สารสกัดจากชาสำหรับการยับยั้ง *Propionibacterium acnes*

นางสาวนวพร วิรัตน์เกษม

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TEA EXTRACTS FOR INHIBITION OF *Propionibacterium acnes*

Miss Nawaporn Wiratkasem

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By	Miss Nawaporn Wiratkasem
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Thesis Advisor	Associate Professor Supason Wanichwecharuang, Ph.D.
Thesis Co-Advisor	Associate Professor Chanpen Chanchao, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr.rer.nat.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Vudhichai Parasuk, Ph.D.)

.....Thesis Advisor
(Associate Professor Supason Wanichwecharuang, Ph.D.)

.....Thesis Co-Advisor
(Associate Professor Chanpen Chanchao, Ph.D.)

.....Examiner
(Yupyn Chintapakorn, Ph.D.)

.....External Examiner
(Prasat Kittakoop, Ph.D.)

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ในปัจจุบันชาอู่หลงเป็นเครื่องดื่มที่ได้รับความนิยมอย่างกว้างขวาง โดยจัดเป็นเครื่องดื่มที่ดีต่อสุขภาพ ชาอู่หลงเป็นชากึ่งหมัก ของใบจากต้นชาที่มีชื่อวิทยาศาสตร์คือ *Camellia sinensis* แม้ว่าจะพบรายงานเกี่ยวกับสารสกัดจากชาถึงฤทธิ์ในการต้านเชื้อแบคทีเรียหลายชนิด แต่ยังไม่มีการรายงานฤทธิ์การยับยั้งเชื้อ *Propionibacterium acnes* ของสารสกัดจากชาอู่หลง ดังนั้นงานวิจัยนี้จึงได้ตรวจสอบฤทธิ์ดังกล่าว งานวิจัยพบว่าสารสกัดที่ได้จากกากชา (ชาที่ผ่านการสกัดน้ำร้อน) มีปริมาณสาร epigallocatechin-3-gallate (EGCG) สูง ให้ฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ต้านการอักเสบ และมีฤทธิ์ต้านเชื้อก่อสิวคือ *P. acnes* โดยให้ค่า MIC ที่ 1.25 มิลลิกรัมต่อมิลลิลิตร สารสกัดมีความเป็นพิษต่อเซลล์เม็ดเลือดขาวต่ำ การใช้สารสกัดชานี้ร่วมกับสารแซนโทนที่ได้จากเปลือกมังคุดพบการเสริมฤทธิ์ของสารทั้งสองในการยับยั้งการเจริญของเชื้อ *P. acnes* นอกจากนี้สามารถสร้างเม็ดบีดเพื่อเพิ่มความเสถียรของสารสกัดชาได้โดยเป็นเม็ดบีดอัลจีเนตที่มีความคงตัวของฤทธิ์ต้านเชื้อ *P. acnes* แม้เก็บไว้ที่อุณหภูมิห้อง

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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Oolong tea is a semi-oxidised Chinese tea obtained from leaves of *Camellia sinensis*. Although anti-bacterial activities of oolong extract against various bacteria have been reported, there is no evidence on anti-bacterial activity against *Propionibacterium acnes (P. acnes),* causative bacterium of skin acne. Here, it was found that the extract from oolong tea residue (the leaves that has been used in hot water) contains high amount of epigallocatechin-3-gallate (EGCG), possessed anti-oxidant, anti-inflammation, and anti-*P. acnes* activity (minimum inhibition concentration of 1.25 mg/ml), and minimal toxicity against mice white blood cells. Combination of this tea extract and mangosteen extract showed enhanced anti-*P. acnes* activity. Lastly, encapsulation of the extract into alginate bead provided beads with anti-*P. acnes* activity, even the beads had been kept at room temperature.

Field of Study: Biotechnology
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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List of Abbreviations

α	Alpha
%	Percent
℃	Degree Celsius
g	Gram
h	Hour
mg	Milligram
mL	Milliliter
mM	Millimolar
min.	Minute
mL	Milliliter
ppm	Parts per million
µg	Microgram
µl	Microliter
µM	Micromolar
BHI	Brain heart infusion
CFU	Colony forming unit

CH_2Cl_2	Dichloromethane
CME-1	Crude methanol extract
CME-2	Crude methanol extract
CWE-1	Crude water extract
CX	Crude methanol plus xanthone
CX_1L	Crude methanol with xanthone with lipoic acid
CX_1E	Crude methanol with xanthone with Vitamin E
DMEM	Dulbecco's modified minimum essential medium
DMSO	Dimethyl sulfoxide
EtOH	Ethanol
FBS	Fetal bovine serum
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
MeOH	Methanol
PBS	Phosphate buffer solution
UV	Ultraviolet
X	Xanthone

CHAPTER I

INTRODUCTION

1.1 Acne Vulgaris

Acne Vulgaris is a common skin disease, involving the physiological change in the pilosebaceous units which contains a hair follicle and sebaceous glands. Acne appears on face, chest and back. Because the skin around those areas contains a lot of sebaceous glands. Acne occurs when hair follicles become plugged with oil and dead skin cells [2]. There are two main types of acne. Acne type one is non-inflammatory acne such as blackheads and whiteheads. And acne type two is inflammatory acne such as papule, pustule, nodule and cyst [3]. Acne can also be aggravated by both internal and external factors. The internal factors include hormonal stimulation of the sebaceous gland, increased sebum production, abnormal keratinization resulting in plugging of follicles, increase of *Propionibacterium acnes* within the hair follicles. *Propionibacterium acnes* usually cause inflammation by secreting lipase enzyme that can break down triglycerides into fatty acids, which subsequently cause inflammation. *P. acnes* also secrete enzymes such as protease, hyaluronidase and neutrophil chemotactic factors [4], which stimulate the irritation and inflammation. The external factors that influence acnes are the environment, UV radiation, temperature, chemical substance,

hypersensitive skin, behavior, stress, nutrition, and lifestyle. A typical acne treatment is the use of antibiotic [5].

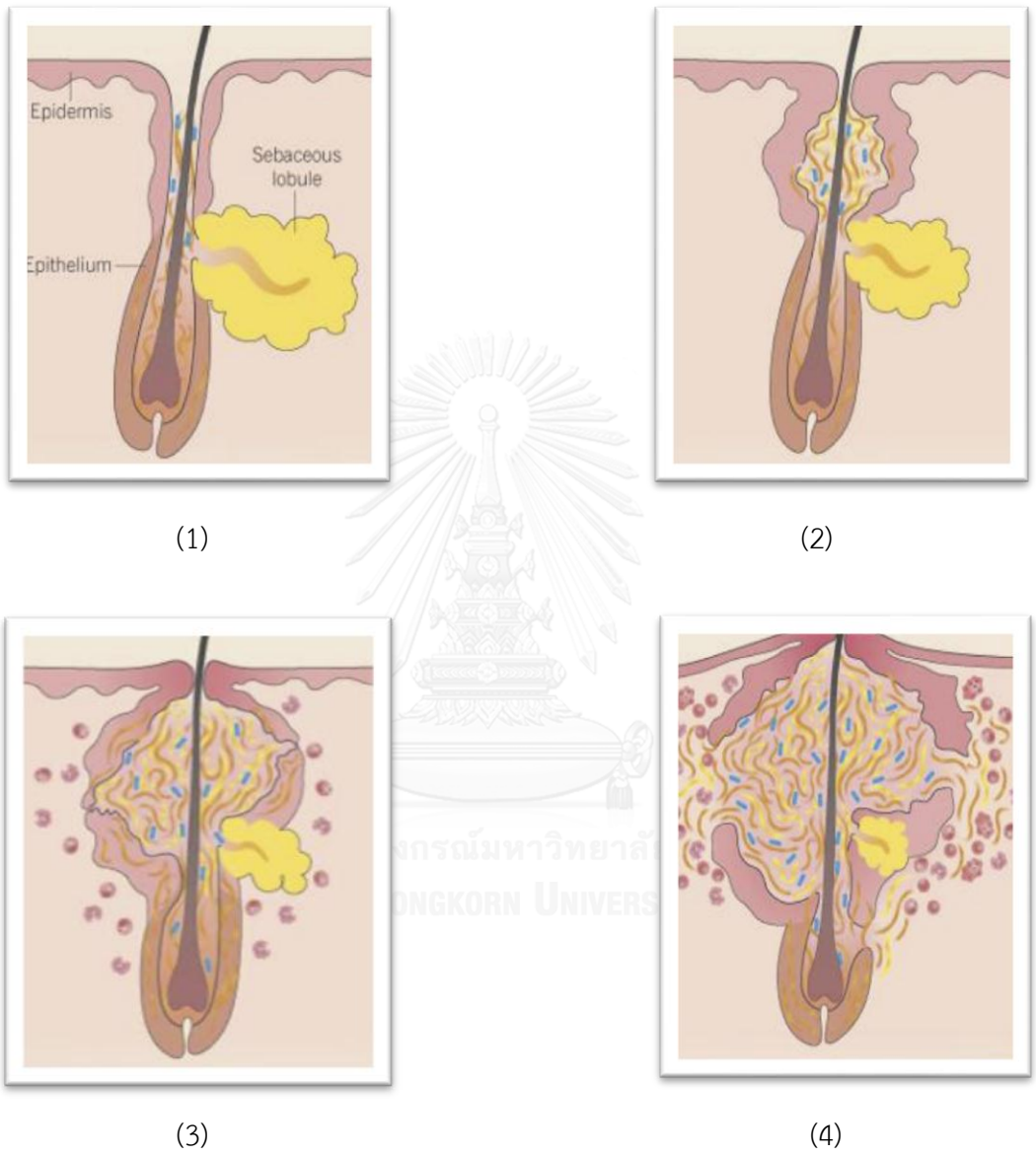


Figure 1.1 The pathogenesis of acne vulgaris [6]

1.2 The pathogenesis of acne vulgaris

Acne is a disorder of the sebaceous follicles, which are special pilosebaceous units located on the face, neck, chest, upper back, and upper arms. These units consist of relatively large sebaceous glands associated with small hair follicles. The most pathogenesis factors of acne have been identified into four major factors which are involved in the pathogenesis of acne vulgaris.

1. Increased sebum production: All acne patients have increased sebum production. And acne generally begins in puberty and adolescent, when androgen increases production and stimulates higher sebum secretion [7]. Moreover, androgens have associated in acne pathogenesis. Hormones not only exert their effects on sebaceous glands but they also may play a role in follicular hyperkeratinization [8].

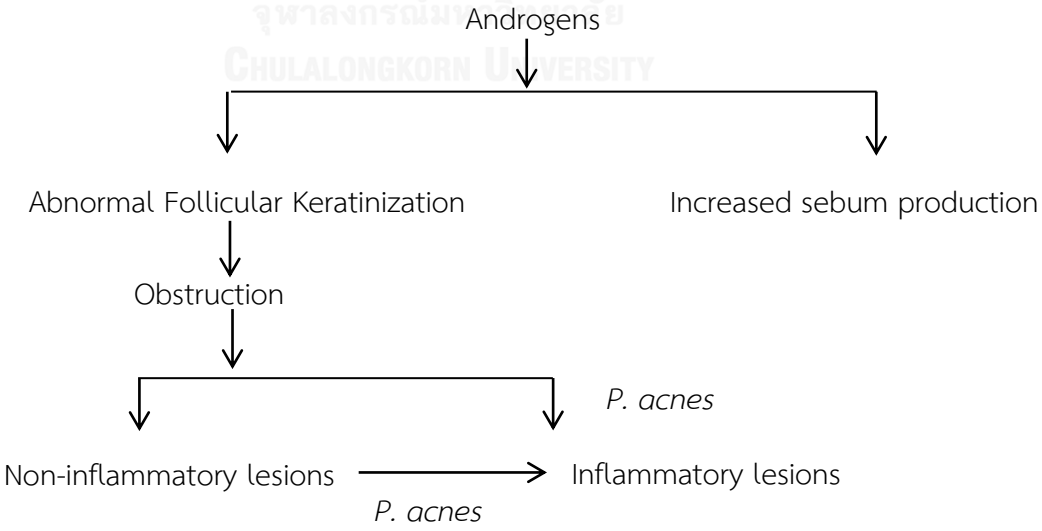


Figure 1.2.1 Basic mechanisms involved in the pathogenesis of acne [1]

2. Abnormal of keratinization: Defects in the proliferation, accumulation, and adhesion of keratinocytes, which are lead to the formation of microcomedones and comedones. Microcomedones are the first type lesions of acne. However, the resulting abnormalities in sebaceous glands influence of hormonal and sebum composition [9].

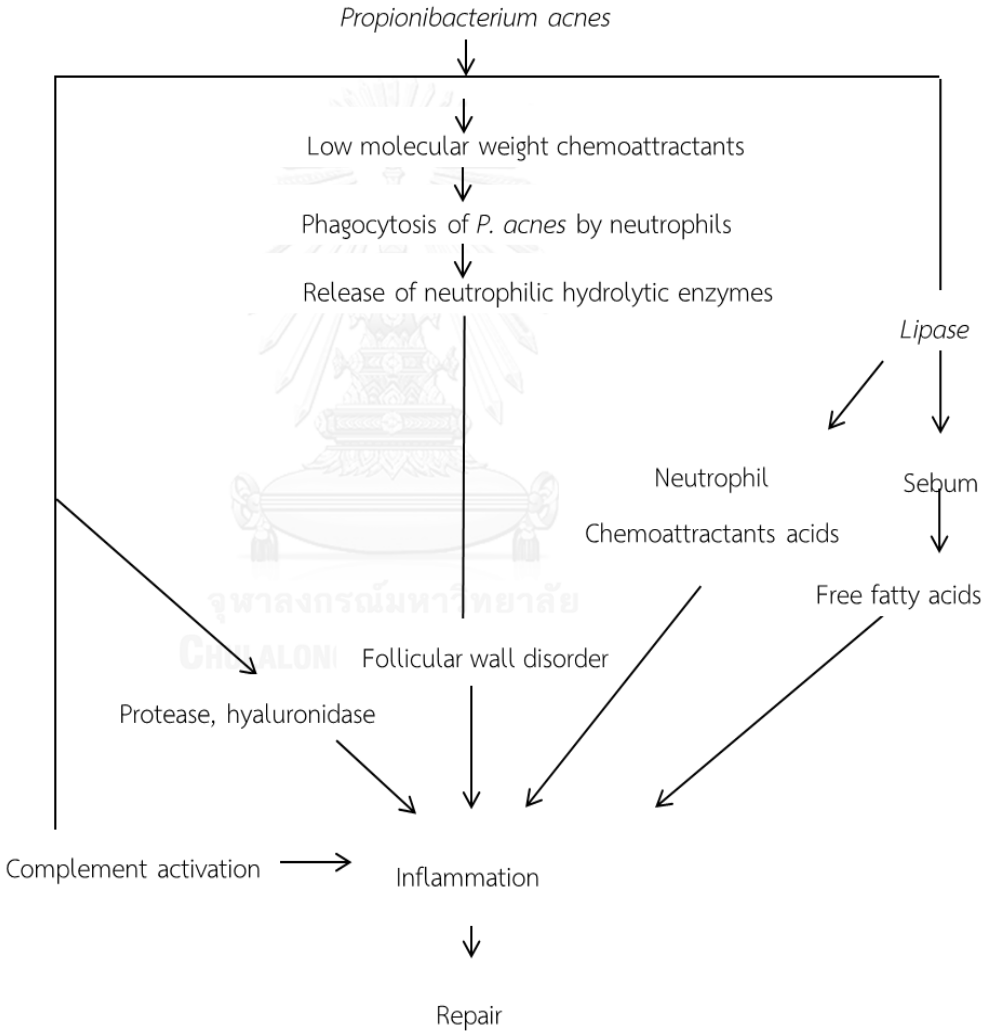


Figure 1.2.3 Sequence of events leading to acne inflammation primarily induced by *P. acnes*.

3. Proliferation of *Propionibacterium acnes*: *P. acnes* is found as commensals on human skin and other keratinized epithelia, which can be significantly increased in the pilosebaceous units of acne valgaris. It also releases lipases, proteases, hyaluronidases, and neutrophil chemotactic factors that lead to tissue injury. Lipases play important hydrolysis sebum triglycerides to free fatty acid and glycerol. And releasing irritating free fatty acids in the pilosebaceous follicles, which are irritants and can induce inflammation of acne vulgaris [10]. Moreover, deficiency of free fatty acids in the follicle also promotes the growth of P. acnes and the releasing of additional free fatty acids from sebum triglycerides. In addition, P. acnes induce the production of tumor necrosis factor-α (TNF-α), interleukin (IL)-1 α, and IL-8, which are proinflammatory cytokines [11].
4. Inflammation: *P. acnes* induce inflammation of acne vulgaris. It leads to inflammation through activation of various chemotactic factors as well as, through promoting abnormal of the comedone [12].

Although, the pathogenesis of acne vulgaris is multifactorial, but normal flora and *P. acnes* are the most important factors to proliferation of acne lesions [13].

1.3 *Propionibacterium acnes*

P. acnes, previously classified as *Corynebacterium parvums* [14] is a gram positive, rod shaped bacterium, anaerobic and non-spore-forming. It is frequently found as normal flora of the oral cavity, large intestine, the conjunctiva, the external ear canal, the deep within the follicles and the pore of the skin [13]. *P. acnes* could grow in anaerobic condition at 37°C. It is commonly isolated from sebum-rich areas of skin, around the pilosebaceous follicle [15].

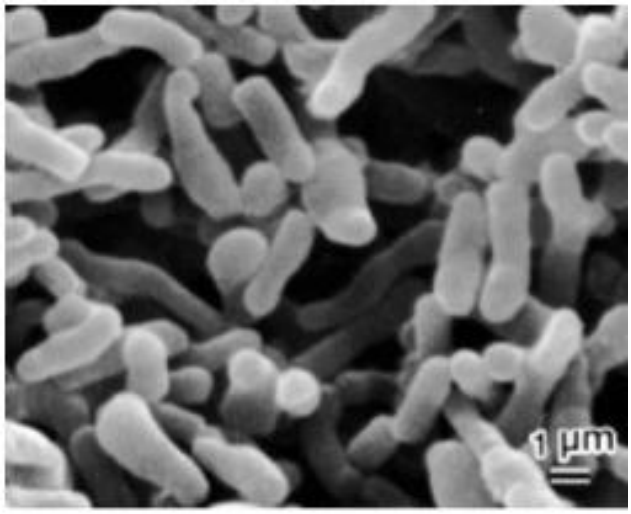


Figure 1.3 Rod shaped *P. acnes* seen through scanning electron microscopy. Lines represent 1µm in length [16].

1.4 Topical treatment for acne

Normal acne treatments usually are directed at reducing sebum production, comedone formation, and inflammation to prevent scars and reduce bacterial counts. Selection of treatment is generally based on lesions of acnes. Nowadays, the most popular topical agents were retinoids, benzoyl peroxide, azelaic acids, and topical antibiotics [17].

Retionoids is keratolytic agents which act by decreasing epidermal proliferation and reducing the abnormal keratinization process in the hair follicle. Retionids work for both comedonal and inflammatory acne [18].

Benzoyl peroxide is an anti-microbial agent destroying both *P. acnes* bacterial and free fatty acids. It improves inflammatory and non-inflammatory lesions by generating reactive oxygen radical in the sebaceous follicle inhibit bacterial organism and anti-inflammatory action [19]. Side effects consist mainly of skin irritation including burning, blistering, crusting, severe redness, and skin rash [20].

Topical antibiotics include tetracycline, clindamycin, and erythromycin. The advantage of topical antibiotics over the oral antibiotics is the reduction in the risk of potential systemic side effects and particularly with clindamycin [21]. At present, the efficacy of erythromycin is low because of bacterial resistance. Side effects are erythema, dryness, itching, peeling and burning, and pseudomembranous colitis [22]. Tetracycline may cause some yellow staining of teeth and nail diseases such as

photo-onycholysis [23]. The long term use of antibiotics against acne have caused antibiotic-resistant *P. acnes* , and it is most common with erythromycin [21]. Therefore, other ways to treat acnes such as hormonal therapy, natural therapy laser and light therapy [5] have been introduced. A natural therapy is a form of alternative medicine employing a wide array of natural treatments, including acupuncture and herbal [24]. Herbal extracts are bioactive compounds derived from plants, and possess properties of natural remedies [25]. Herbal extracts have less side effects and less toxicity when compare with medical drug and can be used alternative medicine for acne treatment.

1.5 Herbal extracts for acne treatment

In 2003, Nam *et al.* reported that *Glycyrrhiza glabra* could inhibit the growth of *P. acnes* with the minimum inhibitory concentrations (MIC) value of 200μg/ml. The author concluded that *G. glabra* could be helpful for the prevention and treatment of acne lesions [26].

In 2003, Gulluce *et al.* evaluated antimicrobial and antioxidant activities of the essential oil, obtained by using a Clevenger distillation apparatus. The essential oil was derived from aerial parts of *Satureja hortensis* L. plants. The results showed that the essential oil of *S. hortensis* could inhibit the growth of against *S. epidermidis* and *Staphylococcus aureus,* which are bacterial causing acne vulgaris. In addition, *S.*

hortensis possessed antimicrobial activities against all 23 bacteria and 15 fungi and yeast species tested [27].

In 2008, Sukatta *et al.* developed anti-acne gel from mangosteen crude extract and investigated antibacterial activity of the gel. The results showed that the optimal formula which contained 94.2% water, 0.5% Carbopol Ultrez-10, 0.5% Triethanolamine, 0.5%, Panthenol, 2.0% Dimethicone, 0.8% Germaben, 1.0 % Polysorbate 20 and 0.5% mangosteen crude extract, has high efficacy in inhibit the growth of against *S. aureus, S. epidermidis* and *P. acnes* in vitro [28].

In 2009, Pothitirat *et al.* reported that α-mangostin could inhibit the growth of *P. acnes* and *S. epidermidis*. The α-mangostin presented high antibacterial efficacy with MIC values of against *P. acnes* and *S. epidermidis* were in the range of 7.81-15.63 and 15.63-31.25 μg/mL, while The minimal bactericidal concentration (MBC) values were in the range of 15.63-31.25 and 62.50-125.00 μg/mL [29].

In 2008, Kim, S. S *et al.* evaluated the antimicrobial activities of Jeju medicinal plants against *Propionibacterium acnes* and *S. epidermidis* by using disc diffusion and broth dilution methods. They found that the JeJu medicinal plants could inhibit the growth of *P. acnes* and *S. epidermidis*. The MIC values against *P. acnes* and *S. epidermidis* were 15.6 and 125 μg/ml, respectively [30].

In 2009, Yamaguchi *et al*. showed that lupulones and xanthohumol were derived from hop plant (*Humulus lupulus* L.) were the highest potent bacterial

compounds against *P. acnes* and *S. epidermidis* at the MIC value of 0.1 µg/ml. Moreover, xanthohumol showed stronger anti-oxidant, and could inhibit *S. epidermidi*s [31].

In 2010, Tsai *et al.* evaluated the antimicrobial activities of herbal extracts against *P. acnes.* They found that duzhong extracts showed strong antimicrobial activity against *P. acnes* with an MIC of 0.5 mg/ml. The MIC of yerba mate extract was 1 mg/ml. The rose extract was less effective against *P. acnes* with an MIC of 2 mg/ml [32].

In 2011, Vijayalakshmi *et al*. reported the antimicrobial activities of developed cream formulations contained flavonoid fraction and other formulations contained tannin fraction of *Terminalia arjuna* bark. *P. acnes* and *S. epidermidis* were investigated by agar well diffusion method. The result showed that cream formulation containing flavonoid fraction has greater activity than the formulation cream containing tanin fraction. The antibacterial activity of cream formulation containing flavonoid showed zones of inhibition >17 mm and >20 mm for *P. acnes* and *S. epidermidis*, respectively [33].

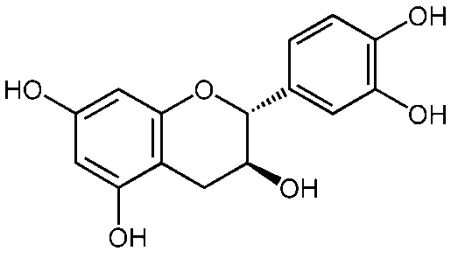
These researches demonstrated that herbal extracts and plant extracts possessed effective antibacterial activity and could reduce inflammatory acne lesions.

1.6 *Camellia sinensis* or Tea

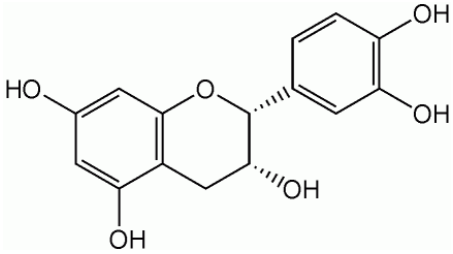
Camellia sinensis or tea is classified in the family of Theaceae. *Camellia sinensis* is a species of plant which leaves and leaf buds are used to produce the popular beverage tea [34]. Two species of tea plants derived from *Camellia sinensis* are Chinese tea; *Camellia sinensis* var. *sinensis* and Assam tea; *Camellia sinensis* var. *assamica*. Tea plants are widely cultivated in Southeast Asia including China, Japan, India, Taiwan and Thailand [35]. The tea plant is an evergreen shrub or small tree that blooms in spring and grows up to 15 m high. The tea leaves show lanceolate or elliptic shape, sizes of them are varying from 2-3 cm depends on the species and variety: the length of Assam and Chinese tea leaves are 15 to 20 cm and 5 to 12 cm long, respectively [36]. Old leaves are dark green whereas the younger are light green. They are, therefore, preferably harvested for tea production. Difference of leaf age provides different tea qualities since dissimilarity of their chemical compositions. Tea plants are very sensitive to any change in their position, temperature, humidity and moisture [36]. Tea is classified into three major types, green, oolong, and black, which essentially depend on the manufacturing process [37]. Green tea is non-fermented tea, whereas Oolong and Black tea are semi-fermented or semi-oxidized and fully fermented tea, respectively. Moreover, these basic types of tea have different characteristics such as sense, taste, and color [38]. Tea leaf composes of various chemical components, i.e., polyphenol, flavonoids, tannin, amino acids, caffeine, mineral, vitamin, and other pigments [39]. Among these components,

polyphenols from the flavonoid, which are the secondary metabolites produced by plants, are important for the biological activity in tea leaves [40]. Furthermore, they also have widely been used in human especially in Asia because they have benefit for health, mainly as antioxidant, anti-inflammatory, anticancer, anti-allergic, antimicrobial and antiviral agents [41].

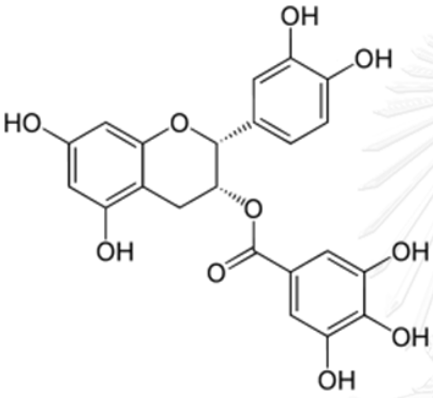
Flavanols (flavan-3-ols or flavans) are a class of flavonoids, which are polyphenols, and present in relatively large amounts in tea. Catechins are divided into four primary compounds, (-)epicatechin (EC), (-)epicatechin gallate (ECG), (-)-epigallocatechin (EGC), and (-)-epigallocatechin gallate (EGCG) [41]. The most active and abundant catechin in tea is epigallocatechin-3-gallate (EGCG) [42].



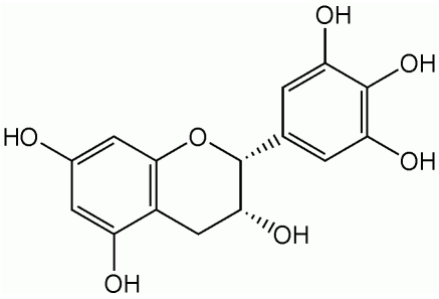
Catechin



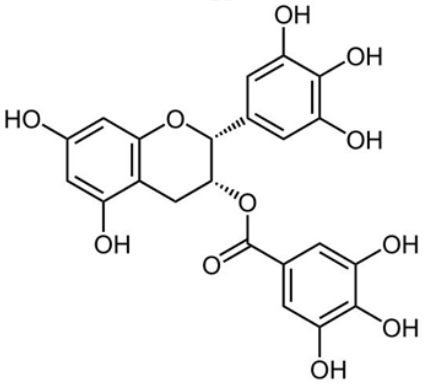
Epicatechin (EC)



Epicatechin-3-gallate (ECG)



Epigallocatechin (EGC)



Epigallocatechin-3-gallate (EGCG)

Figure 1.4 Chemical structure of major catechin found in tea extract.

The EGCG content in tea leaves is about 60 to 70% of total catechins [43]. EGCG is the most active and believed to have a wide range of pharmaceutical activities such as antioxidant, anti-inflammatory, anticancer, anticarcinogenic and antimicrobial activities [44].

1.7 Bioactivity of tea extract

1.7.1 Anti-oxidative activity

Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions [45]. Tea polyphenols, mainly flavonoids, are well known for their antioxidant properties.

In 1996, Nanjo *et al.* determined the scavenging effects of tea catechins and their epimerized products by DPPH radical. They found that epigallocatechin, epicatechin gallate and epigallocatechin gallate are more effective radical scavengers than catechin and epicatechin [46].

In 1997, Gadow et al. reported that the antioxidant activity of aqueous extracts of rooibos tea (unfermented, semi-fermented and fermented), which compared with green tea, oolong tea and black tea, were determined using DPPH radical and β-carotene bleaching method. From the DPPH radical scavenging results, the decrease of the antioxidant activity of all the tea, in the order: green > unfermented rooibos > fermented rooibos > semi-fermented rooibos > black > oolong respectively, was observed. However, the antioxidant activity of all tea

extracts the tests using the β-carotene bleaching method decreased in the order: green > black > fermented rooibos > oolong> unfermented rooibos >semi-fermented rooibos, respectively. All the tea extracts were strong inhibitors of β-carotene bleaching as well as highly active hydrogen donors to the DPPH radical [47].

In 1999, Bors et al. evaluated the antioxidant ability of catechins and gallate esters. They found that epicatechin is capable for scavenging of hydroxyl radicals, peroxyl radicals, superoxide radical, and DPPH radicals [48].

In 2002, Zhu et al. demonstrated DPPH radical scavenging activity, the amount of total phenolic compounds, and the inhibitory effect on erythrocyte hemolysis, of oolong tea water extract (OTE). The OTE showed strong antioxidant activity, which can provide the inhibition erythrocyte hemolysis [49].

In 2011, Chan et al. investigated the role of non-polymeric phenolic (NP) and polymeric tannin (PT) components in the antioxidant and antibacterial properties of six brands of green, black and herbal teas. The results showed that the antioxidant property of green tea extracts was stronger than that of black and herbal tea. For all six brands tests, the antioxidant property of NP fractions was significantly less than crude extracts and PT fractions. Although PT fractions have stronger antioxidant and antibacterial properties, they are only a minor component of the tea [50].

In 2012, Jungmin et al. evaluated the antioxidant and antimicrobial activities of various leafy herbal tea (LHT) extracts. They found that green tea extract showed

the highest antioxidant activity and had potent antimicrobial activity against all five pathogens (*Streptococcus mutans, S. sobrinus, Listeria monocytogenes, Shigella flexneri* and *Salmonella enteria*) [51].

These results of numerous investigations have confirmed that flavanols isolated from green, black, oolong, white and red tea leaves possess very strong antioxidant proprieties.

1.7.2 Anti-inflammatory activity

Inflammation is a physiological response of the immune system to resist pathological states such as irritation and infection caused by chemical, injury and microbial pathogens. However, unbalanced or prolonged inflammation leads to progressive tissue damage and has been involved in development of many diseases [52].

In 2011, Thring et al. evaluated the anti-inflammatory activity of plants extracts using IL-8 inhibition assay. They found that the white tea extract and the witch hazel extracts exhibited the best activity ranging from 85-83% inhibition of IL-8 production. Among various types of plants, rose extract showed the lowest activity ranging from 30-45% inhibition of IL-8 production [53].

In 2012, Chen et al. investigated the anti-inflammatory effect of a crude tea flower extract (TFE) on acute inflammation and *P. acnes* plus LPS which induced liver inflammation in mice. The results showed that tea flower extract with the

concentrations of 100 and 200 mg/kg could inhibit nitric oxide production at the level of 28.6% and 34.9%, respectively [54].

In 2014, Hisanaga et al. investigated the anti-inflammatory and molecular mechanism of oolong tea theasinensin in both cellular and animal models. Theasinesin is considered as bioactive compound in oolong tea. They found that theasinesin could reduce the levels of pro-inflammatory mediators including nitric oxide (NO), interleukin-12, tumor necrosis factor alpha (TNF-α) and monocyte chemotactic protein-1 (MCP-1). Moreover, in the animal model, they found that theasinesin attenuated mouse paw edema induced by LPS, which inhibited the production of IL-12, (TNF-α) and MCP-1. [55].

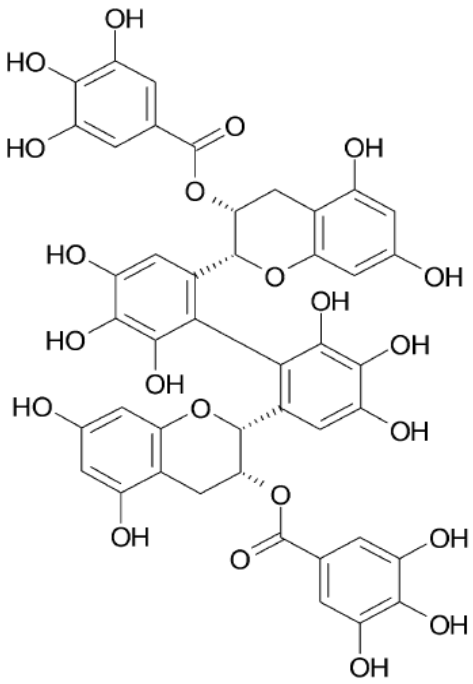


Figure 1.5. The chemical structure of theasinesin A (TSA) [55]

Thus it can be concluded that many researches have demonstrated potent anti-inflammatory activity of the leaves extract of *Camellia sinensis.* .

1.7.3 Antimicrobial activity

Recently the antimicrobial activities of plant extracts and herbs have become more interesting due to theirs bioactivities. The plant extracts are rich in a wide variety of secondary metabolites such as alkaloids flavonoids and tannins, which have shown in vitro antimicrobial properties [56]. Comparing to synthetic antibiotics, several medicinal plants have been reported for therapeutically infectious diseases owing to their fewer side effects and less toxicity [17].

In 2001, Matsunaga et al. reported that EGCG enhanced the *in vitro* resistance of alveolar macrophages from infection of *Legionella Pneumophila,* which selectively alters the immune responses of macrophages to *L. pneumophila*. *L. Pneumophila* is the causative agent of Legionnaires' disease in immune compromised patients [57].

In 2005, Ooshima et al. evaluated the anti-bacterial activity of various types of tea and their polyphenols. They found that the IC_{50} for insoluble the glycan synthesis inhibition activity of purified oolong tea polyphenols was found to be 2 μg/ml, 8 μg/ml for theaflavin purified from black tea leaves, 40 μg/ml for oolong tea extract, 250 μg/ml for a green tea extract, and much higher concentrations were needed with simple catechins. Moreover, oolong tea polyphenols could inhibit the

glucosyltransferases activity of S*treptococci mutans* and decrease the surface hydrophobicity of *S. mutans* [58].

In 2006, Friendman *et al.* evaluated the antimicrobial activities of tea catechins, theaflavins and tea extracts against *Bacillus cereus*. *B. cereus* is a foodborne pathogen, cause foodborne illness. They found that (-)- gallocatechin-3-gallate, (-)-epigallocatechin-3-gallate, (-)-catechin-3-gallate, (-)-epicatechin-3-gallate, theaflavin-3, 3'-digallate, theflavin-3'-gallate and theaflavin-3-gallate showed strong antimicrobial effects against *B. cereus* [59].

In 2006, Si et al. reported that Chinese green tea extract could inhibit the growth of *Escherichia coli* 0157:H7, *Salmonella Typhimurium* DT104, *Listeria monocytogenes*, and *Staphylococcus aureus*, which are foodborne pathogens. The green tea extract exhibits the highest and lowest activity against *S. aureus* and *E. coil* 0157:H7, respectively [60].

In 2009, Stoicov et al. evaluated the bactericidal and bacteriostatic effect of green tea extracts against *Helicobacter felis* and H. *pylori* in vitro. The results revealed the effects of green tea extracts on the development of *Helicobacter*-induced gastritis in an animal model. They found that green tea could inhibit the growth of *Helicobacter* and prevent gastric mucosal inflammation [61].

In 2010, Cho et al. evaluated the antimicrobial activity and biofilm inhibition of tea polyphenols extract (TPP) on human teeth. The results showed that TPP could

inhibit the growth of S*treptococcus sanguis* and *S. mutans*, which are oral pathogens. Moreover, the TPP possesses antimicrobial effects and inhibits the formation of biofilms on human teeth. Therefore, tea polyphenols extract can be developed as a potential antimicrobial agent against oral microorganisms and used for prevention and treatment of dental caries [62].

In 2012, Sharma et al. demonstrated antimicrobial activity of an aqueous extract of green tea leaves against bacteria strains related to skin infection by using disc diffusion assay and MIC assay. The results showed that green tea extract could inhibit growth of *S. epidermidis*, *Microroccus lutens, Brevibacterium linens*, *Pseudomonus fluorescens* and *Bacillus subtilis*. All strains showed more than 7mm inhibition zone s. The strain that exhibited the maximum zone of inhibition (20 mm) was reported to be *S. epidermidis*. The MIC values were determined using NBT dye on 96 well plates. The MIC of an aqueous extract of *M. lutens*, *S. epidermidis, P. fluorescens* and *B. subtilis* were found to be 0.156 mg/ml, whereas 0.313 mg/ml for that of *B. linens* [63].

These researches show that tea catechins and tea extracts containing both classes of polyphenol compounds exhibit strong antibacterial activities against foodborne pathogenic bacteria and pathogenic bacteria that cause infectious illnesses in humans.

1.8 Stability of tea extracts

Epimerization, oxidation and degradation were reported as the main cause of changes in tea catechins during food processing and storage.

Zhu et al. (1997) studied on stability of green tea catechins. The green tea catechins are very stable in the acidic solution (pH < 4). Furthermore, four epicatechin isomers showed variable stability in alkaline solutions with EGCG and EGC being equally instable while EC and ECG were relatively stable [64].

Chen et al. (2001) reported that green tea catechins in soft drink of pH 6 completely degraded after 4 months but partially decomposed around 45 % in a soft drink of pH 3.23 after 6 months of storage [65].

Lun Su et al. (2003) studied the stability of green tea catechins (GTC) and theaflavins (TF) in various solutions and drinks. They found that TF was less stable than GTC. In sodium phosphate buffer (pH 7.4) at room temperature, GTC with EGCG and EGC completely degraded in 6 h of incubation, whereas the degradation with EC and ECG was less than 35%. In boiling water, four GTCs showed similar rate of degradation. Moreover, four TFs also showed different stability. Generally, theaflainv-3, 3'-digallate (TF3) and theaflainv-3'-gallate-B (TF2B) are more stable than theaflavin-1 (TF1) and theaflavin-3-gallate-A (TF2A) in either boiling water or alkaline sodium phosphate buffer [66].

These results suggested that the stabilities of tea chtechins and tea extract are pH and temperature dependent. The tea catechins are more stable at the lower pH during processing and storage. In aqueous solutions, they are very stable when pH is below 4, whereas unstable in solutions with pH>6.

Objective of this research

The goal of this research can be summarized as follow:

1. To find the appropriate tea leaf extraction process in order to obtain extracts with high anti- *P. acnes* activity.
2. To enhance the anti-*P. acnes* activity in the extract by using other plant extract.
3. To determine anti-inflammatory and antioxidant activities of the obtained extracts.
4. To evaluate stability of the obtained extracts.

CHAPTER II

EXPERIMENTAL

Materials and Methods

2.1 Materials and Chemicals

Solvents used in extraction, antibacterial activity, antioxidant activity and encapsulation of tea extracts, and acetic acid were reagent or analytical grades purchased from Labscan (Bangkok, Thailand). *p*-Iodonitrotetrazolium violet, 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) was purchased from Tokyo Chemical Industry Company (Tokyo, Japan). Xanthone powder (±95% purity) extract was supplied by Welltechbiotechnology Company (Bangkok, Thailand). Epigallocatechin-3-gallate was purchased from Tokyo Chemical Industry Company (Tokyo, Japan). Lipoic acid was purchased from Pronova laboratory Company (Bangkok, Thailand). Vitamin E, sodium alginate and caffeine were purchased from Sigma–Aldrich ChemieGmbH (Steinheim, Germany). Metronidazole was purchased from Thai Nakorn Patana Co., Ltd. (Nonthaburi, Thailand). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) used as stable radical in antioxidant activity assay was purchased from Sigma–Aldrich ChemieGmbH (Steinheim, Germany). Butylated Hydroxy Toluene (BHT) used as standard antioxidant was purchased from Sigma–Aldrich ChemieGmbH (Steinheim, Germany). Calcium chloride was purchased from

Carlo erba reagents (Rodano, Italy). Oolong tea leaves were from Boon Rawd Farm, (Chiang Rai, Thailand).

2.1.1 Media culture for antibacterial study

Brain heart infusion broth (BHI broth) and Brain heart infusion agar (BHI agar) were purchased from Difco laboratories (Detroit, MI, USA). GasPak was purchased from Biomerieux (Marcy'Etoile, France).

2.1.2 Media culture and chemical reagent for cell culture and treatment

Dulbecco's modified minimum essential medium (DMEM), fetal bovine serum, 100 mM sodium pyruvate, and HEPES (4-(2-hydroxyethyl)-1-piperazineethane sulfuric acid) (free acid 1 M solution (238.3 g/L)) were obtained from Hyclone (Utah, USA). Streptomycin sulphate and penicillin G (sodium salt) were purchased from M & H manufacturing (Samutprakarn, Thailand). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide or MTT was purchased from USB Corporation (Ohio, USA). Lipopolysaccharide (LPS; *Samonella minnesota*) was purchased from Sigma–Aldrich ChemieGmbH (Steinheim, Germany).

2.2 Instruments and Equipments

2.2.1 Antibacterial determination

The equipments were sterilized with autoclave SX-700 (Tomy Company, USA). Laminar flow used for experiments was supplied from Renowm technical company (Bangkok, Thailand). Bacteria were incubated in shaking incubator THZ-300 purchased

from Chincan company (Shanghai, China). Automatic micropipettes P10, P20, P100, and P1000 used in the experiment were purchased from Gilson company, Inc. (Ohio, USA). Microplate reader was Titertek Multiskan MCC/340 (Düsseldorf, Germany). Petri dish was purchased from Sterilin Limited, (Newport, United Kingdom). Tissue culture plate 96 well was purchased from DSI Thermo Fisher Scientific (Hvidovre, Denmark).

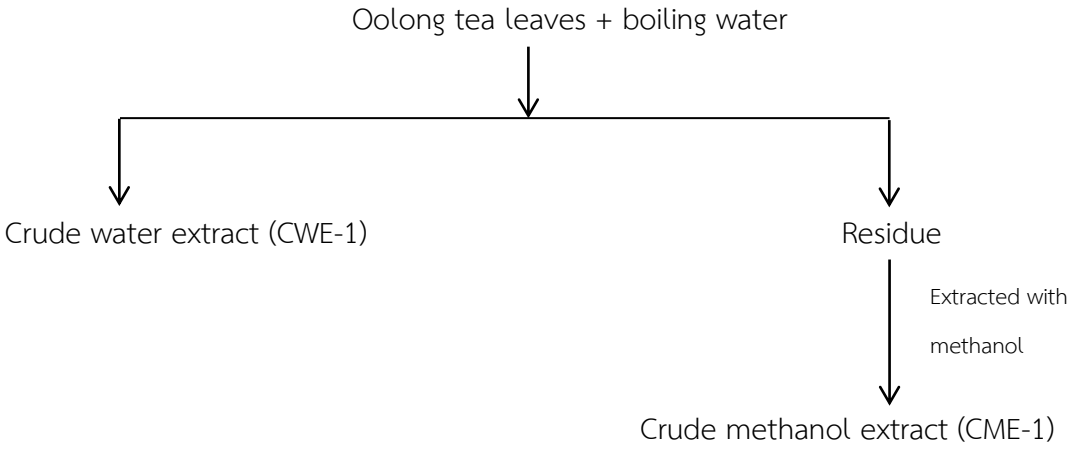
2.2.2 Cytotoxic and anti-inflammatory experiment

The murine macrophage like cell line, RAW 264.7 used in cytotoxic study and anti-inflammatory assay was obtained from the American Type Culture Collection. The raw 264.7 cells were harvested with Rotofix 32 centrifuge (Hettich, Kirchlengern, Germany). Heamotocytometer used for cell count was purchased from Boeco (Hamburg, Germany). Larminar flow carbinet H1 was supplied from Lab service LTD part (Bangkok, Thailand). Tissue culture 96 well plate used for experiment was supplied from NuncTM Brand products (Roskilde, Denmark).

2.3 Preparation of tea extracts

2.3.1 Extraction with water

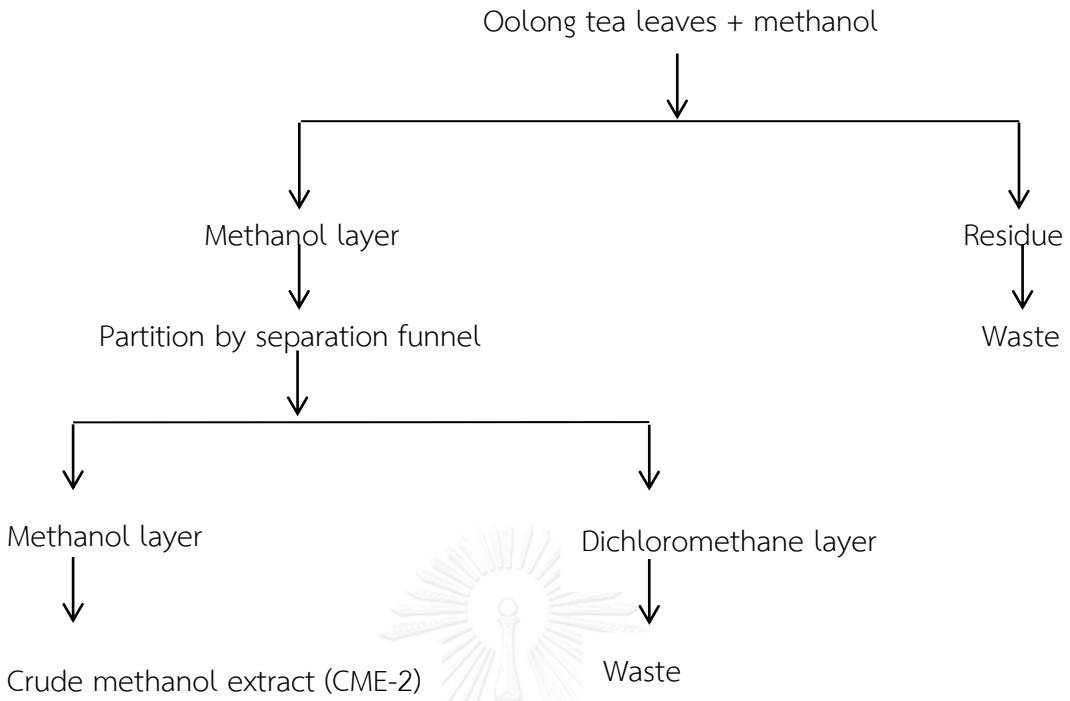
The dried leaves of Oolong tea (74g) were extracted by soaking in boiling water (1500 mL) for 1 h. Later, the extract was filtered and then evaporated in vacuum at below 55°C in a rotary evaporator. The obtained crude water extract (CWE) was kept at -20°C. Next, the residue of tea was dissolved by methanol in the volume of 1000 ml for 24h. Later, it was filtered and then evaporated in vacuum at below 55 °C in a rotary evaporator. The obtained crude methanol extract (CME) was kept at -20°C.



Scheme 1 Extraction with water + methanol

2.3.2 Extraction with methanol

Twenty six grams of Oolong tea leaves were soak in 300 ml methanol at 55 °C. After 3 h, the mixture was cooled, paper filtered and the solvent evaporated under vacuum at 55 °C. Next, the obtained extract was partitioned by shaking in a separating funnel with dichloromethane: methanol: water at the ratio 3:1:0.2 v/v. This mixture was allowed to settle, the lower layer (dichloromethane) was discarded and the upper layer (methanol/water) was evaporated in vacuum at 55 °C in a rotary evaporator. The obtained crude methanol extract of tea (CME-2) was kept at -20°C [67]-[68].



Scheme 2 Extraction with methanol

2.4 HPLC analysis of EGCG in tea extract

Tea extracts were analysed on a reverse phase high performance liquid chromatographic system (Waters 1525 binary HPLC pump) coupled to an ultraviolet detector (Water 2489). The separations were performed using a C18 reverse phase column (100 x 4.6mm i.d), packed with Hypersil C18 (Thermo Fisher Inc, Waltham, Massachusetts, USA). The sample (22 µL) was injected into the column. The flow rate was maintained at 0.7 ml/min and UV-detection was performed at 270 nm. The mobile phase consisted of aqueous formic acid (0.1% in water, solvent A) and acetonitrile (solvent B). Gradient elution started at 95% solvent A and 5% solvent B, increased the solvent B content linearly to 15% within 14min and maintained solvent B content for 11 min. Then the solvent B content was increased to 35% within

28min, and finally to 85% within 12 min. The column was re-equilibrated with 85% mobile phase B for 15 min before the next injection [69].

2.4.1 Calibration curve

The method development was concentrated on establishing the linear calibration curve for the CWE-1, CME-1, CME-2, and EGCG analysed. Analysis of the standards was carried out by triplicate injections of the standard solutions in the range of 1000-125 ppm concentrations.

2.5. Antimicrobial activity assay

2.5.1 Preparation of bacteria

Propionibacterium acnes (DMST 14916) was obtained from the Department of Medical Sciences (Bangkok, Thailand). *P. acnes* was cultured in BHI broth for 72 h at 37 °C under anaerobic condition and adjusted to 0.5 McFarland standard to yield approximately $1x10^8$ CFU/ml.

2.5.2 Preparation of the tested samples

Nine samples, CWE-1, CME-1, CME-2, xanthone, EGCG, caffeine, metronidazole, lipoic acid, and vitamin E were used in the test. Each sample was first prepared as the stock solution in appropriate solvent; CWE-1, CME-1, CME-2, EGCG, caffeine, and metronidazole were dissolved in 5%(v/v) DMSO (the concentration of stock solution were 10 mg/ml); xanthone, lipoic acid and vitamin E were dissolved 200 µl ethanol and 1800 µl of 5% (v/v) DMSO in water (the final concentration of stock solution were 10 mg/ml).

2.5.3. Minimum inhibitory concentration (MIC) assay

Minimum inhibitory concentrations (MIC) of all samples were determined using *p*-iodonitrotetrazolium violet (INT) [70]. Briefly, stock solutions of the 9 samples (CWE-1, CME-1, CME-2, xanthone, EGCG, caffeine, metronidazole, lipoic acid, and vitamin E) (500 µL, concentration of samples = 10 mg/ml) were directly diluted with 5% (v/v) DMSO in water using two fold serial dilutions over a range to give a series of concentrations of samples. In sterile 96 well plates, 50 µl of the sample were added to wells containing 50 µl of the bacterial suspension in BHI broth ($1x10^8$ CFU/ml in BHI). Triplicate samples were performed for each test concentration. Negative controls were prepared using the same solvents employed to dissolve the samples and BHI broth. Metronidazole was used as positive control. After incubation for 72 h at 37 °C under anaerobic condition in shaking incubator, 5 microliters of INT (4 mg/ml) were added to each well. The MIC was determined as the lowest concentration of samples at which no coloration developed within 15 min after adding the dye. All the experiments were performed in triplicate.

2.5.4. Minimum bactericidal concentration (MBC) assay

The minimum bactericidal concentration (MBC) was defined as the minimum level of extracts concentration that produces at least a 99.9% reduction in the numbers of microbial cells. MBC was determined by assaying the live organisms of those tubes from the MIC that showed no growth. A loopful of bacterial broth from each of the well showing no growth was inoculated onto BHI plates and examined

for sign of growth (colonies) after 72 h of incubation at 37 °C. All experiments were performed in triplicate [71].

2.5.5 Determination of the synergism between the crude methanol extract (CME-1) and xanthone

The synergism between the crude methanol extract of tea (CME-1) and xanthone was evaluated by determining the minimal inhibitory concentration (MIC) of the extracts against *P. acnes* strains (using *p*-iodonitrotetrazolium violet (INT)) [70]. Stock solutions of CME-1 (50 mg/ml) and xanthone (6.25 mg/ml) were prepared in DMSO (500 µl). The stock solution was kept at -20°C until used the concentration of stock solution were 5 mg/ml and 0.625 mg/ml. The sample was then subjected to MIC determination, as previously described.

Table 2.1 Concentrations of crude methanol extract of tea (CME-1) and xanthone for anti-*P. acnes* assay

Samples	Contents	Concentrations(mg/ml)
C	CME-1	5
CX1	CME : Xanthone	5 : 0.625
CX2	CME : Xanthone	2.5 : 0.312
CX3	CME : Xanthone	1.25 : 0.156
CX4	CME : Xanthone	1.25 : 0.078
CX5	CME : Xanthone	0.625 : 0.078
CX6	CME : Xanthone	0.312 : 0.078
CX7	CME : Xanthone	0.312 : 0.039
X	Xanthone	0.625

2.6 Antioxidant activity

2.6.1. Scavenging of diphenyl-picrylhydrazyl (DPPH) radicals

The DPPH radical scavenging ability was determined according to the method described by Yuan *et al* [72] with a slight modification. Here, fifty microliters of each concentration of sample were dissolved in methanol and were allowed to react with 200 µl of 0.3 mM DPPH solution. Each mixture was kept in the dark for 30 min. The absorbance of the resulting solution was measured at 517 nm against a blank. BHT was employed as positive control (4, 2, 1, 0.5 and 0.25 mM). Methanol was used as

negative control. All the experiments were performed in triplicate. The percentage % scavenging activity was calculated by the following formula:

$$\% \text{ scavenging activity} = \frac{Abs\,control - Abs\,sample}{Abs\,control} x100$$

Where $Abs_{control}$ and Abs_{sample} are the absorbance of the control (DPPH solution without sample) and the test sample (DPPH solution plus test sample or positive control), respectively. The inhibition curves were prepared and IC_{50} values defined as the amount of antioxidant necessary to decrease the initial DPPH· concentration by 50% was determined.

2.7. Cytotoxicity by MTT assay

2.7.1. Cell culture and treatment

RAW 264.7 cells was cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) FBS, 10 mM HEPES, 100 mM sodium pyruvate, 0.01% (w/v) penicillin G and 0.05% (w/v) streptomycin sulphate (DMEM complete medium). All cells were incubated at 37 °C in a humidified atmosphere enriched with 5% (v/v) CO_2, and were harvested by the addition of 10 mM cold phosphate buffer (pH 7.4) and gently aspirated before washing and dispersion into DMEM complete medium.

2.7.2. Cell viability

The evaluation of cytotoxic activity was based on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to give a blue formazan product, which

can be measured spectrophotometrically. In this experiment, RAW 264.7 cells were seeded in a 96-well plate at a concentration of $1x10^4$ cells/well. Then, 100 µl of sample (dissolved in DMEM at various concentrations) was mixed into the well and cells were incubated at 37 °C for 24 h with the addition of 10 µl of 25 mM MTT reagent in PBS. Incubation was continued for 4 h, there should be formazan crystals formed in the live cells. After incubation, add 200 µl of DMSO to each well and pipette up and down to help dissolve crystals. The absorbance was measured at 540 nm. All the experiments were performed in triplicate. Cell viability was calculated using the formula below:

Cell viability (%) = (Abs test cells/Abs control cells) x100

Cell viability of untreated cells was set as 100%, and water was used as negative control. All the experiments were performed in triplicate [71].

2.8. Anti-inflammatory by nitrite assay

The accumulation of nitrite is an indicator of nitric oxide (NO) synthesis. Here nitrite was measured in the culture medium using the colorimetric test, based on the griess reaction [73]. In this method, RAW 264.7 cells were seeded in a 96-well plate at a concentration of $1x10^4$ cells/well. One hundred µl of the test sample (in DMEM complete) added and the mixture was incubated for 24 h. Cells were activated by adding 100 µl/ml of LPS and 10 µl/ml of Interferon (INF) incubated at 37 °C for 10 min. An aliquot (100 µl) of the supernatant was mixed with an equal volume of griess

reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). The mixture was then subjected to UV-visible absorption spectroscopic analysis. The nitrite concentration was determined by comparison the absorbance at 540 nm with that of the standard solutions of $NaNO_2$ standards prepared in culture medium (constructed as a standard curve). The results were expressed as the percentage of NO production compared to the control, as follows:

$$\% \text{ Inhibition} = \frac{Abscontrol - Abssample}{Abscontrol} x100$$

Where $Abs_{control}$ is the absorbance of the control reaction and Abs_{sample} is the absorbance in the presence of samples with the extracts, respectively.

2.9 Stability of tea extract

2.9.1 Preparation of samples

Test samples are shown in Table 2.2. Each sample was first prepared as the stock solution in appropriate solvent; sample CME-1 was dissolved in 5% DMSO (the concentration of stock solution were 5 mg/ml); Xanthone, CX1, lipoic acid and vitamin E were dissolved in DMSO (the concentrations of stock solution were 0.625 mg/ml, 5 mg/ml and 0.625 mg/ml, 2.5mg/ml and 2.5mg/ml). Two sets of CX1 sample were prepared, the CX1 mixed with lipoic acid (CX_1L) and the CX1 mixed with vitamin E (CX_1E).

2.9.2 Stability of CME-1, xanthone, lipoic acid, vitamin E CX1, CX_1L and CX_1E

Stability of each sample (Table 2.2) was examined at -20°C, and room temperature for 7, 14, 21, and 28 days, using the MIC value as indicator. Samples (Table 2.2) were kept at the corresponding temperature for the indicated period and then the activity of sample was tested by MIC assay as described above.

Table 2.2 Concentration of samples

Samples	Contents	Concentrations(mg/ml)
CME-1	Crude methanol extact-1	5
Xanthone	Xanthone	0.625
LIpoic acid	LIpoic acid	5
Vitamin E	Vitamin E	5
CX1	CME-1 : Xanthone	5 : 0.625
CX_1L	CME-1 : lipoic acid	5 : 0.625 : 2.5
CX_1E	CME-1 : Vitamin E	5 : 0.625 : 2.5

2.10 Antimicrobial activity of the tea extract loaded in alginate bead

2.10.1 Preparation of samples

Samples (Table 2.3) were loaded into alginate beads under pH 3.5, pH 5.5 and unbuffered conditions, using ionic gelation method [74]. To prepare the beads, sodium alginate (0.375 g) was dissolved in 0.1 M acetate buffer pH 3.5 at 50 °C. Then, samples (Table 2.3) (dissolved in 25ml of 0.1 M acetate buffer pH 3.5), were mixed with the alginate solution. The obtained mixture was extruded drop-wise into $CaCl_2$ (2wt %) solution through 0.5 mm inner diameter tube. The beads were collected and washed with distilled water. The beads of distilled water and pH 5.5 buffer were also prepared similarly.

Table 2.3 Concentration of samples

Samples	Contents	Concentrations(mg/ml)
C	CME-1	5
CX1	CME : Xanthone	5 : 0.625
CX1L	CX1 : lipoic acid	5 : 0.625 : 2.5
X	Xanthone	0.625

2.10.2 Antimicrobial activity of the tea extracts loaded in alginate bead

The antimicrobial activities of the samples (Table 2.3) encapsulated in the alginate beads were evaluated by monitoring the inhibition of *P. acnes* growth. The assay was carried out by disc diffusion method. *P. acnes* inoculum was uniformly spread using sterile cotton swab on a sterile BHI agar. In each test, 500 µl of the microbial suspension and determined amounts of beads were placed in a plate. The systems were incubated for 72 h at 37 ℃ under an anaerobic condition. After incubation, confluent bacterial growth was observed. Then the inhibition zones were analyzed. Inhibition of the bacterial growth was measured in cm. Each experiment was performed in triplicate and the results were reported as the mean for all experiment.

2.11 Statistical analysis

All data were presented as means ± standard deviation (SD). Statistical significant difference between experiment and control groups was determined by one-way analysis of variance (ANOVA) with Duncan's multiple range tests used to analyze and compare the data using SPSS Statistic Base 22 (SPSS Co., Ltd., Thailand, Server IP Address; dc1.win.chula.ac.th).

CHAPTER III

RESULTS AND DISCUSSION

3.1 Yield of tea crude extract

The highest yield of tea extract was found in crude methanol extract (CME-2), followed by crude water extract (CWE-1) and crude methanol extract (CME-1), respectively (Table 3.1).

Table 3.1 Yield of tea in the various extracts

Samples	% Yield
CWE-1	22%
CME-1	6%
CME-2	46%

3.2 Determination of epigallocatechin-3-gallate (EGCG) content in tea extracts

Table 3.2 The contents of EGCG in tea extracts

Samples	Concentration of EGCG (%w/w)
CWE-1	6.18
CME-1	42.26
CME-2	22.14

The HPLC method could be used for the determination of EGCG in tea extracts. The calibration curve was obtained by plotting %area versus concentration of EGCG. The correlation coefficient for the EGCG standard was greater than 0.997, suggesting that the HPLC determination for the EGCG demonstrated good linearity within the concentration range of 1000–125 mg/L. As shown in Table 3.2, the content of the EGCG in CME-1 was 42.26 %w/w, while the content in CME-2 and CWE-1 were 22.14 %w/w and 6.18 %w/w of EGCG, respectively. It can be concluded that CME-1 has the highest content of EGCG comparing with CWE-1 and CME-2. Interestingly, the CME-1 is produced by extracting the tea residue with methanol. Since the methanol extract is the best solvent which is highest purity of EGCG. Moreover, these results reveal that the extracted compounds contain many high bioactivities including antimicrobial, antioxidant and anti-inflammation.

Hence, CME-1, which is often considered as industry wastes, has a potential to be utilized as a source of bioactive compounds. These compounds widely used in

food, pharmaceutical and cosmetics industries due to their great variety of biological activities.

3.3 Antimicrobial activity

MIC refers to the lowest concentration of an agent that prevents visible growth of bacteria. In this work, the tea extracts, xanthone, EGCG, caffeine, lipoic acid and Vitamin E were evaluated for growth inhibitory activity against skin pathogens (*P. acnes*). All the MIC values were determined using *p*-iodonitrotetrazolium violet (INT). As shown in Table 3.3, *P. acnes* had susceptibility towards the CME-1 as indicated by the MIC value of 1.25 mg/ml. The MIC values of CWE-1 (2.5 mg/ml) and of CWE-2 (2.5 mg/ml) also indicated anti-*P. acnes* activity of the two fractions, although at a lesser degree than the CME-1. It can be concluded that the CME-1 possesses stronger anti-*P. acnes* than CWE-1 and CME-2, while the corresponding MBC values are 2.5, 5, and 5 mg/ml, for CME-1, CWE-1, and CME-2, respectively. Moreover, the anti-*P. acnes* activity of the CWE-1, CME-1 and CME-2 showed better efficiency than that of metronidazole (MIC value was 5mg/ml). In other studies, the MIC and MBC values of xanthone, lipoic acid, Vitamin E, EGCG and caffeine against *P. acnes* were 0.312, 2.5, 10, 1.25, and 5mg/ml, respectively. The MBC values of xanthone, EGCG and Vitamin e have been reported to be 0.625, 2.5 and 10mg/ml, respectively. Interestingly, the MIC values of CME-1 (1.25 mg/ml) is similar to the MIC value of EGCG (1.25 mg/ml). Although the assays were performed under different conditions, the similar values indicated similar range of activity. Shimamura et al. demonstrated that EGCG could

directly bind to peptidoglycan and induced peptidoglycan precipitation [44]. Moreover, Arakaea et al. suggested that the bacterial action of EGCG might also depend upon the generation of hydrogen peroxide by the reaction of EGCG with reactive oxygen species in the presence of superoxide dismutase [75]. These observations suggested that the antimicrobial activity was directly related to the polyphenol concentration in the tea extracts.

In conclusion, this study confirmed that tea extracts containing EGCG and other polyphenols were effective anti- *P. acnes* agent. For the best of our knowledge, this is the first report on anti- *P. acnes* activity of oolong tea extract.

Furthermore, the anti-*P. acnes* activity of the CME-1 was further investigated when it was mixed with xanthone (extract from mangosteen) in order to evaluate the synergism between the CME-1 and the xanthone.

Table 3.3 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the tea extracts and standard compounds.

Samples	*Propionibacterium acnes*	
	MIC (mg/mL)	MBC (mg/mL)
Crude water extract (CWE-1)	2.5	5
Crude methanol extract (CME-1)	1.25	2.5
Crude methanol extract (CME-2)	2.5	5
Xanthone	0.312	0.625
EGCG	1.25	2.5
Caffeine	5	>10
Metronidazole	5	10
Lipoic acid	2.5	-
Vitamin E	>10	-

3.4 Synergism between crude methanol extract (CME-1) and xanthone against *P. acnes*

The antibacterial activities of CME-1 and xanthone were evaluated in vitro by broth micro-dilution method against *P. acnes* and the results are shown in Table 3.4. The results indicated that combinations between the two plant extracts possessed higher inhibitory activities than the each extract alone. The combination of tea extract and xanthone against *P. acnes*, exhibited a predominantly synergistic effect.

In conclusion, the combination of tea extract and xanthone produced predominantly synergistic interaction and substantial reduction in the MIC values of both the tea extract and the xanthone against *P. acnes.*

Table 3.4 Synergism between crude methanol extract (CME-1) and xanthone against *P. acnes*

Samples	Contents	Concentrations (mg/ml)	Inhibition of *P. acnes* MIC (mg/mL),
CX1	CME-1 : Xanthone	5 : 0.625	√
CX2	CME-1 : Xanthone	2.5 : 0.312	√
CX3	CME-1 : Xanthone	1.25 : 0.156	√
CX4	CME-1 : Xanthone	1.25 : 0.078	√
CX5	CME-1 : Xanthone	0.625 : 0.078	√
CX6	CME-1 : Xanthone	0.312 : 0.078	x
CX7	CME-1 : Xanthone	0.312 : 0.039	x
C	CME-1	5	MIC = 1.25 mg/ml
X	Xanthone	0.625	MIC = 0.312 mg/ml

√ = Samples could inhibit the growth of *P. acnes*

X = Samples could not inhibit growth of *P. acnes*

3.5 Antioxidant activity

Scavenging of diphenyl-picrylhydrazyl (DPPH) radicals

Table 3.5 Antioxidant activity of tea extracts

Sample	IC_{50} (μM/L)
BHT (positive control)	0.309 ± 1.5^{a}
Crude water extract (CWE-1)	0.715 ± 1.0^{a}
Crude methanol extract (CME-1)	0.157 ± 0.9^{a}
Crude methanol extract (CME-2)	0.060 ± 1.1^{b}

[a-b] Statistic analysis was performed using ANOVA with Duncan test (N = 3, values with different superscript were significantly different, ($p < 0.05$). CWE-1: crude water extract, CME-1: crude methanol extract-1, and CME-2: crude methanol extract-2

The DPPH assay method is based on the reduction of DPPH, an organic stable radical, This assay is widely used to determine the antiradical activity of a given compound or an extract by the detection of UV-absorbance of DPPH radicals at 517 nm. The absorption will decrease when DPPH interacts with radical scavenging species. The interaction results in a visually noticeable discoloration from purple (517 nm absorption) to yellow [76].

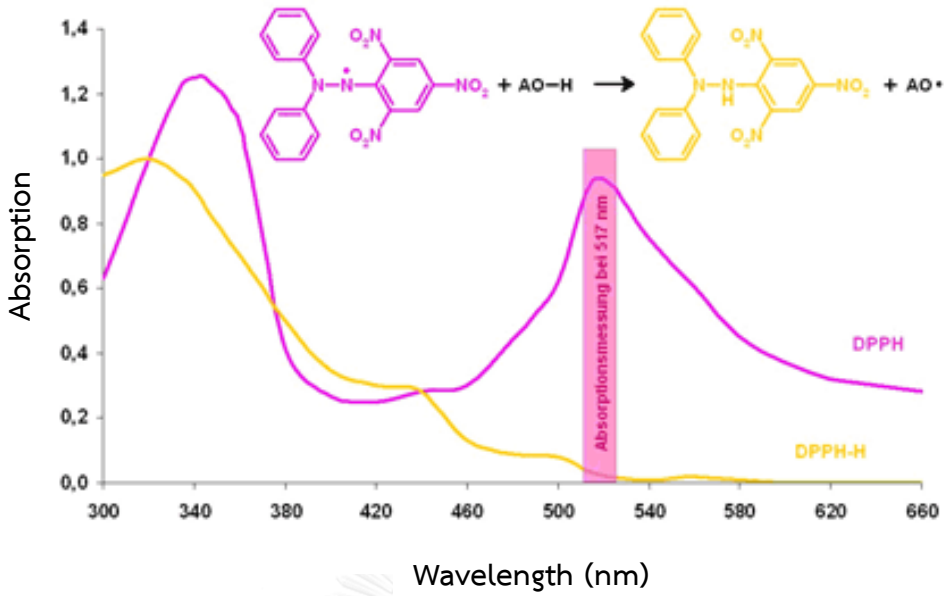


Figure 3.1 The absorption of DPPH free radical [77].

Therefore, the amount of DPPH free radical, which directly relates to the free radical scavenging activity of any tested materials in the system, can be monitored by this assay. Lower value of inhibition concentration at 50% (IC_{50}) indicates higher antioxidant activity. As shown in Table 3.5, the IC_{50} values for CWE-1, CME-1, CME-2 and a standard compound butylated hydroxytoluene (BHT) were 0.715 ± 1.0, 0.157 ± 0.9, 0.060 ± 1.1 and 0.309 ± 1.5 μM/L, respectively. The CME-2 showed significantly (one-way ANOVA followed by Duncan's test; $p < 0.05$) higher activity than BHT and the other extracts. The high temperature used in the extraction process of CWE-1 probably lead to the decrease in the antioxidant activity of the obtained CWE-1 due to, thermal degradation, polymerization and epimerization [78]. Su et al reported the antioxidant activity of oolong tea infusion under various steeping conditions. They found that steeping oolong tea at higher temperature and longer time led to the

degradation of phenolic compounds and, thus reduced antioxidant activity [79]. Chen et al. demonstrated that the antioxidant ability of teas decreased with the increased duration of autoclave treatment. They found that the high temperature could cause chemical change in the tea leaf extracts [80].

Thus it could be concluded that CME-2 was the most potent antioxidation tea extract fraction.

3.6 Cytotoxicity study

The cytotoxicity activity of tea extracts was determined using MTT assay. MTT assay is designed to be used for the quantification of both cell proliferation and cell viability [81]. This test is widely used in the in vitro evaluation of the biosafety of tea extracts [82]. In this study, the cytotoxicity effect of tea extract was evaluated on RAW 264.7 cells. The density of $1x10^4$ cells/well of RAW 264.7 cells was incubated with various concentrations of tea extracts (0–1000 µg/mL) for 24 h. The cell viability was detected by microplate reader at 540 nm of the absorbance. The relative number of viable cells as a percentage of control was calculated, defining as 100%. The MTT assays revealed that there was no significant change in cell viabilities by all tea extract treatment at the tested concentrations (Fig 3.2). The results of cytotoxic activity of all tea extracts are presented in Figure 3.2. The data indicated that these tea extracts were relatively safe for Raw 264.7 cells at the above concentrations. All tea extracts showed that the concentration range of 3 to 1000 µg/ml was non-toxic

due to the cell viability up to 85 percents. These data agree well with the fact that tea is the edible material.

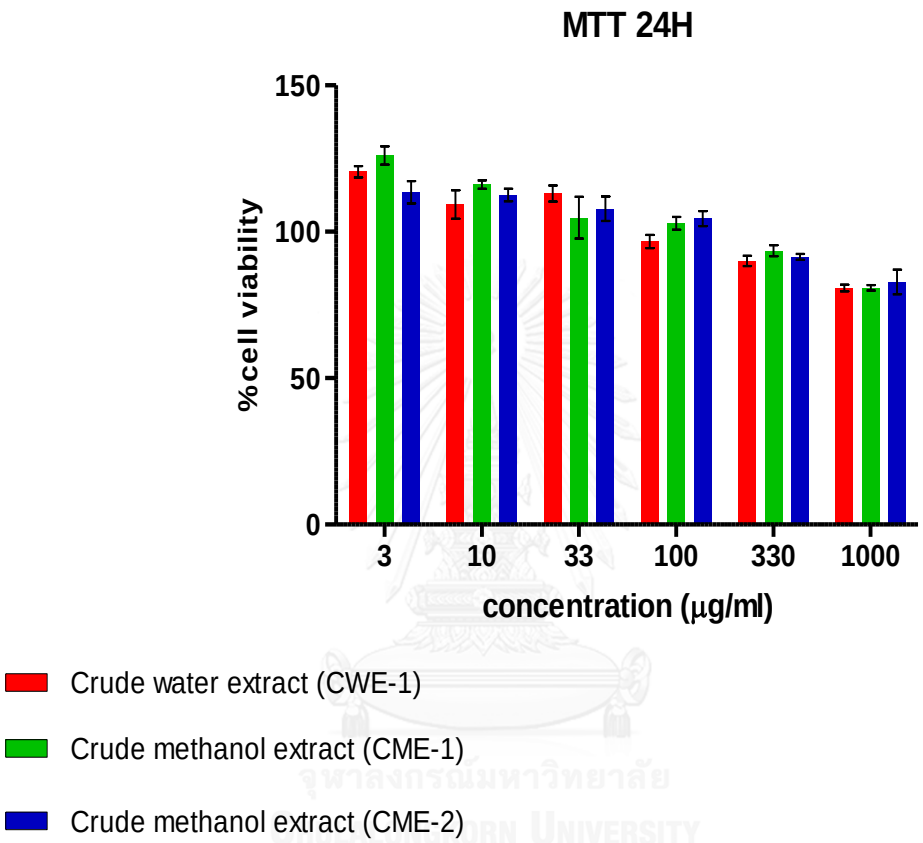


Figure 3.2 Cytotoxicity of tea extracts

3.7 Anti-inflammatory activity of tea extracts

Table 3.6 Anti-inflammatory activity of tea extracts

Sample	%Anti-inflammatory activity (at 10µg/ml)
Crude water extract (CWE-1)	67.53 ± 6.9[a]
Crude methanol extract (CME-1)	84.89 ± 3.9[b]
Crude methanol extract (CME-2)	84.28 ± 5.2[b]

[a-b] Statistic analysis was performed using ANOVA with Duncan test (N = 3, values with different superscript were significantly different, $p < 0.05$).

Anti-inflammatory activity of the tea extracts was investigated with concentration at 10 µg/ml. Anti-inflammatory activity was determined using LPS and INF stimulated murine RAW 264.7 macrophages and NO production by quantification of using Griess reagent. The cytotoxic effect of the extract was evaluated on RAW 264.7 using MTT to ensure that the anti-inflammatory activity was not due to cytotoxicity effect from the tea extracts. As shown in Table 3.6, all tea extracts showed NO inhibitory effect in a dose-dependent manner. CME-1 showed the highest NO inhibition compared to CWE-1 and CME-2 with values of 84.89 ± 3.9%, 67.53 ± 6.9%, and 84.28 ± 5.2% at a concentration of 10 µg/ml. Following the mean comparison of the percentage anti-inflammatory activity of each samples by one-way ANOVA with Duncan test indicated that the percentage anti-inflammatory activity of

tea extracts were significantly different ($p< 0.05$). According to the results obtained from anti-inflammatory activity, tea extracts is a potent anti-inflammatory agent. The anti-inflammatory activity might be from the presence of polyphenols and flavonoid compounds or other tea catechins such as EGCG and ECG in the extract. These compounds have been shown to be able to inhibit NO production by suppressing nitric oxide synthase (iNOS) gene expression [52]. Previously, Chan et al demonstrated the effect of tea catechins on iNOS inhibition. They suggested that iNOS inhibition by the catechins was due to the inhibition of the binding of the substrate L-arginine and the cofactor tetrahydrobiopterin [83]. In addition, Lin et al. reported that EGCG inhibits LPS and interferon-γ-induced nitrite production by mouse peritoneal cells [84]. The catechins were efficient inhibitors. The most active compound is EGCG [85]. Recently, Hisanaga et al. suggested that the inhibition of nitric oxide and iNOS in the RAW 264.7 cells was due to the NO suppressing action of oolong tea extract [55].

Therefore it can be concluded that the tea extracts possess inhibitory activity against LPS and interferon production of NO in the RAW 264.7 cells. Thus, tea extracts are potential anti-inflammatory agents for preventing and treating inflammation-medicated diseases.

3.8 Stability of tea extract

In this work, the thermal stability of tea extract was determined in term of antimicrobial activity against *P. acnes*, as indicated by the MIC value. The tea extract, xanthone, lipoic acid, vitamin E, CX1, CX_1L and CX_1E were prepared and stored at -20 ℃ and room temperature in micro-tubes and then tested periodically for their antimicrobial activity over a period of 28 days. The stability of tea extracts stored at room temperature is shown in Figs. 3.3. On the first day, the MIC values of tea extract, xanthone, lipoic acid, Vitamin E, CX1, CX_1L and CX_1E were 1.25, 0.312, 2.5, 5, 0.625, 2.5 and 5 mg/ml, respectively. After 28 days, the MIC values of tea extract, xanthone, lipoic acid, Vitamin E, CX1, CX_1L and CX_1E were 2.5, 0.625, 2.5, 5, 0.625, 2.5 and 5 mg/ml, respectively. The stability of tea extract kept at -20 ℃ is shown in Figure 3.4. On the first day, the MIC values of tea extract, xanthone, lipoic acid, Vitamin E, CX1, CX_1L and CX_1E were 1.25, 0.312, 2.5, 5, 0.625, 2.5, and 5mg/ml, respectively. After 28 days, the MIC values of tea extract, xanthone, lipoic acid, Vitamin E, CX1, CX_1L and CX_1E were 1.25, 0.312, 2.5, 5, 0.625, 2.5, and 5 mg/ml, respectively.

From the above results, it could be concluded that the stability of the antimicrobial effectiveness of the tea extract depended on storage temperature. The tea extract kept at room temperature loss approximately 50% of their relative activity after 28 days of storage. However, the MIC values of tea extracts kept at -

20°C was unchanged after 28 days. The reason for the decreased antimicrobial activity might be the instability of bioactive compounds at high temperatures [78]

The MIC value of xanthone also increased which mean decreasing of the antimicrobial activity, during the storage at room temperature. This indicates temperature instability of the xanthones.

The CX1 still showed potent antimicrobial activity with MIC of 0.625 mg/mL against *P. acnes* even when kept at room temperature for 28 days (similar to those kept at -20 ℃). These results showed that the bioactive compounds against *P. acnes* in the CX1 were stable during the storage at room temperature.

The anti-*P. acnes* activity of pure vitamin E and CX_1E were tested and the MIC values were more than 5 mg/ml. This indicates ineffective of the two materials in inhibiting *P. acnes*.

P. acnes had susceptibility towards the lipoic acid as indicated by the MIC value of 2.5 mg/ml. These results indicated that the lipoic acid could help preventing acne. The recent agrees well with previous report by Kruszewska and coworkers which indicated that lipoic acid exhibited antimicrobial activity against *S. aureus* (MIC value of 36 mg/ml) and *Candida albicans* (MIC value of 8 mg/ml) [86]. Since lipoic acid is also an antioxidant, we propose here that lipoic acid could be and to increase the stability of tea extracts in the anti-acne formulation containing tea extracts as anti-acne achieve ingredient. The recent of the mixture between the tea extracts and

lipoic acid reflected that the antimicrobial activity with MIC of 2.5 mg/mL against *P. acnes.*

In conclusion, the storage temperature had an influence on the stability of tea extracts, after 7 days. Tea extract stored at room temperature showed less antibacterial activity against *P. acnes* than the tea extract stored at -20 ℃. In addition, the tea extracts showed stability in the room temperature, so this study was not do experiment in 4 ℃.

Table 3.8 Stability of tea extracts

Samples	Contents (mg/ml)	Concentrations (mg/ml)	Minimal inhibitory concentration (MIC) at RT		Minimal inhibitory concentration (MIC) at -20°C	
			0 (days)	28 (days)	0 (days)	28 (days)
CME-1	CME-1	5	1.25	2.5	1.25	1.25
Xanthone	Xanthone	0.625	0.312	0.625	0.312	0.312
Lipoic acid	Lipoic acid	5	2.5	2.5	2.5	2.5
Vitamin E	Vitamin E	5	>5	>5	>5	>5
CX1	CME-1:Xanthone	5 : 0.625	0.625 : 0.078	0.625 : 0.078	0.625 : 0.078	0.625 : 0.078
CX_1L	CME1: xanthone:lipoic acid	5 : 0.625 : 2.5	2.5:0.312:1.25	2.5:0.312:1.25	2.5:0.312:1.25	2.5:0.312:1.25
CX_1E	CME1: xanthone:vitamin E	5 : 0.625 : 2.5	>5 : 0.625 : 2.5	>5 : 0.625 : 2.5	>5 : 0.625 : 2.5	>5 : 0.625 : 2.5

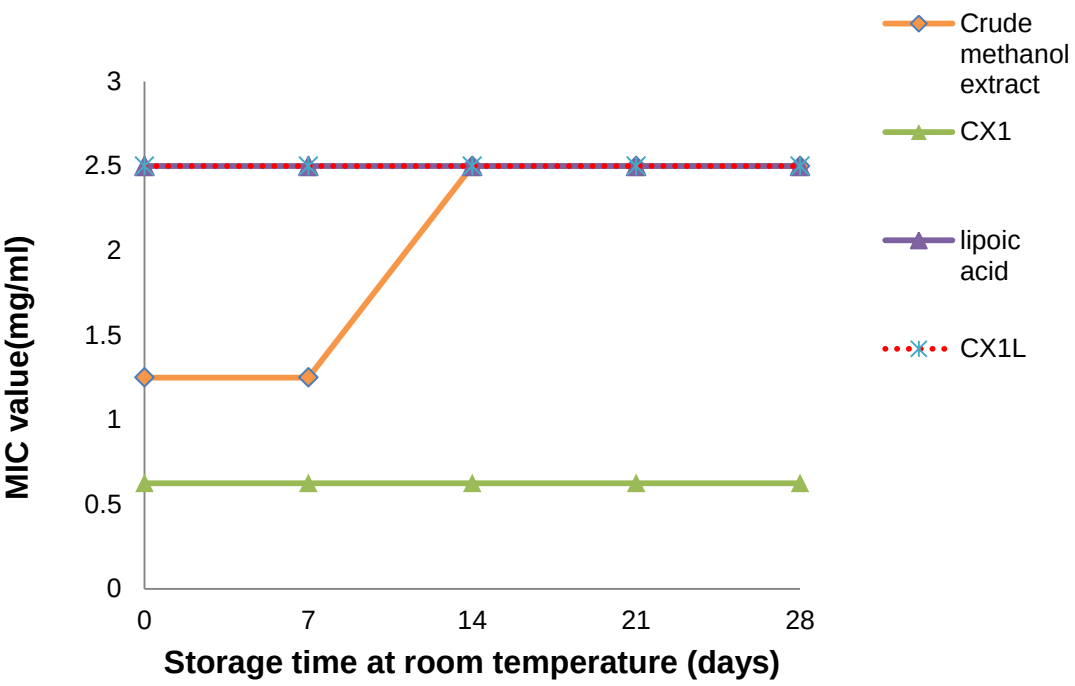
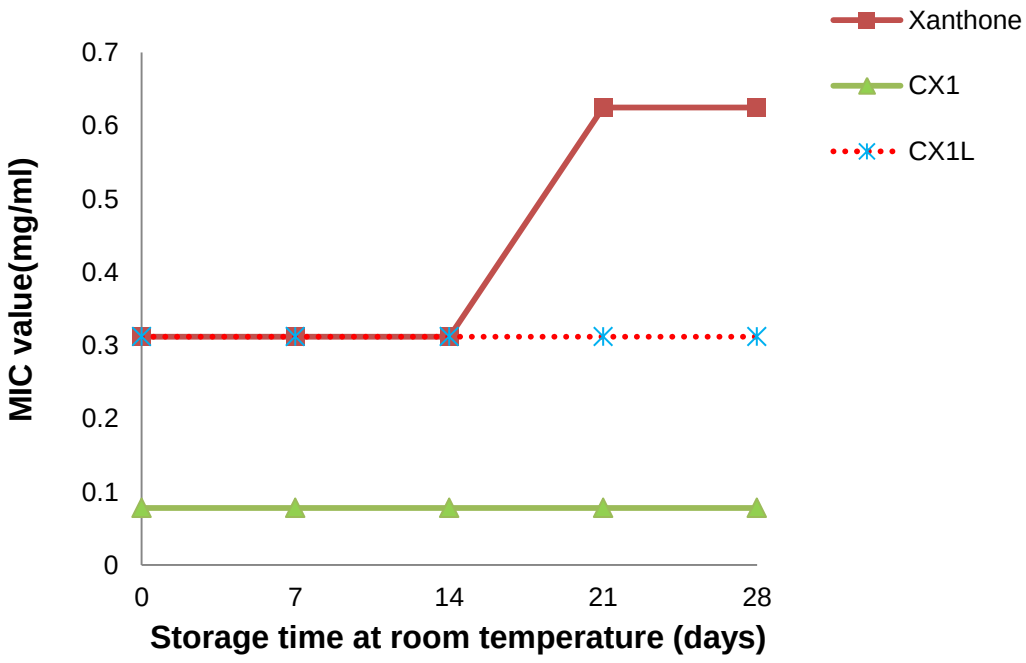


Figure 3.3 The stability of tea extract activities stored at room temperature



Figures 3.4 The stability of xanthone activities stored at room temperature

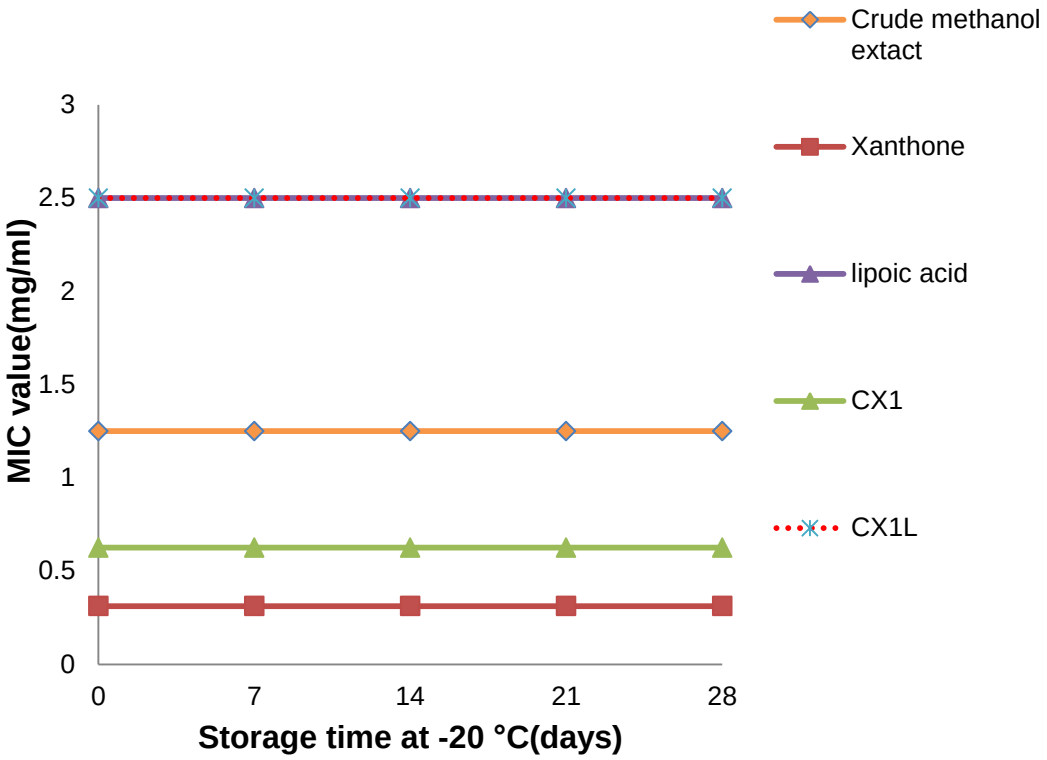


Figure 3.5 The stability of tea extracts activities stored at 20°C

3.9 Antimicrobial activity of the tea extracts loaded alginate bead

The antimicrobial activity of the tea extract, xanthone, CX1 and CX_1L encapsulated in the alginate beads were determined using agar disc diffusion method. The alginate beads were prepared at pH 3.5 and pH 5.5. As shown in Table 3.8, the pH 3.5 alginate beads containing the tea extract, xanthone, CX1 and CX_1L showed the inhibition zone against *P. acnes*. CX_1L bead gave the widest zone of inhibition (1.18±0.1 cm), followed with CX1 bead (1.15±0.1 cm), xanthone bead (0.68±0.1 cm), and tea extract bead (0.53±0.1 cm), respectively. Similarly, at pH 5.5, CX_1L alginate bead also gave the highest zone of inhibition (1.18±0.1 cm) against *P. acnes,* followed by CX1 bead (1.12±0.2 cm), xanthone bead (0.57±0.1 cm) and tea extract bead (0.50±0.0 cm), respectively. The pH 3.5 tea extract beads gave a higher anti-*P. acnes* activity than that of pH 5.5 beads. This is likely a result of better stability of active components at pH 3.5 comparing to the pH 5.5. Zhu et al. reported that the green tea catechins were very stable in the acidic solution (pH < 4) and were very unstable in the alkaline solution [64]. Oryza et al also demonstrated that the stability of purple tea polyphenol was better at low pH (pH <5) [87].

Table 3. 9 Average bacterial of zone inhibition (cm)

Samples	Contents	Concentrations (mg/ml)	Zone of Inhibition (cm)
Bead C DI	CME-1	0.227	-
Bead C pH 3.5	CME-1	0.227	0.53 ± 0.1^{a}
Bead C pH 5.5	CME-1	0.227	0.50 ± 0.0^{a}
Bead X DI	Xanthone	0.042	-
Bead X pH 3.5	Xanthone	0.042	0.68 ± 0.1^{a}
Bead X pH 5.5	Xanthone	0.042	0.57 ± 0.1^{a}
Bead CX1 DI	CME-1:Xanthone	0.2: 0.025	-
Bead CX1 pH 3.5	CME-1:Xanthone	0.2: 0.025	1.15 ± 0.1^{b}
Bead CX1 pH 5.5	CME-1:Xanthone	0.2: 0.025	1.12 ± 0.2^{b}
Bead CX_1L DI	CME-1 : Xanthone : lipoic acid	0.217: 0.027 : 0.109	-
Bead CX_1LpH 3.5	CME-1 : Xanthone : lipoic acid	0.217: 0.027 : 0.109	1.18 ± 0.1^{b}
Bead CX_1L pH 5.5	CME-1 :Xanthone : lipoic acid	0.217: 0.027 : 0.109	1.18 ± 0.1^{b}

[a-b] Statistic analysis was performed using ANOVA with Duncan test (N = 3, values with different superscript were significantly different, $p < 0.05$).

CHAPTER IV

CONCLUSION

The results showed that amount of EGCG, which is the most abundant polyphenols in Oolong tea extract, was 42.26% in the method methanol extract obtained from tea waste. The obtained extract showed antimicrobial activity against *P. acnes* and anti-inflammatory activity (84.89%) at the concentration of 1.25 mg/ml and 10 µg/ml. Interestingly, the obtained tea extract showed synergistic effect with mangosteen extract for the inhibition of *P. acnes* growth. The tea extract exhibited a very low cytotoxicity. Nevertheless, storing the extract at -20 ℃ was required for the stabilization of its anti-*P. acnes* activity. Improved stability at room temperature could be obtained by encapsulating the extract in alginate beads.

REFERENCES

[1] Tahir, C.M. Pathogenesis of acne vulgaris: Simplified Journal of Pakistan Association of Dermatologists 20 (2010): 93-97.

[2] Shalita, A.R. Acne: clinical presentations. Clinics in Dermatology 22(5) (2004): 385-6.

[3] Jacob, C.I., Dover, J.S., and Kaminer, M.S. Acne scarring: A classification system and review of treatment options. Journal of the American Academy of Dermatology 45(1) (2001): 109-17.

[4] Koreck, A., Pivarcsi, A., Dobozy, A., and Kemeny, L. The role of innate immunity in the pathogenesis of acne. Dermatology 206(2) (2003): 96-105.

[5] Sakamoto, F.H., Lopes, J.D., and Anderson, R.R. Photodynamic therapy for acne vulgaris: a critical review from basics to clinical practice: part I. Acne vulgaris: when and why consider photodynamic therapy? Journal of the American Academy of Dermatology 63(2) (2010): 183-93; quiz 193-4.

[6] Studyblue. Pathogenesis of Acne Vulgaris 2013. Available from: https://www.studyblue.com/notes/note/n/dermatologyrheumatology/deck/9921325 [2014]

[7] Thiboutot, D., Gilliland, K., Light, J., and Lookingbill, D. Androgen metabolism in sebaceous glands from subjects with and without acne. Archives of Dermatology 135(9) (1999): 1041-5.

[8] Lever, L. and Marks, R. Current views on the aetiology, pathogenesis and treatment of acne vulgaris. Drugs 39(5) (1990): 681-92.

[9] Eichenfield, L.F. and Leyden, J.J. Acne: current concepts of pathogenesis and approach to rational treatment. Pediatrician 18(3) (1991): 218-23.

[10] Dessinioti, C. and Katsambas, A.D. The role of *Propionibacterium acnes* in acne pathogenesis: facts and controversies. Clinics in Dermatology 28(1) (2010): 2-7.

[11] Vowels, B.R., Yang, S., and Leyden, J.J. Induction of proinflammatory cytokines by a soluble factor of *Propionibacterium acnes*: implications for chronic inflammatory acne. Infection and Immunity 63(8) (1995): 3158-65.

[12] Pochi, P.E. The pathogenesis and treatment of acne. Annual Review of Medicine 41(1) (1990): 187-198.

[13] Bojar, R.A. and Holland, K.T. Acne and Propionibacterium acnes. Clinics in Dermatology 22(5) (2004): 375-9.

[14] Mara, M., Julak, J., Bednar, M., Ocenaskova, J., Mikova, Z., and Dohnalova, A. The influence of Propionibacterium acnes (Corynebacterium parvum) fractions on immune response in vivo. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten Und Hygiene. Zweite Naturwissenschaftliche Abt.: Allgemeine, Landwirtschaftliche und Technische Mikrobiologie 281(4) (1994): 549-55.

[15] Holland, K.T., Ingham, E., and Cunliffe, W.J. A review, the microbiology of acne. Journal of Applied Bacteriology 51(2) (1981): 195-215.

[16] Abate, M.E. Shedding New Light on Acne: The Effects of Photodynamic Therapy on *Propionibacterium acnes* 2009. Available from: http://www.studentpulse.com/articles/763/shedding-new-light-on-acne-the-effects-of-photodynamic-therapy-on-propionibacterium-acnes [2013]

[17] Kanlayavattanakul, M. and Lourith, N. Therapeutic agents and herbs in topical application for acne treatment. International Journal of Cosmetic Science 33(4) (2011): 289-97.

[18] Yentzer, B.A., McClain, R.W., and Feldman, S.R. Do topical retinoids cause acne to "flare"? Journal of Drugs in Dermatology 8(9) (2009): 799-801.

[19] Lyons, R.E. Comparative effectiveness of benzoyl peroxide and tretinoin in acne vulgaris. International Journal of Dermatology 17(3) (1978): 246-51.

[20] Fakhouri, T., Yentzer, B.A., and Feldman, S.R. Advancement in benzoyl peroxide-based acne treatment: methods to increase both efficacy and tolerability. Journal of Drugs in Dermatology 8(7) (2009): 657-61.

[21] Swanson, J.K. Antibiotic resistance of *Propionibacterium acnes* in acne vulgaris. Dermatology Nursing 15(4) (2003): 359-62.

[22] Coates, P., Vyakrnam, S., Eady, E.A., Jones, C.E., Cove, J.H., and Cunliffe, W.J. Prevalence of antibiotic-resistant propionibacteria on the skin of acne patients: 10-year surveillance data and snapshot distribution study. British Journal of Dermatology 146(5) (2002): 840-8.

[23] Shapiro, L.E., Knowles, S.R., and Shear, N.H. Comparative safety of tetracycline, minocycline, and doxycycline. Archives of Dermatology 133(10) (1997): 1224-30.

[24] Xue-mei, H. Treatment of 60 cases of acne by acupuncture and herbal medicine. Journal of Acupuncture and Tuina Science 3(1) (2005): 51-52.

[25] Bent, S. and Ko, R. Commonly used herbal medicines in the United States: a review. American Journal of Medicine 116(7) (2004): 478-85.

[26] Nam, C., Kim, S., Sim, Y., and Chang, I. Anti-acne effects of oriental herb extracts: A novel screening method to select anti-acne agents. Skin Pharmacology and Applied Skin Physiology 16(2) (2003): 84-90.

[27] Gulluce, M., Sokmen, M., Daferera, D., Agar, G., Ozkan, H., Kartal, N., Polissiou, M., Sokman, A., and Sahin, F. In vitro antibacterial, antifungal, and antioxidant activities of the essential oil and methanol extracts of herbal parts and callus cultures of Satureja hortensis L. Journal of Agricultural and Food Chemistry 51(14) (2003): 3958-65.

[28] Sukatta, U., Rugthaworn, P., Pitpiangchan, P., and Dilokkunanant, U. Development of mangosteen anti-acne gel. Kasetsart Journal Natural Science 42 (2008): 163 - 168.

[29] Pothitirat, W., Chomnawang, M.-T., and Gritsanapan, W. Anti-acne inducing bacteria activity and α-mangostin content of Garcinia mangostana fruit rind extracts from different provenience. Songklanakarin Journal of Science and Technology 31((1)) (2009): 41-47.

[30] Kim, S.S., Kim, J.Y., Lee, N.H., and Hyun, C.G. Antibacterial and anti-inflammatory effects of Jeju medicinal plants against acne-inducing bacteria. J Gen Appl Microbiol 54(2) (2008): 101-6.

[31] Yamaguchi, N., Satoh-Yamaguchi, K., and Ono, M. In vitro evaluation of antibacterial, anticollagenase, and antioxidant activities of hop components

(Humulus lupulus) addressing acne vulgaris. Phytomedicine 16(4) (2009): 369-76.

[32] Tsai, T.-H., Tsai, T.-H., Wu, W.-H., Tseng, J.T.-P., and Tsai, P.-J. In vitro antimicrobial and anti-inflammatory effects of herbs against *Propionibacterium acnes*. Food Chemistry 119(3) (2010): 964-968.

[33] Vijayalakshmi, A., Tripura, A., and Ravichandiran, V. Development and evaluation of anti-acne products from *Terminalia arjuna* Bark. International Journal of ChemTech Research (2011): 320-327.

[34] Namita, P., Mukesh, R., and Vijay, K.-J. *Camellia Sinesis* (Green tea): A review. Global Journal of Phamacology 6(2) (2012): 52-59.

[35] Suteerapataranon, S., Butsoongnern, J., Punturat, P., Jorpalit, W., and Thanomsilp, C. Caffeine in Chiang Rai tea infusions: Effects of tea variety, type, leaf form, and infusion conditions. Food Chemistry 114(4) (2009): 1335-1338.

[36] Squire, G.-R. and Callander, B.A. Tea plantation in water deficits and plants growth. Academia Press New york 6 (1981): 471-510.

[37] Tounekti, T., Joubert, E., Hernández, I., and Munné-Bosch, S. Improving the polyphenol content of tea. Critical Reviews in Plant Sciences 32(3) (2012): 192-215.

[38] Senanayake, N. Green tea extract: Chemistry, antioxidant properties and food applications – A review. Journal of Functional Foods 5(4) (2013): 1529-1541.

[39] Graham, H.N. Green tea composition, consumption, and polyphenol chemistry. Preventive Medicine 21(3) (1992): 334-50.

[40] Daglia, M. Polyphenols as antimicrobial agents. Current Opinion in Biotechnology 23(2) (2012): 174-81.

[41] Bansal, S., Choudhary, S., Sharma, M., Kumar, S.S., Lohan, S., Bhardwaj, V., Syan, N., and Jyoti, S. Tea: A native source of antimicrobial agents. Food Research International 53(2) (2013): 568-584.

[42] Du, G.J., Zhang, Z., Wen, X.D., Yu, C., Calway, T., Yuan, C.S., and Wang, C.Z. Epigallocatechin Gallate (EGCG) is the most effective cancer chemopreventive polyphenol in green tea. Nutrients 4(11) (2012): 1679-91.

[43] Ru, Q., Yu, H., and Huang, Q. Encapsulation of epigallocatechin-3-gallate (EGCG) using oil-in-water (O/W) submicrometer emulsions stabilized by iota-carrageenan and beta-lactoglobulin. Journal of Agricultural and Food Chemistry 58(19) (2010): 10373-81.

[44] Shimamura, T., Zhao, W.-H., and Hu, Z.-Q. Mechanism of action and potential for use of tea catechin as an anti-infective agent. AntiInfect AgentsMed Chem 6 (2007): 57-62.

[45] Gramza1, A., Korczak1, J., and Amarowicz, R. Tea polyphenols-their antioxidant properties and biology activity- a review. Polish Journal of Food and Nutrition Sciences 14/55(3) (2005): 219-235.

[46] Nanjo, F., Goto, K., Seto, R., Suzuki, M., Sakai, M., and Hara, Y. Scavenging effects of tea chatechins and their derivatives on, 1-diphenyl-2 -picryhydrazyl radical. Free Radical Biology & Medicin 21(6) (1996): 895-902.

[47] Gadow, A.V., Joubert, E., and Hansmann, C.F. Comparison of the antioxidant activity of rooibos tea (*Aspalathus linearis*) with green, oolong and black tea. Food Chemistry 60(1) (1997): 73-77.

[48] Bors, W. and Michel, C. Antioxidant capacity of flavanols and gallate esters: pulse radiolysis studies. Free Radical Biology and Medicine 27(11-12) (1999): 1413-26.

[49] Zhu, Q.Y., Hackman, R.M., Ensunsa, J.L., Holt, R.R., and Keen, C.L. Antioxidative Activities of Oolong Tea. Journal of Agricultural and Food Chemistry 50(23) (2002): 6929-6934.

[50] Chan, E.W., Soh, E.Y., Tie, P.P., and Law, Y.P. Antioxidant and antibacterial properties of green, black, and herbal teas of *Camellia sinensis.* Pharmacognosy Research 3(4) (2011): 266-72.

[51] Oh, J., Jo, H., Cho, A.R., Kim, S.J., and Han, J. Antioxidant and antimicrobial activities of various leafy herbal teas. Food Control 31(2) (2013): 403-409.

[52] Zhong, Y., Chiou, Y.S., Pan, M.H., and Shahidi, F. Anti-inflammatory activity of lipophilic epigallocatechin gallate (EGCG) derivatives in LPS-stimulated murine macrophages. Food Chemistry 134(2) (2012): 742-8.

[53] Thring, T.S., Hili, P., and Naughton, D.P. Antioxidant and potential anti-inflammatory activity of extracts and formulations of white tea, rose, and witch hazel on primary human dermal fibroblast cells. Journal of Inflammation 8(1) (2011): 27.

[54] Chen, B.T., et al. Anti-inflammatory effects of a polyphenols-rich extract from tea (Camellia sinensis) flowers in acute and chronic mice models. Oxidative Medicine and Cellular Longevity 2012 (2012): 537923.

[55] Hisanaga, A., et al. Anti-inflammatory activity and molecular mechanism of Oolong tea theasinensin. Food Funct 5(8) (2014): 1891-7.

[56] Cowan, M.M. Plant Products as Antimicrobial Agents. Clinical Microbiology Reviews 12(4) (1999): 564-582.

[57] Matsunaga, K., Klein, T., Friedman, H., and Yamamoto, Y. *Legionella pneumophila* Replication in Macrophages Inhibited by Selective Immunomodulatory Effects on Cytokine Formation by Epigallocatechin Gallate, a Major Form of Tea Catechins. Infect. Immun 69(6) (2001): 3947-3953.

[58] Ooshima, T. Anti-caries Activity of Tea Polyphenols. Foods Food Ingredients J. Jpn 210(4) (2005).

[59] Friedman, M., Henika, P.R., Levin, C.E., Mandrell, R.E., and Kozukue, N. Antimicrobial activities of tea catechins and theaflavins and tea extracts against Bacillus cereus. Journal of Food Protection 69(2) (2006): 354-61.

[60] Si, W., Gong, J., Tsao, R., Kalab, M., Yang, R., and Yin, Y. Bioassay-guided purification and identification of antimicrobial components in Chinese green tea extract. Journal of Chromatography A 1125(2) (2006): 204-210.

[61] Stoicov, C., Saffari, R., and Houghton, J. Green tea inhibits Helicobacter growth in vivo and in vitro. International Journal of Antimicrobial Agents 33(5) (2009): 473-478.

[62] Cho, Y.-S., Oh, J., and Oh, K.-H. Antimicrobial activity and biofilm formation inhibition of green tea polyphenols on human teeth. Biotechnology and Bioprocess Engineering 15(2) (2010): 359-364.

[63] Sharma, A., Gupta, S., Sarethy, I.P., Dang, S., and Gabrani, R. Green tea extract: Possible mechanism and antibacterial activity on skin pathogens. Food Chemistry 135(2) (2012): 672-675.

[64] Zhu, Q.Y., Zhang, A., Tsang, D., Huang, Y., and Chen, Z.-Y. Stability of Green Tea Catechins. Journal of Agricultural and Food Chemistry 45(12) (1997): 4624-4628.

[65] Chen, Z.-Y., Zhu, Q.Y., Tsang, D., and Huang, Y. Degradation of Green Tea Catechins in Tea Drinks. Journal of Agricultural and Food Chemistry 49(1) (2000): 477-482.

[66] Lun Su, Y., Leung, L.K., Huang, Y., and Chen, Z.-Y. Stability of tea theaflavins and catechins. Food Chemistry 83(2) (2003): 189-195.

[67] Perva-Uzunalić, A., Škerget, M., Knez, Ž., Weinreich, B., Otto, F., and Grüner, S. Extraction of active ingredients from green tea (Camellia sinensis): Extraction efficiency of major catechins and caffeine. Food Chemistry 96(4) (2006): 597-605.

[68] Yanagimoto, K., Ochi, H., Lee, K.G., and Shibamoto, T. Antioxidative activities of volatile extracts from green tea, oolong tea, and black tea. Journal of Agricultural and Food Chemistry 51(25) (2003): 7396-401.

[69] Wu, C., Xu, H., Héritier, J., and Andlauer, W. Determination of catechins and flavonol glycosides in Chinese tea varieties. Food Chemistry 132(1) (2012): 144-149.

[70] Donaldson, J.R., Warner, S.L., Cates, R.G., and Gary Young, D. Assessment of Antimicrobial Activity of Fourteen Essential Oils When Using Dilution and Diffusion Methods. Pharmaceutical Biology 43(8) (2005): 687-695.

[71] Wangthong, S., Palaga, T., Rengpipat, S., Wanichwecharungruang, S.P., Chanchaisak, P., and Heinrich, M. Biological activities and safety of Thanaka (Hesperethusa crenulata) stem bark. Journal of Ethnopharmacology 132(2) (2010): 466-72.

[72] Yuan, X., Gao, M., Xiao, H., Tan, C., and Du, Y. Free radical scavenging activities and bioactive substances of Jerusalem artichoke (Helianthus tuberosus L.) leaves. Food Chemistry 133(1) (2012): 10-14.

[73] Hendra, R., Ahmad, S., Oskoueian, E., Sukari, A., and Shukor, M.Y. Antioxidant, anti-inflammatory and cytotoxicity of Phaleria macrocarpa (Boerl.) Scheff Fruit. BMC Complementary and Alternative Medicine 11 (2011): 110.

[74] Lee, J.-S., Park, S.-A., Chung, D., and Lee, H.G. Encapsulation of astaxanthin-rich Xanthophyllomyces dendrorhous for antioxidant delivery. International Journal of Biological Macromolecules 49(3) (2011): 268-273.

[75] Arakawa, H., Maeda, M., Okubo, S., and Shimamura, T. Role of hydrogen peroxide in bactericidal action of catechin. Biological and Pharmaceutical Bulletin 27(3) (2004): 277-81.

[76] Hendra, R., Ahmad, S., Oskoueian, E., Sukari, A., and Shukor , M.Y. Antioxidant, Anti-inflammatory and Cytotoxicity of Phaleria macrocarpa (Boerl.) Scheff Fruit. BMC Complementary and Alternative Medicine 11(1) (2011): 110.

[77] Ahmad, I. DPPH Antioxidant assay Method Development 2012. Available from: http://www.researchgate.net/

[78] Ananingsih, V.K., Sharma, A., and Zhou, W. Green tea catechins during food processing and storage: A review on stability and detection. Food Research International 50(2) (2013): 469-479.

[79] Su, X., Duan, J., Jiang, Y., Duan, X., and Chen, F. Polyphenolic profile and antioxidant activities of oolong tea infusion under various steeping conditions Int. J. Mol. Sci 8(12) (2007): 1196-1205.

[80] Chen, B.-L. and Lay, H.-L. Components and antioxidant activity in teas after autoclave treatment. Crop, Environment & Bioinformatics 8 (2011): 109-118.

[81] Hajiaghaalipour, F., Kanthimathi, M.S., Sanusi, J., and Rajarajeswaran, J. White tea (Camellia sinensis) inhibits proliferation of the colon cancer cell line, HT-29, activates caspases and protects DNA of normal cells against oxidative damage. Food Chemistry 169(0) (2015): 401-410.

[82] Silva, A.R., Seidl, C., Furusho, A.S., Boeno, M.M., Dieamant, G.C., and Weffort-Santos, A.M. In vitro evaluation of the efficacy of commercial green tea extracts in UV protection. Int J Cosmet Sci 35(1) (2013): 69-77.

[83] Chan, M.M.-Y., Fong, D., Ho, C.-T., and Huang, H.-I. Inhibition of Inducible Nitric Oxide Synthase Gene Expression and Enzyme Activity by Epigallocatechin

Gallate, a Natural Product from Green Tea. Biochemical Pharmacology 54(12) (1997): 1281-1286.

[84] Lin, Y.L. and Lin, J.K. (-)-Epigallocatechin-3-gallate blocks the induction of nitric oxide synthase by down-regulating lipopolysaccharide-induced activity of transcription factor nuclear factor-kappaB. Molecular Pharmacology 52(3) (1997): 465-72.

[85] Paquay, J.B.G., Haenen, G.R.M.M., Stender, G., Wiseman, S.A., Tijburg, L.B.M., and Bast, A. Protection against Nitric Oxide Toxicity by Tea. Journal of Agricultural and Food Chemistry 48(11) (2000): 5768-5772.

[86] Kruszewska, H., Zarba, T., and Tyski, S. Examination of antimicrobial activity of selected non-antibiotic products. Acta Poloniae Pharmaceutica 67(6) (2010): 733-736.

[87] Oryza.co[Internet]. Purple tea extract 2013. Available from: http://www.oryza.co.jp [2013 October 9]

APPENDIX A

1. Calculation of concentration of EGCG into tea extract

Calibration curve of EGCG

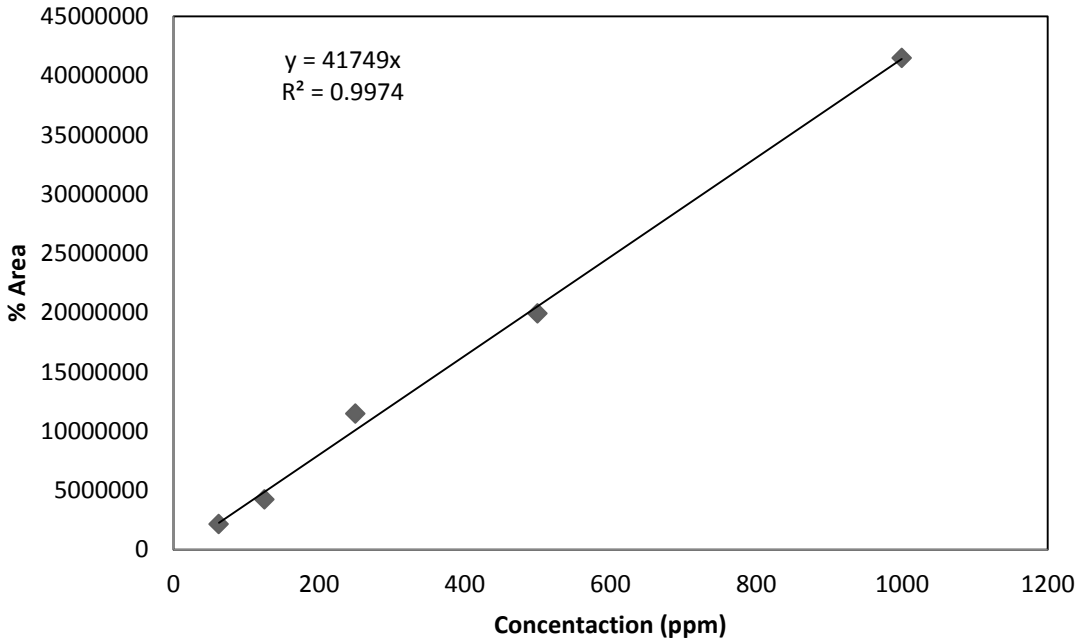


Figure A-1 Calibration curve of EGCG

By plotting a graph between percent %area and concentrations of EGCG solution, a linear relationship was obtained and for calculation of concentrations of tea extracts.

From the equation of calibration curve;

$$Y = 41749x, R^2 = 0.9974 \qquad (1)$$

The amount of CWE-1 was calculated by equation (1);

$$4824013 = 41749x$$

X = 123.56 ppm = 123.56 mg/L

The amount of CME-1 was calculated by equation (1);

35618081 = 41749x

X = 845.14 ppm = 845.14 mg/L

The amount of CME-2 was calculated by equation (1);

18147760 = 41749x

X = 442.7 ppm = 442.7 mg/L

2 Antioxidant activity of tea extracts

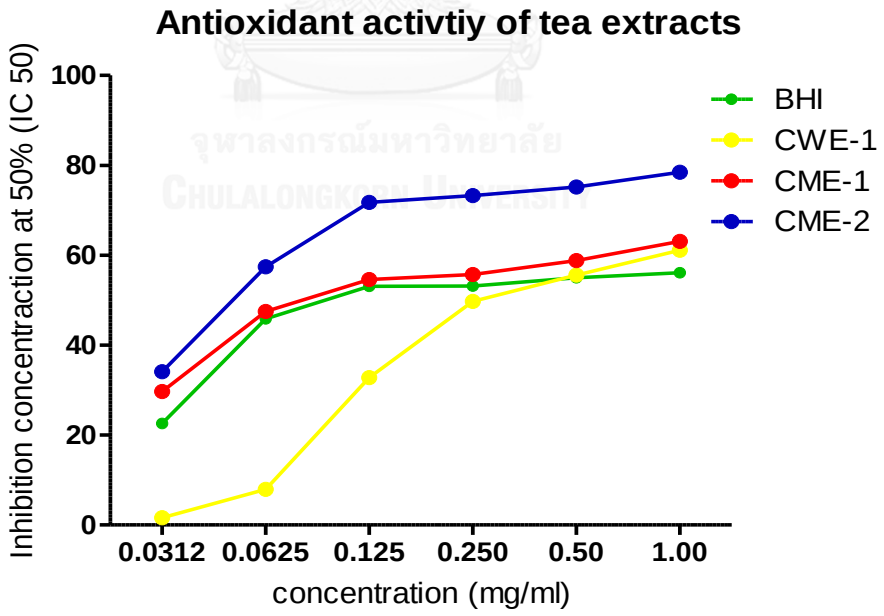


Figure A-2 Inhibition concentration at 50 (%) versus concentration (mg/mL)

Table A-1 Antioxidant activity of tea extracts (concentration at 1mg/ml)

Samples	Absorbance (Control)	Absorbance (Sample)	Equation	%inhibition
BHT	1.501	0.66	y = 8.94ln(x) + 60.496	56.1
CWE-1	1.439	0.56	y = 10.19ln(x) + 53.405	61.1
CME-1	1.537	0.57	y = 9.43ln(x) + 67.443	63.1
CME-2	1.538	0.33	y = 13.03ln(x) + 86.64	78.5

2.1 Calculation of DPPH radical scavenging activity of BHT

DPPH radical scavenging activity $= \frac{Abs\,control\ - Abs\,sample}{Abs\,control} x100$

= (1.501 – 0.66 / 1.501) x100

= 56.1%

2.1.1 Calculation of inhibition concentration at 50% of BHI

y = 8.94ln(x) + 60.496

y = DPPH radical scavenging activity, x = concentration of BHT

50 = 8.94ln(x) + 60.496

x = 0.716

2.2 Calculation of DPPH radical scavenging activity of CWE-1

$$\text{DPPH radical scavenging activity} = \frac{Abs\,control\ - Abs\,sample}{Abs\,control} x100$$

= (1.439 – 0.56 / 1.439) x100

= 61.1%

2.2.1 Calculation of inhibition concentration at 50% of CWE-1

y = 10.19ln(x) + 53.405

y = DPPH radical scavenging activity, x = concentration of CWE-1

50 = 10.19ln(x) + 53.405

x = 0.715

2.3 Calculation of DPPH radical scavenging activity of CME-1

$$\text{DPPH radical scavenging activity} = \frac{Abs\,control\ - Abs\,sample}{Abs\,control} x100$$

= (1.537 – 0.57 / 1.537) x100

= 63.1%

2.3.1 Calculation of inhibition concentration at 50% of CWE-1

y = 9.43ln(x) + 67.443

y = DPPH radical scavenging activity, x = concentration of CME-1

50 = 9.43ln(x) + 67.443

x = 0.157

2.4 Calculation of DPPH radical scavenging activity of CME-2

$$\text{DPPH radical scavenging activity} = \frac{Abs\,control - Abs\,sample}{Abs\,control} x100$$

= (1.538 – 0.33 / 1.538) x100

= 78.5 %

2.4.1 Calculation of inhibition concentration at 50% of CME-2

y = 13.03ln(x) + 86.64

y = DPPH radical scavenging activity, x = concentration of CME-2

50 = 13.03ln(x) + 86.64

x = 0.060

APPENDIX B

1 Chemical and culture medium preparation

1.1. Brain heart infusion (BHI) agar for *P. acnes* culture (500 ml)

Twenty-six grams of BHI a gar was mixed with reverse osmosis water 500 ml. The medium was sterilized using autoclaving at 121°C at 15 psi for 1 hour. The obtained BHI was kept at 4°C until used.

1.2. Brain heart infusion broth (BHI) for *P. acnes* culture (500 ml)

Seventeen-grams of BHI broth was mixed with reverse osmosis water 500 ml. The medium was sterilized using autoclaving at 121°C at 15 psi for 1 hour. The obtained BHI was kept at 4°C until used.

1.3. Isotonic Phosphate Buffer Saline (PBS) pH 7.4

NaCl	8.00 g
KCl	0.20 g
KH_2PO_4	0.20 g
Na_2HPO_4	1.44 g

Dissolve NaCl, KCl, KH_2PO_4 and Na_2HPO_4 with distilled water then adjust volume to 1000 ml with distilled water and the pH is measured with pH meter.

1.4. Preparation of MTT stock solution

Prepare a 12 mM MTT stock solution by 5 mg/ml of MTT reagent was dissolved in 10 ml of PBS. The solution was stirred until completely dissolved and mixed together. The obtained stock solution was sterilized through a 0.5 μm filter and transferred into aliquot tubes. The stock solution was stored at 4°C until used.

1.5. Acetate buffer pH 3.5 and pH 5.5

Acetate acid solution: 0.2 M of acetic acid (11.5 ml acetic acid in 1000 ml of distilled water)

Sodium acetate Solution: 0.2 M of sodium acetate (16.4 g of $C_2H_3O_2Na$ or 27.2 g of $C_2H_3O_2Na{\cdot}3H_2O$ is dissolved in 1000ml distilled water.

The two solutions were stirred until completely dissolved and mixed together (Table B-1) and the pH is measured with pH meter.

Table B-1 The volume (ml) of 0.2M acetic acid solution to be added to 50ml of 0.2M sodium acetate to achieve a particular pH.

pH	ml of 0.2 M of acetic acid	ml of 0.2 M of sodium acetate
3.5	46.3	3.7
5.5	4.8	45.2

APPENDIX C

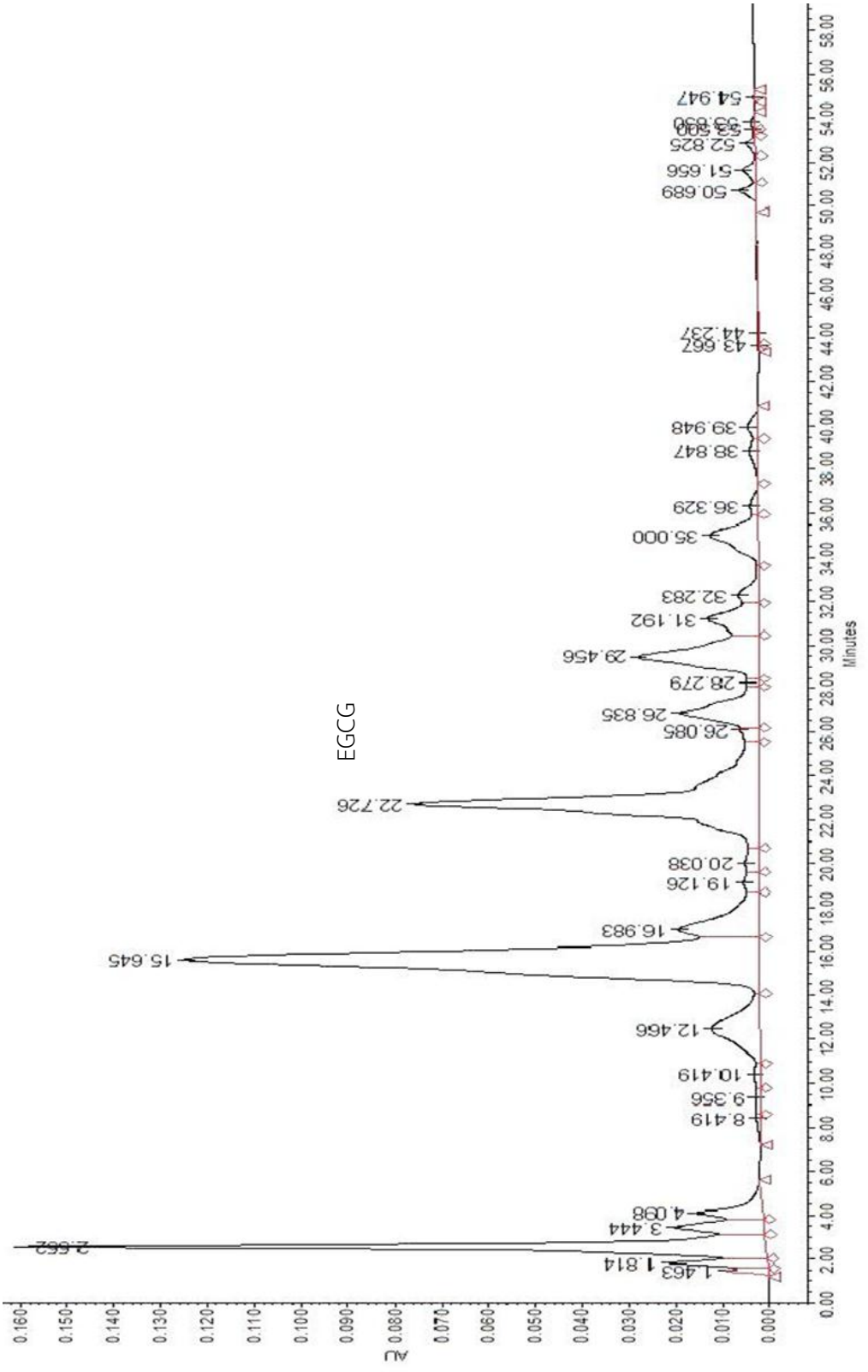


Figure C-1 HPLC chromatogram of crude water extract-1

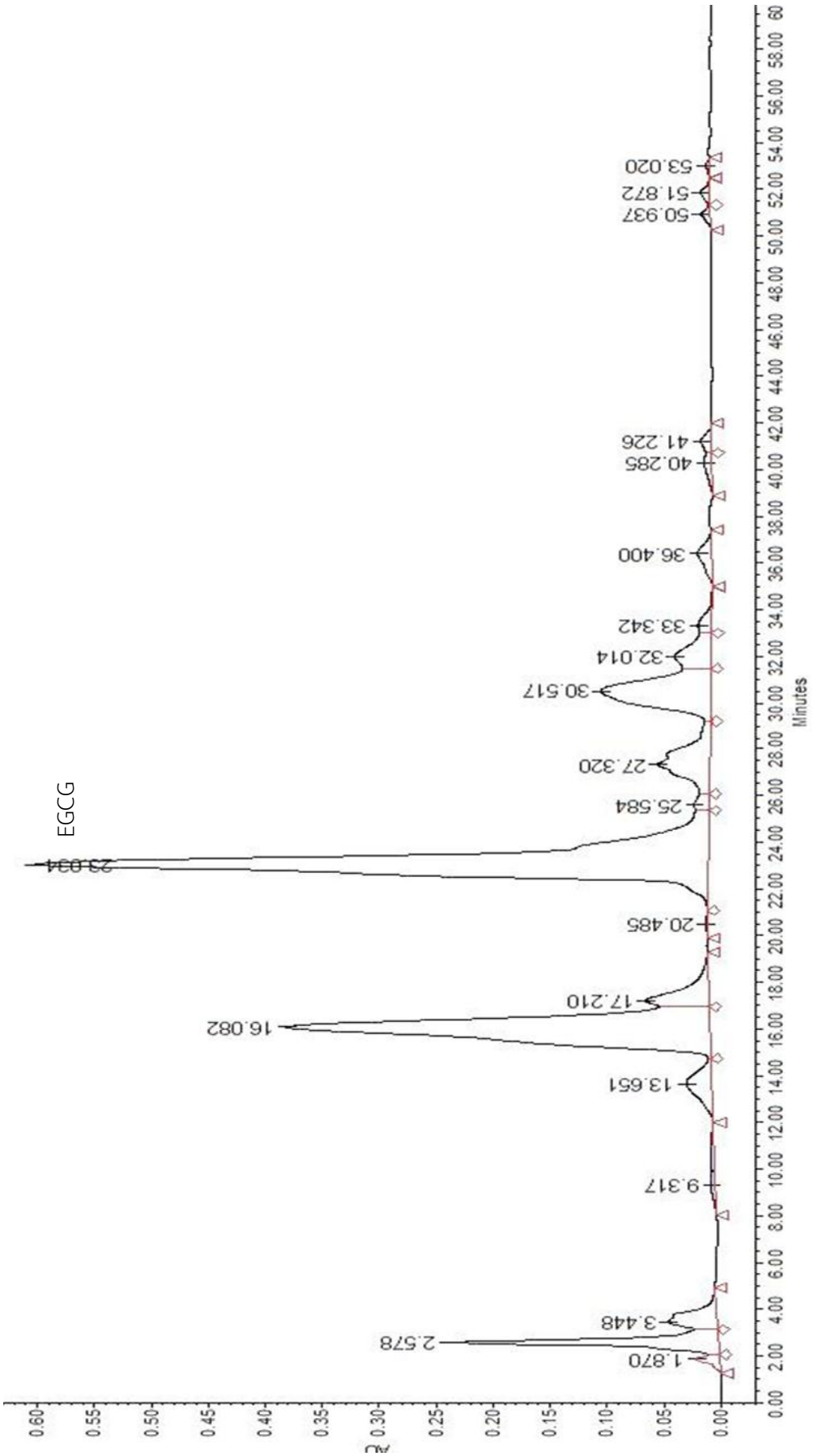


Figure C-2 HPLC chromatogram of crude methanol extract-1

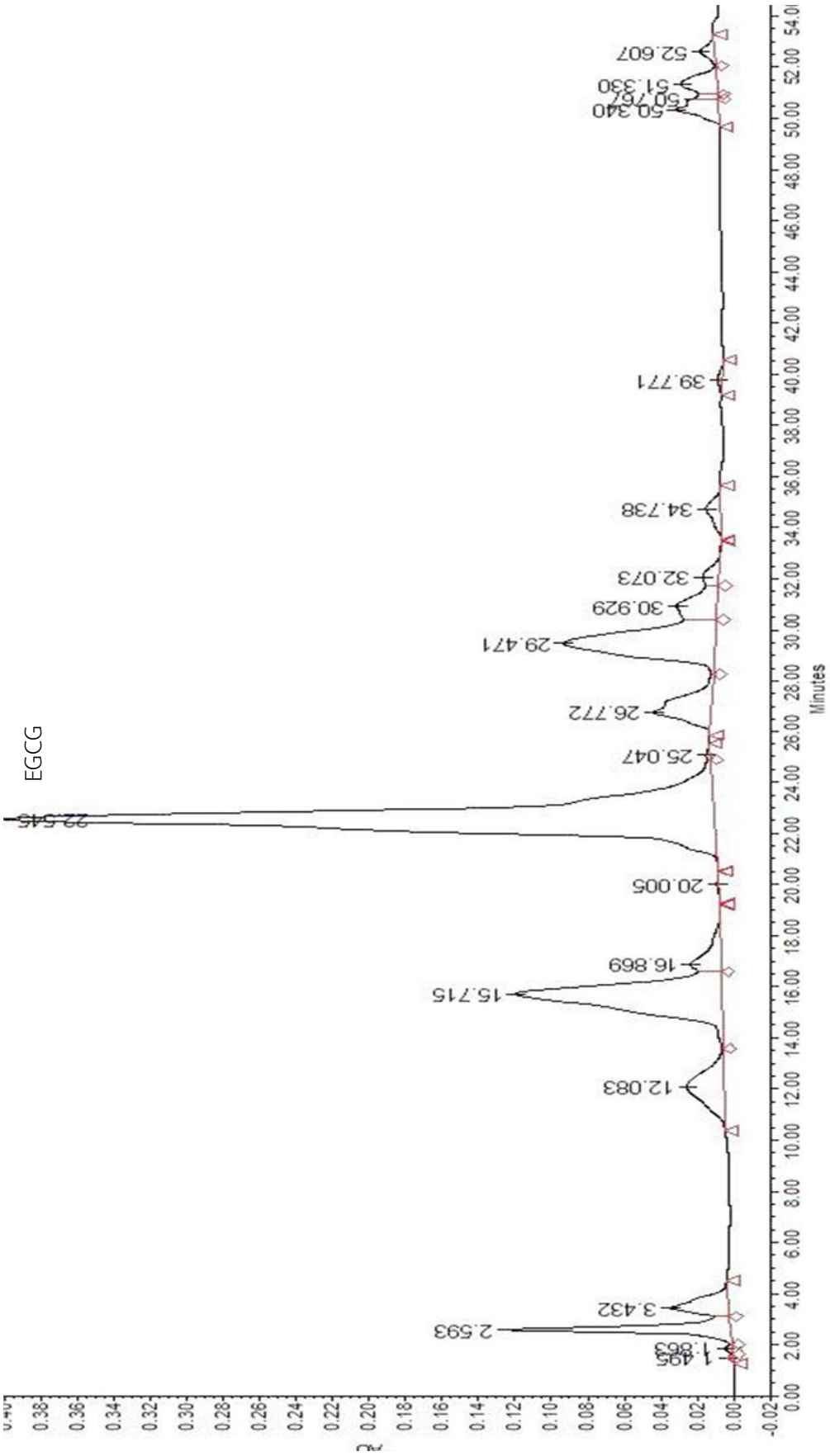


Figure C 3HPLC chromatogram of crude methanol extract-2

VITA

Miss Nawaporn Wiratkasem was born on July 27, 1988 in Phayao. She got a Bachelor's Degree of Science in Cosmetic Science from Mae Fah Luang University in 2010. After that, she started her graduate study on a Master's degree in the Program of Biotechnology, Faculty of Science, Chulalongkorn University. During master study, she had the opportunity to present her work in poster session in the topic of "Tea Extract-Loaded Alginate Beads and Their Biological Activity" at the 2014 IUPAC world the polymer congress (MACRO 2014), Chiang Mai, Thailand. My address is 210/8 Prachakorn Thai Housing Club Soi. 5 ladprao93 Road, Wangthonglang, Bangkok, 10310, Tel. 087-9959390.