynthesis in intact cells. Boswellic acid and its derivatives acetylb-boswellic acid, 11-keto-b-boswellic acid and acetyl-11-keto-b-boswellic acid have been extensively studied. *In vitro*, BAs selectively blocked the leukotriene, IL-12 and IL-6 generation

down regulating NFkB activation. In animal models of inflammation, BA has been shown to be an effective adjuvant mitigating BSA-induced arthritis.

***Myristica fragrans* Houtt**: Macelignan was isolated from *M. fragrans*. It exhibited potent anti-inflammatory activity *in vitro* in microglial cells. One of the important features in neurodegenerative disease was the failure to regulate oxidative stress and inflammation. Macelignan could suppress COX-2 and iNOS expression in microglial cells activated by LPS. A subsequent reduction of NO and significant suppression of pro-inflammatory cytokine TNF-alpha and IL-6 was recorded.

Other plants: Inhibition of DNA-transcription factor (TF) interactions was hypothesized to be a strategy for the development of anti-inflammatory, anti-tumor and anti-viral therapeutic agents. Several TFs including, NFkB, AP-1, STATs, cAMP response element binding protein (CREB) and GATA-1 are involved in inflammatory processes. Their intervention in human pathologies related to inflammation, such as rheumatoid arthritis, chronic asthma and inflammatory bowel diseases was analyzed by electrophoretic mobility shift assay performed using [g32P] 50-end-labeled oligonucleotides. Terminalia arjuna, Saraca asoca and Aphanamixis polystachya extracts were the most effective extracts inhibiting AP-1/ DNA interactions. Extracts of *Embelica officinalis*, *Hemidesmus indicus*, *T. arjuna*, *Aegle marmelos*, *Saraca asoca* and *A. polystachya* showed high NFkB/DNA inhibitory activity. *A. marmelos*, *S. asoca*, *A. polystachya* extracts were the most active in inhibiting interaction between GATA-1 and DNA. *T. arjuna*, *S. asoca* and *A. polystachya* inhibited STAT-3/DNA interaction. *T. arjuna*, *S. asoca* and *E. officinalis* possessed an intermediate activity regarding the CREB/DNA interaction studies.

1.3 Objectives of study

The objectives of the present study are to evaluate effect of miroestrol and deoxymiroestrol on anti-inflammatory effect in RAW 264.7 macrophages and mice.

1.4 Scope of research

Anti-inflammatory effects of miroestrol and deoxymiroestrol were investigated in mice by using LPS induced inflammation in mouse RAW 264.7 macrophages to investigate inflammatory related cytokines, namely TNF alpha and IL-6. Moreover, this

study was investigated anti-inflammatory activities in mice by using paw edema method. The results from the study were useful as supportive studies and information about anti-inflammation property of deoxymiroestrol and miroestrol.

1.5 Research methodology

1.5.1 Chemicals

RPMI 1640 medium, Fetal bovine serum, penicillin a/streptomycin, LPS were supplied by Sigma Chemical Co. (St. Louis, MO). The Takara RT-PCR kit and ReverTraAce was a product of Toyobo Co. Ltd. (Osaka, Japan). Trizol® was obtained from Sigma Chemical Co. (St. Louis, MO). Forward and reverse primers of GAPDH were synthesized by Bio Basic, Inc. (Merkham Ontario, Canada). The TaqMan® Gene Expression Assays were products of Applied Biosystems (Branchburg, NJ). TNF-alpha and IL-6 enzyme-link immunosorbent assay kits were purchase from Bio Basic, Inc. (Merkham Ontario, Canada). Dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT), caragenan, and dexametasone were purchased from Sigma Chemical Co. (St. Louis, MO). All other laboratory chemicals were of the highest purity and from commercial suppliers.

1.5.2 Plant materials

The tuberous roots of *P. mirifica* will be collected in UbonRatchatani, Thailand. Plant material will be identified compare with reference specimen (NI-PSKKU 007-010) at the Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand.

1.5.3 Sample preparation and miroestrol and deoxymiroestrol analysis

Miroestrol and deoxymiroestrol will be extracted from dried powder of tuberous roots of P. mirifica will be extract by hexane and ethylacetate as described previously (Yusakul et al., 2011). The miroestrol and deoxymiroestrol will be indentified using high performance liquid chromatography (HPLC). The mobile phase consisting of 20% acetonitrile containing 1.5% acetic acid at a flow rate of 1.0 ml/minute. HPLC was performed using a PerkinElmer Series 200 LC pump connected to a PerkinElmer 785 A UV/VIS detector (254 nm) and a PE Nelson computer. An RP-18 column (LiChroCART®, 125 mm x 4mm, 5 um particle size, Merck, Germany) will be used.

1.5.4 The RAW 264.7 macrophage cell culture

The RAW 264.7 cells, a mouse macrophage-like cell line, was cultured in RPMI 1640 supplement with 10% fetal bovine serum and 1% penicillin/streptomycin. Cell were incubated at 37°C in a humidified atmosphere (5% carbon dioxide). The test compounds, miroestrol, deoxymiroestrol, dexametasone (positive control), and DMSO were diluted with 1% RPMI to the appropriate concentrations and were added 1 hour before LPS treatment.

1.5.5 MTT assay for cell toxicity and cell viability

The MTT assay was performed to measure cell toxicity and viability. RAW 264.7 cells were mechanically scraped and seeded in 96-well plates. The RAW 264.7 cells (1 x 10^6 cells/ml) were incubated in 37°C in a humidified atmosphere (5% carbon dioxide) overnight. After 24 hours of plating, RAW264.7 cells were treated with different concentration (1,000, 500, 250, 125, 62.5, 31.25 ug/ml, respectively) of miroestrol and deoxymiroestrol for 24 hours. DMSO and RPMI 1640 medium were positive and negative control, respectively. Subsequently, 20 µL of 5 mg/ml MTT in fetal bovine serum-free medium was added to each well, and cells will be incubated for 4 hours. MTT complex was removed and resolved with 150 µl/well DMSO. The optical density was measured at 540 nm using a UV-vis microplate reader. Concentrations were determined for three wells of each sample, and each experiment was done in triplicate.

1.5.6 Determination of TNF-alpha and IL-6 level

Mouse RAW 264.7 cells (1 x 10^6 cells/ml) were seeded on 96 well plates and were treated with miroestrol and deoxymiroestrol at the concentration of 1 mg/ml, 10 mg/ml, and 100 mg/ml, respectively for 1 hour, before treatment of 1 µg/ml of LPS for 24 hours in a 37°C, 5% carbon dioxide incubator. Cell-free supernatants were collected and stored at -20°C until analyzed cytokine expression. The concentration of TNF-alpha and IL-6 in the supernatant of RAW 264.7 cell culture was determined using an ELISA assay kit. Concentrations was determined for the three wells in each sample, and each experiment was done in triplicate.

1.5.7 Semi-quantitative reverse transcription-polymerase chain reaction of TNF-alpha and IL-6

The expression of mRNAs was quantified by RT-PCR. cDNAs are synthesized by ReverTraAce under the conditions recommended (Applied Biosystems) for TNF-alpha, IL-6, and GAPDH as a house keeping gene. PCR will be performed using the ABI (Applied Biosystems). The reaction profile is as follows: 50 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. The primers of TNF-alpha, IL-6, and GAPDH are showed in the Table 2. After separation of the PCR products by 2% agarose gel electrophoresis, the target cDNA was detected under ultraviolet light in the presence of ethidium bromide and semi-quantified by Syngene gel documentation (InGenius L, Cambridge, UK) and the GeneTools program.

Table 2 The primers of TNF-alpha, IL-6, and GAPDH

Gene	Forward primer	Reverse primer
TNF-alpha	5'-CTGGGAACTCCCTTTGTTCA-3'	5'-GGATCCCCTAGGAGTTCGAG-3'
IL-6	5'-AGTTGCCTTCTTGGGACTGA-3'	5'-TCCACGATTTCCCAGAGAAC-3'
GAPDH	5'-TCCACTCACGGCAAATTCAACG-3'	5'-TAGACTCCACGACATACTCAGC-3'

1.5.8 Animal treatments

Male ICR mice 6 weeks were obtained from the National Animal Center, Mahidol University, Nakhon Pathom, Thailand. At all times, mice were housed on wood chipping bedding in plastic cages with the water and commercial mouse diet supplied ad libitum and acclimated for least 7 days in housing with a 12-hour-light and dark cycle under control temperature and humidity before dosing. All handing and the treatment protocol were approved by the Animal Ethic Committee of Khon Kaen University, Khon Kaen, Thailand (AEKKU 2559).

1.5.9 The carrageenan-induced hind paw edema

The carrageenan-induced hind paw edema model was used for determination of anti-inflammatory activity (Chang et al., 2009). Animals were intraperitonial treated with miroestrol or deoxymiroestol (0.1, 1, and 10 mg/kg), indomethecin or normal saline, 30

min prior to injection of 1% carrageenan (50 µl) in the plantar side of right hind paws of the mice. The paw volume were measured immediately after carrageenan injection and at 0, 1, 2, 3, 4, and 5 h intervals after the administration of the edematogenic agent using a plethysmometer (model 7140, Ugo Basile, Varese, Italy). The degree of swelling induced were evaluated by the ratio a/b, whereas a is the volume of the right hind paw after carrageenan treatment and b is the volume of the right hind paw before carrageenan treatment. Indomethecin was be used as a positive control. After 5 h, the animals were sacrificed.

1.5.9 General appearance observation

The general appearance and body weight of all groups of animals were recorded during the time course of the study. Clinical sign of toxicity (i.e., hair loss, weakness, or death) is also observed.

1.5.10 Statistic analysis

The results were expressed as the mean ± SEM for each group (n=5) and analyzed by a one-way analysis of variance (ANOVA) follow by the Tukey post hoc test (SPSS ver. 22.0). Differences at $p<0.05$ or $p<0.01$ was considered to be statistically significant.

Chapter 2 Results

2.1 Effect of miroestrol and deoxymiroestrol on cell viability

The viability of RAW 264.7 cells incubated with different treatment concentrations of miroestrol and deoxymiroestrol (31.25, 62.5, 125, 250, 500, 1,000 ug/ml, respectively) was evaluated through the MTT assay after 24 hours of exposure. The results showed that both of miroestrol and deoxymiroestrol in the range 31.5-125 ug/ml did not depress the cell viability (Figure 2 and Figure 3). Miroestrol at 1,000 ug/ml reduced cell viability to 53.67% (Figure 2), whereas deoxymiroestrol at the concentration of 250, 100, and 1,000 ug/ml decreased cell viability 28.11, 29.82, 46.33 %, respectively (Figure 3).

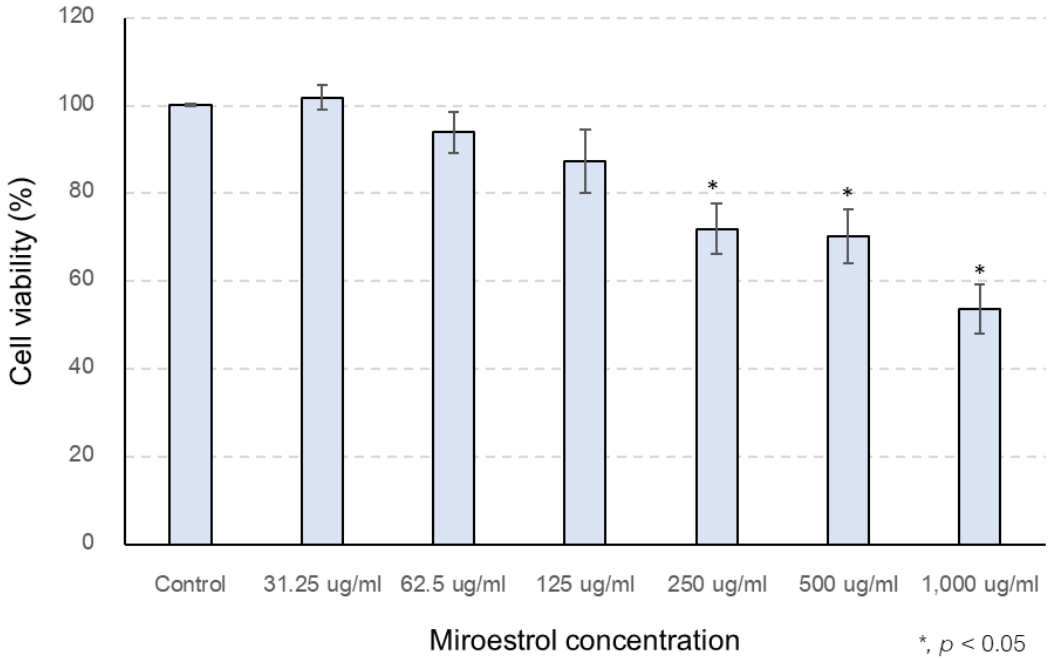

Figure 2 Cell viability of RAW 264.7 cells incubate with different concentrations of miroestrol at 31.25, 62.5, 125, 250, 500, and 1,000 ug/ml, respectively, was evaluated through the MTT assay after 24 hours of exposure. Cell viability are reported as the mean ± SD relative to RPMI 1640 medium control. *, $p < 0.05$ was considered significant using one-way ANOVA, Tukey's *post hoc* test.

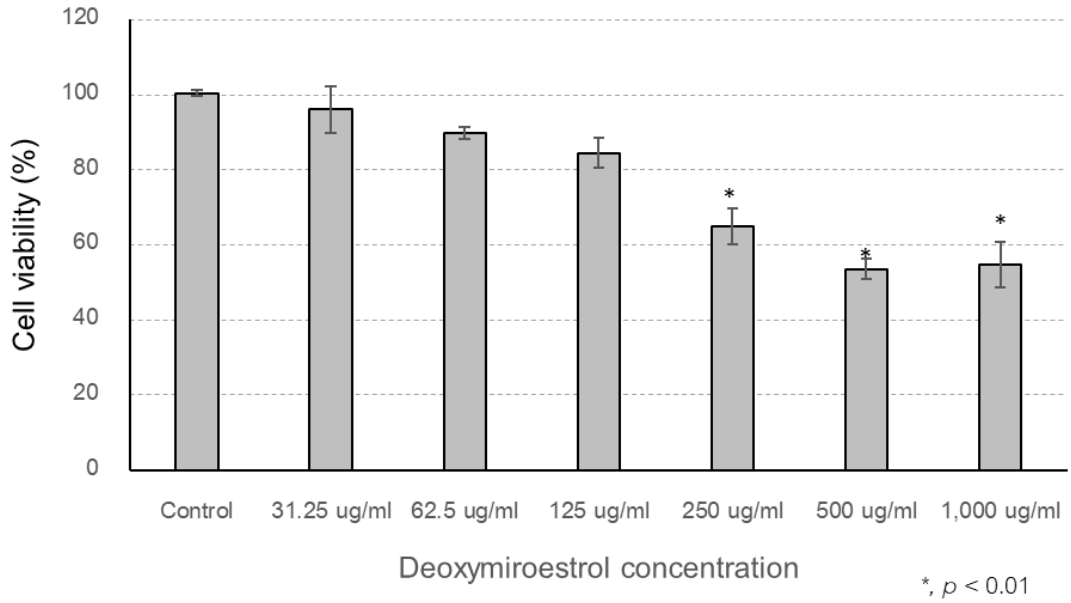

Figure 3 Cell viability of RAW 264.7 cells incubate with different concentrations of deoxymiroestrol at 31.25, 62.5, 125, 250, 500, and 1,000 ug/ml, respectively, was evaluated through the MTT assay after 24 hours of exposure. Cell viability are reported as the mean ± SD relative to RPMI 1640 medium control. *, $p < 0.05$ was considered significant using one-way ANOVA, Tukey's *post hoc* test.

2.2 Effect of miroestrol and deoxymiroestrol on TNF-alpha and IL-6 production in LPS-stimulated RAW 264.7 macrophages

In response to LPS stimulation, macrophages could release pro-inflammatory cytokines, including TNF-alpha and IL-6. The RAW264.7 cells were treated with LPS in the presence or absence of miroestrol and deoxymiroestrol, and the levels of TNF-alpha and IL-6 were measured after 24 hours of treated by ELISA assay kits. 10 uM dexamethasone, standard non-steroidal anti-inflammatory drug, was used as positive control.

Dexamethasone decreased the relative TNF-alpha production to 56.89 ± 5.21 % of control. The results revealed that miroestrol and deoxymiroestrol decrease relative TNF-alpha production with dose response correlation. Miroestrol slightly suppressed TNF-alpha in LPS-stimulated RAW 264.7 macrophages as shown in Figure 4. Deoxymiroestrol was also investigated in LPS-stimulated RAW 264.7 cells, the results

showed that deoxymiroestrol significantly decreased the TNF-alpha level to nearly 73 % and 65 % of control at the dose of 10 ug and 100 ug, respectively (Figure 4).

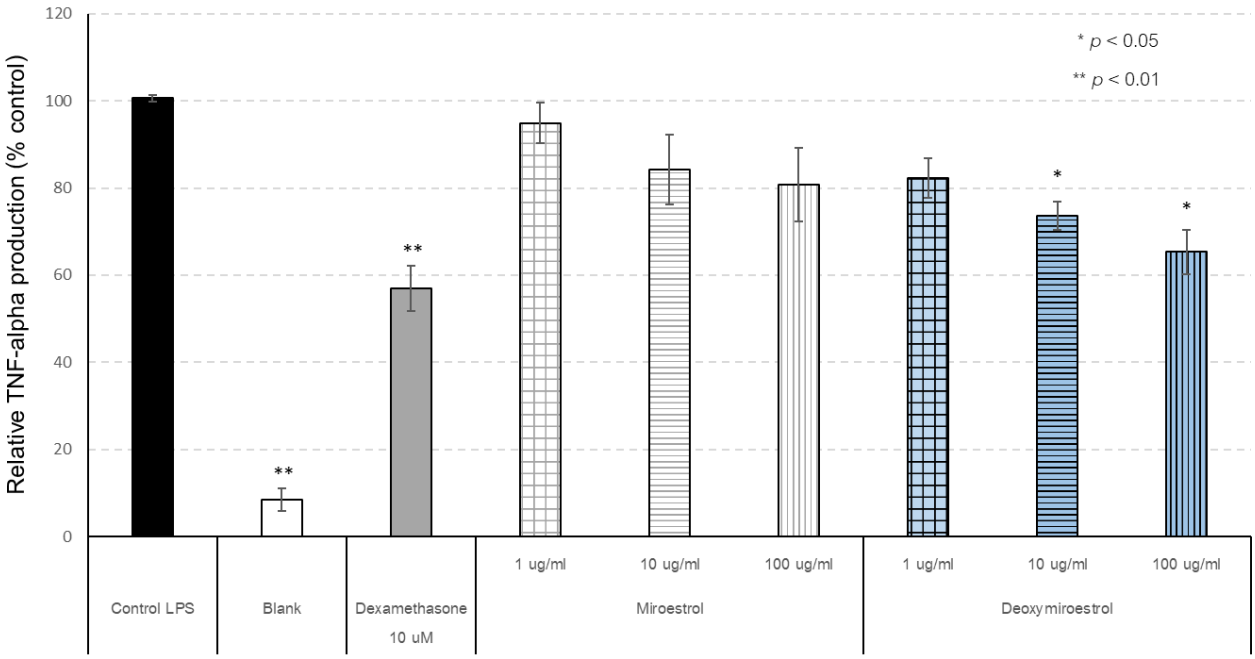

Figure 4 Relative TNF-alpha production (% control) in LPS-stimulated RAW264.7 cells after incubated with different concentrations of miroestrol, deoxymiroestrol at 1, 10, and 100 ug/ml, respectively, was evaluated through the after 24 hours of exposure. Cell viability are reported as the mean ± SD relative to RPMI 1640 medium control. *, $p < 0.05$, and **, $p < 0.01$ was considered significant using one-way ANOVA, Tukey's post hoc test.

Furthermore, the IL-6 production was also evaluated, the results showed that 100 ug of miroestrol and deoxymiroestol significantly decreased IL-6 production. The relative IL-6 production is decreased to 71.93 ± 1.05 % by 10 ug of miroestrol and 63.86 ± 12.36 % by 10 ug of deoxymiroestrol as shown in Figure 5. 10 uM of dexamethasone suppressed the level of IL-6 to 36.29 ± 4.91 % of control. The results from this study reveal that miroestrol and deoxymiroestrol has anti-inflammatory effects with low potency than dexamethasone at standard dose.

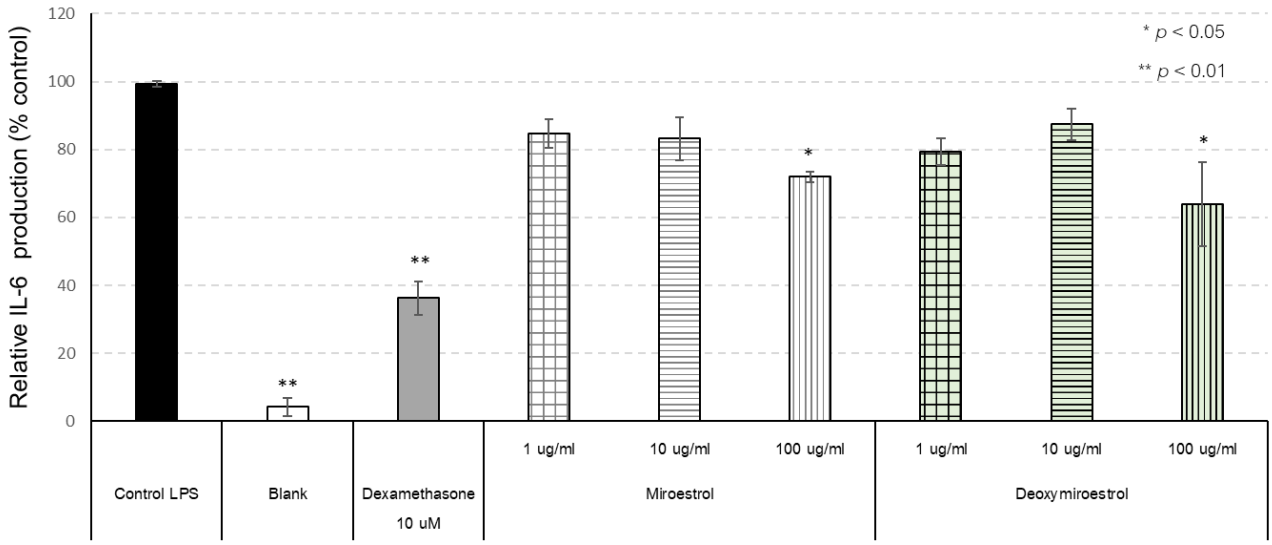

Figure 5 Relative IL-6 production (% control) in LPS-stimulated RAW264.7 cells after incubated with different concentrations of miroestrol, deoxymiroestrol at 1, 10, and 100 ug/ml, respectively, was evaluated through the after 24 hours of exposure. Cell viability are reported as the mean ± SD relative to RPMI 1640 medium control. *, $p < 0.05$, and **, $p < 0.01$ was considered significant using one-way ANOVA, Tukey's post hoc test.

2.3 Effect of miroestrol and deoxymiroestrol on TNF-alpha and IL-6 mRNA expression

The results revealed that miroestrol and deoxymiroestrol decrease relative TNF-alpha mRNA expression with dose response correlation. Miroestrol significantly suppressed TNF-alpha mRNA expression in LPS-stimulated RAW 264.7 macrophages at 10 and 100 ug/ml as shown in Figure 6. Deoxymiroestrol was also investigated in LPS-stimulated RAW 264.7 cells, the results showed that deoxymiroestrol significantly decreased the TNF-alpha mRNA expression to 0.51 ± 0.05 and 0.44 ± 0.10 of control at the dose of 10 ug and 100 ug, respectively (Figure 6). In addition, the IL-6 mRNA expression was also evaluated, the results showed that 10 and 100 ug of miroestrol and deoxymiroestol significantly decreased IL-6 mRNA expression as shown in Figure 7.

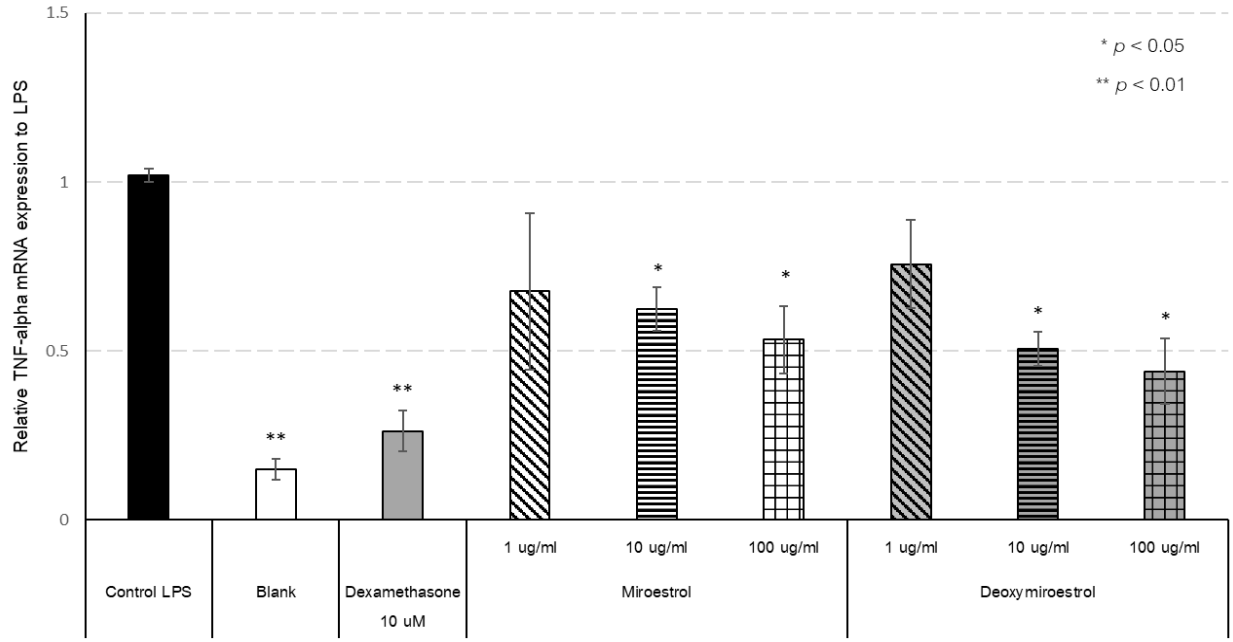

Figure 6 Relative TNF-alpha mRNA expression in LPS-stimulated RAW264.7 cells after incubated with different concentrations of miroestrol, deoxymiroestrol at 1, 10, and 100 ug/ml, respectively, was evaluated through the after 24 hours of exposure. Cell viability are reported as the mean ± SD relative to RPMI 1640 medium control. *, $p < 0.05$, and **, $p < 0.01$ was considered significant using one-way ANOVA, Tukey's post hoc test.

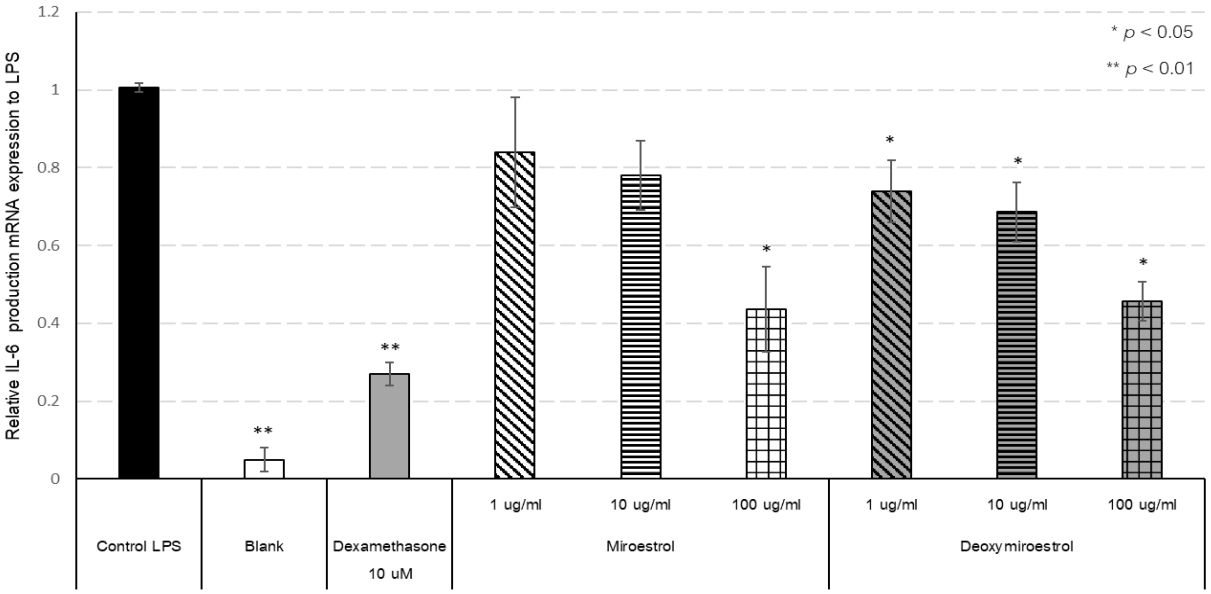

Figure 7 Relative IL-6 mRNA expression in LPS-stimulated RAW264.7 cells after incubated with different concentrations of miroestrol, deoxymiroestrol at 1, 10, and 100 ug/ml,

respectively, was evaluated through the after 24 hours of exposure. Cell viability are reported as the mean ± SD relative to RPMI 1640 medium control. *, $p < 0.05$, and **, $p < 0.01$ was considered significant using one-way ANOVA, Tukey's post hoc test.

2.4 The carrageenan-induced hind paw edema

Male ICR mice fasted overnight were randomly assigned into 8 groups each with 5 mice. Thirty minutes before injection of carrageenan, 0.9% NaCl (normal saline solution: NSS) (10 ml/kg), the test substances and indomethacin (5 mg/kg) were administered orally. The rest of the 6 groups were treated with miroestrol and deoxymiroestrol at doses of 10, 100 and 200 mg/kg, respectively, the leg of each mouse was marked on the skin over the lateralmaleous. The basal volume of the right hind paw of individual mice was measured with a digital plethysmometer. Then, a 0.05 mL of 1% carrageenan in normal saline was injected into the dorsal surface of the right hind paw. The volume of injected paw was measured at 1, 2, 3 and 4 h after carrageenan injection. As indicated in Figure 8 and Table 3, all the tested doses (10, 100, and 200 mg/kg) of the miroestrol significantly decreased paw volume compared to negative control, NSS after 3 and 4 hours of treatment whereas deoxymiroestrol at 100 and 200 mg/kg significantly inhibited edema at 2, 3, 4, and 5 hour of treatment.

Table 3 Determination effect of miroestrol and deoxymiroestrol on percentage of paw volume of mice at different time

Treatment	Concentration	Relative change of edema from carrageenan (%) (mean ± SD, n=5)				
		1 hour	2 hours	3 hours	4 hours	5 hours
NSS	10 ml/kg	55.80 ± 10.06	69.60 ± 5.19	84.80 ± 8.21	94.40 ± 9.51	100.00 ± 9.24
Indomethacin	5 mg/kg	29.80 ± 5.58	40.40 ± 2.55*	49.40 ± 2.24**	55.80 ± 2.61**	75.80 ± 8.71*
Miroestrol	10 mg/kg	44.60 ± 8.25	54.00 ± 4.32	65.80 ± 4.97*	79.60 ± 2.88*	87.40 ± 3.95
	100 mg/kg	44.60 ± 2.94	49.40 ± 1.39*	60.00 ± 2.28*	72.60 ± 4.45*	77.60 ± 5.29*
	200 mg/kg	38.00 ± 7.23	49.80 ± 3.10*	62.40 ± 3.74*	67.40 ± 4.37*	75.60 ± 3.13*
Deoxymiroestrol	10 mg/kg	47.60 ± 7.26	60.40 ± 5.63	73.80 ± 3.83	81.60 ± 5.37	87.60 ± 5.44
	100 mg/kg	39.00 ± 3.09	49.60 ± 3.64*	60.40 ± 6.93*	66.80 ± 4.35*	75.20 ± 3.27*
	200 mg/kg	35.20 ± 3.10	45.40 ± 2.67*	51.20 ± 3.21*	55.00 ± 2.88*	61.80 ± 3.84*

Note: Each value represents the mean ± SD of the mean. *, $p < 0.05$, and **, $p < 0.01$ was considered significant using one-way ANOVA, Tukey's post hoc test.

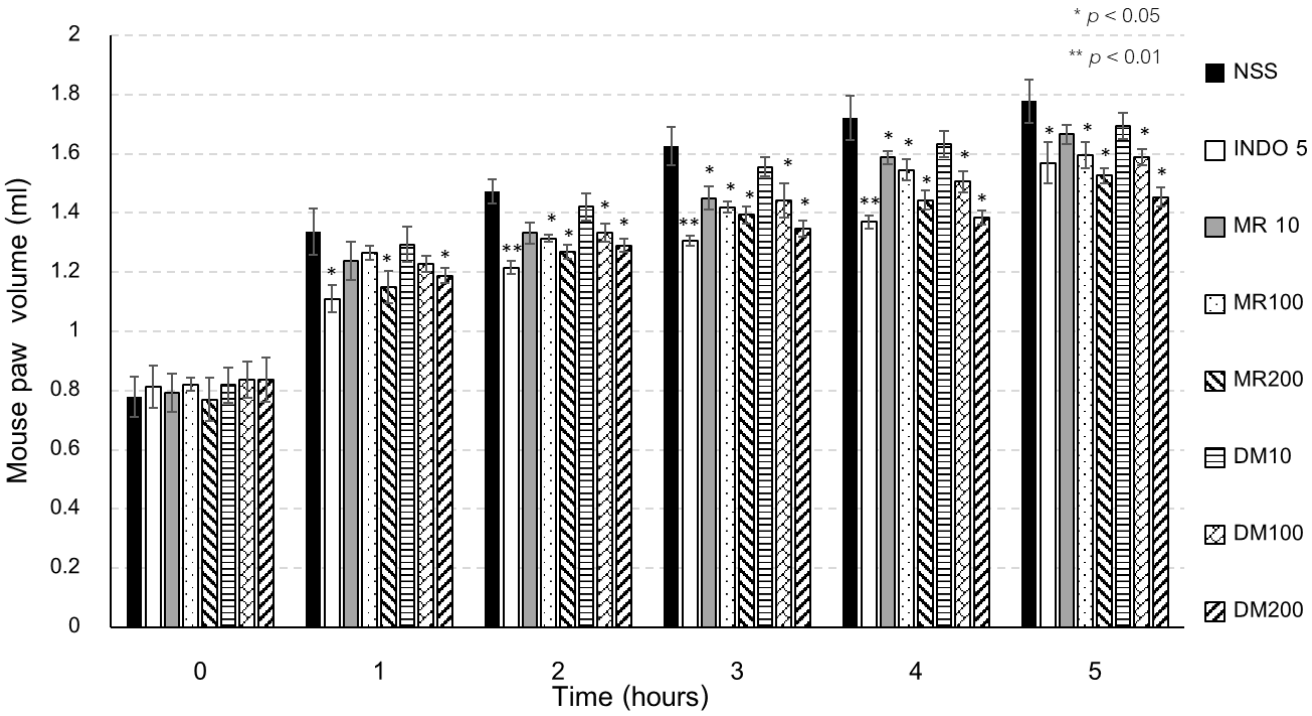

Figure 8 Effects of miroestrol and deoxymiroestrol on hind paw edema induced by carrageenan in mouse. Each value represents the mean ± SD of the mean. *, $p < 0.05$, and **, $p < 0.01$ was considered significant using one-way ANOVA, Tukey's post hoc test. NSS; normal saline solution 10 ml/kg, INDO 5; indomethacin 5 mg/kg; MR; miroestrol 10 , 100, 200 mg/kg, respectively, DM; deoxymiroestrol 10 , 100, 200 mg/kg, respectively.

Chapter 3
Discussion and Conclusion

Miroestrol and deoxymiroestrol are potent active compounds found in *P. mirifica*, which is an important phytoestrogen. *P. mirifica* was used as a source of Kwao Kruea Khaw by Thai herbalists. *P. mirifica* can be found in Thailand and Burma. Because of *P. mirifica* is a phytoestrogen, the estrogenic activity of this plant has been investigated. Their estrogenic activity and the other biological activities have been demonstrated for crude extract and certain purified components in mice, female mice, rats, monkeys, and in clinical trial in human (Cherdshewasart and Sriwatcharakul, 2008), however the anti-inflammatory activity of miroestrol and deoxymiroestrol are unclear. The objectives of the present study are to evaluate effect of miroestrol and deoxymiroestrol on anti-inflammatory effect in RAW 264.7 macrophages and mice. The present study examined the anti-inflammatory effects of miroestrol and deoxymiroestrol using the accepted LPS-induced mouse RAW 264.7 cells. LPS stimulated macrophages trigger the secretion of cytokines and mediators, and upon treatment with LPS, the RAW 264.7 cells produce induced TNF-alpha and IL-6 as well as other inflammatory cytokines (Le et al. 2017). TNF-alpha and IL-6, which are important for immunity in LPS-induced inflammation (Lee et al. 2012). In this study, we found that miroestrol and deoxymiroestrol significantly inhibited TNF-alpha and IL-6 at both of transcription and translation level by using RT-PCR and ELISA assay kits. This study supported the former information about blockage of RAW 264.7 cells, ERK and JNK pathways causes down regulation of COX-2 expression, TNF-alpha and IL-6 production during inflammation (Ruberlei et al. 2017). The mouse RAW 264.7 macrophage cells were initially seeded in microplates followed by different concentrations of miroestrol and deoxymiroestrol. The results showed that treating RAW 264.7 cells with miroestrol and deoxymiroestrol with various concentration did not affect the viability of the RAW 264.7 cells at the concentration 0-125 ug/ml by MTT assay. In response to LPS stimulation, macrophages could release proinflammatory cytokines including TNF-alpha and IL-6. The mouse RAW 264.7 cells were treated with LPS in the presence or absence of miroestrol and deoxymiroestrol, and the levels of TNF-alpha and IL-6 were measured by ELISA assay kits and mRNA expression. The

results showed miroestrol and deoxymiroestrol suppressed the productions in LPS-induced mouse RAW 264.7 cells in a concentration-dependent manner. This report is supporting properties of chemical compounds in plant secondary metabolites has potential to possess anti-inflammatory activity. Plant secondary metabolites known for their antioxidative and anti-inflammatory properties (Dussossoy et al. 2011). In previous studies, quercetin has been reported to exhibit anti-inflammatory effects through regulation of nitric oxide and TNF-a production by NF-jB pathway in LPS-stimulated macrophages (Nakamura and Omura 2008). The effect of ellagic acid on inflammation also has been studied using in vitro and in vivo models (Angeles Rosillo et al. 2012; Anitha et al. 2013). Ellagic acid exerted a renal protective effect in high fat diet/low-dose streptozotocin (HFD/STZ)-induced type 2 diabetic rats by multifactorial approach (Ahad et al. 2014). The anti-inflammatory properties and underlying molecular mechanisms of luteolin in LPS RAW264.7 macrophages have been investigated. Luteolin reduced the expression of pro-inflammatory cytokines and it has potential applications as a functional food component in regulating inflammatory responses (Chen et al.2007).

Furthermore, the presence of edema is one of the prime signs of inflammation. Carrageenan-induced paw edema is a welldefined model of acute inflammation and a variety of inflammatory mediators participate in its development. The evolution of carrageenan-induced acute inflammation indicates the presence of two phases, namely initial and late phases. Following injection of carrageenan, inflammatory mediators such as bradykinin, serotonin and histamine contribute the initial phase occurring from 0 to 2.5 hours as indicated in Table 3, the maximum peak of edema was observed at 180 minute, which is thought to be due to the release of kinin-like substances, particularly of bradykinin. The second phase of edema is a result of overproduction of prostaglandins in tissues and may occur from 2.5 to 6 h post-carrageenan injection. The treatments achieved maximum anti-inflammatory activity at the fifth hour. This is supported by reports that the second phase is proven to be sensitive to the commonly used anti-inflammatory drugs. COX mediated production of different prostaglandins accounted for the emergence of the late phase of induced inflammation but not to lipoxygenase inhibitors. Based on the result of the present study, miroestrol and deoxymiroestrol significantly decreased paw edema in both phases of carrageenan-induced acute

inflammation. This suggests that miroestrol and deoxymiroestrol may suppress both phases of acute inflammation by interfering with the release and/or activity of the chemical mediators, such as histamine, bradykinin, and serotonin in the first phase. In the late phase, a reduction in edema may be attributed to COX inhibitory action of miroestrol and deoxymiroestrol. Previous phytochemical screening on methanol extract has revealed the presence of phenols, tannins, saponins, terpenoids, and flavonoids. The butanolic extract of *Cucumis sativus* demonstrated the presence of flavonoids, saponins, and steroids. In addition, carbohydrates, tannins, alkaloids, saponins, flavonoids, glycosides steroids were found in aqueous extract of *Cucumis melo*. The anti-nociceptive and anti-inflammatory effect ofmany plants has been attributed to their flavonoid, terpenoid, steroid, tannin, phenol, alkaloid and saponin constituents. Flavonoids exhibited anti-inflammatory through their free radical scavenging activity, for example, reactive oxygen species (ROS), and interfere with the action of pro-inflammatory cytokines, such as IL-6, TNF-alpha, and nuclear factor kappa B. Hence the analgesic and anti-inflammatory activities of miroestrol and deoxymiroestrol might be due to the presence of the aforementioned phytoconstituents.

In conclusions, the current main objective of this study is to investigate the mechanisms underlying the anti-inflammatory activity of miroestrol and deoxymiroestrol in LPS-induced mouse RAW 264.7 macrophage cells. The findings suggest that miroestrol and deoxymiroestrol is able to inhibit the production of TNF-alpha and IL-6, as well as the mRNA expression of TNF-alpha and IL-6 in LPS-stimulated RAW 264.7 macrophages. Moreover, miroestrol and deoxymiroestrol treatment has anti-inflammatory activity in carrageenan-induced mice paw edema. Therefore, we suggest that miroestrol and deoxymiroestrol from *P. mirifica* should be considered as candidate potential anti-inflammatory agents for the treatment of inflammation-related diseases.

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